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Investigations of the absorption and metabolism of antioxidant flavonols

by

Azlina Abdul Aziz
BSc (Univ. of Malaya)
MSc (Univ. of Glasgow)

Thesis submitted in partial fulfilment for the degree of Doctor of Philosophy (PhD) in the Faculty of Medicine, University of Glasgow

Department of Human Nutrition,
Yorkhill Hospitals,
University of Glasgow
April 2000

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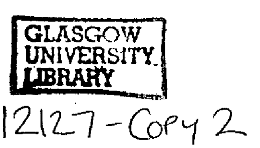

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ABSTRACT

Flavonols are polyphenols, secondary plant metabolites commonly found in plants and foods of plant origin. They have widespread biological properties in the human body. The recent discovery of their potential antioxidant activities has prompted extensive research. Flavonols particularly quercetin are potent antioxidants with higher antioxidant properties than the well known antioxidant vitamins C and E. Several epidemiology studies have demonstrated a strong inverse association between flavonoid intake and risk of coronary heart disease. The association with cancer is less defined with only some studies showing an inverse association and others not. In view of their potential to act as antioxidants and prevent oxidative damage-related diseases, it is important to have an understanding of their bioavailability. Indeed, information of the absorption and metabolism of individual flavonols in man is limited. Previous investigations of their absorption have shown conflicting results. It was previously speculated that flavonol glycosides were not absorbed due to their conjugation to sugar molecules and that only the aglycones were transported into the blood stream. However, recent research has detected the presence of quercetin glycosides in plasma. These findings disagree with the earlier postulation that flavonol glycosides were not absorbed. In addition to absorption, the metabolism of flavonoids is another important area of research. As yet, little firm information is available on the fate of flavonols following absorption. The aim of the present study was to conduct further research on the absorption and metabolism of flavonols and to establish the influence of flavonol structure on the extent of their absorption and metabolism.

In the first study, the accumulation of flavonols in plasma and their excretion in urine was investigated after a meal of lightly fried onions. Five healthy volunteers followed a low flavonol diet for 4 days. On day 3, after an overnight fast, subjects consumed 300 g of lightly fried yellow onions containing quercetin-3,4′-di*O*-β-glucoside, isorhamnetin-4′-*O*-β-glucoside and quercetin-4′-*O*-β-glucoside. Blood was sampled at 0 min, 0.5, 1.0, 1.5, 2, 3, 4, 5 and 24 h after the supplement. In addition, subjects collected all their urine for 24 h following the onion supplements. Isorhamnetin-4′-*O*-β-glucoside, a minor flavonol in onions accumulated in plasma at higher levels than quercetin-4′-*O*-β-glucoside which was a major onion flavonol. The peak concentration

in plasma, expressed as percentage of intake of isorhamnetin-4′-*O*-β-glucoside and quercetin-4′-*O*-β-glucoside was 10.7 ± 2.6% and 0.13 ± 0.03 %, respectively. The time taken to reach peak plasma concentration after ingesting the onions was 1.8 ± 0.7 h for isorhamnetin-4′-*O*-β-glucoside and 1.3 ± 0.2 h for quercetin-4′-*O*-β-glucoside. Excretion in urine, as a proportion of intake, was 17.4 ± 8.3% for isorhamnetin-4′-*O*-β-glucoside and 0.2 ± 0.1% for quercetin-4′-*O*-β-glucoside. It was concluded that flavonols are absorbed into the bloodstream as glucosides. Structural differences in the flavonol molecule appeared to affect the level of accumulation and the extent to which they are excreted.

Further studies investigated the influence of structural modifications on flavonol absorption from the intestine. An *in vitro* everted rat gut model was employed to assess the uptake of several flavonol glycosides as well as free quercetin. Everted rat jejunal segments (6-10 cm) were incubated at 37°C in 20 ml of Krebs buffer containing 10 μM of the test flavonols. The incubation media was continuously gassed with 95% O_2 and 5% CO_2. Incubation was performed for 30 min with samples taken every 5 min from the serosal side. The rate of uptake of flavonols were in the order of quercetin-3,4′-di*O*-β-glucoside > quercetin-3-*O*-β-rutinoside > quercetin-3-*O*-β-glucoside > quercetin-3-*O*-β-rhamnoside > quercetin-4′-*O*-β-glucoside > isorhamnetin-4′-*O*-β-glucoside > quercetin demonstrating the structural dependence of flavonols on their uptake across the intestinal mucosa. Quercetin glycosides and diglucosides as well as isorhamnetin-4′-*O*-β-glucoside were better absorbed than quercetin aglycone. Flavonol glycosides with glucose substitution at C3 or at both C3 and C4′ were better absorbed across the small intestine than those with a glucose substitution only at C4′.

In the light of the differential uptake of flavonols across the intestine, the mechanism of their absorption was explored using the everted rat gut model described above. The uptake of quercetin-3-*O*-β-glucoside appeared to be concentration dependent with evidence of saturation observed in the concentration range of 10 μM to 500 μM. Several experiments were performed to assess the role of the sodium-glucose transport pathway (SGLT1) in flavonol transport. There was no significant difference in the rate of uptake of 10 μM quercetin-3-*O*-β-glucoside in the presence of 0, 1, 10, 50 and 100 mM glucose ($P>0.05$). Phloridzin, an inhibitor of SGLT1 did not reduce the rate of

transport of 10 μM quercetin-3-*O*-β-glucoside at concentration of 1 mM and 5 mM ($P>0.05$) although the same concentration inhibited glucose transport by ca. 33%. A slight inhibition was observed in the uptake of 10 μM quercetin-3,4′-di*O*-β-glucoside in the presence of 1 mM phloridzin, but this was not statistically significant ($P>0.05$). The possibility of quercetin-3-*O*-β-glucoside acting as an inhibitor of SGLT1 was also investigated. This glucoside did not inhibit uptake of 10 mM glucose at concentrations of 10 μM, 50 μM and 500 μM. This study showed that the intestinal uptake of quercetin-3-*O*-β-glucoside and possibly other quercetin glucosides was carrier-mediated. We conclude from this study that SGLT1 did not play a role in the uptake of flavonol glucosides.

The liver metabolism of flavonol glucosides, quercetin-3,4′-di*O*-β-glucoside, quercetin-4′-*O*-β-glucoside, quercetin-3-*O*-β-glucoside and isorhamnetin-4′-*O*-β-glucoside was investigated in an *in vitro* experiment. A 24 nM solution of flavonol was incubated in 10 ml of phosphate buffer (50 mM, pH 7.4, containing 10 mM $MgCl_2$) together with 1 g of rat liver homogenates with or without 24 nM *S*-adenosyl methionine. Incubation was performed for 2 h at 37°C with samples withdrawn every 10 min for the first half an hour followed by every 30 min thereafter. Metabolism of the four flavonol glucosides was seen. The extent of the reaction was highest for quercetin-4′-*O*-β-glucoside (89.1 ± 1.7 %), followed by quercetin-3-*O*-β-glucoside (67.6 ± 2.7 %), isorhamnetin-4′-*O*-β-glucoside (63.6 ± 0.4 %) and quercetin-3,4′-di*O*-β-glucoside (31.9 ± 4.1 %). Extensive methylation of quercetin-3-*O*-β-glucoside occurred with less than 10 % remaining after 2 h. Deglycosylation of the flavonol glycosides occurred, evident from the appearance of deglycosylation products. As the percentage accumulation of metabolites was much lower than the percentage of the substrate metabolised, other metabolic reactions, in addition to deglycosylation and methylation, would appear to have been taking place.

Results obtained from this thesis can partially explain the seemingly low absorption of flavonol glycosides from the small intestine and their accumulation in plasma. The structure of flavonols, particularly the nature and position of the sugar moiety, has a major influence on their absorption and potential bioavailability.

Furthermore, the substantial metabolism of flavonol glucosides by the liver may contribute to the low levels of the parent compounds detected in plasma and urine.

Findings from this present study raised interesting health promotion possibilities as identification of the flavonol conjugates which will be highly absorbed into the bloodstream can be used to encourage the public to increase their intakes of flavonol-rich fruit and vegetables. Identification of the metabolites of flavonol metabolism with potential antioxidant activities can further contribute towards promoting the beneficial effects of increasing intakes of flavonol-rich foods.

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List of Abbreviations

AUC	Area under the curve
HPLC	High performance liquid chromatograph
LDL	Low-density lipoprotein
SAM	S-adenosyl methionine
SGLT1	Sodium-glucose co-transporter
UV	Ultra-violet
Quercetin-3,4′-diglucoside	Quercetin-3,4′-di*O*-β-glucoside
Quercetin-3-glucoside	Quercetin-3-*O*-β-glucoside
Quercetin-4′-glucoside	Quercetin-4′-*O*-β-glucoside
Quercitrin	Quercetin-3-*O*-β-rhamnoside
Isorhamnetin-4′-glucoside	Isorhamnetin-4′-*O*-β-glucoside
Rutin	Quercetin-3-*O*-β-rutinoside
Tamarixetin	4′-*O*-methyl-quercetin

Acknowledgement

I would like to express my sincere gratitude to the following people:

1. Dr Alan Crozier and Dr Christine Edwards for their constant supervision and help throughout my PhD, and for their constructive criticism and helpful advice during the write-up.

2. Staffs and PhD students in the Stevenson Laboratory; Mandie, Jenny, Fiona, Tina, Helena, Claire, Alison, Bill and Morag; for their constant support and for making the past 3 years an enjoyable experience.

3. Mr William Mullen for analysis of samples on the HPLC-MS system.

4. Mr Khalid Khan for performing the *in vitro* digestion and fermentation experiments.

5. Technicians and staff at the Central Research Facility for their help in providing animal tissues.

6. The volunteers who participated in the onion feeding study; many thanks for their time and patience.

7. Dr Margaret Kerr and Dr Martin Christian for performing the blood collection.

8. Staffs at the Department of Human Nutrition, Yorkhill Hospital, in particular Miss Alison Parrett and Mrs Evelyn Smith.

9. University of Malaya, Malaysia for their sponsorship.

10. Last but not least to my family and especially my husband Shafeeq for all his tireless effort, advice and patience throughout my PhD.

Publications

Aziz, A. A., Edwards, C. A., Cahill, A. P., Khan, M. K., Findlay, I. G., Lean, M. E. J., & Crozier, A. 2000, "Absorption and excretion of the conjugated flavonols, quercetin-3,4'-di*O*-β-D-glucoside, quercetin-4'-*O*-β-D-glucoside and isorhamnetin-4'-*O*-β-D-glucoside, in human ileostomy volunteers after the consumption of onions", *FEBS letters*, Submitted.

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Declaration

This thesis represents the original work of the author and has not been submitted in any other form in support of an application for any degree or qualification to any university.

Azlina Abdul Aziz

April 2000

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

The relationship between human diet and disease has been studied for many years. Diet is recognised as one of the main factors influencing disease and life-threatening conditions particularly in the middle aged and elderly. Each year, a significant percentage of the groups suffer from diet-related diseases such as hypertension, cancer and diabetes. Thus, the potential contribution of diet towards prevention of disease is widely studied as one of the means of improving or maintaining quality of life. Furthermore, as prevention is better than treatment, various ways are being investigated to prevent development of diseases.

1.2 Diet and disease

Coronary heart disease results in more deaths than any other single cause in countries around the world, particularly in western populations. The UK has one of the highest deaths from coronary heart disease in the world. Recent Scottish Health Statistics Report (1998) indicated a general fall in the incidence and mortality rates of ischaemic heart disease in Scotland over the period 1987-1996. However, Scotland holds the top position for coronary heart disease in the UK. Smoking, high blood pressure and raised serum cholesterol levels are the major risk factors for heart disease, the last two largely affected by diet.

In addition to coronary heart disease, diet may also be related to the development of cancer at several sites. After heart disease, cancer is the second cause of death in western countries. Scotland for instance, has the world's highest incidence of lung cancer in females (Scottish Health Statistics Report, 1998). Cancer of the breast, stomach and bowel are attributable primarily to diet (Doll and Peto, 1981).

Diet can either promote or prevent heart disease and cancer. Fat, cholesterol, salt and alcohol are possible initiators of heart disease and cancer whereas dietary fibre and

the antioxidant nutrients may be protective. In view of the potential protective effects of diet, extensive research has been carried out to identify these beneficial nutrients, as well as foods in which they are present in high levels. The ability of fruit and vegetables to provide high quantities of the antioxidant nutrients is well established (Williamson, 1996).

1.3 The importance of fruit and vegetables.

Fruit and vegetables have long been known to be beneficial to health. They are a rich source of vitamins and minerals that the body cannot synthesize but which are essential to maintain normal development and function as well as provide protection against a variety of diseases. People with diets rich in fruit and vegetables enjoy better health than those with a low intake. In view of the beneficial health properties of fruit and vegetables, it has been recommended that people should consume at least 5 portions of fruit and vegetables each day (Foerster *et al.*, 1995).

There is emerging epidemiological evidence that low intakes of fruit and vegetables can contribute to several disease conditions including coronary heart disease, cancers, obesity and diabetes (Knekt *et al.*, 1994, Ness and Powles, 1997, Williams *et al.*, 1999). In addition to vitamins and minerals, fruit and vegetables are also low in fat and calories and a good source of dietary fibre. Extensive studies have been performed to identify the compounds in fruit and vegetables thought to provide protection to the body. Among the compounds identified are the antioxidant nutrients, the main ones being vitamins C, E and carotenoids and also the minerals selenium and manganese. Antioxidants are able to scavenge highly reactive free radicals which cause oxidative damage to body tissues (Halliwell, 1996). This helps delay or prevent development of diseases related to free radical damage (see section 1.8). Although the human body has its own defense against oxidative damage, this is inadequate. Thus, diet-derived antioxidants are important in maintaining health.

Attempts have been made to further identify other bioactive compounds in fruit and vegetables with potential health properties. This has led to the discovery of various other non-nutrients with potential antioxidant activities. The most important are

phenolic compounds that are secondary plant metabolites made up of various classes of compounds including hydroxybenzoates, hydroxycinnamates and a range of polyphenols. Flavonoids are the main class of polyphenols. They are potent antioxidants and some are more active than the well-known vitamin antioxidants (Vinson *et al.*, 1995, 1999). This thesis describes the nature and importance of flavonoids and discusses these bioactive compounds and their possible role in providing protection against diseases. Their absorption and metabolism, which is of particular importance will be discussed in detail later in this chapter.

1.4 History of flavonoids

Flavonoids have been reported from as early as 1925, with the description of the anthocyanin pigments in plants (Onslow, 1925). Even before their antioxidant properties were identified, flavonoids had been used for many years in traditional medicine for the purpose of maintaining good health and general well being. Approximately 40 species of plants have been reported to be used as phytomedicines due to their flavonoid content. One of the more popular is *Ginkgo biloba*.

Initially, certain citrus-flavonoids were claimed to have vitamin properties, and were referred to as 'Vitamin P' (Rusznyak and Szent-Gyorgyi, 1936). However, this term was later dropped due to insufficient evidence to support their role as indispensable food components equivalent to vitamins. In recent years, research has been focussed on the identification and quantification of flavonoids from plants. In this way, the physiological and biological properties of individual flavonoids can be evaluated. More importantly, flavonoids that are especially beneficial to health can be identified.

1.5 Chemistry of Flavonoids

Flavonoids are a class of polyphenols that occur ubiquitously in plants and plant products. They are not synthesised in animals, but are found in plant-derived foods. The biological activities of flavonoids at pharmacological doses are well established (Ferry *et al.*, 1996, Herrera *et al.*, 1996) but an important issue is whether they can provide any potential health benefits at normal dietary intakes.

Flavonoids are found in every family and nearly every species of higher plant (gymnosperms and angiosperms). Flavonoids typically occur as conjugates linked to sugars such as glucose, rhamnose or galactose (Cook and Samman, 1996). Relatively small amounts are found in the free form as aglycones.

A basic flavonoid molecule consists of two 6C aromatic rings (A and B rings) linked by a 3C unit (C ring) (Figure 1.1). Ring A is formed by the acetate pathway whereas ring B is formed by the shikimate pathway. Condensation of ring A with the carboxyl group of ring B formed the chalcones which then undergo cyclization to flavane derivatives.

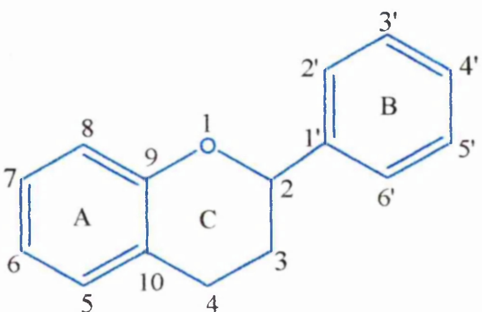

Figure 1.1: Basic flavonoid structure

The structure of the flavonoid molecule allows a multitude of substitution patterns to occur, giving 13 different sub-classes, the main ones being flavones, flavonols, flavan-3-ols, isoflavones, anthocyanidins and flavanones. The substitutions in the flavonoid molecule include hydrogenation, hydroxylation, methylation, sulphation and glycosylation (Cook and Samman, 1996).

To date, over 8000 different flavonoids have been identified and the list still increasing. Not all plant-derived foods have been examined to determine their exact flavonoid content, therefore the information regarding actual flavonoid intake of humans is incomplete. However, this situation is changing as more studies are performed on the identification and quantification of flavonoids in foods commonly consumed in the human diet. Hertog *et al.* (1992) recently reported the flavone/flavonol content of 28 vegetables and 9 fruits consumed in the Netherlands. An extension of such studies will be useful in the estimation of flavonoid intake of the human diet and at the same time, the findings can be used in epidemiological studies to determine the association between flavonoid intake and disease occurrence. Before going into further detail on the beneficial role of this polyphenol, a brief explanation on the different sub-classes and their biosynthesis is discussed.

1.5.1 Sub-classes of flavonoids

1.5.1.1 Flavones

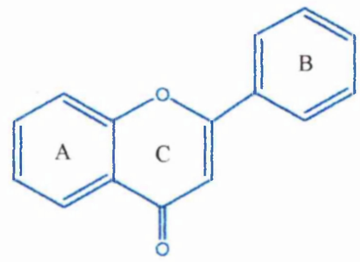

Figure 1.2: Structure of flavone

Flavones consist of a group of pale yellow pigments of flower petals, leaves, seeds and fruits. They are less prominent in fruits compared to other classes of flavonoids, with the exception of citrus fruits. Common flavones include apigenin, luteolin and diosmetin. Their roles in plants are to provide colour and taste to plant tissue (Peterson and Dwyer, 1998). Herbs such as parsley, rosemary and thyme and cereal grains contain flavones (Peterson and Dwyer, 1998).

1.5.1.2 Flavonols

Figure 1.3: Structure of flavonol

The structure of flavonol is very similar to flavones except for the addition of a hydroxyl group at C3 of ring C in the former. Flavonols are pale yellow and sparingly soluble in water. They are abundant in flowers and leaves making the flavonols one of the most widely studied flavonoid groups. Some of their roles include acting as regulators of germination, flowering and pollen tube growth (Furuya *et al.*, 1962). Quercetin, kaempferol and isorhamnetin are some of the more common flavonols and are found in leafy vegetables, berries, herbs and legumes.

1.5.1.3 Flavan-3-ols

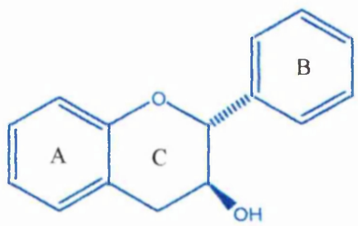

Figure 1.4: Structure of (+)-catechin

Flavan-3-ols such as (+)-catechin (Figure. 1.4) and (-)-epicatechin are colourless, water-soluble and oxygen-sensitive substances. Their structure consists of one OH group in position C3 of ring C as well as saturation of ring C. Catechins represent the group of flavonoids that occur in highest concentrations in green plants especially in teas and fruits such as grapes, black currants and strawberries (Peterson and Dwyer, 1998, van het Hof *et al.*, 1999). Catechin derivatives including epicatechin, epicatechin gallate and epigallocatechin gallate are present in high concentrations in grape seed and black and green tea. The astringent taste of fruits, teas and wine are provided by catechins.

Condensation of catechins leads to the formation of a range of oligomeric proanthocyanidins.

1.5.1.4 Isoflavones

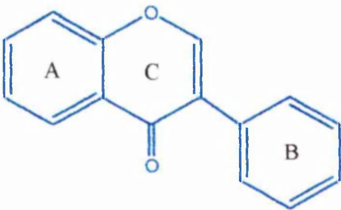

Figure 1.5: Structure of isoflavone

Isoflavones differ from the other sub-classes of flavonoids with attachment of the B ring at the C3 position of ring C. Daidzein and genistein are the most common isoflavones. Isoflavones are water-soluble compounds. This sub-class is found almost exclusively in legumes such as black bean, chickpeas and green split peas. Soybean is the most abundant source of isoflavones and research has been performed on the possible health properties of soybean. Isoflavones are widely known for their estrogenic properties (Setchell and Cassidy, 1999). This has prompted investigation on their role as potential alternative therapies for a range of hormone dependent conditions including cancer, menopause symptoms and osteoporosis.

1.5.1.5 Anthocyanidins

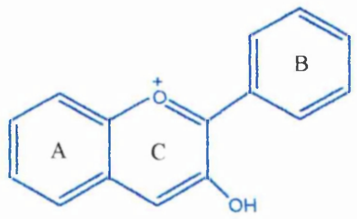

Figure 1.6: Structure of anthocyanidin

Anthocyanidins are water-soluble plant pigments and contain a flavylium structure with a cation group on ring C. Anthocyanidins such as cyanidin, delphinidin and pelargonidin are responsible for most of the red, blue and intermediate colours of

flowers and fruits, their role being associated with pollination or seed distribution. They occur in leafy vegetables, cereals, tubers and bulbs and are also present in fruits such as berries, apples and plums. The most common naturally occurring anthocyanins in the plant kingdom are the 3-glycosides or 3,5-diglycosides of anthocyanidins (Harborne, 1967). Conjugated anthocyanidins are called anthocyanins. The colour of anthocyanins is pH-dependent with anthocyanidins being relatively insoluble whereas their glycosides are easily soluble and stable at pH 3-7.

1.5.1.6 Flavanones

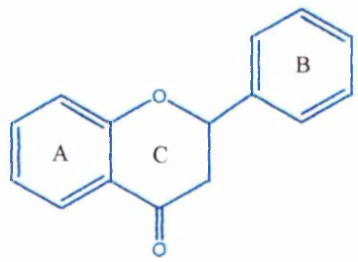

Figure 1.7: structure of flavanone

Flavanone is the precursor for the synthesis of other sub-classes of flavonoids. Flavanones are found mainly in citrus fruits and contribute to the flavour of these fruits. In addition, liquorice roots, cumin and peppermint also contain flavanones. Naringin, which is found in grapefruit, provides the bitter taste whereas hesperidin in oranges is tasteless.

1.6 The biosynthesis of flavonoids

1.6.1 Introduction

The biosynthesis of all flavonoids involves three main pathways consisting of the shikimate pathway for the synthesis of aromatic amino acids, the general phenylpropanoid pathway for the synthesis of the intermediates for various flavonoids and the pathway for the modification of the flavonoid aglycones.

1.6.2 The shikimate pathway

The shikimate pathway, found only in microorganisms and plants is an essential route to the biosynthesis of aromatic amino acids including phenylalanine, tyrosine and tryptophan. It is responsible for the formation of the phenylpropanoid units, the precursors for flavonoid biosynthesis. Intermediates derived from this pathway act as protein building blocks and more important, in the synthesis of various secondary metabolites such as plant pigments and UV light protectants, although in this case, only the biosynthesis of flavonoids will be discussed (Figure 1.8). As only a brief explanation is given, the enzyme involved in each step is not discussed.

The carbon skeleton of the flavonoid molecule is derived from acetate and phenylalanine. Phenylalanine undergoes a series of reactions to form 4-coumaroyl CoA which forms the B ring and parts of the heterocyclic C ring of the flavonoid skeleton. Ring A is formed by condensation of three acetate units via malonyl CoA. Malonyl-CoA and 4-coumaroyl CoA are the precursors for the synthesis of all flavonoids. Condensation of ring A with 4-coumaroyl CoA produces tetrahydroxychalcone which subsequently undergoes isomerization to flavanone (naringenin). Flavanone acts as the precursor for the synthesis of the other sub-classes of flavonoids including flavones, flavonols, anthocyanidins and isoflavonoids.

Flavones (e.g apigenin) are synthesised from flavanones by introduction of a double bond between C2 and C3. On the other hand, oxidative rearrangement of the flavanone with a shift of ring B from position 2 to 3 give rise to isoflavone such as

genistein. Hydroxylation of flavanone in position 3 forms dihydroflavonol, acting as intermediates in the synthesis of flavonols, catechins, proanthocyanidins and anthocyanidins. Introduction of a double bond between C2 and C3 of dihydroflavonols leads to the synthesis of the subclass flavonol.

Reduction of dihydroflavonols in position 4 leads to the formation of leucopelargonidin which act as an intermediate in the synthesis of catechin, proanthocyanidin and anthocyanidin. The synthesis of anthocyanidin involves a series of reactions including reduction of the carbonyl group of dihydroflavonol (Harborne and Grayer, 1986). Further reduction in position 4 of leucoanthocyanidins gives rise to catechins (e.g. Afzelechin). Leucoanthocyanidins are also the precursors for anthocyanin biosynthesis.

1.6.3 Steps to flavonoid modification

Simple flavonoids can be modified to give various aglycones or conjugates within each flavonoid class. This is achieved by further hydroxylation of rings A and B, followed by methylation, glycosylation or sulphation of the various hydroxyl groups. The most common structural modifications of flavonoid aglycones are hydroxylation and subsequent methylation in the 3′ and 5′ positions of ring B (Ebel and Hahlbrock, 1982). Extensive studies have been performed on the biosynthesis of flavone and flavonol glycoside particularly in parsley (Ebel and Hahlbrock, 1982). The sequence of reactions includes substitutions of ring B of the aglycones by hydroxylation and *O*-methylation followed by glycosylation and acylation of the resulting glycosides.

CARBOHYDRATE

Shikimate

Acetyl-CoA

Phenylalanine

Malonyl-CoA

4-Coumaroyl-CoA

2',4',6',4-Tetrahydroxychalcone

Genistein
(5-Hydroxyisoflavonoid)

Naringenin
(Flavanone)

Apigenin
(Flavone)

Dihydrokaempferol
(Dihydroflavonol)

Kaempferol
(Flavonol)

Leucopelargonidin
(Leucoanthocyanidin)

(+)-catechin
(Flavan-3-ol)

Pelargonidin
(Anthocyanidin)

Figure 1.8: A schematic diagram of the pathway of the biosynthesis of flavonoids

1.7 Role of flavonoids

1.7.1 Role of flavonoids in plants

Table 1.1: Summary of the role of flavonoids in plants

Function	Example of flavonoids	Reference
UV protectants	Anthocyanins, flavones, isoflavonoids	Dixon and Paiva, 1995
Regulate auxin levels	Flavones	Moore, 1989
Provide colour to plants	Anthocyanins	Harborne and Grayer, 1986
Attract pollinators	Anthocyanins	Harborne and Grayer, 1986
Aid in seed dispersal	Anthocyanins	Harborne and Grayer, 1986
Feeding deterrents	Anthocyanins	Harborne and Grayer, 1986
Protection against stress:		
i) Pathogen attack	Flavonols, isoflavonoids	Bailey and Mansfield, 1982
ii) Cold temperature	Anthocyanins	Christie *et al.*, 1994
iii)Nutritional stress (e.g. low nitrogen or low phosphate)	Isoflavonoids, anthocyanins	Graham, 1991

The main role of flavonoids in plants is to act as UV protectants and they are synthesised in response to light. They also control the levels of auxins, the regulators of plant growth and differentiation (Moore, 1989) in addition to providing colour, texture and taste to food plants (Harborne, 1986). At the same time, flavonoids can affect pollination by inhibiting or stimulating insect feeding (Hedin and Wangea, 1986).

Flavonoids are synthesised in response to UV-B light. Hence, they accumulated in the upper epidermal cells of leaves (Day *et al.*, 1993) and absorbed UV-B, preventing further penetration into the leaf cells. Mutant plants lacking the ability to synthesise flavonoids are more sensitive to UV-B (Landry *et al.*, 1995). Flavonoids are also produced in plants in response to stress and this includes pathogen attack, high UV light,

cold temperature and nutritional stress (Dixon and Paiva, 1995). Isoflavonoids and flavonols (e.g quercetin and kaempferol) are synthesised in response to pathogen attack (Bailey and Mansfield, 1982) and low levels of nitrogen in nitrogen-fixing plants (Graham, 1991) whereas anthocyanins increase following cold stress (Christie *et al.*, 1994).

Flavonoids can exist in both coloured and colourless forms in plants, and influence the feeding habits of animals, especially insects, birds and herbivores and thereby can indirectly affect pollination. Anthocyanins are the most important flavonoid plant pigments. The flower colours provided by flavonoid pigments provide attraction to pollinating insects with each class of pollinator having its own colour preferences. In addition, flower colour may also signal the availability of the flower for pollination to attract pollinators and may then change colour, lose their attractiveness and hence be avoided by the pollinators (Harborne and Grayer, 1986). This will assist in increasing the efficiency of the pollination process.

Certain flavonoids can act as attractants to stimulate insect feeding and in most cases, the *O*-glycosides are the active compounds rather than the aglycones (Harborne and Grayer, 1986). At the same time, flavonoids that act as attractants for some insects may be a deterrent to others. The structure of the flavonoid molecule together with their substitution pattern helps determine their role either as attractants or deterrents in insect feeding. Flavonoids acting as insect deterrents have the potential to be used as insecticides and confer protection to crops against insect attack.

1.7.2 Role of flavonoids in human health

1.7.2.1 Introduction

Of greater interest is the role of flavonoids in human health and their ability to influence various functions in the human body. Clinically relevant functions have been ascribed to flavonoids including anti-inflammatory, anti-microbial, anti-cancer and hypocholesterolaemic properties (Formica and Regelson, 1995; Table 1.2).

Table 1.2: Summary of the role of flavonoids in human health

Function	Example of flavonoids	Reference
Anti-inflammatory agents	Quercetin, myricetin, fisetin	Koshihara *et al.*, 1983
Vascular responses		
i) inhibits platelet aggregation	Quercetin, myricetin	Lanza *et al.*, 1987
ii) inhibits LDL oxidation	Catechins	Rice-Evans *et al.*, 1996
Anti-microbial		
i) anti-viral	Quercetin, morin, catechin	Selway, 1986
ii)anti-bacterial		Schramm and German, 1998
Anti-cancer		
i) inhibits protein kinase	Quercetin, genistein, daidzein	Srivastava, 1985
ii) inhibits calmodulin	Quercetin	Nishino *et al.*, 1984
iii) prevents oxidative DNA damage	Quercetin, myricetin	Duthie *et al.*, 1997
Inhibitors of enzymes		
i) catechol-*O*-methyl transferase	Quercetin	Borchardt and Huber, 1975
ii) membrane Na^{+}/K^{+} ATPase	Flavonol aglycones	Umarova *et al.*, 1998
Antioxidant See section 1.9	Quercetin, catechin	Rice-Evans *et al.*, 1996

1.7.2.2 Flavonoids as anti-inflammatory agents

The role of flavonoids as anti-inflammatory agents is well established. Flavonoids such as quercetin and catechin can reduce inflammation by suppressing various functions of neutrophils including release of oxidants during phagocytosis (Busse *et al.*, 1984). The mechanism of their action appears to involve the enzyme systems of signal transduction and cell activation processes of the immune system (Formica and Regelson, 1995). This includes the widely reported ability of quercetin to inhibit lipooxygenase and monooxygenase, enzymes of arachidonate metabolism responsible for maintaining integrity of the inflammatory systems (Koshihara *et al.*, 1983, Da Silva *et al.*, 1998).

1.7.2.3 Flavonoids and anti-microbial activity

The antimicrobial activity of flavonoids against a variety of bacteria (*E. coli* and *Salmonella*), viruses (Influenza A, rhinoviruses) and fungi are well documented (Selway, 1986, Schramm and German, 1998). Compounds such as quercetin, morin and catechin possess antiviral activity and the mechanism of action is related to their ability to bind to viral coat protein and interfere with nucleic acid synthesis, therefore damaging DNA (Formica and Regelson, 1995). Damage of bacterial DNA by flavonoids is also postulated as the mechanism for the anti-bacterial activity of flavonoids. The aglycones appear to be the active forms particularly the methylated or lipophilic flavones (Ramaswamy *et al.*, 1972).

1.7.2.4 Flavonoids and vascular responses

Various mechanisms have been ascribed to the role of flavonoids in vascular responses. The ability of flavonoids to act as antioxidants and prevent LDL oxidation is well established (See section 1.9.1). The other important role is their capacity to block platelet aggregation. Flavonoids such as quercetin can inhibit the enzyme phosphodiesterase involved in cAMP breakdown (Lanza *et al.*, 1987) as well as lipid peroxidation in the platelets (Gryglewski *et al.*, 1987), subsequently leading to inhibition of platelet aggregation. In addition, flavonoids are able to promote vascular relaxation, thereby regulating normal blood flow to the heart and this has been postulated as one of the mechanisms of protection against heart disease (Formica and Regelson, 1995).

1.7.2.5 Flavonoids and cancer

The role of flavonoids in the prevention of cancer is associated with their ability to influence cancer-inducing processes in the body. These include the ability of certain flavonoids to inhibit enzymes for instance protein kinase (Srivastava, 1985) as well as the protein calmodulin (Nishino *et al.*, 1984) responsible for regulating many physiological activities such as tumour promotion and regulation of cell transformation and cell growth. In addition, flavonoids particularly quercetin and myricetin can suppress hydrogen peroxide-induced DNA damage in isolated human lymphocytes

(Duthie *et al.*, 1997). Neither α-tocopherol nor β-carotene showed protective effects implying the importance of flavonoids as an alternative source of antioxidants.

1.7.2.6 Flavonoids as inhibitors of enzymes

Other biological effects of flavonoids observed may be largely contributed by their ability to inhibit or promote several enzyme systems involved in major pathways that regulate various processes in the body. Some of the common enzymes inhibited by flavonoids are catechol-*O*-methyl transferase (Borchardt and Huber, 1975), protein kinase C (Srivastava, 1985) and Na^+/K^+-ATPase (Umarova *et al.*, 1998). These enzymes play a role in the function of various mammalian cellular systems including tumour promotion and the regulation of cell transformation and cell growth. In most cases, the aglycones are the active forms whereas the glycosides are less active (Harborne and Grayer, 1986).

Catechol-*O*-methyl transferase is involved in the metabolism and inactivation of the catecholamines epinephrine and norepinephrine which regulate various metabolic processes in the body. Flavonoids such as quercetin, are inhibitors of catechol-*O*-methyl transferase, resulting in prolonging physiological actions of catecholamines. This could subsequently promote anti-histaminic and anti-inflammatory effects as well as reducing capillary permeability (Kuhnau, 1976).

Flavonoids can inhibit membrane-located Na^+/K^+-ATPase responsible for maintenance of transport processes across membranes (Umarova *et al.*, 1998). The Na^+/K^+-ATPase plays an essential part in the mechanism of the inotropic effect of cardiotonic compounds. Several flavonoids particularly quercetin are able to inhibit this enzyme and showed positive inotropic effect. This inhibitory action would restrict membrane permeability.

1.7.2.7 Flavonoids and pro-oxidant activity

Polyhydroxylated flavonoids are susceptible to oxidation reactions, which can lead to formation of oxygen radicals (Canada *et al.*, 1990). This process has been suggested as the basis for the proposed mutagenic properties of flavonoids (Brown,

1980). However, the superoxide anions formed during autooxidation of flavonoids could still be scavenged by the body's defense system including superoxide dismutase, other antioxidants or by flavonoids themselves. Furthermore, at dietary levels (26 mg/day), flavonoids have not been shown to be carcinogenic or mutagenic, in fact several flavonoids possess anti-tumour activities (Kamei *et al.*, 1996).

1.7.2.8 Medicinal properties of flavonoids

Many species of plants with medicinal properties contain flavonoids. Approximately 40 species have been used as phytomedicines due to their flavonoid content. *Ginkgo biloba*, for instance, has various health properties and has been used for generations as a herbal medicine for treating peripheral vascular disease such as intermittent claudication[1] and cerebral insufficiency[2] in the elderly (Kleijnen *et al.*, 1992). This plant contains a number of compounds including flavonols, flavones and proanthocyanidins. Although there is strong evidence that *Ginkgo* can relieve cerebral insufficiency, its role in treating intermittent claudication is inconclusive (Kleijnen and Knipschild, 1992). It is thought that the alleged affects are linked to the antioxidant properties of flavonoids and their interaction with various enzymes in the body (See section 1.7.2.6 and 1.9). Indeed, flavonoids have been shown to exhibit potent action on the nitric oxide system which is involved in various aspects of physiological regulation particularly in the regulation of vascular tone (van Acker *et al.*, 1995).

1.7.3 Summary

Flavonoids are not only important to plants but also play a major role in humans too. However, of particular current interest is their potential to act as antioxidants. As most of the observed beneficial roles of flavonoids are possibly linked to their antioxidative ability, extensive research has been carried out on this topic. Knowledge of the mechanisms of action involved is crucial towards understanding the potential role of

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[1] Pain and cramp in the calf muscles, aggravated by walking and caused by insufficient blood supply.

[2] A collection of symptoms associated with impaired cerebral circulation: include difficulties of concentration and of memory, absent mindedness, confusion, lack of energy, tiredness, decreased physical performance, depressive mood, anxiety, dizziness, tinitus, headache.

flavonoids in preventing oxidative damage and protecting against diseases such as coronary heart disease and cancer. The next section describes the relationship between oxidative damage and these two diseases and the role flavonoids play in providing protection.

1.8 Oxidative damage in the body

In order to assess the antioxidant potential of flavonoids, an understanding of the mechanism of free radical reactions is needed. The human body is constantly under attack from free radicals, causing oxidative stress in tissues and organs. As mentioned previously, free radicals and oxidative stress play a role in the development of several diseases including cancer and coronary heart disease. Free radicals are highly reactive atoms or molecules, capable of existing independently. Electrons in atoms or molecules are usually paired and are thus able to function normally. However, the presence of one or more unpaired electrons create free radicals that are very unstable and have the potential to cause extensive damage to the human body. Examples of free radicals produced in the body include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$) radicals.

Free radical reactions involve either donation or acquisition of a single electron from another atom. This process leads to the generation of a new radical. If this process is repeated, more radicals will be generated, creating a series of reactions known as the chain reaction of oxidation. Chain reactions can cause a lot of damage to cells and tissues in the body, subsequently affecting their normal structure and function. Free radicals can react with either another free radical or a nonradical. When a free radical reacts with another free radical, their unpaired electrons are joined to produce a nonradical. A typical example is the reaction of nitric oxide ($NO^{\bullet}$) with superoxide radical ($O_2^{\bullet -}$) (Figure 1.9a). This type of reaction is desirable as the free radical reaction can be terminated. However, the reaction between a free radical and a nonradical will result in the generation of a new radical. An example is the reaction between superoxide radical ($O_2^{\bullet -}$) and a fatty acid (Figure 1.9b). This type of reaction is more common in the body as most biological molecules are nonradicals.

$$NO^{\bullet} + O_2^{\bullet -} \longrightarrow ONOO^{-} \quad \text{(a)}$$

$$O_2^{\bullet -} + LOOH \longrightarrow LOO^{\bullet} + H_2O \quad \text{(b)}$$

Figure 1.9: Examples of free radical reactions

Sources of free radical production are varied; there are endogenous as well as exogenous sources. Endogenous sources include normal metabolic processes occurring in the body such as aerobic metabolism and the immune system. Approximately 1-3% of the oxygen we breathe into our lungs is turned into free radicals. Some free radicals however are made deliberately. The immune system, for instance, generates free radicals (superoxide and nitric oxide) as part of the body's defence system against foreign materials. However, at the same time, too high a concentration of these radicals can damage nearby cells or tissues.

There are various exogenous sources of free radical production with cigarette smoke, pollution and radiation being the more important. Cigarette smoke is a major hazard in promoting oxidative damage in the body and its role in the development of cancer and heart disease is well established (Kalra *et al.*, 1991). Radiation, especially from sunlight can also contribute significantly to free radical production.

Oxidative stress caused by these free radicals can damage important cells in the body, affecting DNA, proteins and lipids and leading to impairment of their normal function. The human body normally has its own defence system to counteract free radical attack. This defence system is in the form of enzymes and antioxidants. The enzyme systems are synthesised naturally in our body whereas antioxidants are obtained from the diet. Thus, dietary antioxidants are important as intakes of food containing high levels of antioxidants can boost the body's defence system and protect against free radical damage. Ideally, there should be a balance between free radicals and antioxidants in the body to limit damage but when there are more free radicals and insufficient antioxidants to protect against their effects, this will lead to oxidative damage.

Various diseases are associated with free radical damage. Cancer and coronary heart disease are the major diseases associated with free radicals, followed by cataracts and brain dysfunction such as Parkinson's disease and Alzheimer's disease.

1.9 Antioxidant properties of flavonoids

The structure of the flavonoid molecule together with the presence of hydroxyl groups makes them very strong antioxidants. Various experimental studies have been performed to demonstrate the mechanisms of flavonoid action as antioxidants, using both aqueous and lipophilic systems (Cotelle *et al.*, 1992, Salah *et al.*, 1995). Attempts were also made to determine the hierarchy of the antioxidant activity of different flavonoids and to establish their structure-antioxidant activity relationships.

The effectiveness of flavonoids as antioxidants is dependent on their solubility in either the aqueous or lipophilic phase to achieve maximum interaction with the free radicals. Most of the initial research was carried out using *in vitro* systems (Rice-Evans *et al.*, 1996, Frankel *et al.*, 1993) and it was not until recently that more *in vivo* experiments were performed with the development of better and more sensitive analytical procedures (Day *et al.*, 1997, Nigdikar *et al.*, 1998, Serafini *et al.*, 1998). Most of the *in vitro* studies determined the relationship between the structure of the different flavonoids and their effectiveness as antioxidants (Rice-Evans *et al.*, 1996, van Acker *et al.*, 1996). Based on the antioxidant activity of flavonoids in aqueous phase, it has been proposed that the requirements for maximum radical scavenging activities are i) a 3-OH group on ring C and 5-OH group with 4-keto function in the A and C rings, ii) a 2,3-double bond adjacent to the 4-carbonyl in ring C and iii) a catechol moiety on ring B (van Acker *et al.*, 1996). Quercetin, for example, satisfies all the criteria above and is a potent antioxidant (Vinson *et al.*, 1995, van Acker *et al.*, 1996) (Figure 1.10a). Unsaturation in ring C allows electron delocalization across the molecule for stabilisation of the radical form. Catechin on the other hand did not satisfy one of the criteria above and showed a lower antioxidant activity than quercetin (Figure 1.10b). Unsaturation in ring C of catechin resulted in the loss of electron delocalization from the radical on the B ring to the A ring. Most studies have demonstrated strong antioxidant activities of the flavonoid aglycones compared to the glycosides (da Silva *et al.*, 1998, Vinson, 1998). Glycosylation of flavonoids, for example in rutin (quercetin-3-rutinoside), reduces their activity due to blockage of the 3-OH group on ring C, while still retaining the catechol moiety (Rice-Evans *et al.*, 1996) (Figure 1.10c).

