Metabolic fate of [2-^{14}C]quercetin-4'-glucoside in rats and in cultured cells

PhD thesis

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Abstract

Background: Flavonoids are a class of phytochemicals which have been associated with several health benefits, including reduced risk of cancer and cardiovascular disease. Quercetin is one of the most ubiquitous dietary flavonoids with estimated intakes of 3-15 mg/day. The bioactivity of dietary constituents is dependent upon their bioavailability, but the absorption and biotransformation of quercetin is poorly understood as reflected by estimates of absorption rates from <0.1% to 50%. Further investigations of the putative health effects of quercetin require more reliable data on its bioavailability and metabolism in the gastrointestinal tract (GI-tract) and its distribution to body tissues.

Section I and II: Using a radiolabelled quercetin glycoside, the oral absorption and subsequent body distribution of [2-^{14}C]quercetin-4'-glucoside and its metabolites were traced in Rowett Hooded Lister rats. Based on the radioactivity content of plasma and body tissues, ~10% of the oral dose (7.6 mg/kg BW) was absorbed and appeared in tissues almost exclusively in the form of >20 different methylated, glucuronated, and/or sulfated quercetin metabolites. The GI-tract held >85% of the ingested radioactivity at 0.5, 1, 2 and 5 hours after oral administration; at 2 hours all radiolabelled compounds in the GI tract were metabolised forms of the ingested compound. It was hypothesized that quercetin-4'-glucoside was completely metabolised in the GI-tract before absorption into the circulation. Biological effects of dietary quercetin may be due to its metabolites.

Section III: To investigate the individual contribution of the liver (the primary metabolising organ for xenobiotic compounds), GI-tissues or GI-microflora in quercetin metabolism, cultured rat liver (CC-1) and small intestinal (IEC-6) cells were used as a model for pre-systemic metabolism. Both cell lines produced similar types and numbers of quercetin metabolites and 11 of the 16 cell culture metabolites were identical with quercetin metabolites formed in the rat. The degree of chemical instability of [2-^{14}C] quercetin-4'-glucoside in cell culture medium was not anticipated *a priori*. Less than 0.2% of the administered [2-^{14}C]quercetin-4'-glucoside, its radiolabelled break down products or metabolites were associated with the cells. Despite apparent metabolism in the cell culture medium, the presence of cells was essential for the formation of quercetin metabolites, indicating that metabolites had been formed either on the cell membrane or inside the cells with quick subsequent export from cells into the medium. In conclusion, cell culture experiments may be a useful tool to investigate potential health effects of dietary quercetin and *in vivo* metabolites, provided 1) chemical break down of quercetin can be controlled and 2) the cell line can synthesize similar metabolites as found *in vivo*.

Section IV: Previous reports indicate quercetin up-regulates glutathione *in vitro* and *in vivo*, a mechanism by which this flavonoid may increase cellular antioxidant activity. However, glutathione is also up-regulated by oxidative stress and some studies suggest quercetin may possess pro-oxidant activity. Therefore, it was investigated whether dietary glycosylated quercetin up-regulates glutathione in cells that form *in vivo* metabolites of quercetin. We found free and glycosylated quercetin had different effects on glutathione regulation, suggesting that the biological activity of these forms of the flavonoid may be different. We now hypothesize that pro-oxidant by-products formed during quercetin oxidation in cell culture medium may be responsible for many of the observed effects of quercetin *in vitro*.

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List of Abbreviations

amu	atomic mass units
AUC	area under the curve calculation
Bq	becquerel
CC-1	liver epithelial cells
CHD	Coronary Heart Disease
Ci	curie
COMT	catechol-*O*-methyltransferase
dpm	disintegrations per minute
DTNB	5,5'-Dithio-bis[2 nitro benzoic acid] or Ellman's Regagent
EDTA	ethylene-diamine-tetra-acetic acid
FCS	fetal calf serum
GCL	glutamate cysteine ligase
GI-tract	gastrointestinal tract
GSH	reduced glutathione
GSSG	oxidised glutathione
h	hours
HPLC	High Performance Liquid Chromatography
IEC-6	small intestinal epithelial cells
LDL	low density lipoprotein
LPH	lactase phloridzin hydrolase
M	molar
$M^{\cdot}$	molecular ion (in MS analysis)
m/z	mass / charge ratio
min	minutes
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NADPH	β-nicotinamide-adenine-dinucleotide-phosphate in reduced form
NEAA	non essential amino acids
OH	hydroxyl group
PBS	phosphate buffered saline
Q4'G	quercetin-4'-glucoside
RC	radioactivity counting (via on line radioactivity monitor)
RT	retention time
SD	standard deviation
SGLT-1	sodium dependent glucose transporter
SOD	superoxide dismutase
SULT	phenol-sulfo-transferase
UGT	uridine-5'-diphosphate-glucuronyl-transferases
γ-GCS	γ-glutamyl-cysteine-synthetase

Publications from this thesis

Conference abstracts

Graf AB, Wilson R, Caldwell ST, Hartley RC and Lean MEJ. Metabolism of dietary quercetin-4'-glucoside by cultured liver and intestinal cells. *Proceedings of the Nutrition Society* 2003;62:6A.

Graf AB, Mullen W, Caldwell ST, Hartley RC, Duthie GG, Lean MEJ and Crozier A. Fate of quercetin-4'-glucoside in the rat. *Free Radical Research* 2002;36(1):94.

Graf AB, Mullen W, Caldwell ST, Hartley RC, Duthie GG, Lean MEJ and Crozier A. Disposition and metabolism of dietary quercetin-4'-glucoside in the rat. European Postgraduate Poster Symposium, September 2002, Pfizer Global Research and Development, Sandwich, U.K.

Mullen W, Graf AB, Caldwell ST, Hartley RC, Duthie GG, Edwards CA, Lean MEJ and Crozier A. Identification of metabolites of [2-^{14}C]quercetin-4'-glucoside in rats by HPLC-MS/MS with on line radioactivity detection. International Symposium on Dietary Phytochemicals and Human Health, April 2002, Salamanca, Spain.

Peer reviewed papers

Mullen W, Graf AB, Caldwell ST, Hartley RC, Duthie GG, Edwards CA, Lean MEJ and Crozier A. Determination of flavonol metabolites in plasma and tissues of rats by HPLC-radiocounting and tandem mass spectrometry following oral ingestion of [2-^{14}C]quercetin-4'-glucoside. *Journal of Agricultural and Food Chemistry* 2002;50(23):6902-6909.

Pending publications

Graf AB, Mullen W, Caldwell ST, Hartley RC, Duthie GG, Lean MEJ Crozier A and Christine A. Edwards. Gastrointestinal metabolism and absorption of dietary quercetin-4'-glucoside in the rat. *Submitted.*

Graf AB, Wilson R, Crozier A. and Lean MEJ.
Stability and metabolism of [2-^{14}C]quercetin-4'-glucoside in a cell culture model for pre-systemic metabolism.

Graf AB, Wilson R and Lean MEJ.
Quercetin and quercetin-4'-glucoside have different effects on the regulation of intra-cellular glutathione levels in cultured cells.

"... it's sometimes hard to see what the lessons are. (...) Not only may we have difficulty understanding what is being taught, we may never know which lessons we're supposed to master. It would be impossible to master them all perfectly, and there are undoubtedly some dragons we're not supposed to slay this lifetime. Sometimes *not* slaying them is the lesson. Its easy to look at someone else and say, "Oh, its so sad, he didn't get the lesson of forgiveness before he died." But maybe he still learned what he was supposed to. Or perhaps he was presented with opportunities to learn, but chose not to. And who knows? Maybe *he* wasn't supposed to get the lesson by forgiving. Perhaps *you* were offered an opportunity to get the lesson of forgiveness by watching him..."

Elisabeth Kübler-Ross & David Kessler

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Literature Review

1. Flavonoids and Quercetin

1.1. Flavonoids – a large family of polyphenolic compounds

Flavonoids are polyphenolic molecules synthesized in plants, consisting of two aromatic rings which are linked together with a bridge of one oxygen and three carbon atoms (forming an oxygenated heterocycle) (**Figure 1**). Flavonoids have important functions in plant physiology, they are involved in electron transport during photosynthesis (Das, 1994), are powerful UV protectors (Jorgensen, 1993), interact with plant growth hormones and play an important role in the defence against viral or bacterial pathogens (Havsteen, 2002). Flavonoids are synthesised in all vascular plants and are found in considerable quantities in fruits, vegetables, nuts, seeds, herbs, spices, stems and flowers. Flavonoids are present in cooked vegetables and in plant extracts such as tea, coffee and red wine (Beecher, 2003).

Flavonoids are divided into subclasses based on the connection position of the B and C rings as well as the degree of saturation, oxidation, and hydroxylation of the C ring. The subclasses are most commonly categorized as flavan-3-ols (or catechins), flavanones, flavones, isoflavones, flavonols, and anthocyanidins (**Figure 2**). Examples of molecular structures of individual flavonoids are given in **Table 1**. Flavonoids are ubiquitously distributed in the plant kingdom. The content of individual flavonoids and entire flavonoid subclasses can vary markedly, even between similar foods (Sellappan et al, 2002; Crozier et al, 1997). As a result, diverse dietary patterns can provide considerably different intakes of these nutrients. Examples of the principal flavonoids present in some common foods are provided in **Table 2**.

With the exception of some flavan-3-ols, most flavonoids in food are found linked to one or more sugar molecules via β-glycosidic bonds with phenolic hydroxyl groups. The binding site of the sugar moiety (e.g. 3 or 4' position) affects the bioactivity of the glycosilated flavonoid (Murota et al, 2004, Yamamoto et al, 1999; Williamson et al, 1996; Ioku et al, 1995). Sugar conjugation generally increases the water-solubility of the flavonoid, hence it is easier to handle by the plant. Glucose is the most commonly found sugar adduct, although conjugation with galactose, rhamnose, arabinose or xylose is possible, as well as conjugation with glucuronyl and galacturonyl moieties. The diversity of these conjugates contributes to the large number of different naturally occurring flavonoids, estimated at more than 5000 different compounds (Harborne and Baxter, 1999).

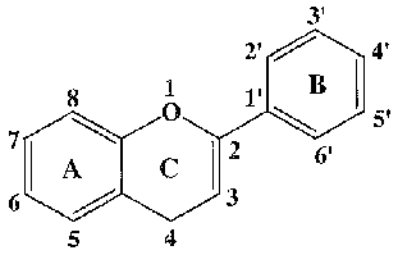


Figure 1: Basic structure and numbering system of flavonoids.
Flavonoids contain two aromatic rings (A and B) that are linked via an oxygenated heterocycle (ring C).

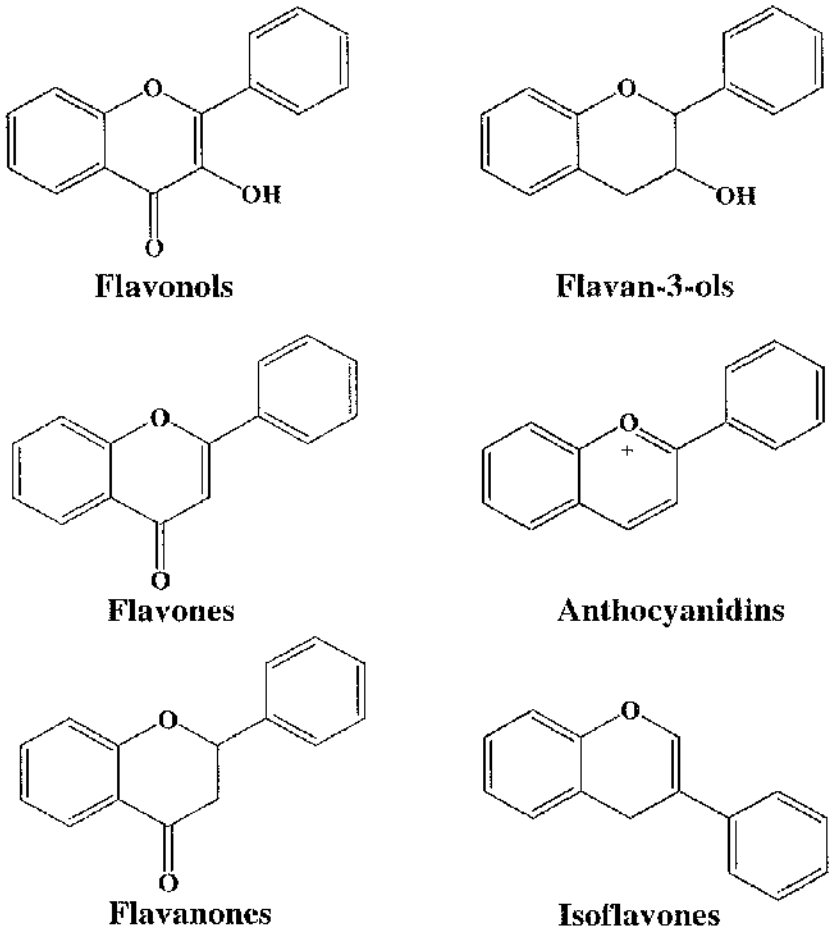


Figure 2: Classes of Flavonoids.
The core structures of six different flavonoid subclasses are shown above. Individual flavonoids are structurally distinct due to different patterns of hydroxylation, methylation and conjugation with various mono and disaccharides. Molecular structures of individual flavonoids are exemplified in Table 1.

Flavonoid Class	Individual Flavonoid	Substituents					
		3	5	7	3'	4'	5'
Flavonols	quercetin	OH	OH	OH	OH	OH	H
	kaempferol	OH	OH	OH	H	OH	H
	myricetin	OH	OH	OH	OH	OH	OH
Flavones	apigenin	H	OH	OH	H	OH	H
	luteolin	H	OH	OH	OH	OH	H
Flavan-3-ols	catechin	OH	OH	OH	OH	OH	H
	epigallocatechin	OH	OH	OH	OH	OH	OH
	epigallocatechin gallate	G	OH	OH	OH	OH	OH
Flavanones	hesperetin	H	OH	OH	OH	OCH_3	H
	naringenin	H	OH	OH	H	OH	H
	eriodictyol	H	OH	OH	OH	OH	H
Anthocyanidins	cyanidin	OH	OH	OH	OH	OH	H
	malvidin	OH	OH	OH	OCH_3	OH	OCH_3
	petunidin	OH	OH	OH	OCH_3	OH	OH
Isoflavones	genistein	H *	OH	OH	H	OH	H
	daidzein	H *	H	OH	H	OH	H

Table 1: Structures of individual flavonoids.
Individual molecular structures of flavonoids are determined by the addition of hydroxyl, methyl and methoxy groups, most commonly at positions, 3, 5, 7, 3', 4' or 5' on the main flavonoid nucleus. Quercetin and catechin have hydroxyl groups at positions 3, 5, 7, 3' and 4', their core structures however are different, with quercetin belonging to the class of flavonols and catechin to the class of flavan-3-ols (Figure 2). The number and positioning of hydroxyl groups together with the degree of saturation of the C ring determines the antioxidant activity of individual flavonoids. G = gallate, H * = in the case of isoflavones, H it attached to position 2, due to the connection of C and B ring at position 3.

Through plant synthesis and also via some types of food processing, flavonoids can be polymerised into larger molecules called tannins. According to their polymerisation pattern and monomeric constituents, tannins are classified into three major groups: proanthocyanidins (or condensed tannins), derived tannins, and hydrolysable tannins (**Table 3**).

Flavonoid Class	Compound Example	Food Sources	Content (mg /100 g)
Flavonols	quercetin	onion, raw	15.36
	kaempferol	broccoli, raw	9.37
	myricetin	apples	4.42
		black grapes	2.99
Flavones	apigenin	celery, raw	5.92
	luteolin	parsley, raw	303.24
		thyme, fresh	56.00
Flavan-3-ols	catechin	apples	9.09
	epigallocatechin	red wine	11.90
	epigallocatechin gallate	green tea	132.12
		black tea	34.26
Flavanones	hesperetin	oranges	43.88
	naringenin	lemons	49.81
	eriodictyol	grapefruit	54.50
Anthocyanidins	cyanidin	blueberries	112.55
	malvidin	raspberries	47.60
	petunidin	red wine	9.19
		red onion	13.14
Isoflavones	genistein	soymilk	9.65
	daidzein	tofu	28.15

Table 2: Flavonoid content of selected foods.
Content of six flavonoid subclasses in the edible portion of selected foods are taken from the USDA Database for the Flavonoid Content of Selected Foods (http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html), except for isoflavone values, which are derived from the Iowa State University Database on the Isoflavone Content of Foods (http://www.nal.usda.gov/fnic/foodcomp/Data/Isoflav.html, April, 2004).

Tannins	Characteristics	Compound names	Food sources
Condensed tannins (pro-anthocyanidins)	convert in part into anthocyanidins after acid catalyzed de-polymerization.	pro-cyanidins pro-delphinidins	apple blueberries dark chocolate
Derived tannins	are formed during food processing, particularly during production of oolong and black tea.	thearubigins theaflavins	green tea black tea red wine
Hydrolyzable tannins	esters of gallic acid or ellagic acid and non-aromatic polyols (mainly glucose).	gallotannins ellagitannins	pomegranate raspberries

Table 3: Tannins are di-, oligo-, or polymers of flavonoids and other polyphenols.
Data is adapted from Beecher, 2003

1.2. Dietary intake of flavonoids

Flavonoids have been a component of human plant foods for millions of years (Swain, 1975) therefore throughout evolution the human organism must have accommodated to the dietary intake of flavonoids. A first step towards exploring the impact of flavonoids on human health was the quantification of the daily exposure to flavonoids. Flavonoid intake (based on aglycones) in today's populations has been estimated to range between 10 and 100 mg daily and is summarized in **Table 4**. These estimates however are likely to be imprecise for the following reasons.

(1) The flavonoid content in food and beverages varies considerably depending on cultivar, growth conditions, ripeness, post-harvest processing, cooking, and storage (Price et al, 1997; Price et al, 1998a; Crozier et al, 1997). For example, quercetin content of 100 g yellow-skinned onion can vary between 8.57 mg (King-Midas, Georgia, USA) and 81.0 mg (Cross Bow, U.K.) and red skinned onions can contain up to 139.0 mg quercetin (Red Baron, U.K.) (Sellappan et al, 2002; Price et al, 1997). In addition, published estimates of flavonoid content in foods are confounded by methodological factors, such as varying analytical procedures and equipment in different laboratories.

(2) Flavonoids are relatively resistant to heat and are stable in lightly fried vegetables, but substantial losses (of up to 75%) may occur due to their solubility in cooking water (Crozier et al, 1997; Price et al, 1997). Therefore when the daily intake of flavonoids is estimated, allowances should be made for losses during food preparation.

(3) Estimates of the daily average flavonoid intake in different countries, were based on the quantification of only selected groups of flavonoids, namely flavonols and flavones, while other important groups of flavonoids, such as catechins and anthocyanidins, were not included.

(4) A single main meal together with a small glass of red wine can contain up to 265 mg of flavonoids (Rechner et al, 2002; Serafini et al, 1998). If this is rounded up by a small piece of dark chocolate (20 mg) and a cup of black tea (200 ml) 50 mg of catechins can be added (Arts et al, 2001), and the total intake was >300 mg. Therefore the calculated daily averages, ranging between 14 and 79 mg (**Table 4**), appear unrealistically low.

Cultural dietary habits influence which foods are most frequently consumed in different countries, so it is not surprising that isoflavone intake from soy is high in Japan (Arai et al, 2000), while in Scotland and Wales, where consumption of black tea is the main source of dietary flavonoids (Kyle et al, 2002; Hertog et al, 1997) and intake of

catechins is highest (Duthie et al, 2003). In addition, the consumption of selected subclasses of flavonoids, rather than the total flavonoid intake, may prove relevant to specific health outcomes. In conclusion, the flavonoid content in foods varies significantly and the actual flavonoid intake of an individual is difficult to determine. Given the above reasons, it is likely that the average flavonoid intake in humans may well be higher than 100 mg per day.

Daily flavonoid intake in different countries

Country	Population	Flavonols	Flavones	Flavanones	Catechins	Anthocyanidins	Iso-flavones	Sum	Reference
Scotland	81 subjects 4 day weighed intake	19	0.1	1	59			79.1	Duthie et al, 2003
U.K. (Wales)	1,900 men, aged 45-59 y FFQ and weighed dietary intake	26.3						26.3	Hertog et al, 1997
Finland	10,054 subjects, 39 ±16 y (dietary history)	4	<1	20				24	Knekt et al, 2002
Holland	4,807 subjects, aged 67.4 ± 7.8 y (FFQ)	26.6						26.6	Geleijnse et al, 2002
Holland	806 men, aged 65-84 y in 1985, (dietary history)				72			72	Arts et al, 2001
USA (Boston)	34,789 men, aged 40-75 y in 1986 (FFQ)	19.9	0.2					20.1	Rimm et al, 1996
USA (Boston)	38,445 women, ≥ 45 y, in 1992, FFQ	23.9	0.7					24.6	Sesso et al, 2003
USA (Iowa)	34,492 postmenopausal women, 55-69 y in 1986, FFQ	13.84	0.06					13.9	Yochum et al, 1999
Japan	115 women, 29-78 y, dietary history	16	<1				47	63	Arai et al, 2000
Germany (Bavaria)	119 adults, 19-49 y, 7 day dietary protocols	12		13.2	8.3	2.7		54	Linseisen et al, 1997

Table 4: Estimated dietary intake of flavonoids in different countries.
Estimates are based on (incomplete) tables of flavonoid content in foods in mg (aglycones)/day. No study provides values for all subclasses of flavonoids, hence all listed values are likely to be underestimates. Method of dietary assessment: Food frequency questionnaire (FFQ), dietary history, and/or weight dietary intake.

Country	quercetin intake (mg/d)	source
Wales, U.K.	14.2	Hertog et al, 1997
Scotland, UK	14.0	Duthie et al, 2000
Boston, USA	15.4	Rimm et al, 1996
Iowa, USA	9.7	Yochum et al, 1999
Finland	3.3	Knekt et al, 2002

Table 5: Estimated quercetin intake in different countries.

Food Sources	quercetin content (mg /100 g)
apples	4.4
black tea, brewed	2.0
broccoli, raw	3.2
capers, canned	180.8
cocoa, dry powder	20.1
cranberries, raw	14.0
elderberry, raw	42.0
fennel leaves, raw	48.8
red grapes	3.5
red onion, raw	19.9
yellow onion, raw	13.3

Table 6: Quercetin rich foods.
Quercetin content in edible portions of selected foods derived from the USDA Database for the Flavonoid Content of Selected Foods (http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html, April, 2004).

1.3. The flavonol quercetin

Certain dietary flavonoids are particularly bioactive and have pronounced effects on mammalian cells. Quercetin, 3, 3', 4', 5, 7-pentahydroxyflavone (**Figure 3**) is one of the most abundant flavonoids in our diet, and estimates of human consumption range from 3 to 15 mg/day (**Table 5**). Some flavonoid families are only present in selected food groups (e.g. isoflavones in soy products, flavanones in herbs, anthocyanidins in red coloured fruit and vegetables). Quercetin however is ubiquitously distributed throughout most plant foods and can be found in onion, apples, berries, grapes, lemons, nuts, grains, tomatoes, broccoli, lettuce, beans, green and black tea, red wine (see **Table 6**). Quercetin displays a wide range of biological effects (reviewed in paragraph 3) and epidemiological studies conclude that quercetin may play a role in disease prevention (reviewed in paragraph 2). In foods quercetin naturally occurs conjugated to sugar molecules, with

quercetin-4'-glucoside and quercetin-3,4'-di-glucoside (**Figure 3**) accounting for 80% of the total flavonoid content in onion, a major source of dietary quercetin (Häkkinen 1999; Rhodes and Price, 1996).

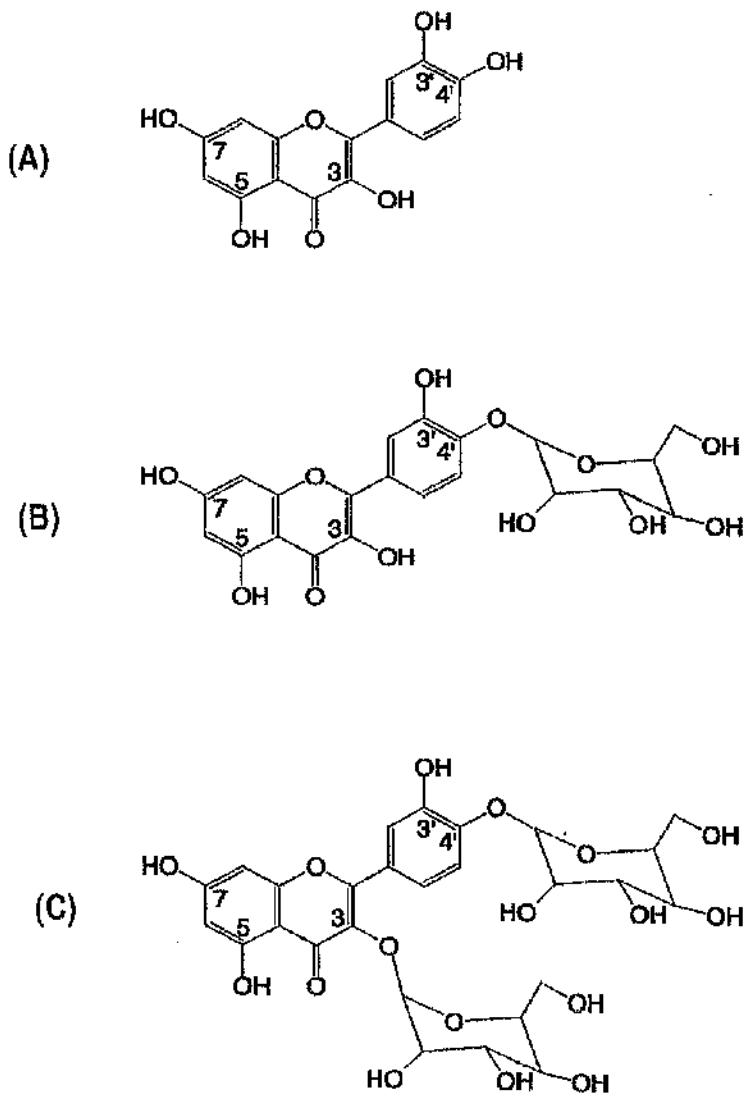


Figure 3: Molecular structures of quercetin and quercetin glycosides.
Structure of quercetin aglycone (A), quercetin-4'-*O*-β-D-glucoside (B) and quercetin-3,4'-di-*O*-β-D- glucoside (C).

2. Flavonoids and human health: Epidemiological evidence

Epidemiological studies consistently show inverse relationships between fruit and vegetable consumption and many degenerative diseases, such as cancer and heart disease (Hu et al, 2003; Riboli and Norat, 2003; World Cancer Res. Fund, 1997; Ness and Powles, 1997; Steinmetz and Potter, 1996). However, epidemiological studies can only point to associations and can not prove which compounds in fruit and vegetables were responsible for the observed health effects. Foods rich in flavonoids also contain other known or unknown nutrients and phytochemicals and it may not be possible to statistically separate their individual effects from effects caused by flavonoids (De Stefani et al, 2000). However, in the past 10 years, the correlation between flavonoid intake and disease incidence was investigated in a number of epidemiological studies (**Tables 7, 8, 9** and **10**). This chapter focuses on the effects of flavonol consumption, particularly quercetin consumption. Epidemiological findings on health protective effects of isoflavones were not included in this chapter, because the main dietary source of isoflavones, soy and soy products, are rarely consumed in European populations. Epidemiological findings on the correlation of catechin intake and disease prevention were recently reviewed by Arts et al. (2001) and Blot et al. (1997). Confounding factors of 'flavonoid epidemiology' are discussed in paragraph 2.5..

2.1. Cardiovascular disease

Four out of eight relevant studies report that a high intake of dietary flavonoids (flavonols, flavones and flavanones) was associated with reduced incidence of heart disease (**Table 7**) (Knekt et al, 2002; Geleijnse et al, 2002; Arts et al, 2001; Hertog et al, 1993). In two studies the consumption of tea was negatively correlated with heart disease (Geleijnse et al, 2002; Arts et al, 2001), however Hertog et al. (1997) reported that in Welsh tea drinkers the intake of >1.2 litres tea /day was correlated with an increased risk, rendering inconsistent results for the association of tea consumption and heart disease. The "Seven Country Study" by Hertog et al (1995) found that 8% of the between country variation of coronary heart disease (CHD) mortality was correlated with a high intake of dietary flavonols (smoking and consumption of saturated fat explained 9% and 73% of the between country variation). In conclusion, the reviewed epidemiological studies suggest that a diet high in flavonoids reduces the incidence of CHD.

2.2. Cerebrovascular disease

Two out of five studies report a significant decline of stroke incidence and mortality in population segments with a high intake of flavonoids (Knekt et al, 2002; Keli et al, 1996) (**Table 8**). The other three studies report no or only a very weak non-significant associations, suggesting that a diet high in flavonoids (namely flavonols, flavones and flavanones) may reduce stroke incidence.

2.3. Cancer

Knekt et al, 2002 reports that dietary intake of flavonoids (flavonols, flavones and flavanones) was inversely correlated with cancer incidence, particularly lung cancer (**Table 9**). Two earlier studies, however, found no correlation between flavonoid intake and cancer incidence (Goldbohm et al, 1997; Hertog et al, 1993). Elevated cancer incidence was observed in a Welsh population with high dietary intake of flavonols and tea (Hertog et al, 1995). High quercetin intake led to a significant reduction of the incidence of esophageal and stomach cancer (De Stefani et al, 1999; Garcia-Closas et al, 1999), however no effect was observed for lung cancer (Garcia-Closas et al, 1998). In conclusion, epidemiological studies on potential effects of flavonoids in cancer prevention are not yet convincing, and further research is needed to clarify this issue.

2.4. Other diseases

A diet rich in quercetin appeared to reduce the incidence of asthma and diabetes (**Table 10**) (Knekt et al, 2002). The putative protective effect of quercetin against the development of diseases, such as heart disease, stroke, cancer, asthma and diabetes, implies that 1) beneficial effects of quercetin are caused by multiple pathways or 2) that the pathologies of the different diseases are based on a similar starting point, e.g. pro-oxidative imbalance in cells and tissues (Halliwell, 1994).

2.5. Confounding factors of epidemiological studies

All epidemiological studies investigating the relationship between food intake and disease development critically depend on an accurate measurement of the consumption of specific foods. In flavonoid epidemiology naturally it is important that major flavonoid rich foods are recorded in the initial dietary snapshot (food frequency questionnaire,

dietary history). In some of the above reviewed studies however, the consumption of some flavonoid rich foods such as onion, berries tea and red wine was not recorded (Knekt et al, 2002; Yochum et al, 1999), which compromises the final results.

Due to the vast number of individual flavonoids, and a lack of adequate food composition data, it is almost impossible to quantify the daily flavonoid intake of humans (paragraph 1.2.). In all the above reviewed epidemiological studies only a very small number of flavonoids, mostly the three flavonols, quercetin, kaempferol and myricetin, have been quantified and the consumption of other flavonoid groups, e.g. anthocyanidins were completely neglected. However, even if adequate food composition data was available, dietary intake of flavonoids does not directly translate into bioavailability. Therefore the correlation of disease outcomes with plasma or tissue levels of flavonoids may focus the outcomes of epidemiological studies.

In conclusion, caution is warranted in the interpretation of epidemiological studies, however, current epidemiological evidence is suggestive that flavonoids including quercetin may play a role in disease prevention, particularly in the prevention of heart disease and stroke.

Flavonoids and CHD – epidemiological data

Country	Study population	Flavonoid intake/d	Compared with	Incident	Relative Risk (95%CI)		Reference
Holland	The Rotterdam Study 4807 subjects ≥55 y (mean 67.4±7.8 y) 5.6 y follow up (1990/93-1997)	>375 ml tea	no tea	Myocardial infarction	0.57 (0.33, 0.98)	○	Geleijnse et al, 2002
				Fatal myocardial infarction	0.30 (0.09, 0.94)	●	
				Nonfatal myocardial infarction	0.68 (0.37, 1.26)	○	
		>32.9 mg flavonols [2]	< 22.8 mg	Myocardial infarction	0.76 (0.49, 1.18)	○	
				Fatal myocardial infarction	0.93 (0.57, 1.52)	○	
				Nonfatal myocardial infarction	0.35 (0.13, 0.98)	●	
Holland	The Zutphen Elderly Study, data re-evaluation 806 men, 65-84 y 1985, 10 y follow up	> 85.9 mg total catechins	< 49.1 mg	Ischemic heart disease mortality	0.49 (0.27, 0.88)	●	Arts et al, 2001
				Ischemic heart disease incidence	0.70 (0.39, 1.26)	○	
Holland	The Zutphen Elderly Study, 805 men aged 65-84 y, 1985, 10 y follow up	> 30 mg flavonols and flavones [3]	< 19 mg	CHD mortality	0.47 (0.27,0.82)	●	Hertog et al, 1993, and 1997
				First myocardial infarction	0.62 (0.24, 1.05)	○	
U.K. (Wales)	The Caerphilly Study 1900 men aged 45-59 y 10-14 y follow up from 1979-83	> 34 mg flavonols [2]	< 19 mg	Ischemic heart disease	1.1 (0.6, 1.6)	○	Hertog et al, 1997
		> 18 mg quercetin	< 10 mg	Ischemic heart disease	1.1 (0.7, 1.7)	○	
		>1200 ml tea	< 300 ml	Ischemic heart disease	2.3 (1.0, 5.1)	●	
Finland	Finnish Mobile Clinic Health Examination Survey 10,054 subjects >15 y 28 y follow up (1967-94)	>27 mg total [1] flavonols (4) flavones (2) flavanones (3)	< 4 mg	Ischemic heart disease mortality	0.93 (0.74-1.17)	○	Knekt et al, 2002
		> 4 mg quercetin	< 1.5 mg	Ischemic heart disease mortality	0.79 (0.63, 0.99)	●	
USA (Boston)	Woman's Health Study 38,445 women ≥ 45 y 6.9 y follow up (1992-ongoing)	47 mg flavonols and flavones [3] (median)	9 mg (median)	Cardiovascular disease	0.88 (0.68, 1.14)	○	Sesso et al, 2003
		> 32.8 mg quercetin (median)	< 6.5 mg (median)	Cardiovascular disease	0.96 (0.74, 1.25)	○	
USA (Iowa)	Postmenopausal women 34,492 55-69 y 1986, 10y follow up	>28.6 mg (median) flavonols and flavones [3]	< 4 mg (median)	Cardiovascular disease, fatal	0.62 (0.44, 0.87)	○	Yochum et al, 1999
		> 18.7 mg quercetin (median)	< 3.3 mg (median)	Cardiovascular disease, fatal	0.74 (0.52, 1.06)	○	
USA (Boston)	Health professionals follow up 34,789 men, 40-75y 5 y follow up (1986-1992)	> 40 mg flavonols and flavones [3]	< 7.1 mg	Myocardial infarction, non-fatal	1.08 (0.81, 1.43)	○	Rimm et al, 1996
				Non-fatal myocardial infarction in men with history of CHD	0.63 (0.33-1.20)	○	

Table 7: Effect of dietary flavonoids on CHD incidence - epidemiological data.
The legend for this table is on the next page.

Flavonoids and stroke - epidemiological data

Country	Study population	Flavonoid intake/d	Compared with	Incident	Relative risk (95% CI)	Reference
Holland	The Zutpen Elderly Study, data re-evaluation 806 men, 65-84 y 1885, 10 y follow up	> 85.9 mg total catechins	< 49.1 mg	Stroke incidence	0.92 (0.51, 1.68) ○	Arts et al, 2001
				Stroke mortality	0.81 (0.36, 1.83) ○	
Holland	The Zutpen Study 552 men 50-69 y 1960-70; 15 y follow up	>28.6 mg flavonols and flavones [3]	< 18.3 mg	Stroke incidence	0.27 (0.11, 0.70) ●	Keli et al, 1996
		≥ 587 g tea (4.7 cups)	< 330 g (2.6 cups)	Stroke incidence	0.31 (0.12, 0.84) ●	
Finland	Finnish Mobile Clinic Health Examination Survey 10,054 subjects >15y 28 y follow up (1967-94)	>27 mg total [1] flavonols (4) flavones (2) flavanones (3)	< 4 mg	Cerebrovascular disease	0.79 (0.64, 0.98) ●	Knekt et al, 2002
		> 4 mg quercetin	< 1.5 mg	Cerebrovascular disease	0.86 (0.70-1.05) ○	
Finland	Alpha Tocopherol Beta Carotene Cancer Prevention Study 26,593 male smokers 50-69y 6.1 y follow up	> 16.4 mg (median) flavonols and flavones [3]	< 4.2 mg (median)	Cerebral infarction	0.98 (0.80-1.21) ○	Hirvonen et al, 2000
USA (Iowa)	Postmenopausal women 34,492 55-69 y 1986, 10y follow up	>28.6 mg (median) flavonols and flavones [3]	< 4 mg (median)	Stroke mortality	1.02 (0.59, 1.79) ○	Yochum et al, 1999

Table 8: Effect of dietary flavonoids on stroke incidence - epidemiological data.
Results are adjusted for dietary and non-dietary confounding factors, however methods of adjustment varied in different studies. [1]Total flavonoids = sum of kaempferol, quercetin, myricetin, isorhamnetin (flavonols), apigenin, luteolin (flavones), hesperetin, naringenin and eriodictyol (flavanones).[3] Sum of quercetin, kaempferol, myricetin, luteolin and apigenin.
● significant result ○ not significant result.

Legend for Table 7 on previous page:

Table 7: Effect of dietary flavonoids on CHD incidence - epidemiological data.
Results are adjusted for dietary and non-dietary confounding factors, however methods of adjustment varied in different studies. Hirvionen et al, 2001 and The Seven Country study (Hertog et al, 1995) is not included because statistical significance of the data was not shown and due to different statistical analysis and data collection.[1]Total flavonoids = sum of kaempferol, quercetin, myricetin, isorhamnetin (flavonols), apigenin, luteolin (flavones), hesperetin, naringenin and eriodictyol (flavanones). [2]Sum of quercetin, kaempferol and myricetin. [3] Sum of quercetin, kaempferol, myricetin, luteolin and apigenin.
● significant result ○ not significant result.

Flavonoids and cancer – epidemiological data

Country	Study population	Flavonoid intake/d	Compared with	Incident	Relative risk (95%CI)		Reference
Finland	Finnish Mobile Clinic Health Examination Survey 10,054 subjects >15y 28 y follow up (1967-94)	>27 mg total [1] flavonols (4) flavones (2) flavanones (3)	< 4 mg	all cancers	0.89 (0.74, 1.06)	○	Knekt et al, 2002
				lung	0.64 (0.39, 1.04)	●	
				colorectum	0.84 (0.43, 1.64)	○	
				breast (in women)	1.23 (0.72, 2.10)	○	
		> 4 mg quercetin	< 1.5 mg	all cancers	0.77 (0.65, 0.92)	●	
				lung	0.42 (0.25, 0.72)	●	
				colorectum	0.62 (0.33, 1.17)	○	
				breast (in women)	0.62 (0.37, 1.03)	○	
U.K. (Wales)	The Caerphilly Study 1900 men aged 45-59 y 14 y follow up from1979-83 FFQ and weighed dietary intake	> 34 mg flavonols [2]	< 19 mg	all cancer mortality	1.3 (0.7, 2.3)	●	Hertog et al, 1997
		> 1200 ml tea	< 300 ml	all cancer mortality	2.2 (1.0, 5.0)	●	
Holland	The Netherlands Cohort Study on Diet and Cancer. 120,852 subjects, 55-69, 1986, 4.3 y follow up, FFQ	43.5 mg median flavonols and luteolin [4]	12.7 mg median	stomach cancer	0.86 (0.47, 1.57)	○	Goldbohm et al, 1997
				colorectal cancer	0.97 (0.71, 1.32)	○	
				lung cancer	0.99 (0.69, 1.42)	○	
				breast cancer	1.02 (0.72, 1.44)	○	
		30.3 mg quercetin (median)	8.4 mg median quercetin	stomach cancer	1.08 (0.56, 2.05)	○	
				colorectal cancer	1.06 (0.77, 1.45)	○	
				lung cancer	0.81 (0.57, 1.17)	○	
				breast cancer	1.00 (0.70, 1.41)	○	
Holland	The Zutpen Elderly Study, 738 men aged 65-84 y, 1985, 5 y follow up, FFQ	> 29.9 mg flavonols and flavones [3]	< 19 mg	all cause cancer incidence	1.21 (0.66, 2.21)	○	Hertog et al, 1994
				lung cancer incidence	1.13 (0.38, 3.40)	○	
				all cause cancer death	1.43 (0.58, 3.54)	○	
Holland	The Zutpen Elderly Study, 728 men aged 65-84 y, 1985, 10 y follow up, FFQ	> 86 mg catechins (6) [5]	< 49 mg	epithelial cancer (oropharynx, esophagus, stomach, colon, rectum, liver, gallbladder, pancreas, kidney, bladder, bronchus and lung)	0.94 (0.56, 1.59)	○	Arts et al, 2001
Spain	Case control study (354 cases, 354 controls,)	highest quartile of flavonol and luteolin [4] intake	lowest quartile	stomach cancer	0.44 (0.25, 0.78)	●	Garcia-Closas et al, 1999
		highest quartile of quercetin intake	lowest quartile	stomach cancer	0.62 (0.35, 1.10)	●	
Spain	Case control study (103 cases, 206 controls, mean age: 63 y)	highest tertile of flavonol and luteolin [4] intake	lowest tercile	lung cancer	0.98 (0.44, 2.19)	○	Garcia-Closas et al, 1998
		> 6.58 mg quercetin	< 2.50 mg quercetin	lung cancer	0.99 (0.44, 2.23)	○	
Uruguay	Case control study (111 cases, 444 controls)	highest quartile of quercetin intake	lowest quartile	esophagus cancer	0.47 (0.23, 0.93)	●	De Stefani et al, 2000

Table 9: Effect of dietary flavonoids on cancer incidence - epidemiological data.
Results are adjusted for dietary and non-dietary confounding factors, however methods of adjustment varied in different studies. [1]Total flavonoids = sum of kaempferol, quercetin, myricetin, isorhamnetin (flavonols), apigenin, luteolin (flavones), hesperetin, naringenin and eriodictyol (flavanones).[2]Sum of quercetin, kaempferol and myricetin. [3]Sum of quercetin, kaempferol, myricetin, luteolin and apigenin. [4]Sum of quercetin, kaempferol, myricetin and luteolin. [5]Sum of catechin, epigallocatechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate.
● significant result ○ not significant result.