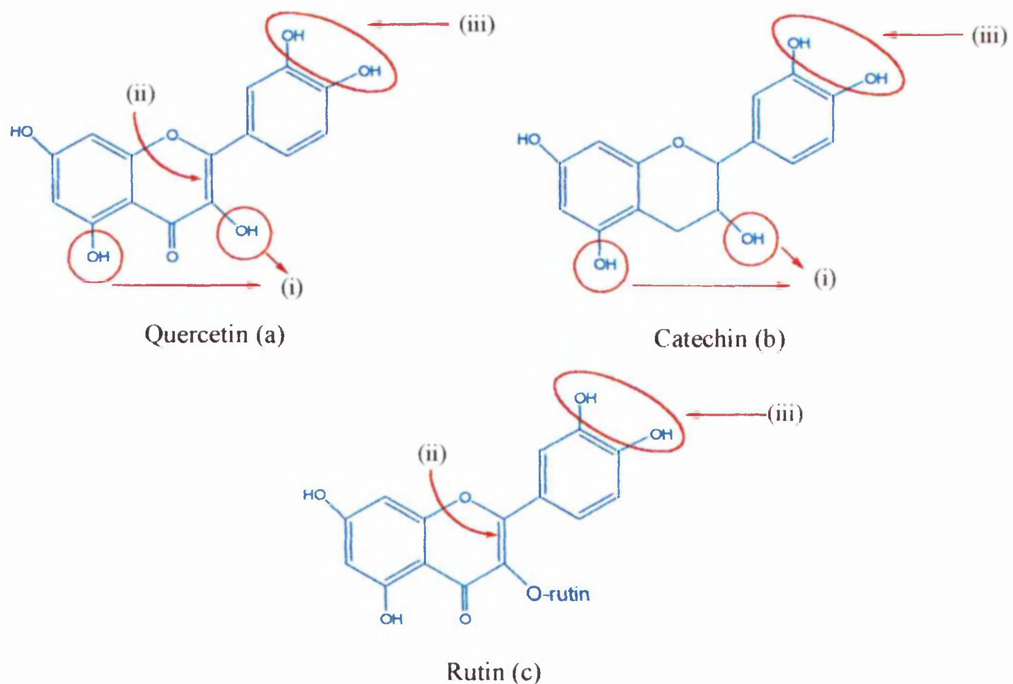

Figure 1.10: Structural modification of flavonoids and their antioxidant activities

The structural features for optimum antioxidant activity of flavonoids in a lipophilic phase requires only the presence of a 3′,4′-orthodihydroxy structure in the B ring and a 4-keto group in the C ring. The *O*-dihydroxy substitution in the B ring is needed for stabilising the resulting free radical form. In general, flavonoids especially the aglycones are not particularly water-soluble and have a more lipophilic characteristic. Thus, they are more likely to act as potent antioxidants in the lipophilic phase depending on their partition coefficient into the lipophilic region and their ability to react with the autooxidising lipids. It has been hypothesised that flavonoids such as catechins might be localised near the membrane surface, scavenging aqueous radicals and preventing consumption of α-tocopherol, whereas α–tocopherol mainly acts as a chain-breaking lipid peroxidation process within the LDL (Salah *et al.*, 1995).

Although extensive research on the *in vitro* antioxidant activity of flavonoids has been performed, very little information is available on the mechanisms through which these compounds act *in vivo*. Ultimately, *in vivo* studies are crucial to assess the bioactive role of flavonoids in the body. Many of the *in vivo* studies have investigated the antioxidant potential of red wine and teas which are known to contain high levels of flavonoids (Princen *et al.*, 1998, Serafini *et al.*, 1998).

Consumption of tea, particularly green tea, protects the plasma against peroxidation (Serafini *et al.*, 1996, Ishikawa *et al.*, 1997). High levels of the tea flavonoid catechins and theaflavins were postulated to prevent LDL oxidation by incorporation into the LDL molecule. In the first study, plasma of human volunteers collected after consumption of 300 ml of green tea significantly increased the lag-phase of 2,2'-diazobis(2-amidinopropane)dihydrochloride (ABAP)-induced peroxidation (Serafini *et al.*, 1996). The second study investigated the antioxidant effect of drinking five cups of tea per day for four weeks (Ishikawa *et al.*, 1997). Plasma of volunteers collected at the end of the study prolonged the lag time of copper-catalysed LDL oxidation. In contrast to this, consumption of six cups per day of green and black tea over four weeks had no effect on copper-induced LDL oxidation in a group of smokers (Princen *et al.*, 1998). However, the large amounts of free radicals in cigarette smoker, which can deplete antioxidant levels as well as cause lipid peroxidation in plasma, may explain the negative results. This may suggest that maximum protection is afforded to healthy humans where flavonoids can act in the prevention of free radical oxidative damage but may not be potent enough to act on damaged cells/tissues. Details on the mechanisms of antioxidant action of flavonoids are discussed in section 1.9.1.

Red wines have long been thought to provide beneficial health properties. They are rich in flavonoids particularly flavonols, anthocyanins and catechins (Burns *et al.*, 2000) and it is thought that these components contribute to the protective effects of red wine against cardiovascular disease, a phenomenon popularised as the 'French Paradox'. As people in France particularly the Southern of France consumed high amounts of red wine, this may explain the low incidence of cancer and coronary heart disease in France compared to the rest of the world (Renaud and De Lorgeril, 1992, Frankel *et al.*, 1993).

Data on the *in vivo* antioxidant activities of red wine are controversial with one study showing no effects (de Rijke *et al.*, 1996) whereas another showed protection against LDL oxidation (Nigdikar *et al.*, 1998). The possibility of alcohol in red wine influencing the *in vivo* antioxidant activities are excluded as similar studies with alcohol-free red wine (Serafini *et al.*, 1998) and concentrated red grape juice (Day *et al.*, 1997) showed enhanced plasma antioxidant capacity following their consumption.

1.9.1 Flavonoids and coronary heart disease

Oxidative damage caused by free radicals has been associated with the development of coronary heart disease (Strain *et al.*, 1991). The three pathological conditions for coronary heart disease are angina pectoris, myocardial infarction and atherosclerosis. Atherosclerosis occurs as a result of narrowing of the blood vessels responsible for carrying blood to the heart. This can lead to heart attack due to impairment in blood flow.

Oxidative damage to low density lipoprotein (LDL) is implicated in the aetiology of atherosclerosis (Regnstrom *et al.*, 1992). Figure 1.11 illustrates a schematic diagram of LDL oxidation. LDL is responsible for transporting cholesterol from the blood to the body's tissues for storage and is susceptible to free radical attack especially in the blood vessel wall. Free radical attack on LDL leads to oxidation of the LDL molecule (Figure 1.11). This is not recognised by the normal LDL receptors and is taken up instead by 'scavenger' receptors on macrophages, a part of the white blood cell. A variety of products are formed as a result of LDL oxidation. Some of these products, for example lysolecithin are capable of inducing adhesion molecules for monocytes on the surface of endothelial cells (Kume *et al.*, 1992). Other compounds such as cytokines can affect the growth of monocytes and their differentiation into macrophages and can also contribute to the disruption of endothelial cell integrity (Galis *et al.*, 1994). At the same time, smooth muscle proliferation occurs leading to injury of the endothelial cells. As more LDL is being oxidised, macrophages will be overladen with oxidised LDL, leading to the formation of foam cells. Foam cells together with smooth muscle cells will subsequently transform to fatty streaks, which over time slowly deposit on the endothelial cells causing narrowing of the blood vessels. A series of reactions occur which ultimately lead to platelet aggregation and subsequently to occlusive thrombosis. Myocardial infarction is caused by coronary thrombotic occlusions whereby the thrombotic clot breaks away and flows to a branch of the coronary arterial tree, thus blocking the artery.

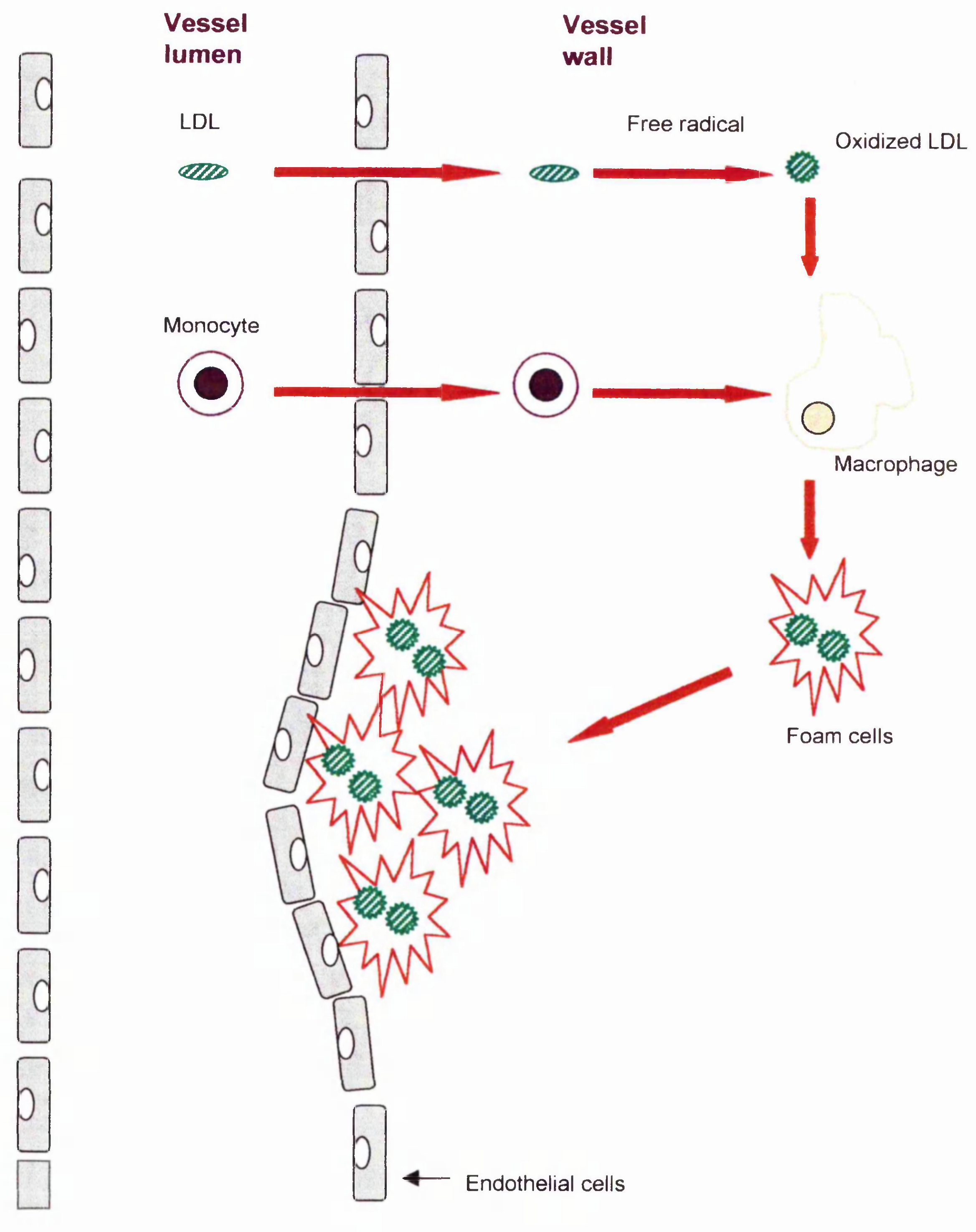

Figure 1.11: A schematic diagram of LDL oxidation

Polyphenols including flavonoids have been shown to inhibit copper-induced or macrophage-induced LDL oxidation *in vitro* (Frankel *et al.*, 1993, de Whalley *et al.*, 1990, Vinson *et al.*, 1995). The proposed mechanism of inhibition of LDL oxidation is via reducing the formation or release of free radicals in macrophages or possibly by protecting α–tocopherols in LDL from oxidation by becoming oxidised themselves (de Whalley *et al.*, 1990). In addition, flavonoids can terminate the chain reaction of lipid peroxidation by hydrogen donation, thus becoming a flavonoid radical and in turn react with free radicals to stop the chain reaction from propagating (Robak and Gryglewski, 1988).

In addition to preventing LDL oxidation, flavonoids can also act as anti-thrombotic agents *in vitro*. Activated platelets lead to the generation of lipid peroxides and superoxide anions. These radicals can destroy the endothelium dependent relaxing factor (EDRF) which are responsible for vasodilatory activity as well as inhibiting platelet aggregation. Flavonoids particularly from the sub-class flavonols are able to bind to platelet membranes, and together with their free-radical scavenging action can act as anti-thrombotic and vasoprotective agents and preventing the generation of lipid peroxides (Gryglewski *et al.*, 1987). At the same time, they can also prolong the half-life of EDRF.

Flavonoids are also scavengers of superoxide anions and have the ability to break the chain of formation of free radicals. They act as hydrogen-donating radical scavengers, for example by scavenging lipid alkoxyl and peroxyl radicals (Robak and Gryglewski, 1988, Salah *et al.*, 1995). This will stop or limit the initiation of free radical chain reaction that can ultimately lead to lipid peroxidation.

Flavonoids also possess metal-chelating properties e.g. towards iron and copper. Iron and copper are known to catalyse many processes leading to the generation of free radicals. Thus, their removal from the cells may inhibit the formation of oxygen radicals. Catechin, quercetin and diosmetin are capable of removing iron from iron-loaded hepatocyte cultures (Morel *et al.*, 1993) possibly by forming an inert complex with iron. Afanas'ev *et al.* (1995) demonstrated the formation of an iron-rutin complex which is inactive and unable to produce oxygen radicals and this may play an important role in the antioxidant action of flavonoids.

1.9.2 Flavonoids and cancer

Oxidative stress by free radicals has also been implicated in the development of cancer at many sites. Sunlight exposure and radiation, some of the sources of free radical production are risk factors for skin and lung cancer.

Cancer occurs as a result of abnormal cell division during growth and renewal of cells. Carcinogens, including free radicals, can initiate cancer by causing changes in DNA, leading to mutation of the DNA. When a mutated DNA is fixed in replication, the damage will be transferred into the new DNA. Accumulation of damaged DNAs ultimately leads to cancer. Various mechanisms have been postulated for the role of antioxidants in preventing cancer. This includes their ability to repair damaged DNA and react with free radicals to limit or stop their action on DNA (Ames *et al.*, 1993). A recent study has demonstrated that diet supplemented with vitamin C, E and β–carotene to a group of smokers and non-smokers resulted in a significant decrease in endogenous oxidative base damage in the lymphocyte DNA (Duthie *et al.*, 1996). Moreover, the lymphocytes from these subjects showed an increase resistance to H_2O_2-induced oxidative damage *in vitro*.

Flavonoids have the potential to provide protection against cancer. Two methods are commonly used to investigate the anti-cancer properties of flavonoids. One involved animal studies whereby the ability of flavonoids to inhibit or reduce chemically-induced cancer was assessed. The second approach involved studying the effect of flavonoids on cultured cancer cells *in vitro*. The anti-carcinogenic properties of quercetin have been extensively studied. Quercetin has been shown to inhibit skin tumours in mice induced by chemicals such as 7,12-dimethylbenz(a)anthracene, 3-methylcholanthrene and benzo(a)pyrene (Mukhtar *et al.*, 1988). In addition, dietary quercetin (2%) and rutin (4%) suppressed hyperproliferation of colonic epithelial cells and ultimately colon tumour incidence induced by azoxymethanol (Deschner *et al.*, 1991). In a recent study, quercetin has been shown to inhibit growth and induce apoptosis in cultured colon carcinoma cells (Richter *et al.*, 1999). Low dosage of quercetin (4-6 μM) was able to reduce cell numbers by 50%.

The anti-carcinogenic properties of flavonoids described above were based on animal studies and their relevance to humans is not known. Obviously, human studies would provide more beneficial information. Recently, a clinical trial was performed on 51 patients with terminal cancer. Following intravenous administration of quercetin at varying doses (60-1700 mg/m^2), the levels achieved in plasma inhibited lymphocyte tyrosine kinase activity, the enzyme capable of overriding growth regulatory control, subsequently leading to cancer (Ferry *et al.*, 1996). This is one of the initial studies describing the possible role of flavonoids as anti-cancer agents in humans.

On the other hand, a few studies have shown that flavonoids, when used at higher doses may promote the development of cancer. Quercetin was employed in many of these studies whereby high concentrations of this compound can increase the incidence of cancer including intestinal bladder tumours (Pamukcu *et al.*, 1980), kidney tubule lesions in male rats (Dunick and Hailey, 1992) and colon cancer (Pereira *et al.*, 1996). However, at the level of dietary intake, mutagenic properties of flavonoids have not been observed.

Several mechanisms have been suggested for the role of flavonoids in cancer prevention. They include the ability of flavonoids to act as modulators of cell differentiation and apoptosis, as antioxidants, as modulators of protein function and as hormones, the last mechanism possibly by the estrogenic properties of flavonoids. Quercetin for example, has antiviral activities, being able to bind viral coat protein and inflict damage to DNA, therefore destroying the proliferative capacity of certain viruses (Castrillo and Carrasco, 1987). Quercetin also possesses anti-tumour activities as demonstrated by its ability to suppress the growth of cultured human tumour cells (Kamei *et al.*, 1996). One of the reported mechanisms of action was by inhibiting protein kinase C (Srivastava, 1985). Quercetin has been shown to inhibit epidermal growth factor (EGF) receptor in cultures of colonic tumour cells, thereby inducing apoptosis (Richter *et al.*, 1999). Flavonoids may also act as antioxidants in protecting cells or tissues against oxidative damage. Quercetin for instance has been shown to protect CaCo-2 cells against oxidative attack (Duthie *et al.*, 1999).

Although the above studies show promising anti-cancer properties of quercetin, the relevance of such studies to the anticarcinogenic properties in humans is

questionable. For one, the high dose and type of carcinogens used in the animals is possibly different from cancer initiators in humans. Thus, the effect of quercetin may vary in human cells. In most cases, high doses of flavonoids were employed to induce an anti-cancer effect and their ability to show the same effect at dietary levels is not known.

1.9.3 Conclusion

Strong evidence exists particularly from animal studies on the ability of flavonoids to modulate various biochemical processes relating to protection against coronary heart disease and cancer. Their potent antioxidant action appears to play a major role in providing the beneficial effects. However, their ability to provide the same effect at the levels of dietary intake has yet to be addressed. Information on their absorption is crucial for this purpose. To address this issue, epidemiology studies investigating intake of dietary flavonoids and risk of coronary heart disease and cancer were performed.

1.10 Epidemiology evidence on flavonoid intake and disease occurrence

1.10.1 Introduction

Earlier studies have suggested that high dietary intake of fruit and vegetables, strongly correlated with the intake of antioxidant vitamins, may protect against cardiovascular diseases (Gaziano *et al.*, 1994). Fruit and vegetables contain amongst others, high levels of flavonoids which may contribute to protection against diet-related diseases. Assessment of the health protective effects of dietary flavonoids requires nutritional epidemiology studies investigating the habitual intake of a certain population and the development of diet-related diseases. Such studies are not easy to evaluate due to the presence of other nutrients including non-nutrients in the diet, as well as flavonoids. Therefore, a detailed protocol is required when conducting epidemiology studies, taking into account these confounding factors, as well as subject bias.

These factors not withstanding, epidemiology studies are useful for investigating the association between the dietary intake of flavonoids and risk of disease. This type of study monitors large populations and can provide important information regarding the flavonoid intake of a population group and occurrence of disease. However, the data should be interpreted with caution taking into account factors such as study design, effects of chance, subject bias, for instance smoking habit and lifestyle which may influence development of diseases studied. Data collection has to be carefully done to ensure maximum information is obtained. Also, reliance on existing food tables which do not have accurate flavonoid content of foods may result in under- or over-estimation of flavonoid intake. In addition, data on the bioavailability of flavonoids is still lacking and such information is important for assessing the relevance of the results obtained. Furthermore, the methods used to assess dietary intake have to be considered. The use of poor or inappropriate methods for assessing dietary intake is a major problem in this type of study.

1.10.2 Epidemiology evidence on flavonoid intake and coronary heart disease

To date, six epidemiological studies have been carried out investigating the association between flavonoid intake and the occurrence of coronary heart disease. Four of the studies established an association between flavonoid intake and coronary heart disease whereby higher intakes of flavonoids were associated with lower incidence of coronary heart disease.

Table 1.3: Summary of epidemiological studies investigating the association between flavonoid intakes and incidence of coronary heart disease

Study	Population	Follow up *(y)*	Flavonoid intake *(mg/d)*	Disease risk (relative risk)
Hertog *et al.*, 1993 *The Zutphen Elderly Study* Netherlands	805 men (65-84 y)	5 (1985-1990)	25.9	0.32
Hertog *et al.*, 1995 *The Seven Countries Study*	16 cohorts from 7 countries 12 763 men (40-59 y)	25 (1960-1985)	2.6 – 68.2	r = -0.50 (p = 0.01)
Keli *et al.*, 1996 *The Zutphen Study* Netherlands	552 men (50-69 y)	15 (1970-1985)	18.3 – 28.6	0.27
Knekt *et al.*, 1996 Finland	5133 men and women (30-69 y)	20 (1967-1992)	0 - 41.4	Women = 0.73 Men = 0.67
Rimm *et al.*, 1996 *Male health professionals* USA	34 789 male (40-75 y)	6 (1986-1992)	20.1	1.08
Hertog *et al.*, 1997 *The Caerphilly Study* UK	1900 men (45-59 y)	14 (1979-1993)	26.3	1.1

The first study was a five-year observation study in the Netherlands, otherwise known as the Zutphen Elderly Study (Hertog *et al.*, 1993). Some of the subjects were recruited from a previous study in 1960, while others were recruited at the beginning of this study in 1985. The age group ranged from 65-84 years and was therefore not representative of the general population. Following a 5-year follow up, a strong inverse association was found between mortality from coronary heart disease and intakes of flavonoids in the highest against the lowest tertile of flavonoid intake with a relative risk of 0.32. The study also suggested that flavonoid intake and tea consumption was

inversely associated with mortality from coronary heart disease (rr=0.45) suggesting tea as a possible major source of dietary flavonoids in protection against coronary heart disease.

In a prospective cross-cultural study, the same investigators looked at 16 different cohorts in seven countries; Finland, Italy, Greece, the former Yugoslavia, Japan, the Netherlands and the United States (Hertog *et al.*, 1995). After 25 years of follow up, average flavonoid intake was inversely associated with mortality from coronary heart disease and this explained about 25% variance in coronary heart disease in the 16 cohorts. The cohort in Japan had the highest flavonoid intake (68.2 mg/d) and the lowest age-adjusted 25-year mortality (4.5-6.3%) compared to the other cohorts. Japanese people are known to drink a lot of tea, particularly green tea, which is rich in flavonoids (Hertog *et al.*, 1995) and this may explain the above results. On the other hand, green tea consumption has been shown to reduce serum concentrations of lipids and lipoproteins, suggesting a different mechanism for their protection against cardiovascular disease (Imai and Nakachi, 1995). The 16 cohorts recruited in this study showed varying background and may not be representative of the general population. In addition, the subjects may also have different lifestyles and dietary habits depending on their countries, which may influence the outcomes measured. The results, therefore, should be interpreted cautiously due to the influence of other dietary or non-dietary factors. Furthermore, although a 7-day dietary record was employed to assess nutrient intake in most of the cohort, in some cases, only a 1-day dietary record was used. The accuracy of the latter method is questionable, as it may not represent the habitual dietary intake of the subjects.

In another study, a cohort in Zutphen, Netherlands, consisting of 552 men aged 50-69 years were monitored for 15 years (Keli *et al.*, 1996). The subjects were followed from 1970 onwards although information on their dietary intake was available from 1960, 1965 and 1970 owing to a previous study. The study demonstrated a strong inverse association between flavonoid intake and risk of stroke (rr=0.27) with participants in the highest quartile of flavonoid intake showing 73% lower risk of stroke. Based on the food data from 1960 to 1970, a strong inverse association was observed between tea consumption and stroke risk. Men who drank 4.7 cups of tea daily had a relative risk of 0.31 compared to those who drank less than 2.6 cups daily.

In Finland, 5133 men and women were observed for 20 years (Knekt *et al.*, 1996). To date, this is the only study which provided information on the association between flavonoid intake and coronary heart disease in women. A weak association was found between the intake of flavonoids and risk of coronary heart disease with a relative risk of 0.54 and 0.78 for women and men, respectively. However, after adjusting for intakes of antioxidant vitamins, the relative risk was higher in women (0.73) but lower for men (0.67). Intake of fruits, which are rich in antioxidant vitamins, was strongly correlated with flavonoids intake. Therefore, the possibility that the inverse association observed in this study was also contributed by intakes of antioxidant vitamins cannot be excluded.

More recently, 34,789 male health professionals in the United States were observed for 6 years (Rimm *et al.*, 1996). The data obtained did not support an inverse association between flavonoid intake and coronary heart disease and a relative risk of 1.08 was attained. In this study, men with a higher flavonoid intake tended to have a healthier lifestyle, drank less alcohol and smoked less. On the other hand, an inverse association was found between total flavonoid intake and coronary mortality in men with previous history of coronary heart disease (rr=0.63), although this was not statistically significant. This could be attributed to changes in lifestyle or dietary habits once diagnosed with cardiovascular disease. Another possible explanation is that flavonoids can be protective in men with prevalent coronary heart disease, probably due to their antioxidative action on thrombosis (Gryglewski, 1987).

The latest prospective cohort study was published three years ago involving 1900 Welsh men (Hertog *et al.*, 1997). After 14 years of follow up, no association was found between the intake of flavonoids and incidence of ischemic heart disease. The relative risk was 1.1 in the highest versus the lowest quartile of flavonoid intake. Instead, men in the group with high tea consumption appeared to have a higher incidence of ischemic heart disease which contrasted with previous results. This study argued that there was no association probably due to the effects of adding milk to tea. This might have caused the protein in milk to bind to flavonoids, thus inhibiting their absorption (Serafini *et al.*, 1996). As this style of drinking tea is common among the British, it may explain the above observation. However, not much information is available on the absorption of flavonoids from tea without milk and available data appear to suggest low bioavailability

of flavonoids in tea (Hollman, 1997). This could be another alternative explanation to the results obtained. Also, in this study, men with high flavonoid intake tend to smoke more, which may have affected the outcomes due to production of free radicals, leading to oxidative damage and subsequently heart disease.

In most of the studies, the average flavonoid intake of the participants was estimated from the study by Hertog *et al.*, (1992) who determined the flavonoid content of several common fruit, vegetables and beverages. Although flavonoid intake was inversely associated with coronary heart disease, the bioavailability of flavonoids from different foods should be taken into account. Furthermore, not much information is available regarding the absorption of dietary flavonoids from different foods and available data appear to indicate either low bioavailability of flavonoids from foods or their extensive metabolism following absorption (Hollman *et al.*, 1995, 1997). Further studies are therefore required to elucidate the absorption of flavonoids from various foods.

Most of the studies mentioned above employed either the dietary history method or a food frequency questionnaire, although in some instances, a 7-day dietary record method was used (Hertog *et al.*, 1995, Rimm *et al.*, 1996). Although ideally the 7-day dietary record is the best method to assess dietary intake, it is often not suitable for large population studies because of its high cost and low compliance from subjects. However, this method allows determination of nutrient intake based on the weight of all the food consumed and recorded by the subjects. The dietary history method or food frequency questionnaire, although less reliable than the 7-day dietary record are more applicable to population studies. Food frequency questionnaires are normally designed to assess intakes of specific foods or nutrients and may not be accurate enough for assessing flavonoid intake whereby certain high-flavonoid foods may have been excluded. On the other hand, the dietary history method requires subjects to recall their food intake, for example what they eat the year prior to the study. Subjects may not be able to recall what they ate last year or may have changed their dietary habits, which may influence the outcomes measured.

At the same time, flavonoid content can also vary significantly between cultivars of the same fruit and vegetables. Yellow and red onions for instance contain much

higher levels of flavonols than white onions (Tsushida and Suzuki, 1996). Lollo Rosso lettuce contains 100 times more flavonols than Round lettuce (Crozier *et al.*, 1997). Therefore, if the specific type of fruit and vegetables or beverages is not recorded, this could result in either under-estimation or over-estimation of flavonoid intake of the participants. Several of the studies found an inverse association between tea consumption and mortality from coronary heart disease (Hertog *et al.*, 1993b, Keli *et al.*, 1996). However, in addition to the flavonols in tea, the above effects could have also been contributed by the presence of other tea polyphenols particularly epicatechin gallate and epigallocatechin gallate. This type of study cannot prove causal relationships between flavonol intake and mortality from heart disease. More epidemiology as well as experimental studies on the mechanisms involved is still needed before definite conclusions can be made.

1.10.3 Epidemiology evidence on flavonoid intake and cancer

A vast number of epidemiological studies have consistently shown an association between high consumption of fruit and vegetables with a reduced risk of cancer at most site (Block *et al.*, 1992). Although the compounds responsible for this have not been identified, it is postulated that several antioxidants present in fruit and vegetables may play a role. These include the well known antioxidants carotenoids and vitamins E and C. Other non-nutrient compounds have also been identified as possible anti-cancer agents including dietary fibre and polyphenols, among which flavonoids are extensively studied. Several large epidemiological cohort studies as well as case-control studies on the cancer protective effects of flavonols and flavones have been conducted, as summarised in Table 1.4.

Table 1.4: Summary of epidemiological studies investigating the association between flavonoid intakes and incidence of cancer.

Study	Population	Follow up *(y)*	Flavonoid intake *(mg/d)*	Disease risk (relative risk)
Hertog *et al.*, 1994 *The Zutphen Elderly Study* Netherlands	738 men (65-84 y)	5 (1985-1990)	25.9	1.21
Goldbohm *et al.*, 1996 *Netherlands Cohort Study*	120,852 men & women (55-69 y)	4.3	n.a	1
Hertog *et al.*, 1995 *The Seven Countries Study*	16 cohorts from 7 countries 12,763 men (40-59 y)	25 (1960-1985)	2.6-68.2	r=0.39 (p=0.86)
Knekt *et al.*, 1997 Finland	9,959 men & women (15-99 y)	24 (1967-1991)	4 (0-41.4)	0.54
Case-control studies				
Garcia-Closas *et al.*, 1998 *Lung cancer risk in women* Barcelona, Spain	103 cases 206 controls (mean age=63 y)	3 (1989-1992)	5.1-5.6	Odds ratio=0.51 (p=0.1)
Garcia-Closas *et al*, 1999 *Risk of gastric cancer* Spain	354 cases 354 controls	2 (1987-1989)	9.5	Odds ratio=0.44 (p=0.003)

n.a = not analysed

The Zutphen Elderly Study, which began in 1960, was a longitudinal study investigating risk factors for chronic diseases (Hertog *et al.*, 1994). In addition to new randomly selected subjects, some subjects from this study were recruited in 1985. This was also a part of the same study which investigated the association between flavonoid intake and coronary heart disease risk reported in the previous section. During the 5 years of follow-up, total flavonoid intake was not related to all-cause cancer incidence or cancer mortality (rr=1.21). Although flavonoids from tea were not associated with all-cause cancer risk, flavonoids from fruit and vegetables were inversely associated with cancer incidence (rr=0.57). This may suggest the contribution of other beneficial compounds in fruit and vegetables including the antioxidant vitamins which are not present in tea. At the same time, men with cancers tended to be older and smoked more than men without cancer. This implies that long-term cigarette smoking may have already caused irreversible damage and flavonoid or other bioactive compounds in foods could not protect against cancer.

In another study, a relationship between flavonoid intake, tea consumption and cancer incidence in 120,852 Dutch men and women aged 55-69 y was observed (Goldbohm *et al.*, 1996). Initially, an inverse association between flavonoid intake and risk of lung and stomach cancer was detected; however, the association disappeared following adjustment for confounding factors including other dietary antioxidants.

The Seven Countries Study which investigated the association between flavonoid intake and coronary heart disease (see section 1.10.2) also looked at the incidence of cancer (Hertog *et al.*, 1995). However, the study did not find any association between flavonoid intake and all-cause cancer mortality, even after adjusting for fat intake and percentage of smokers. The use of dietary records taken at baseline (1960) to estimate flavonoid intake is questionable. This may not reflect long-term intakes of flavonoids and because no new dietary data were taken, any changes in the dietary habits of the subjects will not be recorded.

Another study in Finland followed 9,959 men and women and after 24 years found an inverse association between the intake of flavonoids and incidence of all-cause cancers (Knekt *et al.*, 1997). The relative risk was 0.80 between the highest and lowest quartile of flavonoid intake. The association was particularly strong for lung cancer

(0.54). Participants who had lung cancer tended to be older males who smoked and consumed fewer fruit and vegetables. To date, this is the only epidemiology study in which a protective effect of flavonoids against cancer was obtained and the association still remained significant after adjustment for confounding factors including the intake of other antioxidant vitamins. However, further prospective studies are required, taking into account dietary as well as environmental factors which can influence the aetiology of cancer.

In a recent case-control study in Spain, the association between flavonoid intake and the risk of lung cancer was investigated in 103 cases and 206 controls (Garcia-Closas *et al.*, 1998). The study found a nonsignificant negative association between the intake of kaempferol and the risk of lung cancer (or=0.51). No protective effect was observed for quercetin, luteolin or total flavonoid intake. It should be noted that the food frequency questionnaire used in this study was designed to estimate intakes of carotenoids rather than flavonoids. Only 33 food items were included, most of them high-carotenoid foods and many high-flavonoid foods were excluded, for example onions and teas. Therefore, intake of flavonoids was probably underestimated and may not represent the actual intake of the groups studied. This was evident from the average amount of flavonoids consumed in this study (5.1 mg/d), which was lower than the other previously reported studies.

The same group performed a case-control study investigating the intake of flavonoid and risk of gastric cancer in Spain (Garcia-Closas *et al.*, 1999). A protective effect of flavonoid intake against the risk of gastric cancer was reported between the highest and the lowest quartile of intake with an odds ratio value of 0.44. At the same time, an inverse association was also found between the risk of gastric cancer and the consumption of fruit and vegetables. In addition to flavonoids, fruit and vegetables are also rich in antioxidant vitamins as well as other bioactive compounds which may contribute to the results obtained.

Apart from cohort study, the case-control study is another way to assess the association between diet and disease. In this type of study, people with the disease (known as cases) are compared with individuals without the disease (controls) and ideally, they have to be as closely matched as possible to avoid any confounding factors

or bias. However, one of the disadvantages of this study is the possible misclassification between cases and controls for example, a case may reflect on their past diet differently from a control who has no reason to look back over what they ate.

Therefore, with one exception, the results of epidemiological studies so far do not show any protective effects of flavonoid intake and the risk of cancer at various sites. At the same time, a case-control study also showed a protective effect of flavonoids against gastric cancer. It is more difficult to assess the effects of diet, in particular flavonoids and their association with cancer as various other factors, dietary as well as environmental, can influence the development of cancer. Also, cancer at different sites has different aetiologies. Furthermore, the follow up period of 4-5 years in several of the studies may have been too short to see an effect of flavonoids on carcinogenesis, a process which can take approximately 15-20 y.

1.10.4 Conclusion

The epidemiology studies described in this section showed promising results on the potential of flavonoids to protect against diseases particularly coronary heart disease and to a lesser extent, cancer. However, experimental data are required to support this. Although the *in vitro* antioxidative capability of flavonoids is well established, knowledge on their absorption and metabolism is not well understood. This area of research is important in trying to evaluate the biological properties of flavonoids.

1.11 Absorption and metabolism of flavonoids

1.11.1 The importance of absorption studies

Various beneficial health properties have been attributed to flavonoids. This is largely contributed by their potent antioxidant activities. The ability of flavonoids to limit free radical reaction, which are determinants of diseases, implies their possible role in maintaining health and protect against diseases. Furthermore, epidemiology studies also suggest an inverse association between flavonoid intake and risk of coronary heart disease and to a lesser extent with cancer. To provide maximum protection, flavonoids have to maintain their active form in the body as well as being deposited in the target tissues where they can exert their antioxidative effects. With various complex chemical reactions taking place in the human body, flavonoids need to avoid the metabolic pathways that could transform them into inactive molecules, thus destroying their antioxidant properties.

The increasing interest on the potential role of flavonoids in human health has thus created new prospects for flavonoid research particularly on their absorption and metabolism. Information on the bioavailability of these compounds is essential as the fate of these compounds once absorbed from the intestine can be determined and their health protective effects can then be evaluated.

1.11.2 Historical background on the absorption of flavonoids

Studies investigating flavonoid absorption began as early as the 1950s, concentrating mainly on animals. In most cases, pure flavonoids in high doses were used due to the lack of sensitivity of methods for flavonoid analysis. Over the years, absorption studies have gradually advanced to humans, and the information obtained is more relevant and directly related. Furthermore, the development of better and more sensitive analytical methods for flavonoid identification and quantification has facilitated the advancement of absorption studies.

Attempts to investigate the absorption and metabolism of flavonoids have shown conflicting results. It was previously postulated that only free flavonoids were absorbed and not the glycosides due to their conjugation to sugar moieties (Kuhnau, 1976). However, this perception is slowly changing as more detailed studies are performed. There is emerging evidence that flavonoids appeared to be absorbed as their glycosides (Hollman *et al.*, 1995, 1997). Clearly, additional studies are needed to further establish this, in parallel with the use of improved analytical methodology.

1.11.3 Absorption and metabolism of non-dietary flavonols

Most of the studies have been performed on animals with pure compounds instead of dietary flavonols (Table 1.5). Four separate studies administered quercetin aglycone to rats. In all instances, similar metabolites of quercetin metabolism were detected in plasma, urine and bile. The metabolites consisted of conjugated derivatives of quercetin, isorhamnetin and tamarixetin (Table 1.5). One of the studies which employed radiolabelled quercetin identified the conjugates as glucuronides and/or sulphates (Ueno *et al.*, 1983; Table 1.5). Unchanged quercetin was also detected, mainly in faeces (Ueno *et al.*, 1983, Manach *et al.*, 1995).

In contrast to the animal studies, human studies did not detect conjugates of isorhamnetin or tamarixetin following administration of quercetin aglycone (Table 1.6). Four years ago, a study was performed whereby pure quercetin aglycone was orally administered to nine ileostomy volunteers (Hollman *et al.*, 1995). A small percentage of quercetin (0.12%) was excreted in urine (Table 1.6). Quercetin in urine was analysed after acid hydrolysis. Thus, the presence of specific metabolites was not described. However, when absorption was estimated by subtracting quercetin content of the ileostomy effluent from the oral intake, percentage absorption of 24% was obtained (Table 1.6). This implies absorption of the aglycone and as only 0.12% was excreted, extensive metabolism/sequestration of quercetin has probably occurred. This contrasted with the findings of Gugler *et al* (1975) who did not detect anything in the urine even after a high oral dose of quercetin aglycone (4 g; Table 1.6). However, approximately 50% of unchanged quercetin were excreted in faeces, suggesting low absorption. This

Table 1.5: summary of studies on the absorption and metabolism of non-dietary flavonols in animals

Reference	Compounds administered	Dose	Type of animals	Metabolites	Body fluids
Ueno et al (1983)	[4-^{14}C]quercetin	18.8 mCi/mmol (oral)	Rats	Unchanged quercetin Isorhamnetin & tamarixetin glucuronides Quercetin glucuronides Quercetin sulphates	Urine, faeces Bile, urine Bile, urine Urine
Manach et al (1995)	Quercetin aglycone	0.125-0.5% (oral)	Rats	Unchanged quercetin Quercetin conjugates Isorhamnetin conjugates	Faeces Plasma Plasma
Manach et al (1996)	Quercetin aglycone	0.25% (oral)	Rats	Quercetin conjugates Isorhamnetin conjugates Tamarixetin conjugates	Bile, urine, plasma Bile, urine, plasma Bile, urine
Manach et al (1997)	Quercetin aglycone	0.2% (oral)	Rats	Quercetin conjugates Isorhamnetin conjugates Tamarixetin conjugates	Plasma Plasma Plasma
Manach et al (1995)	Rutin	0.5-1.0% (oral)	Rats	Unchanged rutin Quercetin conjugates Isorhamnetin conjugates	Faeces Plasma Plasma
Manach et al (1997)	Rutin	0.4% (Oral)	Rats	Quercetin conjugates Isorhamnetin conjugates Tamarixetin conjugates	Plasma Plasma Plasma

Table 1.6: Summary of studies of the absorption of non-dietary flavonols in humans

Ref	Supplement	Dose	Metabolites (Expressed as % absorption/excretion)		
			Plasma	**Urine**	**Others**
Gugler et al (1975)	Quercetin aglycone	100 mg – i.v.	Unchanged quercetin [b](11.1%)	Unchanged quercetin (0.6%) Quercetin conjugates (7.4%)	N/A
Gugler et al (1975)	Quercetin aglycone	4 g – oral	n.d	n.d.	**Faeces** Unchanged quercetin (53%)
Hollman et al (1995)	Quercetin aglycone	100 mg – oral	N/A	Unchanged quercetin (0.12%)	*Ileostomy effluent* Unchanged quercetin [a](24%)
Hollman et al (1995)	Rutin	100 mg – oral	N/A	Rutin (0.07%)	*Ileostomy effluent* Rutin [a](17%)

[a] Absorption determined by subtracting flavonol content of ileostomy from dietary intake
[b] Percentage absorption at peak concentration, assuming 3000 ml of plasma in circulation
N/A not analysed
n.d. not detected

group detected excretion of quercetin conjugates in urine only following i.v. administration. However, the validity of the method employed to analyse flavonols was equivocal and probably not sensitive enough to detect low levels of quercetin.

In addition to the aglycone, studies with flavonol conjugates are also needed to establish the differences, if any, between the absorption of flavonol conjugates and the aglycones. Rutin (quercetin-3-rutinoside), orally administered to rats was absorbable and metabolites were present as conjugates in plasma (Manach *et al.*, 1995, 1997; Table 1.5). The rutin supplement produced similar metabolites as the quercetin supplements. However, the plasma concentration of metabolites from the rutin supplement was lower than the quercetin supplement. This raises the possibility that quercetin is absorbed and metabolised at a different rate than rutin. On the other hand, following oral administration of pure rutin compounds to human ileostomy volunteers, none of the metabolites mentioned above were detected (Hollman *et al.*, 1995; Table 1.6). Although the estimated percentage absorption of rutin was 17%, no metabolites were excreted in urine which contained only traces of rutin (<0.1% of dose; Table 1.6).

1.11.4 Absorption and metabolism of dietary flavonols

Although data are available on the absorption of flavonols following administration of the pure compounds, the main issue which needed to be addressed concerns the bioavailability of flavonols from common foods. Such studies are more complex as various factors can influence bioavailability of the dietary flavonols. This includes, for example, the ease in which flavonoids can free themselves from the food matrix and their solubility in the small intestine for the purpose of absorption. In addition, their interaction with other substances in foods may also affect their bioavailability.