Flavonoids and other diseases – epidemiological data

Country	Study population	Flavonoid intake/d	Compared with	Incident	Relative Risk (95%CI)	Reference
Finland	Finnish Mobile Clinic Health Examination Survey 10,054 subjects >15y 28 y follow up (1967-94)	>27 mg total [1] flavonols (4) flavones (2) flavanones (3)	< 4 mg	Rheumatoid arthritis	1.18 (0.62, 2.26) ○	Knekt et al, 2002
				Diabetes	0.98 (0.77, 1.24) ○	
				Cataract	1.36 (0.84, 2.21) ○	
				Asthma	0.65 (0.47, 0.90) ●	
		> 4 mg quercetin	< 1.5 mg	Rheumatoid arthritis	2.64 (1.30, 5.36) ○	
				Diabetes	0.81 (0.64, 1.02) ●	
				Cataract	0.94 (0.57, 1.56) ○	
				Asthma	0.76 (0.56, 1.01) ●	

Table 10: Effect of dietary flavonoids on asthma, cataract, diabetes and rheumatoid arthritis - epidemiological data.
Results are adjusted for dietary and non-dietary confounding factors. [1]Total flavonoids = sum of kaempferol, quercetin, myricetin, isorhamnetin (flavonols), apigenin, luteolin (flavones), hesperetin, naringenin and eriodictyol (flavanones).
● significant result ○ not significant result.

3. Experimental evidence: Biological activity of Quercetin

Epidemiological evidence suggests that dietary intake of quercetin rich foods may contribute to a better maintenance of physiologic function and to a lower prevalence of many degenerative conditions, such as heart disease and cancer. Understanding how dietary quercetin affects the pathogenesis of chronic disease may contribute to the development of alternative therapeutic treatments and new guidelines for dietary intake of flavonoids.

In this chapter three major areas of quercetin bioactivity are reviewed: 1) its antioxidant capacity, 2) its potential role in the prevention of atherosclerosis and heart disease, and 3) its impact on cancer pathogenesis. In addition to these bioactivities, a large number of other pharmacological properties have been ascribed to quercetin, such as antiviral, anti-microbial, anti-inflammatory and anti- or pro-estrogenic effects, which are reviewed elsewhere (Middleton et al, 2000; Ross and Kasum, 2002, Havsteen; 2002).

3.1. Antioxidant activity of quercetin

Free radicals are produced in the body as part of normal metabolism (**Table 11**) and mammalian organisms have developed a complex defence system (**Table 12**) to protect cellular components such as nucleic acids, lipids, carbohydrates and proteins against oxidative damage. Dietary antioxidants are "substances in foods that significantly inhibit adverse effects caused by reactive oxygen and nitrogen species in the body" (Dietary Reference Intakes, Institute of Medicine, Food and Nutrition Board, USA) and oxidative stress is defined as a shift of the fine physiological balance of pro-oxidants and antioxidants towards an excess of pro-oxidants, causing oxidative damage in tissues and cells (Sies et al, 1985). Oxidative damage appears to be involved in the aetiology of degenerative diseases, such as cataract, age related macular degeneration, cancer, diabetes mellitus, cardiovascular disease, several neuro-degenerative diseases and the aging process (Lindsay and Astley, 2002; Halliwell, 1994; Zoch, 1995). Therefore avoidance of oxidative stress may contribute to human health.

3.1.1. Direct and indirect antioxidant mechanisms of quercetin

Quercetin and other flavonoids can function as antioxidants due to their ability to stabilize unpaired electrons within their molecular structure after having donated one or

Pathways of free radical formation *in vivo*

Mitochondiral respiration
Enzymatic oxidation (e.g. by mono- and dioxigenases and cyclooxygenase)
Autoxidation (e.g. of catecholamines)
During immune defence by activated phagocytic cells (monocytes, neutrophiles, macrophages)
Drug metabolism (doxorubin, dithranol and paraquat)
Smoking
UV-irradiation
Environmental pollutants (SO_2, O_3, Al_3^+, Pb_2^+)
Progression of certain diseases (e.g. haemochromatosis)

Table 11: Pathways of free radical formation in vivo.
Data adapted from Babior and Woodman (1990) and Zoch (1995).

Antioxidant defence network

Endogenous	Exogenous
Enzymatic	L-ascorbate
catalase	tocopherols/tocotrienols
glutathione peroxidase	carotenoids
superoxide dismutase	dietary phenolics
Cellular	
glutathione	
α-lipoic acid	
ubiquinone	
uric acid	
Protein	
ceruloplasmin	
ferritin	
transferrin	

Table 12: Antioxidant defence network in mammalian organisms.
Data adapted from Milbury and Blumberg (2003).

more electrons to free radicals. Giving away an electron makes quercetin itself a reactive molecule (free radical), however, due to its aromatic structure the quercetin radical remains relatively stable via de-locating the unpaired electron within its π-electron system (Duthie et al, 2003). Three principal structural properties of quercetin contribute to its antioxidant activity: the O-dihydroxy (catechol) structure of ring B, the 2,3 double bond in conjugation with the 4-oxo group of ring C, and hydroxyl groups at position 3,5,7,3' and 4' (Bors et al, 1990). *In vitro*, quercetin can inactivate peroxyl radicals, alkyl radicals, superoxide, hydroxyl radicals, nitric oxide and peroxynitrite in aqueous and organic environments (Duthie and Crozier 2000). Flavonoids like quercetin and catechin can be more potent antioxidants than vitamin C and E (Gardener et al, 1997), however it

is unclear if flavonoids are equally effective in the human body (O'Reilly et al, 2000; O'Reilly et al, 2001).

Quercetin and other flavonoids can also act indirectly as antioxidants by forming a "redox inactive complex" with metal ions like $Cu^{2+}$and Fe^{3+}, thus preventing free radical generation through Fenton type reactions (Duthie et al, 2000; Ferrali et al, 1997; Zoch, 1995). Quercetin may also interact synergistically with the cellular antioxidant defence network by recycling, sparing or inducing other antioxidants, such as glutathione, α-tocopherol, glutathione-S-transferase, SOD and catalase (Molina et al, 2003; Milbury et al, 2002; Rimbach et al, 2001; Fiander and Schneider, 2000; Middleton et al, 2000; De Whalley et al, 1990). Synergy with other antioxidants implies additional mechanisms beyond stoichiometric quenching of free radicals or sparing of other antioxidants (Milbury et al, 2002), however, the nature of these mechanisms are unclear to date.

3.1.2. Pro-oxidant activity of quercetin ?

Quercetin was reported to induce the formation of free radicals in cell culture environments (Metodiewa et al, 1999; Dickancaite et al, 1998; Hodnick et al, 1986). If quercetin can form free radicals *in vivo*, it could act as pro-oxidant with potential detrimental effects on DNA integrity and carcinogenesis. Indeed, quercetin induced recombinational DNA mutations in cell culture environments (Caria et al, 1995; Rueff et al, 1986; Suzuki et al, 1991; Jurado et al, 1991), hence promoting carcinogenesis. In mammalian organisms however, dietary or supplemented quercetin appears in body tissues almost exclusively as glucuronated, sulfated and methylated quercetin metabolites. Metabolism can significantly alter biological effects of the parent compound, e.g. 3'-O-methylation of quercetin renders a non-mutagenic methyl-quercetin (isorhamnetin) (Zhu et al, 1994). Such metabolic transformation of quercetin *in vivo*, may explain why the vast majority of *in vivo* studies did not confirm the hypothesis that quercetin has carcinogenic properties (Walle et al, 2004).

In addition, quercetin concentrations required to induce mutations and cytotoxicity may not be physiologically achievable through dietary sources. It is a common misconception that if a little of something is good, then more is better. In cell culture experiments often very high doses of quercetin are used. Metabolizing enzymes in both cultured cells and mammalian organisms may be saturated by large doses, and un-metabolized free quercetin may cause unwanted adverse effects. Therefore, commercially available supplements containing mega doses of quercetin or other flavonoids may have biological activities that could adversely affect human health (Skibola et al, 2000) and

Antioxidant activity of quercetin *in vivo* and *ex vivo*

Observed effects	How was it measured	Reference
↑ resistance of LDL against oxidation	Length of lag time before LDL particle gets oxidised.	Chopra et al, 2000 Hayek et al, 1997
↑ plasma antioxidant capacity	ORAC (oxygen radical absorbance capacity) FRAP (ferric reducing antioxidant power) TEAC (trolox equivalent antioxidant capacity) TRAP (total radical trapping parameter)	Moon et al, 2001 McAnlis et al, 1999 Terao et al, 1999 Morand 1998
↓ lipid peroxidation	Malondialdehyde (MDA) concentration in urine, plasma or liver F_2 isoprostane concentration in urine or plasma	Pavanato et al., 2003 Su et al, 2003 Fremont et al, 1998 Rathi et al, 1984
↓ DNA damage	8-hydroxy-deoxy-guanine concentration in plasma and urine DNA strand breaks: Comet assay	Kim et al, 2003 Lodovici et al, 2000 Boyle et al, 2000 Lean et al, 1999
↓ Protein damage	Protein carbonyl concentration in liver and kidney	Kahraman et al, 2003 Funabiki et al, 1999

Table 13: Antioxidant activity of quercetin *in vivo*.
Estimates of the *in vivo* antioxidant activity after ingestion of quercetin as determined by biomarkers of oxidative stress.

quercetin supplements should not be taken by the public until toxicological issues are clarified.

3.1.3. Measuring the antioxidant activity of quercetin *in vivo*

The pro/anti-oxidant balance in human cells and tissues is difficult to analyse. Therefore the antioxidant capacity of dietary compounds is indirectly characterized by measuring biomarkers of oxidative stress in urine and plasma (summarized in **Table 13**).

LDL particles in plasma are susceptible to oxidation, and a common method to measure the efficacy of dietary antioxidants is to isolate plasma LDL and measure its resistance to oxidative stress *ex vivo*. Consumption of quercetin rich foods and quercetin supplements prolonged the resistance of LDL particles against oxidation (Chopra et al, 2000; Hayek et al, 1997). Oxidised, electronegative LDL has been linked to the development of arteriosclerosis with its clinical manifestations, myocardial and cerebral

infarction, therefore increased resistance of LDL to oxidation following consumption of dietary quercetin may slow the initiation and progression of arteriosclerosis.

The ability of blood plasma to withstand artificially generated oxidative stress *in vitro* is defined as plasma "antioxidant capacity" or "total antioxidant power". Using various assay methods, a number of authors reported an increase of plasma antioxidant capacity after the consumption of quercetin rich foods or quercetin supplements (Moon et al, 2001; McAnlis et al, 1999; Terao 1999; Morand 1998). It is generally assumed that an elevated plasma antioxidant capacity is desirable, however it may also reflect a physiological response to oxidative stress. Therefore caution is warranted and plasma antioxidant status alone is not sufficient for determining the antioxidant status on an individual (Prior and Cao, 1999).

Quercetin also affects biomarkers of lipid peroxidation, e.g. malondialdehyde (MDA) and F2-isoprostanes. F2-isoprostanes are free radical derived, cyclo-oxygenase independent, oxidation products of arachidonic acid (Natarajan et al, 1999). Plasma and urinary isoprostanes are currently regarded as the most specific measure of lipid peroxidation compared with MDA formation (Morrow and Roberts, 1996). Several studies have reported that ingestion of quercetin reduced the level of biomarkers of lipid peroxidation *in vitro* (Kahraman and Inal 2003; Pavanato et al, 2003).

3.1.4. Conclusion

Current evidence strongly suggests, but does not prove, that quercetin is a causative agent in maintaining human health via antioxidant mechanisms. Therefore, while quercetin may fit the definition of a dietary antioxidant, further work is necessary to determine its true physiological relevance and bio-availability.

3.2. Quercetin and cancer

3.2.1. Oxidative damage to DNA and lipids

Oxidative DNA damage is thought to play a significant role in the initiation and progression of cancer (Toyokuni et al, 1995; Feig et al, 1994; Ames, 1983). Cancer tissue contains higher concentrations of oxidised DNA than adjacent healthy tissue (Olinski et al, 1992). Consumption of dietary quercetin or quercetin supplements may reduce oxidative DNA damage and lipid peroxidation (Kim et al, 2003; Lodovici et al, 2000). Lipid peroxidation products like MDA can both damage and oxidise DNA. A pro-oxidant milieu in the cytosol also affects the expression of proteins involved in regulating

Putative anti-cancer mechanisms of quercetin

Observed effects	Proposed mechanism	Source
↓ tumour size, number and incidence in rats and mice	possibly a combination of all observed effects listed underneath	Khanduja et al, 1999 Elangovan et al, 1994 Ranelletti et al, 1992 Deschner et al, 1991 Verma et al, 1988 Mukhtar et al, 1988
↓ carcinogen formation	inhibition or activation of enzymes which metabolise carcinogens	Coughtrie et al, 1998
↓ oxidative DNA damage	decrease of mutation	Kim et al, 2003 Lodovici et al, 2000 Boyle et al, 2000 Lean et al, 1999 Kuo et al, 1996
↓ cell proliferation	↑ apoptosis of tumour cells reduced DNA synthesis in cancer cells inactivation or down regulation of pro-oxidant enzymes and signal transduction enzymes cell cycle arrest at G1 phase	Spencer et al, 2004 Shen et al, 2003 Plaumann et al, 1996 Uddin and Choudhry, 1995 Ranelletti et al, 1992
↓ metastasis	reduced ability of breast adeno-carcinoma cells to migrate on a collagen layer	Lu et al, 1996

Table 14: Putative anti-cancer mechanisms of quercetin.

cell proliferation and differentiation (Toyokuni et al, 1995) (e.g. MAP kinases and RTK signal transduction pathways) (Benhar et al, 2002; Chiarugi and Cirri, 2003). Dietary components that help maintain a healthy balance of pro and anti-oxidants are therefore potential anti-cancer agents.

3.2.2. Modulation of enzyme activities

Quercetin and other flavonoids have been suggested to affect carcinogenesis via modulation of the activity and expression of diverse enzyme systems, such as the P450 superfamily, phase II metabolising enzymes or quinone reductase (Duthie et al, 2000).

3.2.3. Apoptosis, malignant transformation and anti-proliferative effect

Quercetin may prevent or reverse carcinogenesis by inducing the apoptosis of transformed cells (Dong et al, 1997). The proposed mechanisms for this are the modulation of gene expression and the regulation of protein activities relevant for apoptosis (such as caspase 3, Mcl-1, NF-κB, AP-1, p53 and others) (Plaumann et al, 1996; Shen et al., 2003; Lee et al., 2003). Quercetin and other flavonoids inhibited malignant transformation in cell culture by 50% (Franke et al, 1998). It is remarkable that quercetin and other flavonoids appear to specifically inhibit cell growth of carcinoma cell lines, compared to cell growth of normal cell lines (Duthie et al, 2000; Kuntz et al, 1999).

3.2.4. Conclusion

Experimental data suggests that quercetin may play a role in cancer prevention, however more studies are required to confirm cancer preventive qualities of quercetin.

3.3. Quercetin and atherosclerosis

3.3.1. LDL oxidation

High plasma concentrations of LDL, particularly oxidised LDL are associated with increased development of arteriosclerosis and its clinical manifestations, myocardial and cerebral infarction (Holvoet et al, 1998; Holvoet et al, 1995). Proteins and polyunsaturated fatty acids in LDL particles are susceptible to free radical mediated oxidation. The consumption of dietary quercetin or quercetin supplements have reduced the susceptibility of LDL to oxidation *ex vivo* (Chopra et al, 2000; Hayek et al, 1997). This suggests that a diet high in quercetin may reduce the development and progression of atherosclerosis via its protective effects on LDL.

3.3.2. Plasma lipids

Apart from its protective effect on LDL, quercetin may also influence the homeostasis of plasma lipids. Reduced serum triglyceride levels were measured in humans and animals after ingestion of quercetin and other flavonoids (Hasumura et al, 2004; Arai et al, 2000; Kaku et al, 1999). The potential clinical significance of these observations is obvious.

Putative anti-atherosclerosis mechanisms of quercetin

Observed effects	Proposed mechanisms	Source
↓ LDL oxidation	antioxidant mechanisms	Chopra et al, 2000 De Whalley et al, 1990 Hayek et al, 1997
↓ plasma cholesterol and triglycerides	unknown	Hasumura et al, 2004 Arai et al, 2000 Kaku et al, 1999
↓ platelet aggregation and thrombus formation in vivo and in vitro	↓ cyclooxygenase: inhibition of arachidonic acid metabolism ↓ thromboxane formation antagonism to thromboxane receptors and other mechanisms.....	Middleton et al, 2000 Gryglewski et al, 1987
↓ atherosclerotic plaques	↓ LDL oxidation	Hayek et al, 1997
↓ inflammation	modulation of cytokines and prostanoid synthesis	Morikawa et al, 2003 Larrick and Wright, 1990
↓ adhesion molecule expression	↓ upregulation of cytokine induced genes	Kobuchi et al, 1999 Gerritsen et al, 1995
↓ blood pressure	↑ endothelial function enhancing nitric oxide release	Ibarra et al, 2003 Taubert et al, 2002

Table 15: Putative mechanisms of quercetin to help prevent atherosclerosis.

3.3.3. Platelet aggregation and thrombus formation

Platelets are key participants in thrombus formation, which can lead to acute myocardial or cerebral infarction. Quercetin and rutin may prevent the formation of platelet thrombi and have been shown to disperse pre-formed platelet thrombi at physiological plasma concentrations (under 0.05 μM) (Gryglewski et al, 1987). Thus, quercetin and other flavonoids may be potent blood "thinning" agents, similar to aspirin, reducing the incidence of infarction in older adults. Mechanisms for quercetin's inhibition of platelet adhesion to the endothelium and platelet aggregation may include its inhibition of arachidonic acid metabolism by cyclooxygenase, inhibition of thromboxane formation, and its antagonism to thromboxane receptors (Middleton et al, 2000). Consequently, flavonoids may be potentially useful anticoagulant drugs.

3.3.4. Inflammation and adhesion molecules

The development of inflammatory processes requires the activation of the vascular endothelium and the expression of adhesion molecules on the cellular surface. Circulating monocytes in plasma attach to adhesion molecules, roll along the vascular endothelium and migrate into the inflammatory site/intima (Aplin et al, 1998) where they transform into macrophages after ingesting a large number of oxidised LDL particles. The increased expression of adhesion molecules is the first morphological change in the development of atherosclerosis (Ross, 1995). Flavonoids, including quercetin can inhibit adhesion molecule expression (Gerritsen et al, 1995) and the adherence of leucocytes to the endothelium (Larrick and Wright, 1990). Thus flavonoids may have the potential to intercept atherogenesis at a very early stage.

3.3.5. Conclusion

A diet rich in quercetin may help reduce the progression of atherosclerosis via various antioxidant and non-antioxidant mechanisms, e.g. by affecting the plasma lipid homeostasis, elevating the resistance of LDL against oxidation and inhibiting blood clotting and thrombus formation.

4. Bioavailability – Key for biological relevance

4.1. The concept of bioavailability

The journey of dietary micronutrients from food into organs and tissues begins with their oral ingestion. Micronutrients are released from the food matrix by "food processing" in the mouth, stomach and intestine. Micronutrients must be presented to the enterocytes in a particular condition to ensure absorption. Absorption into the enterocytes may occur either by passive diffusion or by active transport mechanisms. Once uptaken into enterocytes, lipid soluble micronutrients are packaged into chylomicrones which are secreted into the lymph system, arrive in the blood stream via ductus thoracicus and are further distributed by the liver, responsible for lipid metabolism and distribution of lipid soluble micronutrients within the body. Enterocytes secrete water soluble micronutrients directly into portal blood. However, prior to their secretion, enterocytes metabolize some of these compounds into more "biocompatible molecules" (Stahl et al, 2002).

In pharmacology, the term 'bio-availability' is used to describe the "rate and extent of a drug to reach its site of action". The bioavailability of micronutrients in humans, however, can not be determined according to this concept as it is usually impossible to collect human tissues for this purpose. Therefore, micronutrient bioavailability is commonly estimated from plasma concentrations and has been re-defined as "the fraction of an oral dose (parent compound or active metabolite) that reaches the systemic circulation" (Lin et al, 1999; Schümann et al, 1997).

After its appearance in plasma, the micronutrient's journey through the body, however, continues and 1) it may be ubiquitously distributed throughout the entire organism, 2) it may be transported to specific organs and tissues, 3) it may remain for a different length of time in the blood stream before it is excreted (urine, bile), or 4) it may be sequestered by organs or tissues. Some micronutrients, e.g. folic acid, enter the enterohepatic circulation, where the compound is excreted via the bile into the intestine and subsequently re-absorbed to re-enter the systemic circulation. Therefore, the plasma concentration of a micronutrient, e.g. quercetin, does not sufficiently describe its true bioavailability *in vivo*.

The final mission of a bioactive nutrient is to arrive at the site of action in a bioactive form. The bioactive form can be its natural molecular structure as present in food, or its metabolites. At present we know little about the active forms of many micronutrients, including quercetin. It is important to note that micronutrients do not

always need to enter the systemic circulation to be bioactive (Walle 2004; Stahl et al, 2002). Unabsorbed antioxidant micronutrients may play an important role in limiting oxidative stress in the GI-tract, an important site for immune and inflammatory functions.

In summary, the bioavailability of flavonoids and other micronutrients depends on food processing, composition of the meal, digestion, presence of bile acids and metabolic transformation. Considering all these variables, databases of the micronutrient content in food only provide an estimate of the amount ingested, and the true availability of bioactive compounds at target tissues remains unclear for many micronutrients.

4.2. Methods to determine bioavailability

In humans, it is normally not possible to determine the micronutrient concentration in target tissues. Therefore, the micronutrient concentration or the concentration of its active metabolites is determined in accessible fluids or tissues and the concentration at target tissues is an estimate.

Many investigators regard peak plasma concentrations as a indicator of micronutrient absorption. These values however can not reflect the true absorption or bioavailability, as some compounds are absorbed gradually and have a low peak plasma concentration over a long period of time, whereas other compounds are absorbed rapidly with a high peak plasma concentration for only a very short time. The peak plasma concentration however, is useful to determine physiological micronutrient concentrations in human plasma and is important for the design of *in vitro* and cell culture studies.

The area under the plasma concentration time curve (AUC) is most commonly used as a tool to determine bioavailability after oral administration. For this approach it is essential to consider plasma clearance, e.g. the rate and extent of urinary and bilary excretion and the rate of exchange of the micronutrient between the plasma compartment and storage or target compartments. If either the plasma clearance or the tissues sequestration is unclear, the AUC calculation can not be used to determine micronutrient bioavailability.

In some studies bioavailability is determined by quantifying the urinary excretion of the ingested and absorbed compound. However, this method is only applicable for selected micronutrients that are exclusively excreted via urine, that are not metabolised and not retained in the body (Rechner et al, 2002). As quercetin is found almost exclusively in conjugated and oxidised (degraded) forms in urine, and since bilary excretion of flavonoids has been reported to be significant (Rechner et al, 2002), the

quantification of quercetin conjugates in urine is an insufficient method to determine quercetin bioavailability.

Radiolabelled micronutrients or stable isotopes have been the method of choice for bioavailability studies. The body-distribution of labelled compounds, reflects un-ambiguously the absorption of the ingested compound including its potentially unknown metabolites. Whole-body auto-radiography or timed sample collection can help to identify target tissues (Deprez and Scalbert, 1999). Due to the radiation hazard, radiolabelled micronutrients are usually administered only to animals, however, stable isotopes can be used in human intervention studies, although the detection of small quantities or unknown metabolites is easier if the compound under investigation is radiolabelled. In both cases the number and positioning of labelled atoms within the micronutrient molecule is crucial, as it determines the strength of the signal, and the metabolite fraction which can be identified after metabolic catabolism. In conclusion, by using labelled micronutrients, definite information on the absorption and body distribution can be obtained, especially for heavily metabolised compounds. However the synthesis of both stable isotopes and radiolabelled compounds, is expensive and time consuming and the radiation hazard can be problematic.

In summary, no common approach can be recommended for measuring the bioavailability of micronutrients. This is due to substantial differences in absorption mechanism, metabolism, body distribution and excretion pathways. Each micronutrient may require an individually tailored strategy for the correct determination of its bioavailability (Schümann et al, 1997). Many commonly used methods for the determination of bioavailability are likely to give incomplete results.

4.3. Absorption

4.3.1. How much is absorbed?

In recent years the absorption of dietary quercetin has been controversially discussed. Studies with ileostomy patients, where ileal fluid was analysed, suggested that over 50% of ingested quercetin was absorbed from a meal containing fried onion (Walle et al, 2000; Hollman et al, 1995). However, much less than 10% of ingested quercetin is found in plasma and urine, which suggests a much lower absorption (Goldberg et al, 2003; Graefe et al, 2001; Erlund et al, 2000; Manach et al, 1998). There are three possible explanations for the high disappearance of quercetin from the small intestine in the ileostomy studies and the low quercetin recovery in plasma, and urine: 1) only a

small amount of quercetin is absorbed, but whilst in the GI-tract most of the quercetin is metabolised into other compounds, 2) substantial quantities of quercetin are absorbed and rapidly metabolised by internal tissues, or 3) large amounts of quercetin are absorbed and rapidly sequestered by body tissues.

4.3.2. Factors affecting quercetin absorption

Pre-absorption events are important as they determine if and in what form a nutrient reaches the enterocytes where it may be absorbed. Quercetin glycosides, like other micronutrients in plants, need to be released mechanically from plant cells where flavonoids accumulate in the cell wall and cell vacuole (Markham et al, 2000). Differences in chewing time or the ingestion of puree and juices can therefore affect the level of quercetin release and its subsequent bioavailability.

Quercetin absorption seems to be greatly influenced by its solubility in the food matrix. Data from Azuma et al (2002) indicates that a combination of lipids and emulsifiers can elevate the absorption of quercetin aglycone in rats. This assumption is supported by findings from Cermak et al (2003) where quercetin absorption in pigs was increased when ingested with beef, suggesting that quercetin bioavailability depends on the composition of the meal.

Quercetin can react irreversibly with proteins (Walle et al, 2003; Zsila et al, 2002), however, reports on the bioavailability of tea flavonoids from black tea, consumed either with or without milk, are controversial (Kyle et al, 2003; Holiman et al 2001; Hertog et al, 1997; van het Hof et al, 1998).

The position and type of the sugar moiety attached to the quercetin molecule not only determines the speed and the site of absorption (as described in the next section), it also affects the total amount that is absorbed. The reason for the preferred absorption of particular quercetin glycosides (quercetin-4'-glucoside ≥ quercetin-3-glucoside > quercetin > rutin) remains to be determined. (Murota and Terao, 2003; Cermak et al, 2003; Morand et al, 2000; Day et al, 2003, Olthof et al, 2000). Also, whether the rate of quercetin absorption is dependent on the dose remains to be determined, as reports are controversial (Cermak et al, 2003; Erlund et al, 2000). In summary, the chemical structure, degree and postion of glycosylation and solubility in dietary matrix may determine the bioavailability of quercetin.

4.3.3. Mechanism of absorption

Three mechanisms for the absorption of quercetin and its glycosides have been proposed in recent years: active transport via SGLT-1 (Walgren et al, 2000; Gee et al, 1998), hydrolysis by lactase phloridzin hydrolase (LPH) or by colon micro flora (Day et al, 2000; Macdonald et al, 1983) followed by subsequent absorption of the released aglycone via passive diffusion into the enterocytes (Williamson et al, 2000; Day et al, 2003; Murota and Terao, 2003). The timing of the hydrolysis affects both bioavailability and pharmacokinetics, as quercetin from rutin (quercetin-3-β-O-rhamnosylglucoside) reaches its peak plasma concentrations at 6-8 h after ingestion presumably due to its hydrolysis by colonic micro-flora and subsequent absorption from the colon (Graefe et al, 2001; Hollman et al, 1999; Hollman et al, 1997; Griffiths and Barrow, 1972). Quercetin glycosides such as quercetin-4'-glucoside and quercetin-3-glucoside however appear to be absorbed from the upper GI-tract, as peak plasma levels are reached after 15-45 minutes (Cermak et al, 2003; Graefe et al, 2001; Hollman et al, 1999). It has been postulated that LPH, a β-glucosidase located on the outside of the enterocyte brush border (Day et al, 2000) releases quercetin from quercetin-4'-glucoside and quercetin-3-glucoside, and the aglycone is subsequently absorbed by passive diffusion (Williamson et al, 2000; Day et al, 2003). Only quercetin-4'-glucoside (not rutin and quercetin-3-glucoside) was transported into intestinal enterocytes with the involvement of the Na^+/glucose co-transporter SGLT-1. (Walgren et al, 2000; Gee et al, 1998; Day et al, 2003). A greater understanding of the absorption mechanism of quercetin and its dietary quercetin glycosides is essential to customise supplements or flavonoid rich meals to achieve maximal quercetin bioavailability.

4.4. Metabolism

4.4.1. Metabolism – the enemy of bioavailability?

The term metabolism embraces all processes that change the molecular structure of the parent compound. This may be degradation of its molecular structure or the addition of other molecules. Metabolic transformation may considerably change the function and activity of a compound, as they may become more water soluble, less toxic or more suitable for excretion (Coughtrie et al, 1998). Enzyme catalysed detoxification reactions are one of the primary defence mechanisms against toxic or xenobiotic compounds, and are classified into phase I and phase II metabolites. The cytochrome P-450 complex, the most important phase I metabolising enzyme family, primarily hydroxylates, de-

hydroxylates, or de-methylates compounds, generally to activate compounds for subsequent phase II metabolism.

Phase II metabolising enzymes are widely distributed among tissues, including liver, lung, intestine and kidney. Glucuronidation occurs on the luminal side of the endoplasmatic reticulum by uridine-5'-diphosphate-glucuronyl-transferases (UGTs), a large family of enzymes (Donovan and Waterhouse, 2003). Cytosolic phenol-sulfo-transferases (SULTs) transfer sulfate groups from 3'-phosphoadenosine-5'-phosphosulfate to phenolic compounds (Duffel et al, 2001). Catechol-O-methyltransferases (COMTs), also located in the cytosol, catalyse the S-adenosyl-L-methionine-dependent methyl group transfer to OH groups of flavonoid acceptor molecules (Willits et al, 2004). The addition of a sulfate or glucuronyl group results in metabolites that are negatively charged at physiological pH (Donovan and Waterhouse, 2003).

The amount of orally administered micronutrients or drugs to reach the systemic circulation can be reduced by both intestinal and hepatic metabolism, called first-pass or pre-systemic metabolism. It is believed that the liver is the major site for pre-systemic metabolism due to its size and high content of metabolising enzymes. However, in recent years the role and contribution of intestinal metabolism has been increasingly recognized (Lin et al, 1999). In the classic view, pre-systemic metabolism is regarded as mechanism of the body to limit the bioavailability of xenobiotic and potentially toxic compounds (Coughtrie et al, 1998). Recent studies indicate that all absorbed quercetin underwent phase II metabolism, raising the question whether phase II metabolism activates or inactivates absorbed quercetin. Therefore, knowledge about metabolism and the identification of quercetin metabolites is vital in the effort to explore mechanisms of bioactivity and ultimately the identification of active compounds.

4.4.2. Characterisation of quercetin metabolites in the body

Absorbed quercetin appears in plasma conjugated with glucuronide, sulfate and methyl moieties (Ueno et al, 1982). Plasma quercetin concentrations are therefore routinely measured after the release of free quercetin from its conjugates by enzymatic or acid hydrolysis. This indirectly proves the presence of phase II quercetin metabolites in plasma, even if individual quercetin metabolites were not characterised by LC-MS/MS analysis (Moon et al, 2000; Manach et al, 1999; Spencer et al, 1999). In recent years, several investigators have identified various quercetin metabolites and results are summarized in **Table 16.** A convincing number of studies report that no or very little free

quercetin is detected in human or animal plasma and phase II metabolites of quercetin are clearly the dominating form of absorbed quercetin *in vivo* (Crespy et al, 2003; Terao, 1999; Manach et al, 1999; Morand et al, 1998).

Absorbed forms of quercetin *in vivo*: quercetin metabolites

Quercetin metabolites	Sample	Administered form of quercetin	Reference
Quercetin-3'-sulphate Quercetin-3-mono-glucuronide Quercetin-4'-mono-glucuronide Quercetin-3'-mono-glucuronide 3'-Methyl-quercetin-3-mono-glucuronide 3'-Methyl-quercetin-4'-mono-glucuronide* Quercetin-di-glucuronides Sulfated quercetin mono-glucuronide	human plasma	onion	Day et al, 2001
Quercetin-3-mono-glucuronide Quercetin-4'-mono-glucuronide	rat plasma	quercetin	Moon et al, 2001
5 isoforms of quercetin mono-glucuronide, no sulfates	human plasma	food or herbal medicine	Witting et al, 2001

Table 16: Absorbed forms of quercetin *in vivo*: quercetin metabolites.
Positive identification of phase II metabolites of quercetin by LC-MS/MS analysis. The conjugation position of methyl, sulfate or glucuronide moieties were determined by ^{1}H NMR analysis or by comparison of the HPLC retention time with an authentic standard. * clear identification of this compound was not possible

4.4.3. First step in metabolising quercetin: hydrolysis of quercetin glycosides

While dietary anthocyanidins appear to be absorbed intact, e.g. complete with various sugar moieties that are attached to the anthocyanidin structure (Milbury et al, 2002; Rechner et al, 2002; Cao et al, 2002), most other flavonoids are extensively metabolised prior to their distribution throughout the body. The first step in metabolism is most likely de-glycosylation of quercetin glycosides (Walle, 2004; Day et al, 2003; Murota and Terao, 2003). Flavonoid glycosides don't seem to be hydrolysed in the acidic environment of the stomach (Crespy et al, 2002; Rechner et al, 2002; Hollman et al, 1999). However hydrolysis occurs either before, during or after absorption from the small intestine or colon (Aura et al, 2002; Williamson, 2000; Day et al, 2003; Walle et al, 2000). Indeed, recent studies clearly showed β-glycosidase activity (cytosolic β-

glycosidase and LPH which is attached to the luminal side of enterocytes) in the small intestinal epithelium and in cultured liver and intestinal cells (Day et al, 2000; Lambert et al, 1999; Ioku et al, 1998; Day et al, 1998).

Despite these findings, there has been considerable debate over whether or not quercetin glycosides are absorbed intact. Some reports claim the presence of intact quercetin glycosides in human plasma (Aziz et al, 1998; Paganga and Rice Evans, 1997; Hollman et al, 1997). Initially it was hypothesised that in these studies glucuronides were mistaken for glycosides due to their similar absorption spectrum and HPLC retention time. However, two studies positively identified small amounts of quercetin glycosides in plasma using MS technology (Oliveira et al, 2002; Mauri et al, 1999). While absorption of some quercetin glycosides may be possible, most studies report that no quercetin glycosides were found in plasma or intestinal mucosa (Day et al, 2003; Manach et al, 1998).

4.4.4. Phase I and II metabolism of quercetin

In analogy with the metabolism of drugs and other foreign compounds, flavonoids are metabolised by phase I and phase II metabolising enzymes. After the initial de-glycosylation step, free quercetin appears to be re-conjugated with glucuronide, methyl and sulfate moieties (Crespy et al, 1999; Vargas et al, 1997), presumably in the enterocytes directly after absorption (Crespy et al, 1999; Spencer et al, 1999). It has been shown that quercetin is a substrate for UDP-glucuronyl transferases (Oliveira and Watson, 2000), catechol-O-methyltransferases (De Santi et al, 2002; Okushio et al, 1999b) and phenol sulfotransferases which are present in human and animal small intestine and liver (Donovan et al, 2001; Kuhnle et al, 2000; Piskula et al, 1998).

Human and animal liver cytochrome P-450 mono-oxygenases can de-methylate and hydroxylate flavonols *in vitro* (Breinholt et al, 2002; Nielsen et al, 1998). To date however there is no *in vivo* evidence of quercetin metabolism by cytochrome P-450 enzymes (Walle, 2004) therefore, conjugation of quercetin with glucuronide, methyl and sulphate groups appears to be the most likely metabolic route (Walle 2004; Williamson et al, 2000).

Significant inter species differences have been reported for phase II metabolism. Sulfation appears to be a minor pathway in the rat small intestine, in humans however, the intestinal SULT activity is much higher then in rats (Dunn et al, 1998; Pacifici et al, 1988). Pigs can not form sulfate conjugates at all, and in cats glucuronidation is rare (Gibson and Skett, 1996). In comparison to humans, microflora in the rat stomach may

cause microbial degradation of compounds prior to their entry into the small intestine (Williamson et al, 2000). Therefore an informed choice of the model organism and cautious interpretation of the results are important.

4.4.5. Site of quercetin metabolism

The liver was always considered to be the chief organ for xenobiotic metabolism including flavonoid metabolism (Griffiths, 1982; Graefe et al, 1999; Williamson et al, 2000). The abundance and activity of the three main families of metabolising enzymes (cytochrome P-450 complex, UDP glucuronyl-transferases and sulfo-transferases) is superior in the liver in comparison to other tissues, e.g. kidney and intestinal mucosa (Lin et al, 1999). However metabolic transformation of flavonoids in the intestinal mucosa and enterocytes (Crespy et al, 1999) has been reported.

4.4.6. Quercetin catabolism

The extent of the absorption of quercetin and other dietary polyphenols from the small intestine appears to be relatively small (estimates range from <1% to 50%) (Erlund et al, 2000; Walle et al, 2000; Hollman et al, 1995). Therefore it is expected that the majority of the ingested quercetin reaches the large intestine either un-metabolised or in the form of quercetin conjugates. In humans, colon microflora holds a high degree of bio-catalytic power, with 10^{12} micro-organisms per g tissue, in comparison with 10^{5} in the small intestine and 10^{3} in the stomach (Frank, 1992). Colonic micro flora has enormous potential to transform flavonoids into lower molecular weight phenolics, which may be bio-active in the colon. Initially, the aglycone is released from quercetin glycosides or quercetin conjugates by microbial β-glycosidases, α-rhamnosidases and β-glucuronidases (Aura et al, 2002; Manach et al, 1995; Bokkenheuser et al, 1987; McDonald et al, 1983). Colon microflora can open the C ring of the flavonol structure via hydrolytic cleavage of the ether bond at the 1,2 position, to catabolise quercetin into phenolic acids (**Figure 4**) (Rechner et al, 2002; Justesen et al, 2000). Phenolic acids are derived from the B ring therefore the type of phenolic acid depends on the hydroxylation pattern of the B ring (Justesen et al, 2000). Phenolic acids derived from quercetin are phenyl-acetic and phenyl-propionic acids (**Table 17**). The major A ring product is phloroglucinol (1,3,5 trihydroxybenzene), which is thought to oxidise further forming butyrate and acetate (Pillai et al, 2002).

Colonic ring fission of quercetin

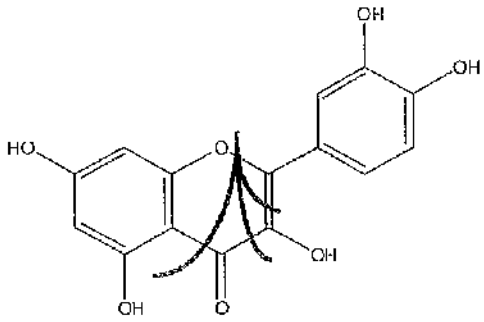


Figure 4: Ring cleavage positions in quercetin catabolism by colon microflora.
Colonic micro flora oxidises quercetin by opening the C ring. The figure shows the three possible types of ring fission. Adapted from Justesen et al,(2000).

Ring fission products of quercetin

colonic metabolites of quercetin	identified by
3,4-di-hydroxy-toluol	Justesen et al, 2000
3,4-di-hydroxy-benzaldehyde	Justesen et al, 2000 Schneider et al, 1999
3- or 4- hydroxy-phenyl-acetic acid	Aura et al, 2002 Justesen et al, 2000 Kim et al, 1998 Gross et al, 1996 Winter et al, 1989
3, 4-di-hydroxy-phenyl-acetic acid (homo-proto-catechuic-acid)	Aura et al, 2002 Justesen et al, 2000 Schneider et al, 1999 Kim et al, 1998 Gross et al, 1996 Winter et al, 1989
3- or 4- hydroxy-phenyl-propionic acid	Justesen et al, 2000

Table 17: Colonic ring fission products of quercetin.

It has been shown that phenolic acids, such as phenyl-propionic-acid are absorbed from the colon (Sawai et al, 1987; Jacobson et al, 1983), and may be further metabolised in the liver (Rechner et al, 2002). The plasma concentrations of phenolic acids can be up to 10 times higher than for conjugated quercetin metabolites (Rechner et al, 2002). It has

also been reported that these phenolic acids may have potential health beneficial effects *in vivo*, hence they might be in part responsible for the reported health beneficial effects of quercetin (Kim et al, 1998; Merfort et al, 1996).