Flavonoids in foods are normally present as glycosides. Although data are available on the absorption of pure flavonoid glycosides (e.g. rutin), their absorption from dietary sources needs to be established, particularly in humans. This type of study began only a few years ago and results are summarised in Table 1.7. The pioneer research was performed by a group in the Netherlands who demonstrated the absorption

Table 1.7: Summary of studies on the absorption of dietary flavonols in humans

Ref	Dietary supplement	Flavonol content	Metabolites (Expressed as % absorption/excretion)		
			Plasma	Urine	Others
Hollman et al (1995)	215 g fried onions	89 mg quercetin	N/A	Quercetin glucosides (0.31%)	*Ileostomy effluent* Quercetin glucosides [a](52%)
Hollman et al (1996)	150 g fried onions	64.2 mg quercetin	Quercetin conjugates [b](1%)	N/A	N/A
McAnlis et al (1999)	225 g fried onions	50.4 mg quercetin	Quercetin conjugates [b](1.5%)	N/A	N/A
Nielsen et al (1997)	500 g broccoli	9 mg quercetin	N/A	n.d.	N/A
		12.5 mg kaempferol	N/A	Kaempferol conjugates Kaempferol aglycone	N/A
Young et al (1999)	750-1500 ml/d (1 week) black currant and apple juice	4.8-9.6 mg/d quercetin	Same as baseline	Quercetin conjugates (0.3-0.5%)	N/A

[a] Absorption determined by subtracting flavonol content of ileostomy from dietary intake
[b] Percentage absorption at peak concentration, assuming 3000 ml of plasma in circulation
N/A not analysed
n.d. not detected

of quercetin glycosides following a meal of lightly fried onions (Hollman *et al.*, 1995; Table 1.7). However, they only measured quercetin after acid hydrolysis (which included estimates of free quercetin as well as the aglycone liberated from the glycosides following acid hydrolysis). Therefore, it was not entirely certain that all the quercetin was present as conjugates and not the aglycone. Ileostomy subjects with minimal resection were employed to overcome the problem of colonic degradation of flavonoids by microorganisms. In this way, absorption could be estimated by subtracting the amount of flavonols recovered in the ileostomy effluent from total intake. They reported that quercetin glucosides from the onion meal were highly absorbable at 52% of the total intake. However, only 0.31% was recovered in urine. Thus, more than 50% of quercetin glucosides was unaccounted for. This study determined quercetin content following acid hydrolysis, therefore the presence of any quercetin metabolites such as glucuronides or sulphates will not be detected as these conjugates are also broken down to the aglycone form by the acid hydrolysis procedure.

A more recent study performed by the same group reported 1% absorption of quercetin glucosides in plasma of human volunteers when measured at peak plasma concentrations (Hollman *et al.*, 1996; Table 1.7). This was detected following consumption of high-flavonol fried onions. This was the first study that demonstrated absorption of quercetin glucosides into plasma. However, as only 2 subjects were used in the study, the validity of the results have to be evaluated using a bigger sample size. The most recent feeding experiment with onions was published this year using similar protocol to the ones used by Hollman (McAnlis *et al.*, 1999). The percentage absorption of 1.5% in plasma for quercetin conjugates was similar to Hollman's (1996; Table 1.7). This supports the findings of Hollman *et al* (1996) as bigger number of subjects were used this time. It should be noted that the above studies reported the presence of quercetin conjugates based on the liberation of the aglycone compound after acidic or enzymic hydrolyses and did not analyse the non-hydrolysed samples for presence of free quercetin. Thus, it is not clear if the quercetin detected were actual conjugates liberated by the hydrolysis procedure or infact free quercetin already present in the samples.

In addition to onions, feeding studies with other high-flavonol foods or beverages were also performed. Consumption of broccoli, rich in kaempferol and quercetin, resulted in excretion of the conjugates of kaempferol as well as lower levels of its

aglycone (Nielsen *et al.*, 1997; Table 1.7). No quercetin or its metabolites were detected in urine suggesting either their extensive metabolism or their presence at minute levels, below the limits of detection.

In another study, human volunteers consumed black currant and apple juice for 1 week, after which 0.3-0.5% of the dose was excreted in urine as quercetin conjugates (Young *et al.*, 1999). The excretion in urine was similar to the ones obtained in the ileostomy study where subjects consumed fried onions (Hollman *et al.*, 1995; Table 1.7). The only difference is that in the ileostomy study, only a single dose was used whereas in the study by Young *et al.*, the juice was consumed daily over a period of one week. However, the total intake of quercetin was similar in both studies.

Flavonols are also able to undergo ring fission in the large intestine (Booth *et al.*, 1956, Griffiths and Smith, 1972). This process is performed by the intestinal microorganisms, leading to production of phenolic acids. Phenolic acids can still be absorbed from the large intestine and this process is discussed further in Section 1.12.4.

The study performed by Hollman *et al* (1995) appeared to indicate the ability of flavonols to be absorbed as glycosides from foods, thus contradicting the theory by Kuhnau (1976) that flavonoid glycosides are not absorbed. However, based on levels detected in plasma and urine, the bioavailability of flavonols appears to be low. If the ileostomy study showed 52% absorption of quercetin glucosides (Hollman *et al.*, 1995) whereas only 1% was detected in plasma (Hollman *et al.*, 1996), thus a large percentage of the compounds were unaccounted for. This would suggest possibly extensive metabolism of the absorbed flavonols, the most likely reaction being glucuronidation, sulphation or methylation and/or rapid removal from the blood stream via organs such as the liver.

1.11.5 Absorption and metabolism of catechins

Extensive information is available on the absorption and metabolism of catechin although most of the studies were carried out using pure compounds rather than dietary sources (Table 1.8). Catechins appeared to be more readily absorbed compared with

Table 1.8: Summary of studies on the absorption and metabolism of catechins

Ref	Compounds administered	Dose	Species	Metabolites	Body Fluids
Hackett et al (1983)	(+)-[$U^{14}C$]catechin	2 g (oral)	Humans	Unchanged (+)-catechin (+)-catechin metabolites (+)-catechin glucuronides 3'-O-methyl-(+)-catechin glucuronides and sulphates	Plasma Plasma Urine Urine
Wermeille et al (1983)	(+)-catechin	1 g (oral)	Humans	(+)-catechin glucuronides 3'-O-methyl-(+)-catechin glucuronides	Urine Urine
Das and Sothy (1971)	(+)-[$U^{14}C$]catechin	25-50 mg (i.v.)	Rats	Catechin metabolites (+)-catechin glucuronides	Urine Bile
Shaw and Griffiths (1980)	(+)-[$U^{14}C$]catechin	10 mg (oral)	Rats	3'-O-methyl-(+)-catechin glucuronides	Urine, bile
Wermeille et al (1983)	3-O-methyl-(+)-catechin	1 g (oral)	Humans	3-O-methyl-(+)-catechin glucuronides 3,3'-O-dimethyl-(+)-catechin glucuronides	Urine Urine
Hackett et al (1985)	3-O-methyl-(+)-[$U^{14}C$]catechin	2 g (oral)	Humans	3-O-methyl-(+)-catechin glucuronides and sulphates 3,3'-O-dimethyl-(+)-catechin glucuronides	Urine Urine

Ref	Compounds administered	Dose	Species	Metabolites	Body Fluids
Hackett and Griffiths (1981)	3-O-[^{14}C]methyl-(+)-catechin	25 or 125 mg/kg (oral, ip, iv)	Rats	3,3'-dimethyl-(+)-catechin glucuronides	Urine, bile
Hackett and Griffiths (1981)	3-O-[^{14}C]methyl-(+)-catechin	25 or 125 mg/kg (ip, iv)	Mice Marmoset	Free dimethyl-(+)-catechin Unchanged 3-O-methyl-(+)-catechin	Urine Faeces
Hackett and Griffiths (1982)	3-palmitoyl-(+)-[U^{14}C]catechin	10 mg (oral)	Rats	(+)-catechin glucuronides 3'-O-methyl-(+)-catechin glucuronides 3'-O-methyl-(+)-catechin sulphates 3'-O-methyl-(+)-catechin Unchanged 3-palmitoyl-(+)-catechin	Urine Urine, bile Urine Faeces Faeces
Piskula and Terao (1998)	(-)-epicatechin	172 μmol/kg (oral)	Rats	(-)-epicatechin sulphates and glucuronides Methyl-(-)-epicatechin sulphates and glucuronides	Plasma Plasma
Okushio et al (1999)	(-)-epicatechin	100 mg (oral)	Rats	3'-O-methyl-(-)-epicatechin conjugates (-)-epicatechin conjugates	Plasma, urine, bile
Richelle et al (1999)	Epicatechin	82 mg 184 mg	Humans	Unchanged epicatechin	Plasma
Unno and Takeo (1995)	(-)-epigallocatechin gallate	50 mg (oral)	Rats	(-)-epigallocatechin gallate	Plasma

quercetin. This was evident from several studies that reported approximately 41-50% excretion of the total radioactivity in urine following administration of radioactive catechin (Das and Sothy, 1971, Shaw and Griffiths, 1980, Hackett and Griffiths, 1981; Table 1.8). Roughly 34-51% of the administered radioactivity were excreted into the faeces of rats (Hackett and Griffiths, 1981).

Following administration of the catechin supplements, the metabolites detected were mostly conjugated derivatives of the original supplement (Table 1.8). In most cases, the conjugates were glucuronides and to a lesser extent, sulphate conjugates. Shaw and Griffiths (1980) detected the glucuronide of 3′-*O*-methyl-(+)-catechin following oral administration of (+)-catechin to rats. Following the oral administration of (+)-catechin to human volunteers, the major urinary metabolites were the glucuronides and sulphates of the administered compound (Hacket *et al.*, 1983). Humans also excreted glucuronides and sulphates of 3-*O*-methyl-(+)-catechin in addition to the glucuronides of 3,3′-dimethyl –(+)-catechin following an oral dose of 3-*O*-methyl-(+)-catechin (Hackett *et al.*, 1985). This suggests methylation as another important pathway in the metabolism of catechins whereby the formation of methyl and dimethyl catechin metabolites was common. The most common position for methylation to occur was either at C3 of ring C or C3′ of ring B; or in the case of dimethylation, both C3 and C3′ (see Table 1.8). In addition to methylation, glucuronidation and sulphation, ring fission of catechins also occurred by bacteria in the large intestine. This reaction generated phenolic acids which are readily absorbed. This is discussed in more detail in Section 1.12.4.

A recent study investigated the absorption of epicatechin from dark chocolate. This study demonstrated the absorption of epicatechin in plasma of human volunteers following ingestion of the chocolate (Richelle *et al.*, 1999; Table 1.8). Absorbed epicatechin was estimated after enzymic hydrolysis and did not describe the metabolites formed. Therefore, any glucuronides or sulphates of epicatechin cleaved by the enzymes as well as free epicatechin will be included. Methylation products were not reported.

1.11.6 Absorption and metabolism of other flavonoids

Table 1.9 summarises the studies on the metabolites formed following administration of flavone, flavanone or anthocyanin. The pathway for the metabolism of diosmetin (5,7,3′-trihidroxy-4′-methoxy flavone) was followed in rats following oral treatment. Diosmetin was observed to circulate as glucuronides as well as diglucuronides in plasma. No free diosmetin was present in blood or urine (Boutin *et al.*, 1993). This indicated a rapid glucuronidation process. In another study, no unchanged compound was detected in plasma after oral treatments of diosmin (diosmetin-7-rutinoside) to human volunteers although its aglycone diosmetin was present (Cova *et al.*, 1992). Both diosmin and diosmetin were not detected unchanged in urine, however their minor metabolites were excreted in urine, mainly as glucuronic acid conjugates. Degradation products such as alkyl-phenolic acids were present suggesting ring fission had occurred (See section 1.12.4).

Following administration of luteolin (5,7,3′,4′-tetrahydroxyflavone) to rats, methylated metabolites of luteolin (9-14% of dose) as well as the unchanged compound (6-15% of dose) were excreted in urine and bile (Liu *et al.*, 1995; Table 1.9). The presence of catechol group on ring B of luteolin probably promotes its methylation.

The metabolic fate of anthocyanin is not as widely researched as the other flavonoids despite their wide presence in the plant kingdom. Recently published data reported absorption of intact cyanidin-3-glucoside and cyanidin-3,5′-diglucoside following oral administration of these compounds to rats and humans (Miyazawa *et al.*, 1999; Table 1.9). No metabolites of the parent compounds were detected. Unchanged cyanidin-3-glucoside was also present in plasma of rats following oral administration (Tsuda *et al.*, 1999). The authors also reported the presence of protocatechuic acid, a metabolite formed from the degradation of cyanidin-3-glucoside. Levels of protocatechuic acid exceeded that of cyanidin-3-glucoside suggesting the high absorption or extensive degradation of cyanidin-3-glucoside. It was postulated that the structure of anthocyanins with the flavylium cation group confers resistance against conjugation or hydrolysis reaction. At the same time, this group also detected O-methylation products of cyanidin-3-glucoside in the liver and kidneys (Tsuda *et al.*, 1999). However, these

Table 1.9: Summary of studies on the absorption and metabolism of other flavonoids

Ref	Compounds Administered	Dose	Species	Metabolites	Body fluids
Boutin et al (1993)	Diosmetin (5,7,3'-trihydroxy-4'-methoxy flavone)	100 mg/kg (oral)	Rats	Diosmetin-7,3'-diglucuronides Diosmetin-3'-glucuronides Diosmetin glucuronides	Plasma Plasma Urine
Cova et al (1992)	Diosmin (Diosmetin-7-rutinoside)	10 mg/kg (oral)	Humans	Diosmetin Glucuronides of diosmin metabolites	Plasma Urine
Liu et al (1995)	Luteolin (5,7,3',4'-tetrahydroxyflavone)	40 mg/kg (ip)	Rats	Methylated metabolites of luteolin Unchanged luteolin	Urine, bile Urine, bile
Miyazawa et al (1999)	Cyanidin-3-glucoside (Cy-g) & Cyanidin-3,5-diglucoside (Cy-dg)	Cy-g – 160 mg/kg Cy-dg – 20 mg/kg (oral)	Rats	Cyanidin-3-glucoside Cyanidin-3,5-diglucoside Cyanidin	Plasma Plasma Plasma
Miyazawa et al (1999)	Cyanidin-3-glucoside (Cy-g) & Cyanidin-3,5-diglucoside (Cy-dg)	Cy-g – 2.7 mg/kg Cy-dg – 0.25 mg/kg (oral)	Humans	Cyanidin-3-glucoside Cyanidin-3,5-diglucoside	Plasma Plasma
Tsuda et al (1999)	Cyanidin-3-glucoside	0.9 mmol/kg (oral)	Rats	Protocatechuic acid Cyanidin-3-glucoside	Plasma Plasma
Hackett and Griffiths (1977)	3',4',7-tri-O-(-β-hydroxy[$^{14}C_2$]ethyl)rutoside	25 mg/kg (iv).	Mice	Glururonides of Tri-HR Unchanged Tri-HR Tri-hydroxyethyl quercetin	Urine, bile Urine, bile Faeces

Ref	Compounds Administered	Dose	Species	Metabolites	Body fluids
Hackett and Griffiths (1977)	7-mono-O-(β-hydroxy[$^{14}C_2$]ethyl) rutoside	25 mg/kg (iv)	Mice	Mono-HR glucuronides Unchanged mono-HR Monohydroxyethyl quercetin	Urine, bile Urine, bile Faeces
Hackett and Griffiths (1979)	7-mono-O-(β-hydroxyethyl[$^{14}C_2$]) rutoside	250 mg (oral)	Dogs	Unchanged mono-HR Mono-HR glucuronides Monohydroxyethyl quercetin	Urine Plasma, urine Faeces
Hackett and Griffiths (1979)	7-mono-O-(β-hydroxyethyl[$^{14}C_2$]) rutoside	250 mg (iv)	Dogs	Mono-HR Mono-HR glucuronides Monohydroxyethyl quercetin	Plasma, urine Plasma, urine Faeces

methylated metabolites were not present in plasma, suggesting they were probably retained in the tissues. Oral and i.v. administration of either mono- or tri-hydroxyethyl rutoside led to the urinary and biliary excretion of glucuronides of the parent compounds (Hackett and Griffiths, 1977, 1979). The unchanged compound was also absorbed and excreted in urine without modification (Table 1.9). In most instances, more than 50% of the radioactivity was excreted in faeces.

1.11.7 Biliary excretion of flavonoids

In addition to urine, another pathway for the excretion of flavonoids is through bile. Absorbed flavonoids and their conjugates can be reexcreted into the bile where they will flow into the duodenum. Flavonoids from biliary excretion may be subjected to further metabolism by the intestinal microorganisms. Alternatively, they may be reabsorbed from the intestine, transferred via the hepatic portal vein to the liver, metabolised and re-excreted in bile, creating an enterohepatic recirculation. Enterohepatic circulation may be important for bioactive substances including flavonoids as they can maintain flavonoid concentration in target tissues

Biliary excretion has been demonstrated in several animal experiments. Conjugates of quercetin, isorhamnetin and tamarixetin were excreted in bile following administration of quercetin to rats (Ueno *et al.*, 1983, Manach *et al.*, 1996). Biliary excretion was also observed in rats after administration of catechins, methyl catechins and epicatechin (Das and Sothy, 1971, Hackett and Griffiths, 1981, Okushio *et al.*, 1999). In most instances, the conjugated forms of the parent compounds (including glucuronides and sulphates) or their metabolites were excreted in bile (see Table 1.5 and 1.8). Administration of labelled mono- and tri-hydroxyethyl rutoside to mice led to extensive biliary excretion of the unchanged rutosides as well as their glucuronides (71% of dose; Hackett and Griffiths, 1977). The extent of biliary excretion of flavonoids is comparable or maybe even higher than their excretion in urine. This implies the importance of this pathway in flavonoid metabolism as these beneficial compounds can be subjected to enterohepatic circulation, thus maintaining a constant level in the general circulation.

1.11.8 The kinetics of flavonoid absorption in humans

Investigations of the kinetics of flavonoid absorption are useful to address their bioavailability. Bioavailability is defined as percentage of the ingested flavonoids that enters the blood circulation and becomes available for utilisation. The plasma profile of flavonoid accumulation can be used to establish various kinetic parameters including the absorption, distribution and elimination phases of flavonoids. Such information can give an insight into the pattern of flavonoid absorption, for instance whether flavonoids persist in the circulation following absorption or whether they are rapidly removed from the blood for excretion.

Absorbed flavonoids have to be transported by the blood to the corresponding tissues or organ to provide a biological response. Thus, it is possible that the concentration and persistence of flavonoids in the blood is a direct measure of their intensity and duration in the target tissues. One way of estimating this is by quantifying the area under the curve (AUC) of plasma flavonoid concentration versus time. The size and duration of the biological response should be related to the AUC. AUC is also useful for evaluating the bioavailability of flavonoids and their clearance. When a single oral dose of flavonoids is administered, absorption as well as elimination can occur simultaneously in blood. The peak flavonoid levels in plasma only represents the time where the rate of absorption equals the rate of elimination and does not necessarily indicate the end of the absorption phase. In theory, AUCs of flavonoids administered intravenously as well as orally are required to determine their absolute bioavailability. However, this is not always possible and only relative bioavailability can be estimated from AUC of plasma flavonoids from oral doses.

1.11.8.1 Quercetin

So far, only three studies have followed the kinetics of quercetin absorption in humans. The first study used pure quercetin aglycone and failed to detect the unchanged compound in plasma following oral administration (Gugler *et al*., 1975). It is possible that a large fraction of the absorbed quercetin was conjugated with glucuronic acid or sulphate. As the authors only analysed plasma without prior deconjugation of quercetin conjugates, this could explain why they did not detect quercetin aglycone in plasma.

Two studies reported the kinetics of quercetin glycosides absorption following a fried onion meal (Hollman *et al.*, 1996, McAnlis *et al.*, 1999). Both studies consumed similar amounts of quercetin from the onions. When absorption was expressed as proportion of intake, approximately 1-1.5% of quercetin glycosides was present in plasma at peak concentration. The time taken to reach peak concentration varied from 2 to 2.9 h between the two studies. The plasma profile of quercetin indicates a rapid absorption phase followed by a slower elimination period. Low levels of quercetin could still be detected 24-48 h after the onion meal. As these studies only measured quercetin after hydrolysis, information on the absorption of quercetin aglycone was excluded.

Studies with ileostomy subjects found 52% absorption of quercetin glycosides from onions (estimated after acid hydrolysis; Hollman *et al.*, 1995). However, the figures obtained in plasma (1-1.5%) were nowhere near this (Hollman *et al.*, 1996, McAnlis *et al.*, 1999). The low values obtained in plasma implied possibly extensive metabolism of this compound to its metabolites or their effective removal from the general circulation.

The profile for absorption of pure rutin indicated a slower absorption phase than quercetin with peak concentration achieved 9 h after taking the supplement (Hollman, 1997). This suggests slower absorption from the intestine or possibly absorption taking place further down the gastrointestinal tract. The author also compared the profiles of the absorption of quercetin from apples and from onions. Similar profiles were noted, although at peak plasma concentration, levels in the former was half that of the latter. Apples contained different types of quercetin glycosides compared to onions which maybe absorbed at different rates.

In most cases, flavonols were still detectable in plasma up to 24 h after the supplements. Thus, repeated intake of high-flavonol foods may lead to their build-up in plasma. Such studies are needed to confirm this.

Absorption studies reported above only determined absorption of flavonols from foods and did not specify or identify the absorption kinetics of the specific flavonols present in the dietary sources. Onions for example contain a wide variety of quercetin glucosides particularly quercetin-3,4′-diglucoside, quercetin-3-glucoside and quercetin-

4′-glucoside. Investigation of the kinetics and bioavailability of these compounds as well as other ubiquitous flavonol glycosides can provide better information on the structure-absorption relationship of flavonols. At the same time, knowledge on the flavonols that are highly absorbable can be used to encourage the general population to consume foods containing high levels of these compounds.

1.11.8.2 Catechins

Catechins were readily absorbed and in most instances, peak plasma concentration was reached within 1 to 3 h, implying rapid absorption. Plasma analysis showed the compound was still present 12 h after the start of the supplement (Hackett *et al.*, 1983, 1985), indicating a slow elimination phase. Longer periods of blood collection could provide further information on the existence of catechins in the general circulation. When areas under the plasma were estimated, the values obtained for 3-*O*-methyl-(+)-catechin (Hackett *et al.*, 1985) was 5 fold more than (+)-catechin (Hackett *et al.*, 1983; Table 1.10). Thus, introduction of a methyl group substantially increased their absorption, possibly altering their stereospecificity, favouring their uptake from the intestine. Alternatively, methylated catechin may be less extensively metabolised in the body. However, as areas under the curve of plasma levels were determined only up to 12 h, it may not be truly representative of the complete elimination phase.

In another study, a proportionate increase between AUC of plasma of epicatechin and the administered dose was observed (Richelle *et al.*, 1999; Table 1.10). This implies a non-saturable effect on the absorption of epicatechin up to a dose of 164 mg. Maximum concentration in plasma was reached between 2 to 3 h, followed by rapid elimination.

Comparing the AUC for epicatechin from chocolate and quercetin from onions, the latter appear to be more bioavailable than the former (Table 1.10). The structural variation of quercetin and epicatechin may have an impact on their absorption. At the same time, influence of the nature and/or position of the sugar moiety on quercetin absorption should not be dismissed.

Table 1.10: Areas under the plasma of flavonoids following consumption of the supplements

Flavonoid supplements	Dosage	Peak plasma concentration (% of intake)	AUC (ng/ml/h)	Reference
Quercetin glucosides (from onions)	68 mg	224 ng/ml (1.5%)	2330	Hollman's thesis 1997
Rutin	100 mg	90 ng/ml (0.27%)	983	Hollman's thesis 1995
3-O-methyl-(+)-catechin	2 g	18000 ng/ml (3%)	41600	Hackett *et al* 1985
(+)-catechin	2 g	1507 ng/ml (0.2%)	8970	Hackett *et al* 1983
Epicatechin (from chocolate-40 g)	82 mg	103 ng/ml (0.4%)	445	Richelle *et al* 1999
Epicatechin (from chocolate-80 g)	164 mg	196 ng/ml (0.4%)	1069	Richelle *et al* 1999
Diosmin	10 mg/kg body wt.	400 ng/ml (0.24%)	5617	Cova *et al* 1992

1.11.8.3 Flavones

Interestingly, following the administration of diosmin, (diosmetin-7- rutinoside), no unchanged compound could be detected in plasma (Cova *et al.*, 1992). Instead the aglycone, diosmetin, was present. Absorption was rapid with peak plasma concentration reached 1 h after dosage. This was followed by a slower elimination period with diosmetin still present above baseline levels at 48 h. Although similar dosage were consumed, a higher peak plasma concentration was obtained for diosmetin (400 ng/ml) compared to quercetin (248 ng/ml; Table 1.10) suggesting a higher rate of absorption of the former compound. The presence of the aglycone instead of the parent compound implies deglycosylation had taken place. At similar dosage, flavones appear to be more bioavailable than quercetin glucosides from onions, rutin and epicatechin (Table 1.10).

1.11.8.4 Anthocyanins

Following oral ingestion of cyanidin glucoside and cyanidin diglucoside, high levels of the former was detected in plasma of human volunteers with a peak concentration of 13 ng/ml (Miyazawa *et al.*, 1999). In contrast, only trace levels of the latter were observed. Absorption of cyanidin glucoside was rapid with peak concentration reached 60 min after the supplement. However, when compared to quercetin, the percentage of anthocyanin absorbed from total intake was relatively low (0.02%) compared to 1.5% for quercetin.

1.11.8.5 Conclusion

Excluding 3-*O*-methyl-(+)-catechin and (+)-catechin, diosmin from the sub-class flavones appeared to be more bioavailable than the other flavonoids. Comparison of the relative bioavailability of 3-*O*-methyl-(+)-catechin and (+)-catechin with the other flavonoids could not be made due to the higher dosage of the two compounds (2 g; Table 1.10). The AUC of plasma for epicatechin may have been underestimated as blood was collected only up to 8 h when levels of epicatechin was still above baseline.

It is evident that various factors play a role in determining the absorption/bioavailability of flavonoids from the small intestine. Modification in the

structure of the flavonoid molecule appears to have a big impact on their absorption. This could be as a consequence of changes in stereospecificity of the molecule or their solubility in either the lipid or aqueous phase. The mechanism of flavonoid uptake from the small intestine needs to be established. Hollman *et al.*, (1995) postulated involvement of the Sodium-Glucose Co-transport system (SGLT1) in the absorption of quercetin glucosides. This area of research merits further investigation as determination of the mechanism of flavonoid absorption can provide information on the mode and extent of their absorption from the intestine.

Information obtained so far are still limited and there are still many missing pieces to be put together to get a clearer picture. However, with the development of better procedure for flavonoid identification, rapid progress can be made. At the same time, identification of individual flavonoids following consumption of high-flavonoid foods can provide valuable information on the bioavailability of the different flavonoids present in the foods. Eventually, this can be used to encourage high consumption of foods containing highly bioavailable flavonoids.

1.12 Sites for flavonoid metabolism

1.12.1 Introduction

Little information is available on the metabolic pathway of dietary flavonols in humans following their absorption from the intestine. Knowledge on the fate of flavonoids following their absorption is relevant to assess their potential biological effects in the human body. Absorption studies indicated rapid absorption of the unchanged flavonoids followed by a slower elimination phase (see section 1.11.8). When absorption or excretion was expressed as percentage of the amount ingested, it appears that very little flavonoids are absorbed from the intestine. It is not known if this is a result of low bioavailability of flavonoids or their extensive modification before appearing in the general circulation. Another alternative explanation is their effective removal from the bloodstream for the purpose of sequestration in tissues or excretion. The extent of flavonoid metabolism determines their ability to maintain their bioactive form in order to provide protection to human health. Furthermore, the metabolites formed need to have the capability to be stored in a significant amount in the body to exert their beneficial effects. The nature of the flavonoid molecule with multiple hydroxyl groups and substitution patterns makes them susceptible to various enzymes and modification reactions.

Animal studies indicated that several sites are responsible for the metabolism of flavonoids and this may be true in humans too. The liver and the large intestine are the most common sites for flavonoid metabolism (Hackett, 1986). At the same time, the small intestine and kidney may to a certain extent contribute to the biotransformation of flavonoids. The metabolic reactions which occur on the flavonoid molecule include conjugation (e.g. with glucuronic acid or sulphate), methylation, oxidation, reduction and ring fission. Animal studies particularly with radioactively labeled flavonoids are ideal for metabolic studies. Such methods allow monitoring of the radioactive flavonoids following their absorption as well as estimation of the radioactivity in various cells and tissues.

1.12.2 Liver metabolism of flavonoids

The liver is probably the main organ responsible for the metabolism of flavonoids and contains various enzymes for these reactions. Some of the more important enzymes include UDP-glucuronyl transferases and catechol-O-methyl transferases. Furthermore, animal studies with radioactively labeled quercetin have reported a rapid decrease of radioactivity in the blood following absorption, accompanied by short-term increase in the levels in the liver and kidneys (Ueno *et al.*, 1983).

1.12.2.1 Methylation

Phenolic compounds, including flavonoids with adjacent hydroxyl groups, can undergo *O*-methylation. An *in vitro* experiment with rat liver microsomes demonstrated the metabolism of flavonoid aglycones by enzymes of the microsomes, the main reactions being hydroxylation, demethylation and methylation (Nielsen *et al.*, 1998). Modification in the structure of the flavonoid molecule particularly in the B ring appears to influence their metabolism by the microsomes. The extent of flavonoid metabolism is highly dependent on the number and position of the hydroxy and methoxy groups in the B-ring. Flavonoids without hydroxyl groups or with a 4′-OH group in the B-ring were hydroxylated to give the catechol structure (3′,4′-dihydroxylation). On the other hand, flavonoids with *O*-methylation at the 4′-position (but not the 3′-position) were demethylated to the corresponding hydroxylated compound. The catechol moiety on the flavonoid molecule then becomes the substrate for catechol-*O*-methyl transferase, causing *O*-methylation at the 3′-position. This structure may be more biologically stable.

Piskula and Terao (1998) detected activities of catechol-*O*-methyl transferase in the liver. This enzyme catalyses the transfer of methyl groups from S-adenosyl methionine to a variety of substrates containing a catechol group. Methylation has only been observed at the 3′ position and not the 4′ implying specific affinity of catechol-*O*-methyl transferase for its substrate.

Several flavonoids have been reported to inhibit catechol-*O*-methyl transferase in rat liver and also in human liver (Kuhnau, 1976). This inhibitory action is largely

dependent on the structure of the flavonoid molecule with flavonoid glycosides less active than the corresponding aglycones. However, the 3′,4′-*O*-dihydroxylation of ring B of flavonoids appears to increase the affinity of flavonoids for this enzyme.

1.12.2.2 Conjugation

In addition to methylation, a conjugation reaction also occurs in the liver. The conjugation of flavonoids mainly involved the introduction of glucuronic acids and sulphates. These reactions feature prominently in flavonoid metabolism particularly of catechin and quercetin (See Table 1.5 and 1.8). This perhaps is the most common final step in the metabolic pathway of flavonoids.

Certainly, glucuronidation and sulphation of quercetin and catechin has been demonstrated using isolated perfused rat liver system (Shali *et al.,* 1991). In addition, activities of glucuronosyltransferases and phenolsufotransferases, the enzymes responsible for glucuronidation and sulphation respectively, has also been detected in the liver of rats (Piskula and Terao, 1998).

Conjugation of flavonoids increases their molecular weight and at the same time creates more polar molecules which subsequently favours elimination in urine or via the biliary route where they can be further metabolised by the colonic microorganisms. In rats orally fed [^{14}C]quercetin, 20% of the absorbed quercetin was excreted in bile and urine as glucuronide and sulphate conjugates of [^{14}C]quercetin, [^{14}C]3′-*O*-monomethyl quercetin and [^{14}C]4′-*O*-monomethyl quercetin (Ueno *et al.,* 1983).

1.12.2.3 Deglycosylation

A recent study reported deglycosylation of flavonoid glycosides in cell free extracts of human liver (Day *et al.,* 1998). This demonstrated the presence of glycosidases in liver which has never been detected before. The rate and extent of flavonoid deglycosylation appears to be dependent on the structure and type or position of the sugar moiety. Quercetin with sugars at either the 3′ position or the 3,4′-positions (e.g. quercetin-3,4′-diglucoside) were resistant to hydrolysis. On the other hand, quercetin-4′-glucoside and several 7-glucosides of isoflavonoids were deglycosylated by

the liver enzymes (Day *et al.,* 1998). Perhaps binding of sugars at the 3′-position or 3,4′-position reduces the affinity of the glycosidases towards these flavonoids.

1.12.2.4 Hydroxylation

The liver has also been suggested as the site for hydroxylation of flavonoids and monooxygenases are widely reported to catalyse this reaction (Hackett, 1986). Indeed, several flavonoids did undergo hydroxylation following incubation with rat liver microsomes (Nielsen *et al.,* 1998). The structure of the flavonoid molecule appears to affect their hydroxylation. Flavonoids lacking hydroxyl groups in the B ring are more prone to this reaction. The end product appeared to be formation of the catechol structure.

1.12.3 Small intestinal metabolism of flavonoids

1.12.3.1 Deglycosylation

It was previously postulated that only flavonoid aglycones were absorbed, and not the glycosides, due to the lack of glycosidases in the small intestine capable of breaking the sugar bonds. Lately, however, *in vitro* incubations of flavonoid glycosides with small intestinal contents have led to the formation of aglycones of the parent compounds (Day *et al.,* 1998) suggesting the presence of glycosidases. The structure of flavonoids as well as the nature/position of the sugar residues determines the extent of their deglycosylation. Similar to liver, isoflavonoids with glucose at the C-7 position and quercetin-4′-glucoside were extensively deglycosylated (>60%). This was also observed by Ioku *et al* (1998). Quercetin-3-glucoside, which was resistant to deglycosylation by the liver enzymes, was deglycosylated, albeit at a lower proportion (16%), by enzymes of the small intestine. Previous incubations of flavonoids with small intestinal contents as well as digestive enzymes demonstrated their stability in this environment (Hollman *et al.,* 1995). This implies that the glycosidases are possibly located at the brush border membrane and that deglycosylation takes place during passage of the flavonoids through the intestinal membrane. This is supported by recent publications which detected absorption of quercetin aglycone in the serosal side following perfusion of isolated rat intestines with quercetin-3-glucoside (Spencer *et al.*, 1999, Noteborn *et al.*, 1997).

1.12.3.2 Glucuronidation

In addition to deglycosylation, glucuronidation of flavonoids has also been described in the small intestine. Until recently, liver was assumed to be the only site for glucuronidation of flavonoids. However, the recent detection of glucuronosyl transferase activities in the intestinal mucosa meant that the first step of flavonoid metabolism might already occur at the level of intestinal mucosa (Piskula and Terao, 1998). Indeed, the ability of the intestinal mucosa to glucuronidate other compounds such as naphthyl glucosides during their absorption have been reported (Mizuma *et al.*, 1994). Thus, the possibility of flavonoid glucuronidation is also highly likely. It was only recently that the ability of the intestinal mucosa to glucuronidate flavonoids was demonstrated *in in vitro* experiments with rat intestines (Shimoi *et al.*, 1998, Spencer *et al.*, 1999, Walle *et al.*, 1999). In all cases, glucuronidation appeared to take place during the passage of flavonoids through the intestinal membrane. Glucuronidation includes the aglycone as well as the glycosides of flavonoids.

However, the extent of intestinal glucuronidation of flavonoids *in vivo* is not known. Such findings question the role of the liver in glucuronidation of flavonoids. Intestinal glucuronidation has only been described for a small number of flavonoids (mainly flavonols and isoflavonoids). This area merits further research particularly the effect on catechins which are extensively glucuronidated upon their absorption.

1.12.4 Colonic bacterial metabolism

The colon is probably the main site for the metabolism of flavonoids that remain unabsorbed after passing through the small intestine. In addition, absorbed flavonoids can be reexcreted into the duodenum in bile and thus be subjected to further metabolism. The colon contains a large population of anaerobic microorganisms, widely responsible for the biotransformations of unabsorbed substances including flavonoids. The colonic microflora such as Bacteroides, Bifidobacterium and Eubacterium contain several enzymes capable of deconjugating the glycosidic as well as the glucuronide bonds of flavonoid conjugates to liberate the free form. The resulting aglycones of flavonoids are either absorbed or subjected to further metabolism by the intestinal microorganisms. The

enzymes glycosidase and glucuronidase have been detected in colonic microorganisms (Kim *et al.*, 1998).

1.12.4.1 Ring fission of flavonoids

Ring fission is perhaps the most important step in the colonic bacterial metabolism of flavonoids. This reaction ultimately results in the split of the heterocyclic oxygen-containing ring of flavonoids to yield metabolites known as phenolic acids. Table 1.11 summarizes the animal and human studies on ring fission products following administration of flavonoids while the chemical structure of the resulting phenolic acids are illustrated in Figure 1.12. Evidence that ring fission of flavonoids required the presence of colonic microorganisms was obtained using rats treated with the antibiotic neomycin. The generation of ring fission products of flavonoids was not observed in these rats supporting the importance of colonic microorganisms (Nakagawa *et al.*, 1965, Griffiths and Smith, 1972).

In most cases, flavonoid glycosides as well as their aglycone generated the same type of phenolic acids. Kaempferol and robinin (kaempferol-7-rhamnosyl-3-galactorhamnoside) for instance, underwent a cleavage reaction to generate a similar phenolic acid, hydroxyphenylacetic acid (Griffiths and Smiths, 1972; Table 1.11). The same was reported for quercetin and rutin (quercetin-3-rutinoside) (Booth *et al.*, 1956) as well as apigenin and naringin (apigenin-7-rhamnoglucoside) (Griffiths and Smiths, 1972; see Table 1.11). This implies that flavonoid glycosides are initially hydrolysed by the intestinal microorganisms to the respective aglycones prior to the ring fission reaction.

Ring fission products are largely dependent on the structure of the flavonoid molecule, therefore the phenolic acids formed varied depending on sub-classes of flavonoids. Investigations of the bacterial metabolism of flavonols, in particular quercetin, rutin and kaempferol showed that the compounds were degraded mainly into hydroxyphenylacetic acids (Booth *et al.*, 1956, Nakagawa *et al.*, 1965, Griffiths and Smith, 1972a, Baba *et al.*, 1981; Table 1.11). Oral administration to rats of certain flavone and flavanone, for instance apigenin and naringin resulted in the excretion of

Table 1.11: Summary of studies on the ring fission products of colonic microorganisms excreted in urine following administration of flavonoids

	Reference	Flavonoid administered	Dose (Species)	Phenolic acids excreted in urine
1.	Booth et al., 1956	Rutin Quercetin	Rabbits, rats, guinea pigs, humans	3-hydroxylphenylacetic acid 3-methoxy-4-hydroxyphenylacetic acid 3,4-dihydroxyphenylacetic acid
2.	Masri et al., 1959	Quercetin	80 mg – oral (Rats)	3-hydroxylphenylacetic acid and its glucuronides 3-methoxy-4-hydroxyphenylacetic acid 3,4-dihydroxyphenylacetic acid
3.	Petrakis et al., 1959	^{14}C-quercetin	5 mg – oral (Rats)	3-hydroxylphenylacetic acid 3-methoxy-4-hydroxyphenylacetic acid
4.	Nakagawa et al., 1965	Quercetin	Rats	3-hydroxylphenylacetic acid 3-methoxy-4-hydroxyphenylacetic acid 3,4-dihydroxyphenylacetic acid 3-hydroxybenzoic acid 3-hydroxycinnamic acid 3-hydroxyphenylpropionic acid
5.	Baba et al., 1981	[2',5',6'-d_3]rutin	10 mg/kg – oral (Humans)	3-hydroxylphenylacetic acid 3-methoxy-4-hydroxyphenylacetic acid 3,4-dihydroxyphenylacetic acid
6.	Griffiths and Smith, 1972	Myricetin Myricitrin (Myricetin-3-rhamnoside)	200 mg – oral 100 mg – oral (Rats)	3,5-dihydroxyphenylacetic acid 3-hydroxylphenylacetic acid

	Reference	Flavonoid administered	Dose (Species)	Phenolic acids excreted in urine
7.	Griffiths and Smith, 1972a	Kaempferol Robinin (Kampferol-7-rhamnosido-3-galactorhamnoside)	100 mg – oral 100 mg – oral (Rats)	Hydroxyphenylacetic acid
8.	Griffiths and Smith, 1972a	Apigenin Naringin (Apigenin-7-rhamnoglucoside)	200 mg – oral 200 mg – oral (Rats)	p-hydroxyphenylpropionic acids
9.	Cova et al., 1992	Diosmin	10 mg/kg body wt (Humans)	Hydroxyphenylpropionic acid 3-hydroxy-4-methoxybenzoic acid 3-methoxy-4-hydroxyphenylacetic acid 3,4-dihydroxybenzoic acid
10.	Das and Sothy, 1971	(+)-[U-^{14}C]catechin	25 mg – i.v. (Rats)	Hydroxyphenylacetic acid Hydroxyphenylpropionic acids Hydroxyphenyllactic acid δ-(3-hydroxyphenyl)-γ-valerolactone δ-(3,4-dihydroxyphenyl)-γ-valerolactone
11.	Hackett et al., 1983	(+)-[^{14}C]catechin	Humans	3-hydroxybenzoic acid 3-hydroxyhippuric acid 3-hydroxyphenylpropionic acid
12.	Hackett and Griffiths, 1981	3-O-methyl-(+)-catechin	Mice, rats, marmoset	No ring fission products

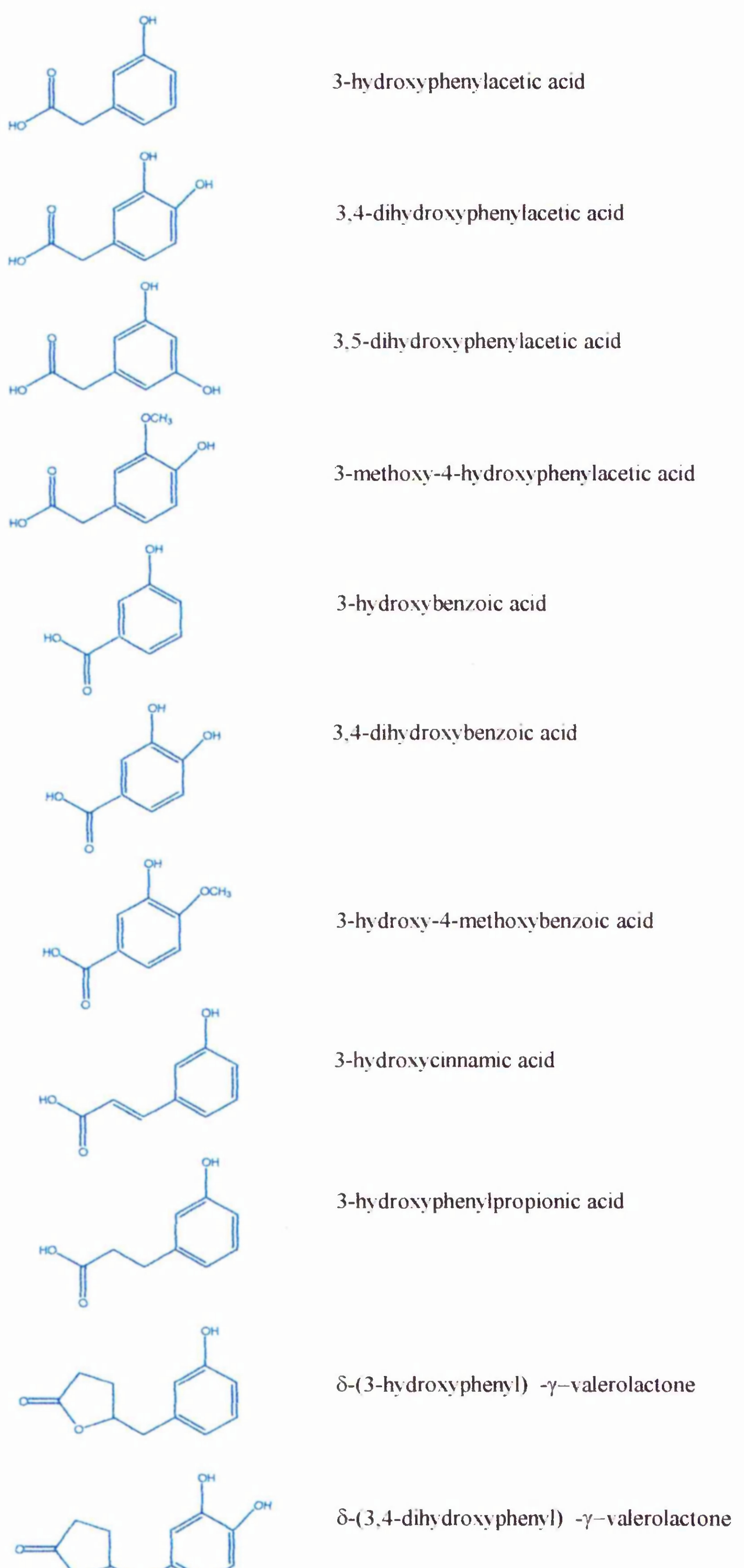

Figure 1.12: Structure of ring fission products of colonic bacterial metabolism of flavonoids

hydroxyphenyl propionic acids (Griffiths and Smith, 1972a). Ring fission reaction on (+)-catechin generated hydroxyphenylpropionic acids, hydroxybenzoic acids and valerolactones (Das and Sothy, 1971, Hackett *et al.*, 1983). In contrast to (+)-catechin, 3-O-methyl-(+)-catechin was resistant to ring fission (Hackett and Griffiths, 1981). The presence of a methyl group at C-3 possibly confers resistance towards ring fission. Clearly, structural features of the flavonoid molecule determine the type of ring fission products. The presence of hydroxyl groups appears to be necessary for ring fission and their numbers as well as position determine their susceptibility to degradation by the intestinal microorganisms.