4.5. Quercetin excretion

Absorbed flavonoids are quickly eliminated from plasma. Few studies are able to detect appreciable levels of quercetin in plasma 24 h after consumption. Three major mechanisms for the excretion of flavonoids including quercetin have been reported: 1) elimination by the kidneys via urine, 2) elimination by the liver in bile and 3) elimination by the small intestine by active efflux transport mechanisms. The unabsorbed (majority of) ingested quercetin is are excreted via faeces. Before excretion, the quercetin content in the large intestine is subject to ring fission and further metabolism by colonic micro flora.

4.5.1. Urinary excretion of quercetin

After consumption of foods containing quercetin glycosides, 1-10% of the dose is typically recovered in urine as phase II metabolites (Graefe et al, 2001; Hollman et al, 1997). Urine contains no or very little un-metabolised free quercetin (Rechner et al, 2002; Petrakis et al, 1959). Quantifying urinary quercetin metabolites with intact ring structure (e.g. phase II metabolites) cannot be considered a reliable indicator for absorption as 1) urinary excretion may be only a minor route of excretion and 2) ring fission products constitute a large proportion of the urinary metabolites. The presence of ring fission products in urine appears to be dependent on microbial action in the lower intestine, therefore their presence in urinary may be an indicator of gut micro flora status. If quercetin absorption is to be quantified by urinary excretion, both phase II metabolites and ring fission products of quercetin need to be taken into account.

4.5.2. Quercetin in bile and enterohepatic circulation

A major route of flavonoid and quercetin excretion may be the secretion of quercetin conjugates (particularly glucuronides) via the bile into the small intestine (Donovan and Waterhouse, 2003; Rozman et al, 1996). Excretion from liver into the bile depends partly upon molecular weight, and usually compounds above 350 kDa are filtered from the blood stream (Spencer et al, 2004). Once excreted with the bile into the intestine, quercetin conjugates may reach the colon where gut micro flora could release

the aglycone, which may be re-absorbed and participate in enterohepatic cycling (Scalbert and Williamson, 2000; Scheline, 1973).

4.5.3. Quercetin in faeces

Studies using radiolabelled quercetin have reported that after an oral dose of ^{14}C-labelled quercetin (1%) by rats, 45% of the administered radioactivity appeared in faeces and two thirds of the radiolabelled compounds in faeces were identified as unchanged quercetin (Ueno et al, 1983). Human volunteers also excreted radiolabelled compounds in faeces, both, after oral ingestion and i.v. administration of ^{14}C labelled quercetin, however in both cases faeces contained only 5% of the administered radioactive dose (Walle et al, 2001). Other studies report that quercetin or rutin was completely degraded by colonic microflora (Aura et al, 2002; Justesen et al, 2000). The rate of orally ingested quercetin which is excreted unchanged in faeces, may depend on the activity of colonic microflora and the ingested dose.

5. Aims of this thesis

The aims of this thesis were to address following questions:

A. What is the true bioavailability of dietary quercetin?

- Estimates of quercetin absorption in humans range from 1% to 50%. Which of these estimates are realistic?
- How is absorbed quercetin distributed within the body?
- Does quercetin accumulate in specific organs?

B. In what form is dietary quercetin bio-available?

- Does dietary quercetin-4'-glucoside appear in plasma unchanged?
- What metabolites are found in plasma?
- Could unknown metabolites explain controversial reports of quercetin absorption?
- Are different metabolites found in different organs?

C. What are the active forms of quercetin *in vivo*?

- Is dietary quercetin contributing to human health?
- What are the mechanisms?
- Can we formulate recommendations for dietary intake?

Section I:

Biological fate of [2-^{14}C]quercetin-4'-glucoside in the rat

Abstract

Background: Quercetin is a bioactive food compound whose intestinal absorption and subsequent biological fate are still controversial.

Objective: To investigate the absorption, body distribution and biotransformation of dietary quercetin using radiolabelled quercetin-4'-glucoside, a major flavonol glucoside in onions.

Design: Twelve rats received an oral dose of 3 mg radiolabelled quercetin-4'-glucoside and were sacrificed at 0.5, 1, 2 and 5 hours after the meal. The distribution of radioactivity within the whole body (brain, GI-tract, heart, kidneys, liver, lungs, muscle, plasma, red blood cells, spleen and testes) was determined at 0.5, 1, 2 and 5 hours after the meal. Radiolabelled compounds were extracted from plasma and tissues and identified using HPLC with online radioactivity detection and tandem mass spectrometry.

Results: Two hours after dosing all ingested quercetin-4'-glucoside had been metabolised. Over 85% of the ingested radioactivity was present in the GI-tract at all time points. Only extremely small amounts of radiolabelled metabolites of quercetin-4'-glucoside were detected in blood and internal organs, mainly in the liver and kidney. Over 95% of the absorbed quercetin was in the form of over 20 different methylated glucuronated and/or sulfated quercetin conjugates. The main metabolic end products, 5 h after ingestion of [2-^{14}C] quercetin-4'-glucoside, were quercetin di-glucuronides in liver, kidney and the GI-tract, and glucuronated sulfates of methylated quercetin in plasma.

Conclusions: About 10% of dietary quercetin-4'-glucoside was absorbed in rats. Quercetin occurs in the body almost exclusively as conjugates with the attachment of one or more methyl, sulfate or glucuronide groups. The main site of quercetin metabolism appears to be the GI-tract. Thus the metabolism of dietary quercetin may be primarily pre-systemic, e.g. a defence strategy. Any systemic biological effects previously attributed to free quercetin must be due to its metabolites.

Key words: [2-^{14}C]quercetin-4'-glucoside, absorption, body-distribution, metabolism, rat-tissues, HPLC-tandem mass spectrometry.

1. Background and Aim

The inferred benefits of dietary quercetin depend on its consumption, its intestinal absorption and subsequent distribution of active compound(s) throughout body tissues, where anti-carcinogenic and anti atherogenic effects may occur at a cellular level (Stahl et al, 2002). In recent years the absorption of dietary quercetin has been controversially discussed. Studies with ileostomy patients suggested that over 50% of ingested quercetin was absorbed from fried onion (Walle et al, 2000; Hollman et al, 1995), however the vast majority of studies report that < 10 % of the ingested quercetin is found in plasma or urine, suggesting a much lower absorption (Goldberg et al, 2003; Graefe et al, 2001; Erlund et al, 2000; Manach et al, 1998).

There are three possible explanations for the high disappearance of quercetin from the small intestine in ileostomy patients and a low quercetin recovery in plasma, and urine: 1) only a small amount of quercetin is absorbed, but whilst in the GI-tract most of the quercetin is metabolised into other compounds, 2) large amounts of quercetin are absorbed and rapidly metabolised by internal tissues, or 3) large amounts of quercetin are absorbed and rapidly sequestered by body tissues.

The focus of this study was to determine the dynamics of quercetin absorption, metabolite formation, distribution and accumulation in plasma and body tissues using radiolabelled quercetin-4'-glucoside, a quercetin glycoside naturally present in onion.

2. Materials and Methods

2.1. Materials

[2-^{14}C]quercetin-4'-O-β-D-glucoside (**Figure 1**) was synthesised by S.T. Caldwell and R. C. Hartley (Department of Chemistry, University of Glasgow, Glasgow G12 8QQ) using the previously reported method for the synthesis of [2-^{13}C]quercetin 4'-O-β-D-glucoside (Caldwell et al, 2000). The specific activity of [2-^{14}C]quercetin-4'-glucoside was 3.75 mCi/mmol. The standards quercetin and kaempferol were purchased from Sigma-Aldrich Co. Ltd. (Poole, Dorset, U.K.). Isorhamnetin was obtained from Apin Chemicals Ltd. (Abingldon, Oxon, U.K.). A sample of quercetin-3-glucuronide was kindly supplied by Prof. Gary Williamson (Food Research Institute, Norwich, UK.) Quercetin-4'-glucoside was generously provided by Dr. T. Tsushida (National Food Research Institute) Ibaraki, Japan. All other chemicals were of analytical grade and solvents were of HPLC grade purchased from Rathburn Chemicals Ltd. (Walkerburn, Peebleshire, U.K.).

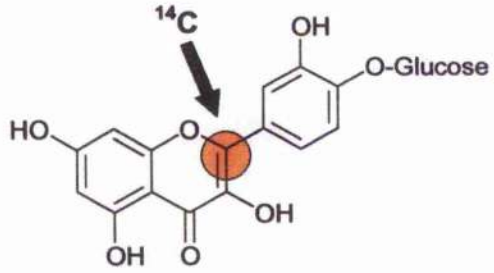


Figure 1: Radiolabelled [2-^{14}C]quercetin-4'-glucoside.
[2-^{14}C]quercetin-4'-glucosid was synthesised by S.T. Caldwell and R. Hartley, Department of Chemistry, University of Glasgow (specific activity 3.75 mCi / mmol).

2.2. Animals and oral administration of [2-^{14}C]quercetin-4'-glucoside

After an overnight fast, 12 male rats (Rowett Hooded Lister strain; mean weight 430 ± 4 g) were offered 1 g of stock rat feed (CRM, Special Diet Services, Witham, Essex, UK) containing 3.26 mg (7.6 mg/kg body weight) of [2-^{14}C]quercetin-4'-glucoside to give a radioactivity dose of 58.5 x 10^6 dpm. The rats consumed all of the ration within 2 min (as they were very hungry, after the overnight fast). The rat feeding and sample collection was carried out at the Rowett Research Institute by Phil Morrice and Dr. Garry G. Duthie.

2.3. Sample collection and preparation

After 0.5, 1, 2 and 5 h, three rats were terminally anaesthetised with isofluorane and blood was removed by cardiac puncture into heparinised evacuated tubes (Becton Dickinson, Oxford, U.K.). Plasma was obtained by centrifugation at 1000 g for 10 min at 4 °C. The pelleted red blood cells were re-suspended in 9 ml of phosphate buffered saline (pH 7.4). Livers were perfused *in situ* with chilled 0.15 M KCl and then removed along with brain, heart, kidney, lung, muscle (gastrocnaemius), spleen, and testes. The gastrointestinal tract (GI-tract) was removed intact including its contents. The sample collection was carried out at the Rowett Research Institute by Phil Morrice and Dr. Garry G. Duthie. All samples were immediately frozen in liquid nitrogen and stored at –80 °C. Upon arrival in Glasgow, all organs were freezedried and ground to a powder using mortar and pestle and again stored at –80 °C. The weights of the fresh and freezedried organs are summarized in the appendix (Appendix, Table 1).

2.4. Measurement of radioactivity

Aliquots of the freezedried and homogenized tissues (10 mg), whole GI-tract (50 mg) plasma (200µl) and red blood cell suspension (150µl) were digested in 0.5 ml Biosol tissue solubilizer (National Diagnostics, Hull, UK) for 3 h at 50 °C in a shaking water bath. With the exception of red blood cells, which were bleached using 1.75 ml of a 25% solution of sodium hypochlorite, the solublization treatment produced clear solutions. Aliquots of the solubilized tissue were added to scintillation fluid (Optiflow Safe One, Fisons, Longhborough, UK) and the radioactivity was determined using a Wallac 1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden). Representative samples of all analysed tissues were assayed for quench interference. The tissue-scintillation fluid mix was diluted six fold with additional scintillation fluid and the radioactivity was measured by liquid scintillation counting. When the measured radioactivity was equivalent in diluted samples and undiluted samples, it was assumed that quenching had not interfered with the measurement.

2.5. Extraction of metabolites from tissues, red blood cells and plasma

2.5.1. Method development

Aliquots of lyophilised and homogenised liver tissue (from liver samples collected at 0.5 h) were extracted under continuous shaking using different extraction solvents (**Figure 2**). After 30 min, the mixture was centrifuged at 2000 g for 20 min, the supernatant was collected and the pellet re-extracted with the same solvent two more times. The recovery of radiolabelled compounds was determined by the choice of the extraction solvent. 99% of the radioactivity, which was originally present in the tissue sample, went into solution when a 50% methanol/50% phosphate extraction buffer was used (**Figure 2**). The phosphate buffer was made up by adding a solution of 0.1 M K_2HPO_4 to a solution of 0.1 M KH_2PO_4 until a pH of 7.0 was obtained. The phosphate buffer was diluted by 50% with methanol and 20 mM sodium diethyldithiocarbamate was added as an antioxidant. To obtain a clear separation of the individual radiolabelled compounds by HPLC analysis the extracts were further purified as described in the next section.

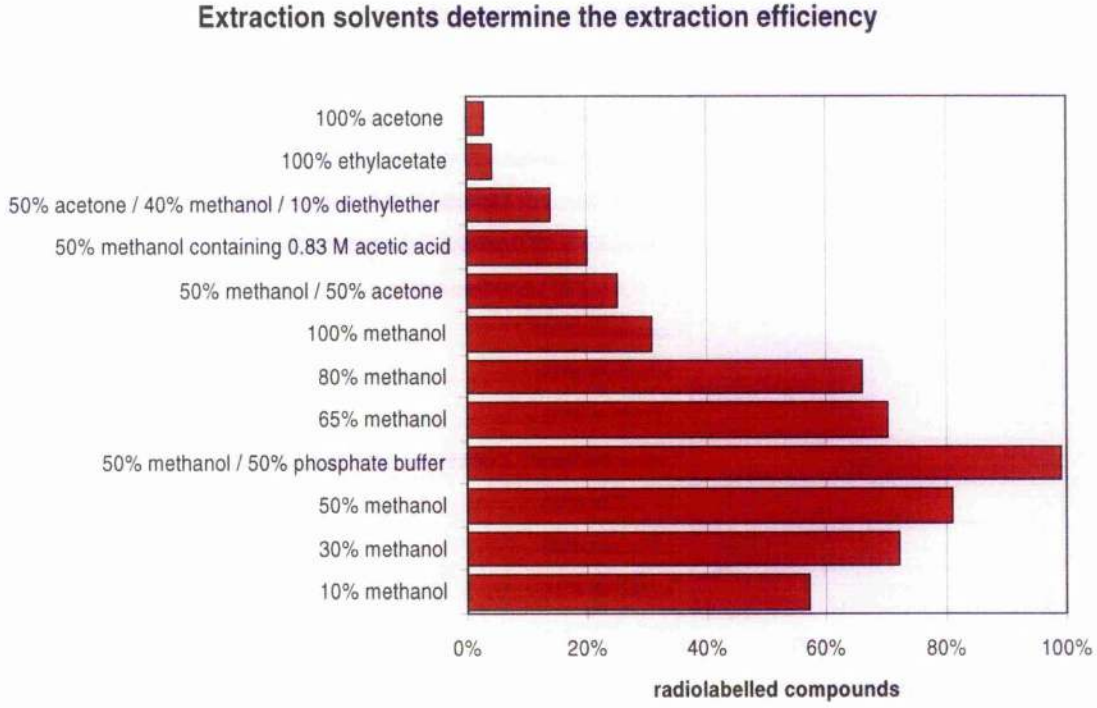


Figure 2: Extraction of radiolabelled compounds with different solvents.
Lyophilised and homogenised liver tissue was extracted with different extraction solvents under continuous shaking. After centrifugation, the supernatant was collected and the pellet was extracted with the same solvent for two more times. The graph shows the sum of the recovered radioactivity from three consecutive extractions.

2.5.2. Final tissue and plasma extraction method

Freezedried and homogenised tissues from rats sacrificed at the same timepoint were pooled, and 1 g aliquots were extracted (**Figure 3**) by continuous shaking with 15 ml of 50% methanol in 0.1 M phosphate buffer (pH 7.0) containing 20 mM sodium diethydithiocarbamate as an antioxidant. After 30 min, the mixture was centrifuged at 2000g for 20 min. The methanolic supernatant was decanted and the pellet re-extracted a further two times. The three methanolic supernatants were combined, and the methanol was removed *in vacuo*. The remaining aqueous phase was adjusted to pH 3 using 25% H_2SO_4 and partitioned three times with an equal volume of ethyl acetate.

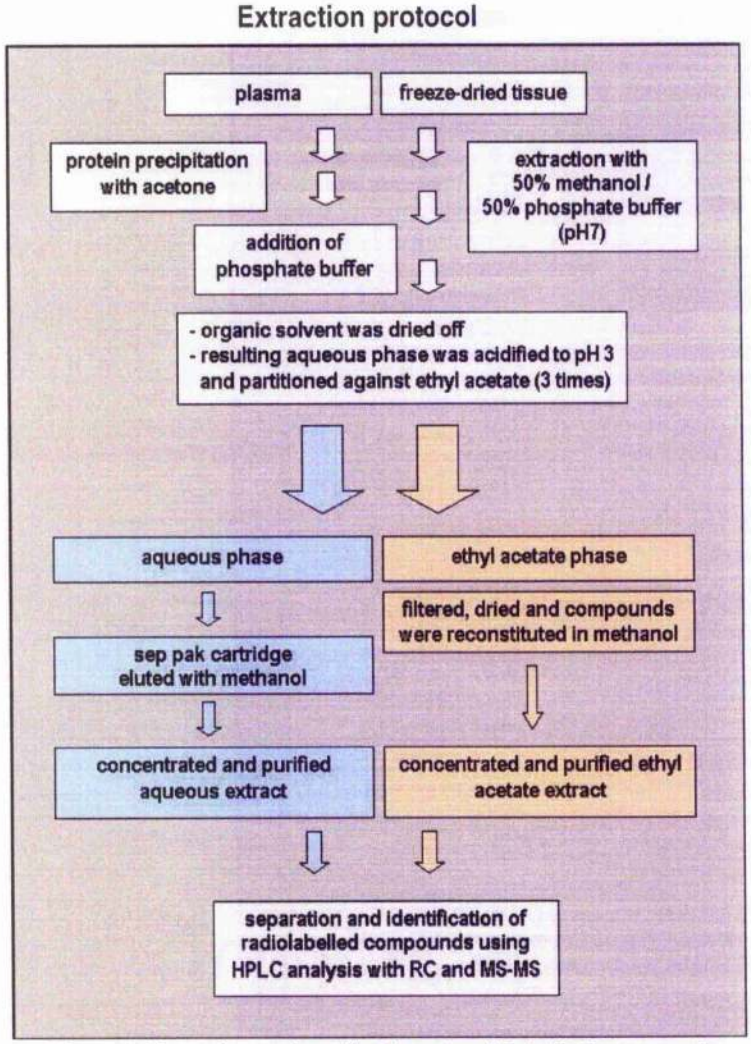


Figure 3: Extraction and purification of radiolabelled compounds from rat tissues and plasma. Schematic depiction of the extraction protocol.

Extraction efficiency in %:
Radiolabelled compounds extracted from rat tissues and plasma

organ	extraction				aqueous phase			ethyl acetate phase	extraction efficiency
	I	II	III	sum I - III	after drying organic solvent off	after partitioning	after elution from sep pak	after partitioning	final recovery
GI-tract	68 (±6)	19 (±3)	5 (±1)	92 (±10)	79 (±16)	14 (±10)	8 (±7)	56 (±10)	64 (±14)
liver	75 (±6)	16 (±4)	4 (±2)	94 (±11)	70 (±8)	43 (±3)	28 (±8)	19 (±11)	46 (±9)
kidney	84 (±7)	18 (±6)	4 (±3)	104 (±12)	94 (±11)	34 (±9)	18 (±4)	24 (±18)	42 (±20)
plasma	65 (±1[illegible])	0 (±1)	0 (±0)	66 (±11)	66 (±13)	51 (±14)	42 (±9)	6 (±4)	48(±9)
			average:	89 %					average: 50 %

Table 1: Extraction efficiency of radiolabelled compounds from rat tissue and plasma.
Quercetin and its metabolites were extracted from rat tissue (GI-tract, liver and kidney) and plasma collected at 0.5, 1, 2 and 5 h after oral ingestion of [2-^{14}C]quercetin-4'-glucoside. The table shows the extraction efficiency (recovery of radiolabelled compounds in the extracts) at various stages of the purification and concentration process (Figure 3). The extraction efficiency (in %) is calculated as the ratio: activity in the extract / initial activity in each sample x 100. All radioactivity measurements were carried out in duplicate, by adding a 50 µl aliquot of the extract to 5 ml scintillation fluid followed by liquid scintillation counting. Values in the table are the mean (±SD) of four extractions.

The ethyl acetate extracts were combined and reduced to dryness *in vacuo*, then re-suspended and stored in methanol at –80 °C. A gentle stream of nitrogen was used to remove residual ethyl acetate from the aqueous phase before it was loaded onto a 2 g C_{18} Sep Pak cartridge (Waters, Milford, Massachusetts, USA). The cartridge was washed with 15 ml of distilled water adjusted to pH 3.0 (with H_2SO_4) and the radiolabelled compounds were eluted with 30 ml of methanol. The methanolic eluent was reduced to dryness *in vacuo*, re-suspended in methanol, and stored at –80 °C.

To precipitate proteins, plasma was treated twice for 10 min with 2.5 volumes of acetone. After centrifugation the two acetone extracts were combined, 20 ml of 0.1 M phosphate buffer (pH 7.0) was added and *in vacuo* reduced to the aqueous phase. The ethyl acetate and aqueous extracts were obtained as described above. Over 89% of the radioactivity, which was originally present in the tissues/plasma, went into solution during the extraction process (**Table 1**). The concentrated purified extracts contained on average 50% of the radioactivity originally present in the tissues and plasma.

The concentrated ethyl acetate and aqueous extracts were analysed individually (extracts from organs collected at 1 and 5 h), or were combined before analysis (extracts from organs collected at 0.5 and 2 h). The samples were dissolved in the HPLC mobile phase and analysed as described below.

2.6. Identification of radiolabelled compounds by LC-MS/MS analysis

Aliquots of plasma and tissue extracts containing ca. 30,000 dpm of radioactivity, were analysed on a P4000 high performance liquid chromatograph (HPLC) fitted with an AS 3000 autosampler and with the initial detection by a UV6000 diode array absorbance monitor scanning from 250 to 700 nm (Thermo Finnigan, San Jose, CA, USA). Separation of the radiolabelled compounds was carried out using a 240 x 4.6 mm i.d. 4 µM Synergy RP-Max column (Phenomenex, Macclesfield, UK), eluted with a 60 min gradient of 5-40% acetonitrile in 1% formic acid at a flow rate of 1 ml/min at 40 °C. After passing through the flow cell of the UV absorbance monitor, the column eluate was split by 50%, and simultaneously directed to a radioactivity monitor (Reeve Analytical Model 9701, Lab Logic, Sheffield, UK), and to a Finnigan LCQ Duo tandem mass spectrometer with an electrospray interface in negative ion mode operating in full scan mode from 150 to 2000 amu. The parallel connection of the radioactivity monitor with the tandem mass spectrometer was set up by Mr. Bill Mullen, who also maintained the equipment and injected the samples. The radiolabelled compounds were identified by MS-MS analysis, comparison of HPLC retention times, the elution pattern, UV absorption values at 365 nm and their UV diode array spectra. The MS-MS data was analysed by Mr. Bill Mullen and Brigitte A. Graf using LCQ™ Deca XP (Revision 1.2) software for Xcalibur™ (Thermo Finnigan, San Jose, CA, USA).

2.7. Quantification of radiolabelled compounds

2.7.1. Initial peak quantification

Our MS/MS software package did not include integration software for peak quantification, therefore all radiolabelled compounds (=peaks) were quantified by measuring peak heights in HPLC-RC chromatograms (**Figure 6**).

The measured peak heights (in cm) were converted into 'y-axis-scale' units, considering the individual scales of the y-axis' of each chromatogram (**Table 2**, step 1, 2 and 3). The 'y-axis-scale' units were transformed into dpm (disintegrations per minute), a standard measure for radioactivity, based on the assumption that the sum of all peaks in one chromatogram, was identical with the injected radioactivity in dpm (**Table 2**, step 4). The radioactivity (in dpm) represents the number of molecules which contain a radiolabelled C atom, e.g. 1 µmol of [2-^{14}C]quercetin-4'-glucoside or any [2-^{14}C]metabolite has a radioactivity of 8.3 x 10^6 dpm.

The result of the quantification calculation at this stage (**Table 2**, step 4) is the actual quantity of each metabolite (in dpm which is convertible into μmol) in the analysed aliquot of the extract. To calculate the total amount of metabolites per whole organ (**Table 2**, step 5), the amount of radioactivity which was recovered in the final extract was considered, together with the weights of the freezedried and fresh organs (Appendix, Table 1).

The final quantification results (Appendix, Table 4) are the amounts of the individual metabolites per whole organ. On average 50% of the original radioactivity was present in the purified and concentrated extracts. However for every extracted organ the recovery rate varied slightly (**Table 1** and Appendix, Table 2). In order to compare the quantities of the individual metabolites between different organs, in Table 6, 8 and 9; Figure 7 the metabolite quantities are given as a percentage of the extracted and analysed radioactivity (radioactivity present in the purified concentrated extract ≈ 50% of the original radioactivity = analysed radioactivity = 100%). This way the results reflect accurately our data but remain comparable. We assume that the losses of radioactive compounds during the purification and concentration process were ubiquitous not specific, e.g. not selected compounds, but a small quantity of all compounds were lost equally in the purification process.

Five step metabolite quantification

Step 1: Measurement of peak height:
e.g. peak 5 in chromatogram obtained from liver 0.5 h after dosing:
peak 5 = 8.50 cm (on an A4 print out of the chromatogram)

Step 2: Conversion of peak height into 'y-axis-scale' units:
8.50 cm = 0.15758 'y-axis-scale' units / injection of 75,385 dpm

Step 3: Adjustment of injected radioactivity:
peak 5 = 0.15758 'y-axis-scale' units / injection of 75,385 dpm ⇒
peak 5 = 0.06303 'y-axis-scale' units / injection of 20,000 dpm

Step 4: Convert radioactivity units into dpm:
The sum of all peaks in this chromatogram = 0.54431 'y-axis-scale' units.
⇒ 0.54431 'y-axis-scale' units = 20,000 dpm
peak 5 = 0.06303 'y-axis-scale' units = 2,316 dpm

Step 5: Quantity of metabolites per whole organ:
The whole organ contained 1,126,615 dpm,
50% of the original activity was present in the purified concentrated extract.
100% = 1,126,615 dpm
50 % = 563,307.5 dpm
>
if the whole tissue would have been extracted (recovery 563,307.5 dpm),
peak 5 would have a quantity of 65,231 dpm

Table 2: Five step peak quantification.
All individual radiolabelled compounds (=peaks in chromatograms obtained with the radioactivity detector) were quantified by measuring their peak heights. Subsequently, the quantity of each metabolite per whole organ was calculated using a 5 step quantification method as shown above. The calculation is demonstrated for metabolite peak 5 detected in liver, 0.5 h after dosing. For the final peak quantification, an abbreviated quantification method was used as described underneath and a calculation example is shown in Table 3.

2.7.2. Abbreviated method for metabolite quantification

The peak heights were directly converted into metabolite quantity per whole organ, using a simple 3 step method, devised by Mr. Bill Mullen. The sum of the measured peak heights (in cm) was assumed to be 100% (**Table 3**, step II), and each peak was given a percentage value reflecting its quantity in the analysed sample. This percentage value was directly used to calculate the amount of the radiolabelled compound per whole organ (**Table 3**, step III), assuming that the extracted aliquot of freezedried tissue represented the metabolite spectrum in the whole organ. The abbreviated 3 step quantification method gave identical results as the above described 5 step method, hence the shorter method was used for the final peak quantification of all samples.

Three step metabolite quantification

Step I: **Measurement of peak height:**
e.g. peak 5 in chromatogram obtained from liver 0.5 h after dosing:
peak 5 = 8.50 cm (on an A4 print out of the chromatogram)

Step II: **Conversion of peak height into a percentage of the injected sample:**
Sum of all peak heights = 73.40 cm = 100%
peak 5 = 8.50 cm = 11.58 %

Step III: **Quantity of metabolites per whole organ:**
The whole organ contained 1,126,615 dpm,
50% of the original activity was present in the purified concentrated extracts.
100% = 1,126,615 dpm
50 % = 563,307.5 dpm
⇒
if the whole tissue would have been extracted (recovery 563,307.5 dpm),
peak 5 would have a quantity of 11.58% = 65,233 dpm

Table 3: Abbreviated method for metabolite quantification (by Bill Mullen).
All individual radiolabelled compounds (=peaks in chromatograms obtained with the radioactivity detector) were quantified by measuring their peak heights. Subsequently, the quantity of each metabolite per whole organ was calculated using the abbreviated three step quantification method (devised by Bill Mullen). The calculation is demonstrated for metabolite peak 5 detected in liver, 0.5 h after dosing.

3. Results

3.1. Distribution of radioactivity in body tissues and plasma

The distribution of the radioactivity in the rats was determined at 0.5, 1, 2 and 5 h after the meal. At all time points, 86%-93% of the ingested radioactivity was present in the GI-tract (**Figure 4**), which included the stomach, small and large intestine, and their contents. Thirty minutes after the meal, 7.2% of the ingested radioactivity was present in internal organs (liver, kidneys, heart, lungs, spleen, testes and brain) and blood (**Table 4**), indicating early absorption from the upper GI-tract. The absorbed radioactivity was distributed throughout all analysed organs, but the amounts present in the spleen, red blood cells and the brain were minute. Highest levels were detected in the plasma, liver and kidney with a peak concentration of 2.9% (14 µM), 1.9% and 1.0% of the ingested radioactivity at 0.5 hours (**Table 4**). The total radioactivity present in internal organs and plasma was highest at 0.5 h and decreased thereafter over the time course of the experiment, indicating no retention and quick excretion of radiolabelled compounds.

3.2. Separation and identification of radiolabelled metabolites

HPLC-RC analysis of methanolic extracts of the rat feed confirmed that the only radiolabelled compound present in rat feed was [2-^{14}C]quercetin-4'-glucoside, and no metabolites had formed prior to dosing (**Figure 5**). The HPLC-RC profiles obtained from tissue and plasma extracts (**Figure 6**) show a complex pattern of radiolabelled metabolites with 27 major peaks being detected. Using negative ion, electrospray, tandem mass spectrometry, 22 of the 27 detected metabolites were identified as quercetin conjugates with one or two glucuronyl, methyl or sulfate groups attached as detailed in **Table 5**.

3.2.1. Ingested [2-^{14}C]quercetin-4'-glucoside:

Peak 18 (retention time 33.87 min) was identified as the ingested [2-^{14}C]quercetin-4'-glucoside on the basis of its co-elution with an authentic standard and MS analysis. MS analysis detected a molecular ion (M^-) with a mass to charge ratio *(m/z)* of 463, which disintegrated into a fragment ion at *m/z* 301. Hence, a neutral molecule of the size of 162 atomic mass units (amu) was split from the molecular ion, corresponding to the

Body distribution of [2-^{14}C]quercetin-4'-glucoside or its metabolites

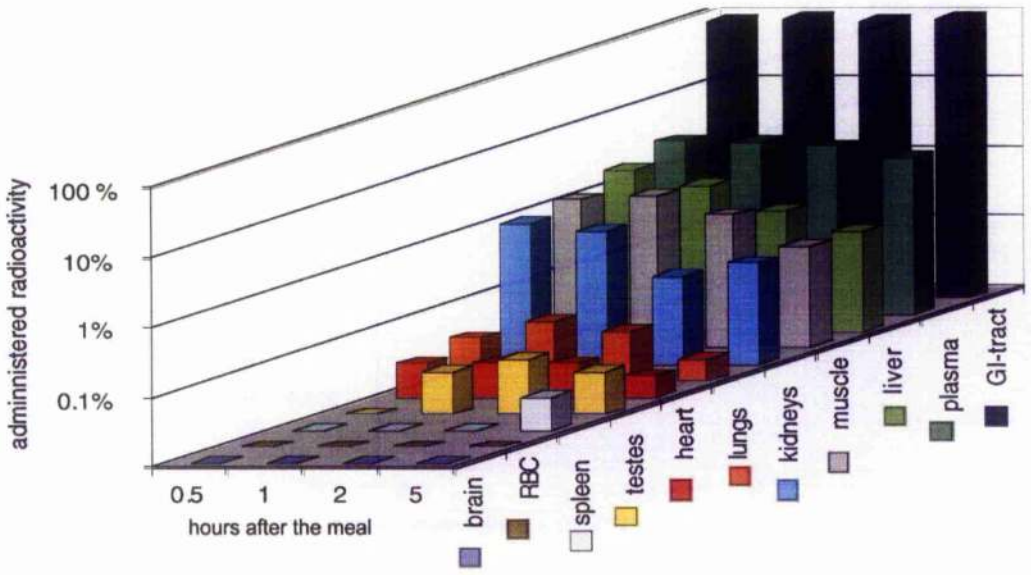


Figure 4: Body distribution of [2-^{14}C]quercetin 4'-glucoside or its metabolites in rats.
Graphical depiction of data given in Table 4 (underneath). The y-axis has a logarithmic scale.

Tissue/fluid	0.5 hours		1 hour		2 hours		5 hours	
plasma	1,696 ± 401	(2.90 %)	1,644 ± 157	(2.81 %)	1,460 ± 35	(2.50 %)	982 ± 149	(1.68 %)
Red blood cells	7 ± 2	(0.01 %)	4 ± 4	(0.01 %)	5 ± 2	(0.01 %)	5 ± 2	(0.01 %)
Kidneys	595 ± 346	(1.02 %)	468 ± 295	(0.80 %)	104 ± 12	(0.18 %)	163 ± 85	(0.28 %)
Liver	1,127 ± 623	(1.93 %)	684 ± 240	(1.17 %)	304 ± 78	(0.52 %)	155 ± 22	(0.27 %)
Spleen	5 ± 1	(0.01 %)	6 ± 1	(0.01 %)	5 ± 1	(0.01 %)	18 ± 30	(0.03 %)
Brain	0 ± 0	(0.00 %)	1 ± 1	(0.00 %)	1 ± 2	(0.00 %)	1 ± 2	(0.00 %)
lungs	25 ± 3	(0.04 %)	38 ± 10	(0.07 %)	27 ± 7	(0.05 %)	14 ± 3	(0.02 %)
Heart	15 ± 2	(0.03 %)	18 ± 2	(0.03 %)	16 ± 4	(0.03 %)	9 ± 3	(0.02 %)
Muscle	753 ± 289	(1.29 %)	839 ± 310	(1.43 %)	471 ± 76	(0.81 %)	130 ± 51	(0.22 %)
testes	7 ± 3	(0.01 %)	25 ± 3	(0.04 %)	33 ± 6	(0.06%)	24 ± 7	(0.04 %)
Sum	4,230 ± 1,376	(7.23 %)	3,727 ± 405	(6.37 %)	2,426 ± 193	(4.15 %)	1,501 ± 315	(2.57 %)
Gastro-intestinal-tract (including contents)	50,303 ± 4,460	(85.99 %)	54,530 ± 4,728	(93.21 %)	51,494 ± 8,091	(88.02 %)	53,870	(92.09 %)
Recovered radioactivity from rats	54,533 ± 5,569	(93.22 %)	58,257 ± 4,804	(99.58 %)	53,920 ± 8,143	(92.17 %)	55,371	(94.65 %)

Table 4: Body distribution of [2-^{14}C]quercetin 4'-glucoside or its metabolites in rats.
Distribution of radioactivity in rats 0.5, 1, 2, and 5 h after ingestion of [2-^{14}C]quercetin 4'-glucoside (58.5 x 10^6 dpm). Radioactivity was measured by liquid scintillation counting after treating tissues/plasma with tissue solublizer. Radioactivity is expressed as dpm x 10^3 per tissue/fluid per whole rat ± standard deviation (n = 3), and in parenthesis as a percentage of the administered radioactivity.

molecular weight of the glucosyl unit of [2-^{14}C]quercetin-4'-glucoside. The fragment ion at *m/z* 301 corresponds with the molecular weight of quercetin: 302 amu less 1 amu due to the ionisation process during the MS analysis. The specific activity of the [2-^{14}C]quercetin-4'-glucoside was 3.75 mCi/mmol, indicating that only about 8% of the quercetin-4'-glucoside molecules were radiolabelled, thus heavier by 2 amu. Therefore in the MS analysis, the major ions come from the "cold" quercetin-4'-glucoside not the radiolabelled quercetin-4'-glucoside, which would display a *m/z* value of 303 (molecular weight: 302 amu + 2 amu for the heavier C atom – 1 amu due to ionisation = *m/z* value of 303).

[2-^{14}C]quercetin-4'-glucoside was essentially present only in the GI-tract at 0.5 and 1 h after ingestion (**Table 6**). No intact [2-^{14}C]quercetin-4'-glucoside was detected in the kidney and plasma, however the liver contained a very small amount at 0.5 h.

3.2.2. Quercetin

Free quercetin (peak 24, retention time 43.73 min) was identified on the basis of its coelution with an authentic standard and MS analysis. A molecular ion (M^-) at *m/z* of 301 was detected and no fragments were formed. Quercetin was present in the GI-tract at all time points. No free quercetin was detected in plasma, although small amounts were present in liver and kidney at some time points.

3.2.3. Mono-glucuronides

Seven different radiolabelled compounds (peak 12, 15, 19, 20, 21, 22 and 23) were identified as glucuronide conjugates of quercetin and methyl-quercetin. Quercetin mono-glucuronides (peak 12, 15, 22 and 23) had a M^- at *m/z* 477 which fragmented into *m/z* of 301, indicating that a neutral molecule with a molecular weight of 176 amu had been attached to quercetin. According to its molecular weight, it was concluded that the attachment was a glucuronyl group. Peak 12 was identified as quercetin-3-glucuronide on the basis of its coelution with an authentic standard. To further confirm that a neutral loss of 176 resembled the cleavage of a glucuronyl group, tissue extracts were treated with β-glucuronidase, which would selectively split glucuronyl units from the main molecule. If the enzyme treatment would release free quercetin or methyl quercetin, (which would have been readily detectable using HPLC-RC) the 176 unit could have been only a glucuronyl group. Unfortunately, the hydrolysed samples were lost in the Bower Building fire, and it was not possible to repeat the experiment.

Peaks 19, 20 and 21 had a M^- at *m/z* 491 which fragmented into a *m/z* ion of 315.

The cleaved neutral molecule had a molecular weight of 176 amu indicating that a glucuronyl unit had been attached. From previous analysis of flavonol standards we know that isorhamnetin (3'-O-methyl-quercetin) and tamarixetin (4'-O-methyl-quercetin) has a M^- of *m/z* 315, therefore peaks 19, 20 and 21 were identified as glucuronides of methylated quercetin. The different retention times of the mono-glucuronide metabolites indicated different positioning of the methyl and/or glucuronide groups on the quercetin molecule, changing the polarity and the elution behaviour of that molecule.

Mono-glucuronides were present in all organs, but only at 0.5, 1 and 2 h after the ingestion of [2-^{14}C]quercetin-4'-glucoside. After 5 h mono-glucuronides were found only in the liver in relatively small amounts.

3.2.4. Di-glucuronides

Nine of the radiolabelled metabolites (peak 3, 5, 6, 7, 8, 9, 10, 11, 14) were identified as di-glucuronide conjugates of quercetin and methyl-quercetin. Quercetin di-glucuronides (peak 3, 6, 7, 9, 10 and 11) had a M^- at *m/z* 653 which fragmented to produce ions at *m/z* 477 and 301. Two neutral molecules have been cleaved from the M^-, both with a molecular weight of 176 amu, indicating that two glucuronyl have been attached to the quercetin molecule. Mullen et al, 2002 assumed that the two glucuronyl-units were attached at two different positions on the basis of previous results showing that disaccarides conjugated to anthocyanins fragmented with a loss of the intact disaccaride unit (Giusti et al, 1999). Peak 5, 8 and 14, were identified as methyl-quercetin with the attachment of two glucuronyl units, as they displayed a M^- at *m/z* 667 which fragmented to produce ions at *m/z* 491 and 315.

Di-glucuronides were the major group of metabolites in liver and kidney at all timepoints, and in the GI-tract at 5 h. In plasma the quantity of di-glucuronides was highest at 0.5 h and decreased thereafter.

3.2.5. Sulfates

Five different radiolabelled compounds contained a sulfate moiety. Peaks 13,16 and 17 were identified as glucuronated and sulfated quercetin or methyl-quercetin. With a M^- at *m/z* 557 which fragmented to produce ions at *m/z* 477, 381 and 301, peaks 13 and 17 were identified as conjugates of quercetin with a glucuronyl unit and a sulfate unit attached. A sulfate (SO_3) adduct is characterised by a neutral loss of 80 amu. The fragmentation pattern indicates that the glucuronyl unit and the sulfate unit are cleaved off separately (see Table I-3). Peak 16 had a M^- at *m/z* 571 which fragments at *m/z* 491,

395 and 315, revealing that it was a conjugate of methyl-quercetin with the attachment of a sulfate and a glucuronyl unit. Small amounts of glucuronated sulfates were present in liver, kidney and the GI-tract at most timepoints. In plasma, the quantity of glucuronated sulfates increased with time, and after 5 hours, 76% of all metabolites present in plasma were glucuronated sulfates. Peak 25 was a methyl-quercetin sulfate (M^- at *m/z* 395 which fragmented to *m/z* 315) and peak 26 was a quercetin sulphate (M^- at *m/z* 381 which fragmented to *m/z* 301). Both of these sulphated metabolites were found only in the intestine at 0.5, 1 and 2 h.

3.2.6. Un-identified metabolites

Metabolite peaks 1, 2 and 4 were not identified, as they did not yield recognisable mass spectra. All three metabolites had relatively short HPLC retention times indicating that they are relatively polar molecules. Metabolite peak 4 appears in small quantities in intestine, liver and kidney. Metabolites 1 and 2 appear only in the intestine 5 h after ingestion.

3.3. Metabolite spectra in tissues and plasma

3.3.1. GI-tract

At 0.5 h half of the total ingested dose was metabolised (**Table 4 and 8**). At this early stage, mono-glucuronides, di-glucuronides and sulfated quercetin and/or methyl-quercetin conjugates were detected in the GI-tract together with free quercetin and its methylated derivative isorhamnetin. At one and two hours after the meal, mono-glucuronides formed a major portion of the metabolites. Five hours after the meal the mono-glucuronides had disappeared and di-glucuronides formed the major class of metabolites.