Depending on their structure, some of these phenolic acids show considerable antioxidant activities and thus may contribute towards the biological properties of flavonoids. Phenolic acids such as 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid showed higher anti-platelet aggregation activities than rutin and quercetin (Kim *et al.*, 1998). Studies have shown that phenolic acids were capable of absorption in the colon as they could be detected in urine of animals and humans following administration of the parent compounds (Griffiths and Smith, 1972, Hackett *et al.*, 1983, Cova *et al.*, 1992). Thus, further information on the extent of phenolic acid absorption as well as their metabolism is useful to evaluate their potential contribution as antioxidants in the human body.

1.12.5 Renal metabolism of flavonoids

The major purpose of the kidneys is to produce urine. They therefore act as a vehicle for the excretion of metabolic products which need to be disposed of by the body and at the same time maintain osmolarity of the body fluids. The role of the kidney in the metabolism of flavonoids is probably the least studied.

Animal studies with radiolabelled flavonoids have detected radioactivity in kidney following flavonoid absorption (Ueno *et al.*, 1983). The kidney may contain enzymes capable of biotransformation of flavonoids. Indeed, methylation of flavonoids was observed in the kidney of rats (Piskula and Terao, 1998). The investigators also detected low levels of the enzyme catechol-*O*-methyl transferase responsible for this reaction.

Flavonoid metabolism in kidney is not widely studied. As their main purpose is in the excretion of substances, it is possible that they play only a minor role in flavonoid metabolism.

1.13 The mechanism of flavonoid absorption

1.13.1 Introduction

Information regarding the uptake of flavonoids across the small intestine is limited. Such information is useful to provide an insight into the bioavailability of various flavonoids from the diet, their ability to be absorbed and subsequently provide beneficial health properties. Previously, flavonoids were regarded as unabsorbable although recent evidence reported the selective absorption of quercetin glucosides from onions as well as lower levels of quercetin aglycone (Hollman *et al.*, 1995).

Recent interest in the mechanism of flavonoid uptake from the small intestine has sparked widespread research in this area. Evaluation of the mechanisms of flavonoid uptake are important because:

i) it can provide a better understanding of the role of flavonoids in human health
ii) the bioavailability of various flavonoids from dietary sources can be determined
iii) the relationship between flavonoid structure, their cellular location and biological function in the human body can be addressed.

1.13.2 Background on intestinal absorption

In order to study the mechanism of flavonoid absorption, an understanding of the process that occurs in the small intestine during absorption of substances is important. The small intestine can be divided into 3 segments consisting of the duodenum, jejunum and ileum. Jejunum is the main segment where most of the absorption of compounds occurs. Its mucosal surface is enriched with villus as well as transport molecules to enhance the efficiency of the absorption process.

Intestinal absorption of substances can occur either by passive or active transport. Passive transport involves the flow of specific substances from high concentration to low concentration so as to equilibrate their concentration gradients. There are 2 mechanisms of passive transport, by simple diffusion or by facilitated diffusion. Simple diffusion involves direct diffusion of specific molecules across the membrane, for example fatty

acids. Facilitated diffusion requires a specific carrier protein to transport specific molecules. GLUT5 for instance is a protein carrier for fructose as the cell membrane is not permeable to hexoses. At the same time, GLUT5 may also be able to transport glucose, albeit at a lesser extent than fructose (Gould and Holman, 1993, Mueckler, 1994). A simplified diagram of the various transport mechanisms in the small intestine is illustrated in Figure 1.13.

Compounds that are hydrophilic are mainly transported via carrier proteins as they are not soluble in the lipid phase of the membrane. On the other hand, lipophilic compounds can diffuse across the membrane depending on the partition coefficient between water and the non-polar phase or the compound's tendency to transfer from the aqueous phase to the membrane's non-polar core. It was postulated that quercetin glucosides maybe transported via a carrier-mediated system as they are more polar and have low solubility in lipids.

In active transport, specific molecules are transported against their concentration gradients, from low concentration to high concentration. An example is the Sodium-Glucose Co-transport protein (SGLT1) responsible for the active transport of glucose and galactose. These hexoses are transported across the brush border membrane mediated by the simultaneous movement of sodium ions into the basolateral membrane (Figure 1.13). Energy for this active transport is provided by a concentration gradient of sodium ions across the membrane, maintained by the enzyme Na^+/K^+-ATPase.

Once in the enterocytes, absorbed compounds are transported essentially by the same mechanism across the basolateral membrane into the blood stream. Fatty acids can diffuse directly across the membrane (Figure 1.13). Another carrier, GLUT2, which is not Na-dependent, is present in the basolateral membrane for the transport of glucose. GLUT2 is also able to handle fructose and galactose.

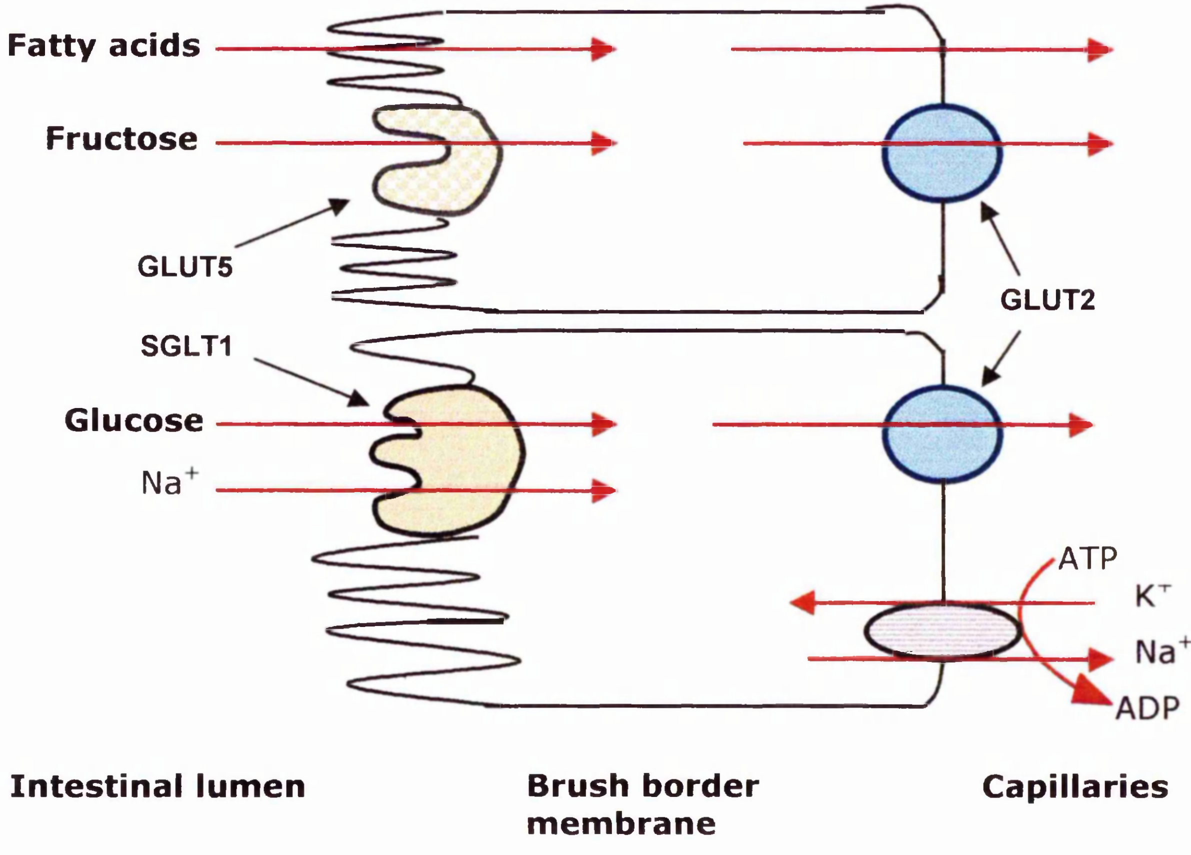

Figure 1.13: A schematic diagram illustrating the various transport mechanisms involved in the uptake of specific substances across the intestinal membrane

1.13.3 Approach in the study of flavonoid transport

Transport studies are somewhat intricate as various factors have to be taken into account when conducting such studies. Movement of substances across the small intestine is defined as their passage through the epithelial barrier between the intestinal lumen and the blood vessels. The small intestine can transfer substances in 2 directions, (i) from the lumen to the bloodstream (absorption), and (ii) from the bloodstream to the lumen (secretion). Substances have to pass through 2 membranes during transport, the luminal (or mucosal) and the basolateral (or serosal) membranes. These 2 membranes have different characteristics and the mechanism of transport for one substance may differ between the 2 membranes.

Although many techniques are available to study intestinal absorption, there is not one perfect technique. *In vivo* methods are ideal due to the presence of blood circulation and maintenance of the physiological parameters. However, this is not always practical as several aspects of absorption cannot be studied, such as collection of the absorbed products. Although not without its disadvantages, *in vitro* methods are more popular as the experimental conditions can be easily controlled and at the same time allows better sampling from the mucosal as well as the serosal side. Perhaps a combination of different methods is best to provide better information on absorption and mechanism of transport, taking into account variations in physiological as well as intestinal function in the methods.

1.13.4 Flavonoid uptake from the small intestine

Various studies have investigated the uptake of several flavonoids from the small intestine, using different *in vitro* techniques. This is summarised in Table 1.12. It is hoped that such studies could provide better understanding of the relationship between the structure of flavonoids and the extent of their absorption from the intestine.

A study used CaCo-2 cells as a model of human intestinal absorption and measured the permeability of various flavonoids in both luminal to basal and basal to luminal directions (Walgren *et al*., 1998). Quercetin was the most permeable compound

Table 1.12: Summary of the various techniques used to investigate the *in vitro* absorption of flavonoids from the small intestine

Ref.	Methods Employed	Incubation compounds	Metabolites/compounds absorbed into the serosal side		
			Unchanged compound	Aglycone of incubation compound	Glucuronide
1. Gee et. al. 1998	Everted segments of rat jejunum, preloaded with ^{14}C galactose	Quercetin-3,4'-diglucoside	✓	–	–
		Quercetin-3-glucoside	✓	–	–
		Quercetin-4'-glucoside	✓	–	–
		Quercetin-3-rutinoside	✓	–	–
2. Shimoi et al 1998	Everted rat intestine	Luteolin	✓	–	✓
		Luteolin-7-glucoside			✓
3. Walle et al. 1999	Human CaCo-2 cells	Chrysin (5,7-dihydroxyflavone)	✓	–	✓
4. Walgren et. al. 1998	Human Caco-2 cells	Quercetin-3,4'-diglucoside	✓	–	–
		Quercetin-4'-glucoside	n.d.	–	–
		Quercetin aglycone	✓	–	–

Ref.	Methods Employed	Incubation compounds	Metabolites/compounds absorbed into the serosal side		
			Unchanged compound	Aglycone of incubation compound	Glucuronide
5. Noteborn et. al. 1997	i). Perfusion of Rat jejunal segments. ii). Human CaCo-2 cells	Quercetin-3-rutinoside	✓	–	–
		Quercetin-3-glucoside	–	✓	–
		Quercetin-4'-glucoside	n.d.	–	–
		Quercetin aglycone	✓	–	–
6. Spencer et al. 1999	In vitro perfusion of isolated rat intestine	Luteolin	✓	–	✓
		Quercetin aglycone	✓	–	✓
		Kaempferol	–	–	✓
		Hesperetin	✓	–	✓
		Luteolin-7-glucoside	–	–	✓
		Quercetin-3-glucoside	✓	✓	✓
		Kaempferol-3-glucoside	–	–	✓

n.d. – not detected

in the luminal to basal directions followed by much lower permeability of quercetin-3,4′-diglucoside and no absorption of quercetin-4′-glucoside (Table 1.12). It is possible that the glucose moiety in the 3-position promotes absorption whereas inhibits it in the 4′-position. The same method was used to study the uptake of chrysin (5,7-dihydroxyflavone; Walle *et al.*, 1999). Movement from basolateral to luminal was two fold higher than from luminal to basolateral. This suggest bidirectional movement of certain flavonoids implying that not all the flavonoids absorbed will be available as a proportion of it might be excreted back into the intestinal lumen. However, the correlation between the cell culture technique and *in vivo* absorption is questionable. CaCo-2 cells are cancerous cells and any modification in the transport system as a result of cancer in not known. The relevance to uptake in normal subjects is open to argument. As the cell culture conditions may not be physiologically identical to the *in vivo* system, the observed basolateral to luminal movement of flavonoids may not occur in the actual human body as they might be taken up straight into the general circulation. Furthermore, in the *in vivo* system, the luminal concentration of ingested flavonoids would be expected to be much higher than the basolateral concentration, thus promoting passive diffusion. This gradient is expected to be maintained by removal of the flavonoids from the basolateral side by blood flow and plasma protein binding.

One other study indicated intestinal absorption of quercetin aglycone (Noteborn *et al.*, 1997) with another detecting low absorption, only 60 min after the start of incubation (Spencer *et al.*, 1999; Table 1.12). However, Spencer *et al* found much higher presence of quercetin glucuronides following incubation with quercetin aglycone. This may explain the low absorption of the unchanged compound.

Recently published studies claimed the ability of the small intestine to both absorb and glucuronidate several flavonoids (Spencer *et al.*, 1999, Shimoi *et al.*, 1998; Table 1.12). This was observed in the glycosides as well as the aglycone of flavonoids although the structure of the molecule may affect the extent of their absorption and glucuronidation. The other previous studies only looked at absorption of the parent compound following their incubation. Therefore, the presence of glucuronides, if any were not included.

In contrast to the above findings, following perfusion of isolated rat intestine with quercetin-3-glucoside, Spencer *et al* (1999) detected higher absorption of the aglycone compound compared to the parent compound and the glucuronides. This implies possibly deglycosylation of quercetin-3-glucoside while being transported through the intestinal tissue. Mizuma *et al* (1994) reported similar deglycosylation of naphthyl glucosides during intestinal absorption.

Most of the studies reported intestinal absorption of quercetin glycosides although one detected no absorption of quercetin-4′-glucoside (Walgren *et al.*, 1998; Table 1.12). The aglycone form of several flavonol and flavone were also absorbed (Noteborn *et al.*, 1997, Walgren *et al.*, 1998, Spencer *et al.*, 1999). However, one study reported glucuronidation of the aglycone of quercetin and kaempferol during their passage through the intestinal wall (Spencer *et al.*, 1999). Quercetin-3-glucoside was both deglycosylated and glucuronidated during intestinal absorption with higher levels of the aglycone detected than the glucuronides (Spencer *et al.*, 1999).

All the *in vitro* techniques employed to study the absorption of flavonoids showed some discrepancies in the results obtained (Table 1.12). As mentioned before, various factors can contribute to this, particularly the set up of the experiments and control of the physiological conditions. Therefore, at this point in time, it is difficult to come to a definite conclusion regarding the extent of flavonoid uptake from the small intestine and the factors influencing them. It appears that only a very limited amount of flavonoids was transported (Noteborn *et al.*, 1997, Spencer *et al.*, 1999), suggesting low bioavailability of these compounds. However, this cannot be directly compared with *in vivo* system as lower absorption is expected from *in vitro* systems due to the lack of blood supply and other physiological conditions.

1.13.5 Mechanisms of flavonoid absorption from the small intestine

Hollman and his colleagues (1995) in their absorption study, found that quercetin glycosides from onions were better absorbed than the aglycone. They proceeded to postulate that the SGLT1 might be involved in the transport of quercetin glucosides.

Indeed, SGLT1 has been shown to be able to transport naphthyl glycosides in addition to glucose, their primary substrate (Mizuma *et al.*, 1994). A few studies have investigated this hypothesis although no strong evidence was obtained. One study reported interaction of several quercetin glucosides (1 mM) including quercetin-3,4'-diglucoside, quercetin-3-glucoside and quercetin-4'-glucoside with SGLT1 based on an everted rat gut model (Gee *et al.*, 1998). However, in contrast to this, quercetin-3-glucoside as well as quercetin aglycone behaved as glucose transport carrier inhibitors at flavonol concentration of 10 μM (Noteborn *et al.*, 1997). It is rather puzzling that at low concentration, quercetin and its glucosides can inhibit SGLT1 but not so at high concentrations suggesting possible discrepancies in the two methods employed.

Obviously, more studies are needed in this area, using different approaches. Phloridzin, an inhibitor of SGLT1 could be employed to block the transporter and thus study their influence on flavonol glucosides uptake. As yet, it cannot be confirmed that quercetin glucosides are transported by SGLT1 in the small intestine.

1.13.6 Limitations of *in vitro* techniques to study intestinal absorption

Obviously, with *in vivo* techniques, the physiological parameters are maintained, such as blood flow and hormone levels. However, with *in vitro* methods, it is difficult to maintain the exact physiological conditions and admittedly, some of it will be lost. Thus, the relationship between the transport mechanisms and the overall absorption may not be clear. Preparations of intestinal tissues from animals are also an important determinant particularly in maintaining tissue viability. Furthermore, speed and the use of well-oxygenated buffer are crucial in working with surviving intestines. However, the importance of this type of study cannot be denied in order to evaluate the exact mechanism and the extent of flavonoid absorption from the intestine. Researchers will need to be aware of the confounding factors and limitations of such studies.

1.13.7 Conclusion

The potential for flavonoids to provide beneficial health properties is very promising. The next step is the determination of their absorption and metabolism. Of particular importance is the ability of flavonoids to provide protection at the levels of dietary intake rather than at pharmacological dose. Although substantial research have been carried out on this field, several gaps or loopholes still exist particularly on the mechanism of absorption and the fate of these compounds following their absorption. Inevitably, the flavonoids that circulates in the blood stream, either as the unchanged compound or as the metabolites will have the biggest impact on human health. Thus, evaluation of the factors influencing the mode and extent of flavonoid uptake from the small intestine together with their metabolism will provide a better understanding on the interaction between these compounds and the biological system.

2 Aims of Study

CHAPTER 2: AIMS OF STUDY

The widespread potential of flavonoids in humans particularly as antioxidants is widely recognised. This has opened up the prospect for more research especially regarding their absorption and metabolism. As yet, information is still limited and further studies are required to provide a better understanding of their beneficial health properties *in vivo*.

Therefore, the main objectives of this thesis were:

1. To investigate the extent of accumulation of flavonols in plasma and their excretion in urine following consumption of a high-flavonol food.

2. To establish the mode and extent of flavonol absorption from the small intestine and evaluate the effect of structural modification as well as the nature/position of sugar moiety on their absorption.

3. To investigate the influence of flavonol glycoside structure on the extent of their metabolism by the liver.

3 Materials and Methods

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CHAPTER 3: MATERIALS AND METHODS

This chapter describes the general materials and methodologies applied in conducting experiments. An automated high performance liquid chromatograph system (HPLC) was extensively used for the analysis of flavonoids from various sources. Methods for extracting flavonoids from plant tissues as well as biological samples are explained. In addition, optimization of flavonoid analysis in plasma is also detailed in this chapter as information obtained is useful for the feeding studies described in Chapter 4.

3.1 MATERIALS

3.1.1 Flavonoid standards

Quercetin-3,4′-diglucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside were a gift from Dr T. Tsushida, National Food Research Institute, Ibaraki, Japan. Kaempferol, quercetin, and morin were obtained from Sigma Aldrich (Poole, Dorset, UK). Quercetin-3-glucoside and isorhamnetin were purchased from Apin Chemicals Ltd. (Oxon, UK).

3.1.2 HPLC solvents

Methanol and acetonitrile were of HPLC grade and obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). Aluminium nitrate nonahydrate and trifluoroacetic acid were purchased from Sigma Aldrich (Poole, Dorset, UK). Glacial acetic acid was obtained from Fischer Scientific (Leicestershire, UK).

3.1.3 Plant Materials

Yellow onions for flavonol analysis and the human feeding trials were purchased at different dates at Safeway plc. (Byres Road, Glasgow). Previous analyses have shown

that the flavonol content of the same cultivar of onions did not vary significantly from month to month (Crozier *et al*., 1997).

3.2 METHODS

3.2.1 Flavonoid analysis by High Performance Liquid Chromatography

Samples were analysed using a Shimadzu (Kyoto, Japan) LC-10A series automated liquid chromatograph comprising a SCL-10A system controller, two LC-10A pumps, a SIL-10A auto injector with sample cooler, a CTO-10A column oven and SPD-10A UV-VIS detector and an RF-10A fluorimeter linked to Reeve Analytical (Glasgow, UK) 2700 data handling system. Reversed phase separations were carried out at 40°C using a 150 x 3.0 mm i.d., 4 um Genesis C_{18} cartridge column fitted with a 10 x 4.0 mm i.d. C_{18} Genesis guard cartridge in an integrated holder (Jones Chromatography, Mid-Glamorgan, UK). The mobile phase was a 25 min, 20-40% gradient of acetonitrile in water adjusted to pH 2.5 with trifluoroacetic acid, eluted at a flow rate of 0.5 ml/min. Column eluent was first directed to the SPD-10A absorbance monitor operating at 365 nm, after which post-column derivatization was achieved by the addition of methanolic, 0.1M aluminium nitrate containing 7.5% glacial acetic acid, as described by Hollman *et al* (1996) and pumped at a flow rate of 0.5 ml/min by a pulse-free Model 9802 precision mixer/splitter (Reeve Analytical). The mixture was directed to a RF-10A fluorimeter and fluorescent flavonol complexes detected at excitation 420 nm and emission 485 nm. The limit of detection at A365 nm was <5 ng and linear 5-250 ng calibration curves were obtained for morin, quercetin, kaempferol, isorhamnetin, quercetin-3,4′-diglucoside, quercetin-3-glucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside. The fluorescence intensity of the individual flavonoid derivatives varied, however, 0.1-100 ng linear calibration curves were obtained for morin, quercetin, kaempferol, isorhamnetin, quercetin-4′-glucoside and isorhamnetin-4′-glucoside.

Figure 3.1 illustrates typical HPLC traces of standards of flavonol aglycones and flavonol conjugates detected at absorbance 365 nm on the UV detector (Figure 3.1 A) and after post-column derivatization, detected by the fluorimeter (Figure 3.1 B). Peaks

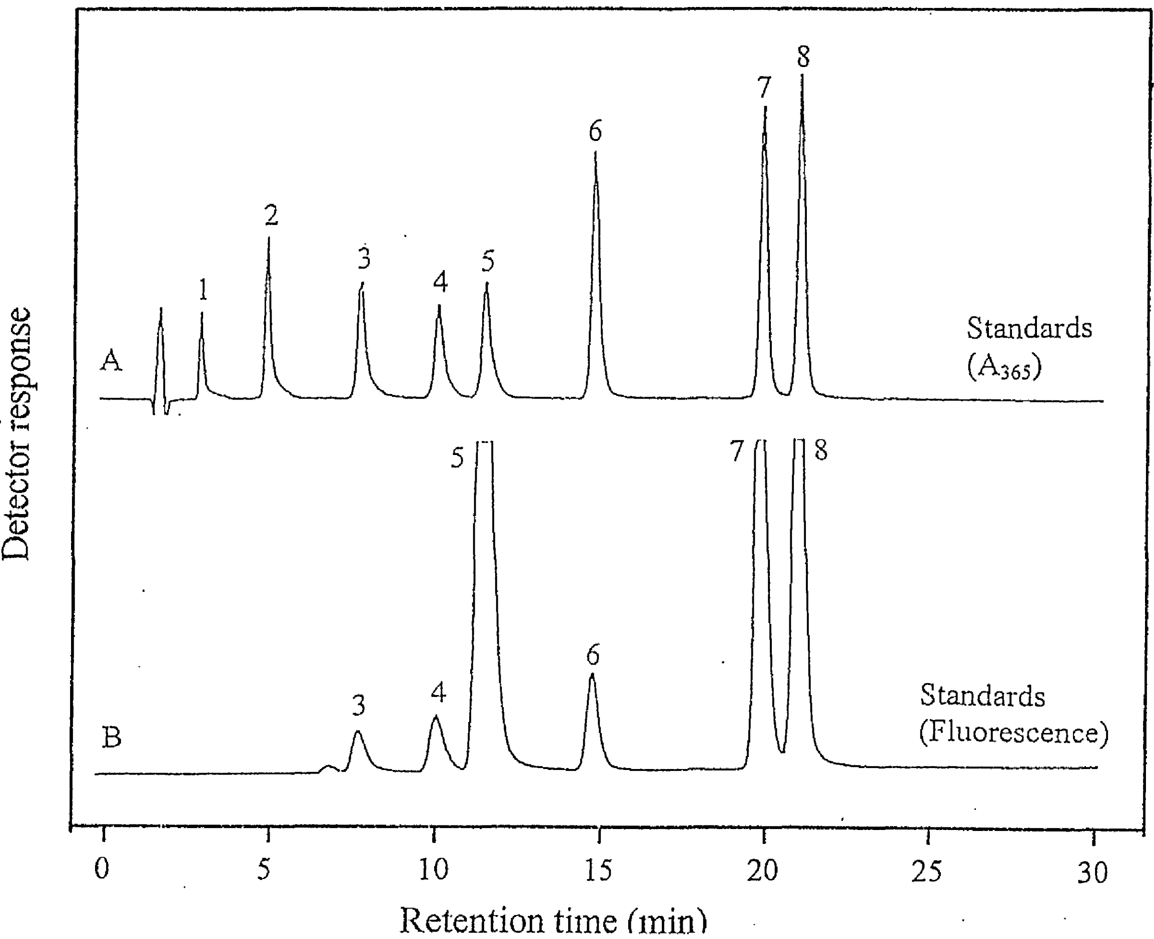

Figure 3.1: Gradient reverse phase HPLC analysis of flavonols.

Column: 150 x 3.0 mm i.d. 4-µm Genesis C_{18} cartridge column with a 10 x 4.0 mm 4-µm Genesis C_{18} guard cartridge. Mobile phase: 20 min gradient of 20-40% acetonitrile in water containing 0.5% trifluoroacetic acid. Flow rate: 0.5 ml/min. Detector: absorbance monitor operating at 365 nm and, after on–line post-column derivatization with methanolic aluminium nitrate, a fluorimeter operating at excitation 420 nm and emission 485 nm. Samples: (A) 50 ng flavonol standards: (1) quercetin-3,4′-diglucoside, (2) quercetin-3-glucoside, (3) quercetin-4′-glucoside, (4) isorhamnetin-4′-glucoside, (5) morin, (6) quercetin, (7) kaempferol and (8) isorhamnetin detected at $A_{365\ nm}$; **(B)** as (A) but with fluorescence detection after post-column derivatization. The numbers corresponded with peaks for standards listed in (A)

for all the flavonol standards could be detected at A_{365nm} whereas following post-column derivatization, the only flavonols that form fluorescent complexes were morin, quercetin, kaempferol, isorhamnetin, quercetin-4′-glucoside and isorhamnetin-4′-glucoside.

3.2.2 Flavonol extraction from samples

3.2.2.1 Hydrolysis of flavonol conjugates in samples

The optimization of acidic conditions for the hydrolysis of flavonol conjugates in a range of plant tissues has been described by Hertog *et al* (1992) following an earlier detailed study by Harborne (1965) on the release of free flavonols by acid and enzymic hydrolyses. For this study, 600 µl of plasma, 750 µl of urine and 20 mg of lyophilised fried onions were hydrolysed in a total of 2 ml of 50% methanol containing 1.2 M HCl and 20 mM sodium diethyldithiocarbamate as an antioxidant. The procedure was performed in a 3 ml glass v-vial whereby a teflon-coated magnetic stirrer was placed in the vial, which was then tightly sealed with a PTFE-faced septum prior to heating in a Reacti-Therm Heating/Stirring Module (Pierce, Rockford, Il, USA). Hydrolysis was carried out at 90°C for 2 h (urine and fried onions) and 3 h (plasma). Extract aliquots were taken before acid hydrolysis to allow for the quantification of flavonol conjugates as well as any free flavonols present. Following acid hydrolysis, samples were centrifuged for 10 min at 3000 x g and the clear supernatant used to quantify for flavonol content.

In the case of onion hydrolysis, morin was used as the internal standard whereby 10 µg of the standard was added to a total volume of 2 ml in the hydrolysis mixture and hydrolysis performed as mentioned above. Recovery of the morin internal standard was approximately 74% following a 2 h acid hydrolysis, indicating roughly 26% losses of flavonols from hydrolysis.

Extract aliquots of 75 µl taken both before and after acid hydrolysis, were made up to 250 µl with distilled water containing 0.5% trifluoroacetic acid (TFA) prior to analysis of 100 µl volumes (fried onions) and 200 µl volumes (plasma and urine) by

gradient elution reversed phase HPLC. Figure 3.2 shows a simplified diagram for the extraction and HPLC analysis of flavonols in plant tissues and biological samples.

The non-hydrolysed samples will contain all the flavonoid conjugates as well as any free flavonoids present whereas the acid hydrolysed samples will contain the aglycone as well as the free flavonoids liberated from their sugar conjugates. Therefore, the amount of conjugated flavonoids can be estimated by subtracting the levels of free flavonoids in the non-hydrolysed samples from total flavonoids obtained in the hydrolysed samples.

Conjugated flavonoids	=	Total flavonoids	–	Free flavonoids
		(Hydrolysed samples)		*(Non-hydrolysed samples)*

3.2.2.2 Stability of flavonols in plasma during acid hydrolysis

For the routine analysis of flavonols in plasma, morin could not be used as the internal standard to account for losses that occur during acid hydrolysis. This is due to the presence of contamination peaks co-eluting with the morin internal standard which would affect the accuracy of the recovery of the standard. Therefore, a separate experiment was performed to establish the stability of flavonols in plasma during the hydrolysis process. For this purpose, standards of quercetin, kaempferol and isorhamnetin (2 μg) were added into 600 μl of plasma containing 50% methanol and 1.2M HCl in a total volume of 2 ml. Plasma samples were then subjected to acid hydrolysis as mentioned in Section 3.2.2.1. Aliquots of 300 μl samples were taken at 1 h, 2 h, 3 h and 4 h during the hydrolysis process and content of the flavonol standards analysed.

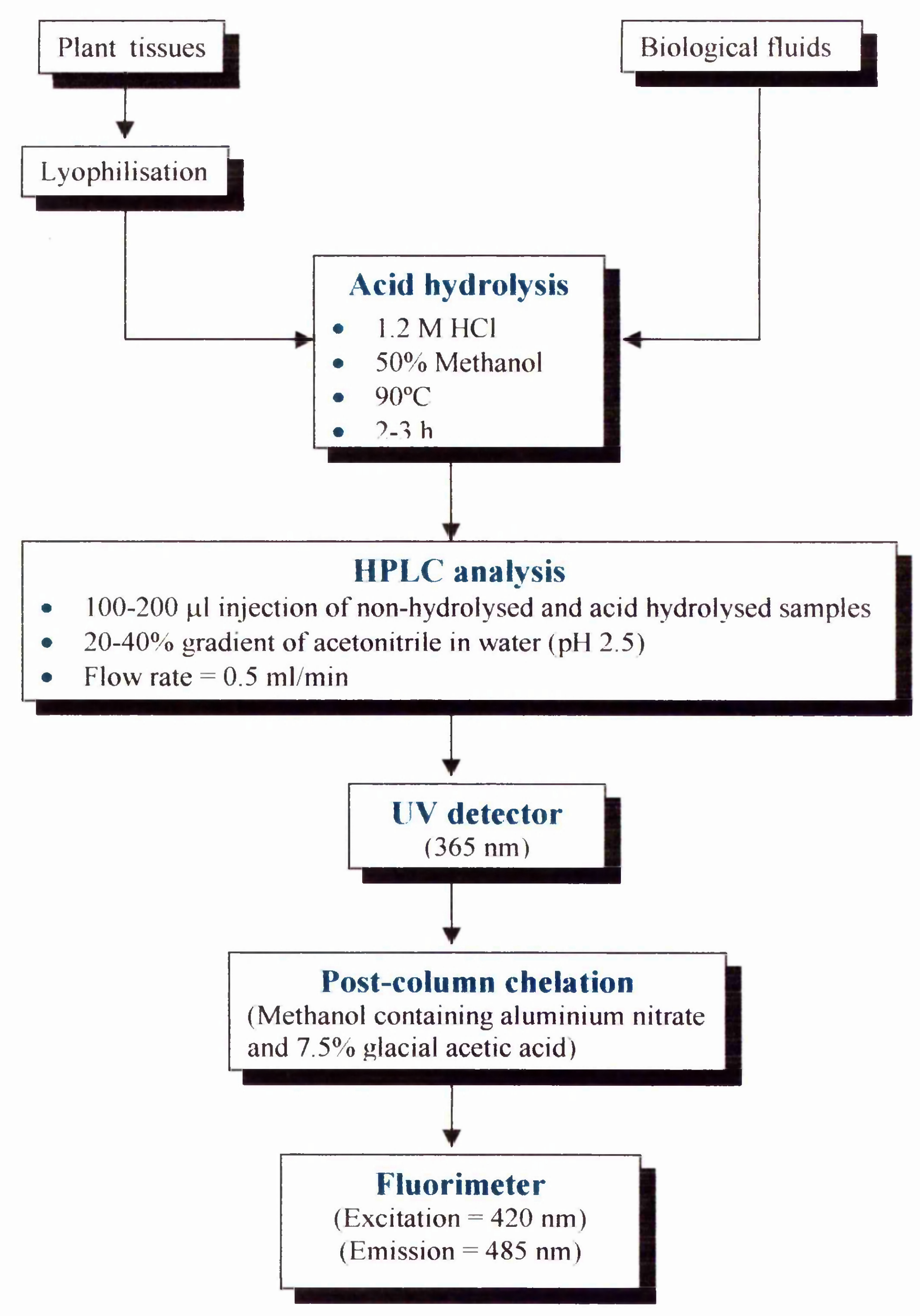

Figure 3.2: a simplified diagram of flavonol extraction and HPLC analysis

3.2.3 The effect of temperature and storage time on the flavonol content of plasma

Some of the plasma samples from human volunteers collected after they had ingested 300 g of fried onions were used for this study (see section 4.2.1). The plasma samples were divided into two groups and each group of samples was stored at either –20°C or –80°C. Flavonols in the plasma samples were analysed following a 1 week, 5 weeks and 3 months storage and after being left for 3 h at room temperature. The procedure for acid hydrolysis of the samples is described in section 3.2.2.1. Following acid hydrolysis, the flavonol content of the plasma samples was analysed on the HPLC system (section 3.2.1).

3.3 RESULTS

3.3.1 Stability of flavonols in plasma during acid hydrolysis

Results of the stability of flavonols in plasma during acid hydrolysis are illustrated in Figure 3.3. The content of quercetin, kaempferol and isorhamnetin appear to increase from 1 h to 2 h hydrolysis after which the level stabilized before decreasing at the 4 h time point. Overall, quercetin, kaempferol and isorhamnetin were stable during acid hydrolysis for up to 3 h, after that, degradation of the standards was observed. The low recoveries of quercetin, kaempferol and isorhamnetin at the 1 h time point could be a result of some of the flavonols still binding to plasma protein. Boulton *et al* (1998) reported extensive binding of quercetin to plasma proteins and the same may be true for kaempferol and isorhamnetin. Therefore, a 1 h hydrolysis of plasma is not sufficient to liberate the flavonols binding to plasma proteins.

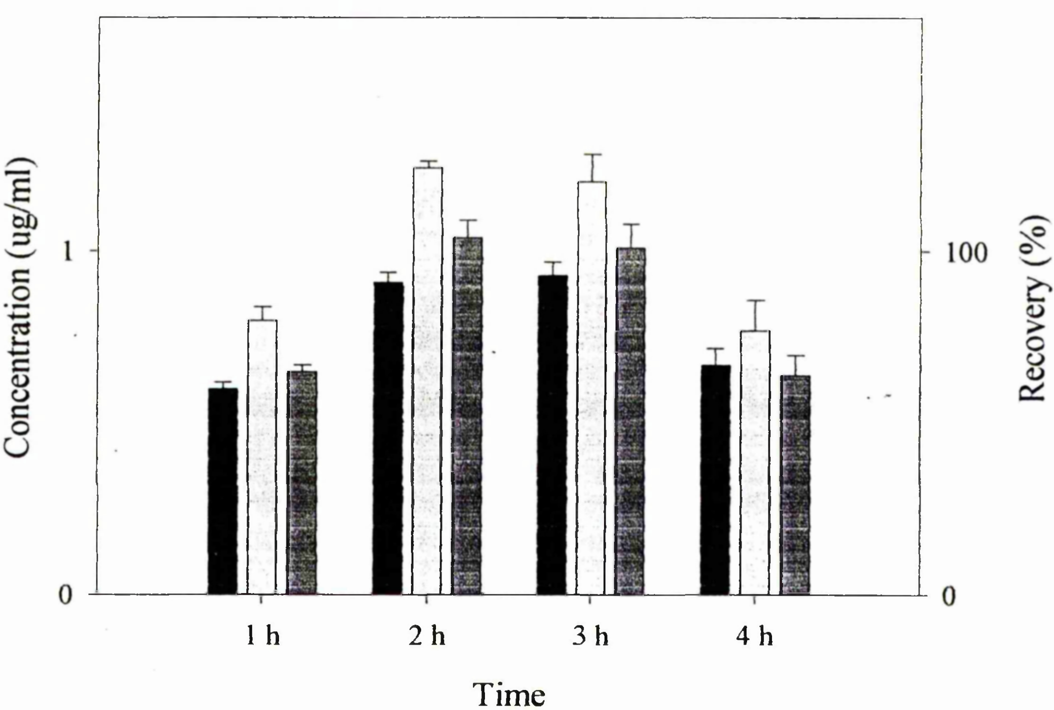

Figure 3.3: The stability of flavonols in plasma during acid hydrolysis

Standards of quercetin, kaempferol and isorhamnetin (2 μg) were added into plasma samples and subjected to acid hydrolysis for 4 h and stability of the added standards were estimated at every hour. Results are expressed as concentration (μg/ml ± standard error) and percentage recovery.

■ Quercetin □ Kaempferol ▒ Isorhamnetin

3.3.2 The effect of temperature and storage time on the flavonol content of plasma

Table 3.1 summarizes the effect of temperature and storage time on the flavonol content of plasma. Free quercetin in plasma was stable up to 5 weeks in storage with concentration ranging from 8.1 to 9.8 ng/ml. During these 3 storage times, the levels of conjugated quercetin did not change significantly and remained the same. This was evident in both samples stored at –20°C and –80°C. When left at room temperature for 3 h, levels of free quercetin increased by about 6 ng/ml although conjugated quercetin was the same as in the other 3 storage times. The slight increase in free quercetin could be a result of low level hydrolysis of conjugated quercetin.

There was not much difference in the content of conjugated kaempferol and isorhamnetin in the 4 storage times although the level of conjugated isorhamnetin analysed 1 week after storage at –80°C was slightly lower than the others. There were no detectable levels of free kaempferol and isorhamnetin in all the samples analysed.

Overall, there was no significant difference in the flavonol content of plasma stored at –20°C, –80°C and at room temperature for 3 h. Generally, flavonols in plasma are stable during storage for up to 3 months. The levels of free quercetin were slightly higher when left at room temperature for 3 h. Thus, plasma samples analysed within 3 months will still give reliable flavonol values.

Table 3.1: The effect of temperature and storage time on the flavonol content of plasma

Plasma samples were collected from human volunteers following the consumption of 300 g of lightly fried onions. Samples were stored at –20°C and –80°C and analysed at times described in the table. Results are expressed as ng/ml ± standard error.

Flavonol	1 week		5 weeks		3 months		Room T 3 hr
	-20°C	-80°C	-20°C	-80°C	-20°C	-80°C	
Quercetin	8.71 ± 0.41	9.83 ± 0.83	8.08 ± 0.2	9.32 ± 0.6	na	na	14.95 ± 1.08
*Conjugated quercetin	323.36 ± 49.75	200.36 ± 10.82	299.36 ± 27.3	336.5 ± 10.3	na	na	330.41 ± 15.49
Total quercetin	332.07 ± 49.95	210.19 ± 10.44	307.44 ± 27.50	345.82 ± 10.88	340.85 ± 30.68	348.95 ± 9.05	345.36 ± 14.41
Kaempferol	nd	nd	nd	nd	nd	nd	nd
*Conjugated kaempferol	2.92 ± 0.26	1.67 ± 0.09	1.93 ± 0.20	2.03 ± 0.08	2.59 ± 0.04	2.40 ± 0.19	2.38 ± 0.35
Total kaempferol	2.92 ± 0.26	1.67 ± 0.09	1.93 ± 0.20	2.03 ± 0.08	2.59 ± 0.04	2.40 ± 0.19	2.38 ± 0.35
Isorhamnetin	nd	nd	nd	nd	nd	nd	nd
*Conjugated isorhamnetin	12.16 ± 1.65	7.58 ± 0.5	11.85 ± 1.1	11.37 ± 0.4	9.05 ± 1.32	10.66 ± 0.22	10.73 ± 1.47
Total isorhamnetin	12.16 ± 1.65	7.58 ± 0.5	11.85 ± 1.1	11.37 ± 0.4	9.05 ± 1.32	10.66 ± 0.22	10.73 ± 1.47

* Conjugated quercetin, kaempferol and isorhamnetin were analysed after acid hydrolysis
nd = not detected
na = not analysed

3.4 Conclusion

Acid hydrolysis of plant tissues and biological fluids is widely used as one of the procedure for the hydrolysis of conjugates of flavonoids (Hertog *et al.*, 1992, Hollman *et al.*, 1995). Flavonoid standards are generally stable up to 3 h when heated at 90ºC in the presence of 1.2M HCl. The stability of flavonols in plasma up to 3 months in storage provides the ease in sample analysis as immediate analysis is not required. A study reported that quercetin added to plasma samples was stable for 5 h at 37°C and for 2 months when stored at –20°C (Liu *et al.*, 1995). HPLC procedure is also a very reliable method for the separation and identification of flavonols. The development of the post-column derivatization procedure (Hollman *et al.*, 1996) has enabled the estimation of minute quantities of flavonols normally present in biological fluids due to increased sensitivity and selectivity. Acid hydrolysis of samples and HPLC analysis were commonly used throughout this PhD project and this made up the main methods used in this thesis. Other methods relevant to each project will be described in detail in the relevant result chapter.