3.3.2. Plasma

Neither quercetin nor the ingested quercetin-4'-glucoside were detected in plasma at any time point. In plasma, the amount of di-glucuronides decreased over the time course of the experiment and mono-glucuronides were present only at 0.5 and 1 h after the meal. However, the amount of glucuronidated sulfates increased steadily and 5 h after the meal glucuronated sulfates form the major group of metabolites in plasma in the form of peak 13, a sulfated quercetin glucuronide, and peak 16 a methylated, sulfated quercetin glucuronide. Peak 16 was the predominant metabolite in plasma at 5 hours,

representing 62% of the radioactivity in plasma at that time point.

3.3.3. Liver and kidney

In both liver and kidney the most abundant group of metabolites were di-glucuronides followed by mono-glucuronides and sulfated glucuronides. The amount of di-glucuronides increased steadily over the time of the experiment. Small amounts of free quercetin were detected in the liver, and in kidney. In the liver a small amount of quercetin-4'-glucoside was present at 0.5 h.

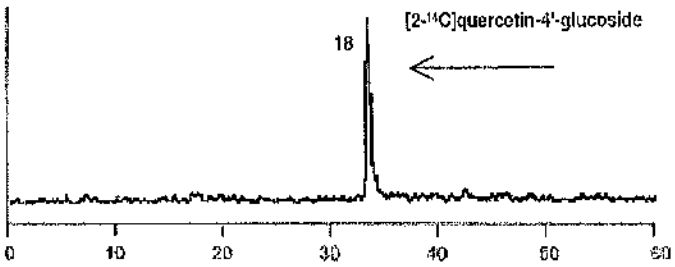


Figure 5: HPLC-RC trace of rat feed containing [2-^{14}C]quercetin-4'-glucoside.
The rat feed was extracted and analysed by gradient, reverse phase HPLC-RC to confirm that [2-^{14}C]quercetin-4'-glucoside was stable in the food matrix and that no metabolites had formed prior to dosing.

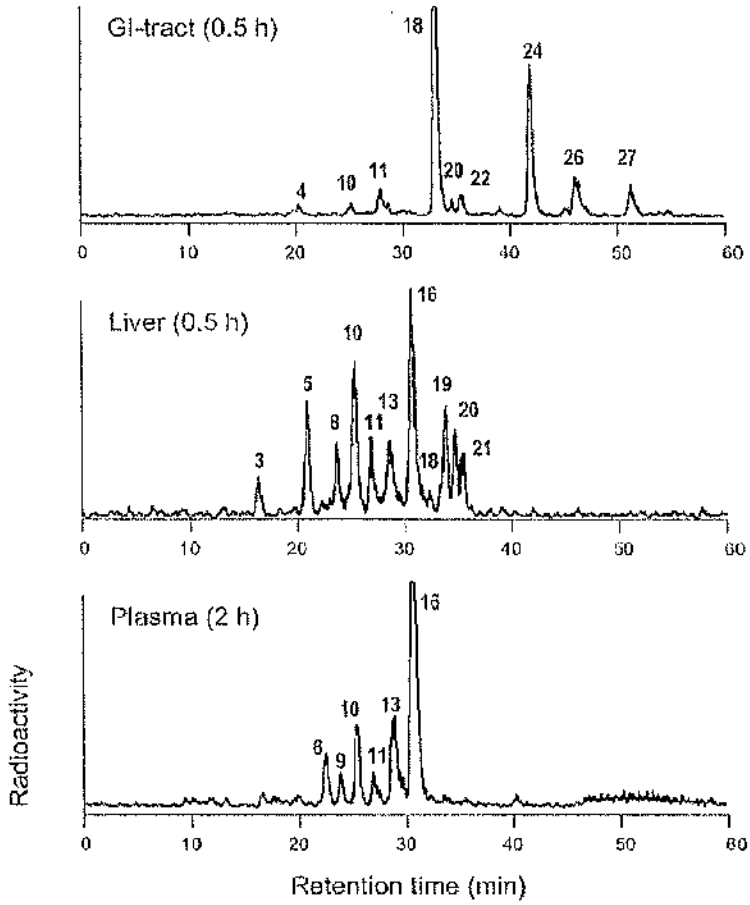


Figure 6: HPLC-RC traces of plasma and tissue extracts after oral ingestion of [2-^{14}C]quercetin 4'-glucoside by rats.
Extracts were analysed by gradient, reverse phase HPLC-MS/MS with online radioactivity detection. The above chromatograms show radiolabelled compounds in the GI-tract and liver at 0.5 h and in plasma at 2 h after ingestion of [2-^{14}C]quercetin 4'-glucoside by rats. HPLC retention times and MS-MS data on these and other metabolite peaks are given in Table 5.

Identification of radiolabelled compounds by tandem mass spectrometry

peak	RT (min)	compound	$[M-H]^-$ (m/z)	MS^2 fragment ions (m/z)		
1	9.30	Not identified				
2	12.04	Not identified				
3	17.22	Quercetin di-glucuronide	653	477 ([M-H]$^-$-GlcUA)	301 ([M-H]$^-$-GlcUA -GlcUA)	
4	20.05	Not identified				
5	21.32	Methylated quercetin di-glucuronide	667	491 ([M-H]$^-$-GlcUA)	315 ([M-H]$^-$-GlcUA -GlcUA)	
6	23.10	Quercetin di-glucuronide	653	477 ([M-H]$^-$-GlcUA)	301 ([M-H]$^-$-GlcUA -GlcUA)	
7	24.24	Quercetin di-glucuronide	653	477 ([M-H]$^-$-GlcUA)	301 ([M-H]$^-$-GlcUA -GlcUA)	
8	24.40	Methylated quercetin di-glucuronide	667	491 ([M-H]$^-$-GlcUA)	315 ([M-H]$^-$-GlcUA -GlcUA)	
9	24.92	Quercetin di-glucuronide	653	477 ([M-H]$^-$-GlcUA)	301 ([M-H]$^-$-GlcUA -GlcUA)	
10	26.27	Quercetin di-glucuronide	653	477 ([M-H]$^-$-GlcUA)	301 ([M-H]$^-$-GlcUA -GlcUA)	
11	27.95	Quercetin di-glucuronide	653	477 ([M-H]$^-$-GlcUA)	301 ([M-H]$^-$-GlcUA -GlcUA)	
12	29.38	Quercetin-3-glucuronide	477	301 ([M-H]$^-$-GlcUA)		
13	29.54	Quercetin mono-glucuronide sulfate	557	477 ([M-H]$^-$-SO_3)	381 ([M-H]$^-$-GlcUA)	301 ([M-H]$^-$-SO_3-GlcUA)
14	30.26	Methylated quercetin diglucuronide	667	491 ([M-H]$^-$-GlcUA)	315 ([M-H]$^-$-GlcUA -GlcUA)	
15	31.12	Quercetin mono-glucuronide	477	301 ([M-H]$^-$-GlcUA)		
16	31.77	Methylated quercetin mono-glucuronide sulfate	571	491 ([M-H]$^-$-SO_3)	395 ([M-H]$^-$-GlcUA)	315 ([M-H]$^-$-SO_3-GlcUA)
17	33.16	Quercetin mono-glucuronide sulfate	557	477 ([M-H]$^-$-SO_3)	381 ([M-H]$^-$-GlcUA)	301 ([M-H]$^-$-SO_3-GlcUA)
18	33.87	Quercetin-4'-glucoside	463	301 ([M-H]$^-$-Glc)		
19	35.01	Methylated quercetin mono-glucuronide	491	315 ([M-H]$^-$-GlcUA)		
20	35.89	Methylated quercetin mono-glucuronide	491	315 ([M-H]$^-$-GlcUA)		
21	36.70	Methylated quercetin mono-glucuronide	491	315 ([M-H]$^-$-GlcUA)		
22	36.74	Quercetin mono-glucuronide	477	301 ([M-H]$^-$-GlcUA)		
23	38.70	Quercetin mono-glucuronide	477	301 ([M-H]$^-$-GlcUA)		
24	43.73	Free quercetin	301			
25	50.36	Methylated quercetin sulfate	395	315 ([M-H]$^-$-SO_3)		
26	50.56	Quercetin sulfate	381	301 ([M-H]$^-$-SO_3)		
27	52.38	Isorhamnetin (methylated quercetin)				

Table 5: Identification of metabolites of [2-^{14}C]quercetin-4'-glucoside isolated from rat plasma and tissues. Extracts were analysed by gradient, reverse phase HPLC with online radioactivity detection and paralell MS/MS detection. The above table shows HPLC retention times, negative ion MS-MS fragmentation patterns and identification of metabolites isolated from rat tissues and plasma after oral ingestion of [2-^{14}C]quercetin 4'-glucoside. Peak numbers refer to the order of elution from the column. (RT = retention time, $[M-H]^-$ = molecular ion, m/z = mass to charge ratio, GlcUA = glucuronyl unit, Glc = glucosyl unit). The identity of quercetin-3-glucuronide (peak 12) was confirmed by an authentic standard kindly provided by Prof. Gary Williamson. Examples of typical MS trances of quercetin metabolites are shown in Appendix Figure 1.

Relative quantity of radiolabelled metabolites in rat tissues and plasma

peak	Compound	0.5 hours				1 hour				2 hours				5 hours			
		intestine	plasma	liver	kidneys	intestine	plasma	liver	kidneys	intestine	plasma	liver	kidneys	intestine	plasma	liver	kidneys
1	Not identified	-	-	-	-	-	-	-	-	-	-	-	-	10.6	-	-	-
2	Not identified	-	-	-	-	-	-	-	-	-	-	-	-	10.8	-	-	-
3	Quercetin di-glucuronide	-	-	3.8	6.3	0.8	-	6.4	7.4	-	-	-	25.4	-	-	35.5	40.3
4	Not identified	1.8	-	-	3.4	0.8	-	-	4.5	3.0	-	-	-	-	-	4.7	3.4
5	Methylated quercetin di-glucuronide	-	-	11.6	15.7	1.3	-	13.0	17.0	5.5	-	4.8	15.3	-	-	-	-
6	Quercetin di-glucuronide	-	1.2	-	3.1	0.2	6.4	-	-	2.9	9.7	3.1	-	2.4	7.8	42.5	17.4
7	Quercetin di-glucuronide	-	4.4	-	-	-	-	-	-	-	-	-	-	-	3.2	-	-
8	Methylated quercetin di-glucuronide	-	-	6.8	12.1	0.6	-	-	12.5	7.2	-	-	3.6	2.1	-	-	2.5
9	Quercetin di-glucuronide	-	6.2	-	5.4	-	3.7	-		-	5.4	6.8	16.6	-	3.2	-	-
10	Quercetin di-glucuronide	2.2	30.0	15.0	1.3	2.5	20.5	33.5	11.7	14.9	14.9	29.9	19.1	66.7	2.1	8.4	33.7
11	Quercetin di-glucuronide	4.5	10.6	7.1	5.9	0.6	8.2	4.0	8.4	3.6	5.7	7.7	3.4	-	1.8	-	-
12	Quercetin-3-glucuronide	-	9.2	-	5.9	10.6	2.1	6.9	7.9	8.6	-	-	-	-	-	-	-
13	Quercetin glucuronide sulfate	-	1.6	6.8	-	6.9	13.8	3.0	-	7.0	16.8	8.3	-	1.2	14.1	-	-
14	Methylated quercetin di-glucuronide	-	-	-	-	-	-	-	-	-	-	-	-	-	5.7	-	-
15	Quercetin mono-glucuronide	-	16.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	Methylated quercetin glucuronide sulfate	-	9.4	22.5	2.3	0.7	43.6	12.8	3.1	7.1	47.5	20.1	-	-	62.2	-	-
17	Quercetin glucuronide sulfate		3.3	-	-	-	-	-	-	-	-	-	-	-	-	1.7	1.0
18	Quercetin-4'-glucoside	49.0	-	1.8	-	26.2	-	-	-	-	-	-	-	-	-	-	-
19	Methylated quercetin mono-glucuronide	-	1.1	10.5	9.1	6.5	0.5	13.4	7.7	7.2	-	8.3	7.8	-	-	2.9	-
20	Methylated quercetin mono-glucuronide	2.5	2.1	8.3	22.4	4.9	0.4	5.6	16.8	7.0	-	3.9	8.9	-	-	-	-
21	Methylated quercetin mono-glucuronide	-	2.6	5.9	7.0	12.2	0.8	-	3.1	9.2	-	7.1	-	-	-	-	-
22	Quercetin mono glucuronide	3.3	-	-	-			-		-	-	-	-	-	-	-	-
23	Quercetin mono-glucuronide	-	2.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	Free quercetin	25.1	-	-	-	7.2	-	1.5	-	6.7	-	-	-	6.1	-	4.3	1.8
25	Methylated quercetin sulfate	-	-	-	-	17.9	-	-	-	-	-	-	-	-	-	-	-
26	Quercetin sulfate	6.5		-	-		-	-	-	9.1	-	-	-	-	-	-	-
27	Isorhamnetin	5.1		-	-	-	-	-	-	1.0	-	-	-	-	-	-	-

Table 6: Relative quantity of radiolabelled metabolites in rat plasma and tissues after oral ingestion of [2-^{14}C]quercetin-4'-glucoside.
Radiolabelled metabolites were extracted from the GI-tract, plasma, liver and kidney, and identified using HPLC-RC-MS. The quantity of each metabolite is expressed as a percentage of the total recovered radioactivity from each organ (given in Table 4).

Four types of quercetin conjugates

Di-glucuronides (9)	**Di-glucuronides** (6 isoforms)	Quercetin	+ Glucuronic acid + Glucuronic acid
	Methylated Di-glucuronides (3 isoforms)	Quercetin	+ Methyl group + Glucuronic acid + Glucuronic acid
Mono-glucuronides (7)	**Mono-glucuronides** (4 isoforms)	Quercetin	+ Glucuronic acid
	Methylated mono-glucuronides (3 isoforms)	Quercetin	+ Methyl group + Glucuronic acid
Glucuronated sulfates (3)	**Glucuronated sulfates** (2 isoforms)	Quercetin	+ Sulfate group + Glucuronic acid
	Methylated glucuronated sulfate (1 isoform)	Quercetin	+ Methyl group + Sulfate group + Glucuronic acid
Sulfates (2)	**Sulfate** (1 isoform)	Quercetin	+ Sulfate group
	Methylated sulfate (1 isoform)	Quercetin	+ Methyl group + Sulfate group

Table 7: Four major types of quercetin conjugates found in rat tissues and plasma.
Using HPLC with on line radioactivity detection and tandem mass spectrometry, 22 different quercetin conjugates were detected (Table 5) which were classified into four major groups of quercetin conjugates (methyl-quercetin, peak 27 not included). The number of individual metabolites belonging to each group is given in parenthesis.

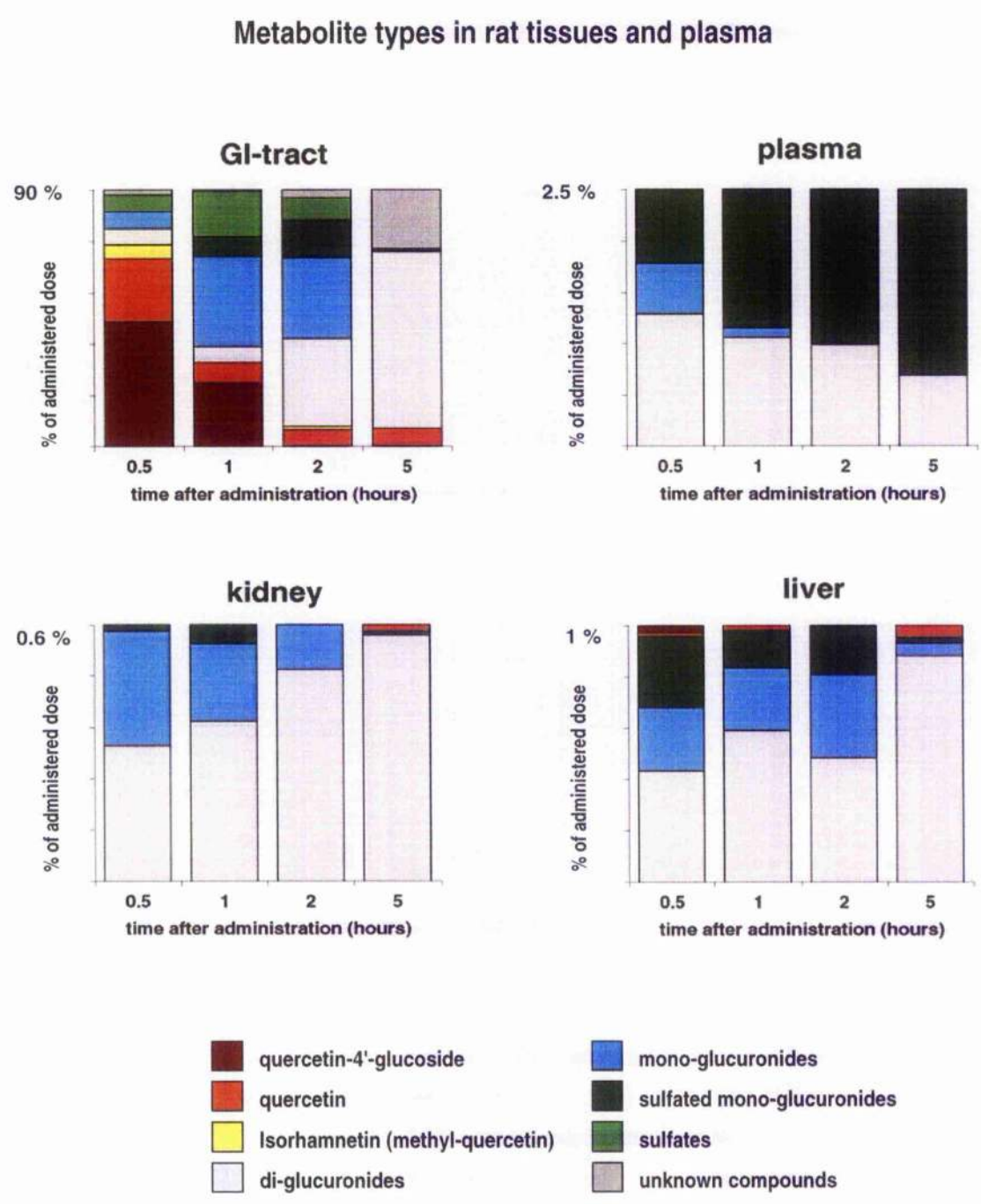


Figure 7: Metabolite types found in rat GI-tract, plasma, liver and kidney. Using HPLC-RC with MS/MS, 27 different radiolabelled compounds were detected in rat tissues and plasma after oral ingestion of [2-^{14}C]quercetin-4'-glucoside and classified into 8 major groups (Table 7). The relative quantities of each metabolite group are shown in relation to the administered dose.

Abundance of different metabolite types in rat tissue and plasma

	% of ingested dose				% of tissue content			
	GI-tract				GI-tract			
Types of metabolites	0.5 h	1 h	2 h	5 h	0.5 h	1 h	2 h	5 h
Quercetin-4'-glucoside	42.2	24.4	-	-	49.0	26.2	-	-
Quercetin	21.6	6.7	5.9	5.6	25.1	7.2	6.7	6.1
Isorhamnetin	4.4	-	0.8	-	5.1	-	1.0	-
Di-glucuronides	5.7	5.6	30.0	65.6	6.7	6.0	34.1	71.2
Mono-glucuronides	5.1	31.9	28.2	-	5.9	34.2	32.1	-
Glucuronated sulfates	-	7.2	12.4	1.1	-	7.7	14.1	1.2
Sulfates	5.6	16.7	8.0	-	6.5	17.9	9.1	-
unknown	1.5	0.8	2.6	19.8	1.8	0.8	3.0	21.5
sum	86.1	93.3	87.9	92.1	100	100	100	100

	plasma				plasma			
Types of metabolites	0.5 h	1 h	2 h	5 h	0.5 h	1 h	2 h	5 h
Quercetin-4'-glucoside	-	-	-	-	-	-	-	-
Quercetin	-	-	-	-	-	-	-	-
Isorhamnetin	-	-	-	-	-	-	-	-
Di-glucuronides	1.5	1.1	0.9	0.4	52.3	38.8	35.6	23.7
Mono-glucuronides	1.0	0.1	-	-	33.3	3.8	-	-
Glucuronated sulfates	0.4	1.6	1.6	1.3	14.4	57.4	64.4	76.3
Sulfates	-	-	-	-	-	-	-	-
unknown	-	-	-	-	-	-	-	-
sum	2.9	2.8	2.5	1.7	100	100	100	100

	liver				liver			
Types of metabolites	0.5 h	1 h	2 h	5 h	0.5 h	1 h	2 h	5 h
Quercetin-4'-glucoside	-	-	-	-	1.8	-	-	-
Quercetin	-	trace	-	trace		1.5	-	4.3
Isorhamnetin	-	-	-	-	-	-	-	-
Di-glucuronides	0.9	0.7	0.3	0.2	44.3	56.9	52.3	86.5
Mono-glucuronides	0.5	0.3	0.1	trace	24.7	25.8	19.3	2.9
Glucuronated sulfates	0.6	0.2	0.2	trace	29.3	15.8	28.4	1.7
Sulfates	-	-	-	-	-	-	-	-
unknown	-	-	-	trace	-	-	-	4.7
sum	2.0	1.2	0.6	0.2	100	100	100	100

	kidneys				kidneys			
Types of metabolites	0.5 h	1 h	2 h	5 h	0.5 h	1 h	2 h	5 h
Quercetin-4'-glucoside	-	-	-	-	-	-	-	-
Quercetin	-	-	-	trace		-	-	1.8
Isorhamnetin	-	-	-	-	-	-	-	-
Di-glucuronides	0.5	0.5	0.2	0.3	49.8	56.9	83.1	93.9
Mono-glucuronides	0.5	0.3	trace	-	44.5	35.5	16.6	-
Glucuronated sulfates	trace	trace	-	trace	2.3	3.1	-	1.0
Sulfates	-	-	-	-	-	-	-	-
unknown	trace	trace	-	trace	3.4	4.5	-	3.4
sum	1.0	0.80	0.2	0.3	100	100	100	100

Table 8: Abundance of different metabolite types in rat tissues and plasma.
This table shows the large difference between the amounts of metabolites present in the GI-tract and the other organs, including plasma. Using HPLC-MS-RC, 27 different radiolabelled compounds were detected in rat tissues and plasma after oral ingestion of [2-^{14}C]quercetin-4'-glucoside (Table 5). The radiolabelled compounds were classified into 8 major groups (Table 7). The quantity of each group of metabolites is given as a percentage of the ingested dose (left column) and as a percentage of the radioactivity in the respective tissues and plasma (right column).

Methylated quercetin metabolites in rat tissue and plasma

	% of ingested dose				% of tissue content			
	GI-tract				GI-tract			
Types of metabolites	0.5 h	1 h	2 h	5 h	0.5 h	1 h	2 h	5 h
Methylated metabolites	6.6	13.1	38.9	1.9	7.6	14.1	44.2	2.1
Un-methylated metabolites	35.7	55.0	46.6	70.4	41.6	59.0	52.9	76.5
Unchanged or unknown *	43.7	25.2	2.6	19.8	51.0	27.0	3.0	21.5
sum	86.0	93.3	88.1	92.1	100	100	100	100
	plasma				plasma			
Types of metabolites	0.5 h	1 h	2 h	5 h	0.5 h	1 h	2 h	5 h
Methylated metabolites	0.4	1.3	1.2	1.1	15.2	45.3	47.5	67.8
Un-methylated metabolites	2.5	1.5	1.3	0.5	84.8	54.7	52.5	32.2
Unchanged or unknown *	-	-	-	-	-	-	-	-
sum	2.9	2.8	2.5	1.6	100	100	100	100
	liver				liver			
Types of metabolites	0.5 h	1 h	2 h	5 h	0.5 h	1 h	2 h	5 h
Methylated metabolites	1.3	0.5	0.2	trace	65.5	44.8	44.2	2.9
Un-methylated metabolites	0.6	0.7	0.3	0.3	32.7	55.2	55.8	92.4
Unchanged or unknown *	trace	-	-	trace	1.8	-	-	4.7
sum	1.9	1.2	0.5	0.3	100	100	100	100
	kidneys				kidneys			
Types of metabolites	0.5 h	1 h	2 h	5 h	0.5 h	1 h	2 h	5 h
Methylated metabolites	0.7	0.5	0.1	trace	68.7	57.0	35.5	2.5
Un-methylated metabolites	0.3	0.3	0.1	0.3	28.0	38.5	64.5	94.1
Unchanged or unknown *	trace	trace	-	trace	3.4	4.5	-	3.4
sum	1.0	0.8	0.2	0.3	100	100	100	100

Table 9: Abundance of methylated quercetin metabolites in rat tissues and plasma.
Radiolabelled compounds from rat tissue and plasma were extracted and 27 different compounds were detected using HPLC-RC-MS/MS (Table 5). The data shown in Table 8 was assembled without considering the methylation status of the metabolites. Therefore, the table above shows the abundance of methylated quercetin metabolites as a percentage of the administered dose (left column) and as a percentage of the total radioactivity in tissues and plasma (right column). The 27 radiolabelled compounds were grouped into methylated metabolites (8 metabolites) and un-methylated metabolites (15 metabolites) and *unchanged or unknown compounds (quercetin-4'-glucoside and 3 un-identified compounds).

4. Discussion

4.1. Absorption: less then 10%

The administered dose of 3 mg [2-^{14}C]quercetin-4'-glucoside to rats corresponds with the quercetin-4'-glucoside content in 250 g fresh onion consumed by a 70 kg human subject (Crozier et al, 2000; Tsushida et al, 1995). About 10% of the administered radioactivity was absorbed into internal organs or blood (peak plasma concentration: 14 µM at 0.5 h). This is in keeping with human and animal studies which report low peak plasma concentrations of quercetin conjugates, suggesting a low absorption of quercetin or its metabolites from supplements or quercetin rich foods (Graefe et al, 2001; Erlund et al, 2000; Hollman et al, 1997). In contrast, two studies with ileostomy patients (Walle et al, 2000; Hollman et al, 1995) found that over 50% of the ingested quercetin (in fried onion) disappeared between oral ingestion and ileostomy effluent collection. As quercetin proved to be relatively stable in ileal fluid (only 5% degraded), it was concluded that the 'missing quercetin' must have been absorbed (Hollman et al, 1995). Our data show that up to 44% of the quercetin metabolites in the GI-tract of the rats were methylated (**Table 9**). The methylation of quercetin, presumably to isorhamnetin, or tamarixetin, and its further conjugation with glucuonic acid/sulfate groups has been described in a number of human and animal studies (DuPont et al, 2002; Manach et al, 1999; Manach et al, 1997). The ileal fluid however was only analysed for the presence of quercetin or quercetin conjugates, not for the presence of methylated quercetin or its conjugates (Walle et al, 2000; Hollman et al, 1995). The metabolism of quercetin into methylated quercetin and its conjugates might therefore provide an explanation for the high disappearance of quercetin in the ileostomy model (Walle et al, 2000; Hollman et al, 1995) and the absorption of quercetin might be substantially lower then suggested by these two studies.

High absorption of quercetin has also been reported by Walle et al (2001), on the basis of a study where human volunteers ingested [^{14}C]quercetin aglycone. The absorption of radiolabelled quercetin was estimated to be 36–53% of the ingested dose (100 mg) by calculating the "area under the curve" of the total radioactivity present in plasma over 72 h. During this period 4.2 % of the orally ingested radioactivity was excreted in urine, while 52% was exhaled as $^{14}CO_2$. It was assumed that the exhaled $^{14}CO_2$ originated from the portion of [^{14}C]quercetin which was not absorbed from the

small intestine but underwent bacterial degradation in the colon (Ueno et al, 1983; Petrakis et al, 1959). About 70% of the CO_2 in venous blood is usually transported in the form of HCO_3^- ions which are dissolved in plasma (Rehner and Daniel, 1999). Therefore, radioactivity detected in plasma by Walle et al (2001) is likely to include $^{14}CO_2$, en route to the lungs for exhalation, together with other radiolabelled products of intermediary metabolism derived from hydroxy-phenylacetic acids (Aura et al, 2002) and related ring fission products of [^{14}C]quercetin. Consequently, we believe that quercetin absorption of 36-53%, based on the "area under the curve" calculation of plasma radioactivity by Walle et al (2001) was an overestimate.

4.2. Site of metabolite formation: the GI-tract ?

The catalytic activity of drug metabolising enzymes in the small intestine are generally lower then the corresponding values in the liver (Lin et al, 1999). Therefore, it is usually assumed that the liver is the major site for metabolism of xenobiotics such as quercetin (Griffiths, 1982; Hackett, 1986). However, from our results we conclude that most of the metabolites must have been formed in the GI-tract. This conclusion is based on following observations: 1) all ingested radiolabelled quercetin-4'-glucoside had undergone metabolic changes within 2 h, and 2) at all timepoints most of the radiolabelled compounds were present in the GI-tract (86%-93% of administered dose) (**Table 4** and **Table 6**). If the liver were the main site of quercetin metabolism, and the metabolites were immediately excreted into the intestine via the bile, the liver should contain higher levels of radioactivity, at least at one time point. In practice, the liver contained no more than 2% of the ingested radioactivity at any time point (**Figure 8**).

Our hypothesis is supported by Crespy et al, (2003) who perfused rat intestine *in situ* with quercetin and collected intestinal eluent and bile separately. Bile contained only 10% of the quercetin conjugates, intestinal eluent contained 90% of the quercetin conjugates, indicating that the majority of the metabolism had occurred in the gut not in the liver. Preliminary data by Cermak et al. (2003) supports our conclusion, as it was reported that portal blood of pigs contained exclusively quercetin metabolites, and that quercetin was metabolically transformed before reaching the liver.

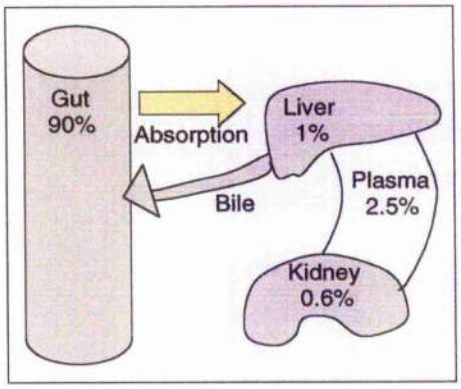


Figure 8: Is the GI-tract the main site of quercetin metabolism *in vivo*?

Although the primary function of the small intestine is to absorb nutrients and water, it also has the ability to metabolise food constituents and drugs by numerous pathways (Suzuki et al, 2000). From our results and other reports we conclude that most of the ingested dietary quercetin is metabolised directly in the GI-tract. Thus gastrointestinal quercetin metabolism may be a pre-systemic mechanism to reduce the bioavailability of quercetin, e.g. a defence strategy (Schuelz and Schinkel, 1999).

4.3. Quercetin metabolism by internal tissues

In agreement with previous reports (O'Leary et al, 2003; Donovan et al, 2001; Day et al, 2000; Manach et al, 1999) our data suggests that internal tissues were capable of quercetin metabolism.

Glucuronidation: At 0.5 h, the GI-tract contained two quercetin di-glucuronides (peak 10 and 11) whereas kidneys contained seven different di-glucuronides (peak 3, 5, 6, 8, 9, 10 and 11) and liver and plasma contained both five different di-glucuronides (liver: peak 3, 5, 8, 10 and 11, plasma: peak 6, 7, 9, 10 and 11) (**Table 6**). This indicates that internal tissues, most likely the liver and kidney, may further modify absorbed quercetin mono-glucuronides.

Methylation: Methylated forms of different types of quercetin metabolites (mono-glucuronides, di-glucuronides, glucuronated sulfates, sulfates) were predominant in liver and kidney at 0.5 h, whereas the GI-tract contained only a small proportion of methylated metabolites at this timepoint (**Table 9**). This either indicates selective absorption and/or transport of methylated metabolites into liver and kidney tissues or post-absorptive

methylation as previously suggested by a number of studies (De Santi C et al, 2001, Piskula and Terao 1998; Zhu et al, 1994).

In conclusion, our data suggests that internal tissues can metabolise quercetin. However, *in vivo*, quercetin metabolism in the liver and kidney may only play a minor role since only small amounts of the ingested dose appears to be absorbed.

4.4. Types and isoforms of quercetin metabolites

In most previous studies the presence of quercetin metabolites in human and animal plasma has been shown indirectly, by releasing free quercetin and methyl-quercetin from glucuronyl and sulfate moieties via enzymatic or acid hydrolysis before HPLC analysis (Manach et al, 1999; Spencer et al, 1999; Crespy et al, 1999; Manach et al, 1998). In this study the advantage of radiolabelled metabolites, good compound separation on the HPLC column, and on line radioactivity detection in parallel with tandem mass spectrometry (HPLC-RC-MS/MS) allowed us to characterize 22 different types and isoforms of glucuronated, sulfated and/or methylated quercetin conjugates: Seven different quercetin mono-glucuronides and nine different quercetin di-glucuronides have been detected together with three glucuronated sulfates, two quercetin sulfates and isorhamnetin (methylated quercetin) (**Table 6 and 7**).

It has been previously reported that glucuronidation of quercetin may occur at different and multiple hydroxyl groups within the quercetin molecule (Day et al, 2000). Quercetin conjugates and isomers have identical molecular mass and fragmentation patterns on mass spectrometry, therefore positional isomers could only be distinguished by their different elution times from the HPLC column (e.g. column retention time).

Assuming that all 5 OH groups on the quercetin molecule are possible binding positions for glucuronyl units, and OH groups at 3' and 4' position are possible binding positions for methyl groups, 8 isoforms of methylated quercetin mono-glucuronide and 4 isoforms of quercetin mono-glucuronide could theoretically occur (**Figure 9**). In agreement with our findings, two other laboratories have recently detected 3 methylated quercetin mono-glucuronides and 4 un-methylated quercetin mono-glucuronides which were formed in liver cell free extracts and by HepG2 cells (Day et al, 2000; O'Leary et al, 2003). In addition Oliveira et al, (2002) also reported the formation of 4 un-methylated quercetin mono-glucuronides by rat hepatocytes in suspension. The fact that all four laboratories report the same number of isoforms despite the use of different

Binding sites and theoretical number of isomers of quercetin conjugates

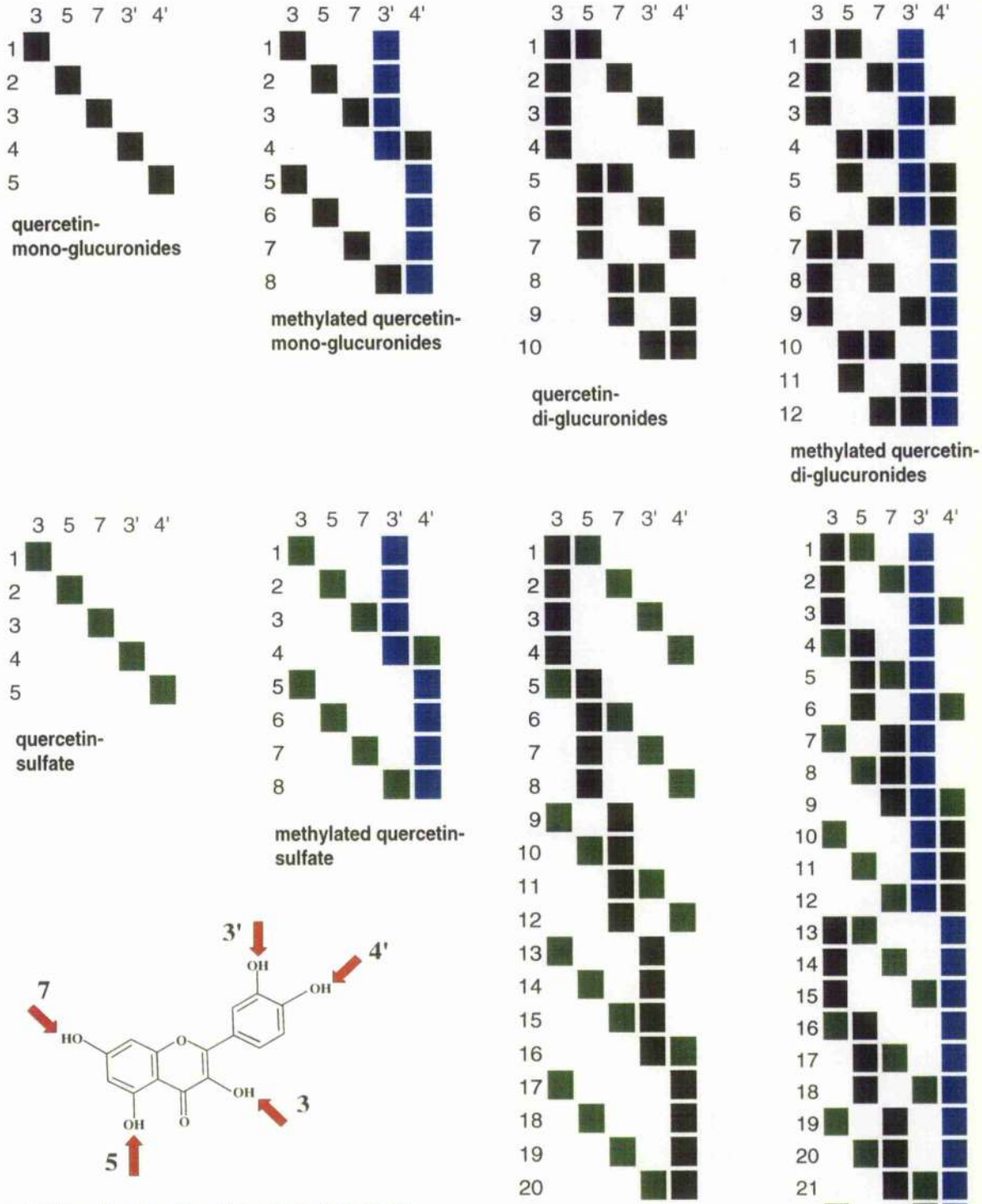


Figure 9: Theoretical number of isomers of quercetin conjugates.
Red arrows highlight putative binding positions of glucuronide, sulfate and methyl moieties to the quercetin molecule. Current literature suggests that methyl groups only bind to positions 3' and 4'. On the x axis of the figures: binding positions (3, 5, 7, 3', 4'), y axis: number of possible isoforms. blue = methyl group, black = glucuronide group, green = sulfate group.

chromatographic conditions and equipment, suggests that glucuronyl transferases (UGTs) and methyl transfereases (COMTs) selectively conjugate particular hydroxyl groups on quercetin and that not all possible isomers are formed.

4.5. Pathways of metabolite formation

4.5.1. Cleavage of the glycoside

Plasma or tissue metabolites did not contain the original glucose moiety of quercetin-4'-glucoside, therefore it is evident that the glucose unit was efficiently hydrolysed before or during absorption and before further metabolism. Two enzymes are believed to hydrolyse quercetin glycosides in the small intestine: lactase phloridizin hydrolase (LPH) on the enterocyte brush border and cytosolic β-glucosidases inside enterocytes (Day et al, 2000; Day et al, 2003). These findings support the view that ingested quercetin-4'-glucoside is hydrolysed in the small intestine, before, during or immediately after the absorption step.

4.5.2. Glucuronidation: part of the absorption process?

All quercetin metabolites found in plasma and internal tissues (liver and kidney) contained a glucuronyl unit. This may suggest that glucuronidation either (1) permits or is part of the absorption process of quercetin, or (2) occurs immediately after absorption. This conclusion is supported by Inoue et al (1999), reporting that both naphthyl glucoside and p-nitrophenol glucoside are metabolised to the corresponding glucuronides during passage through rat intestinal mucosa.

4.5.3. Is sulfation only occurring in the GI-tract?

Quercetin sulfates (without the attachment of a glucuronide moiety) were only present in the GI-tract at 0.5, 1 and 2 h. Plasma, kidney and liver only contained quercetin sulfates with the attachment of a glucuronide group. Thus it is hypothesized that in the rat, quercetin or methyl-quercetin might be first sulfated in either the lumen or enterocytes of the GI-tract, followed by glucuronidation either during absorption or immediately after absorption.

4.5.4. Are metabolites further metabolised?

The distribution of metabolite types and numbers in tissues and plasma changed dramatically over the monitored 5 h period (**Table 6, Figure 7**). In the GI-tract, the

number of individual metabolites increased from 8 to 15 at 0.5 and 1 h, and decreased to 14 and 7 at 2 and 5 h. The increased number of metabolites may be due to prolonged exposure to metabolising enzymes and possibility differences of pH and enzyme activity as the chymus moves down the GI-tract. In this short time frame, it is unlikely that the increased number of metabolites was due to quercetin induced upregulation of phase II enzymes (Galijatovic et al, 1999).

However at 5 h, the number of metabolites was drastically reduced to 4 main quercetin conjugates (peaks 3, 6, 10, 16) (**Table 10**). The explanation that in an absorptive organ, the metabolites disappeared because they had been absorbed and then subsequently sequestered by tissues, must be excluded, as radioactivity levels in all internal organs continually decreased. The only logical explanation seemed to be that quercetin conjugates underwent further metabolism, e.g. de-conjugation with subsequent re-conjugation at a different binding position.

This hypothesis is in agreement with recent studies (O'Leary et al 2003; Crespy et al, 2003; Oliveira et al, 2002; Donovan et al, 2001) therefore it is assumed that the main metabolite at 5 h, (60% of total radioactivity dose) had been formed in the lower GI-tract after de-conjugation and subsequent re-conjugation to form the quercetin di-glucuronide isoform which elutes as peak 10.