Absorption and Excretion of Flavonols Including Conjugated Flavonols by Human Volunteers After the Consumption of Onions

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CHAPTER 4: ABSORPTION AND EXCRETION OF FLAVONOLS INCLUDING CONJUGATED FLAVONOLS BY HUMAN VOLUNTEERS AFTER THE CONSUMPTION OF ONIONS.

4.1 Introduction

Recent evidence has strongly supported the antioxidative role of flavonoids and their protections against LDL oxidation (de Whalley *et al.*, 1990, Vinson *et al.*, 1995). Quercetin, which in conjugated forms is commonly present in fruits and vegetables in high concentrations, possesses strong antioxidative properties (Rice-Evans *et al.*, 1996). Epidemiological evidence suggests a protective effect of flavonoid intake against coronary heart disease and to a lesser extent, against cancer (Chapter 1, section 11.10). The average intake of all flavonols has been estimated at 23 mg/d in the Netherlands diet with tea and onions being the major sources at 48% and 29% of total intake respectively (Hertog *et al.*, 1993). Estimating the intakes of flavonoids and the major dietary sources is not enough if their bioavailability is not known. From the estimated intake of 23 mg/d, it is important to know how much of it is actually made available or capable of intestinal absorption and subsequently utilise by the body. Determination of the bioavailability of different types of flavonoids can be used to identify foods containing the highly bioavailable flavonoids, which when consumed, will accumulate in the bloodstream and provide potential health benefits.

The absorption and metabolism of individual flavonols in man is however still poorly understood. Attempts to investigate their absorption have shown conflicting results. It was previously speculated that only free flavonols were absorbed and not the glycosides due to their conjugation to sugar residues (Kuhnau, 1976). However, recent research has reported the absorption of rutin in human plasma (Paganga and Rice-Evans, 1997) while data obtained with ileostomy patients has been interpreted as indicating that conjugated forms of quercetin may be better absorbed than the aglycone (Hollman *et al.*, 1995).

Onions contain high concentrations of flavonols in the form of quercetin-3,4′-diglucoside and quercetin-4′-glucoside as well as smaller amounts of quercetin-3-glucoside, quercetin-7,4′-diglucoside, quercetin-7-glucoside and isorhamnetin-4′-glucoside (Tsushida and Suzuki, 1995, Price and Rhodes, 1997; Figure 4.1) . Onions therefore are useful materials for the investigation of the absorption of flavonols as recently developed HPLC techniques (Crozier *et al.*, 1997) with post-column derivatization (Hollman *et al.*, 1996) can be used to quantify the overall levels of free and conjugated quercetin and isorhamnetin. This sensitive and selective method of analysis can also be used to monitor trace levels of quercetin-4′-glucoside and isorhamnetin-4′-glucoside in body fluids and, as such, facilitates more detailed studies on the absorption of flavonol conjugates than has previously been achieved. The aim of this study was to investigate the extent of accumulation of flavonols in plasma and their excretion in urine after a meal of lightly fried onions and to establish whether any of the different flavonol conjugates present in the dietary supplement were absorbed without undergoing structural modifications. At the same time, the stability of the onion flavonols was investigated in an *in vitro* model of small intestinal digestion and colonic fermentation experiments.

Quercetin

Quercetin-3,4'-diO-β-glucoside

Quercetin-4'-O-β-glucoside

Quercetin-3-O-β-glucoside

Isorhamnetin-4'-O-β-glucoside

Quercetin-7,4'-diO-β-glucoside

Quercetin-7-O-β-glucoside

Figure 4.1: Structures of flavonol conjugates detected in onions

4.2 Materials and Methods

4.2.1 Study design

Five healthy volunteers (1 male, 4 female), mean age 29.4 years (range 23-37 y), who were not on any medication and were non-smokers participated in this study. All subjects gave a written informed consent. Volunteers were asked to follow a low flavonoid diet for 4 days prior to the experiment and this included avoiding foods and beverages containing more than 15 mg/kg of flavonoids (Hertog *et al.*, 1992; See Appendix I). On day 4, after an overnight fast, volunteers were fed 300 g of lightly fried onions between 9.30 to 10.00 a.m. An i.v cannula was inserted into each subject's arm and venous blood samples were withdrawn at 0, 0.5, 1, 1.5, 2, 3, 4, 5 and 24 h after completing the meal. 10 ml of blood were collected at each time point into heparinised tubes and immediately centrifuged at 3000 x g for 10 min at 0°C. Aliquots of 1 ml plasma were placed into eppendorf tubes and stored at –80°C until analysis. Volunteers also collected all their urine over a 24 h period following the meal and collection was divided into 3 time points, 0-6 h, 6-12 h and 12-24 h. Urine was collected into plastic containers which were kept in a cooler on ice. Aliquots of 20 ml urine at the various time points were stored at -80°C prior to analysis. The study protocol was approved by the University of Glasgow Human Ethics Committee for Non-Clinical Research.

4.2.2 Preparation of Onions

Yellow onions were purchased from Safeway plc. (Byres Road, Glasgow). Following removal of the dry outer scales, the onions were chopped into slices and lightly fried in olive oil before 300 g samples were consumed by the volunteers. Previous analyses have shown yellow and red onions to contain much higher levels of flavonols than white onions (Crozier *et al.*, 2000). The same group also showed that much more extensive frying of onions resulted in only a 21% loss of flavonols (Crozier *et al.*, 1997). Volunteers consumed the onions over a period of 20 min after collection of the fasting blood sample. Triplicate samples of fried onions from each feeding experiment were lyophilised for quantitative analysis of their flavonol content.

4.2.3 *In Vitro* digestion and fermentation of fried onions[1]

The digestion or intestinal breakdown of flavonols in onions was investigated in an *in vitro* model. Yellow onions were purchased from Safeway plc (Byres Road, Glasgow). The dry skin was removed before they were chopped into slices and a 1 kg sample lightly fried in 250 ml olive oil. 50 g of fried onions were treated with 1 g of pepsin in 50 ml of 0.1 M HCl (pH 1) for 1 h at 37°C in a shaking water bath. This was followed by the addition of 30 ml of 0.1 M NaOH (pH 7.0-7.5) containing 10 g of Creon 25000 pancreatin (Solvay Healthcare, UK). Digestion was continued for a further 3 h.

Colonic bacterial metabolism of flavonoids was tested in an *in vitro* model modified from the method of Adiotomre *et al* (1990). Faecal samples were obtained from 4 healthy volunteers and processed within 1 h of defecation. A 32% faecal slurry was prepared in phosphate buffer, pH 7.0. 1 ml faecal slurry was added to 9 ml of pre-reduced fermentation medium (pH 7.0) containing basic salts and 100 mg of either digested or undigested fried onions. Cultures were incubated in anaerobic jars at 37°C for 24 h. In addition, a control culture without onions was carried out.

Following the digestion and fermentation processes, samples were immediately frozen and lyophilised, after which they were ground to fine powder and stored at -20°C prior to analysis.

4.2.4 Flavonoid extraction from samples

Extraction and hydrolysis procedure of flavonoids from plasma, urine, lyophilised onions, digested and fermented onions are described in section 3.2.2.

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[1] The *in vitro* digestion and fermentation of fried onions was performed by Mr Khalid Khan, Department of Human Nutrition, Yorkhill Hospitals.

4.2.5 Flavonol analysis by high performance liquid chromatography

The separation and quantification of flavonols in the samples were performed on the high performance liquid chromatograph system as detailed in section 3.2.1.

Where necessary, identification of flavonols in the biological samples and plant materials were performed by co-chromatography of the samples with flavonol standards. This was carried out on the HPLC system.

4.3 Results

4.3.1 Flavonols in fried onions

Quantitative estimates of quercetin-3,4′-diglucoside, quercetin-4′-glucoside, isorhamnetin-4′-glucoside, quercetin, isorhamnetin and kaempferol in the five lightly fried onions consumed by the volunteers are described in Table 4.1. There was a variation in the flavonol content of the different onion samples which were purchased over a 6-month period and this is in keeping with previous observations (Crozier *et al.*, 1997). The levels of free quercetin for example, varied from as low as 0.41 ± 0.03 mg/g fresh weight in one sample to as high as 7.4 ± 0.2 mg/g fresh weight in another. A 3 to 6 fold difference was observed between the lowest and the highest values of the other flavonols in samples of the five lightly fried onions. In all instances, quercetin-3,4′-diglucoside and quercetin-4′-glucoside were the major flavonols present contributing approximately 71% and 25% of total flavonols respectively. At the same time, lower levels of isorhamnetin-4′-glucoside was also detected together with the aglycone forms of quercetin, isorhamnetin and kaempferol. The flavonol aglycone represented less than 1% of the total flavonols in the onion samples.

Table 4.1: Flavonol content of 300 g samples of lightly fried onions eaten by five volunteers

Results are expressed as mg/g fresh weight ± standard error (μmoles ± standard error).

Flavonol	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Mean
Quercetin	0.41 ± 0.03 (1.21 ± 0.1)	0.46 ± 0.08 (1.4 ± 0.2)	0.94 ± 0.07 (2.8 ± 0.2)	7.4 ± 0.2 (21.9 ± 0.6)	2.6 ± 0.1 (7.8 ± 0.4)	2.8 ± 1.3 (7.0 ± 3.9)
Quercetin-3,4'-diglucoside	228 ± 2 (326 ± 3)	133 ± 9 (190 ± 12)	361 ± 2 (517 ± 2)	374 ± 1 (535 ± 2)	340 ± 2 (487 ± 3)	287 ± 46 (411 ±67)
Quercetin-4'-glucoside	65 ± 2 (126 ± 4)	37 ± 4 (70 ± 7)	111 ± 3 (215 ± 5)	147 ± 6 (283 ± 11)	150 ± 1 (290 ± 3)	102 ± 22 (197 ± 43)
Isorhamnetin	n.d	n.d	0.06 ± 0.01 (0.11 ± 0.01)	0.33 ±0.01 (0.66 ± 0.02)	0.13 ± 0.02 (0.26 ± 0.04)	0.10 ± 0.06 (0.21 ± 0.12)
Isorhamnetin-4'-glucoside	10.0 ± 0.4 (20.2 ± 0.8)	5.5 ± 0.2 (11.1 ± 0.4)	10.9 ± 0.1 (22.1 ± 0.3)	14.3 ± 0.4 (28.8 ± 0.8)	12.0 ± 0.1 (24.1 ± 0.2)	10.5 ± 1.5 (21.3 ± 2.9)
Kaempferol	0.29 ± 0.06 (1.01 ± 0.21)	0.52 ± 0.02 (1.82 ± 0.07)	0.17 ± 0.02 (0.59 ± 0.07)	0.28 ± 0.001 (0.98 ± 0.03)	0.21 ± 0.01 (0.73 ± 0.02)	0.29 ± 0.06 (1.03 ± 0.21)
Conjugated kaempferol	0.77 ± 0.09 (2.69 ± 0.31)	0.26 ± 0.02 (0.91 ± 0.07)	0.52 ± 0.02 (1.82 ± 0.07)	0.98 ± 0.06 (3.42 ± 0.21)	0.51 ± 0.06 (1.78 ± 0.06)	0.61 ± 0.12 (2.12 ± 0.43)

n=3
n.d = not detectable

Our HPLC procedure provided an effective separation of flavonol standards with quercetin-3,4′-diglucoside, quercetin-3-glucoside, quercetin-4′-glucoside, isorhamnetin-4′-glucoside, morin, quercetin, kaempferol and isorhamnetin being detected with an absorbance monitor operating at 365 nm (Figure 4.2 A). With the exception of quercetin-3,4′-diglucoside and quercetin-3-glucoside, all of the above compounds form fluorescent complexes following a post-column reaction with aluminium nitrate (Figure 4.2 B). This method provided a more sensitive and selective approach for the identification of flavonols. Figure 4.2 C and D illustrated typical HPLC traces obtained for the analysis of unhydrolysed fried onion extracts. Peaks that co-chromatographed with quercetin-3,4′-diglucoside, quercetin-4′-glucoside, isorhamnetin-4′-glucoside and morin internal standard were detected at A_{365nm} (Figure 4.2 C) whereas peaks that corresponded to the 4′-glucosides of quercetin and isorhamnetin as well as small amounts of quercetin and kaempferol were present after postcolumn derivatization (Figure 4.2 D).

4.3.2 Identification of flavonols in plasma and urine

Typical HPLC traces of plasma after postcolumn derivatization collected at 0 and 1.5 h after the ingestion of onions are illustrated in Figures 4.3 C and D. The 0 h sample of plasma contained only very minor fluorescent components and there were no peaks that co-chromatographed with the flavonol standards. On the other hand, plasma collected at 1.5 h after eating 300 g of fried onions contained isorhamnetin-4′-glucoside peak in far higher sensitivity than quercetin-4′-glucoside which was present in only trace quantities as a shoulder on an impurity (Figure 4.3 D) despite being found in onions in much larger amounts than the isorhamnetin conjugate (Table 4.1). In some plasma samples, kaempferol and isorhamnetin were also found but in trace amounts close to the limit of detection. The major onion flavonol, quercetin-3,4′-diglucoside, does not fluoresce following postcolumn derivatization and was not detected at A_{365nm} in any of the plasma samples analysed. This does not necessarily mean it was not present in levels broadly comparable to quercetin-4′-glucoside because the limit of detection at A_{365nm} for quercetin-3,4′-diglucoside was 500 ng/ml while postcolumn derivatization enabled quercetin-4′-glucoside to be monitored at

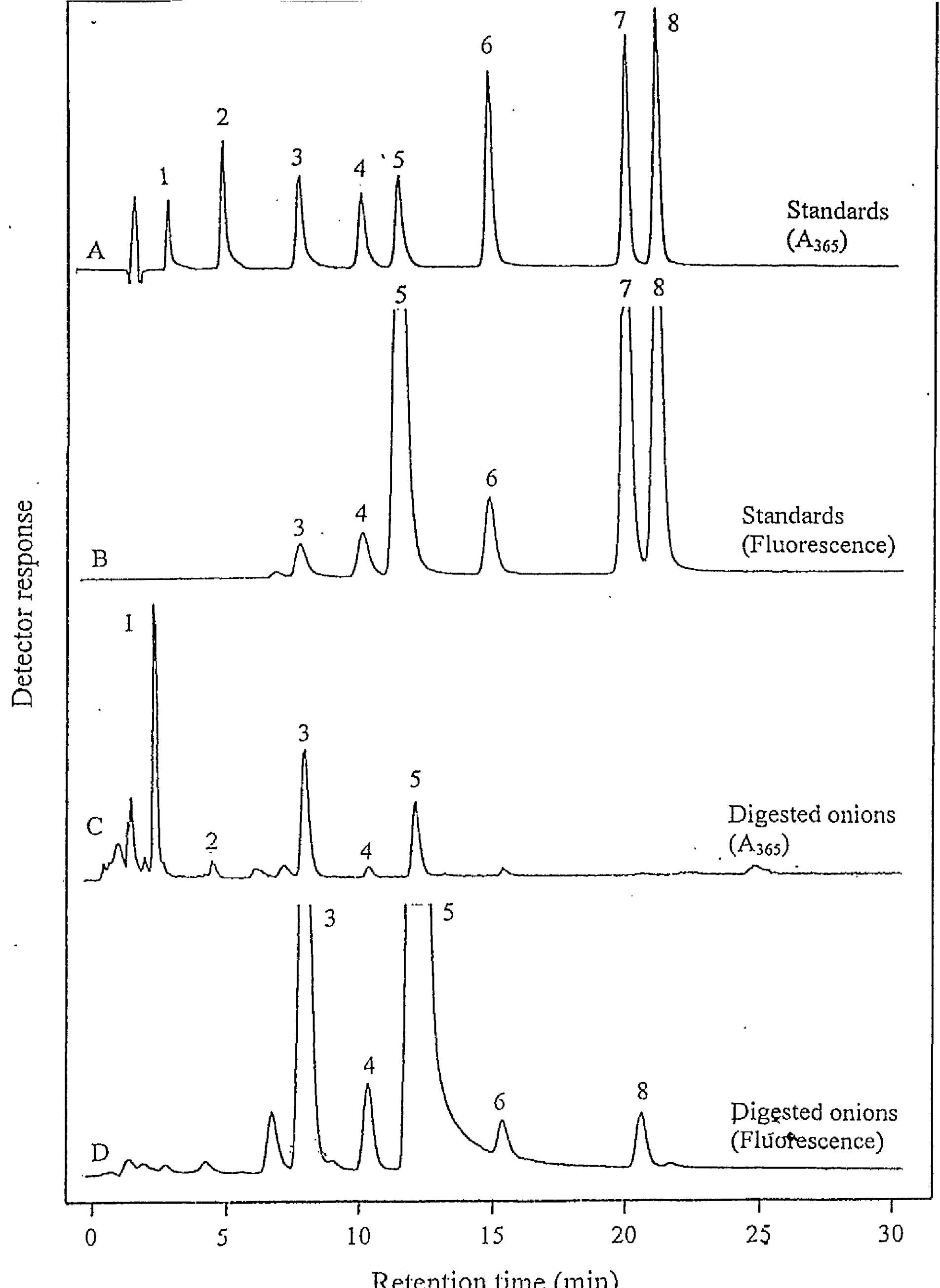

Figure 4.2: Gradient reverse phase HPLC analysis of flavonols in onions.

Column: 150 x 3.0 mm i.d. 4-µm Genesis C_{18} cartridge column with a 10 x 4.0 mm 4-µm Genesis C_{18} guard cartridge. Mobile phase: 20 min gradient of 20-40% acetonitrile in water containing 0.5% trifluoroacetic acid. Flow rate: 0.5 ml/min. Detector: absorbance monitor operating at 365 nm and, after on-line post-column derivatization with methanolic aluminium nitrate, a fluorimeter operating at excitation 420 nm and emission 485 nm. Samples: **(A)** 50 ng flavonol standards: (1) quercetin-3,4′-diglucoside, (2) quercetin-3-glucoside, (3) quercetin-4′-glucoside, (4) isorhamnetin-4′-glucoside, (5) morin, (6) quercetin, (7) kaempferol and (8) isorhamnetin detected at $A_{365\ nm}$; **(B)** as (A) but with fluorescence detection after post-column derivatization; **(C)** aliquot of a non-hydrolysed extract of yellow onions detected at A_{365nm}; **(D)** same as (C) but with post-column derivatization and fluorescence detection. The numbers corresponded with peaks for standards listed in (A).

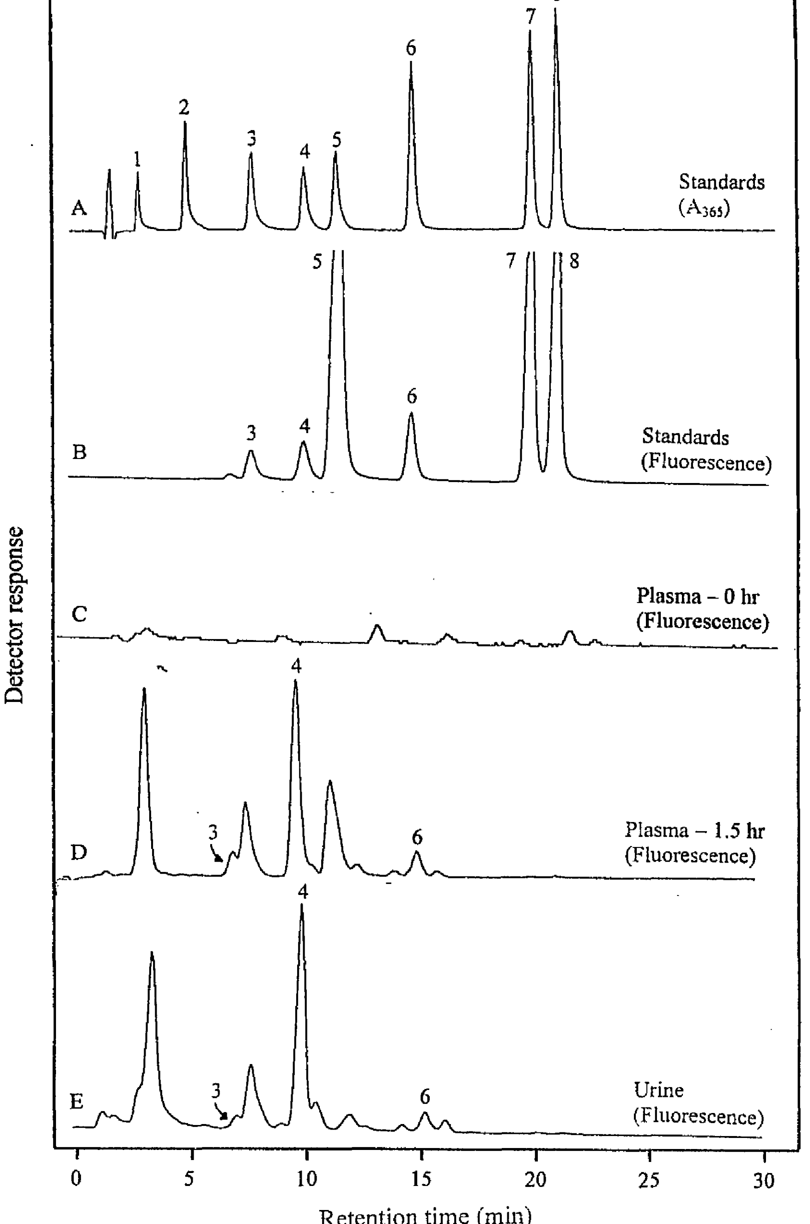

Figure 4.3: Gradient reverse phase HPLC analysis of flavonols in plasma and urine.

Column: 150 x 3.0 mm i.d. 4-µm Genesis C_{18} cartridge column with a 10 x 4.0 mm 4-µm Genesis C_{18} guard cartridge. Mobile phase: 20 min gradient of 20-40% acetonitrile in water containing 0.5% trifluoroacetic acid. Flow rate: 0.5 ml/min. Detector: absorbance monitor operating at 365 nm and, after on–line post-column derivatization with methanolic aluminium nitrate, a fluorimeter operating at excitation 420 nm and emission 485 nm. Samples: **(A)** 50 ng flavonol standards: (1) quercetin-3,4′-diglucoside, (2) quercetin-3-glucoside, (3) quercetin-4′-glucoside, (4) isorhamnetin-4′-glucoside, (5) morin, (6) quercetin, (7) kaempferol and (8) isorhamnetin detected at $A_{365\ nm}$; **(B)** as (A) but with fluorescence detection after post-column derivatization; **(C)** aliquot of a non-hydrolysed plasma collected immediately prior to the consumption of 300 g of lightly fried onions, with post-column derivatization and fluorescence detection; **(D)** as C but plasma collected 1.5 h after eating fried onions; **(E)** aliquot of a non-hydrolysed urine after consumption of fried onions with post-column derivatization and fluorescence detection. The numbers corresponded with peaks for standards listed in (A).

concentrations as low as 10 ng/ml. In all instances, identifications of quercetin-4′-glucoside, isorhamnetin-4′-glucoside, quercetin, kaempferol and isorhamnetin were confirmed by co-chromatography and the peaks corresponded with the flavonol standards (Figure 4.3 A and B).

Figure 4.3 E showed typical HPLC traces obtained for the analysis of flavonols in urine after ingestion of the fried onions, detected following post-column derivatization. The trace was quite similar to plasma collected at 1.5 h (Figure 4.3 D) with the presence of isorhamnetin-4′-glucoside in higher quantities than quercetin-4′-glucoside which was a shoulder on an impurity peak. No flavonol peaks in both plasma and urine samples were detected on the UV detector at A_{365nm}. This is due to the high limits of detection of flavonols and the UV detector is not sensitive enough to detect flavonols which were normally present in low levels in biological samples.

4.3.3 Baseline flavonol levels in plasma of volunteers on a normal diet

A separate study was performed whereby three healthy subjects who participated in the fried onions study were asked to follow their normal diet for five days, after which a fasting blood sample was collected on the 6th day. This was to determine the baseline level of flavonols of subjects on a normal diet. Table 4.2 represents the flavonols detected in plasma of the three subjects. Quercetin and isorhamnetin were detected in plasma although there were no detectable levels of isorhamnetin in subject 1. The conjugated forms of flavonols were predominant compared to the aglycones. Quercetin was the main flavonol, contributing almost 80% of the total flavonols. The concentration of quercetin in these subjects is in agreement with a study reporting a mean baseline value of 28.4 ng/ml in plasma of 5 subjects following consumption of fried onions (McAnlis *et al.*, 1999).

Table 4.2: flavonol content of plasma following a normal diet

Results expressed as ng/ml ± standard error

Subject	Free quercetin	Conjugated quercetin	Free isorhamnetin	Conjugated isorhamnetin
1	2.22 ± 1.15	18.91 ± 0.56	n.d.	n.d.
2	6.45 ± 0.58	18.85 ± 0.73	n.d.	5.87 ± 1.07
3	10.55 ± 2.07	13.20 ± 2.81	n.d.	3.96 ± 0.38

n=3

4.3.4 Flavonol accumulation in plasma

The fried onion supplement was well accepted and tolerated by the human volunteers and no adverse effects were reported. The five subjects followed a low-flavonol diet for three days and fasted overnight prior to being fed the onions. The time course profiles of the appearance of free and conjugated quercetin, quercetin-4′-glucoside and isorhamnetin-4′-glucoside in plasma are presented in Figures 4.4 A and B. As the amounts of flavonols in the onions consumed varied (see Table 4.1), flavonol levels in the plasma are expressed as percent of the amount ingested.

Due to the significant variation in the accumulation of flavonols in plasma of the five subjects, the profile of each individual was illustrated (Figure 4.4 A and B). Despite following a low flavonol diet 3 days before the experiment, trace levels of free quercetin could be detected in the fasting (0 h) plasma samples of subjects 2 and 5 (9.9-11.4 ng/ml) (Figure 4.4A). Low levels of conjugated quercetin (6.8 ± 2.8 ng/ml) was also present in the 0 h sample of subject 2. Levels detected in fasting plasma samples were much lower than baseline plasma levels of subjects on a normal diet (Table 4.2). This indicates that the low flavonol diet managed to eliminate most of the flavonols in the circulation. In other instances, there were no detectable quantities of flavonols in the fasting plasma samples. After 24 h, only conjugated quercetin could still be detected (above the baseline) in plasma of the 5 subjects whereas there were no detectable levels of the other flavonols.

Subjects 1, 2 and 3 exhibited similar profiles with a rapid increase of flavonol levels after onion consumption followed by a rapid decline in their concentration. On the other hand, flavonols in subjects 4 and 5 appeared to have a second peak concentration later on in the time course of the experiment and had a slower decline in flavonol content than subjects 1, 2 and 3. Based on the profile of flavonol accumulation in plasma of the five subjects, the pattern of flavonol accumulation could be divided into 2 groups, the first being rapid absorption followed by rapid decline (as in subjects 1,2 and 3) and the second being moderate absorption and slower decline in flavonol levels (subjects 4 and 5). Subjects in the

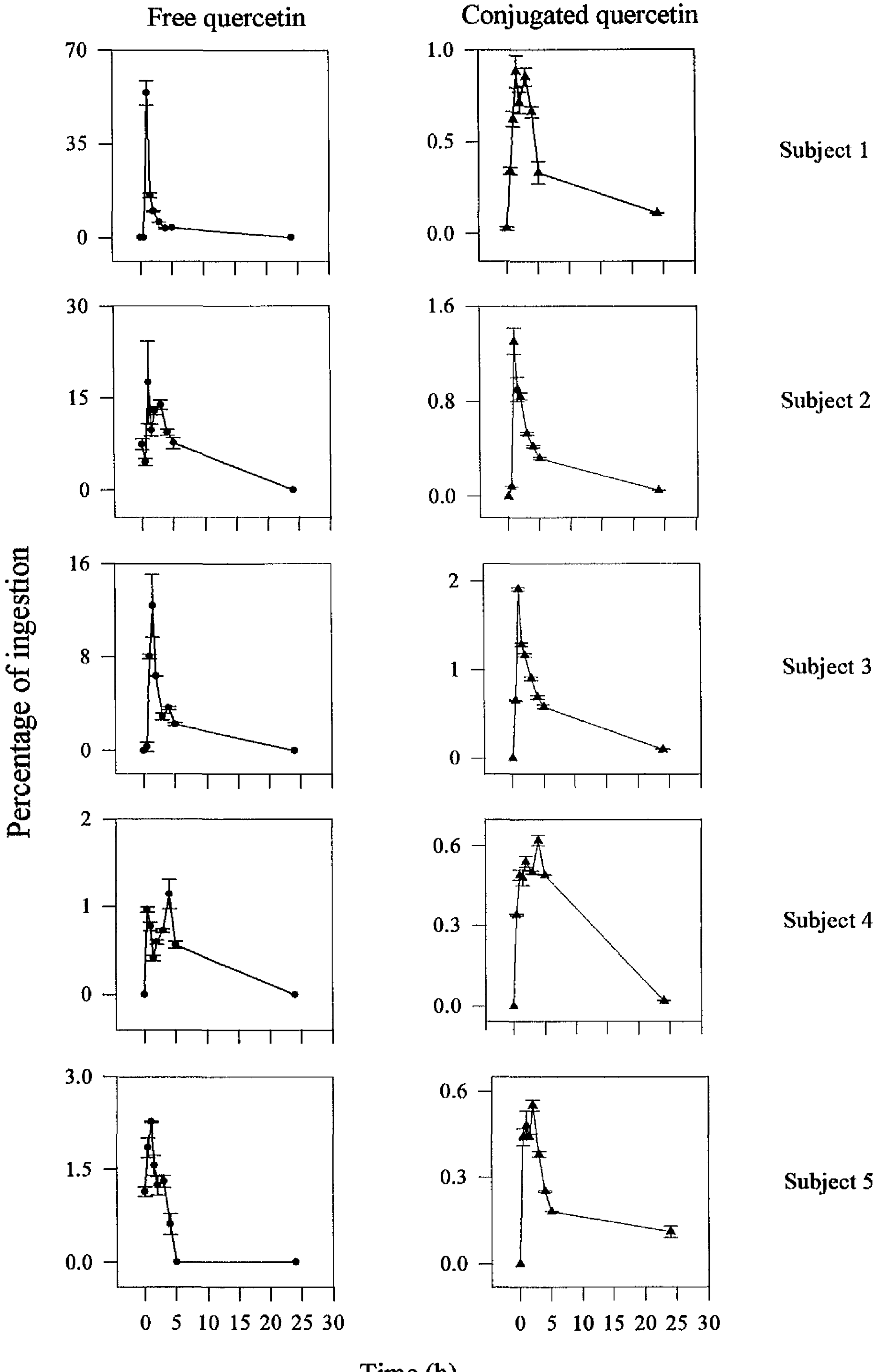

Figure 4.4 A: Free and conjugated quercetin in plasma collected from five human volunteers after the ingestion of 300 g of lightly fried onions.

Data expressed as percentage of the intake based on flavonol content of onions ± standard error (n=5) and calculated on the basis of 3000 ml of plasma per person.

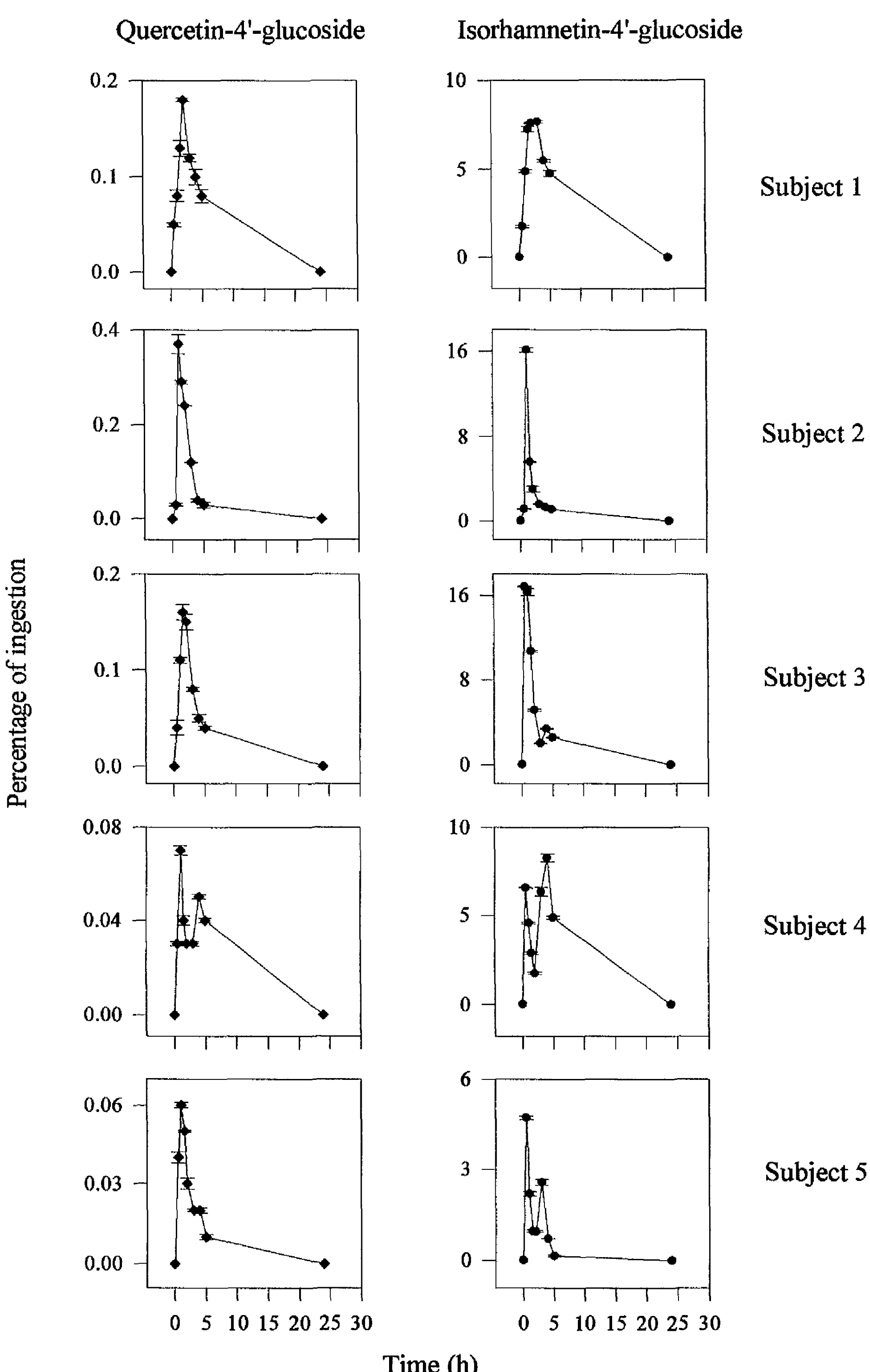

Figure 4.4 B: Quercetin-4′-glucoside and isorhamnetin-4′-glucoside in plasma collected from five human volunteers after the ingestion of 300 g of lightly fried onions.

Data expressed as percentage of the intake based on flavonol content of onions ± standard error (n=5) and calculated on the basis of 3000 ml of plasma per person.

latter category appeared to have lower values of flavonols at peak plasma concentration when expressed as a proportion of intake compared to the first category. Overall, the absorption of flavonols was moderately rapid with peak plasma concentrations being reached in the five subjects at times ranging from 0.5-4.0 h after ingestion of the fried onions, although in most cases, a figure of 1.0-2.0 h was typical (Table 4.3).

There was a large variation in the profile for plasma accumulation of free quercetin among the 5 volunteers with subjects 1, 2 and 3 showing peak concentration as percentage of ingestion in the range of 12-53% whereas with subjects 4 and 5, it was between 1.1-2.3%, a difference of approximately 15 fold (Figure 4.4A). Similar profiles were observed for conjugated quercetin with subjects 1,2 and 3 showing higher peak plasma concentration expressed as percentage of ingestion (0.8-2.0%) compared to subjects 4 and 5 (0.6%). It is notable that compared with the levels present in the ingested onions, isorhamnetin-4′-glucoside accumulated in plasma in amounts ca. 10 times greater than conjugated quercetin and 50-fold more than quercetin-4′-glucoside. This was evident with all five subjects (Figure 4.4). In addition to free and conjugated quercetin, variations was also noticed in the accumulation of 4′-glucosides of isorhamnetin and quercetin, as a percentage of the amount ingested, between the five subjects. With subjects 2 and 3, the peak isorhamnetin-4′-glucoside level was ca. 16% and that of quercetin-4′-glucoside ca. 0.2 –0.4 % while the equivalent figures for subject 5 were ca. 5% and ca. 0.1% respectively (Figure 4.4 B).

The mean values for the key features of flavonol accumulation in plasma are presented in Table 4.3. A peak concentration of 452 ± 100 ng/ml was obtained for the overall level of quercetin conjugates while with quercetin-4′-glucoside it was 45 ± 11 ng/ml, which was less than 10% of the total quercetin conjugate concentration. The mean maximum concentration of isorhamnetin-4′-glucoside was 370 ± 91 ng/ml which when expressed as a proportion of intake from the onions was 10.7 ± 2.6% compared to values of 0.13 ± 0.03% and 0.97 ± 0.21% from quercetin-4′-glucoside and quercetin conjugates, respectively. In addition to the conjugated flavonols, we could also detect the accumulation

Table 4.3: Mean values ± standard error of key features of flavonol accumulation in plasma following the consumption of 300 g of lightly fried onions by five subjects.

Flavonol	Intake (mg)	Peak plasma concentration (ng/ml)	Time of peak plasma concentration (h)	Peak plasma concentration as a proportion of intake* (%)
Quercetin-4'-glucoside	102 ± 22	45 ± 11	1.3 ± 0.2	0.13 ± 0.03
Isorhamnetin-4'-glucoside	10.5 ± 1.5	370 ± 91	1.8 ± 0.7	10.7 ± 2.6
Free quercetin	2.8 ± 1.3	37.5 ± 9.5	1.7 ± 0.6	17.4 ± 9.6
Conjugated quercetin[a]	139 ± 25	452 ± 100	1.9 ± 0.6	0.97 ± 0.21

*Calculated on the basis of 3000 ml plasma/person.

[a] Determined following acid hydrolysis.

of trace amounts of free quercetin in plasma although at peak concentration, the levels were much lower than the other flavonols detected, i.e ca 10% of conjugated quercetin. However, the levels of free quercetin detected could be a result of hydrolysis of very large pool of conjugated quercetin rather than their direct absorption or metabolism.

The area under the curve for plasma accumulation of the different flavonols after the fried onion meal was estimated (Table 4.4). Conjugated quercetin showed the highest AUC whereas free quercetin had the lowest. The relative accumulation of quercetin-4′-glucoside and free quercetin was 5% that of conjugated quercetin. On the other hand, isorhamnetin-4′-glucoside showed bioavailability approximately 50% that of conjugated quercetin.

4.3.5 Urinary excretion of flavonols

Similar to plasma, there was a higher excretion of isorhamnetin-4′-glucoside with a percentage excretion of 17.4 ± 8.3% compared to 0.2 ± 0.1% for quercetin-4′-glucoside (Table 4.5). Free quercetin and isorhamnetin were also detected in urine of the five subjects with percentage excretion of 30.2 ± 11.8% and 12.0 ± 9.0% respectively. Again, figures for free quercetin and isorhamnetin could be exaggerated because of large conjugate pools with trace levels of hydrolysis. Quercetin conjugates in the hydrolysed samples showed a percentage excretion of 0.8 ± 0.4% which was 4 times the percentage excretion of quercetin-4′-glucoside. In most cases, a high proportion of the flavonols was excreted in the first collection period (0-6 h) whereby approximately 57% of the cumulative excretion were reached. Urine samples from the last collection period, contained on average, 4% of the total daily output of the flavonols indicating that the peak of urinary flavonol excretion lay well within the 12 h period.

Table 4.4: Area under the curve of flavonols in plasma following ingestion of 300 g of fried onions.

Flavonol	Peak plasma concentration (ng/ml)	AUC $_{0-24h}$ (h.ng/ml)
Quercetin-4'-glucoside	45 ± 11	211 ± 36
Isorhamnetin-4'-glucoside	370 ± 91	1617 ± 524
Free quercetin	37.5 ± 9.5	149 ± 30
Conjugated quercetin[a]	452 ± 100	3331 ± 700

[a] Determined following acid hydrolysis
n=5 ± standard error

Table 4.5: Mean values ± standard error for the excretion of flavonols in urine following the consumption of 300 g of lightly fried onions by five subjects.

Results expressed as μg ± standard error.

Flavonol	Intake (mg)	Excretion period			Total excreted as a proportion of intake (h)
		0-6 h	6-12 h	12-24 h	
Quercetin-4'-glucoside	102 ± 22	100 ± 27	65 ± 43	4.8 ± 2.6	0.2 ± 0.1
Isorhamnetin-4'-glucoside	10.4 ± 1.5	1175 ± 482	620 ± 374	23 ± 15	17.4 ± 8.3
Quercetin	2.8 ± 1.3	134.9 ± 46.7	101.7 ± 61.6	5.6 ± 2.3	30.2 ± 11.8
Isorhamnetin	0.10 ± 0.06	4.6 ± 2.1	3.9 ± 1.8	0.3 ± 0.2	12.0 ± 9.0
Conjugated quercetin[a]	139 ± 25	661 ± 281	348 ± 184	66 ± 29	0.8 ± 0.4

n.d. = not detected

[a] Determined following acid hydrolysis

4.3.6 *In vitro* digestion of fried onions

Table 4.6 shows the flavonol content of fried onions before and after *in vitro* digestion. There was no significant difference in the flavonol content of onions before and after digestion. However, a slight decrease was observed in the levels of conjugated quercetin including quercetin-3,4′-diglucoside and quercetin-4′-glucoside analysed in the nonhydrolysed samples. The levels of free quercetin were slightly higher after digestion compared to before. This suggests hydrolysis of trace amounts of the glucosides to liberate free quercetin. Isorhamnetin-4′-glucoside was the most stable compound with only 7% losses occurring during the digestion process. Overall, flavonols in fried onions are generally stable to the acidic and alkaline conditions of digestion with approximately 7-18% losses observed.

4.3.7 *In vitro* fermentation of fried onions

In vitro fermentation was performed on the digested as well as the undigested fried onions (Table 4.6). Following fermentation, there were no detectable levels of quercetin-3,4′-diglucoside and quercetin-4′-glucoside in both the undigested as well as the digested onions. The levels of free quercetin did not increase after fermentation and less than 3% of conjugated quercetin was recovered following the fermentation process. As for isorhamnetin-4'-glucoside, there was almost complete degradation with less than 2% recovered after fermentation. Overall, fermentation of fried onions led to the disappearance of conjugated flavonols including quercetin-3,4′-diglucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside.

Table 4.6: The effect of *in vitro* digestion and colonic fermentation on the flavonol content of lightly fried onions.

Data are expressed as μg flavonol g^{-1} onion (fresh weight) ± standard error (n=3).

Treatment	Quercetin-3,4'-diglucoside	Quercetin-4'-glucoside	Quercetin	Isorhamnetin-4'-glucoside	Isorhamnetin
In vitro digestion					
Undigested	877 ± 7.4	329 ± 5.0	6.3 ± 0.2	292 ± 4.7	n.d
Digested	804 ± 50	272 ± 17	12 ± 0.4	270 ± 18	n.d
In vitro fermentation					
Undigested	n.d	n.d	4.0 ± 1.3	0.5 ± 0.1	0.7 ± 0.2
Digested	n.d	n.d	2.7 ± 0.0	0.4 ± 0.1	0.7 ± 0.2
Blank fermentation medium	n.d	n.d	n.d	n.d	n.d

n.d – not detected.

4.4 Discussion

Although the literature contains much information on the seemingly low levels of absorption of the aglycone quercetin in a variety of rat test systems, there are few studies on the absorption of flavonol conjugates, the typical constituents of foods, by humans (see Chapter 1, section 1.11). Hollman and co-workers (1995) have investigated the absorption of quercetin glucosides by humans with an ileostomy. The subjects were fed quercetin glucoside-rich onions, rutin or free quercetin, after which the flavonol content of ileostomy effluent and rutin were monitored over a 13 h period. *In vitro* incubations of the three sources of flavonols with gastrointestinal fluids showed minimal degradation and extremely low levels of flavonols were excreted in urine. After corrections for sample handling losses and low level degradation in the ileostomy bag, absorption was estimated by subtracting the flavonol content of the ileostomy effluent from the oral intake. This albeit indirect procedure, indicated surprisingly high levels of absorption, 52% of onion quercetin glucosides, 17% for rutin and 24% for quercetin. Subsequently, the same group, who analysed samples only after acid hydrolysis, which does not allow distinction between free and conjugated quercetin pools, monitored flavonol levels in plasma after the ingestion of onions (Hollman *et al.*, 1996). The time course profiles obtained with two volunteers were similar to those obtained in the present study (Figure 4.3), as was the peak quercetin (free plus conjugated) plasma concentration of 196 ng/ml. This is equivalent to ca. 0.9% of the flavonol content of the ingested onion flavonols and comparable to figures in Table 4.3. However, our results presented a more reliable figure as more subjects were used (n=5) compared to 2 in the study by Hollman *et al.* Detection of low plasma concentrations imply that quercetin/quercetin conjugates, if they are absorbed into the bloodstream in the quantities reported by Hollman *et al.* (1995), are being rapidly metabolised and/or removed from the bloodstream, presumably by the liver.

In the present study, it has been demonstrated for the first time that the onion flavonol glucosides, quercetin-4′-glucoside and isorhamnetin-4′-glucoside accumulate in the bloodstream and are excreted in urine without seemingly undergoing structural modification. The main flavonol in onions, quercetin-3,4′-diglucoside, was not detected in body fluids but this is likely to be a consequence of the relative lack of sensitivity of the HPLC detection

systems for this conjugate. The level of quercetin released from conjugated forms by acid hydrolysis, although low, was invariably several-fold higher than the concentration of quercetin-4′-glucoside in both plasma and urine (Table 4.3 and 4.5). This may be due to the presence of metabolites such as quercetin glucuronide and sulphate conjugates, which release free quercetin when acid hydrolysed (Hollman *et al.*, 1996, 1997), as would trace levels of quercetin-3,4′-diglucoside, which may also have been present. However, when compared to the levels present in the ingested onions, it is evident that isorhamnetin-4′-glucoside accumulated in both plasma and urine in proportionally far higher amounts than quercetin-4′-glucoside and other quercetin conjugates (Table 4.3 and 4.5). Further study is required to determine whether this is due to more effective absorption of the isorhamnetin conjugate or whether it is a consequence of the absorbed quercetin conjugates being removed from the bloodstream more rapidly than isorhamnetin-4′-glucoside. There is however, an alternative possibility that at least part of the isorhamnetin-4′-glucoside pool is formed by 3′-*O*-methylation of quercetin-4′-glucoside. Isorhamnetin is one of a number of metabolites that appear in the bile of rats after oral intake of quercetin (Manach *et al.*, 1996).

After the consumption of onions, flavonols accumulated rapidly in plasma with peak concentrations being reached within 1-2 h in most instances (Table 4.3, Figure 4.4). This observation agrees with previous findings by Hollman *et al.* (1996) discussed above and implies that absorption of flavonol conjugates occurs primarily from the stomach and/or the small intestine. The variation in the profile of absorption of the subjects (Figure 4.4) may be due to the differences in their intestinal physiology and habitual diet, which influence the extent of flavonol absorption, or alternatively they could be a consequence of different rates of metabolism/sequestration of the absorbed conjugates. Interestingly, the mean peak plasma concentration for conjugated quercetin and isorhamnetin-4′-glucoside (Table 4.3) are both in excess of the levels of β–carotene that are typically found in human plasma (Stocker and Frei, 1991).

Based on the *in vitro* digestion of fried onion, the onion flavonols were generally stable to the acidic and alkaline condition in the small intestine, leaving the flavonols unchanged for absorption. This also suggests that no modification or degradation of flavonols occurred in the small intestine. The same observation was reported by Hollman *et*

al (1995). *In vitro* fermentation of onions resulted in almost complete degradation of flavonols by the faecal microorganisms. This suggests that no absorption of the unchanged flavonols take place in the large intestine. Hydrolysis of the conjugated flavonols by enzymes of faecal bacteria should have led to the increase in levels of free flavonols (Table 4.6). However, this was not the case and there were low levels of free flavonols present following fermentation indicating the free flavonols were also subjected to degradation by the microorganisms. The ability of colonic microorganisms to metabolise flavonoids to phenolic acids is widely recognised (Griffiths, 1982, Hackett, 1986). Quercetin for example undergoes ring fission to form phenolic acids (Booth *et al.*, 1956, Nakagawa *et al.*, 1965).

The presence of flavonols in urine indicated their absorption from the small intestine. However, the levels obtained are not directly comparable to the values in plasma because absorbed flavonols maybe metabolised or sequestered in other parts of the body.

In this study, based on the AUC values, conjugated quercetin appears to be the most bioavailable flavonol relative to the other flavonols detected after the onion meal (Table 4.4). The value for conjugated quercetin is similar to the ones reported by Hollman (1997). Conjugated quercetin in the hydrolysed samples may contain other glycosides, glucuronides or sulphates in addition to quercetin-4′-glucoside, contributing to the high AUC observed. Although ideally, determinations of absolute bioavailability require AUC for both flavonoids administered intravenously and orally, this is not always practical. However, relative bioavailability of various flavonoids can be estimated based on AUC from oral dose.

In their reports on the absorption of flavonols derived from onions and other vegetables, the Dutch group fitted data on flavonol levels in plasma and urine into a two-open compartment model using the equation $C(t)=Ae^{-kt}+Be^{-\alpha t}+Ce^{-\beta t}$ (Shargel and Yu, 1992) where t equals time and k is the absorption rate constant and α and β being the slope for the distribution and elimination phases respectively. The different parameters calculated from the equation were used to estimate the half-lives of the absorption, distribution and elimination phases and the bioavailability of total quercetin was calculated by comparing the areas under the percentage flavonol ingested-time curve (Hollman *et al.*, 1996, 1997). The validity of such extrapolations is, however, open to question. Although the figures for

isorhamnetin-4′-glucoside were higher, the peak quercetin levels detected in plasma and urine were low, ca. 1% of the amounts in the ingested onions. Homeostasis of plasma flavonol pools is almost certainly in a state of flux because of the combined effects of transport through the gut wall into the bloodstream and removal by sequestration, metabolism and excretion. In the circumstances, figures obtained from the two-open compartment model are likely to be of little value until much more is known about the underlying physiological and metabolic events.

Information regarding the mechanism of absorption of flavonols is still not well understood. It has been postulated that the Na^+-glucose co-transport system may play a role in flavonol absorption (Hollman, 1997). This co-transport system is involved in the transport of glucose across the intestinal wall (Mizuma *et al.*, 1994) and since the present study has provided unequivocal evidence for the absorption of the flavonol glucosides, the possible involvement of this method of transport merits investigation.

4.5 Conclusion

In conclusion, this study has shown that following the ingestion of lightly fried onions, there is a proportionally higher accumulation of quercetin and isorhamnetin-4′-glucoside than quercetin conjugates, including quercetin-3,4′-diglucoside and quercetin-4′-glucoside, in plasma and urine of humans. This is likely to be a consequence of either preferential absorption of isorhamnetin-4′-glucoside or, a post-absorption conversion of quercetin-4′-glucoside to isorhamnetin-4′-glucoside via 3′-*O*-methylation. Distinguishing between these processes and clarification of the mechanisms involved requires further detailed metabolic studies including using radiolabelled flavonols. Identification of flavonols that are highly absorbable can be used to encourage increased consumption of food containing high levels of these compounds, which when consumed will accumulate in the blood stream.

5 The Uptake of Individual Flavonols in an Everted Rat Gut Model

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CHAPTER 5: THE UPTAKE OF INDIVIDUAL FLAVONOLS IN AN EVERTED RAT GUT MODEL

5.1 Introduction

Investigation of the absorption of flavonols is crucial in evaluating their biological role. The process that occurs during the movement of flavonols across the intestinal membrane is the first important step in determining how much of the ingested compound is absorbed. *In vivo* studies with animals and humans have described absorption of dietary as well as non-dietary flavonols (see section 1.11). Studies with ileostomy volunteers demonstrated higher absorption of quercetin glycosides (52%) than the aglycone quercetin (24%) (Hollman *et al.*, 1995). At the same time, there is a preliminary report on the presence of rutin in plasma of non-supplemented subjects (Paganga and Rice-Evans, 1997). On the other hand, conjugated quercetin was detected in plasma of human volunteers following the consumption of rutin-rich cherry tomatoes (Crozier *et al.*, 2000). The absorption of specific flavonols is not known apart from our study which detected accumulation of higher levels of isorhamnetin-4′-glucoside in plasma compared to quercetin-4′-glucoside following ingestion of fried onions (Aziz *et al.*, 1998).

In vivo experiments to investigate intestinal absorption of flavonols are complex due to the difficulty of sampling the absorbed flavonols from the serosal side of the gastrointestinal tract. Ultimately, *in vitro* systems provide the best approach in evaluating the relative rate and extent of absorption of individual flavonols from the small intestine (see Chapter 1, section 1.13.3). This chapter describes the use of an everted rat gut model to investigate the intestinal transport of several flavonols.

The everted intestine method employed in this study is well established and is widely used to investigate *in vitro* intestinal transport. This model which uses rat intestine, originally developed by Wilson and Wiseman (1954) is capable of surviving *in vitro* over extended periods of time. It has the advantage that several segments can be obtained from one animal, allowing randomisation as well as several replicates to be

performed. In addition, the ability to incubate everted segments in the mucosal buffer allows the ease of testing various compounds as these can be added into the incubation media. Also, eversion of the intestine exposes the highly active mucosal cells to the well-oxygenated incubation media, thus prolonging the viability of the absorptive cells.

The objective of this study was to evaluate the effect of structural modification of the flavonol molecule on their uptake across the rat intestine. At the same time, the influence of different sugars and their position on the flavonol molecule was determined. The flavonols tested were quercetin-3,4′-diglucoside, rutin, quercetin-3-glucoside, quercitrin, quercetin-4′-glucoside, isorhamnetin-4′-glucoside and quercetin (Figure 5.1). These flavonols showed variation in terms of position and type of sugar moiety attached to the molecule as well as the presence of a methoxy group (isorhamnetin-4′-glucoside). Information obtained will be useful to establish if there is any preferential uptake of flavonols and subsequently determine their potential to act as antioxidants and their role in promoting human health.

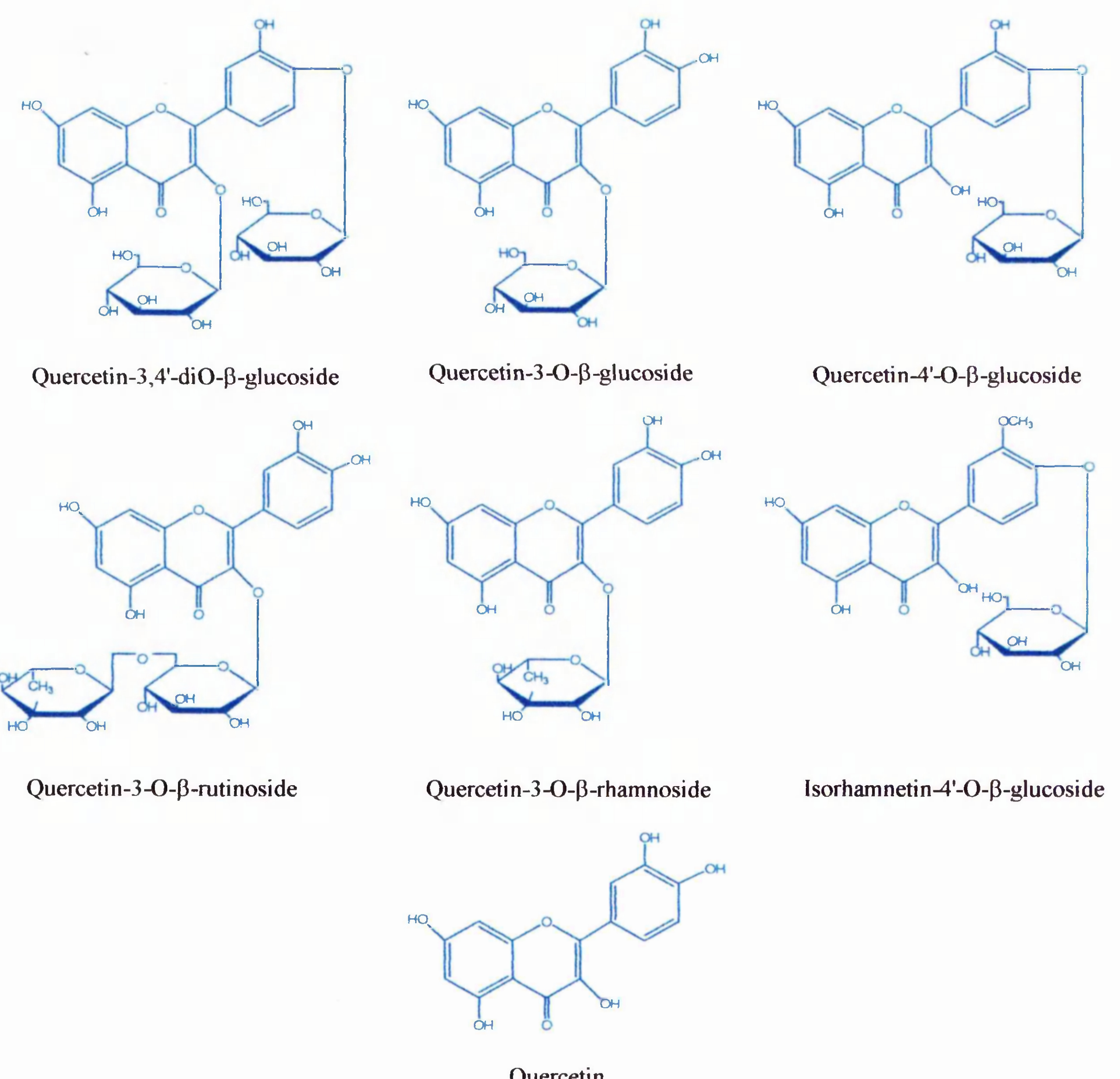

Figure 5.1: The chemical structures of the flavonols employed in the everted rat intestine model for investigation of their intestinal transport.

5.2 Materials and methods

5.2.1 Preparation of Krebs incubation buffer

All incubation experiments were done in Krebs-Ringer buffer. The Krebs incubation media were made up of 113.3 mM NaCl, 4.83 mM KCl, 1.214 mM KH_2PO4, 1.205 mM $MgSO_4$, 16.96 mM $NaHCO_3$, 10.18 mM Na_2HPO_4, 0.645 mM $CaCl_2$ (Mizuma *et al.*, 1994).

5.2.2 Preparation of rat intestinal tissues

Sprague-Dawley rats (male and female) were obtained from the animal unit at the Central Research Facility, University of Glasgow. Their average weight was 298 g. Animals followed the standard rat and mouse expanded diet obtained from B and K Universal Limited (Hull, UK) and water was provided ad lib. The non-fasted rats were stunned by a blow to the head prior to killing by cervical dislocation. An incision was made on the abdomen and the small intestine was immediately removed by cutting from the upper end of the duodenum to the upper end of the colon and was kept on ice-cold saline gassed with 95% O_2 and 5% CO_2. The small intestine was cleaned by removal of fatty tissues and mesentery. Eversion was carried out by tying one end of the segment onto a piece of glass rod (30 cm length, 2 mm diam) and pushing the intestine into the rod until the mucosal side was exposed (Mizuma *et al.*, 1994). The segments were then cut to the desired length (6-10 cm) and placed in ice-cold saline infused with 95% O_2 and 5% CO_2. Before the start of incubation, the segments were ligatured at both ends after the insertion of plastic cannulas, which in turn were connected to 3-way taps. A 1 ml plastic disposable syringe could then be conveniently attached to the 3-way taps for rinsing the segments as well as sampling from the serosal side.

5.2.3 Incubation procedures for the everted segments

The incubation procedure was performed according to the method of Mizuma *et al.* (1994) with slight modifications. Briefly, after rinsing with Krebs buffer, the

segments were filled with buffer using a 5 ml syringe and the tissue was placed in a 50 ml beaker containing 20 ml Krebs incubation buffer with or without flavonol standards. The incubation medium was continuously gassed with 95% O_2 and 5% CO_2 and placed in a 37°C water bath. Using the 1 ml syringe, approximately 150 μl was sampled from the serosal side and the same volume of oxygenated Krebs was replaced. Sampling was performed at 5 min intervals over a 30-minute incubation period. The same volume of sample was also taken from the incubation media at the start and the end of the incubation experiment. The samples were then subjected to flavonol extraction. Between five and eight segments were used for each experiment.

5.2.4 Uptake of individual flavonols

Standards of quercetin-3,4′-diglucoside, quercetin-3-glucoside, quercitrin, rutin, quercetin-4′-glucoside, isorhamnetin-4′-glucoside and quercetin aglycone were employed to establish the effects of structural modification on flavonol uptake by the small intestine. A concentration of 10 μM was used for each compound and standards were dissolved in methanol. The percentage of methanol in the incubation media was kept at a minimum (<0.7%).

The 10 μM concentration used for each flavonol was estimated based on the reported daily quercetin intake of 16.3 mg (Hertog *et al.*, 1993). Assuming a gastric fluid volume of 100 to 500 ml and complete availability of flavonoids in the intestine, the final concentration of quercetin will roughly be between 100 to 500 μM in the intestine. Considering a divided intake of flavonoids throughout the day as well as the use of segments of rat intestine with less fluid volume, a flavonol concentration of 10 to 50 μM was considered a physiological level. In addition, variability in the extraction of the different flavonols as well as fluid volumes along the intestine should also be considered.

Control experiments were performed without addition of flavonols in the incubation media. Similar sampling procedure as the test samples was carried out. This is to establish that no peaks corresponding to the added flavonols appear during the incubation process.

5.2.5 Uptake of flavonol conjugates added into the same incubation media

A separate experiment was performed to establish if any competition existed between the uptake of flavonols across the rat intestinal wall when several were present together in the incubation media. For this purpose, standards of quercetin-3,4′-diglucoside, quercetin-3-glucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside were added into the same incubation media. A 10 μM concentration of each flavonol was used. Incubation of the intestinal segments and sampling procedure is as described in section 5.2.3.

5.2.6 Flavonol extraction from serosal and mucosal samples

Flavonols in the serosal and incubation media (150 μl) were extracted in 50% methanol. Samples were centrifuged for 5 min at 5000 x g and extract aliquots of 75 μl were taken and made up to 250 μl with distilled water containing 0.5% trifluoroacetic acid. Volumes of 200 μl were subsequently analysed by gradient elution reversed phase HPLC as described in section 3.2.1

5.2.7 Statistics

Where necessary, results were compared by Mann Whitney U non-parametric statistical test. The Minitab version 12.21 statistical software for Windows was used for all analyses.

5.3 Results

All the flavonols used in this study could be detected on the serosal side following incubation of the jejunal segments in incubation media containing the appropriate flavonol standards. Control samples run without the presence of flavonols in

the incubation media did not show any contaminating peaks co-eluting with the flavonols tested in this experiment. The profiles for the uptake of the flavonols across the intestinal wall over the 30 min time course are illustrated in Figure 5.2. For the sake of clarity, as well as comparative purposes, the uptake of flavonols was expressed as pmoles of flavonol detected in the serosal samples per 10 cm of jejunal segments. The profiles for the uptake of the different flavonols showed some variation. In most cases, a linear-like profile was observed for uptake of most of the flavonols. In this instance, the amount of flavonols transferred was still increasing and had not reached its maximum values at the 30 min time point. On the other hand, two of the test flavonols, isorhamnetin-4′-glucoside and quercetin aglycone showed a sigmoid profile with slower initial uptake, followed by a rapid increase and subsequently reaching a maximal value where it plateau-ed at this point (Figure 5.2).

In most instances, flavonols could already be detected in the serosal samples at the 5 min time point. The uptake of isorhamnetin-4′-glucoside and quercetin appeared to reach their maximal values in the serosal side after 20 to 25 min incubation. When transport of flavonols was expressed as cumulative uptake over the 30 min incubation period, the uptake of quercetin-3,4′-diglucoside was highest, followed in descending order by rutin, quercetin-3-glucoside, quercitrin, quercetin-4′-glucoside, isorhamnetin-4′-glucoside and lastly, quercetin (Table 5.1). The above observations corresponded with the rate of uptake of the flavonols (Table 5.1). Quercetin-3,4′-diglucoside showed the highest rate of uptake (2.99 ± 0.62 nmoles/10 cm/h) whereas quercetin had the lowest (0.01 ± 0.006 nmoles/10 cm/h). With the exception of rutin and quercetin-3-glucoside, the rate of uptake of quercetin-3,4′-diglucoside was significantly different from the other flavonols tested ($P<0.05$, Table 5.1).

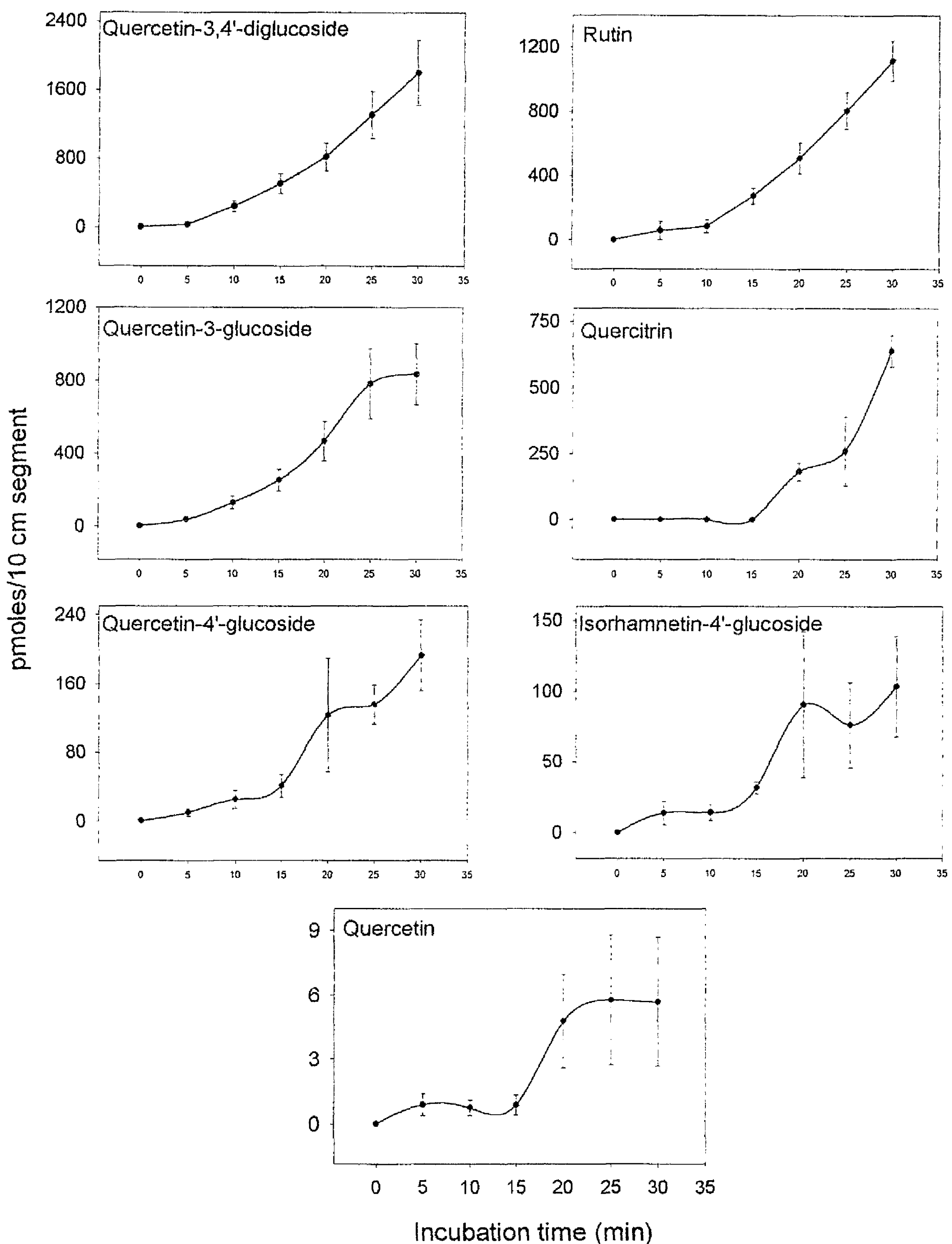

Figure 5.2: The profiles for the uptake of individual flavonol across the epithelial membrane over a 30 min time course.

Results are expressed as pmoles of flavonols detected in the serosal samples per 10 cm of jejunal segments.

Table 5.1: The main features of the uptake of flavonols across rat intestinal segment

Flavonol	Rate of uptake (nmoles/10cm/hr)	Cumulative uptake (nmoles/10 cm)	Recovery in incubation media (%)	95% CI[a]
Quercetin-3,4'-diglucoside	2.99 ± 0.62	1.80 ± 0.38	92 ± 2	
Rutin	1.83 ± 0.26	1.12 ± 0.13	81 ± 3	(-0.87, 3.09)
Quercetin-3-glucoside	1.56 ± 0.34	0.83 ± 0.17	85 ± 2	(-0.12, 3.14)
Quercitrin	0.77 ± 0.07	0.64 ± 0.06	94 ± 2	(0.47, 3.99)
Quercetin-4'-glucoside	0.32 ± 0.08	0.19 ± 0.04	63 ± 8	(1.12, 4.38)
Isorhamnetin-4'-glucoside	0.22 ± 0.04	0.10 ± 0.04	48 ± 16	(1.15, 4.41)
Quercetin	0.01 ± 0.006	0.01 ± 0.003	34 ± 8	(1.35, 4.63)
Flavonol conjugates mix				**95% CI[b]**
Quercetin-3,4'-diglucoside	2.63 ± 0.12	1.56 ± 0.17	97 ± 0.1	(-1.24, 2.06)
Quercetin-3-glucoside	3.03 ± 0.47	1.84 ± 0.41	89 ± 2	(-3.24, 0.27)
Quercetin-4'-glucoside	0.49 ± 0.13	0.29 ± 0.09	44 ± 21	(-0.60, 0.21)
Isorhamnetin-4'-glucoside	0.34 ± 0.04	0.23 ± 0.03	47 ± 7	(-0.30, 0.04)

[a] = 95% CI for significant difference between the rate of uptake between quercetin-3,4'-diglucoside and the other flavonols tested.

[b] = 95% CI for significant difference between the rate of uptake between individual flavonol and flavonol conjugates in the same incubation media.

n = 5-8 segments ± SEM

Figure 5.3 (C and D) shows an example of a typical HPLC trace of a serosal sample collected 30 min following incubation of an everted segment with quercetin-3-glucoside. Peak 2, detected by the UV monitor (Figure 5.3 C) co-chromatographed with quercetin-3-glucoside standard (Figure 5.3 A). Except for possibly some trace impurities, no other putative metabolite peaks were present on the UV trace (Figure 5.3 C). When the same sample was analysed by the fluorimeter following post-column derivatization, trace levels of non-polar peaks were detected (Figure 5.3 D), none of which correspond to reference compounds (Figure 5 A and B). Free quercetin was not present, suggesting that deglycosylation of quercetin-3-glucoside was at best a peripheral event. No early eluting peaks were present in Figures 5.3 C and D indicating that quercetin-3-glucoside was not converted to glucuronide conjugates.

5.3.1 Uptake of flavonol conjugates from the same incubation media

Figure 5.4 illustrates the profiles for the uptake of the four flavonol conjugates following incubation of jejunal segments in incubation media containing all four flavonols. Quercetin-3,4′-diglucoside and quercetin-3-glucoside presented linear profiles for their uptake whereas isorhamnetin-4′-glucoside and quercetin-4′-glucoside showed an initial slow uptake. Uptake of flavonol conjugates in a mixture in the incubation media was compared to uptake of individual compounds. When incubation was performed with all four flavonol conjugates, the uptake of quercetin-3-glucoside was slightly higher compared to its estimated uptake as an individual flavonol. However, this difference was not statistically significant (Table 5.1). The relative rate of uptake of quercetin-3,4′-diglucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside remained similar between the two experiments ($P>0.05$). Quercetin-3-glucoside showed the highest rate (3.03 ± 0.47 nmoles/10 cm/h) followed by quercetin-3,4′-diglucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside with values of 2.63 ± 0.12, 0.49 ± 0.13 and 0.34 ± 0.04 nmoles/10 cm/h respectively.

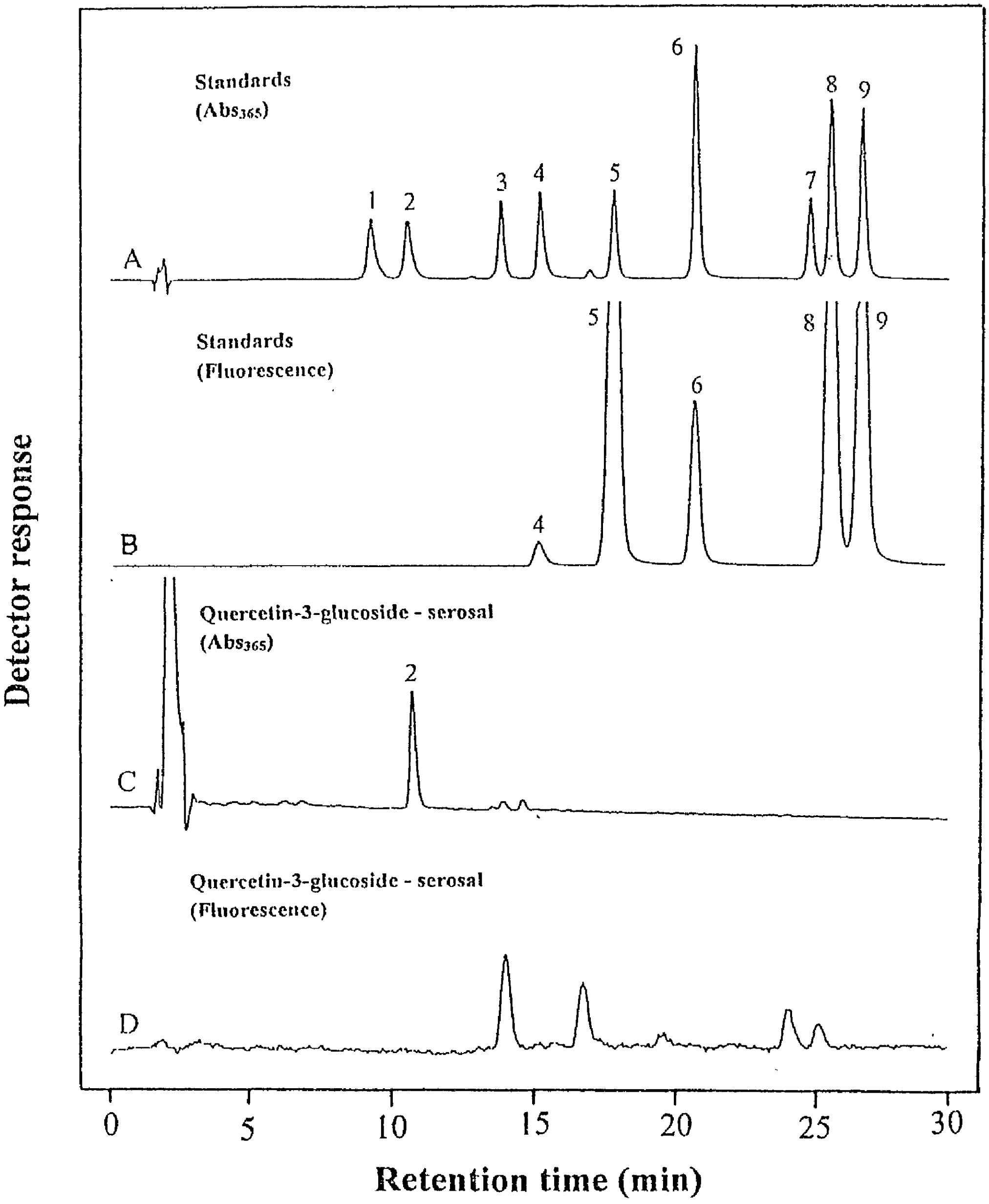

Figure 5.3: Gradient reverse phase HPLC analysis of flavonols in serosal samples

Column: 150 x 3.0 mm i.d. 4-µm Genesis C_{18} cartridge column with a 10 x 4.0 mm 4-µm Genesis C_{18} guard cartridge. Mobile phase: 25 min gradient of 15-40% acetonitrile in water containing 0.5% trifluoroacetic acid. Flow rate: 0.5 ml/min. Detector: absorbance monitor operating at 365 nm and, after on-line post-column derivatization with methanolic aluminium nitrate, a fluorimeter operating at excitation 420 nm and emission 485 nm. Samples: (**A**) 100 ng flavonol standards: (1) rutin, (2) quercetin-3-glucoside, (3) quercitrin, (4) myricetin, (5) morin, (6) quercetin, (7) apigenin, (8) kaempferol and (9) isorhamnetin detected at $A_{365\ nm}$; (**B**) as (A) but with fluorescence detection after post-column derivatization; (**C**) aliquot of a non-hydrolysed serosal samples collected 30 min after incubation with quercetin-3-gluoside, detected at $A_{365\ nm}$; (**D**) as C but with post-column derivatization and fluorescence detection. The numbers corresponded with peaks for standards listed in (A).

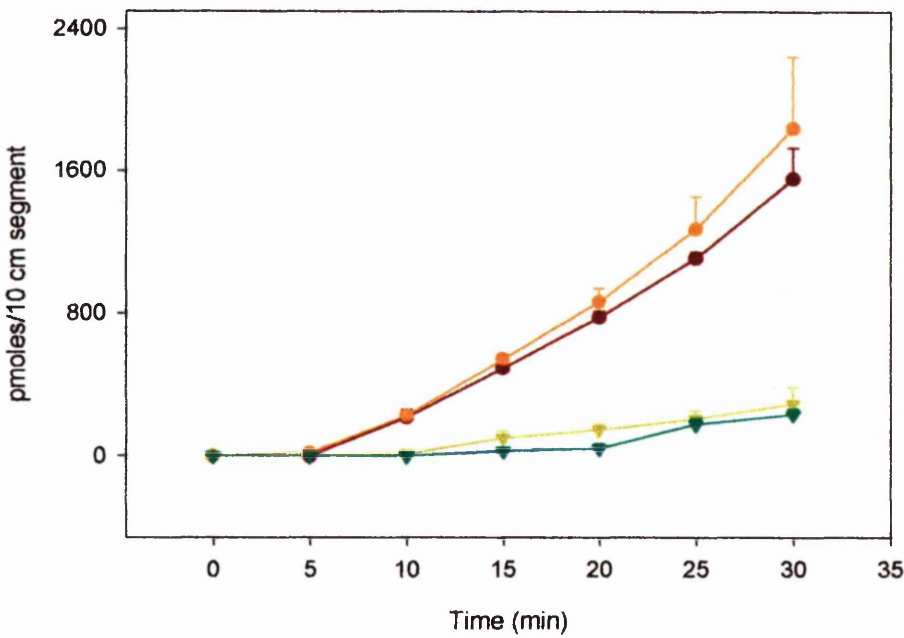

Figure 5.4: The profiles for the uptake of flavonol conjugates, quercetin-3,4′-diglucoside, quercetin-3-glucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside following incubation of the four flavonols in the same incubation media. Results are expressed as pmoles of flavonols detected in the serosal samples per 10 cm of jejunal segments.

- Quercetin-3,4'-diglucoside
- Quercetin-3-glucoside
- Quercetin-4'-glucoside
- Isorhamnetin-4'-glucoside

5.3.2 Percentage absorption and residuals of flavonols

The amount of flavonols remaining in the incubation media at the end of the 30 min incubation period was determined. The residual flavonols remaining after 30 min was high in most instances with more than 80% detected in the incubation media (Table 5.1). However, compared to the low levels detected in the serosal samples, recoveries of quercetin-4′-glucoside, isorhamnetin-4′-glucoside and quercetin in the incubation media were low (<60%). This was observed in experiments with the individual flavonols as well as when all the flavonol conjugates were present in the incubation buffer.

Overall, compared to the amount of flavonol present in the incubation media, the total amount transferred to the serosal side at the end of the incubation period was low, with less than 0.5% being transported across the gut wall.

5.4 Discussion

Previous *in vivo* studies with humans and animals reported variation in the absorption of different flavonoids. Hollman *et al* (1995) reported higher absorption of quercetin glucosides (52%) compared to quercetin aglycone (24%). Rutin, a quercetin conjugate with glucose-rhamnose sugar moiety had an even lower absorption at 17% (Hollman *et al.*, 1995). In a recent report, it has been shown that quercetin-3-glucoside accumulates in plasma much more rapidly than rutin (Hollman *et al.*, 1999). In our study, we detected higher levels of isorhamnetin-4′-glucoside than quercetin-4′-glucoside in plasma after a fried onion meal to human volunteers (Chapter 4). Isorhamnetin-4′-glucoside was only a minor flavonol in onion whereas quercetin-4′-glucoside was present in much higher concentrations. In addition to flavonols, flavonoids from the sub-class catechins were also absorbed at different rates with 3-*O*-methyl-(+)-catechin being absorbed (Hackett *et al.*, 1985) more effectively than (+)-catechin (Hackett *et al.*, 1983). This observation implies the possible influence of the structural modification of flavonol on their intestinal absorption. At the same time, the nature/position of sugar moiety on flavonols may also affect the extent of their absorption.

However, it is not always straightforward to determine the extent of intestinal absorption of flavonols based on *in vivo* feeding experiments. Various physiological factors can play a role in flavonoid absorption. In *in vivo* experiments, absorbed flavonoids are subject to modification and metabolism reactions, producing metabolites different from the original compound. The rate and extent of metabolism of the absorbed flavonoids may vary from one flavonoid to the other. Thus, the concentration of flavonoids in plasma may not be directly related to their rate of absorption due to the influence of metabolic processes. A high plasma flavonoid concentration could mean two things; either it is a consequence of their effective uptake from the intestine or their less effective metabolism or removal from the bloodstream. Clearly, *in vivo* methods using plasma measurements are not ideal in evaluating flavonoid absorption from the small intestine. Rather, they give a measure of their relative absorption compared to other flavonoids.

With this in mind, researchers have developed various *in vitro* methods to study the uptake of compounds across the intestinal wall. Although the advantages far outweigh the disadvantages, limitations may still arise while using *in vitro* intestinal preparations. Removal of intestine from the animal will result in loss of blood supply, subsequently causing loss of nucleotides from the mucosal cells and a decrease in the rate of oxygen consumption (Bronk and Leese, 1973). To overcome this problem, efforts were made to ensure minimal loss of intestinal function. This included placing the extracted intestinal tissue in well-oxygenated medium immediately after removal from the animal to ensure an uninterrupted oxygen supply. In addition, all our *in vitro* experiments were completed in the shortest time possible to minimise loss of tissue viability. With this method, the extent of absorption of various flavonols can be determined and their possible biological response investigated. Also the structure-absorption relationship of flavonols can be evaluated.

5.4.1 Structural modification and flavonol transport

All flavonols employed in this study could be detected in the serosal side indicating they were able to undergo transepithelial transport, i.e. crossing the luminal as well as the basolateral membrane. We found clear a distinction in the uptake of different

flavonols. The glycosides of quercetin were more highly absorbable than isorhamnetin-4′-glucoside. However, variation in the amount transported was also observed among the different glycosides of quercetin. Quercetin-3,4′-diglucoside and quercetin-3-glucoside were both absorbed rapidly in contrast to quercetin-4′-glucoside and isorhamnetin-4′-glucoside which in turn are both absorbed more effectively than quercetin (Figures 5.2 and 5.4). It would therefore appear that a C-4′ glucose moiety attached to quercetin results in an improved rate of absorption compared to the aglycone. However, the presence of a C-3 glucose markedly enhances the transport of quercetin conjugates. 3′-*O*-methylation, as observed in isorhamnetin-4′-glucoside did not seem to greatly affect its transport when compared to quercetin-4′-glucoside ($P<0.05$). Two published studies, one using perfusion of the rat intestine and CaCo-2 cells (Noteborn *et al.*, 1997) and the other using CaCo-2 cells (Walgren *et al.*, 1998) reported no absorption or only trace absorption of querccetin-4′-glucoside.

When rhamnose was present instead of glucose at C-3, as in quercitrin, the rate of uptake appeared lower than that of quercetin-3-glucoside, although this difference was not significant ($P>0.05$). Nevertheless, when both rhamnose and glucose were present at C-3, as in rutin, uptake was higher than quercitrin ($P<0.05$). This suggests that in addition to their position on the flavonol molecule, type of sugar moiety also plays a role in influencing the rate of uptake of flavonol glycosides. These observations would imply a structural specificity of the carrier responsible for flavonol uptake across the intestinal wall, assuming flavonol transport is carrier-mediated. Possibly, a glucose moiety at C3 of flavonol glucosides increased their affinity for interaction with the carrier protein for their uptake.

Results obtained in this study were in agreement with a previous study by Gee *et al.* (1998). These authors used a slightly different method to study intestinal absorption whereby transport of several flavonol glucosides were determined based on their ability to displace preloaded [^{14}C]galactose in everted segments of rat jejunum. Thus, they did not directly measure concentrations of flavonols transported, but the amount of [^{14}C]galactose displaced by the flavonols. They found that cumulative efflux of [^{14}C]galactose was highest when everted sacs were incubated with quercetin-3,4′-diglucoside, and this was followed by quercetin-3-glucoside and rutin. Efflux of galactose by quercetin-4′-glucoside was lower than the above flavonol glucosides. The

same was also observed for quercitrin which had a lower cumulative efflux than quercetin-4′-glucoside. This further supports the proposed importance of glucose in enhancing intestinal transport of flavonol glucosides and that glucose substitution at C-4′ of flavonol conjugates can substantially reduce intestinal transport of these flavonols.