Peak	Metabolite	GI-tract	Plasma	Liver	Kidney	Sum
3	Quercetin di-glucuronide	-	-	0.1 (35.5)	0.1 (40.3)	0.2
6	Quercetin di-glucuronide	2.2 (2.4)	0.1 (7.8)	0.1 (42.5)	0.0 (17.4)	2.4
10	Quercetin di-glucuronide	61.5 (66.7)	0.0 (2.1)	0.0 (8.4)	0.1 (33.7)	61.6
16	Methylated quercetin glucuronide sulfate	-	1.0 (62.2)	-	-	1.0

Table 10: Four predominant metabolites at 5 h.
The quantity of each metabolite is given as a % of the ingested dose, and in parenthesis as a percentage of the radioactivity, which was extracted from each organ.

4.5.5. Catabolism of quercetin metabolites in the colon?

Two major radiolabelled metabolites, accounting for 21% of the radioactivity in the GI-tract at 5 h after [2-^{14}C]quercetin-4'-glucoside administration, were not identified as they did not yield recognisable mass spectra. Their relatively short HPLC retention times suggested that they were polar molecules, possibly phenolic acids which may have been formed by colonic microflora (Rechner et al, 2002; Justesen et al, 2000). Catabolism of

quercetin into phenolic acids would therefore be expected to occur in the caecum and colon of the rat at timepoints around 5 h after the oral dose (Manach et al, 1997; Brown et al, 1988). The present study was focussed on quercetin absorption and metabolism from the small intestine, and for the investigation of quercetin metabolism and/or catabolism in the large intestine it would have been necessary to collect samples at later time points.

4.6. Discussion of the applied methods

4.6.1. Extraction of quercetin metabolites from biological samples

In a number of studies acidified solvents were used for the extraction of flavonoid metabolites from biological samples (Manach et al 2000, Alder et al 2000, Boulton et al 1999, Manach et al 1999). Therefore this approach was tested (materials and methods, Figure 2), however, when our extraction solvent (50% methanol/phosphate buffer) was acidified, the extraction efficiency of radiolabelled quercetin metabolites from liver was only 20% compared to > 95% when the extraction solvent (50% methanol/phosphate buffer) was kept at pH 7.0.

It is evident, that both the extraction solvent and technique determines the extraction efficiency, and low findings of plasma metabolites in previous studies may be due to inadequate extraction techniques. Internal standards, added to plasma samples before the extraction, may not reflect the true extraction efficiency, as virtually all plasma quercetin is metabolised and bound to plasma proteins (Walle et al, 2003; Zsila et al, 2002, Boulton et al, 1998). Thus the true value of absorbed quercetin may be significantly higher as previously reported.

4.6.2. 50% recovery

Using 50% methanol/phosphate buffer as extraction solvent, over 95% of the radiolabelled quercetin metabolites were released from the tissue matrix and went into solution. During the subsequent multi step purification and concentration process (**Figure 2**), on average 50% of the original radioactivity was lost (**Table 1**), however the final extract was sufficiently concentrated and pure for MS/MS analysis. It was assumed that a similar quantity of all compounds was lost equally, and no specific compound was lost selectively.

4.6.3. Metabolites in urine and faeces ?

As the main focus of this study was the investigation of absorption and metabolism of [2-^{14}C]quercetin-4'-glucoside, urine and faeces samples had not been collected. However, as >92% of the ingested radioactivity was accounted for by measuring the radioactivity concentration in plasma, whole GI-tract and eight other tissues we assume that up to 5 h post ingestion no significant amounts of [2-^{14}C]quercetin-4'-glucoside or its metabolites were excreted in urine or faeces.

5. Conclusion

This study supports the view that ~10% of dietary quercetin is absorbed from the GI-tract. Absorbed quercetin occurs in the body almost exclusively metabolised with one or more methyl, sulfate or glucuronide groups attached. We hypothesise that most of the ingested dietary quercetin is metabolised directly in the GI-tract. Thus the metabolism of dietary quercetin is primarily pre-systemic, e.g. may be a defence strategy. Any systemic biological effects previously attributed to free quercetin must be due to its metabolites.

Section II:

Distribution of [2-^{14}C]quercetin-4'-glucoside and its metabolites within the GI-tract

Abstract

Background: Previous results suggested that quercetin is extensively metabolised before or during its absorption. It was hypothesized that the GI-tract was the major organ of quercetin bio-transformation.

Objective: Determine where in the GI-tract quercetin metabolites may have been formed by comparing the metabolite profiles of physiologically distinct GI-tissues and contents.

Design: Rat GI-tracts were divided into 5 physiologically distinct segments (stomach, proximal small intestine, distal small intestine, caecum and colon), and separated from their luminal contents. Radiolabelled compounds were extracted and stored for LC-MS/MS analysis.

Results: Half an hour after ingestion >90% of the radioactivity was found in the stomach and small intestine and at 5 hours >80% was found in caecum and colon. Small intestinal tissue held the highest concentration of radiolabelled compounds (4.3% of the ingested dose at 0.5 hours) compared to other body tissues. Data on the identity of radiolabelled compounds in different GI-segments were lost in the Bower Building fire.

Conclusions: The time-dependent distribution of radiolabelled compounds throughout the GI-tract confirms our hypothesis that metabolites at 0.5 hours after oral ingestion of [2-^{14}C] quercetin-4'-glucoside were located in the stomach and small intestine. Similarly, the metabolite composition found in the GI-tract at 5 hours reflected the situation in caecum and colon.

Key words: [2-^{14}C] quercetin-4'-glucoside, radioactivity-distribution, GI-tissues, chymus.

1. Background and Aim

Epidemiological and experimental studies suggest that dietary quercetin may reduce the risk of heart disease and certain cancers, but an incomplete understanding of the bioavailability and metabolism of dietary quercetin confounds the interpretation of these results. Results in Section I suggest that dietary quercetin is completely metabolised before its entry into systemic circulation, implying that the GI-tract may be the major site of quercetin metabolism.

Due to the early appearance of quercetin metabolites in the GI-tract at 0.5 h after the oral ingestion (Section I, Table 6, Figure 7) it was hypothesized that quercetin metabolites had been formed in the upper GI-tract. However, the speed of passage of ingested [2-^{14}C]quercetin-4'-glucoside through the rat GI-tract was not known. In Section I, complete GI-tracts (GI-tissues and contents) were analysed as one homogenized unit, and the distribution of radiolabelled compounds between GI-tissues and luminal contents was not determined.

To explore the metabolic fate of dietary quercetin in different GI-segments, the GI-tracts of two rats were divided into five physiologically distinct gut segments and the GI-tissues were separated from their luminal contents. The aim of the study was to identify and compare quercetin metabolites in GI-tissues and their respective luminal contents, to test the hypothesis that specific GI-tissues are the primary location of quercetin metabolism *in vivo*.

2. Materials and Methods

2.1. Materials, animals and sample collection

Two GI-tracts that had been collected in the previous experiment (Section I) were used for this study. As described in Section I, rats received an oral dose of 3.26 mg [2-^{14}C]quercetin-4'-glucoside (7.6 mg/kg body weight) (radioactivity dose: 58.5 x 10^6 dpm). The GI-tracts were snap frozen in liquid nitrogen and stored at –80 °C. The animal feeding and sample collection was carried out at the Rowett Research Institute by Phil Morrice and Dr. Garry G. Duthie. After their arrival in Glasgow, the GI-tracts were divided into stomach, proximal small intestine (first 1/3 of the small intestine), distal small intestine (second 2/3 of the small intestine), caecum and colon (**Figure 1**). Luminal

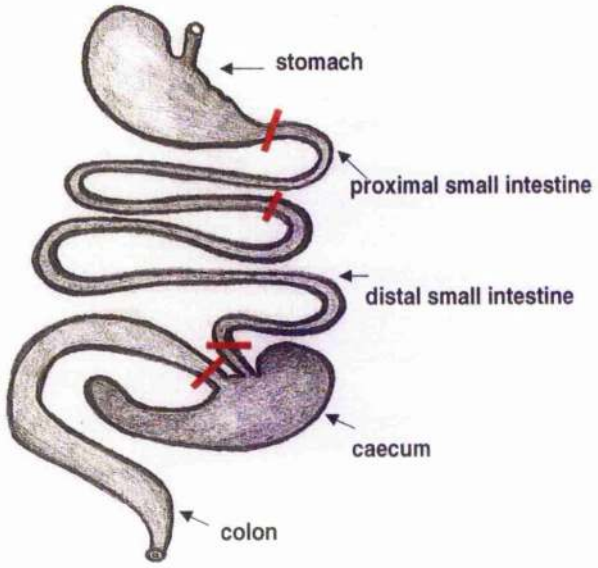


Figure 1: Rat GI-tract.
The rat GI-tract was divided into 5 physiologically distinct segments, as shown in the figure. .

contents were collected by gently squeezing out the segments and GI-tissues were rinsed with PBS until the rinsing solution was free of radioactivity, which indicated that luminal contents were completely removed from GI-tissues (the radioactivity content of the rinsing solution was monitored by liquid scintillation counting). GI-tissues were homogenized in extraction buffer, and luminal contents were first freezedried and then homogenized using mortar and pestle.

2.2. Measurement of radioactivity content

Aliquots of tissue homogenates (300 mg) and luminal contents (30 mg) were digested using Biosol tissue solubilizer, and analysed by liquid scintillation counting as previously described in Section I, paragraph 2.4.

2.3. Extraction of radiolabelled metabolites

All samples containing >100.000 dpm were extracted following the same extraction protocol as described in Section I, paragraph 2.5.2. Samples containing <100.000 dpm did not contain a sufficient amount of radiolabelled compounds for extraction and HPLC-RC analysis. The recovery of radiolabelled compounds from tissues and luminal contents (extraction efficiency) was 50%±15% and 66%±12%,

respectively, with exception of 94% for radiolabelled compounds from luminal contents of the stomach at 0.5 h after oral ingestion of [2-^{14}C]quercetin-4'-glucoside.

2.4. Identification of radiolabelled compounds by LC-MS analysis

It was intended to analyse the extracts containing radiolabelled compounds from GI-tissues and their respective luminal contents by HPLC-RC-MS/MS as described previously (Section I, paragraph 2.6). Unfortunately, however, all extracts were lost in the Bower Building fire, (October 24th 2001), and it was not possible to repeat the animal experiments in the time frame of this PhD.

3. Results

3.1. Distribution of radioactivity within GI-tissues and luminal contents

The distribution of radiolabelled compounds within the GI-tract of rats was determined at 0.5 and 5 hours after oral ingestion of [2-^{14}C]quercetin-4'-glucoside (**Figure 2**). At both time points ~92% of the ingested radioactivity was associated with the whole GI-tract, which included the stomach, small intestine, caecum, colon and their contents (Section I, Table 4). When the GI-tract was divided into physiological segments, at 0.5 h after ingestion, 91% of the ingested radioactivity was found in the stomach and small intestine, with 6% present in tissues and 85% in luminal contents. Compared with all other analysed body tissues (liver, kidney, lung, heart, testes, spleen brain, muscle and plasma, Section I, Table 4), small intestine tissue held the highest concentration of radiolabelled compounds (4.3% of the ingested dose at 0.5 h, 2,513 x 10^3 dpm/3.6 g wet tissue) (**Table 1**).

Five hours after oral ingestion of [2-^{14}C]quercetin-4'-glucoside 80% of the ingested radioactivity was associated with caecum and colon, e.g. present in the lower GI-tract. Tissues of the stomach and small intestine did not retain the amounts of radiolabelled compounds which they held at 0.5 h (Table 1).

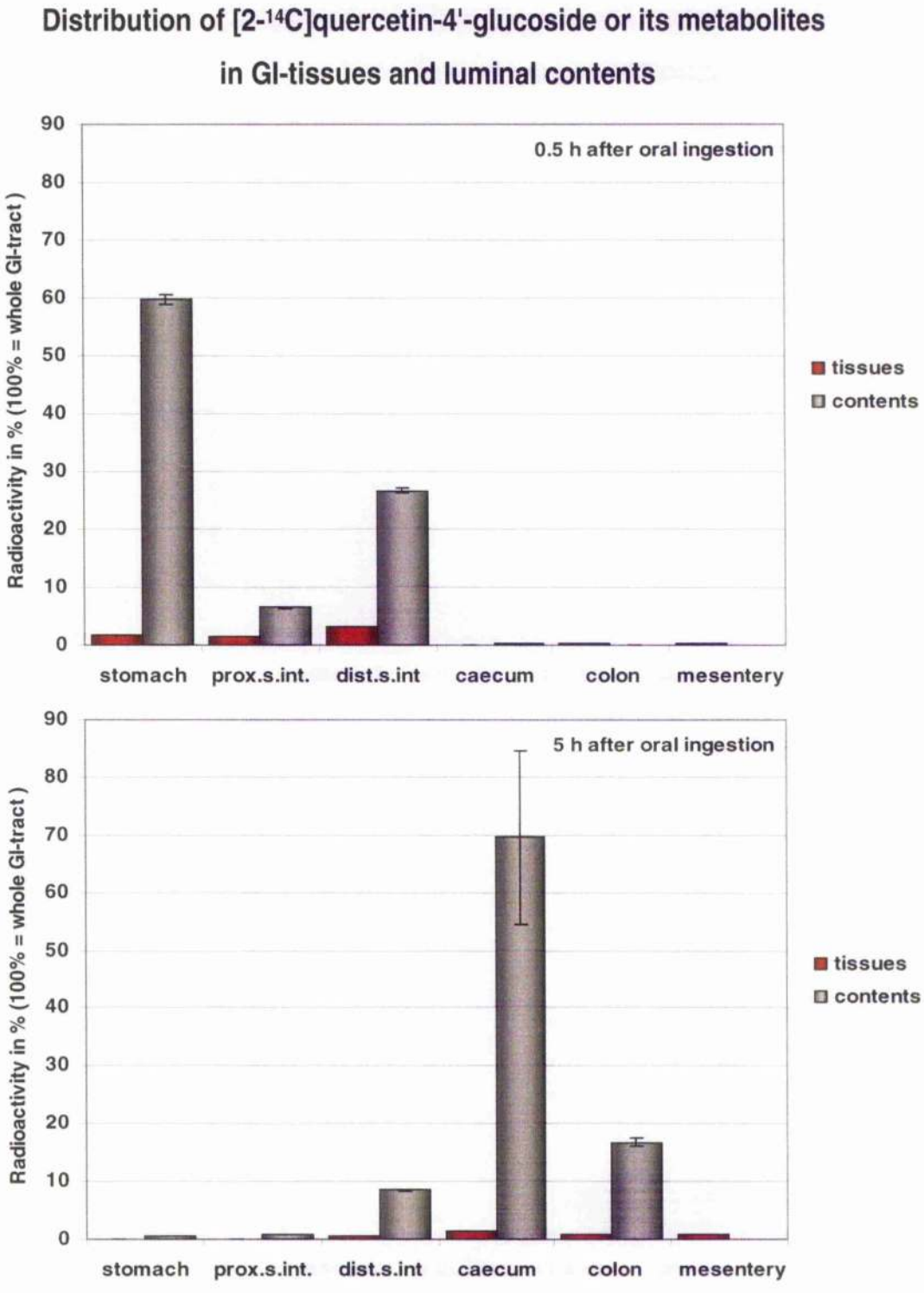


Figure 2: Distribution of radioactivity in GI-tissues and luminal contents.
Distribution of radioactivity in the GI-tract of rats, 0.5 and 5 h after ingestion of 3 mg [2-^{14}C]quercetin-4'-glucoside. GI-tissues and luminal contents were collected separately, and tissues were thoroughly washed to completely remove luminal contents (until the wash was free of radioactivity). Radioactivity was measured by liquid scintillation counting after solubilising samples with tissue solubilizer. This figure graphically shows data given in Table 1. Abbreviations: prox.s.int = proximal small intestine, dist.s.int.= distal small intestine.

Distribution of [2-^{14}C]quercetin-4'-glucoside and its metabolites in GI-tissue and its luminal contents

0.5 h after oral ingestion		dpm	± SD	% of whole GI-tract	% of oral dose
Tissues	stomach	896	± 56	1.66	1.53
	proximal small intestine	807	± 23	1.50	1.38
	distal small intestine	1,706	± 45	3.17	2.92
	caecum	73	± 3	0.14	0.13
	colon	131	± 6	0.24	0.22
	mesentery	140	± 0.6	0.26	0.24
	Sum: GI-tissues	**3,753**		**6.97**	**6.42**
Contents	stomach	32,086	± 424	59.65	54.85
	proximal small intestine	3,469	± 73	6.45	5.93
	distal small intestine	14,323	± 205	26.63	24.48
	caecum	113	± 10	0.21	0.19
	colon	42	± 4	0.08	0.07
	Sum: GI-contents	**50,033**		**93.02**	**85.52**
	Sum: GI-tissues + contents	**53,786**		**100.00**	**91.94**

5 h after oral ingestion		dpm	± SD	% of whole GI-tract	% of oral dose
Tissues	stomach	35	± 0.3	0.06	0.06
	proximal small intestine	36	± 3	0.07	0.06
	distal small intestine	250	± 2	0.46	0.43
	caecum	805	± 12	1.49	1.38
	colon	447	± 52	0.83	0.76
	mesentery	473	± 51	0.88	0.81
	Sum: GI-tissues	**2,046**		**3.79**	**3.50**
Contents	stomach	302	± 23	0.56	0.52
	proximal small intestine	472	± 11	0.88	0.81
	distal small intestine	4,607	± 84	8.55	7.87
	caecum	37,461	± 8,092	69.54	64.04
	colon	8,981	± 369	16.67	15.35
	Sum: GI-contents	**51,823**		**96.20**	**88.59**
	Sum: GI-tissues + contents	**53,869**		**100.00**	**92.09**

Table 1: Distribution of radioactivity in GI-tissues and luminal contents.
Distribution of radioactivity in the GI-tract of rats, 0.5 and 5 h after ingestion of 3.26 mg [2-^{14}C]quercetin-4'-glucoside (58.5 x 10^6 dpm). GI-tissues and luminal contents were collected separately, and tissues were thoroughly washed to completely remove luminal contents (until the wash was free of radioactivity). Radioactivity was measured by liquid scintillation counting and results are shown in dpm x 10^3 (± SD, n=3) (left column), as a percentage of the radioactivity content of the whole GI-tract (*middle column*) and as percentage of the administered radioactivity (right column).

3.2. Identification of radiolabelled compounds in GI-tissues and contents

The Identification of radiolabelled compounds in GI-tissues and luminal contents by HPLC-RC-MS/MS analysis was not possible due to the loss of the samples in the fire.

4. Discussion

4.1. Passage of radiolabelled compounds through the rat GI-tract

Half an hour after oral ingestion of [2-^{14}C]quercetin-4'-glucoside >90% of the ingested radioactivity was found in the stomach and small intestine, and at 5 hours >80% of the ingested dose was found in caecum and colon. Therefore, it can be concluded that metabolites which were present in the whole homogenized GI-tract at 0.5 h (Section I, Figure 7) were located in stomach or small intestine. Similarly, metabolites extracted from the whole homogenized GI-tract at 5 hours (Section I, Figure 7) most likely reflect the situation in caecum and colon. If metabolites were formed in the GI-tract, those metabolites found at 0.5 h must have been formed either in stomach or small intestine.

5. Conclusion

The tissue of the small intestine held the largest concentration of radiolabelled compounds compared with all other analysed body tissues. Thus, intestinal epithelia may be the part of the body that is most exposed to ingested flavonoids, such as quercetin-4'-glucoside. Dietary flavonoids may play a role in the maintenance of GI-health.

Section III:

Metabolism of [2-^{14}C]quercetin-4'-glucoside by cultured cells

Abstract

Background: The determination of the putative roles of dietary quercetin and its *in vivo* metabolites in disease prevention requires information on its bioavailability and biotransformation. Thus, we examined the contribution of the liver and small intestine to quercetin metabolism.

Objective: To compare the ability of cultured liver and intestinal cells to synthesize quercetin metabolites.

Design: Cultured liver (CC-1) and intestinal cells (IEC-6) were exposed to 5 μM [2-^{14}C]quercetin-4'-glucoside for 0.5, 2, 5, 24, 48 and 72 hours. Radiolabelled compounds were collected, extracted, and analyzed by LC-MS/MS with on-line radioactivity detection.

Results: Both cell lines produced similar types and numbers of quercetin metabolites. Eleven of the 16 quercetin metabolites were identical with metabolites formed *in vivo* in the rat. Four of the *in vitro* metabolites not identical to rat metabolites were new isoforms of previously detected metabolites. The degree of chemical instability of [2-^{14}C]quercetin-4'-glucoside in cell culture medium was not anticipated *a priori*. Less than 0.2% of [2-^{14}C]quercetin-4'-glucoside, its break down products or metabolites were associated with the cells. Culture medium contained >99.8% of the administered radioactivity, but the presence of the cells was essential for the formation of the metabolites.

Conclusions: Intestinal and liver cells form similar types and numbers of quercetin metabolites. These results support the hypothesis that the GI-tract is involved in quercetin metabolism *in vivo*. We now hypothesize that quercetin metabolites are quickly exported from cells after their synthesis. Provided the stability of the flavonoid under investigation is ensured (e.g., cell independent break down is controlled), *in vitro* experiments can provide a useful tool for investigating the effects of quercetin metabolites *in vivo*.

Key words: [2-^{14}C] quercetin-4'-glucoside, liver cells, intestinal cells, *in vitro* metabolism, compound-stability.

1. Background and Aim

Ingested dietary quercetin is quickly metabolised in animals and humans (Section I; Crespy et al, 2003; Sesink et al, 2001; Graefe et al, 2001). To explore the potential role of quercetin metabolites in disease prevention, it is essential to determine where quercetin metabolites are formed and which metabolites are produced. The liver is regarded as the primary metabolising organ for xenobiotic compounds, including flavonoids (Lin et al, 1999; Griffiths, 1982; Hackett, 1986). However, our results (Section I of this thesis) support the hypothesis that in rats, the GI-tract is the major site of quercetin metabolite formation, as at 2 h >90% of orally ingested [2-^{14}C]quercetin-4'-glucoside was found metabolised in the GI-tract, while the metabolite concentration in liver tissues never exceeded 2%. However, the possibility that quercetin metabolites had been synthesized in the liver and transported into the small intestine via the bile, could not be entirely ruled out by this data. To estimate the individual contribution of the liver and the GI-tract towards quercetin metabolism, the quantity and nature of synthesized metabolites must be determined separately. *In vivo* this question is usually resolved by collection and analysis of the bile during bile duct cannulation experiments in animals.

The aim of this study was 1) to investigate whether cultured liver or intestinal cells were capable of metabolising quercetin-4'-glucoside in a similar manner to that seen in the rat and 2) to investigate whether there is potential for establishing a cell culture model for *in vivo* metabolism of dietary quercetin. Cultured liver and intestinal cells were incubated with [2-^{14}C]quercetin-4'-glucoside and radiolabelled compounds were collected, purified and analysed following the same metabolite extraction protocol as previously used for rat samples.

2. Materials and Methods

2.1. Cell culture

2.1.1. Cell lines

The cell lines IEC-6 (small intestinal epithelial cells) and CC-1 (liver epithelial cells) were purchased from The European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, U.K.) Both cell lines were derived from normal (non-cancerous) tissue. Rat cell lines were chosen to allow comparison with previous results from the rat feeding study (Section I) without interspecies extrapolation.

2.1.2. Materials and equipment

IEC-6 cells were maintained in Dulbeco's Modified Eagle Medium (D 5671, Sigma, Poole, Dorset, U.K) supplemented with 0.3 IU insulin/ml, 2 mM L-glutamine, 50 units penicillin/ml, 0.05 mg streptomycin/ml and 5% (v/v) fetal calf serum (Sigma). CC-1 cells were cultured in Minimum Essential Medium (M 7278, Sigma) containing 1% (v/v) MEM Non Essential Amino Acid Solution (M 7145, Sigma), 2 mM L-glutamine, 50 units penicillin/ml, 0.05 mg streptomycin/ml and 10% (v/v) fetal calf serum (Sigma). All manipulations of CC-1 and IEC-6 cells were carried out under sterile conditions in a class-2 laminar flow cabinet (BHA 48, Faster, Ferrara, Italy). The cells were incubated at 37 °C and 5% CO_2 in a IG50 Jouan incubator (St.Herblain, France) and kept in uncoated plasticware (75 cm^2 and 25 cm^2 flasks, 6, 12 and 24 well plates) purchased from Greiner-bio-one (Frickenhausen, Germany). The morphology of growing and confluent cells was closely monitored, under 20 x magnification using a Nikon Microscope (Tokyo, Japan).

2.1.3. Cell passage

At 90% confluence cell monolayers were rinsed twice with PBS and covered with 1-2 ml 0.25% trypsin/EDTA for 1 min. After removal of excess trypsin, flasks were incubated at 37 °C for 10 min and detached cells were dispersed in cell culture medium and split by a ratio of 1 to 20.

2.2. Experiments and sample collection

[2-^{14}C]quercetin-4'-glucoside (3.75 mCi/mmol) was solubilized in methanol at a concentration of 0.5 g/ml. CC-1 and IEC-6 cells were seeded into 25 cm^2 flasks (at 3 x 10^4 cells/ml medium, 10 ml medium was used for a 25 cm^2 flask) and incubated with 5 μM [2-^{14}C]quercetin-4'-glucoside (8.3 KBq) immediately after seeding or after reaching confluence. The final methanol concentration in culture medium was 0.5% (v/v) which had no adverse effect on cell viability. After incubation for 0.5, 2, 5, 24, 48 and 72 hours at 37 °C, cell morphology was closely monitored at 20 x magnification, culture media was collected and the cell monolayer was rinsed twice with PBS and trypsinised as described above. To stop trypsin activity, detached cells were dispersed in 20% (v/v) foetal calf serum in PBS. The cell pellet was collected after centrifugation, re-suspended in 200 μl 20% (v/v) methanol in PBS and lysed by three repeated freeze-thawing cycles.

For control experiments 5 μM [2-^{14}C]quercetin-4'-glucoside was added to fresh cell culture medium and incubated at 37 °C for 5 and 72 h.

To determine whether metabolite formation had occurred in the cell culture medium by substrates and/or enzymes released from the cells, medium was separated from cell monolayers after 72 h co-culture and placed in fresh culture flasks (containing no cells). 5 μM [2-^{14}C]quercetin-4'-glucoside was added to the culture medium and incubated at 37 °C for 24 and 72 h.

2.3. Extraction of radiolabelled metabolites

The radioactivity content of cell lysate and cell culture medium was determined by dissolving the total sample, or an aliquot, in 5 ml of scintillation fluid, and counting the radioactivity using a Multi Purpose Liquid Scintillation Counter (LS6500, Beckmann Instruments, Fullerton, CA 92634-3100, U.S.A.).

The radioactivity content in the cell lysates (< 0.2% of the administered dose) was in-sufficient for extraction and analysis, therefore only radiolabelled compounds in culture medium were extracted and analysed, following the previously developed method for the analysis of [2-^{14}C]quercetin-metabolites in rat tissues and plasma (Section I). Cell culture media was adjusted to pH 3 (using 25% H_2SO_4) and partitioned three times with an equal volume of ethyl acetate. The three ethyl acetate extracts were combined and stored at –80 °C. Any residual ethyl acetate was removed from the aqueous phase by drying it for 1 h using a centrifugal evaporator (GyroVap, Howe, U.K.) at 40 °C, before

it was loaded onto a 2 g C_{18} Sep Pak cartridge (Waters, Milford, Massachusetts, USA). The cartridge was washed with 15 ml of distilled water adjusted to pH 3.0 (with H_2SO_4) and radiolabelled compounds were eluted with 30 ml of methanol. The methanolic eluent was combined with the ethyl acetate extracts and reduced to dryness *in vacuo*, re-suspended in methanol, and stored at –80 °C.

Radiolabelled compounds extracted from cell culture medium: extraction efficiency in %

Sample ID	aqueous phase		ethyl acetate phase	extraction efficiency
	after partitioning	after elution from sep pak	after partitioning	aqueous phase + ethyl acetate phase
CC-1 confluent 0.5 h	12.4	14.4	75.7	90.1
CC-1 confluent 2 h	9.5	17.8	73.1	90.9
CC-1 confluent 5 h	34.3	30.9	50.7	81.6
CC-1 confluent 72 h	66.4	50.2	40.0	90.2
CC-1 growing 24 h	37.0	37.5	30.7	68.1
CC-1 growing 48 h	48.8	32.2	32.7	64.9
CC-1 growing 72 h	54.3	54.6	26.3	80.8
CC-1 in or out 24 h	32.4	20.8	48.9	69.7
CC-1 in or out 72 h	32.8	21.3	34.8	56.0
CC-1 control medium 5 h	16.1	14.6	71.7	86.4
CC-1 control medium 72 h	18.6	20.9	27.7	48.6
IEC-6 confluent 0.5h	14.9	15.8	71.6	87.4
IEC-6 confluent 2 h	12.2	16.4	61.5	77.8
IEC-6 confluent 5h	68.7	76.9	21.8	98.7
IEC-6 confluent 72 h	41.9	39.5	47.5	87.0
IEC-6 growing 24 h	35.1	30.2	46.0	76.1
IEC-6 growing 48 h	28.9	28.1	50.2	78.3
IEC-6 growing 72 h	21.4	34.1	41.6	75.7
IEC-6 in our out 24 h	19.1	21.4	73.4	94.9
IEC-6 in our out 72 h	66.7	57.7	17.7	75.4
IEC-6 control medium 5 h	16.8	28.9	48.3	77.3
IEC-6 control medium 72 h	68.2	55.7	9.5	65.2
			average recovery:	78.2

Table 1: Extraction efficiency of radiolabelled compounds from cell culture medium.
Cultured liver and intestinal cells were incubated with 5 µM [2-^{14}C]quercetin-4'-glucoside and after 0.5, 2, 5, 24, 48 or 72 h radiolabelled compounds were extracted from cell culture medium, according to the extraction protocol which was previously used for extracting quercetin metabolites from rat tissues and plasma (Section I, Figure 3 and Table 1). The extraction efficiency (in%) is the ratio: activity in the extract / initial activity in each sample x 100. The initial activity in each sample, e.g. cell culture medium was on average 90% of the administered dose = 450,000 dpm. Sample ID (left column of the table): Cell type (CC-1 / IEC-6), experiment (confluent cells, growing cells, are metabolites formed inside or outside of cells, abbreviated as "in or out", control medium which was never in contact with cells) and incubation times (0.5, 2, 5, 24, 48 and 72 hours).

2.4. Separation and identification of radiolabelled metabolites

2.4.1. HPLC analysis with online radioactivity detection (HPLC-RC)

Cell culture medium extracts (containing ca. 50.000 dpm) were analysed using a P4000 HPLC fitted with an AS 3000 autosampler, a UV 6000 diode array absorbance monitor (Thermo Finnigan, San Jose, CA, USA) and a radioactivity monitor (Model 9701, Reeve Analytical, Glasgow, U.K.) fitted with a 200 µl heterogeneous flow cell. Separation was carried out at 1 ml/min using a 250 x 4.6 mm, i.d. 4 µm, Polar-RP column (Phenomenex, Macclesfield, U.K.) eluted at room temperature with a gradient of 5 to 25% acetonitrile/2%formic acid over 60 minutes. Signals from both, the diode array and radioactivity monitor were processed using Chromquest 3.0 software (Thermo Finnigan).

2.4.2. MS/MS analysis with online radioactivity detection (LC-MS/MS-RC)

Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, diode array absorbance detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C (Thermo Finnigan, San Jose, CA, USA). Separation was carried out using a 250 x 4.6 mm i.d. 4 µm Synergy RP-Max column (Phenomenex, Macclesfield, U.K.) eluted with a gradient over 60 minutes of 5-40% acetonitrile in 1% formic acid at a flow rate of 1 mL/min and maintained at 40 °C. After passing through the flow cell of the diode array detector the column eluate was directed to a radioactivity monitor (Model 9701, Reeve Analytical, Glasgow, U.K.) fitted with a 500µl heterogeneous flow cell packed with cerium activated lithium glass scintillant, before being split with 50% entering a LCQ DecaXP ion trap mass spectrometer with an electrospray interface (Thermo Finnigan, San Jose, CA, USA) operating in negative ion mode and scanning from 100-2000 amu.

Relating retention times in HPLC-RC and LC-MS/MS-RC chromatograms by their R_f values.

	HPLC-RC analysis gradient 5-25% / 60 mins, column: Phenomenex RP Polar		LC-MS/MS-RC analysis gradient 5-40% / 60 mins, column: Phenomenex RP Max		
radiolabelled peaks	RT	R_f value	R_f value	RT	compound identity
cells 1	16.17	0.40	0.47	15.88	Quercetin di-glucuronide
cells 5	24.47	0.60	0.61	20.59	Unknown
cells 6	25.81	0.63	0.68	23.00	Methylated quercetin di-glucuronide
cells 10	34.16	0.84	0.77	25.95	Quercetin di-glucuronide
cells 12	35.18	0.87	0.86	28.83	Quercetin di-glucuronide
cells 13	37.00	0.91	0.91	30.50	Quercetin mono-glucuronide
cells 15	43.93	1.08	1.02	34.45	Methylated quercetin mono-glucuronide
cells 16	44.71	1.10	1.04	35.00	Methylated quercetin mono-glucuronide
cells 17	46.85	1.15	1.06	35.74	Quercetin

Table 2 A: R_f values of metabolites formed by liver cells. Chromatograms are shown in Figure 3.

	HPLC-RC analysis		LC-MS/MS-RC analysis		
radiolabelled peaks	RT	R_f value	R_f value	RT	compound identity
cells 1	21.73	0.53	0.48	16.29	Quercetin di-glucuronide
cells 4	23.30	0.57	0.57	19.08	Unknown
cells 5	25.70	0.63	0.62	20.96	Methylated quercetin di-glucuronide
cells 10	29.00	0.71	0.77	25.98	Quercetin di-glucuronide
cells 11	31.07	0.76	0.82	27.50	Quercetin di-glucuronide
cells 12	34.05	0.84	0.86	28.94	Quercetin mono-glucuronide
cells 15	43.06	1.06	1.02	34.42	Methylated quercetin mono-glucuronide
cells 17	43.95	1.08	1.07	36.00	Methylated quercetin mono-glucuronide
cells 18	47.41	1.17	1.27	42.73	Quercetin

Table 2 B: R_f values of metabolites formed by intestinal cells. Chromatograms shown in Figure 3.

	HPLC-RC analysis		LC-MS/MS-RC analysis		
radiolabelled peaks	RT	R_f value	R_f value	RT	compound identity
cells 1	20.63	0.51	0.49	16.44	Quercetin di-glucuronide
cells 5	24.84	0.61	0.62	20.82	Methyl-quercetin di-glucuronide
cells 6	28.02	0.69	0.68	23.01	Quercetin di-glucuronide
standard	41.25	1.00	1.00	33.84	Quercetin-4'-glucoside
cells18	52.96	1.30	1.29	43.48	Quercetin

Table 2 C: R_f values of metabolites formed in cell culture media, pre-exposed to liver cells. Chromatograms are shown in Figure 4.

Table 2 A, B and C: Metabolites formed in cell culture - R_f values.
Radiolabelled compounds were extracted from cell culture medium and analysed by HPLC-RC and LC-MS/MS-RC. The retention times (RT) of peaks in HPLC-RC and LC-MS/MS-RC chromatograms (Figure 3 and 4) was different due to different separation conditions (column and gradient) and different equipment. Radioactive peaks in HPLC-RC and LC-MS/MS-RC chromatograms were matched by comparing their R_f values, elution pattern and MS identity. The R_f value is the ratio of RT (unknown compound) / RT ([2-^{14}C]quercetin-4'-glucoside). The reference RT for quercetin-4'-glucoside in HPLC-RC chromatograms was 40.67 min, and in LC-MS/MS-RC chromatograms 33.68 min. Calculation example: [RT (radiolabelled peak "cells 1" in intestinal cells) = 21.73] / [RT ([2-^{14}C]quercetin-4'-glucoside) = 40.67] = R_f value of 0.53.

2.5. Data analysis

The relative quantity of radiolabelled compounds was determined by measuring the height of peaks in chromatograms obtained from the radioactivity monitor by HPLC-RC. The retention time of peaks in HPLC-RC chromatograms and LC-MS/MS-RC chromatograms was different due to the use of different separation conditions (column and gradient) and equipment. Radioactive peaks in HPLC-RC and LC-MS/MS-RC chromatograms were matched by comparison of Rf values, MS/MS identification and elution pattern. The Rf value is the ratio of the retention time of the unknown compound and the retention time of quercetin-4'-glucoside. The reference retention time for quercetin-4'-glucoside in HPLC-RC chromatograms was 40.67 min and in LC-MS/MS-RC chromatograms 33.68 min.

3. Results

3.1. Radiolabelled metabolites formed in cell culture

[2-^{14}C]quercetin-4'-glucoside was not stable in cell culture medium. When 5 µM [2-^{14}C]quercetin-4'-glucoside was exposed to cell culture medium for 72 h, it decomposed completely into unidentified relatively polar compounds (**Figure 1**). After incubating confluent or growing liver (CC-1) and intestinal (IEC-6) cells for 0.5, 2, 5, 24, 48 and 72 h with culture medium containing 5 µM [2-^{14}C]quercetin-4'-glucoside (8.3 KBq), < 0.2 % of the administered radioactivity was associated with the cells and > 99.8 % was found in culture medium either unchanged or as radiolabelled metabolites and break down products. The uptake of radiolabelled compounds into cells was not increased in sub-confluent, growing cells. In addition, growing cells produced fewer metabolites and the formation of break down products was increased, therefore all further experiments were carried out using confluent cell monolayers.

When confluent monolayers of liver and intestinal cells were incubated with 5 µM [2-^{14}C]quercetin-4'-glucoside for 72 h, cell culture medium contained radiolabelled compounds which eluted at retention times different to decomposition products and unchanged [2-^{14}C]quercetin-4'-glucoside (**Figure 2**). To identify the nature of these radiolabelled peaks, samples were analysed using LC-MS/MS with on-line radioactivity detection (**Figure 4, Table 3**). Sixteen radiolabelled compounds were identified as different types and isoforms of quercetin conjugates with the addition of one methyl or sulfate group and/or one or two glucuronyl groups (**Table 3**). Eleven of these metabolites were identical with metabolites previously detected in the rat (**Table 6**). Four of the five cell culture metabolites not identical with rat metabolites were new isoforms of di-glucuronides (metabolite peaks 2, 3 and 9, **Table 3** and **5**), and a new isoform of methylated and sulfated quercetin mono-glucuronide (peak 8). Cultured intestine cells produced one minor metabolite with a molecular ion (M^-) at *m/z* 639 which fragmented to produce ions at *m/z* 477, 463 and 301. A neutral loss of 162 amu (*m/z* 639 – 477 = 162 amu, and *m/z* 463 – 301 = 162 amu) indicates the presence of a glycosyl adduct. A neutral loss of 176 amu (*m/z* 639 – 463 = 176 amu, and *m/z* 477 – 301 = 176 amu) indicates the presence of a glucuronyl unit (as previously reported in Section I). Therefore metabolite peak 7 was identified as glucuronated quercetin glucoside

(presumably glucuronated quercetin-4'-glucoside), a type of metabolite that was previously not detected.

3.2. Stability of [2-^{14}C]quercetin-4'-glucoside in cell culture medium

[2-^{14}C]quercetin-4'-glucoside decomposed completely into unidentified relatively polar compounds when exposed to cell culture medium for 72 h ('control medium' experiment) (**Figure 1**). The decomposition rate of [2-^{14}C]quercetin-4'-glucoside was different in culture medium formulas for liver or intestinal cells. When 5 µM [2-^{14}C]quercetin-4'-glucoside was incubated with fresh medium for 5 h, all [2-^{14}C]quercetin-4'-glucoside decomposed in medium for intestinal cells, whereas medium for liver cells contained intact [2-^{14}C]quercetin-4'-glucoside together with degradation products (**Figure 1**). The presence of cells appeared to have an effect on the stability of [2-^{14}C]quercetin-4'-glucoside in culture medium (**Figure 1** and **Figure 2**).

3.3. Time of metabolite formation

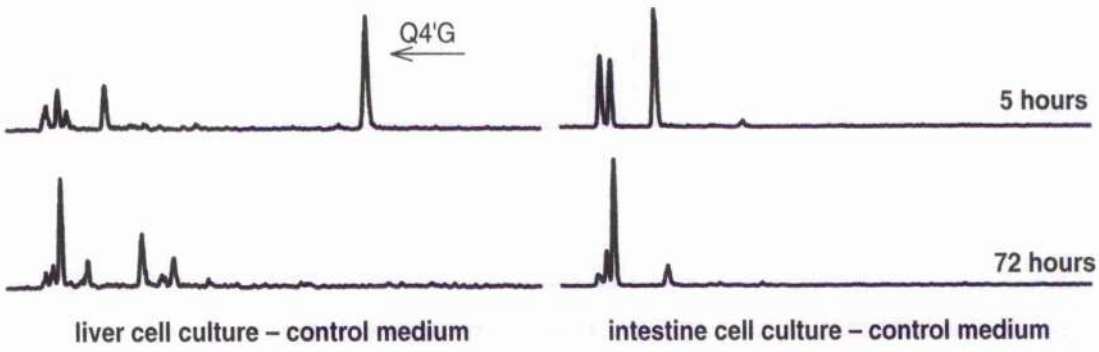
After incubating confluent cells for 0.5, 2 and 5 hours, only degradation products and unchanged [2-^{14}C]quercetin-4'-glucoside were found in cell culture medium (**Figure 2**). However, quercetin metabolites were found after 24 hour incubation of sub-confluent, growing cells (data not shown), indicating that metabolite formation occurred between 5 and 24 hours after addition of [2-^{14}C]quercetin-4'-glucoside. Thus, metabolic transformation of [2-^{14}C]quercetin-4'-glucoside was significantly slower in the employed cell culture model compared with the rat, where 3 mg [2-^{14}C]quercetin-4'-glucoside was completely metabolised by 2 h after ingestion (Section I).