In contrast to this, a study on intestinal absorption of flavonoids reported glucuronidation of several flavonoids, in particular quercetin-3-glucoside and quercetin following perfusion of the rat gut with these compounds (Spencer *et al.*, 1999). However, at the 10 μM concentration used in our study, we did not detect the presence of glucuronide peaks. This was evident from the HPLC traces of serosal samples following incubation with quercetin-3-glucoside (Figure 5.2 C). The HPLC trace detected by the UV monitor at $Abs_{365\ nm}$ only showed a peak corresponding to quercetin-3-glucoside (peak 2). If glucuronides of quercetin-3-glucoside were formed with more polar characteristics than the parent compound, these metabolites would elute from the reversed-phase HPLC column before quercetin-3-glucoside, but there was no evidence of this on the UV trace (Figure 5.3C). Spencer *et al* (1999) detected the presence of glucuronides after 60 min incubation whereas our incubation was performed only up to 30 min. The incubation time used in this study may be insufficient for the formation of glucuronide conjugates and may explain the lack of glucuronides in our study. In addition, they also used a higher quercetin-3-glucoside concentration of 47.6 μM compared to 10 μM used in our study. Even at this high concentration, glucuronidation of quercetin-3-glucoside was low with less than 1.5 nmol detected per 20 cm jejunum per 5 μmol of the perfused compound. These factors may explain why with the more physiologically relevant flavonol concentrations used in our study, there was no evidence of the formation of glucuronide metabolites.

With the aglycone quercetin, total uptake was only 0.01 nmoles/10 cm over the 30 min incubation period. These observations again indicate structural-dependence of the flavonol molecule on their uptake across the rat intestinal wall. More importantly, the presence of sugar moiety as well as their position greatly determines the extent of uptake of the flavonols. Existing literature on the uptake of quercetin have shown varying results. Two previous studies reported *in vitro* transport of quercetin which contrasted with our findings (Noteborn *et al.*, 1997; Walgren *et al.*, 1998). Noteborn *et al* (1997), after a 2 h perfusion of rat jejunal segments with quercetin, detected 0.3-0.4%

absorption of the parent compound, expressed as percentage of the initial dose. Walgren and colleagues studied intestinal transport of flavonols using human intestinal epithelial Caco-2 cells and reported very high flux of quercetin from the apical to basolateral membrane. However, the validity of using a cell-culture method is open to question and several factors have to be considered such as reliability and the extent to which the results are indicative of *in vivo* transport. Furthermore, cancerous cells may have several structural and physiological changes which can affect the normal absorption process of the small intestine. The ability of this cell culture model to successfully mimic a biological barrier like the intestinal mucosa has to be carefully assessed. Several factors can influence the transport and metabolic properties of cultured cells such as the stage of cellular differentiation and the availability of essential nutrients (Meunier *et al.*, 1995). Furthermore, composition of the incubation media e.g. concentration of substrates, temperature and pH may influence transport properties of the cells.

In contrast to this, a rat gut perfusion experiment detected uptake of small amounts of quercetin, but only 60 min after the start of perfusion (Spencer *et al.*, 1999). However, these authors detected the presence of high levels of quercetin glucuronides following perfusion with quercetin. We did not detect HPLC peaks likely to correspond to glucuronides of quercetin in our study. Furthermore the study described above which investigated intestinal transport of quercetin using CaCo-2 cells did not detect peaks which may have corresponded to glucuronides of quercetin in the basolateral samples 1 h after incubation of quercetin in the apical side (Walgren *et al.*, 1998). But these results should be interpreted with caution taking into account the limitations in using cell culture. Again, Spencer *et al* (1999) detected the glucuronides of quercetin only after 60 min of incubation. Thus, the 30 min incubation period employed in our study may exclude any glucuronide formation. We detected low recovery of quercetin in the incubation media at the end of the experiment which could be a result of their instability during the incubation process. This has been reported in another study whereby low recovery of quercetin was detected at the end of the incubation period without evidence of the presence of its metabolites (Walgren *et al.*, 1998).

Two different studies also reported intestinal glucuronidation of flavonoids, this time following incubation of luteolin-7-glucoside with everted rat intestinal segments (Shimoi *et al.*, 1998) and the other following incubations of chrysin (5,7-

dihydroxyflavone) with CaCo-2 cells (Walle *et al.,* 1999). However, the structural difference, coupled with usage of a higher substrate concentration (1 mM; Shimoi *et al.*, 1998) or difference in method for studying intestinal transport (Walle *et al.*, 1999) may explain the differences in the results obtained.

Results obtained from this present study demonstrated the ability of several flavonols to undergo intestinal transport without being metabolised by enzymes. At the 10 μM concentration of test flavonols used, no evidence of glucuronidation and/or other metabolic reactions could be seen. In most instances, the published articles which used non-physiological concentration of flavonols (0.05 – 1 mM), reported lower extent of glucuronidation of the flavonols (Shimoi *et al.*, 1998, Spencer *et al.*, 1999). If this was the case, it would be highly unlikely that glucuronidation could be detected at the concentration we used (10 μM) which was reflective of the normal dietary intake of flavonoids. Furthermore, the relevance of studies using such high concentrations to normal *in vivo* transport is questionable as such a high concentration is not physiological and may affect their uptake. Compounds/metabolites detected could be artefacts which under normal physiological conditions may not be present. High concentrations may also have overloaded the intestinal segments, thus giving a false value of the extent of absorption of the flavonols. In some instances, longer incubation time was used (> 1h; Noteborn *et al.*, 1997, Walle *et al.*, 1999, Spencer *et al.*, 1999). When incubation was performed up to 1 h, reliability of the method used may be affected due to cell death, loss of tissue viability due to prolonged loss of blood supply and reduced oxygen consumption.

When the four flavonols, quercetin-3,4′-diglucoside, quercetin-3-glucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside were added into the same incubation media, their rate of uptake across the intestinal wall remained roughly the same as when transport of the individual flavonols were investigated (Table 5.1). This suggests that transport of these particular flavonols were not dependent on each other and interaction or competition for uptake of these flavonols across the intestinal wall did not occur. This may also imply that a different mechanism of uptake for these flavonols exists. Alternatively, if they were absorbed by the same mechanism, no competition in their transport was observed at the 10 μM concentration used in this study.

Structural modification of flavonoids may alter their stereospecificity, thereby possibly influencing their transfer across the intestinal wall. If transport occurs via a carrier protein, alteration in flavonol structure may change the binding site for the carrier protein, ultimately reducing or inhibiting their transport.

5.5 Conclusion

Results from this study indicated intestinal transport of the flavonol glucosides, albeit at different rates. Flavonols, when present as glycosides were more highly transported compared to the aglycone. It is likely that the nature/position of the sugar moiety, to a certain extent, determine the rate of uptake of the flavonol conjugates. Evidence of glucuronidation or sulphation/methylation of quercetin-3-glucoside and the other flavonols tested was not seen in our study at the concentration used. Further studies are required to determine the mechanism of flavonol transport across the epithelial membrane.

Investigation of the Mechanism of flavonol uptake across the Small Intestine in an Everted Small Intestine Rat Gut Model

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CHAPTER 6: INVESTIGATION OF THE MECHANISM OF FLAVONOL UPTAKE ACROSS THE SMALL INTESTINE IN AN EVERTED SMALL INTESTINE RAT GUT MODEL.

6.1 Introduction

In view of the overwhelming evidence of the biological and antioxidant potential of flavonoids (Chapter 1), research has focused on their absorption and metabolism. Ingested flavonoids need to maintain their bioactive form following absorption to provide beneficial health properties. The first important stage of flavonol absorption is their passage across the wall of the small intestine. As mentioned before in Section 1.13.2, substances can be absorbed from the small intestine via active or passive transport. Hydrophilic compounds are more likely to be transported via carrier proteins as they are not able to diffuse across the lipid phase of the membrane. In contrast, lipophilic compounds do not require carrier proteins as they can easily diffuse across the membrane.

Flavonol glycosides are more polar than their aglycones and are thus thought to be transported by carrier proteins. It has been proposed that the sodium-glucose co transporter (SGLT1) might play a role in the transport of flavonols particularly flavonol glucosides (Hollman *et al.*, 1995). This prompted research to investigate this hypothesis and to date, two papers have presented contrasting results. One study showed interaction of several flavonol glucosides with SGLT1 (Gee *et al.*, 1998) whereas another demonstrated that certain flavonol glucosides behaved as glucose carrier inhibitors (Noteborn *et al.*, 1997). Clearly more work is needed to further establish the role of SGLT1 in flavonol transport across the small intestine. The aim of this study was to investigate the mechanism of the uptake of flavonols across the small intestine and to further elucidate the involvement of glucose transporters in the transport of flavonols.

Several experiments were performed to establish the mode of transport of flavonol glucosides and whether SGLT1 played a role in mediating their uptake. In order to identify if uptake of flavonol glucosides was carrier or non-carrier mediated, the transport of increasing concentrations of flavonoids was investigated. Glucose, together with flavonols was used to investigate the possible involvement of SGLT1 by measuring the possible competition or inhibition for SGLT1. In addition, phloridzin, a known inhibitor of SGLT1 was used to block this carrier and the effect on uptake of flavonols was measured.

6.2 Materials and Methods

6.2.1 Materials

Standards of quercetin-3-glucoside and quercetin-3,4′-diglucoside were dissolved in 100% methanol. Phloridzin and glucose were made up in Krebs buffer. Preparation of Krebs incubation media was described in section 5.2.1. D[$U^{14}C$] glucose was purchased from Amersham (Buckinghamshire, UK) with a specific activity of 310 μCi/mmol.

6.2.2 Animal study

The everted rat gut model previously described was used to investigate the mechanism of flavonol transport across the small intestine. Preparation of the rat intestines and procedure for the eversion of the jejunal segments was as described in Chapter 5, section 5.2.2.

6.2.3 Incubation procedures for the everted segments

The incubation procedure was performed according to the method of Mizuma *et al.* (1994) with slight modifications. Similar procedures as described in section 5.2.3 were

applied. Sampling from the serosal side was performed at 5 min intervals over a 30 minute incubation period.

6.2.4 The mechanism of flavonol uptake by the small intestine

Several experiments were performed to determine the mode of uptake of flavonols from the everted rat jejunal segments. Quercetin-3-glucoside was used in most cases due to its high uptake across the intestinal wall and also the availability of this standard. Figure 6.1 shows a flowchart summarising the various approaches used in determining the mechanism of flavonol absorption from the small intestine.

6.2.4.1 Effect of substrate concentration on the uptake of quercetin-3-glucoside

In the first experiment, the rate of uptake of quercetin-3-glucoside from the mucosal to serosal side was determined. Everted segments of rat jejunum were incubated in 20 ml of Krebs buffer containing quercetin-3-glucoside at concentrations of 1, 5, 10, 50, 100 and 500 μM. Sampling from the serosal side was performed every 5 min for 30 min according to the procedure described in section 5.2.3.

6.2.4.2 The effect of glucose on the uptake of quercetin-3-glucoside across the small intestinal wall

Competition for the glucose carrier was studied by measuring the uptake of quercetin-3-glucoside in the presence of increasing concentrations of glucose. For this purpose, everted segments were incubated in incubation media containing 10 μM quercetin-3-glucoside and glucose at concentrations of 0, 1, 10, 50 and 100 mM. Sampling from the serosal side was performed every 5 min for 30 min as described in section 5.2.3. Eight jejunal segments were used for each concentration.

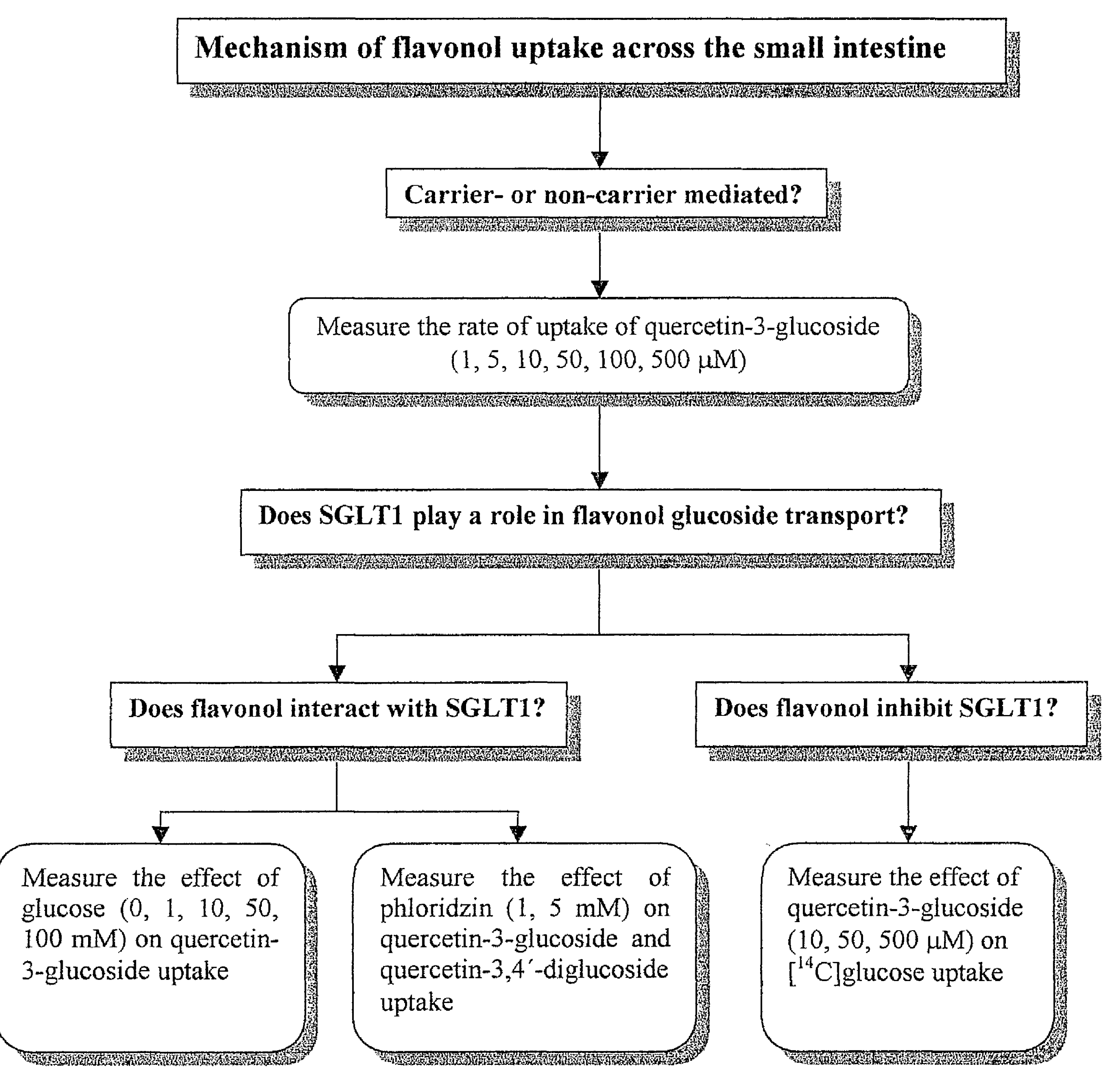


Figure 6.1: A flow chart summarizing the various approaches used to investigate the mode of flavonol glycoside absorption from the small intestine

6.2.4.3 The effect of phloridzin on the uptake of quercetin-3-glucoside and quercetin-3,4′-diglucoside

The involvement of SGLT1 in flavonol uptake was examined by measuring the uptake of quercetin-3-glucoside and quercetin-3,4′-diglucoside in the presence of phloridzin. Phloridzin is a known inhibitor of SGLT1 (Dorando and Crane, 1984). It has the ability to bind to the glucose co-transport carrier and inhibits glucose transport. Everted rat jejunal segments were incubated in Krebs buffer containing 10 μM quercetin-3-glucoside, in the presence or absence of 1 mM and 5 mM phloridzin. In the case of quercetin-3,4′-diglucoside, due to limited availability of the flavonol standard, only 1 mM phloridzin was used. Everted jejunal segments were suspended in incubation media containing 10 μM quercetin-3,4′-diglucoside, with or without 1mM phloridzin. The procedure for sampling from the serosal side was as described in section 5.2.3.

6.2.4.4 The effect of phloridzin on the uptake of glucose

The reliability of the model was established by measuring the uptake of glucose in the presence of 1 mM phloridzin. Everted jejunal segments were placed in the incubation buffer containing 1 mM glucose, 3 μCi D[$U^{14}C$] glucose and with or without 1 mM phloridzin. Sampling from the serosal side was performed every 5 min for 30 min as described in section 5.2.3.

One hundred μl of serosal samples and 20 μl of the incubation media were transferred to scintillation vials, 5 ml of scintillation cocktail (Optisafe 1, Leicestershire, UK) was added to each vial and the amount of radioactivity measured in a liquid scintillation counter.

6.2.4.5 The effect of quercetin-3-glucoside on the uptake of [^{14}C]glucose

The possibility that flavonols may act as inhibitors of SGLT1 was investigated by measuring the uptake of glucose in the presence of increasing concentrations of quercetin-3-

glucoside. In this experiment, the incubation media contained 20 ml of Krebs buffer, 10 mM glucose, 3 μCi D[$U^{14}C$] glucose and quercetin-3-glucoside at concentrations of 10, 50 and 500 μM. The concentration of flavonols used in this experiment was in keeping with the estimated daily intake of dietary quercetin of 16.3 mg/d (Hertog *et al.*, 1993). The highest concentration of flavonol used at 500 μM was to ensure that enough flavonols were available to inhibit SGLT1, if indeed they act as inhibitors of this carrier protein. Incubation was performed by placing the everted jejunal segments in incubation media containing the compounds understudy. A control experiment was carried out by measuring the uptake of 10 mM glucose without the presence of quercetin-3-glucoside. Sampling was performed according to the method described in section 5.2.3. One hunderd μl of the serosal samples and 20 μl of the incubation media were transferred into scintillation vials. 5 ml of Optisafe 1 scintillation cocktail was added into each vial and the amount of radioactivity measured in a liquid scintillation counter.

A preliminary experiment was performed to establish the minimal concentration of glucose required for the incubation experiments. For this purpose, everted segments were incubated in Krebs buffer containing varying concentrations of glucose. 10 mM glucose in the incubation medium produced glucose that could be detected in the serosal samples.

6.2.5 Flavonol extraction from the mucosal and serosal samples and HPLC analysis

Extraction of flavonols from the samples was as described in section 5.2.6. A 200 μl volume of the supernatant was subsequently analysed by gradient elution reversed phase HPLC (see section 3.2.1).

6.2.6 Statistical analysis

Where necessary, results were compared by Mann Whitney U non-parametric statistical test. Minitab statistical software release 12.21 for Windows, was used for all analyses.

6.3 Results

6.3.1 Uptake of varying concentrations of quercetin-3-glucoside

When quercetin-3-glucoside was used to establish the mechanism of flavonol transport across the intestinal wall, the uptake of this flavonol appeared to be concentration dependent. Evidence of saturation of the transport of quercetin-3-glucoside was observed in the concentration range of 10 μM to 500 μM (Figure 6.2). The rate of uptake of this flavonol was linear up to a concentration of 100 μM, after which a hyperbolic curve was seen up to 500 μM. When the results were expressed as cumulative uptake over the 30 min incubation period, the same trend was observed as that seen in the rate of uptake (Table 6.1). The rate of uptake and cumulative uptake of quercetin-3-glucoside increased with concentration.

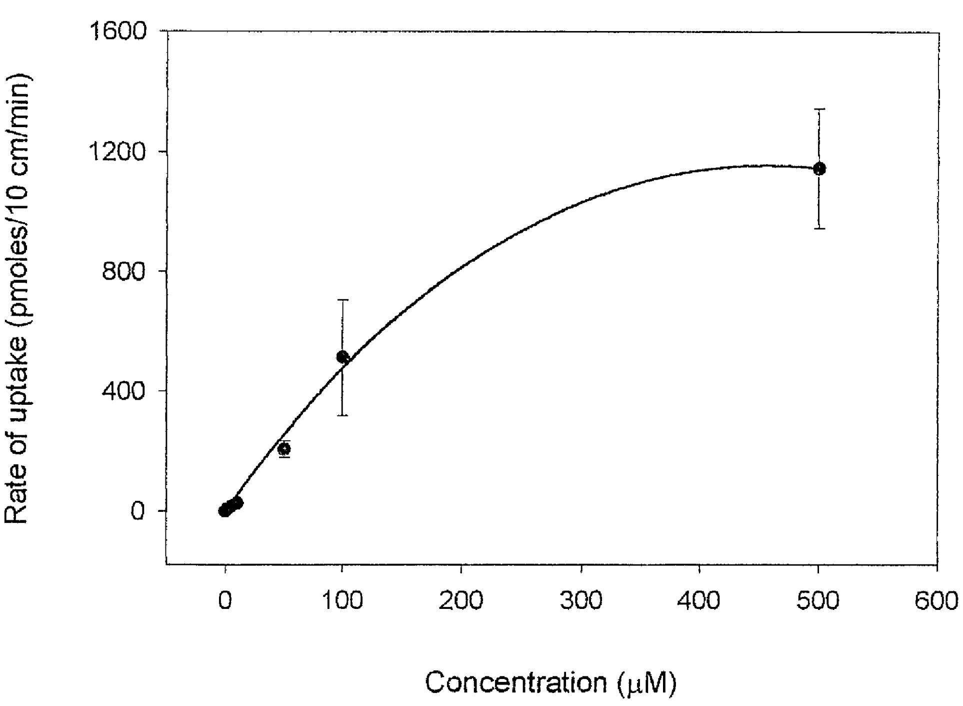


Figure 6.2: The rate of uptake of quercetin-3-glucoside across everted rat jejunal segments.
The rate of quercetin-3-glucoside transport was measured by incubating everted segments in incubation media containing 0, 1, 5, 10, 50, 100 and 500 μM quercetin-3-glucoside over a period of 30 min. The rate of uptake is expressed as pmoles/10 cm/min ± SEM.

Table 6.1: Mean values of the key features of quercetin-3-glucoside uptake across rat jejunal segments

Quercetin-3-glucoside (μM)	Rate of uptake (nmoles/10cm/hr)	Cumulative uptake (nmoles/10cm)	Recovery[a] (%)
1	0.31 ± 0.08	0.12 ± 0.02	75.86 ± 3.81
5	0.95 ± 0.49	1.10 ± 0.48	86.82 ± 0.78
10	1.56 ± 0.34	0.83 ± 0.17	85.00 ± 2.00
50	12.38 ± 1.68	8.31 ± 0.87	85.40 ± 3.80
100	30.72 ± 11.63	21.69 ± 2.88	90.00 ± 1.76
500	68.70 ± 11.88	41.31 ± 6.13	89.89 ± 4.8

n = 6-8 segments ± SEM

[a] = residuals of quercetin-3-glucoside remaining in the incubation media at the end of the 30 min incubation.

6.3.2 The effect of glucose on the uptake of quercetin-3-glucoside across the small intestine

Figure 6.3 shows the uptake of quercetin-3-glucoside over 30 min in the presence of glucose at the concentrations indicated. There was no significant difference in the uptake of quercetin-3-glucoside with and without the presence of 1 mM, 10 mM, 50 mM and 100 mM glucose. Although the rate of uptake of this flavonol glucoside decreased slightly in the presence of 100 mM glucose (1.21 ± 0.32 nmoles/10 cm/h) compared to without glucose (1.56 ± 0.34 nmoles/10 cm/h), these figures were not significantly different (Table 6.2). Essentially the same pattern was seen when results were expressed as cumulative uptake over the 30 min incubation period.

6.3.3 The effect of phloridzin on the uptake of [^{14}C]glucose, quercetin-3-glucoside and quercetin-3,4′-diglucoside

[^{14}C]Glucose

Phloridzin, an inhibitor of SGLT1 was employed to investigate the possible involvement of this carrier on the uptake of flavonol glucosides across the intestinal wall. Figure 6.4 shows the profile for the uptake of glucose with and without the presence of phloridzin. At the 30 min time point, 2.3% and 3.4% of the radioactivity was transported into the serosal side with and without the presence of 1 mM phloridzin, respectively. A slight inhibition (33%) in the uptake of glucose was observed when 1 mM phloridzin was present in the incubation media ($P>0.05$). Nevertheless, phloridzin did not fully inhibit the uptake of glucose as radioactivity could still be detected in the serosal side at the 30 min time point.

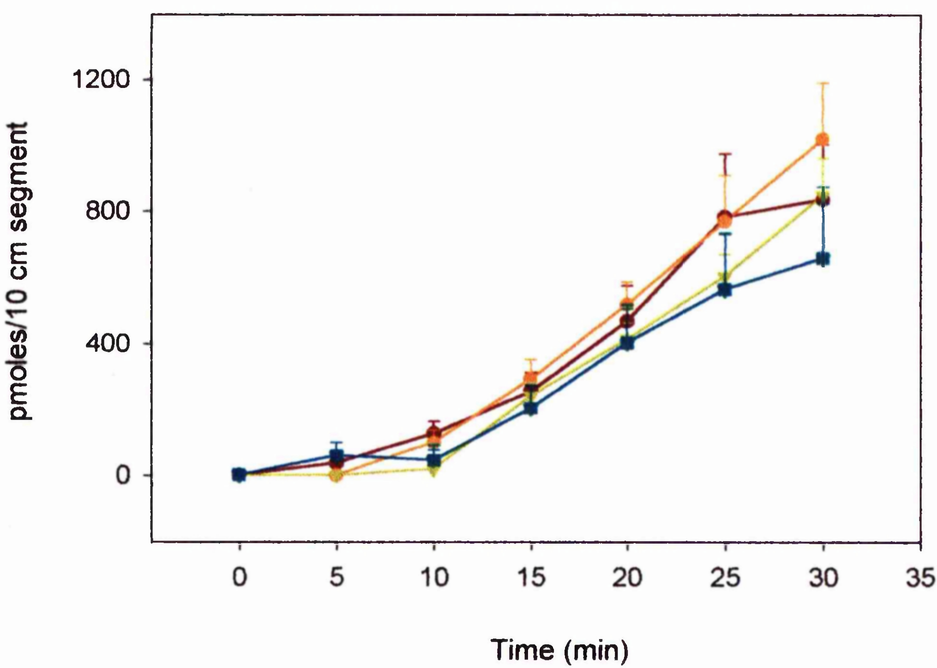


Figure 6.3: The effect of glucose on intestinal absorption of quercetin-3-glucoside

Uptake of 10 μM quercetin-3-glucoside was investigated in the presence of 1 mM, 10 mM, 50 mM and 100 mM glucose in the incubation media. Results are expressed as pmoles quercetin-3-glucoside detected in the serosal samples per 10 cm segment. (n=6-8 segments ± SEM).

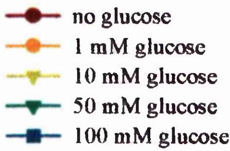


Table 6.2: The effect of glucose on the uptake of quercetin-3-glucoside.

Results represent mean values of the key features of quercetin-3-glucoside uptake across rat jejunal segments.

Glucose (mM)	Rate of uptake (nmoles/10cm/hr)	Cumulative uptake (nmoles/10cm)	Recovery[a] (%)	95% CI[b]
No glucose	1.56 ± 0.34	0.83 ± 0.17	85 ± 2.0	-
1	1.73 ± 0.29	1.02 ± 0.17	86.18 ± 2.31	(-1.23, 1.01)
10	1.37 ± 0.17	0.85 ± 0.11	86.11 ± 3.55	(-0.78, 1.19)
50	1.30 ± 0.37	0.75 ± 0.21	85.27 ± 1.30	(-0.67, 1.61)
100	1.21 ± 0.32	0.66 ± 0.16	85.45 ± 2.71	(-0.69, 1.54)

[a] = residuals of quercetin-3-glucoside remaining in the incubation media at the end of the 30 min incubation.

[b] = 95% CI for significant inhibition of rate of quercetin-3-glucoside uptake by glucose.

n = 6-8 segments ± SEM

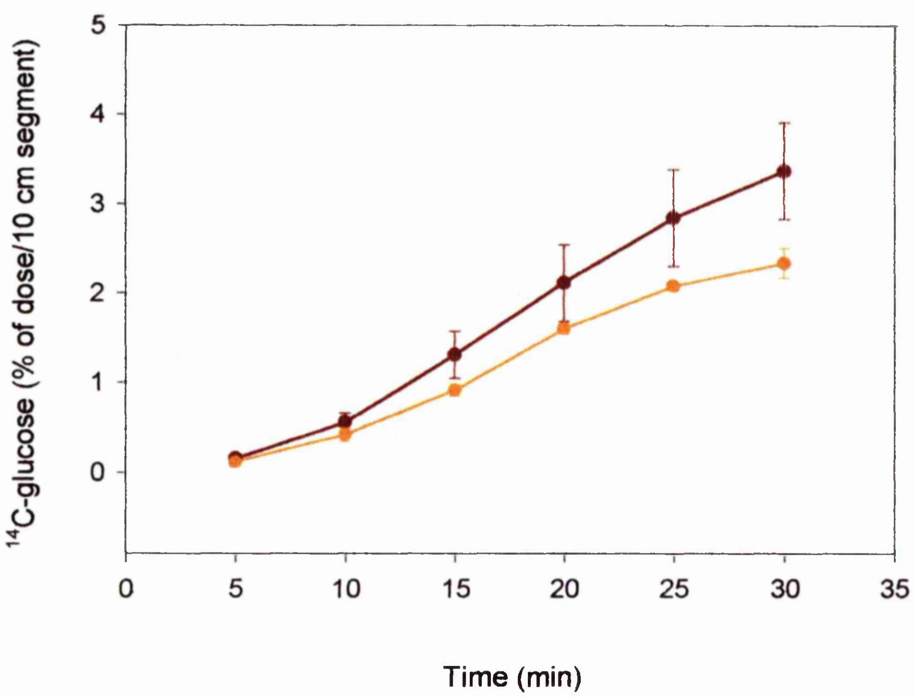
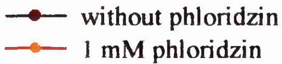


Figure 6.4: The effect of phloridzin on the uptake of ^{14}C-glucose

Uptake of 1 mM ^{14}C-glucose was investigated in the presence of 1 mM phloridzin in the incubation media. Results are expressed as % of radioactivity absorbed from the incubation media. (n=6-8 segments ± SEM).



without phloridzin

1 mM phloridzin

Quercetin-3-glucoside

Figure 6.5 shows the effect of phloridzin (1 mM and 5 mM) on the rate of uptake of quercetin-3-glucoside across the intestinal wall. This was compared with the rate of uptake of quercetin-3-glucoside in the absence of phloridzin. From the graph, compared with the uptake of quercetin-3-glucoside without phloridzin, no inhibition was observed in the transport of quercetin-3-glucoside with the presence of 1 mM phloridzin. A higher phloridzin concentration (5 mM) was also tested. Again, no difference was observed in the rate of uptake of this glucoside. It was evident that phloridzin had no significant effect on the rate of uptake of quercetin-3-glucoside (Table 6.3).

Quercetin-3,4′-diglucoside

Figure 6.6 illustrates the transport of 10 μM quercetin-3,4′-diglucoside with and without the presence of 1 mM phloridzin. Unlike quercetin-3-glucoside, a slight inhibition in the transport of this diglucoside was observed when 1 mM phloridzin was added into the incubation media. Furthermore, the rate of uptake of quercetin-3,4′-diglucoside with phloridzin was approximately half of that of their uptake without phloridzin (Table 6.3). However, this inhibition was not statistically significant.

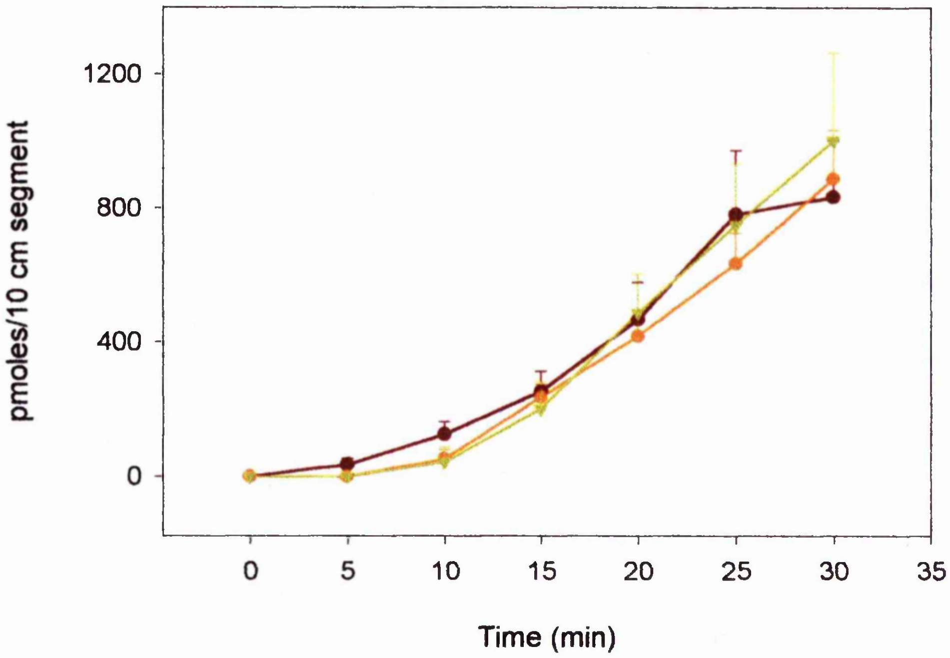


Figure 6.5: The effect of phloridzin on the uptake of quercetin-3-glucoside

Uptake of 10 μM quercetin-3-glucoside was investigated in the presence of 1 mM and 5 mM phloridzin in the incubation media. Results are expressed as pmoles quercetin-3-glucoside detected in the serosal samples per 10 cm segment. (n=6-8 segments ± SEM).

—●— without phloridzin
—●— 1 mM phloridzin
---▼--- 5 mM phloridzin

Table 6.3: The effect of phloridzin on the uptake of quercetin-3-glucoside and quercetin-3,4′-diglucoside.

Results represent mean values of the key features of quercetin-3-glucoside and quercetin-3,4′-diglucoside uptake across rat jejunal segments.

Flavonol	Phloridzin (mM)	Rate of uptake (nmoles/10cm/h)	Cumulative uptake (nmoles/10cm)	Recovery[a] (%)	95% CI
Quercetin-3-glucoside	-	1.56 ± 0.34	0.83 ± 0.17	85 ± 2	-
	1	1.40 ± 0.23	0.89 ± 0.15	86.34 ± 2.24	(-0.67, 1.17)[b]
	5	1.64 ± 0.43	1.00 ± 0.26	89.81 ± 3.02	(-1.91, 1.43)[b]
Quercetin-3,4′-diglucoside	-	2.99 ± 0.62	1.80 ± 0.38	92 ± 2	-
	1	1.76 ± 0.27	0.99 ± 0.16	90.02 ± 3.12	(-0.67, 2.91)[c]

[a] = residuals of quercetin-3-glucoside or quercetin-3,4′-diglucoside remaining in the incubation media at the end of the 30 min incubation.

[b] = 95% CI for significant inhibition of rate of quercetin-3-glucoside uptake by phloridzin.

[c] = 95% CI for significant inhibition of rate of quercetin-3,4′-diglucoside uptake by phloridzin.

n = 6-8 segments ± SEM

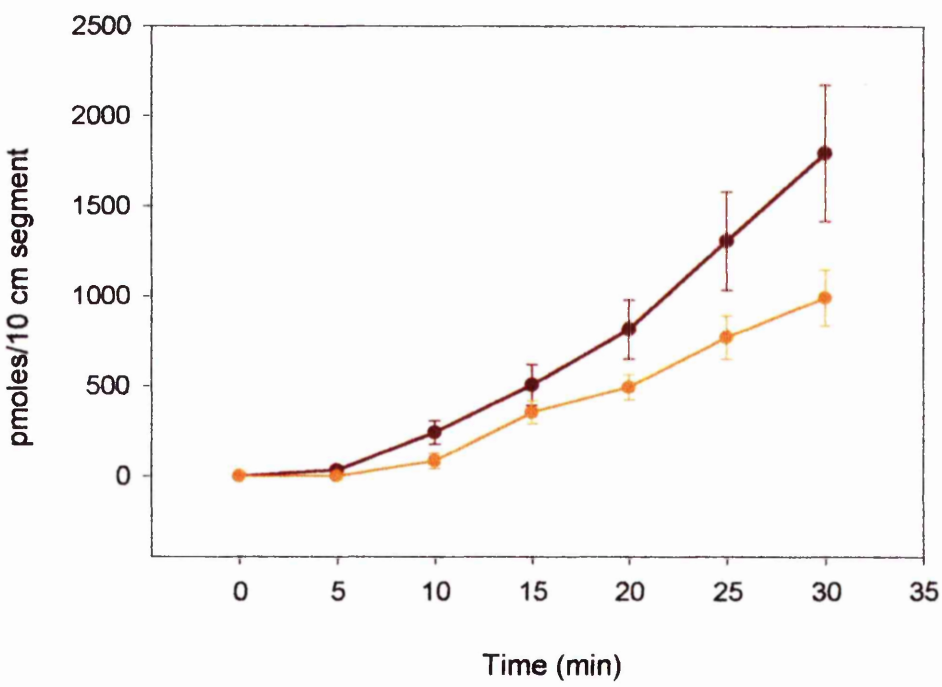
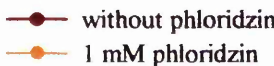


Figure 6.6: The effect of phloridzin on the uptake of quercetin-3,4′-diglucoside

Uptake of 10 μM quercetin-3,4′-diglucoside was investigated in the presence of 1 mM phloridzin in the incubation media. Results are expressed as pmoles quercetin-3-glucoside detected in the serosal samples per 10 cm segment. (n=6-8 segments ± SEM).



6.3.4 The effect of quercetin-3-glucoside on the uptake of [^{14}C]glucose

In this experiment, the possibility of flavonol glucosides acting as inhibitors of SGLT1 was investigated by examining the uptake of glucose in the presence of varying concentrations of quercetin-3-glucoside. The transport of [^{14}C]glucose into the serosal side was expressed as percentage of the initial dose absorbed per 10 cm segment. No difference was observed in the uptake of glucose without the presence of quercetin-3-glucoside and when 10 μM, 50 μM and 500 μM quercetin-3-glucoside were present in the incubation media (Figure 6.7). Statistical comparison of the rate of uptake of glucose with quercetin-3-glucoside did not show any difference from with their uptake without quercetin-3-glucoside (Table 6.4). Furthermore, similar values for the cumulative uptake were obtained for the four conditions used (Table 6.4) suggesting no difference in their uptake.

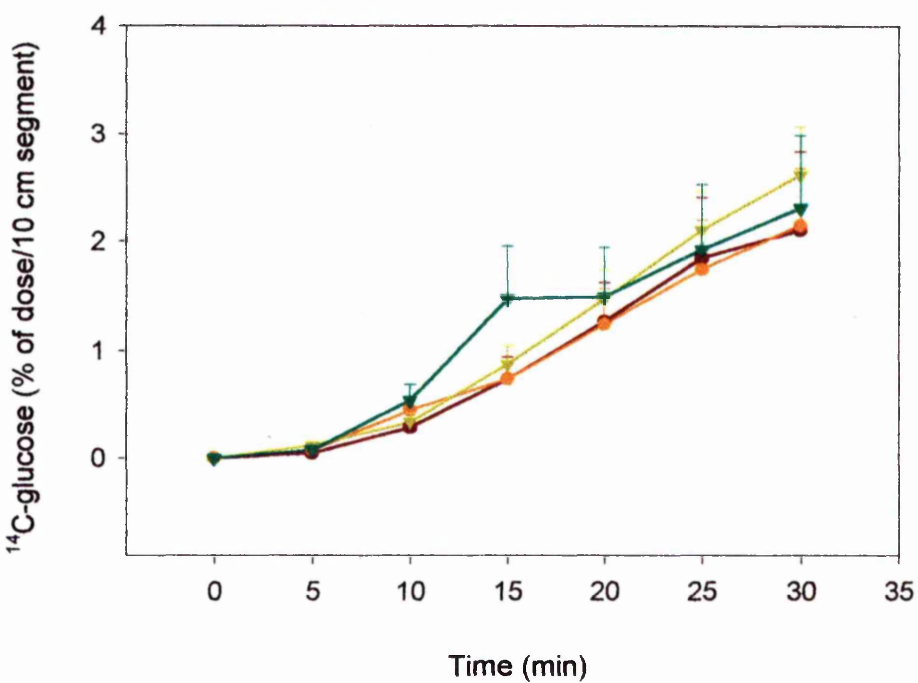


Figure 6.7: The effect of quercetin-3-glucoside on the uptake of ^{14}C-glucose

The possibility that quercetin-3-glucoside may inhibit SGLT1 was investigated by measuring the rate of uptake of ^{14}C-glucose in the presence of quercetin-3-glucoside at the concentrations stated. Results are expressed as % of radioactivity absorbed from the incubation media. (n=6-8 segments ± SEM).

- control
- 10 uM quercetin-3-glucoside
- 50 uM quercetin-3-glucoside
- 0.5 mM quercetin-3-glucoside

Table 6.4: The effect of quercetin-3-glucoside on the uptake of ^{14}C-glucose

Results represent mean values of the key features of glucose uptake across rat jejunal segments.

Quercetin-3-glucoside (μM)	Rate of uptake (%dose/10cm/h)	Cumulative uptake (%dose/10cm)	Recovery[a] (%)	95% CI[b]
-	4.24 ± 1.25	3.15 ± 0.92	82.88 ± 10.91	-
10	4.00 ± 1.06	2.83 ± 0.81	91.70 ± 4.44	(-4.95, 5.36)
50	5.00 ± 0.83	3.71 ± 0.62	93.33 ± 2.42	(-4.96, 5.04)
500	4.61 ± 1.39	3.34 ± 1.08	80.26 ± 7.29	(-7.41, 4.80)

[a] = residuals of quercetin-3-glucoside remaining in the incubation media at the end of the 30 min incubation.

[b] = 95% CI for significant inhibition of rate of glucose uptake in the presence of quercetin-3-glucoside.

n = 6-8 segments ± SEM.

6.4 Discussion

In order to establish the mechanism of flavonol transport across the small intestine, the first step to evaluate is whether their uptake is carrier-mediated or non-carrier mediated. Most polar molecules are transported across the epithelial membrane via a carrier protein as they are not able to diffuse across the lipid membrane. To determine if a carrier is involved in the transport of flavonols, the uptake of increasing concentrations of quercetin-3-glucoside across the intestinal wall was measured. Results revealed that the rate of uptake of quercetin-3-glucoside was linear up to 100 μM. A linear plot of 1/rate of uptake against 1/concentration was also obtained ($y=3.899x + 0.199$, $r^2=0.949$ and correlation coefficient=0.974), suggestive of a hyperbolic relationship between these two parameters and for saturation of uptake at higher concentrations. However, the absence of results for concentrations between 100 and 500 μM means that the data cannot be assigned with certainty to a hyperbolic curve. Nevertheless, the evidence suggests that quercetin-3-glucoside is conveyed across the intestinal membrane in association with carrier molecules. This is highly likely as the glycosylated forms of the flavonol conjugates will give rise to hydrophilic polar molecules which are not able to diffuse passively across the epithelial membrane. Evidence of saturation was also reported in the transport of quercetin diglucoside across the rat intestine up to a concentration of 1 mM (Gee *et al.*, 1998), further strengthening the possible role of a carrier in mediating flavonol glucoside uptake. However, the relevance of the observed saturation at 500 μM and 1 mM concentrations to in vivo physiological condition is questionable as such high concentration is not likely to be achieved at normal dietary intake.