3.4. Accumulation of radiolabelled metabolites in cell culture medium

At all time points, neither growing nor confluent cells accumulated more then 0.2% of the administered radioactivity, casting doubt whether metabolic transformation had occurred inside the cells. Therefore it was investigated whether quercetin metabolites were formed in cell culture medium by enzymes and substrates released by the cells. Cell culture medium was exposed to liver and intestine cell monolayers for 72 hours, transferred to fresh flasks and incubated with 5 µM [2-^{14}C]quercetin-4'-glucoside for 24 and 72 h at 37 °C. No metabolites were formed in medium which was pre-exposed to

intestinal cells, however, medium which was pre-exposed to liver cells contained very low concentrations of three quercetin di-glucuronides after 24 h (<5% of the administered radioactivity dose) (**Figure 3**). The metabolite formation in liver cell medium may have been caused by a few detached floating cells that may have been present in the medium after its separation from the cell monolayer. However, in both experiments over 95% of the administered [2-^{14}C]quercetin-4'-glucoside was recovered from the cell culture medium either unchanged or in the form of degradation products, suggesting that quercetin metabolism occurred on the cell surface or inside the cells with immediate excretion of metabolites into the cell culture medium.

Stability of [2-^{14}C]quercetin-4'-glucoside in cell culture medium



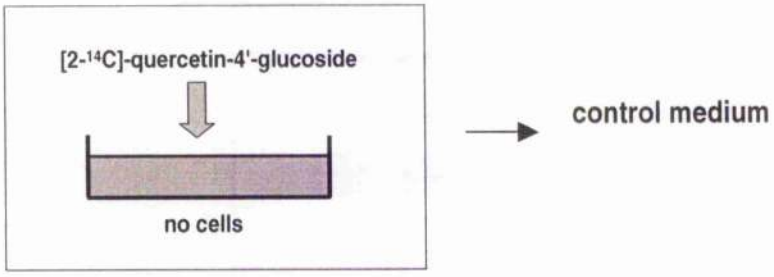


Figure 1: [2-^{14}C]quercetin-4'-glucoside is not stable in culture medium formulas for liver and intestinal cells.
Fresh cell culture medium was incubated for 5 and 72 h with 5 µM [2-^{14}C]quercetin-4'-glucoside. Radiolabelled compounds were extracted from the medium and analysed by HPLC-RC.

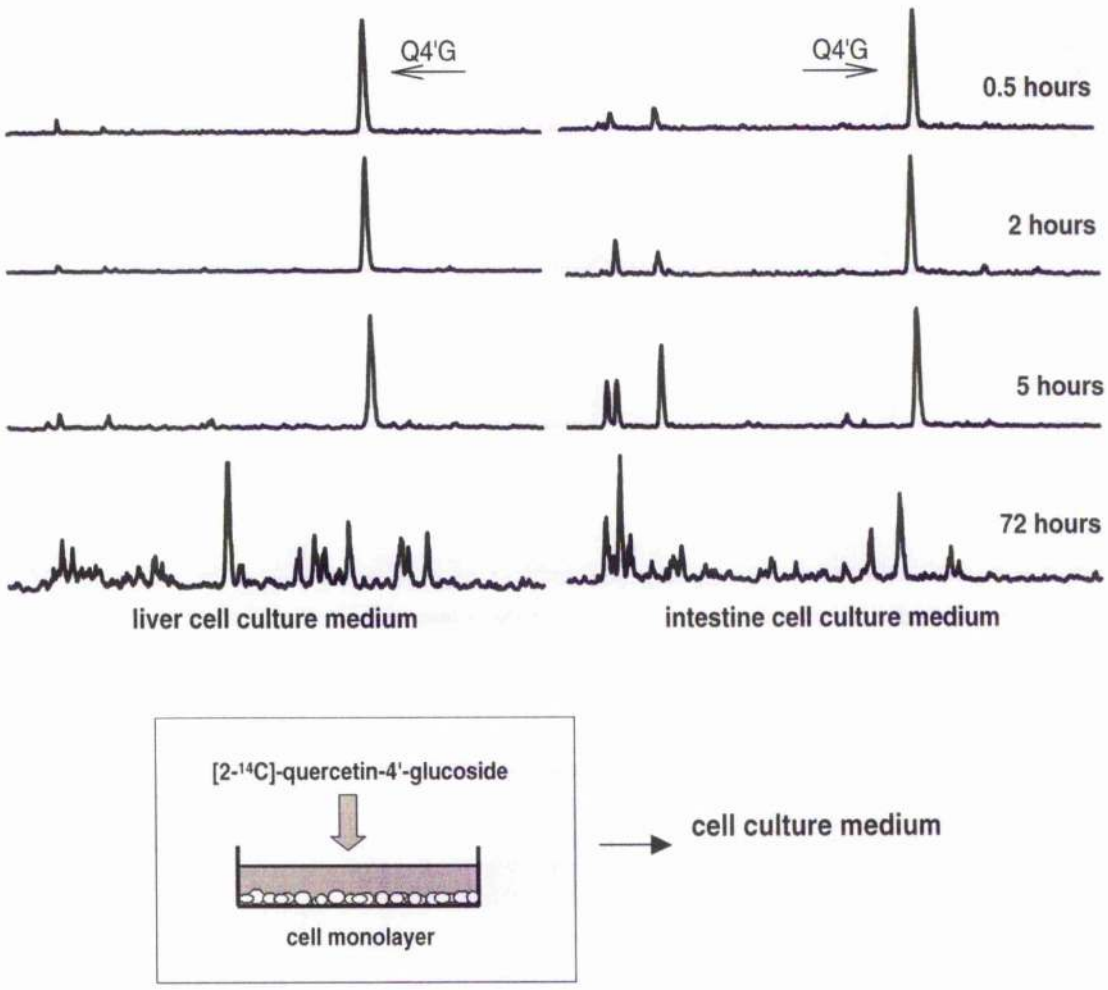


Figure 2: Radiolabelled compounds in cell culture medium after incubating liver and intestinal cells with [2-^{14}C]quercetin-4'-glucoside.
Confluent monolayers of liver or intestinal cells were incubated for 0.5, 2, 5 or 72 h with 5 µM [2-^{14}C]quercetin-4'-glucoside. Radiolabelled compounds were extracted from the medium and analysed by HPLC-RC.

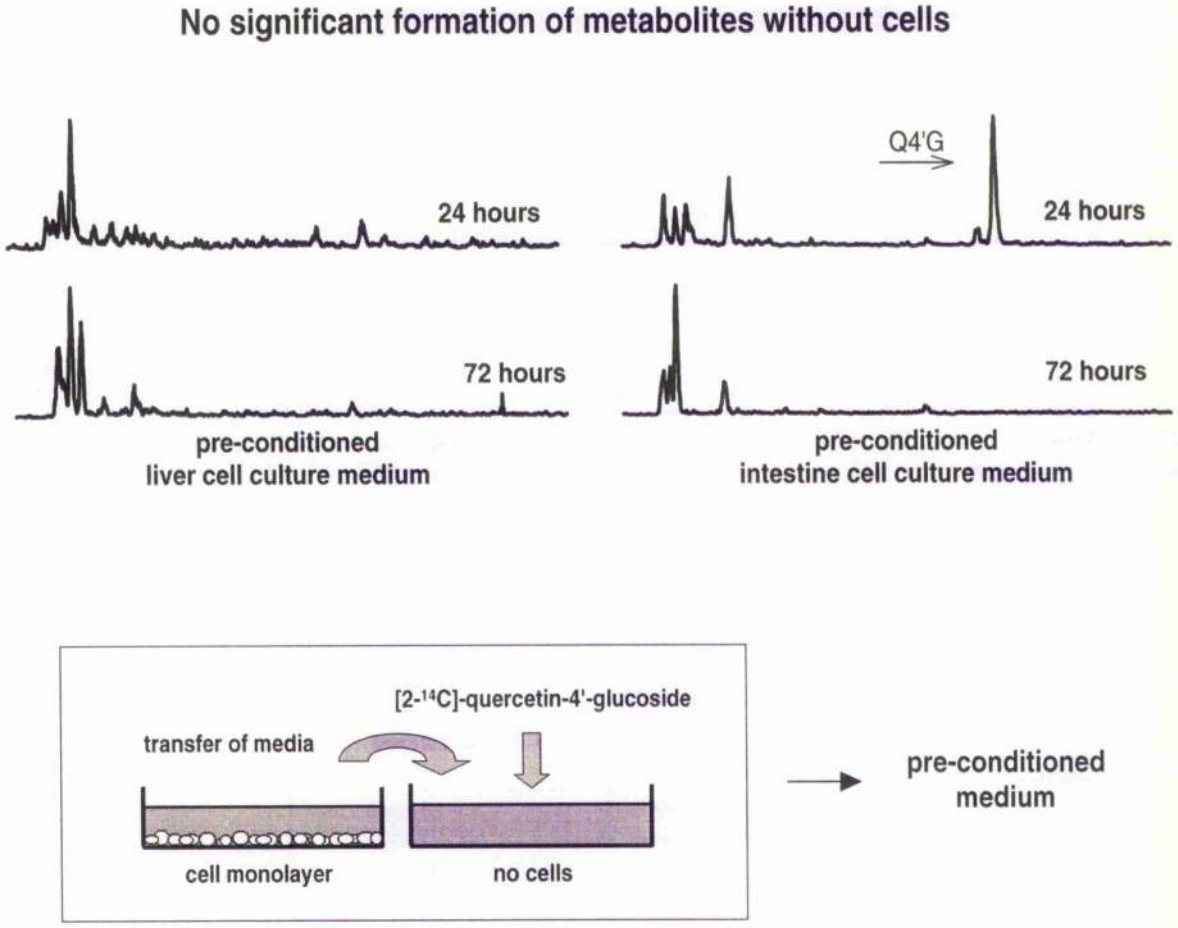


Figure 3: No significant formation of quercetin metabolites without cells.
To investigate whether metabolites were formed in the culture medium or inside the cells, culture medium was exposed to confluent cell monolayers for 72 h, cells were removed and the "pre-conditioned medium" was incubated with [2-^{14}C]quercetin-4'-glucoside for 24 and 72 h. Radiolabelled compounds were extracted and analysed by HPLC-RC and LC-MS/MS-RC.

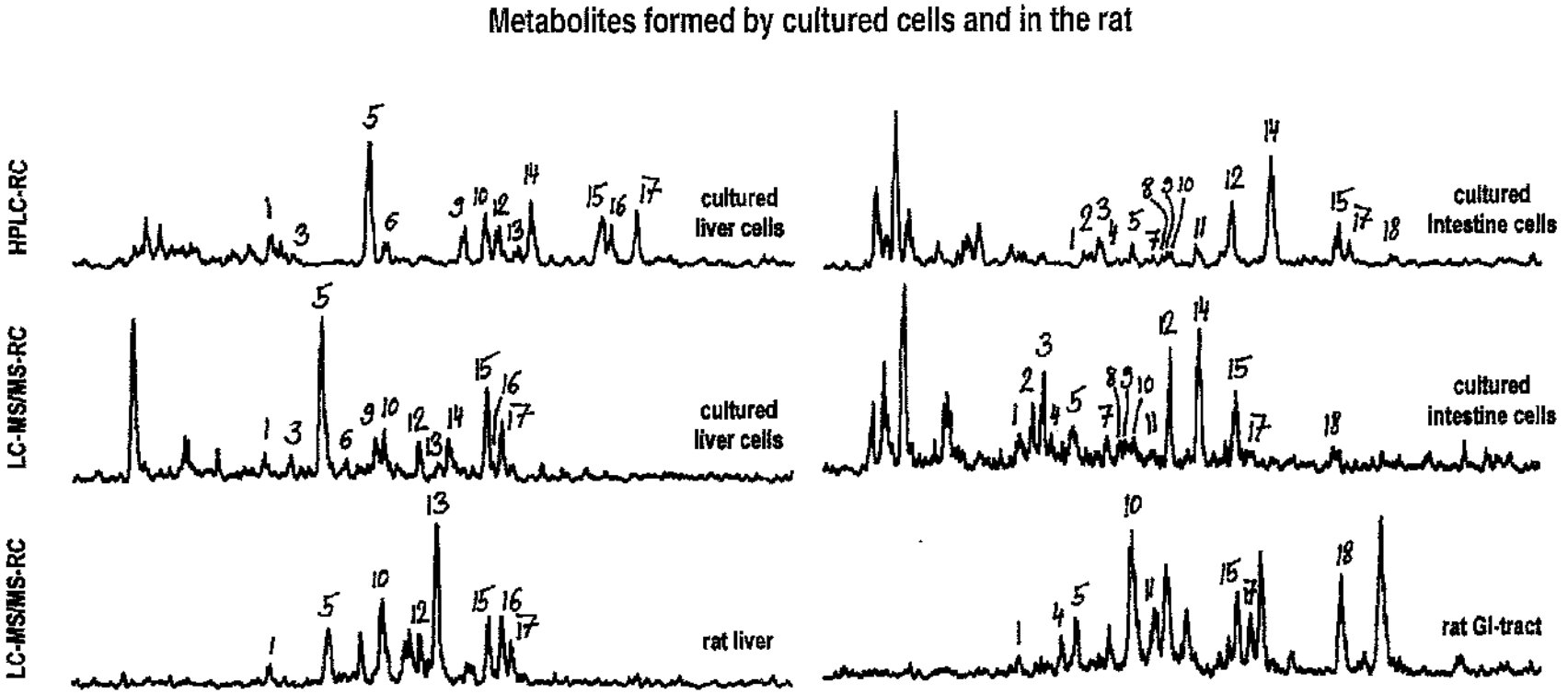


Figure 4: Metabolite formation by cultured cells and in the rat.
Radiolabelled compounds were extracted from cell culture medium (after 72 h incubation with 5 µM [2-^{14}C]quercetin-4'-glucoside), rat liver and rat GI-tract (collected at 0.5 and 2 h after ingestion of 3 mg [2-^{14}C]quercetin-4'-glucoside) and analysed by HPLC-RC and LC-MS/MS-RC. Radiolabelled metabolites were numbered according to order of elution and break down products were not regarded as metabolites. HPLC-RC and LC-MS/MS analysis was carried out using different separation parameters (gradient and column), and peaks in chromatograms from the two different machines were matched by their Rf values, as described in materials and methods..

LC-MS/MS identification of metabolites formed by cultured cells

peak	RT (min)	compound	$[M-H]^-$ (m/z)	MS^2 fragment ions (m/z)		
1	15.88	Quercetin di-glucuronide	653	477 ([M-H]-GlcUA)	301 ([M-H]-GlcUA-GlcUA)	
2	17.46	Methylated quercetin di-glucuronide	667	491 ([M-H]-GlcUA)	315 ([M-H]-GlcUA-GlcUA)	
3	18.27	Quercetin di-glucuronide	653	477 ([M-H]-GlcUA)	301 ([M-H]-GlcUA-GlcUA)	
4	19.08	not identified				
5	20.78	Methylated quercetin di-glucuronide	667	491 ([M-H]-GlcUA)	315 ([M-H]-GlcUA-GlcUA)	
6	23.00	Quercetin di-glucuronide	653	477 ([M-H]-GlcUA)	301 ([M-H]-GlcUA-GlcUA)	
7	23.73	Glycosilated quercetin mono-glucuronide	639	477 ([M-H]-glucosyl)	463 ([M-H]-GlcUA)	301 ([M-H]-SO_3-GlcUA)
8	24.73	Methylated quercetin mono-glucuronide sulfate	571	491 ([M-H]-SO_3)	395 ([M-H]-GlcUA)	315 ([M-H]-SO_3-GlcUA)
9	25.17	Methylated quercetin diglucuronide	667	491 ([M-H]-GlcUA)	315 ([M-H]-GlcUA-GlcUA)	
10	25.97	Quercetin di-glucuronide	653	477 ([M-H]-GlcUA)	301 ([M-H]-GlcUA-GlcUA)	
11	27.80	Quercetin di-glucuronide	653	477 ([M-H]-GlcUA)	301 ([M-H]-GlcUA-GlcUA)	
12	28.89	Quercetin mono-glucuronide	477	301 ([M-H]-GlcUA)		
13	30.50	Methylated quercetin mono-glucuronide sulfate	571	491 ([M-H]-SO_3)	395 ([M-H]-GlcUA)	315 ([M-H]-SO_3-GlcUA)
14	31.35	Quercetin mono-glucuronide sulfate	557	477 ([M-H]-SO_3)	381 ([M-H]-GlcUA)	301 ([M-H]-SO_3-GlcUA)
15	34.48	Methylated quercetin mono-glucuronide	491	315 ([M-H]-GlcUA)		
16	35.00	Methylated quercetin mono-glucuronide	491	315 ([M-H]-GlcUA)		
17	35.87	Methylated quercetin mono-glucuronide	491	315 ([M-H]-GlcUA)		
18	42.73	Free quercetin	301			

Table 3: Identification of [2-^{14}C] labelled metabolites formed by cultured liver and intestinal cells. Radiolabelled compounds were extracted from cell culture medium, and analysed by LC-MS/MS with online radioactivity detection. The table shows LC retention times (RT), negative ion MS/MS fragmentation patterns and putative identities of the radiolabelled metabolites. Abbreviations: GlcUA = glucuronyl unit, $[M-H]^-$ (m/z) = mass to charge ratio of the molecular ion).

Types of quercetin conjugates formed by cultured cells

Di-glucuronides (8)	**Di-glucuronides** (5 isoforms)	Quercetin	+ Glucuronic acid + Glucuronic acid
	Methylated di-glucuronides (3 isoforms)	Quercetin	+ Methyl group + Glucuronic acid + Glucuronic acid
Mono-glucuronides (4)	**Mono-glucuronides** (1 isoform)	Quercetin	+ Glucuronic acid
	Methylated mono-glucuronides (3 isoforms)	Quercetin	+ Methyl group + Glucuronic acid
Sulfated Mono-glucuronides (3)	**Glucuronated sulfates** (1 isoform)	Quercetin	+ Sulfate group + Glucuronic acid
	Methylated glucuronated sulfate (2 isoforms)	Quercetin	+ Methyl group + Sulfate group + Glucuronic acid
Glycosylated Mono-glucuronide (1)	no methylated compound found	Quercetin	+ Glucose + Glucuronic acid

Table 4: Classification of quercetin metabolites into four major groups.
Sixteen different quercetin conjugates were identified as metabolites of [2-^{14}C]quercetin-4'-glucoside formed by cultured liver and intestinal cells. The individual metabolites were classified into four major groups as shown above and the number of individual metabolites is given in parenthesis).

Relative quantities of radiolabelled metabolites formed by cultured cells

			IEC-6 cells	CC-1 cells
peak	compound		relative quantity (in %)	relative quantity (in %)
cells 1	quercetin di-glucuronide		1.20	3.42
cells 2	methyl quercetin di-glucuronide		1.00	-
cells 3	quercetin di-glucuronide		3.01	0.95
cells 4	unknown		2.41	-
cells 5	methyl quercetin di-glucuronide		2.41	17.65
cells 6	quercetin di-glucuronide		-	2.66
cells 7	glycosilated quercetin mono-glcuronide		0.40	-
cells 8	methylated quercetin mono-glcuronide sulfate		0.80	-
cells 9	methyl quercetin di-glucuronide		1.00	3.61
cells 10	quercetin di-glucuronide		1.00	6.83
cells 11	quercetin di-glucuronide		2.21	-
cells 12	quercetin mono-glucuronide		7.43	5.12
cells 13	methylated quercetin mono-glcuronide sulfate		-	1.90
cells 14	quercetin mono-glucuronide sulfate		12.85	8.92
cells 15	methyl querceitn mono-glucuronide		3.01	6.07
cells 16	methyl querceitn mono-glucuronide		-	5.12
cells 17	methyl querceitn mono-glucuronide		2.61	7.02
cells 18	quercetin		0.60	-
		sum	41.94	69.27
		di-glucuronides	11.85	35.10
		mono-glucuronides	13.05	23.34
		glucuronated sulfates	13.65	10.82
		glucuronated glucoside	0.40	-
		phase II metabolites	38.95	69.26
		free quercetin	0.60	-
		unknown	2.41	-
		break down products	58.03	30.74
		sum	100.00	100.00

Table 5: Relative quantities of radiolabelled metabolites formed by cultured cells.
Radiolabelled metabolites in cell culture medium were extracted and analysed by LC-MS/MS with radioactivity detection. Relative quantities of individual metabolites were determined by measurement of peak height. 100% in the table = radioactivity present in cell culture medium = 98.2% of the administered 5 µM [2-^{14}C]quercetin-4'-glucoside. 0.2 % of the administered radioactivity was associated with the cells.

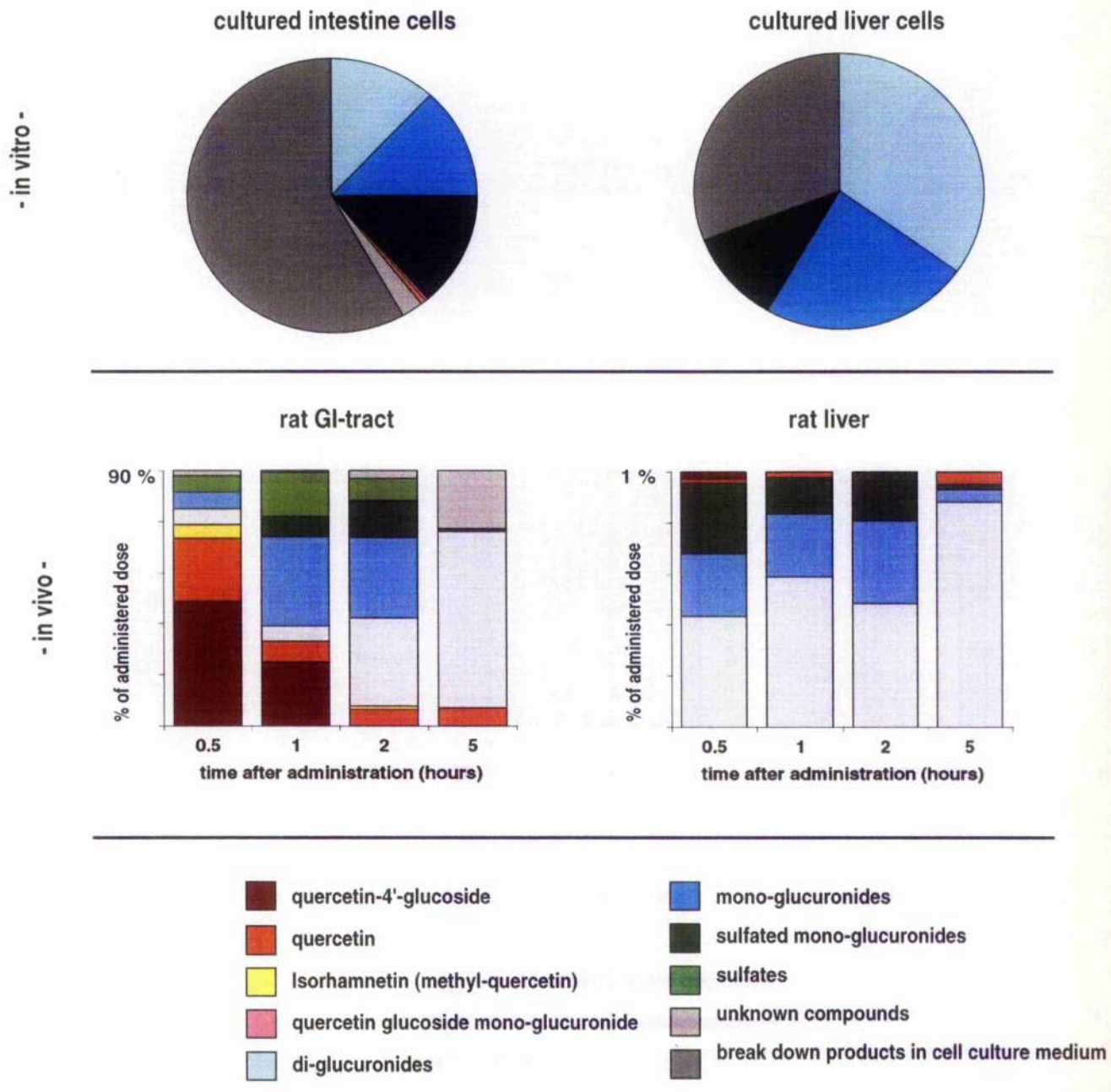


Figure 5: Metabolite classes formed by cultured cells and in rat tissues.
The graph summarizes relative quantities of formed metabolites and break down products formed by cultured cells and in rat GI-tract and liver. Radiolabelled compounds formed in cell culture and in the rat were classified into 10 major groups. Data of rat GI-tract and liver is taken from Section I, Figure 7.

Comparison of quercetin metabolites in cultured cells and in the rat

metabolites formed in rats	rat liver	liver cells	rat GIT	intestinal cells	metabolites formed in cultured cells	Identity of metabolites
rat 1			■			unknown
rat 2			■			unknown
rat 3	■	■	■	■	cells 1	Quercetin di-glucuronide
				■	cells 2	Methylated quercetin di-glucuronide
		■		■	cells 3	Quercetin di-glucuronide
rat 4	■		■	■	cells 4	unknown
rat 5	■	■	■	■	cells 5	Methylated quercetin di-glucuronide
rat 6	■	■	■		cells 6	Quercetin di-glucuronide
				■	cells 7	Glycosylated quercetin glucuronide
rat 7						Quercetin di-glucuronide [1]
				■	cells 8	Methylated quercetin mono-glucuronide sulfate
rat 8			■			Methylated quercetin di-glucuronide
rat 9	■					Quercetin di-glucuronide
		■		■	cells 9	Methylated quercetin di-glucuronide
rat 10	■	■	■	■	cells 10	Quercetin di-glucuronide
rat 11	■		■	■	cells 11	Quercetin di-glucuronide
rat 12	■	■	■	■	cells 12	Quercetin mono-glucuronide
rat 13	■		■			Quercetin mono-glucuronide sulfate
rat 14						Methylated quercetin di-glucuronide [1]
rat 15						Quercetin mono-glucuronide [1]
rat 16	■	■	■		cells 13	Methylated quercetin mono-glucuronide sulfate
rat 17	■	■		■	cells 14	Quercetin mono-glucuronide sulfate
rat 18	■		■			Quercetin-4'-glucoside
rat 19	■	■	■	■	cells 15	Methylated quercetin mono-glucuronide
rat 20	■	■	■		cells 16	Methylated quercetin mono-glucuronide
rat 21	■	■	■	■	cells 17	Methylated quercetin mono-glucuronide
rat 22			■			Quercetin mono-glucuronide
rat 23						Quercetin mono-glucuronide [1]
rat 24	■		■	■	cells 18	Quercetin
rat 25			■			Methylated quercetin sulfate
rat 26			■			Quercetin sulfate
rat 27			■			Isorhamnetin (methylated quercetin)

Table 6: List of quercetin metabolites formed by cultured cells and in the rat.
Comparison of metabolites formed by cultured cells (Table 3) and in the rat (Section I, Table 5).
[1] = metabolite was only found in plasma

■ metabolites found in rat liver and rat GI-tract (Section I, Table 5)

■ metabolites formed in cell culture by the rat liver cell line CC-1

■ metabolites formed in cell culture by the small intestinal cell line IEC-6

Radioactivity associated with the cells

Sample (cell type and confluence, incubation time with Q4'G)			radioactivity associated with the cells (dpm)	% of administered dose	quercetin-4'-glucoside equivalents (ng / 10^6 CC-1 cells)
IEC-6 cells	confluent	0.5 h	304	0.061	3.388
IEC-6 cells	confluent	2 h	319	0.064	3.554
IEC-6 cells	confluent	5 h	270	0.054	3.008
IEC-6 cells	confluent	72 h	675	0.135	7.522
IEC-6 cells	growing	72 h	300	0.060	3.344
CC-1 cells	confluent	0.5 h	578	0.116	4.601
CC-1 cells	confluent	2 h	421	0.084	3.348
CC-1 cells	confluent	5 h	639	0.128	4.537
CC-1 cells	confluent	72 h	570	0.114	4.537
CC-1 cells	growing	72 h	320	0.064	2.547
average			440	0.088	4.039

Table 7: Less then 0.2% of the administered radioactivity was associated with the cells.

Confluent or growing liver (CC-1) and intestinal cells (IEC-6) were incubated with 5 µM [2-^{14}C]quercetin-4'-glucoside. After 0.5, 2, 5 and/or 72 h cell culture medium was collected separately, cells were washed with PBS, trypsinised, suspended in 200 µl 20% (v/v) methanol and lysed by repeated freeze thawing cycles. The radioactivity in total samples was counted with a scintillation counter, and above values are the mean of two measurements. All cell culture incubations were carried out in singlicate.

4. Discussion

4.1. Quercetin metabolism *in vivo* and *in vitro*

The metabolites produced by liver and intestinal cells suggest that both cell types express the relevant metabolising enzymes: β-glycosidase, to release quercetin from its glucose adduct and glucuronyl-transferases (UGT's), sulfo-transferases (SULTs) and catechol-O-methyltransferases (COMT's) to conjugate free quercetin with methyl, sulfate or glucuronide units (Stahl et al, 2002). The synthesis of quercetin conjugates by enterocytes was independent of intestinal micro flora, as a similar pattern of metabolite types and isoforms were formed by cultured cells and in rat GI-tract.

Eleven of the 16 quercetin conjugates formed by cultured cells were identical with conjugates formed in rats (**Table 6**). Four of the five cell culture metabolites not identical with rat metabolites were new isoforms of metabolite types that had been found in the rat. Two main differences were observed between metabolite synthesis in the rat and by cultured cells: 1) Formation of numerous break down products of [2-^{14}C]quercetin-4'-glucoside due to its instability in cell culture medium, 2) All cell culture metabolites contained a glucuronide moiety, whereas rat GI-tract also contained quercetin and methyl-quercetin sulfates without a glucuronide adduct (**Figure 5**). However the presence of un-glucuronated quercetin sulfates in the GI-tract was transient, and quercetin sulfates were not observed at any time point in liver, kidney or plasma (Section I, Figure 7). Therefore, if the formation of break down products in cell culture medium can be controlled, quercetin metabolism by cultured cells appears to be comparable to that observed in the rat, and cell culture experiments may provide a meaningful, expedient and economical alternative to animal experiments.

4.2. Metabolite formation by liver and intestinal cells

Cultured intestinal and liver cells produce similar types and numbers of quercetin metabolites. The length of exposure of the cells to intact [2-^{14}C]quercetin-4'-glucoside may determine the quantity of formed metabolites. Liver cells converted 70%, and intestinal cells converted 40%, of the administered [2-^{14}C]quercetin-4'-glucoside into (an equal number of) phase II metabolites by 72 h. A higher rate of break down product

formation in cell culture medium for intestinal cells may have contributed to the lower overall quantity of formed metabolites in the intestinal cell culture.

In conclusion, the results confirm that intestinal epithelial cells are capable of metabolising quercetin-4'-glucoside, supporting the hypothesis that intestinal tissues may be a major site of quercetin metabolism.

4.3. Stability and availability of quercetin-4'-glucoside in cell culture

[2-^{14}C]quercetin-4'-glucoside was not stable in cell culture medium, and after 72 h it was completely decomposed into relatively polar compounds. Instability of quercetin and other flavonoids in cell culture medium and aqueous solutions has also been reported by Boulton et al, (1999), Kuo (1996) and Nordstroem (1968). Decomposition appeared to be slower in liver cell culture medium than in intestinal cell culture medium, probably due to a higher concentration of FCS (10% compared to 5% respectively) and supplementation with 1% (v/v) non-essential amino acids (NEAA). Serum proteins and supplemented amino acids may preserve quercetin-4'-glucoside from oxidation by becoming surrogate targets. Proteins, such as bovine serum albumin are commonly used in just this manner to stabilize small amounts of valuable enzymes or proteins (personal communication, Sigma, Technical Service, St. Louis, MO, USA) and amino acids, especially those with thiol moieties, have also been shown to function as antioxidants (Levine 1996).

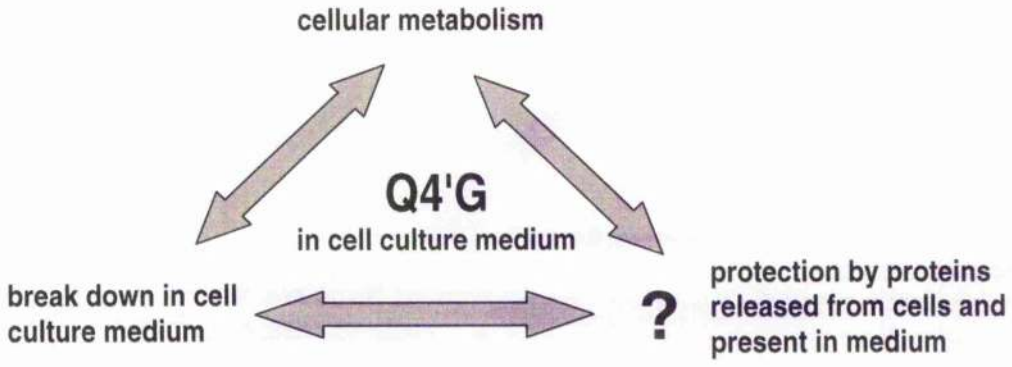


Figure 6: Fate of [2-^{14}C]quercetin-4'-glucoside in cell culture medium.
This figures summarizes the hypothesis, that the availability of quercetin-4'-glucoside to cultured cells depends on its degree of chemical break down in the culture medium, preservation against this break down and the formation of cellular metabolites.

The presence of cells themselves also appeared to have a protective effect. When cells were present, [2-^{14}C]quercetin-4'-glucoside was stable after 5 hour incubation in culture medium for intestinal cells, however when quercetin-4'-glucoside was incubated with medium alone, all [2-^{14}C]quercetin-4'-glucoside was broken down. This protective effect may have been caused by substrates which were secreted by the cells into the culture medium. Liver cells appeared to protect [2-^{14}C]quercetin-4'-glucoside better than cultured intestinal cells, possibly due to active secretion of protective compounds, such as extracellular superoxide dismutase (SOD) and other matrix proteins.

These results illustrate the complexity of the situation in cell culture medium, and emphasise the need to be aware that a cell culture model may include such confounding factors. Degradation of quercetin-4'-glucoside and other flavonoids will reduce the effective dose of the initial compound and observed cellular responses may be caused by a combination of break down products, cellular metabolites and/or the administered compound. It is also important to note that free quercetin aglycone was not found in cell culture medium at any time point. Because synthesized quercetin metabolites were present at 72 h, they appeared to be more stable compared with [2-^{14}C]quercetin-4'-glucoside.

4.4. Metabolite synthesis inside the cells ?

At all time points >90% of the administered radioactivity was present in cell culture medium, and only <0.2% was associated with the cells, independently of confluence level or cell type (**Table 7**, legend of Table 1). However, at 72 h 40% and 70% of the administered dose was found metabolised in the cell culture medium (of intestinal and liver cells respectively). We therefore investigated whether quercetin metabolites were formed in the cell culture medium rather then inside the cells. However, the presence of cells was essential for the formation of metabolites, suggesting that metabolites were either formed within the cells or on the cell surface. As metabolising enzymes are reportedly located in the cytosol (Donovan and Waterhouse, 2003; Duffel et al, 2001; Willitis et al, 2004). We hypothesize that metabolites are formed within the cells with quick subsequent export into cell culture medium.

Recent studies report the active export of flavonoid metabolites from cultured cells into culture medium and from intestinal tissue into intestinal lumen (Spencer et al, 2004; Crespy et al, 2003; Day et al, 2003) via multidrug-resistance-associated transporters (e.g.

MRP-1, MRP-2 and MDR-1) (Spencer et al, 2004; Walgren et al, 2000b; Walle et al, 1999).

The uptake of quercetin into cells has been reported by Kuo et al (1996) who observed quercetin accumulation in Caco-2 cells after exposure to 100 μM quercetin for 40 min using fluorescence microscopy (quercetin naturally fluoresces). This observation was confirmed four years later by Walgren et al (2000) after incubating Caco-2 cells for 15 min with 50 μM quercetin. Murota et al, (2000) detected quercetin metabolites in Caco-2 cells using HPLC-UV (after hydrolysis) and Salucci et al, 2002 reported the association of 0.5 ng quercetin with 10^6 Caco-2 cells after a 48 h incubation with 70 μM quercetin-3-glucoside. Our results are in the same order of magnitude with Salucci's findings, as 0.2% of the administered dose (associated with the cells, Table 7), corresponds to 4 ng quercetin/10^6cells. Thus evidence reported here and in the literature supports the conclusion that quercetin metabolites are formed inside the cells and then quickly excreted via active transport systems.

While metabolites may be formed within the cells, they don't appear to accumulate inside the cells (Spencer et al, 2004, Murota et al, 2003; Shirari et al, 2002). Recently it has been suggested that quercetin and other flavonoids may exhibit their bioactivity via interactions with membrane-phospholipids or membrane-receptors. If these theories hold true, the retention of quercetin and/or quercetin metabolites within the cells would therefore not be essential for their bioactivity (Shirai et al, 2002; Spencer et al, 2004).

4.5. Discussion of the applied methods

4.5.1. Recovery of radiolabelled metabolites

To compare *in vitro* and *in vivo* metabolite formation, metabolites in cell culture medium were extracted following the same extraction protocol as previously used for rat tissue samples. The extraction efficiency of radiolabelled compounds was 80% from cell culture medium and 50% and from rat tissues (**Table 1** and Section I, Table 1). The differences in recovery may be explained by an inverse correlation of the extraction efficiency and sample protein content, as quercetin and its metabolites have been reported to bind to proteins (Walle et al, 2003; Zsila et al, 2002; Boulton et al, 1998). Cell culture medium contains less protein compared with plasma and tissue samples, therefore the recovery of quercetin metabolites would be expected to be higher.

The detection of a relatively large number of novel quercetin metabolites in this study (Section I and Section II of this thesis), may be due to the customised extraction

procedure, which was tailored using the radiolabel as guide for the development of an extraction technique for unknown (but radiolabelled) compounds.

4.5.2. Reproducibility of the results

Due to the very limited availability of [2-^{14}C]quercetin-4'-glucoside, experiments were designed in singlicate and were directed at qualitative outcomes. However, similar results at different time points confirm the consistency of the reported observations.

4.5.3. Stability of [2-^{14}C]quercetin-4'-glucoside in culture medium

The addition of flavonoid aglycones or glycosides to cell culture medium is a commonly used procedure. Therefore, the extent of quercetin break down was not anticipated. Adjustment of the formulation of cell culture medium may ensure the stability of the compound under investigation. Also, if metabolite synthesis by different cell lines is to be directly compared it is advisable to use identical medium formulations, to exclude variations caused by different culture medium.

5. Conclusion

Cell culture experiments may provide useful model systems for the investigation of potential health effects of flavonoid metabolites *in vivo*, provided that cell-independent break down of the studied compound can be controlled or eliminated, and the compound under investigation is stable in culture medium for the duration of the experiment. Intestinal and liver cells formed similar types and numbers of quercetin metabolites, supporting the view that GI-epithelia significantly contribute to flavonoid metabolism. Liver cells however formed a larger quantity of metabolites, possibly due to a better stability of [2-^{14}C]quercetin-4'-glucoside in liver cell culture medium. From our results we can not conclude that metabolite formation occurred inside the cells, however, in accordance with recent publications our results support the hypothesis that quercetin metabolites are formed inside the cells with quick and efficient subsequent export into the cell culture medium.

Section IV:

Effect of quercetin and quercetin-4'-glucoside on cellular glutathione levels

Abstract

Background: Quercetin up-regulates glutathione levels *in vitro* and *in vivo*. This mechanism may partly explain the antioxidant activity of quercetin *in vivo*.

Objective: To investigate whether free quercetin and quercetin glycosides have a similar effect on intra-cellular glutathione concentration *in vitro*.

Design: Rat liver (CC-1) and small intestinal (IEC-6) cells were incubated with 5-100 μM quercetin, quercetin-4'-glucoside and H_2O_2, and intra-cellular glutathione levels were monitored from 3-72 h.

Results: Quercetin up-regulated intra-cellular glutathione in IEC-6 cells by 50%, but quercetin-4'-glucoside and H_2O_2 had no effect. In CC-1 cells, none of these treatments had an effect on glutathione concentration.

Conclusions: Free quercetin and glycosylated quercetin have different biological effects *in vitro*. Thus, the biological activity of free quercetin may not reflect that of the usual glycoslyated forms found in the diet.

Key words: glutathione-regulation, quercetin, quercetin-4'-glucoside, antioxidant-activity

1. Background and Aim

The antioxidant activity of dietary flavonoids is determined by the number and positioning of free hydroxyl groups on the flavonoid structure (Rice-Evans et al, 1996). However, quercetin appears in plasma and body tissues exclusively in the form of quercetin conjugates, where some of the functional hydroxyl groups are inactivated by the attachment of sulfate, methyl or glucuronyl moieties (Section I, Crespy et al, 2003). Thus, quercetin conjugates have a reduced potency to function as antioxidants (Begum and Terao, 2001). Nevertheless, *in vivo* studies reported a variety of antioxidant effects after ingestion of quercetin (Natarajan et al, 1999; Rathi et al, 1984). It is not clear how these *in vivo* antioxidant effects were achieved, although it has been hypothesized that quercetin metabolites may affect the pro- and anti-oxidant balance by interacting synergistically with other endogenous or exogenous antioxidants via mechanisms that do not involve stoichiometric antioxidant quenching (Milbury et al, 2002; Sciuto, 1997).

In recent years, it has been reported that cultured cells responded to quercetin exposure with up-regulation of the intra-cellular glutathione concentration (Cipak et al, 2003; Scharf et al, 2003; Isighe et al, 2001). Animal studies also showed that glutathione levels in tissues were up-regulated after oral ingestion of quercetin and rutin (Cruz et al, 1998; Martin et al, 1998, Gandhi and Khanduja, 1993). Glutathione is a small intracellular tri-peptide, consisting of three amino acids (γ-glutamyl-cysteinyl-glycine), and unlike most other peptides and proteins it is synthesized enzymatically via the γ-glutamyl cycle. Glutathione is the most abundant antioxidant in cells, and plays a major role in the cellular defense against oxidative stress (Deleve and Kaplowitz, 1990; Meister and Anderson, 1983). It can directly scavenge free radicals (Kalyanaraman et al, 1996) or act as a substrate for glutathione peroxidase and glutathione-S-transferases during the detoxification of electrophilic compounds (Armstrong et al, 1997; Ursini et al, 1995). If dietary quercetin and/or its *in vivo* metabolites could modulate the cellular homeostasis of pro- and anti-oxidants via the up-regulation of glutathione synthesis, this could provide an explanation for the antioxidant mechanism of glycosylated quercetin and its metabolites *in vivo*.

The aim of this study was to investigate whether dietary quercetin-4'-glucoside triggers an increase of cellular glutathione similar to that previously reported for free quercetin. As the GI-tract and the liver are the primary organs exposed to dietary

quercetin, small intestinal epithelial cells (IEC-6) and liver epithelial cells (CC-1) were used to investigate the effect of quercetin-4'-glucoside on intra-cellular glutathione levels.

2. Materials and Methods

2.1. Cells and materials

Small intestinal epithelial cells (IEC-6) and liver epithelial cells (CC-1) were maintained using materials and equipment as previously described. DTNB (5,5'-Dithio-bis[2 nitro benzoic acid] or Ellman's Regagent), glutathione, glutathione reductase, H_2O_2, NADPH (β-nicotinamide adenine dinucleotide phosphate in reduced form) and quercetin were purchased from Sigma, (Poole, Dorset, U.K.). Quercetin-4'-glucoside was obtained from Rotec Scientific Ltd. Milton Keynes, U.K. Working solutions for the glutathione assay were made up as follows: "Buffer B (1%)" and "Buffer B (0.1%)": 67 mM potassium di-hydrogen orthophosphate solution and 83 mM di-sodium hydrogen orthophosphate solution were combined at a ratio of 1.64:8.36 and 5 mM EDTA was added. This mixture had a pH of 7.5, if not, it was adjusted accordingly. Then either 1% or 0.1 % (w/v) 5-sulfosalicylic acid was added. "NADPH solution": Buffer B (0.1%) containing 0.5 mM NADPH and 1.5 mM DTNB.

2.2. Cell viability and growth inhibition after exposure to flavonols and H_2O_2

Rat small intestinal cells (IEC-6) or rat liver cells (CC-1) were seeded into 6 well plates at 3 x 10^4 cells/ml medium. After overnight incubation, the medium was removed and replaced with fresh medium containing 5, 10, 20, 50 or 100 μM quercetin, quercetin-4'-glucoside or H_2O_2. Ethanol was used to dissolve quercetin and the final ethanol concentration in all treatments was 0.25% (v/v). After 3 days incubation, the medium was removed, and the cells were detached from the bottom of the 6 well plates using 0.5 ml 1 x trypsin/EDTA for 10 min at 37 °C. Then 0.75 ml culture medium containing FCS was added to stop the enzymatic reaction. The cell-media suspension was mixed, transferred into a microtube and the well was washed with 0.75 ml medium, which was collected into the same microtube. To assess the number of viable cells 50 μl trypan blue was added to an equal volume of cell suspension and an aliquot of the dyed

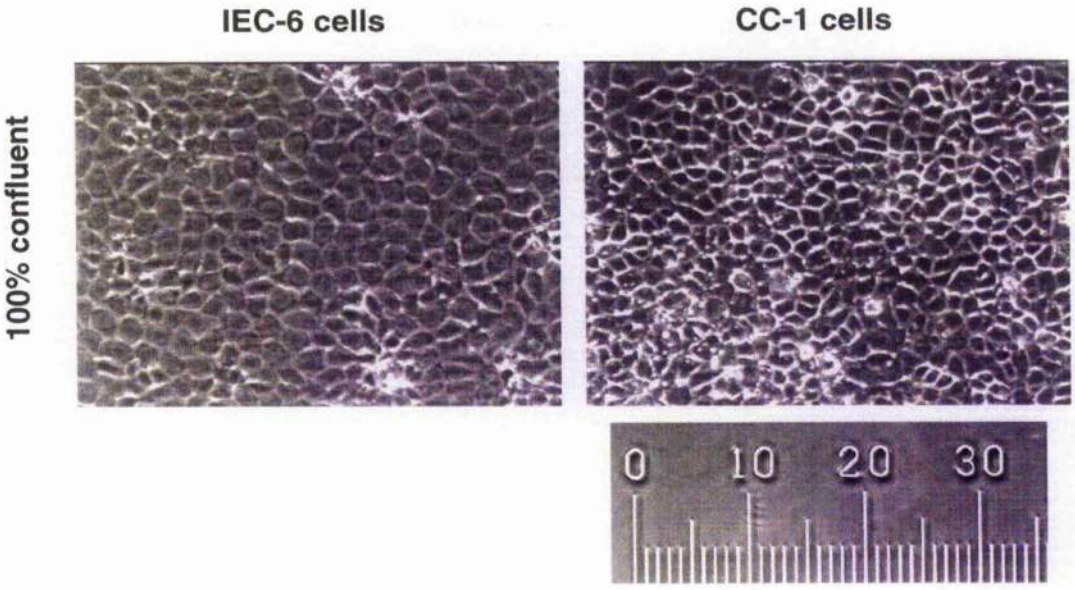


Figure 1: IEC-6 and CC-1 cells.
Cultured rat small intestine (IEC-6) and rat liver (CC-1) cells were visualized at 20 x magnification using a IMT-2, Olympus phase contrast microscope (Optical Co. Ltd, Japan). The pictures were taken with a XC-57CE digital camera (Sony Japan) and Amigo Dazzle Multimedia software. To depict the size of the cells, a 1mm graticule was photographed under the same magnification as the cells (Graticule Ltd, Tonbridge, Kent, England, 100 x 0.01 = 1mm).

cell suspension was transferred to a Neubauer haemocytometer. According to Freshney et al (1986) cells with damaged membrane integrity stain blue, whilst viable cells remain unstained and appear white when viewed under the microscope. In this assay the number of unstained white cells was counted on four haemocytometer squares, and the number of viable cells per 1 ml cell suspension was determined. Each experiment was carried out in duplicate, and each sample was counted twice.

Using the trypan blue assay, both the cytotoxicity and growth inhibition were assessed, which are both reflected by the number of viable cells. In this study, the glutathione content of all cells in one well was compared with the glutathione content of cells from different wells. Therefore it was essential to keep the number of cells per well constant, as a different number of cells would have resulted in a different quantity of glutathione per well.

For subsequent experiments only treatments were used which had no significant effects on cell viability or growth (viability >80%) (**Table 1**). The ethanol vehicle used to solubilize quercetin and quercetin-4'-glucoside had no effect on growth or viability in both cell lines (**Table 2**).

Cell Viability

	% of cells found to be viable	
	IEC-6 cells	CC-1 cells
control	100 %	100%
ethanol control	105 %	102 %
5 µM Q4'G	104 %	78 %
10 µM Q4'G	109 %	101 %
20 µM Q4'G	-	82 %
50 µM Q4'G	97 %	22 %
100 µM Q4'G	83 %	5 %
5 µM Quercetin	105 %	104 %
10 µM Quercetin	105 %	93 %
20 µM Quercetin	108 %	72 %
50 µM Quercetin	77 %	38 %
100 µM Quercetin	35 %	10 %
5 µM H_2O_2	95 %	95 %
10 µM H_2O_2	100 %	102 %
20 µM H_2O_2	97 %	102 %
50 µM H_2O_2	23 %	98 %
100 µM H_2O_2	0 %	43 %

Table 1: Cell viability.
Freshly seeded CC-1 or IEC-6 cells were incubated with quercetin-4'-glucoside (Q4'G), quercetin and H_2O_2 for 72 h. Ethanol control and all flavonol treatments contained 0.25% (v/v) ethanol. The result (% viability) is the mean of two experiments which were both counted in duplicate.

2.3. Experiments and sample collection

IEC-6 and CC-1 cells were seeded into 6 well plates at a concentration of 3 x 10^4 cells/cm^2. At confluence (after 3 days) the media was removed and fresh media containing 5-100 µM quercetin-4'-glucoside, 5-20 µM quercetin or 10-50 µM H_2O_2 was added. Quercetin and quercetin-4'-glucoside were dissolved in ethanol and further diluted directly in the cell culture media. The ethanol concentration was kept constant at 0.25% (v/v) throughout all pilot experiments and at 0.05% (v/v) throughout all subsequent experiments. The relatively high ethanol concentration was unavoidable, since 0.5 g quercetin dissolves in 35 ml ethanol, and a final media concentration of 100 µM quercetin was required. However, the ethanol vehicle had no effect on inter-cellular glutathione levels or cell viability (p=0.64 for IEC-6 cells and p=0.52 for CC-1 cells) (**Table 1** and **Table 2**).

After 3, 6, 12, 24, 48 and 72 h the cell culture media was removed and cell extracts were obtained by incubating the cell monolayer for 20 min with 900 µl 1% 5-

sulfosalicylic acid. The cell extract was diluted in "Buffer B (1%)" by 1:3, and stored at – 80 °C until analysis. Initially the media was examined for its glutathione content, but since no glutathione was present in the cell culture media in all subsequent experiments only the cell extract was analysed.

	intra-cellular glutathione (in nmol / well)	
	untreated cells	0.25% (v/v) ethanol
IEC-6 cells	53.33 ± 6.12	51.12 ± 6.36
CC-1 cells	108.46 ± 7.22	104.94 ± 7.40

Table 2: Ethanol vehicle has no influence on intra cellular glutathione level.
IEC-6 and CC-1 cells were grown to confluence in 6 well plates, and incubated with no or 0.25% (v/v) ethanol for 72 hours. Results are the mean of 4 separate experiments (±SD). One cell culture well had a surface area of 9.6 cm^2, containing 177 ± 18 x 10^4 IEC-6 cells or 269 ± 34 x 10^4 CC-1 cells at confluence. (p=0.64 for IEC-6 cells and p=0.52 for CC-1 cells).

2.4. Glutathione assay

2.4.1. Assay principle

Total glutathione was measured using the enzymatic colorimetric recycling assay devised by Tietz 1969, with modifications described in Punchard et al (1994). The assay is based on the oxidation of glutathione (GSH) by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and resulting oxidised glutathione (GSSG) is reduced by NADPH in the presence of glutathione reductase. The rate of 2-nitro-5-thiobenzoic acid formation from DTNB is monitored at 410 nm over 10 minutes, and is proportional to the total glutathione concentration (GSH and GSSG) of the samples. The quantity of total glutathione per sample was determined by reference to a standard curve.

2.4.2. Assay protocol

An aliquot of 50 µl of the diluted samples was transferred to a flat-bottomed 96 well plate already containing standards at a concentration of 1-10 µM glutathione (1-10 nmol/ml or 0.05 – 0.5 nmol/50 µl) and two quality control samples. To the samples, standards and quality controls, 100 µl of 0.5 mM NADPH (containing 1.5 mM DTNB in "Buffer B (0.1%)") was added. The enzymatic reaction was initiated by the addition of 50 µl 1 U/ml glutathione reductase to all wells excluding the blank. The reaction was monitored at 410 nm over 10 minutes using a Dynatech Microplate Reader (MR 5000,

Guernsey, Channel Islands). Different concentrations of 5-sulfosalicylic acid affected the glutathione measurements, therefore glutathione standards were made up to contain the same 5-sulfosalicylic acid concentration as the samples.

Quality control samples were prepared from confluent IEC-6 cells, by obtaining a cell extract as described above, which was then aliquoted and stored at -80 °C. The detection minimum was 1 µM total glutathione or 0.05 nmol/50 µl analysed sample aliquot.

2.5. Statistical analysis

Results were analysed using the software STATGRAPHICS Plus for Windows 4.0. One-way analysis of variance (ANOVA) was applied to determine whether there was a significant difference between treatments and control experiments. If the p value obtained was smaller than 0.05, the statistical difference between individual groups was determined using the method of Fisher's protected least significant difference (LSD).

As common for bioassays, the results had considerable within and between "batch" variation (**Table 3**). "Batch" was defined as a cluster of cell culture experiments that were seeded and analysed at the same time. All results were always compared to the appropriate control experiment within the same "experimental batch".

Intra and inter experiment variation
of intra-cellular glutathione levels (in nmol/cell culture well)

	mean	intra assay variation	inter assay variation
untreated IEC-6 cells	47.19	3.03 (6.4 %)	6.90 (14.6 %)
untreated CC-1 cells	103.47	7.16 (6.9 %)	15.37 (14.9 %)

Table 3: inter and intra assay variation.
The cellular glutathione concentration in untreated CC-1 and IEC-6 cells (nmol/well) is given as the mean of ALL experiments. The intra and inter assay variation was calculated using ANOVA and the method of Fischer's protected least significant differences.

3. Results

3.1. Effect of quercetin and quercetin-4'-glucoside on cellular glutathione levels

3.1.1. Pilot experiments (n=3)

IEC-6 and CC-1 cells were incubated with various concentrations of quercetin, quercetin-4'-glucoside and H_2O_2 and levels of intra-cellular glutathione were determined after 3, 6, 12, 24, 48 and 72 hours. In both cell lines, no change of intra-cellular glutathione was measured after incubation with quercetin-4'-glucoside. However, glutathione levels were up-regulated by 30% after exposing IEC-6 cells to 20 µM quercetin for 24 hours (p=0.0011) (**Table 4**). No change of the intra-cellular glutathione concentrations was observed when CC-1 cells were incubated with quercetin.

Treatment with 20 µM H_2O_2 for 6 h increased the intracellular glutathione content in CC-1 cells by 10% (p=0.014). However when IEC-6 cells were exposed to the same dose no effect was measured.

3.1.2. Main experiments (n=9)

The result of the pilot experiments was confirmed by repeat experiments using a greater sample size (n=9). IEC-6 cells responded to 24 hour incubation with 10 and 20 µM quercetin with a dose dependent increase of intra-cellular glutathione of 17% ± 9% and 49% ± 13% (p=0.0001) (**Figure 2** and **Table 5**). However, when IEC-6 cells where treated with 20 µM quercetin-4'-glucoside or 20 µM H_2O_2 for the same length of time (24 hours), no change of the glutathione level was observed.

CC-1 cells showed no significant change of intra-cellular glutathione levels after the 24 hour treatment period (**Figure 2** and **Table 5**).

Pilot experiments: Intra-cellular glutathione concentration (in nmol / well) (n=3)

IEC-6 cells

experiments	3 h	6 h	12 h	24 h	48 h	72 h
untreated	49.88 ± 4.29	57.40 ± 0.64	63.87 ± 4.74	50.04 ± 2.18	54.06 ± 3.66	53.61 ± 3.46
5 µM Q	-	-	-	**55.62 ± 1.97** [1]	55.01 ± 4.25	52.92 ± 3.43
10 µM Q	-	-	-	-	-	-
20 µM Q	47.58 ± 2.35	62.14 ± 1.84	67.86 ± 4.41	**64.28 ± 0.05** [1]	59.65 ± 2.19	48.96 ± 3.81
untreated	44.68 ± 2.69	47.31 ± 4.03	54.02 ± 4.00	42.82 ± 2.22	42.71 ± 3.60	44.49 ± 1.60
5 µM Q4'G	42.87 ± 4.84	46.48 ± 3.74	55.04 ± 4.21	40.02 ± 4.16	43.82 ± 2.22	42.13 ± 7.05
10 µM Q4'G	41.70 ± 5.81	46.92 ± 0.93	53.97 ± 4.23	43.23 ± 1.25	44.01 ± 1.96	43.18 ± 1.74
20 µM Q4'G	47.20 ± 3.33	47.40 ± 8.86	54.48 ± 1.14	44.67 ± 1.73	42.82 ± 2.53	45.34 ± 1.94
50 µM Q4'G	44.50 ± 3.68	41.97 ± 3.20	52.90 ± 2.60	45.60 ± 2.00	42.94 ± 1.96	38.43 ± 5.28
100 µM Q4'G	41.58 ± 3.18	45.15 ± 1.52	58.94 ± 2.99	47.29 ± 3.47	41.91 ± 6.42	43.92 ± 2.52
untreated	39.04 ± 2.05	39.35 ± 2.92	40.62 ± 2.45	42.59 ± 1.42	45.12 ± 3.06	49.99 ± 2.03
5 µM H_2O_2	-	-	-	-	-	-
10 µM H_2O_2	36.47 ± 2.76	41.74 ± 2.10	40.14 ± 4.14	42.88 ± 0.89	42.54 ± 2.77	46.42 ± 3.73
20 µM H_2O_2	39.25 ± 1.99	42.71 ± 1.60	42.03 ± 3.22	43.94 ± 1.82	44.12 ± 0.36	49.29 ± 1.76
50 µM H_2O_2	R.V.	R.V.	R.V.	R.V.	R.V.	R.V.

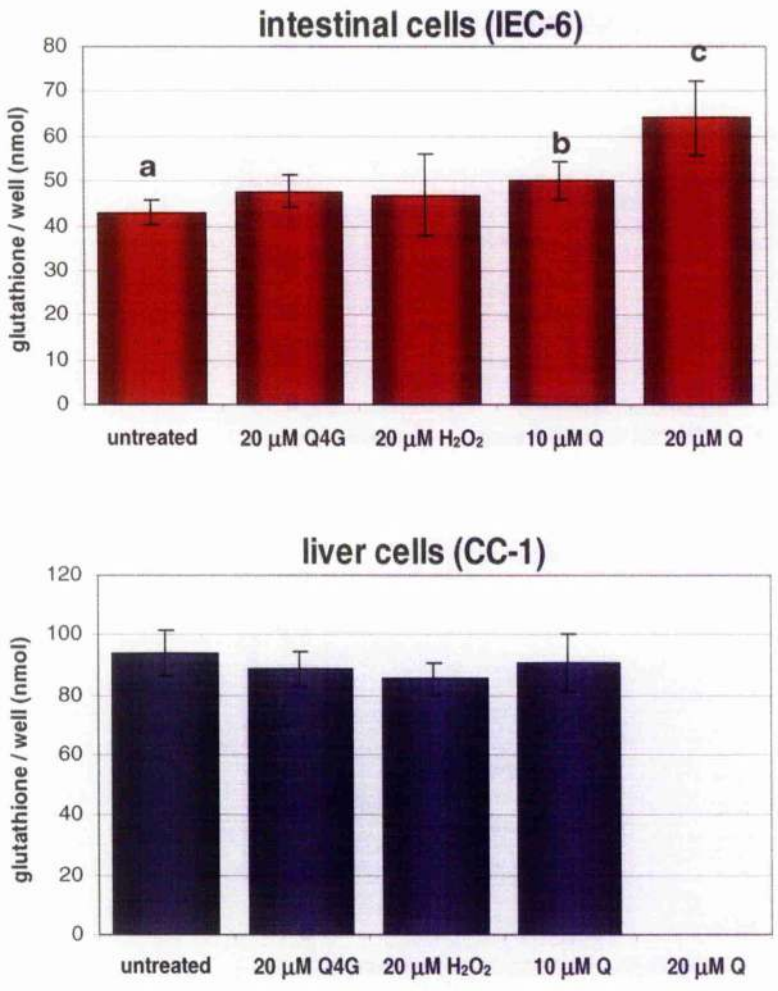
CC-1 cells

experiments	3 h	6 h	12 h	24 h	48 h	72 h
untreated	87.32 ± 9.17	106.30 ± 8.86	109.81 ± 7.92	112.21 ± 6.53	104.55 ± 7.59	104.48 ± 11.11
5 µM Q	-	-	-	-	93.99 ± 5.33	98.77 ± 7.49
10 µM Q	95.75 ± 5.72	121.23 ± 3.74	108.16 ± 4.51	100.21 ± 5.72	-	-
20 µM Q	R.V.	R.V.	R.V.	R.V.	R.V.	R.V.
untreated	97.49 ± 2.55	114.00 ± 2.10	129.11 ± 5.62	103.55 ± 3.16	108.55 ± 7.73	96.67 ± 14.45
5 µM Q4'G	92.88 ± 4.54	106.59 ± 7.89	124.28 ± 4.74	103.05 ± 1.28	112.53 ± 6.17	82.59 ± 8.01
10 µM Q4'G	84.51 ± 8.81	111.02 ± 8.67	129.05 ± 8.76	103.89 ± 7.19	109.81 ± 5.11	98.80 ± 1.42
20 µM Q4'G	100.17 ± 3.86	108.94 ± 5.49	125.06 ± 9.18	107.40 ± 5.29	114.97 ± 1.22	84.00 ± 7.43
50 µM Q4'G	R.V.	R.V.	R.V.	R.V.	R.V.	R.V.
100 µM Q4'G	R.V.	R.V.	R.V.	R.V.	R.V.	R.V.
untreated	73.84 ± 5.04	76.71 ± 1.30	122.31 ± 1.39	118.57 ± 9.02	105.71 ± 4.20	94.85 ± 4.85
5 µM H_2O_2	-	-	-	-	-	-
10 µM H_2O_2	64.80 ± 6.58	73.26 ± 2.05	120.67 ± 4.64	112.15 ± 4.02	98.25 ± 7.45	89.38 ± 3.51
20 µM H_2O_2	70.10 ± 3.21	**84.43 ± 3.49** [2]	117.43 ± 2.86	112.28 ± 6.58	94.52 ± 4.87	90.58 ± 5.28
50 µM H_2O_2	76.78 ± 3.39	**88.03 ± 4.57** [2]	124.04 ± 5.08	116.11 ± 6.45	111.99 ± 4.13	94.35 ± 6.52

Table 4: Intra-cellular glutathione in intestinal (IEC-6) and liver (CC-1) cells after treatment with quercetin, quercetin-4'-glucoside and H_2O_2.

Confluent monolayers of IEC-6 and CC-1 cells were cultured in 6 well plates and incubated with quercetin (Q), quercetin-4'-glucoside (Q4'G) and H_2O_2. At 3, 6, 12, 24, 48 and 72 hours the glutathione concentration in the cell extract was determined in nmol/well ±SD (n=3).

Due to large inter-assay variations, all results were compared to the appropriate control (untreated cells) from the same "experiment batch". Comparison is only possible within one experimental batch, e.g. dose comparison is possible, but comparison between different time points is not possible. Statistical differences between treatments and the untreated control (of the same "experimental batch") were calculated as described in materials and methods and are highlighted in the table: [1] p= 0.0011, [2] p= 0.014. R.V.= reduced viability: these concentrations were not used in this experiment as they affected the viability and growth rate of the cells (Table 1)..



	IEC-6 cells	CC-1 cells
	24 h	24 h
untreated	42.92 ± 2.75 a	93.81 ± 7.65
20 µM Q4G	47.62 ± 3.73	88.87 ± 5.79
20 µM H_2O_2	46.83 ± 9.07	85.43 ± 5.40
10 µM Q	50.11 ± 4.33 b	90.53 ± 9.55
20 µM Q	64.05 ± 8.42 c	R.V.

Figure 2 with Table 5: Glutathione concentration in intestinal (IEC-6) and liver (CC-1) cells after incubation with quercetin, quercetin-4'-glucoside and H_2O_2.
Confluent monolayers of IEC-6 and CC-1 cells were cultured in 6 well plates and incubated with quercetin (Q), quercetin-4'-glucoside (Q4'G) and H_2O_2. After 24 h intracellular glutathione concentrations were determined (in nmol /well). Results are the mean of 9 experiments (±SD). Statistical differences between treatments are indicated with "a", "b" and "c" ($p = < 0.0001$). R.V.= reduced viability: this concentration was not used since it affected the viability and growth rate of the cells (Table 1).

4. Discussion

IEC-6 and CC-1 cell monolayers contained 53±6 and 108±7 pg total glutathione (GSH and GSSG) per culture well (9.6 cm^2), which compares to 301±34 and 403±27 pg total glutathione in 10^4 cells, respectively. CC-1 cells contained 33% more glutathione compared with the IEC-6 cells, in agreement with previous reports that liver cells contain higher levels of glutathione compared with other cells (Locigno and Castronovo, 2001; Sciuto, 1997).

4.1. Quercetin, but not quercetin-4'-glucoside, up-regulated intra-cellular glutathione in IEC-6 cells

Quercetin and quercetin-4'-glucoside had different effects on the regulation of intra-cellular glutathione levels in IEC-6 cells. Combining the results from this Section with our previous results on the stability, metabolism and break down of [2-^{14}C]quercetin-4'-glucoside (Section III, paragraph 4.3), none of the compounds (unchanged quercetin-4'-glucoside, quercetin metabolites or decomposition products of quercetin-4'-glucoside) in the cell culture medium after the addition of quercetin-4'-glucoside affected the regulation of glutathione in IEC-6 cells. Free quercetin however increased cellular glutathione levels by 50%, suggesting that after application of quercetin to cell culture medium, either quercetin itself or derived bioactive metabolites caused the up-regulation of glutathione (**Figure 3**).

De-novo glutathione synthesis is primarily determined by increased expression of the two subunits of the key enzyme, glutamate cysteine ligase (GCL) formerly known as γ-glutamyl-cysteine-synthetase (γ-GCS) (Dickinson and Forman, 2002, Rahman and MacNee, 2002). One major factor for the up-regulation of this enzyme is oxidative stress (Rahman and MacNee, 2002). Recently it has been shown that quercetin can be oxidised *in* cell culture environments forming quercetin-semi-quinone radicals, H_2O_2 and $O_2^{\bullet}$ (Awad et al, 2002; Long et al, 2000; Awad et al, 2000, Metodiewa et al, 1999). Therefore, *de novo* synthesis of glutathione in IEC-6 cells may have been up-regulated by oxidative stress due to the formation of quercetin-quinones. DMNQ, a quinone which continuously generates H_2O_2 and $O_2^{\bullet}$ through redox cycling, similar to quercetin, was shown to elevate intra-cellular glutathione levels and upregulate activity, protein and mRNA content of γ-GCS, in rat lung epithelial cells (Shi et al, 1994).

In conclusion, cultured intestinal cells (IEC-6 cells) up-regulated intra-cellular glutathione after quercetin was added to the cell culture medium. However, addition of quercetin-4'-glucoside had no significant effect on glutathione levels. From our results, it is not obvious why quercetin and glycosylated quercetin had such profoundly different effects. However, it has been suggested, that contact of quercetin with culture medium can result in the formation of free radicals, which could have an effect on glutathione synthesis.

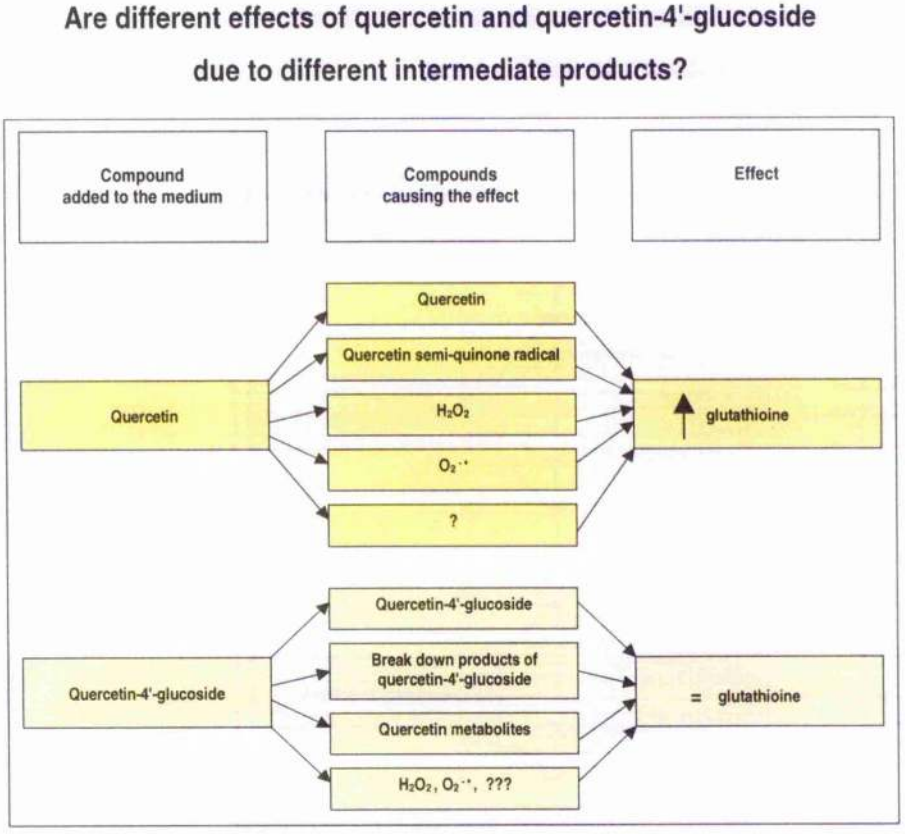


Figure 3: Different effects of quercetin and quercetin-4'-glucoside on IEC-6 cells. Quercetin treatment increased intracellular glutathione levels by 50%, whereas treatment with quercetin-4'-glucoside had no effect. The figure summarizes the hypothesis that different effects may be caused by different intermediate products of quercetin and quercetin-4'-glucoside.

4.2. Effect of quercetin and quercetin-4'-glucoside on intra-cellular glutathione of CC-1 cells

After exposure to quercetin, cultured liver cells (CC-1) did not up-regulate intra-cellular glutathione in a similar way as IEC-6 cells. The reasons for this are unclear and were not further investigated in this study. However, if the up-regulation of glutathione was triggered by increased oxidative stress, then higher concentration of serum (FCS) and amino acids (NEAA) in liver cell culture medium may have buffered pro-oxidative effects caused by reactive quercetin molecules. As previously discussed (Section III, paragraph 4.3), proteins and amino acids, particularly those with thiol groups, can function as antioxidants *in vivo* and *in vitro* (Acworth et al, 1997; Levine et al, 1996; Gutteridge et al, 1994; Ross, 1988). Therefore the different composition of the two cell culture mediums may explain the differential effects of quercetin on glutathione regulation in CC-1 and IEC-6 cells. The up-regulation of intra-cellular glutathione γ-glutamyl-cysteine-synthetase (the key enzyme for glutathione synthesis) has been reported in another liver cell line, HepG2, after incubation with 10-50 μM quercetin (Scharf et al, 2003).

4.3. Effect of H_2O_2 on intra-cellular glutathione

According to stoichiometry, a maximum of 20 μM H_2O_2 could have been formed in cell culture medium during oxidation of 20 μM quercetin (Award et al, 2000). We therefore tested the effect of H_2O_2 on the regulation of intracellular glutathione in our cell culture model. However, the addition of 10 and 20 μM H_2O_2 did not trigger significant up-regulation of intra-cellular glutathione levels in CC-1 and IEC-6 cells (after 24 hours). Therefore if glutathione up-regulation in IEC-6 cells was caused by oxidative stress, the other two reactive molecules, quercetin-semi-quinone radicals and superoxide ($O_2^{\bullet}$), may be responsible. However, quercetin may also trigger the up-regulation of intra-cellular glutathione via other pathways, e.g. via modulation of cytokine expression (Nair et al, 2002).

4.4. Discussion of the applied methods

4.4.1. Inter assay variation

The variation of intra-cellular glutathione levels in untreated "control" cells was significant and ranged from 43-63 nmol/well and 87-112 nmol/well for IEC-6 and CC-1 cells respectively (**Table 4** and **Table 5**). These variations occurred when different batches of cells were seeded on different days and may be due to slightly varying confluence levels e.g. different numbers of cells in one well. All experiments were carried out at 95-100% confluence, determined by visual observation using phase contrast microscopy (**Figure 1**). However, more consistent results may have been achieved, if the exact cell number per well would have been determined, e.g. by total protein determination per well. Due to the high inter assay variation, the obtained results were always compared to the appropriate control within the same "experimental batch".

4.4.2. Toxicity and growth inhibition of quercetin and quercetin-4'-glucoside in cell culture

Intestinal cells (IEC-6) proved to be more resistant against incubation with quercetin and quercetin-4'-glucoside in comparison with rat liver cells (CC-1). In both cell lines, free quercetin had a greater effect on cell viability compared with quercetin-4'-glucoside. Conjugated quercetin may be less reactive than free quercetin (Boersma et al, 2001; Ioku et al, 1995), and this may be the reason why plant and animal organisms contain the "reactivity" of quercetin by conjugating it with sugar, glucuronyl, sulfate and methyl moieties.

4.5. Conclusion

The regulation of intra-cellular glutathione was "compound specific" and "tissue specific": Incubation with quercetin-4'-glucoside had no effect on intra-cellular glutathione levels in both liver and intestinal cells (CC-1 and IEC-6 cells). Incubation with quercetin up-regulated intra-cellular glutathione in IEC-6 cells by 50%, but had no effect in CC-1 cells. These results indicate that free quercetin and glycosylated quercetin have different biological effects in cell culture.

Final comments and conclusions

1. Quercetin bioavailability

To explore potential health effects of dietary quercetin it is crucial to obtain reliable data on its absorption and subsequent availability at target tissues. Estimates of the absorption rate of quercetin from the GI-tract, published in the last 10 years, range from <0.1% to 50%. Therefore, in this study the dynamics of quercetin absorption, metabolite formation and distribution throughout body tissues and plasma was investigated using radiolabelled quercetin-4'-glucoside in an animal model. Our results indicate that ~10% of ingested dietary quercetin is absorbed in rats. It appears that quercetin absorption was previously both significantly over and underestimated, probably because *in vivo* metabolism of quercetin was not known or not considered. As detailed in Section I (paragraph 4.1), Hollman's and Walle's conclusion that 50% of the ingested dietary quercetin was absorbed from the GI-tract may have been an overestimation, because quercetin may have "disappeared" from the ileostomy fluid not because it was absorbed, but because it was methylated. Our data also provides evidence that quercetin bioavailability was previously underestimated. Plasma quercetin is usually quantified after releasing free quercetin from its conjugation with glucosyl, glucuronyl and sulfuryl moieties by acid or enzymatic hydrolysis. This method however does not cleave methyl groups from the quercetin molecule (Hollman et al, 1997). In our study 70% of plasma quercetin was found to be methylated (Section I, Table 9). If after the hydrolysis process, only free *quercetin* is quantified, all *methylated* quercetin remains un-accounted for, resulting in a significant underestimation of plasma quercetin, e.g. in our experiment, plasma quercetin would have been underestimated by 70%.

Another confounding factor in determining the true plasma concentration of absorbed flavonoids is the recovery rate of quercetin or its metabolites during the extraction process. It is evident that both the extraction solvent and technique determines the extraction efficiency (Section I, Figure 2), and low findings of plasma metabolites in previous studies may be due to inadequate extraction techniques. Internal standards, added to plasma samples before extraction, may not reflect the true extraction efficiency, as 1) virtually all plasma quercetin is metabolised (Section I) and 2) quercetin in plasma is bound to plasma proteins (Walle et al, 2003; Zsila et al, 2002, Boulton et al, 1998).

Thus, reported plasma quercetin values reflect a combination of the plasma quercetin concentration AND the effectiveness of the extraction technique.

➡ Quercetin absorption and bioavailability was frequently under or over-reported. From our results we conclude that 5 - 10% of a dietary dose of quercetin is absorbed into the systemic circulation.

2. Consequences of pre-systemic metabolism of quercetin

From our results, and a number of recent reports (Crespy et al, 2003; Cermak et al, 2003), we conclude that most of the ingested dietary quercetin is metabolised directly in the GI-tract (Section I, paragraph 4.2, Section III, paragraph 4.2). The physiological purpose of pre-systemic metabolism (e.g. metabolism before entry into systemic circulation) is the reduction of toxicity and quick excretion of potentially toxic substances. Thus, dietary quercetin is treated *in vivo* like a potentially harmful substance.

Only GI-tissues appear to be exposed to significant quantities of dietary quercetin and its metabolites. Because intestinal epithelia are in direct contact with the potential antioxidant, it is possible that dietary quercetin has an effect on physiological or pathological processes in GI-tissues and on GI-micro flora. In some animal studies, quercetin intake has reduced the incidence and development of colon cancer (Deschner et al, 1991; Yang et al, 2000).

➡ GI-tissues appear to be the "primary target organ" for potential bioactive effects of ingested dietary quercetin or its *in vivo* metabolites. Further research is needed to elucidate the potential of dietary quercetin to contribute to intestinal health.

3. Biological relevance of quercetin metabolites

Orally ingested quercetin is extensively metabolised in the body by two distinct pathways: 1) phase II metabolism in body tissues and 2) ring fission and decomposition into phenolic acids by colonic micro flora (**Figure 1**). The work presented in this thesis (Section I and II) suggests that phase II metabolism may be predominant in the immediate postprandial phase, and phenolic acids appear later due to their formation by micro flora in the large bowel. Absorbed quercetin appears in body tissues, plasma and

urine almost exclusively in the form of phase II and phenolic acid metabolites, suggesting that *in vivo,* health effects are caused by metabolites, not the ingested quercetin glycosides or free quercetin.

However, it is also possible that phase II metabolites of quercetin act as quercetin carriers or pro-drugs, which are transformed into active compounds when needed. Very recent research suggests that many body tissues are capable of effectively hydrolysing quercetin glucuronides (O'Leary et al, 2003; O'Leary et al, 2001), releasing free quercetin from its conjugation adducts (sulfate, methyl and glucuronyl groups). Thus, quercetin metabolites may be hydrolysed under certain circumstances, e.g. during inflammation (Shimoi et al, 2001).

➡ To date, the biological relevance of quercetin metabolites is still unclear, and further research is needed to clarify whether and how quercetin metabolites contribute to human health.

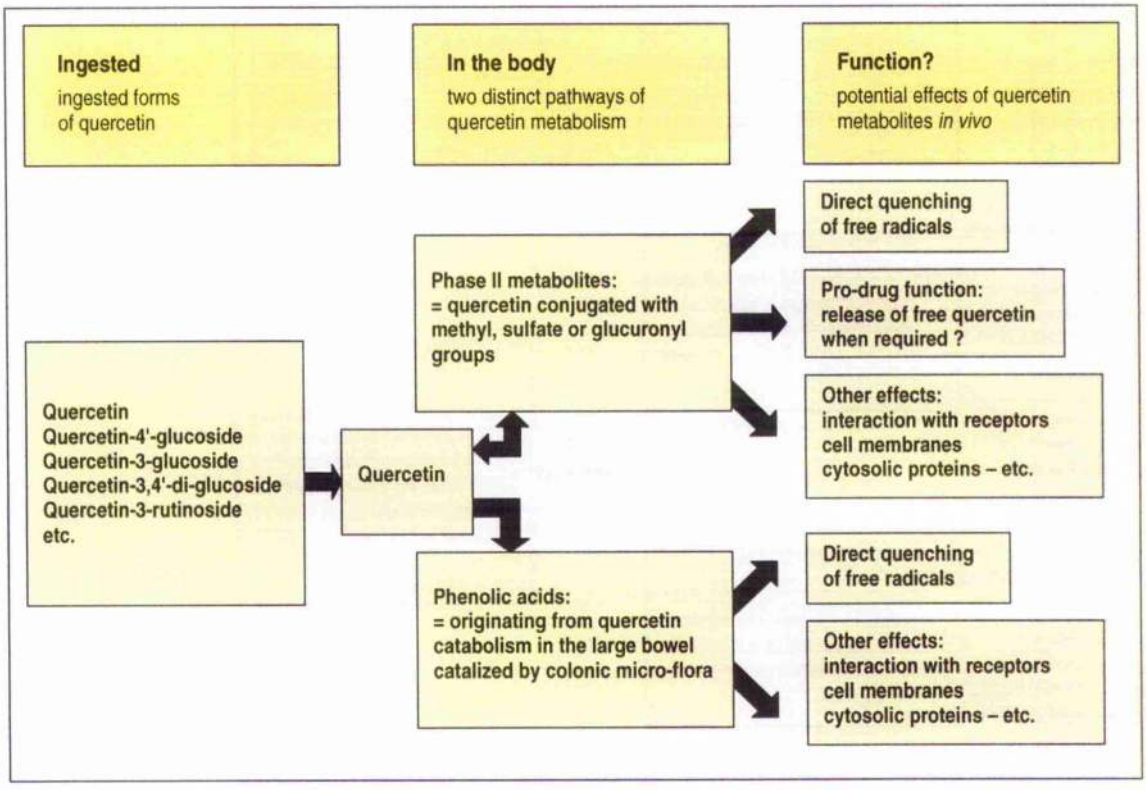


Figure 1: Biological relevance of quercetin metabolites.

4. Caution with cell culture experiments

To accept *in vitro* experiments as an alternative to animal testing, it is essential that the compound under investigation (dietary quercetin) reaches the cells *in vitro* in the same form as *in vivo*. The work presented in this thesis, in agreement with many other studies, show that human and animal plasma exclusively contains quercetin metabolites no matter whether the oral dose was free quercetin or dietary quercetin (e.g. quercetin glycosides). Therefore, if the bioactivity of orally ingested quercetin is investigated using a cell culture model, cultured cells must be exposed to quercetin metabolites, as present in human or animal plasma. As quercetin metabolites are currently not available commercially these would have to be synthesized in the laboratory.

Previously it was assumed that flavonoids which are resistant to high temperatures, acids, etc., would be stable in cell culture medium. However, results shown in Section III, in agreement with other recent reports, strongly suggest that neither quercetin aglycone nor quercetin glycosides are stable in cell culture medium. Therefore the response of cultured cells to quercetin or quercetin glycosides in cell culture medium may be due to either 1) the activity of the original compound, 2) the activity of its cellular metabolites 3) the activity of cell independent break down products formed in culture medium or 4) oxidative stress due to H_2O_2 , $O_2^{-\bullet}$ and quercetin-semi-quinone radicals.

➡ Biological effects ascribed to quercetin based on cell culture experiments need to be re-evaluated using controlled conditions to 1) ensure the stability of the compound under investigation, 2) to eliminate confounding factors such as H_2O_2 formation and to 3) expose cells to quercetin metabolites as present *in vivo*. Cell culture experiments that disregard these factors are likely to report artefacts with have no relevance to the situation in the human body.

Appendix

Appendix Table 1: Animal and organ weights.
At 0.5, 1, 2 and 5 hours after ingestion of [2-^{14}C]quercetin-4'-glucoside rats were terminally anaesthetised and blood was removed by cardiac puncture. The livers were perfused *in situ* and then removed along with brain, heart, kidney, lung, muscle, spleen, testes and the whole GI-tract (stomach, small and large intestine including its contents). The sample collection was carried out at The Rowett Research Institute, Aberdeen, by Phil Morrice and Dr. Garry G. Duthie. All samples were frozen in liquid nitrogen and weighed before and after lyophilization.

organs	animal 0.5 A: 415.5 g			animal 0.5 B: 430.1 g			animal 0.5 C: 429.7 g		
	fresh organ (in gram)	freezedried organ (in gram)	% dry weight	fresh organ (in gram)	freezedried organ (in gram)	% dry weight	fresh organ (in gram)	freezedried organ (in gram)	% dry weight
brain	1.8588	0.4055	21.82	1.7648	0.3839	21.75	1.7371	0.3708	21.35
GIT	24.905	9.4349	37.88	25.2598	9.9056	39.21			
heart	1.1416	0.2456	21.51	1.1685	0.2588	22.15	1.2498	0.2686	21.49
kidney I	1.6658	0.3642	21.86	1.4668	0.3470	23.66	1.5067	0.3118	20.69
kidney II	1.9429	0.6837	35.19	1.3804	0.3575	25.90	1.5395	0.3150	20.46
liver lobe I	7.7974	1.3918	17.85	7.5352	1.6077	21.34	7.3333	1.7555	23.94
liver lobe II	12.315	2.2849	18.55	9.1715	2.0419	22.26	7.5103	1.7489	23.29
lungs	1.7280	0.3245	18.78	1.8101	0.3546	19.59	1.6593	0.3189	19.22
muscle sample	1.6589	0.4214	25.40	1.2639	0.3256	25.76	1.8047	0.4690	25.99
spleen	0.7265	0.1555	21.40	0.7515	0.1828	24.32	0.8022	0.1767	22.03
testes	2.7732	0.4045	14.59	2.8033	0.4058	14.48	2.5345	0.3565	14.07

organs	animal 1 A: 453.3 g			animal 1 B: 436.7 g			animal 1 C: 403.8 g		
	fresh organ (in gram)	freezedried organ (in gram)	% dry weight	fresh organ (in gram)	freezedried organ (in gram)	% dry weight	fresh organ (in gram)	freezedried organ (in gram)	% dry weight
brain	1.7078	0.3716	21.76	1.4717	0.3125	21.23	1.9296	0.4374	22.67
GIT	25.9288	9.2695	35.75	23.9615	8.8825	37.07	24.9518	9.4742	37.97
heart	1.3194	0.2804	21.25	1.2886	0.2809	21.8	1.2796	0.2759	21.56
kidney I	1.5538	0.3576	23.01	1.5775	0.3260	20.67	1.5814	0.3348	21.17
kidney II	1.6243	0.3712	22.85	1.6680	0.3580	21.46	1.6284	0.3455	21.22
liver lobe I	4.6042	1.0950	23.78	8.9322	1.6984	19.01	6.5897	1.2354	18.75
liver lobe II	10.6130	2.3378	22.03	10.3414	1.9245	18.61	12.4208	2.3409	18.85
lungs	2.0082	0.3962	19.73	1.6124	0.3023	18.75	1.7360	0.3399	19.58
muscle sample	2.2020	0.5653	25.67	1.9405	0.5036	25.95	1.7174	0.4506	26.24
spleen	1.0680	0.2709	25.37	0.8377	0.2020	24.11	0.7433	0.1737	23.37
testes	3.0226	0.4361	14.43	3.1566	0.4827	15.29	2.7665	0.4195	15.16

organs	animal 2 A: 421.6 g			animal 2 B: 416.3 g			animal 2 C: 430.4 g		
	fresh organ (in gram)	freezedried organ (in gram)	% dry weight	fresh organ (in gram)	freezedried organ (in gram)	% dry weight	fresh organ (in gram)	freezedried organ (in gram)	% dry weight
brain	1.7376	0.3745	21.55	1.7262	0.3655	21.17	1.7706	0.3752	21.19
GIT	34.4723	13.4767	39.09	35.2468	13.6107	38.62			
heart	1.2357	0.2846	23.03	1.3157	0.3049	23.17	1.3198	0.2845	21.56
kidney I	1.3443	0.3298	24.53	1.4822	0.3009	20.30	1.5360	0.3107	20.23
kidney II	1.4643	0.3575	24.41	1.4288	0.2800	19.60	1.5186	0.3286	21.64
liver lobe I	6.9333	1.7694	25.52	8.5931	1.7380	20.23	7.2786	1.8451	25.35
liver lobe II	7.7392	2.0297	26.23	8.7162	1.7051	19.56	8.8628	2.1527	24.29
lungs	1.5505	0.3842	24.78	1.6245	0.2687	16.54	1.5622	0.3152	20.18
muscle sample	1.4488	0.3584	24.74	1.8866	0.4750	25.18	1.9374	0.4781	24.68
spleen	0.8107	0.1929	23.79	0.7986	0.1902	23.82	0.8441	0.1906	22.58
testes	2.7710	0.4589	16.56	2.7343	0.3969	14.52	2.5099	0.3680	14.66

organs	animal 5 A: 442.4 g			animal 5 B: 433.2 g			animal 5 C: 413.4 g		
	fresh organ (in gram)	freezedried organ (in gram)	% dry weight	fresh organ (in gram)	freezedried organ (in gram)	% dry weight	fresh organ (in gram)	freezedried organ (in gram)	% dry weight
brain	1.7005	0.3677	21.62	1.7225	0.2506	14.55	1.7903	0.3938	22.00
GIT	39.8925	14.3343	35.93						
heart	1.3033	0.2970	22.79	1.2020	0.2665	22.17	1.1544	0.2669	23.12
kidney I	1.5108	0.3271	21.65	1.5825	0.3239	20.47	1.4105	0.2939	20.84
kidney II	1.5804	0.3317	20.99	1.6679	0.3344	20.05	1.4130	0.2958	20.93
liver lobe I	8.1624	1.7243	21.12	7.6208	1.8168	23.84	8.3850	1.9706	23.50
liver lobe II	12.3076	2.5970	21.10	10.1523	2.3416	23.06	10.1273	2.3002	22.71
lungs	1.8368	0.4049	22.04	1.8765	0.3693	19.68	1.8291	0.3357	18.35
muscle sample	2.3210	0.5893	25.39	2.1143	0.5362	25.36	1.7053	0.4333	25.41
spleen	0.7914	0.1871	23.64	0.8562	0.2031	23.72	0.8380	0.2022	24.13
testes	2.5447	0.3894	15.30	2.7543	0.3838	13.93	2.6695	0.3997	14.97

Appendix Table 2: Extraction of quercetin metabolites:
Recovery of radiolabelled compounds in the aqueous and ethyl acetate phase of the extract.
Radiolabelled quercetin metabolites were extracted from freezedried tissue and plasma as described in materials and methods (Figure 2, Section I). The table shows the distribution of radiolabelled compounds (in dpm) between aqueous phase extract and organic phase extract, per whole organ/total plasma (15 ml).

	whole organ (= 100%)	aqueous phase		ethyl acetate phase		extraction efficiency (aqueous + ethyl acetate phase)	
		%	dpm	%	dpm	%	dpm
GI-tract 0.5 h	50,303,345	4%	2,012,134	65%	32,697,174	69%	34,709,308
GI-tract 1 h	54,529,546	4%	2,181,181	46%	25,083,591	50%	27,264,772
GI-tract 2 h	51,494,265	18%	9,268,968	64%	32,956,330	82%	42,225,297
GI-tract 5 h	53,870,401	6%	3,232,224	49%	26,396,496	55%	29,628,721
plasma 0.5 h	1,695,731	30%	508,719	9%	152,616	39%	661,335
plasma 1 h	1,643,770	50%	821,885	2%	32,875	52%	854,760
plasma 2 h	1,460,021	40%	584,008	3%	43,801	43%	627,809
plasma 5 h	982,226	49%	481,291	9%	88,400	58%	569,691
liver 0.5 h	1,126,615	37%	416,848	13%	146,460	50%	563,308
liver 1 h	684,153	17%	116,306	29%	198,404	46%	314,710
liver 2 h	303,530	28%	84,988	6%	18,212	34%	103,200
liver 5 h	155,129	28%	43,436	26%	40,334	54%	83,770
kidney 0.5 h	594,715	22%	130,837	31%	184,362	53%	315,199
kidney 1 h	468,065	20%	93,613	19%	88,932	39%	182,545
kidney 2 h	103,846	12%	12,462	2%	2,077	14%	14,538
kidney 5 h	162,726	16%	26,036	44%	71,599	60%	97,636

Appendix Table 3: Peak numbering in Mullen et al, 2002 and thesis / Graf et al, 2004.
Initially only plasma and tissues samples collected at 1 h after [2-^{14}C]quercetin-4'-glucoside ingestion were analysed, and the detected radiolabelled compounds were numbered 1-19 according to their order of elution from the column. Later, plasma and tissue samples from the other timepoints (0.5, 2 and 5 h) were analysed and 8 additional compounds were identified. For the subsequent publication (Graf et al, 2004) and this thesis the 27 detected radiolabelled compounds were numbered continually according to their order of elution from the column. n.d. = not detected.

Compound	Mullen et al, 2002	thesis / Graf et al, 2004
Unknown	n.d.	1
Unknown	n.d.	2
Quercetin di-glucuronide	1	3
Unknown	2	4
Methylated quercetin di-glucuronide	3	5
Quercetin di-glucuronide	4	6
Quercetin di-glucuronide	n.d.	7
Methylated quercetin di-glucuronide	5	8
Quercetin di-glucuronide	6	9
Quercetin di-glucuronide	7	10
Quercetin di-glucuronide	8	11
Quercetin-3-glucuronide	9	12
Quercetin glucuronide sulfate	10	13
Methylated quercetin di-glucuronide	n.d.	14
Quercetin mono-glucuronide	n.d.	15
Methylated quercetin glucuronide sulfate	11	16
Quercetin glucuronide sulfate	n.d.	17
Quercetin-4'-glucoside	12	18
Methylated quercetin mono-glucuronide	13	19
Methylated quercetin mono-glucuronide	14	20
Methylated quercetin mono-glucuronide	15	21
Quercetin mono-glucuronide	16	22
Quercetin mono-glucuronide	n.d.	23
Quercetin	17	24
Methylated quercetin sulfate	18	25
Quercetin sulfate	19	26
Isorhamnetin	n.d.	27

Appendix Table 4: Quantity of radiolabelled metabolites extracted from rat tissues and plasma.

4 A: whole GI-tract
4 B: plasma
4 C: liver
4 D: kidneys

Radiolabelled compounds were extracted from tissues and plasma, identified using HPLC-RC-MS/MS, and numbered 1-27 according their order of elution from the column. Each metabolite was quantified as outlined in Materials and Methods (Section I). In this table the amount of each metabolite is given in dpm per extracted radioactivity from the whole organ, assuming the extracted aliquot of homogenized and freezedried tissue fully represented the metabolite spectrum of the whole organ.
On average 50% of the original radioactivity was recovered in the purified, concentrated extracts, in practice the extracted radioactivity varied and the exact recoveries are shown in Appendix Table 2. All quantities were calculated using the exact recovery and then normalized as if the extraction efficiency was always 50% to ensure comparability between different samples. The column labelled ' % ' shows the metabolite quantity as a percentage of the extracted radioactivity from each organ.

Appendix Table 4 A: Quantity of radiolabelled metabolites extracted from rat GI-tract.
For Legend please see previous page.

No.	Compound	GI-tract 0.5 h	%	GI-tract 1 h	%	GI-tract 2 h	%	GI-tract 5 h	%
1	unknown	-	-	-	-	-	-	3,150,823	10.6
2	unknown	-	-	-	-	-	-	3,206,052	10.8
3	Quercetin di-glucuronide	-	-	223,013	0.8	-	-	-	-
4	unknown	612,517	1.8	223,013	0.8	1,256,223	3.0	-	-
5	Methylated quercetin di-glucuronide	-	-	364,436	1.3	2,309,829	5.5	-	-
6	Quercetin di-glucuronide	-	-	59,832	0.2	1,215,700	2.9	715,094	2.4
7	Quercetin di-glucuronide	-	-	-	-	-	-	-	-
8	Methylated quercetin di-glucuronide	-	-	163,180	0.6	3,039,249	7.2	607,830	2.1
9	Quercetin di-glucuronide	-	-	-	-	-	-	-	-
10	Quercetin di-glucuronide	748,632	2.2	685,358	2.5	6,281,114	14.9	19,774,612	66.7
11	Quercetin di-glucuronide	1,565,322	4.5	152,301	0.6	1,539,886	3.6	-	-
12	Quercetin-3-glucuronide	-	-	2,876,558	10.6	3,647,099	8.6	-	-
13	Quercetin glucuronide sulfate	-	-	1,892,253	6.9	2,958,202	7.0	357,547	1.2
14	Methylated quercetin di-glucuronide	-	-	-	-	-	-	-	-
15	Quercetin mono-glucuronide	-	-	-	-	-	-	-	-
16	Methylated quercetin glucuronide sulfate	-	-	201,256	0.7	2,998,726	7.1	0	0.0
17	Quercetin glucuronide sulfate	-	-	-	-	-	-	-	-
18	Quercetin-4'-glucoside	17,014,367	49.0	7,133,865	26.2	-	-	-	-
19	Methylated quercetin mono-glucuronide	-	-	1,783,466	6.5	3,039,249	7.2	-	-
20	Methylated quercetin mono-glucuronide	884,747	2.5	1,323,217	4.9	2,958,202	7.0	-	-
21	Methylated quercetin mono-glucuronide	-	-			3,890,238	9.2	-	-
22	Quercetin mono-glucuronide	1,156,977	3.3	3,336,808	12.2	-	-	-	-
23	Quercetin mono-glucuronide	-	-	-	-	-	-	-	-
24	Quercetin	8,711,356	25.1	1,956,059	7.2	2,836,632	6.7	1,816,763	6.1
25	Methylated quercetin sulfate	-	-	-	-	-	-	-	-
26	Quercetin sulfate	2,245,896	6.5	4,890,149	17.9	3,849,715	9.1	-	-
27	Isorhamnetin	1,769,494	5.1	-	-	405,233	1.0	-	-
	Total sum	34,709,308	100.0	27,264,764	99.9	42,225,297	100.0	29,628,720	100.0
	Diglucuronides	2,313,954	6.7	1,648,120	6.0	14,385,778	34.1	21,097,535	71.2
	Monoglucuronides	2,041,724	5.9	9,320,049	34.2	13,534,788	32.1	-	-
	Glucuronated sulfates	0	0.0	2,093,509	7.7	5,956,928	14.1	357,547	1.2
	Sulfates	2,245,896	6.5	4,890,149	17.9	3,849,715	9.1	-	-
	Sum (conjugates)	6,601,574	19.0	17,951,827	65.8	37,727,209	89.3	21,455,082	72.4
	Isorhamnetin (methylated quercetin)	1,769,494	5.1	-	-	405,233	1.0	-	-
	Sum (phase II metabolites)	8,371,068	24.1	17,951,827	65.8	38,132,442	90.3	21,455,082	72.4
	unknown	612,517	1.8	223,013	0.8	1,256,223	3.0	6,356,875	21.5
	Sum (metabolites)	8,983,586	25.9	18,174,840	66.7	39,388,665	93.3	27,811,957	93.9
	Q aglycone	8,711,356	25.1	1,956,059	7.2	2,836,632	6.7	1,816,763	6.1
	Sum (transformed quercetin-4'-glucoside)	17,694,941	51.0	20,130,899	73.8	42,225,297	100.0	29,628,720	100.0
	Quercetin-4'-glucoside	17,014,367	49.0	7,133,865	26.2	-	-	-	-
	Total sum	34,709,308	100.0	27,264,764	100.0	42,225,297	100.0	29,628,720	100.0
	methylated metabolites	884,747	2.5	3,835,555	14.1	18,235,493	43.2	607,830	2.1
	not methylated metabolites	5,716,827	16.5	14,116,272	51.8	19,491,716	46.2	20,847,252	70.4
	(Q and I not included)								
	methylated conjugates	2,654,241	7.6	3,835,555	14.1	18,640,726	44.1	607,830	2.1
	not methylated conjugates	14,428,183	41.6	16,072,331	58.9	22,328,348	52.9	22,664,015	76.5
	(including Q and I)								

Appendix Table 4 B: Quantity of radiolabelled metabolites extracted from rat plasma.
For Legend please see previous page.

No.	Compound	Plasma 0.5 h	%	Plasma 1 h	%	Plasma 2 h	%	Plasma 5 h	%
1	unknown	-	-	-	-	-	-	-	-
2	unknown	-	-	-	-	-	-	-	-
3	Quercetin di-glucuronide	-	-	-	-	-	-	-	-
4	unknown	-	-	-	-	-	-	-	-
5	Methylated quercetin di-glucuronide	-	-	-	-	-	-	-	-
6	Quercetin di-glucuronide	4,748	1.2	54,489	6.4	60,605	9.7	44,287	7.8
7	Quercetin di-glucuronide	17,805	4.4	-	-	-	-	18,117	3.2
8	Methylated quercetin di-glucuronide	-	-	-	-	-	-	-	-
9	Quercetin di-glucuronide	25,119	6.2	31,785	3.7	34,188	5.4	18,117	3.2
10	Quercetin di-glucuronide	121,982	30.0	174,820	20.5	93,239	14.9	12,078	2.1
11	Quercetin di-glucuronide	43,184	10.6	70,382	8.2	35,742	5.7	10,065	1.8
12	Quercetin-3-glucuronide	37,490	9.2	18,053	2.1	-	-	-	-
13	Quercetin glucuronide sulfate	6,592	1.6	118,060	13.8	105,671	16.8	80,522	14.1
14	Methylated quercetin di-glucuronide	0	0.0	-	-	-	-	-	-
15	Quercetin mono-glucuronide	65,317	16.0	-	-	-	-	-	-
16	Methylated quercetin glucuronide sulfate	38,371	9.4	372,345	43.6	298,365	47.5	354,295	62.2
17	Quercetin glucuronide sulfate	13,515	3.3			0	0.0	0	0.0
18	Quercetin-4'-glucoside	0	0.0	0	0.0	0	0.0	0	0.0
19	Methylated quercetin mono-glucuronide	4,638	1.1	4,457	0.5	-	-	-	-
20	Methylated quercetin mono-glucuronide	8,503	2.1	3,677	0.4	-	-	-	-
21	Methylated quercetin mono-glucuronide	10,435	2.6	6,684	0.8	-	-	-	-
22	Quercetin mono-glucuronide	-	-	-	-	-	-	-	-
23	Quercetin mono-glucuronide	9,276	2.3	-	-	-	-	-	-
24	Quercetin	-	-	-	-	-	-	-	-
25	Methylated quercetin sulfate	-	-	-	-	-	-	-	-
26	Quercetin sulfate	-	-	-	-	-	-	-	-
27	Isorhamnetin	-	-	-	-	-	-	-	-
	Total sum	406,974	100.0	854,752	100.0	627,809	100.0	569,691	100.0

Diglucuronides	212,838	52.3	331,476	38.8	223,774	35.6	134,874	23.7
Monoglucuronides	135,658	33.3	32,871	3.8	-	-	-	-
Glucuronated sulfates	58,478	14.4	490,405	57.4	404,035	64.4	434,817	76.3
Sulfates	-	-	-	-	-	-	-	-
Sum (conjugates)	406,974	100.0	854,752	100.0	627,809	100.0	569,691	100.0
Isorhamnetin (methylated quercetin)	-	-	-	-	-	-	-	-
Sum (phase II metabolites)	406,974	100.0	854,752	100.0	627,809	100.0	569,691	100.0
unknown	-	-	-	-	-	-	-	-
Sum (metabolites)	406,974	100.0	854,752	100.0	627,809	100.0	569,691	100.0
Q aglycone	-	-	-	-	-	-	-	-
Sum (transformed quercetin-4'-glucoside))	406,974	100.0	854,752	100.0	627,809	100.0	569,691	100.0
Quercetin-4'-glucoside	-	-	-	-	-	-	-	-
Total sum	406,974	100.0	854,752	100.0	627,809	100.0	569,691	100.0

methylated conjugates	61,947	15.2	387,163	45.3	298,365	47.5	386,504	67.8
not methylated conjugates	345,027	84.8	467,589	54.7	329,444	52.5	183,187	32.2
(Q and I not included)								
methylated conjugates	61,947	15.2	387,163	45.3	298,365	47.5	386,504	67.8
not methylated conjugates	345,027	84.8	467,589	54.7	329,444	52.5	183,187	32.2
(including Q and I)								

Appendix Table 4 C: Quantity of radiolabelled metabolites extracted from rat liver.
For Legend please see previous page.

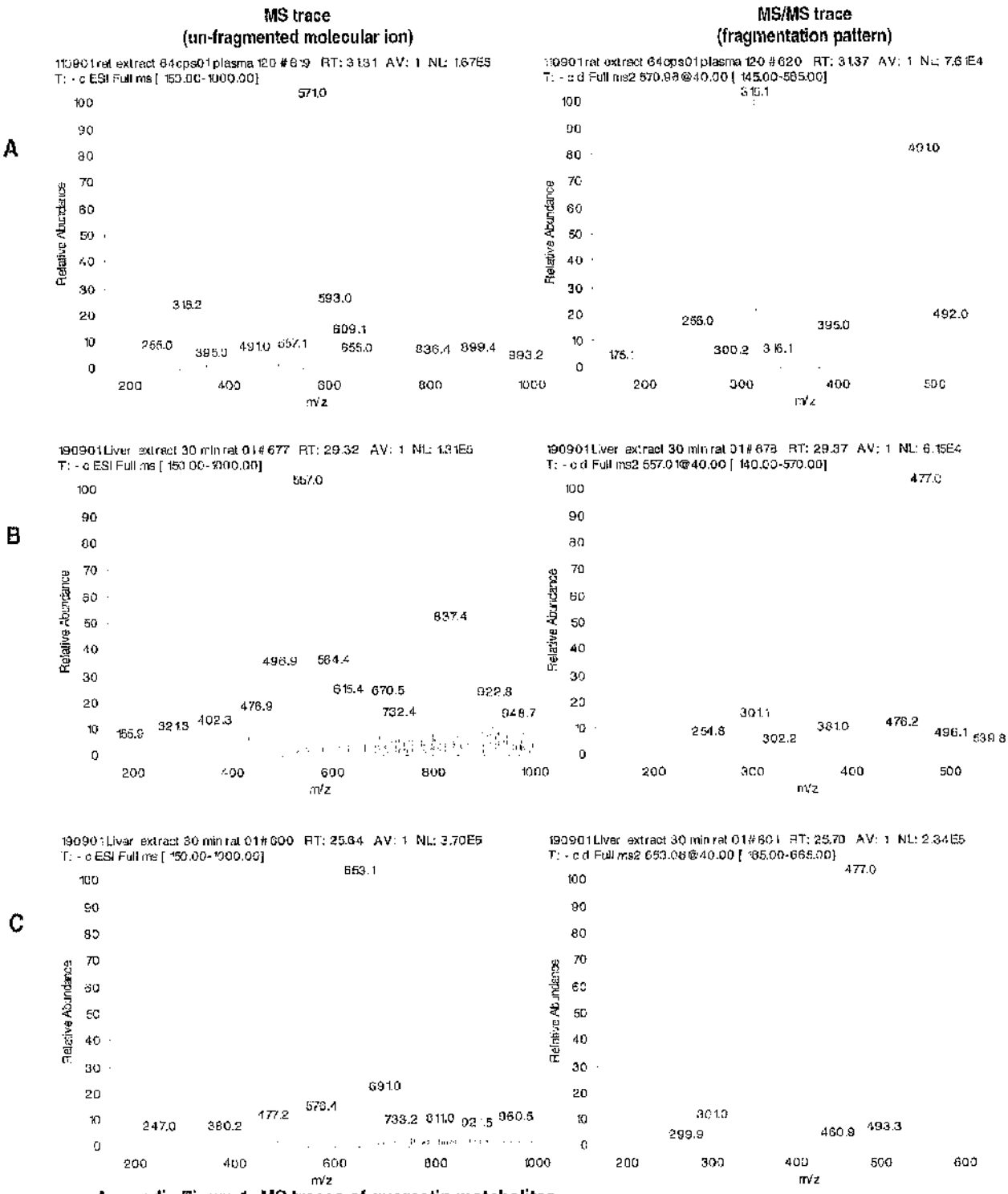
No.	Compound	Liver 0.5 h	%	Liver 1 h	%	Liver 2 h	%	Liver 5 h	%
1	unknown	-	-	-	-	-	-	-	-
2	unknown	-	-	-	-	-	-	-	-
3	Quercetin di-glucuronide	21,489	3.8	20,293	6.4	-	-	29,730	35.5
4	unknown	-	-	-	-	-	-	3,950	4.7
5	Methylated quercetin di-glucuronide	65,233	11.6	40,773	13.0	4,981	4.8	-	-
6	Quercetin di-glucuronide	-	-	-	-	3,188	3.1	35,608	42.5
7	Quercetin di-glucuronide	-	-	-	-	-	-	-	-
8	Methylated quercetin di-glucuronide	38,372	6.8	-	-	-	-	-	-
9	Quercetin di-glucuronide	-	-	-	-	6,973	6.8	-	-
10	Quercetin di-glucuronide	84,419	15.0	105,383	33.5	30,880	29.9	7,061	8.4
11	Quercetin di-glucuronide	39,907	7.1	12,607	4.0	7,969	7.7	-	-
12	Quercetin-3-glucuronide	0	0.0	21,565	6.9	-	-	-	-
13	Quercetin glucuronide sulfate	38,372	6.8	9,289	3.0	8,567	8.3	-	-
14	Methylated quercetin di-glucuronide	0	0.0	-	-	-	-	-	-
15	Quercetin mono-glucuronide	-	-	-	-	-	-	-	-
16	Methylated quercetin glucuronide sulfate	126,629	22.5	40,428	12.8	20,720	20.1	-	-
17	Quercetin glucuronide sulfate	-	-	-	-	-	-	1,436	1.7
18	Quercetin-4'-glucoside	9,977	1.8	-	-	-	-	-	-
19	Methylated quercetin mono-glucuronide	59,094	10.5	42,135	13.4	8,567	8.3	2,394	2.9
20	Methylated quercetin mono-glucuronide	46,814	8.3	17,584	5.6	3,985	3.9	-	-
21	Methylated quercetin mono-glucuronide	33,000	5.9	-	-	7,371	7.1	-	-
22	Quercetin mono-glucuronide	-	-	-	-	-	-	-	-
23	Quercetin mono-glucuronide	-	-	-	-	-	-	-	-
24	Quercetin	-	-	4,644	1.5	-	-	3,591	4.3
25	Methylated quercetin sulfate	-	-	-	-	-	-	-	-
26	Quercetin sulfate	-	-	-	-	-	-	-	-
27	Isorhamnetin	-	-	-	-	-	-	-	-
	Total sum	563,308	100.0	314,701	100.1	103,200	100.0	83,770	100.0

Diglucuronides	249,421	44.3	179,056	56.9	53,991	52.3	72,400	86.4
Monoglucuronides	138,908	24.7	81,284	25.8	19,923	19.3	2,394	2.9
Glucuronated sulfates	165,002	29.3	49,717	15.8	29,286	28.4	1,436	1.7
Sulfates	-	-	-	-	-	-	-	-
Sum (conjugates)	553,331	98.2	310,057	98.5	103,200	100.0	76,230	91.0
Isorhamnetin (methylated quercetin)	-	-	-	-	-	-	-	-
Sum (phase II metabolites)	553,331	98.2	310,057	98.5	103,200	100.0	76,230	91.0
unknown	-	-	-	-	-	-	3,950	4.7
Sum (metabolites)	553,331	98.2	310,057	98.5	103,200	100.0	80,179	95.7
Q aglycone	-	-	4,644	1.5	-	-	3,591	4.3
Sum (transformed quercetin-4'-glucoside))	553,331	98.2	314,701	100.0	103,200	100.0	83,770	100.0
Quercetin-4'-glucoside	9,977	1.8	-	-	-	-	-	-
Total sum	563,308	100.0	314,701	100.0	103,200	100.0	83,770	100.0

methylated conjugates	369,143	65.5	140,920	44.8	45,623	44.2	2,394	2.9
not methylated conjugates	184,188	32.7	169,137	53.7	57,577	55.8	73,836	88.1
(Q and I not included)								
methylated conjugates	369,143	65.5	140,920	44.8	45,623	44.2	2,394	2.9
not methylated conjugates	184,188	32.7	173,781	55.2	57,577	55.8	77,427	92.4
(including Q and I)								

Appendix Table 4 D: Quantity of radiolabelled metabolites extracted from rat kidney.
For Legend please see previous page.

No.	Compound	Kidney 0.5 h	%	Kidney 1 h	%	Kidney 2 h	%	Kidney 5 h	%
1	unknown	-	-	-	-	-	-	-	-
2	unknown	-	-	-	-	-	-	-	-
3	Quercetin di-glucuronide	19,912	6.32	13,446	7.4	3,687	25.4	39,342	40.3
4	unknown	10,591	3.36	8,153	4.5	-	-	3,274	3.35
5	Methylated quercetin di-glucuronide	49,568	15.73	31,012	17	2,231	15.3	-	-
6	Quercetin di-glucuronide	9,744	3.09	-	-	-	-	16,963	17.4
7	Quercetin di-glucuronide	-	-	-	-	-	-	-	-
8	Methylated quercetin di-glucuronide	38,129	12.10	22,736	12.5	517	3.55	2,466	2.53
9	Quercetin di-glucuronide	16,946	5.38	-	-	2,419	16.6	-	-
10	Quercetin di-glucuronide	4,237	1.34	21,405	11.7	2,771	19.1	32,858	33.7
11	Quercetin di-glucuronide	18,641	5.91	15,345	8.4	493	3.4	-	-
12	Quercetin-3-glucuronide	18,641	5.91	14,441	7.9	-	-	-	-
13	Quercetin glucuronide sulfate	-	-	-	-	-	-	-	-
14	Methylated quercetin di-glucuronide	-	-	-	-	-	-	-	-
15	Quercetin mono-glucuronide	-	-	-	-	-	-	-	-
16	Methylated quercetin glucuronide sulfate	7,202	2.28	5,645	3.1	-	-	-	-
17	Quercetin glucuronide sulfate	-	-	-	-	-	-	986	1.0
18	Quercetin-4'-glucoside	-	-	-	-	-	-	-	-
19	Methylated quercetin mono-glucuronide	28,809	9.14	14,061	7.7	1,127	7.75	-	-
20	Methylated quercetin mono-glucuronide	70,750	22.45	30,594	16.8	1,292	8.89	-	-
21	Methylated quercetin mono-glucuronide	22,030	6.99	-	-	-	-	-	-
22	Quercetin mono-glucuronide	-	-	5,700	3.1	-	-	-	-
23	Quercetin mono-glucuronide	-	-	-	-	-	-	-	-
24	Quercetin	-	-	-	-	-	-	1,746	1.79
25	Methylated quercetin sulfate	-	-	-	-	-	-	-	-
26	Quercetin sulfate	-	-	-	-	-	-	-	-
27	Isorhamnetin	-	-	-	-	-	-	-	-
	Total sum	315,199	100.0	182,538	100.1	14,538	100.0	97,635	100.0
	Diglucuronides	157,176	49.9	103,944	56.9	12,119	83.4	91,628	93.8
	Monoglucuronides	140,230	44.5	64,796	35.5	2419.08562	16.6	-	-
	Glucuronated sulfates	7,202	2.3	5,645	3.1	-	-	986	1.0
	Sulfates	-	-	-	-	-	-	-	-
	Sum (conjugates)	304,608	96.6	174,385	95.5	14,538	100.0	92,614	94.9
	Isorhamnetin (methylated quercetin)	-	-	-	-	-	-	-	-
	Sum (phase II metabolites)	304,608	96.6	174,385	95.5	14,538	100.0	92,614	94.9
	unknown	10591.3642	3.36	8153	4.47	-	-	3274.34451	3.35
	Sum (metabolites)	315,199	100.0	182,538	100.0	14,538	100.0	95,889	98.2
	Q aglycone	-	-	-	-	-	-	1746.31707	1.79
	Sum (transformed quercetin-4'-glucoside))	315,199	100.0	182,538	100.0	14,538	100.0	97,635	100.0
	Quercetin-4'-glucoside	-	-	-	-	-	-	-	-
	Total sum	315,199	100.0	182,538	100.0	14,538	100.0	97,635	100.0
	methylated conjugates	216,487	68.7	109,748	60.1	5,167	35.5	2,466	2.5
	not methylated conjugates	88,120	28.0	70,337	38.5	9,371	64.5	90,149	92.3
	(Q and I not included)								
	methylated conjugates	216,487	68.7	109,748	60.1	5,167	35.5	2,466	2.5
	not methylated conjugates	88,120	28.0	70,337	38.5	9,371	64.5	91,895	94.1
	(including Q and I)								

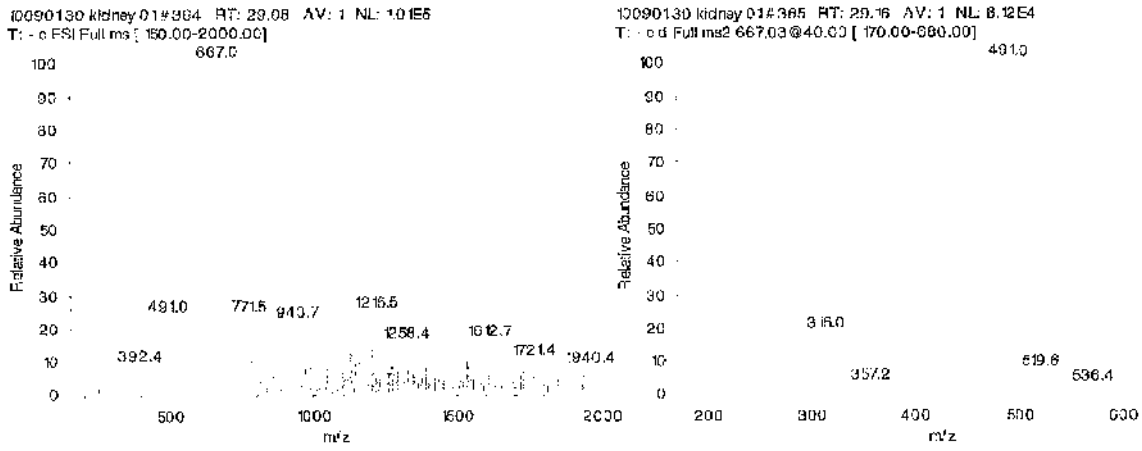


Appendix Figure 1: MS traces of quercetin metabolites.
Radiolabelled compounds were extracted from rat tissue and plasma after an oral dose of [2-^{14}C]quercetin-4'-glucoside. Quercetin metabolites were identified by gradient, reverse phase HPLC with online radioactivity detection and tandem MS/MS, operating in negative ion mode, and complete results are shown in Section I, Table 5. **A:** MS trace of methyl-quercetin-mono-glucuronide-sulfate, peak 16, detected in plasma at 2 h. The m/z of the molecular ion $[M-H]^-$ was 571, with fragment ions at m/z of 491 (=$[M-H]^-$ -sulfate group), 395 (=$[M-H]^-$ -glucuronyl group) and 315 (=$[M-H]^-$ -glucuronide group, and -sulfate group). The fragment ion at m/z 315 corresponds with methyl quercetin. **B:** MS trace of quercetin-mono-glucuronide-sulfate, peak 13, in liver extract at 0.5 h. The m/z for the molecular ion $[M-H]^-$ was 557, the fragment ions had a m/z of 477 (=$[M-H]^-$ - sulfate group), 381 (=$[M-H]^-$ - glucuronyl group) and 301 (=$[M-H]^-$ - glucuronide group and - sulfate group). The fragment ion at m/z 301 corresponds with quercetin. **C:** MS trace of quercetin-di-glucuronide, peak 10, in liver extract at 0.5 h. The m/z for the molecular ion $[M-H]^-$ was 653, the fragment ions had a m/z of 477 (=$[M-H]^-$ - glucuronyl group) and 301 (=$[M-H]^-$ - two glucuronyl groups). The fragment ion at m/z 301 corresponds with quercetin.

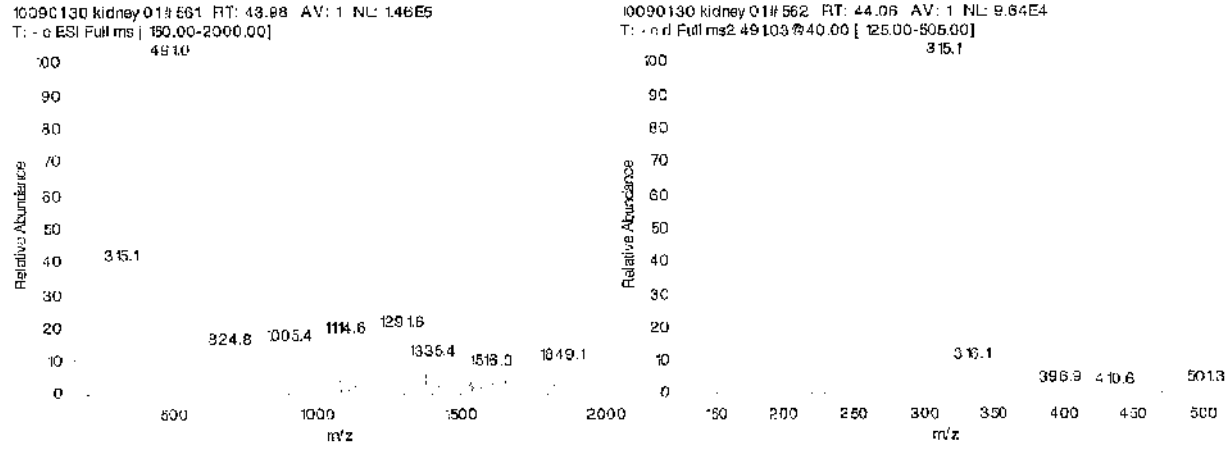
MS trace (un-fragmented molecular ion)

MS/MS trace (fragmentation pattern)

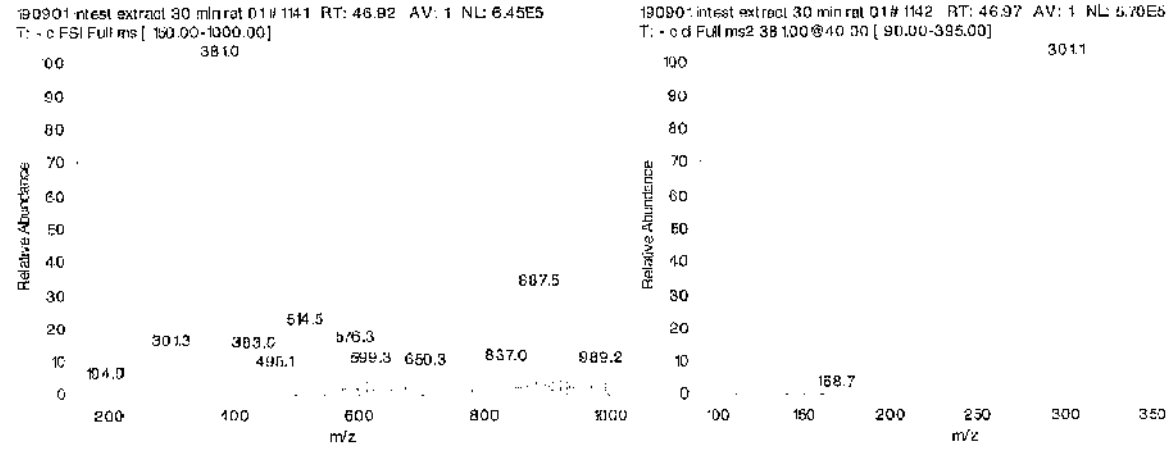
D



E



F



Continuation of Appendix Figure 1: MS traces of quercetin metabolites.

D: MS trace of methyl-quercetin-di-glucuronide, peak 8, in kidney extract at 0.5 h. The m/z for the molecular ion $[M-H]^-$ was 667, the fragment ion had a m/z of 477 (=$[M-H]^-$ - glucuronyl group) and 315 (=$[M-H]^-$ - two glucuronyl groups). The fragment ion at m/z 315 corresponds with methylated quercetin.

E: MS trace of methyl quercetin mono-glucuronide, peak 20, kidney extract at 0.5 h. The m/z for the molecular ion $[M-H]^-$ was 491, the fragment ion had a m/z of 315 (=$[M-H]^-$ - glucuronyl group). The fragment ion at m/z 315 corresponds with methylated quercetin.

F: MS trace of quercetin sulfate, peak 26, intestine extract at 0.5 h. The m/z for the molecular ion $[M-H]^-$ was 381, the fragment ion had a m/z of 301 (=$[M-H]^-$ - glucuronyl group). The fragment ion at m/z 301 corresponds with quercetin.

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Author's Declaration

This thesis and the work described within was carried out entirely by myself unless otherwise cited or acknowledged. Its contents have not previously been submitted for any other degree.

Signed: . ....

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31st of May 2004