6.4.1 The Involvement of SGLT1 in the transport of flavonol glucosides

It was previously postulated that SGLT1 might be involved in the transport of flavonol glucosides across the intestinal membrane (Holmann *et al.*, 1995). This carrier, located in the brush border membrane of the small intestine is responsible for the secondary active transport of glucose and galactose (Hediger and Rhoads, 1994). However, SGLT1 has been shown to be able to transport other compounds apart from glucose. SGLT1 has been shown to mediate uptake of the highly toxic compound cyacasin, a D-glucoside derivative of methylazoxymethanol (Kisby *et al.*, 1992). In addition, this carrier was also

reported to play a role in the transport of 2-naphthyl glycosides (Mizuma *et al.*, 1994). This group used similar everted rat gut model and reported a decrease in the transport of 2-naphthyl glycoside in the absence of Na^+, a co-substrate of SGLT1.

Based on these observations, SGLT1 was hypothesised to play a role in mediating the uptake of flavonol glucosides from the small intestine. It was postulated that the sugar moiety on the flavonol glucosides could bind to the active site on the glucose carrier protein in a similar manner to glucose, and so be transported across the intestinal membrane. If this was true, the presence of glucose together with flavonol glucosides in the incubation media would result in the two compounds competing with each other for binding to the carrier protein. However, when everted jejunal segments were incubated with quercetin-3-glucoside together with glucose, no significant difference was observed in the amount and rate of quercetin-3-glucoside transported across the intestinal membrane (Table 6.2). The presence of glucose, even at the highest concentration of 100 mM did not inhibit or reduce the amount of flavonol glucoside transported. This suggests that no competition exists between quercetin-3-glucoside and glucose for the glucose carrier. As the rate of uptake of quercetin-3-glucoside remained the same, it is possible that other carrier proteins may be involved.

To further establish the possible involvement of SGLT1, phloridzin, a known inhibitor of this carrier protein was employed to investigate its effects on flavonol glucoside uptake. Once more, no inhibition was seen in the amount of quercetin-3-glucoside transported when 1 mM phloridzin was present together with the flavonol in the incubation media. Even a higher concentration of phloridzin (5 mM) did not inhibit the uptake of quercetin-3-glucoside implying no interaction between this glucoside and SGLT1. As for quercetin-3,4′-diglucoside, there was also no significant inhibition in their uptake across the intestinal membrane in the presence of phloridzin. Although the error bars in the graph appeared to suggest a significant inhibition (*Figure*. 6.6), this was not so, possibly due to the small number of segments employed in the experiments.

When uptake of [^{14}C]glucose was measured in the presence of 1 mM phloridzin, only 33% inhibition was observed compared to without phloridzin. This implies that complete inhibition of SGLT1 did not occur in the model used as glucose was still transported across the intestinal membrane. Thus, it is possible that this particular method is not suitable to assess the role of SGLT1 in mediating flavonol uptake. Perhaps a combination of several techniques can provide a more definite answer.

A possible explanation for the lack of inhibition of glucose uptake in the presence of phloridzin could be that, in addition to the transcellular route of glucose absorption, glucose might also pass from the intestinal lumen to the circulation via the paracellular pathway. This route involves movement through the tight junction, the site between two adjacent enterocytes. The highly dynamic structure of the tight junction is subject to regulation, and activation of SGLT1 has been shown to cause contraction of the tight junction, leading to their increased permeability (Philpott *et al.*, 1992). This may allow molecules to cross the epithelium via tight junction by solvent drag. Certainly, 30% of glucose has been shown to be absorbed this way (Asitook *et al.*, 1990). The possibility that some flavonol glucosides might also be transported through this pathway is, however, unlikely given their polar properties. Transport via another carrier system is a more likely explanation.

Our findings contrasted with those of Gee *et al* (1998) who demonstrated that several quercetin glycosides including quercetin-3-glucoside and quercetin-3,4'-diglucoside accelerated the carrier-mediated efflux of pre-loaded galactose. This occurred via a sodium-dependent pathway, implying the involvement of SGLT1. The fact that the rate of uptake of some of these flavonol glucosides was higher than that of glucose signifies that the quercetin conjugates have a higher affinity to the carrier than glucose. In contrast to the findings of Gee *et al* (1998), our results suggest that the glucose carrier protein has a lower affinity for the quercetin conjugates than it does for glucose. It may be of relevance that Gee *et al* did not actually measure the amounts of flavonol glucosides transported in their incubation experiments but estimated uptake based on the efflux of galactose. At the 1 mM concentration used, measurable transport of the flavonols should have occurred and it would be interesting to see if there was any correlation between the cumulative efflux of both the galactose and also the flavonol glucosides tested. It should also be noted that the 1 mM concentration was non-physiological and is not representative of a normal dietary intake. The validity of this study and its relation to actual *in vivo* absorption requires careful evaluation.

To further investigate the possible interaction between flavonol glucosides and the glucose transport pathway, a different approach was used. In this instance, the possibility of flavonol glucosides acting as inhibitors of SGLT1 was investigated. The hypothesis was

based on the structural similarity between phloridzin and quercetin-3-glucoside (Figure 6.8). When the glucose moiety on phloridzin binds to the receptor on SGLT1, it inhibits glucose transport by acting as a competitive inhibitor (Dorando and Crane, 1984). In view of the structural similarity between phloridzin and quercetin-3-glucoside, the latter may possibly have the same action as phloridzin. However, this was not evident in our study as we did not find any inhibition in glucose transport in the presence of up to

Quercetin-3-O-β-glucoside Phloridzin

Figure 6.8: The structures of quercetin-3-glucoside and phloridzin

500 μM concentration of quercetin-3-glucoside. This implies that quercetin-3-glucoside did not inhibit the glucose carrier protein at the concentrations used.

Publications on the mechanism of flavonol transport across the small intestine are sparse as this area of research has received somewhat limited attention. To date, there are only three reports on very preliminary studies (Noteborn *et al.*, 1997, Gee *et al.*, 1998 and Walgren *et al.*, 1998).

Walgren *et al* (1998) used CaCo-2 cells, which were able to express SGLT1, to examine the uptake of several quercetin glucosides. They reported no transport of quercetin-4′-glucoside from the apical to basolateral side of the CaCo-2 cells and concluded that there was no interaction between SGLT1 and the flavonol glucoside. In another study, Noteborn *et al* (1997) studied flavonol uptake using perfusion of surviving rat jejunal

segments. Their results suggested that some quercetin monoglucosides, e.g. quercetin-4′-glucoside might interact with SGLT1 based on the flavonols ability to stimulate glucose transfer. However, at the same time, they also reported that other quercetin glucosides including quercetin-3-glucoside, at concentrations of 10 μM, behaved as inhibitors of this glucose carrier. We did not observe this using the same concentration of quercetin-3-glucoside. However, Noteborn and colleagues measured flavonol absorbed after 2 h perfusion of the gut segments. At this point, the reliability of the segments and their ability to transport molecule is questionable. Cells in the mucosal side may not be viable and may affect transport of molecules across the intestine. Therefore, estimation of true intestinal absorption may not be accurate. Certainly further studies are needed to evaluate this matter.

Other techniques exist to further evaluate the possible role of SGLT1 in mediating the uptake of flavonols. Expression of SGLT1 or other specific transporters in a cell system for example in oocytes of *xaenopus laevis* (Hediger *et al.*, 1987) can provide a more definite answer on the ability of this carrier protein to mediate flavonol uptake. Furthermore, other transporters exist in epithelial cells with the possibility of mediating flavonol uptake. This includes the Na^+-dependent amino acids transporters which share 50-60% sequence identity as SGLT1 (Hediger and Rhoads, 1994). On the other hand, there may exist other, yet to be identified, carrier proteins in the small intestine with the ability to mediate flavonol uptake.

6.4.2 Percentage recovery following incubation experiment

After the 30 minute incubation time, recovery of quercetin-3-glucoside and quercetin-3,4′-diglucoside in the incubation media was more than 80% and 90% respectively. Overall, when cumulative uptake of both the flavonol glucosides was expressed as percentage absorbed, less than 0.5% was detected in the serosal side. At the same time, low absorption of flavonols or other compounds into the serosal side is a typical characteristic of *in vitro* intestinal transport preparations. This can be partly attributed to the different hydrodynamic properties to that which would occur *in vivo*, for instance the absence of blood circulation in *in vitro* preparations. A few studies have demonstrated

glucuronidation of flavonol glucosides including quercetin-3-glucoside during their transport across the epithelial membrane (Shimoi *et al.*, 1998, Spencer *et al.*, 1999). However, we did not detect the presence of HPLC peaks which correspond with putative glucuronide conjugates of quercetin-3-glucoside (see Chapter 5).

6.5 Conclusion

This study established that the uptake of flavonol glucosides across the intestinal membrane occurred via a carrier-mediated pathway. From the various experiments performed, we have established that quercetin-3-glucoside neither interacts with SGLT1 for their transport nor does it inhibit the glucose transport pathway at the concentrations used. Further research are needed to identify the carrier protein responsible for flavonol uptake across the intestinal membrane as this will enable investigations on the structural requirements for the carrier protein. In this way, flavonols that are highly absorbable can be identified and their biological properties determined.

7 *In vitro* Metabolism of Flavonols using Rat Liver Homogenates

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CHAPTER 7: *IN VITRO* METABOLISM OF FLAVONOLS USING RAT LIVER HOMOGENATES

7.1 Introduction

Available evidence on absorption studies points to extensive metabolism of flavonoids following their absorption. We have shown that quercetin glucosides and diglucoside were better absorbed from the intestine than the aglycone, quercetin (Chapter 5). In studies with ileostomy volunteers, based on the amount excreted in the ileal effluent, it was estimated that 52% of quercetin glycosides (Hollman *et al.*, 1995) and 89% and 5% respectively for isorhamnetin-4′-glucoside and quercetin-4′-glucoside (Aziz *et al.*, 2000) were absorbed following a fried onion meal. However, when percentage absorption was measured in plasma following the same type of diet, only low levels of flavonols were detected at peak concentration (<2%) (Hollman *et al.*, 1995, Aziz *et al.*, 1998, McAnlis, 1999). Thus, a large percentage of the absorbed flavonols is unaccounted for which raises the possibility that substantial post-absorption metabolism of the quercetin glycosides may be occurring.

Not much information is available regarding the pathway of flavonol metabolism after their absorption. Several studies have reported glucuronidation as well as methylation of quercetin following oral administration of the aglycone to rats (Ueno *et al.*, 1983, Manach *et al.*, 1997). The liver has been suggested as the major organ responsible for the metabolism of flavonoids and various reactions can occur including hydroxylation, methylation and the formation of sulphate and glucuronide conjugates (Griffiths, 1982, Hackett, 1986) (See Chapter 1, Section 1.12.2). Although some research has been performed on the hepatic metabolism of flavonols, they have always concentrated on aglycones rather than flavonol glycosides. As flavonols in food are predominantly found conjugated to sugar conjugates (Cook and Samman, 1996), which appear to be more absorbable than their aglycones (Chapter 5), investigation of their post-absorptive metabolism is required. The main objective of this study was to investigate the metabolism of flavonol conjugates particularly those that were found in abundance in onions. In addition, the proposal that quercetin-4′-glucoside undergoes

methylation to isorhamnetin-4′-glucoside in the liver was also investigated. For this purpose, *in vitro* incubations of the test flavonols with rat liver extracts were carried out. Similar procedures have been used to study the metabolism of flavonoids such as quercetin and fisetin (Zhu *et al.*, 1994) and (-)-epicatechin (Piskula and Terao, 1998) by hepatic enzymes.

7.2 Materials and Methods

7.2.1 Preparation of flavonol standards and incubation buffer

Standards of quercetin-3,4′-diglucoside, quercetin-3-glucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside (Figure 7.1) were dissolved in methanol. All incubation experiments were performed in 50 mM phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$.

7.2.2 Preparation of liver homogenates

Liver samples were obtained from Sprague-Dawley rats (average weight 286.5 g) kept on a standard rat and mouse expanded diet (B and K Universal Limited, Hull, UK). Rats were stunned with a blow to the head prior to killing by cervical dislocation. An incision was made on the abdomen and the liver was removed immediately and placed in saline solution on ice. The livers were cleaned and divided into 1 g portions which were frozen in liquid nitrogen and stored at –80°C prior to the experiment.

7.2.3 Deglycosylation of flavonol glucosides

A method developed by Shaw and Griffiths (1980) with slight modifications was used for the incubation assay. Rat liver extracts were prepared by homogenising 1 g of thawed liver in 5 ml of ice-cold phosphate buffer (50 mM, pH 7.4) containing 10 mM $MgCl_2$. The incubation medium comprised 1 g of homogenated liver and 24 nM of

Quercetin-3,4'-diO-β-glucoside

Quercetin-3-O-β-glucoside

Quercetin-4'-O-β-glucoside

Isorhamnetin-4'-O-β-glucoside

Isorhamnetin-3-O-β-glucoside

Isorhamnetin

Quercetin

Figure 7.1: Structures of flavonol conjugates and aglycone

flavonol in 10 ml phosphate buffer (50 mM, pH 7.4, containing 10 mM $MgCl_2$). The reaction was started by the addition of the flavonol substrate and the incubation was carried out in a shaking water bath for 2 h at 37°C. Half ml samples were withdrawn at 0 min and every 10 min for the first half an hour and every 30 min thereafter. Triplicate experiments were performed for each flavonol. The flavonols tested were quercetin-3,4′-diglucoside, quercetin-4′-glucoside, quercetin-3-glucoside and isorhamnetin-4′-glucoside. Control experiments were conducted in the absence of either the flavonol or the liver homogenate.

7.2.4 Methylation of flavonol glucosides

The same incubation procedures as those outlined in section 7.2.3 were performed but with the addition of 24 nM *S*-adenosyl methionine (SAM) as a methyl donor (Shaw and Griffiths, 1980). Triplicate incubations were carried out using quercetin-3,4′-diglucoside, quercetin-4′-glucoside, quercetin-3-glucoside and isorhamnetin-4′-glucoside as substrates.

7.2.5 Flavonol extraction from lyophilised tissues

Samples obtained were immediately frozen with liquid nitrogen prior to lyophilisation. The freeze dried incubates were extracted in 0.5 ml of 100% methanol for 30 min at 4°C and the extracts were centrifuged at 3000 x g for 10 min to sediment particulate matter. Aliquots of supernatant, 37 µl in volume, were then made up to 250 µl with distilled water containing 0.5% trifluoroacetic acid prior to the analysis of 200 µl volumes by gradient elution reversed phase HPLC (section 3.2.1).

7.2.6 Identification of isorhamnetin-3-glucoside in the liver extract[1]

Standards of isorhamnetin-3-glucoside and liver extract samples were analysed on a Shimadzu 10Avp liquid chromatograph, with an absorbance monitor operating at 365 nm. A Genesis 4 µm 150 x 3.0 mm C18 reversed phase column, maintained at 40°C was eluted at 0.8 ml/min with a 45 minute gradient of 10-45% acetonitrile in water containing 1% formic acid. After passing through the flow cell of the absorbance monitor, the column eluate was directed to a Shimadzu LCQ8000 quadropole mass spectrometer with an atmospheric pressure chemical ionisation (APCI) interface in positive ion mode operating in full scan mode from 300 to 700 amu.

7.3 Results

7.3.1 Metabolism of quercetin-3,4′-diglucoside

In the presence of liver homogenates, quercetin-3,4′-diglucoside underwent deglycosylation as depicted by the gradual decrease in its concentration over the incubation period and the concomitant increase in the concentration of quercetin-3-glucoside which reached levels of 175 ± 21 ng/g (1.9 ± 0.2 % of the initial substrate concentration) after 2 h (Figure 7.2). Essentially the same pattern was observed when liver extracts and quercetin-3,4′-diglucoside were incubated in the presence of SAM. This suggests that no methylation of this diglucoside occurred. Quercetin-4′-glucoside was not detected in any of the incubation mixtures and no quercetin-3-glucoside was detected in the control samples.

A typical HPLC trace obtained from the incubation of quercetin-3,4′-diglucoside with liver extracts at 0 min and 90 min, detected on the UV monitor at $A_{365\ nm}$ is illustrated in Figure 7.3 (A and B). Co-chromatography established that peaks 1 and 2 corresponded to quercetin-3,4′-diglucoside and quercetin-3-glucoside, respectively.

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[1] HPLC and MS identification of isorhamnetin-3-glucoside in liver extract was performed by Mr William Mullen, Department of Biochemistry, University of Glasgow.

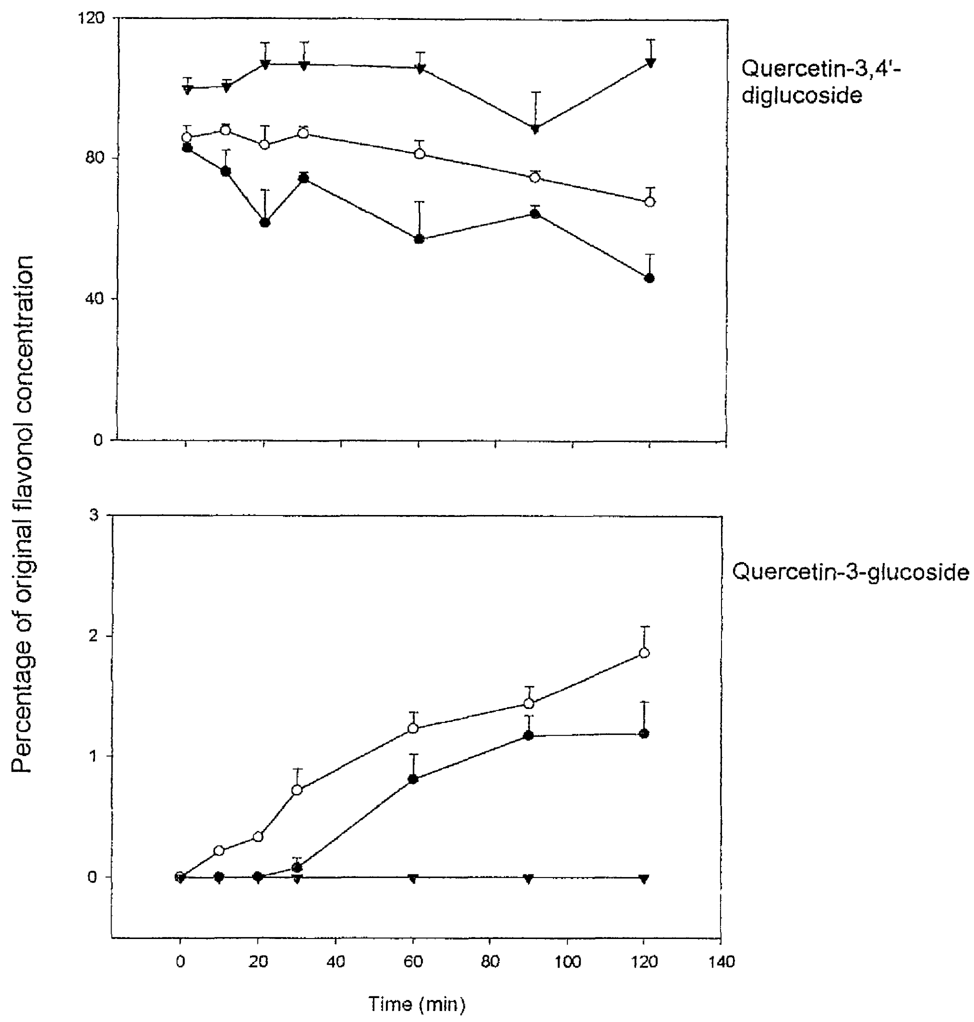

Figure 7.2: The profiles of quercetin-3,4′-diglucoside and its metabolites following incubation with liver homogenates under the conditions stated.

Incubations were performed for 2 h at 37°C and sampling every 10 min for the first 30 min and every half hour thereafter. Following lyophilisation and methanolic extraction, samples were analysed for flavonol content on a reversed phase HPLC system. Flavonol content is expressed as ng/g f.w ± SEM. Each condition is a mean of 3 experiments. Graphs show liver extract profiles of quercetin-3,4′-diglucoside and quercetin-3-glucoside.

—•— Contained SAM
—○— Without SAM
—▾— Without liver homogenates

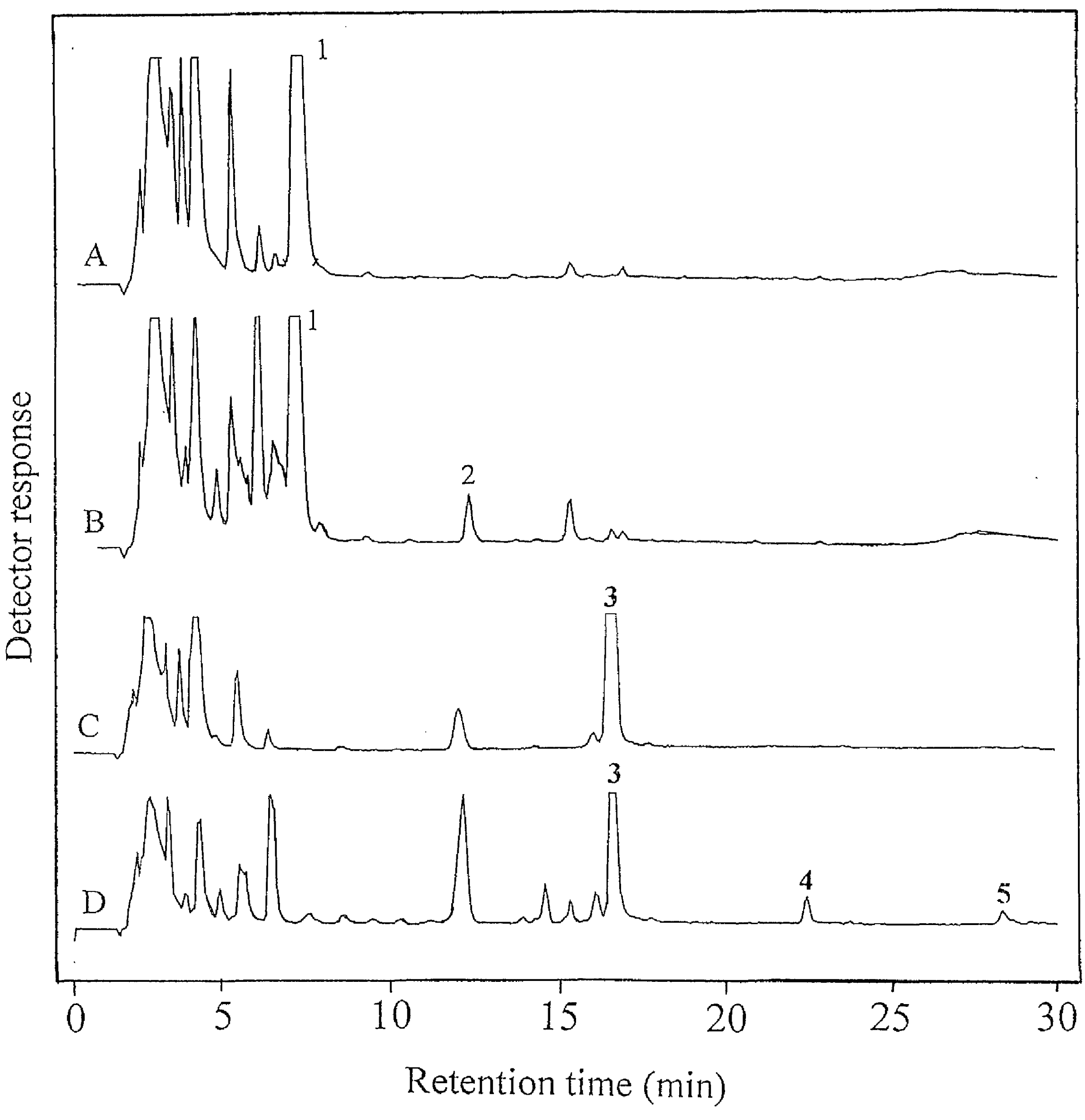

Figure 7.3: Gradient reverse phase HPLC analysis of flavonols in liver extracts

Column: 150 x 3.0 mm i.d. 4-µm Genesis C_{18} cartridge column with a 10 x 4.0 mm 4-µm Genesis C_{18} guard cartridge. Mobile phase: 25 min gradient of 15-40% acetonitrile in water containing 0.5% trifluoroacetic acid. Flow rate: 0.5 ml/min. Detector: absorbance monitor operating at 365 nm. Samples: (**A**) aliquot of sample collected immediately after the incubation of quercetin-3,4′-diglucoside with liver extracts; (**B**) as (A) but collected 90 min after the incubation; (**C**) aliquot of sample collected immediately after the incubation of quercetin-4′-glucoside with liver extracts; (**D**) as (C) but samples collected 90 min after the incubation. Peaks 1=quercetin-3,4′-diglucoside; 2=quercetin-3-glucoside; 3=quercetin-4′-glucoside; 4=quercetin; 5=isorhamnetin.

Quercetin-3-glucoside accumulated over the 90 min incubation period along with other metabolite peaks which did not correspond with any of the available flavonol standards (Figure 7.3 B).

7.3.2 Metabolism of quercetin-3-glucoside

Rapid metabolism of quercetin-3-glucoside occurred when SAM was added to the liver preparations with 4049 ± 55 ng/g f.w at 0 h falling to 354 ± 89 ng/g f.w at the last sampling point (Figure 7.4). Three metabolites were detected, isorhamnetin-3-glucoside and the aglycones isorhamnetin and quercetin (Figure 7.4). The formation of isorhamnetin-3-glucoside was extremely rapid as it was detected at the 0 min time point, which was sampled within seconds of the addition of quercetin-3-glucoside. This flavonol glucoside appeared to peak around 20-30 min with a concentration of 914 ± 26 ng/g f.w (23 ± 0.7 % of the original substrate concentration) before gradually decreasing. Free isorhamnetin was also detected in the presence of SAM, with levels peaking at 23.3 ± 3.4 ng/g f.w (0.6 ± 0.1 % of the original substrate) after 30 min, after which it declined steadily. Trace levels of free quercetin gradually decreased to less than 5 ng/g f.w at the end of the 2 h incubation period.

The data demonstrate that in the liver preparations, quercetin-3-glucoside is subject primarily to 3′-*O*-methylation in the presence of SAM. The detection of trace levels of quercetin and isorhamnetin indicate that both the substrate quercetin-3-glucoside and its major metabolite isorhamnetin-3-glucoside are subject to deglycosylation. The levels of quercetin-3-glucoside in the control samples without liver homogenate remained high during the 2 h experiment. The three metabolites were not detected in the control incubations (Figure 7.4).

Traces obtained in an typical HPLC analysis, with detection at $A_{365\ nm}$ and by fluorescence detection following on-line post-column derivatization, with a liver preparation collected after a 20 min incubation are illustrated in Figure 7.5 B and C. Peak 1 was identified as quercetin-3-glucoside by co-chromatography whereas peaks 2, 4 and 5 corresponded to isorhamnetin-3-glucoside, quercetin and isorhamnetin respectively. Although traces of isorhamnetin-3-glucoside (peak 2) and the unidentified

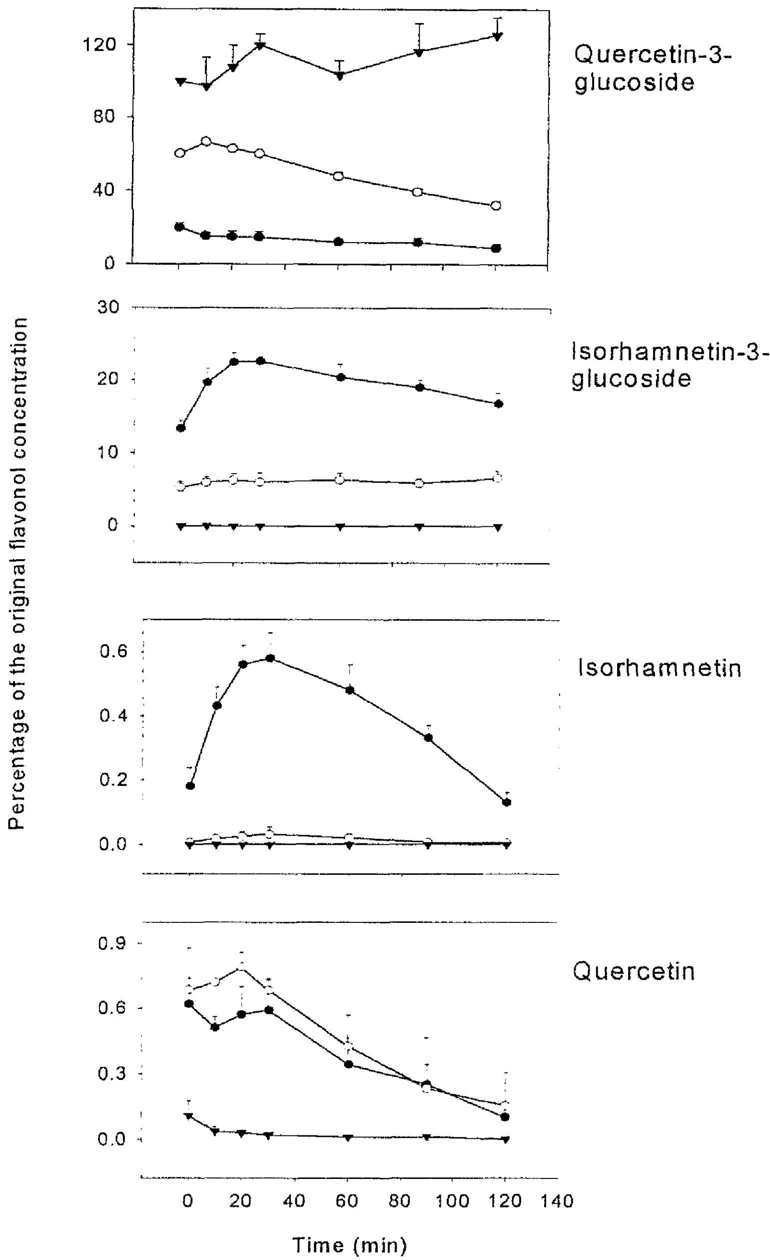

Figure 7.4: The profiles of quercetin-3-glucoside and its metabolites following incubation with liver homogenates under the conditions stated.

Incubations were performed for 2 h at 37°C and sampling every 10 min for the first 30 min and every half hour thereafter. Following lyophilisation and methanolic extraction, samples were analysed for flavonol content on a reversed phase HPLC system. Flavonol content is expressed as ng/g f.w ± SEM. Each condition is a mean of 3 experiments. Graphs show liver extract profiles of quercetin-3,-glucoside, isorhamnetin-3-glucoside, isorhamnetin and quercetin.

—•— Contained SAM
—○— Without SAM
—▾— Without liver homogenates

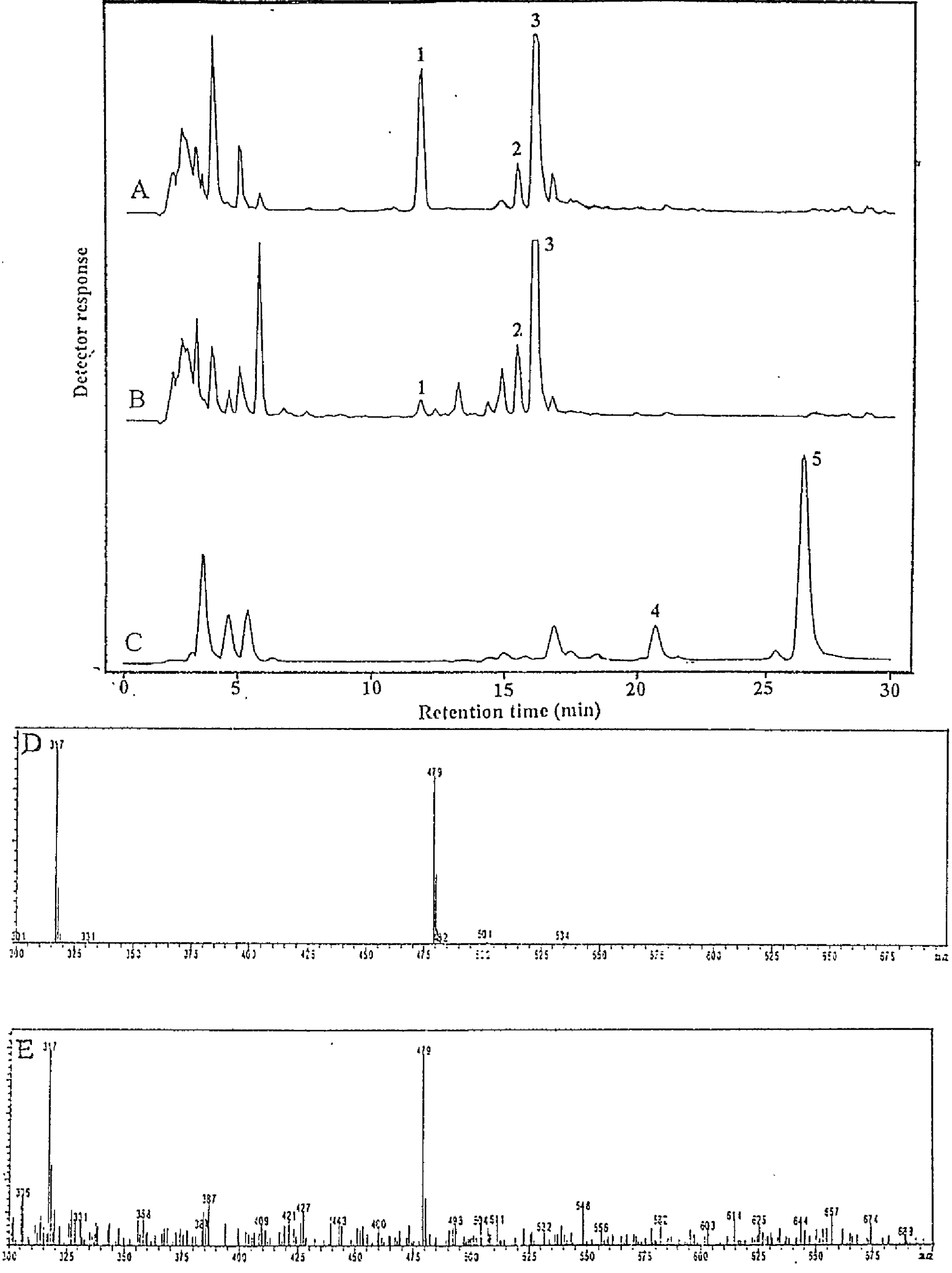

Figure 7.5: Reverse phase HPLC and mass spectrum analysis of a liver extract following the incubation of quercetin-3-glucoside in the presence of S-adenosyl methionine.

HPLC analysis. Column: 150 x 3.0 mm i.d. 4 μm genesis C_{18} cartridge column. Mobile phase: 25 minute gradient of 15-40% acetonitrile in water containing 0.5% TFA. Flow rate: 0.5 ml/min. Detector: absorbance monitor operating at 365 nm and following post-column derivatization, a fluorimeter at excitation 425 nm and emission 480 nm **HPLC-MS analysis**. Shimadzu LCQ8000 quadropole mass spectrometer with an Atmospheric Pressure Chemical Ionisation (APCI) interface in positive ion mode operating in full scan mode from 300 to 700 amu. **(A)** HPLC trace of flavonols in liver extract collected at 0 min following incubation with quercetin-3-glucoside and SAM, detected at A_{365}; **(B)** as (A) but collected 20 min after the incubation, detected at A_{365}; **(C)** same as (B) but with post-column derivatization and fluorescence detection; **(D)** APCI positive ion spectrum of a 50 ng standard of isorhamnetin-3-glucoside, M+-m/z 479; aglycone base peak-m/z 317. **(E)** APCI positive ion spectrum of isorhamnetin-3-glucoside obtained from HPLC peak 1 in (B), M+-m/z 479; aglycone base peak-m/z 317.

HPLC peak 3 were present in the 0 min sample (Figure 7.5A), they were not detected in the control sample without the presence of liver extracts. This implies either rapid metabolism of quercetin-3-glucoside or the presence of trace impurities from the liver preparations.

Isorhamnetin-3-glucoside has the same retention time as quercetin-4′-glucoside (see Figure 7.3) but the identity of the isorhamnetin conjugate was confirmed by LC-MS (Figure 7.5 D-E). The isorhamnetin-3-glucoside standard (Figure 7.5 D) and the metabolite (Figure 7.5 E) both yielded spectra with major ions at m/z 479 (M^+) and m/z 317. The mass spectrum of quercetin-3-glucoside contains the equivalent fragments ions 14 amu lower at m/z 465 and m/z 303 (W. Mullen, unpublished).

7.3.3 Metabolism of quercetin-4′-glucoside

Metabolism of quercetin-4′-glucoside by liver homogenates was observed with the level of the glucoside decreasing gradually over the 2 h incubation period with approximately 175-250 ng/g f.w detected in the last sampling point compared to the initial concentration of 5857 ng/g f.w at 0 min (Figure 7.6). Deglycosylation of quercetin-4′-glucoside occurred as indicated by the build up of low levels of free quercetin, equivalent to ca. 1% of the original substrate concentration after 2 h (Figure 7.6). Incubation of quercetin-4′-glucoside in the presence of SAM resulted in the appearance of isorhamnetin in the liver preparations (Figure 7.6). Isorhamnetin levels peaked around 60 min, giving approximately 43.3 ± 3.7 ng/g f.w (0.7 ± 0.1%), after which it started to decrease. Trace levels of isorhamnetin also formed in preparations to which SAM was not added, possibly as a consequence of endogenous SAM acting as methyl donor. Isorhamnetin-4′-glucoside was not detected as a metabolite of quercetin-4′-glucoside. This suggests that the isorhamnetin that accumulated was formed by 3-*O*-methylation of quercetin. Levels of quercetin-4′-glucoside in the control samples were generally stable and only fell slightly over the 2 h incubation period (Figure 7.6). Neither quercetin nor isorhamnetin were detected when quercetin-4'-glucoside was incubated without the liver preparation.

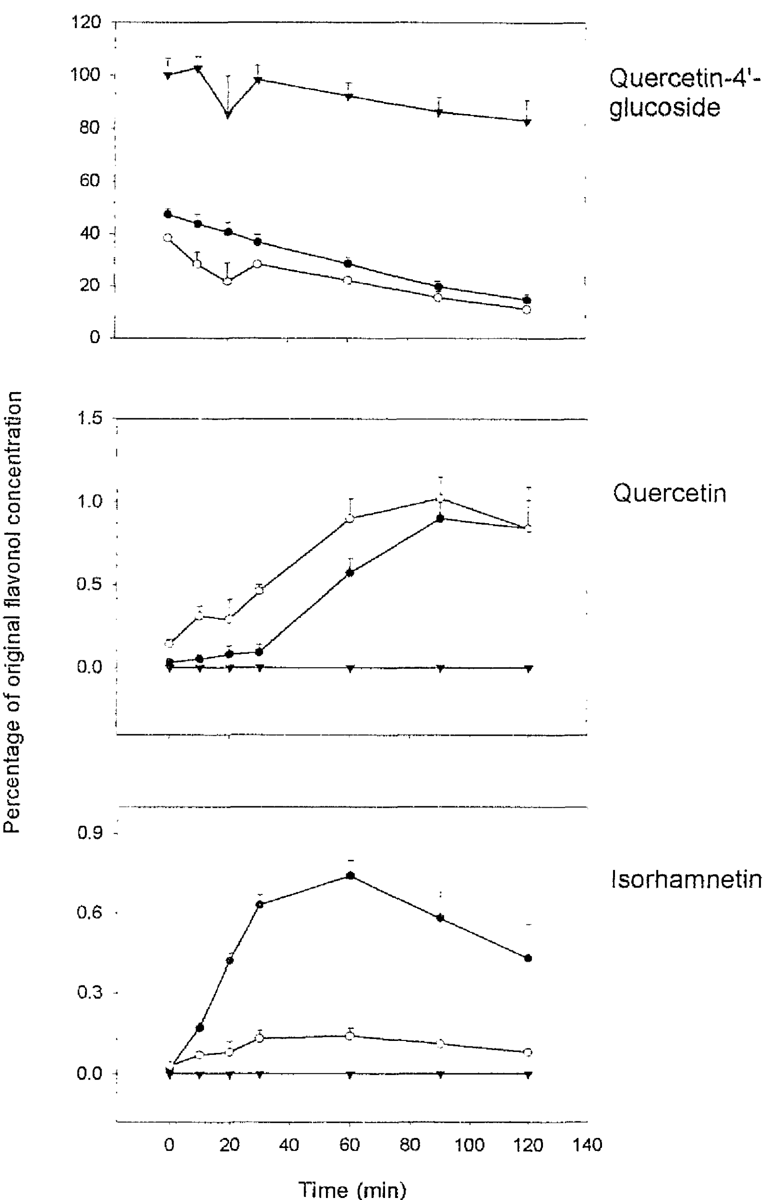

Figure 7.6: The profiles of quercetin-4′-glucoside and its metabolites following incubation with liver homogenates under the conditions stated.

Incubations were performed for 2 h at 37°C and sampling every 10 min for the first 30 min and every half hour thereafter. Following lyophilisation and methanolic extraction, samples were analysed for flavonol content on a reversed phase HPLC system. Flavonol content is expressed as ng/g f.w ± SEM. Each condition is a mean of 3 experiments. Graphs show liver extract profiles of quercetin-4′-glucoside; quercetin and isorhamnetin.

—●— Contained SAM
—○— Without SAM
—▼— Without liver homogenates

Figure 7.3 (C and D) illustrate typical HPLC traces of a sample collected at the 0 min and 90 min time point, detected by the UV monitor at 365 nm. Peaks 3, 4 and 5 co-chromatographed with quercetin-4′-glucoside, quercetin and isorhamnetin, respectively. Quercetin and isorhamnetin gave higher peaks when detected by fluorimeter after post-column derivatization (data not shown). Other unknown peaks not present at 0 min could also be detected on the UV trace 90 min after the incubation. These peaks did not correspond to any of the available standards.

7.3.4 Metabolism of isorhamnetin-4′-glucoside

Deglycosylation of isorhamnetin-4′-glucoside was observed when the conjugate was incubated with the liver homogenates. There was a ca. 50% decline in the level of the glucoside over the 2 h incubation period (Figure 7.7) that was associated with a concomitant increase, albeit at a lower level, of isorhamnetin. Free isorhamnetin was not detected in the control experiments (Figure 7.7). The rate of decline in the level of isorhamnetin-4′-glucoside was not affected by the presence of SAM in the incubation medium (Figure 7.7).

7.3.5 Summary of metabolism of flavonols by rat liver homogenates

The methylation and deglycosylation of the flavonol glucosides by rat liver homogenates is summarised in Table 7.1. For comparative purposes, accumulation of the metabolites at the end of 2 h was expressed as a percentage of the initial substrate concentration. Generally, flavonols in the control samples were stable throughout the 2 h incubation period with more than 80% of the parent compounds recovered at the end of the experiment.

All four flavonol glucosides underwent metabolism over the 2 h incubation period. Metabolism was highest for quercetin-4′-glucoside (10.9 $\pm$ 1.7 % recovered after 2 h) followed by quercetin-3-glucoside (32.4 $\pm$ 2.7 %), isorhamnetin-4′-glucoside (36.4 $\pm$ 0.4 %) and quercetin-3,4′-diglucoside (68.1 $\pm$ 4.1 %) (Table 7.1). Interestingly, when

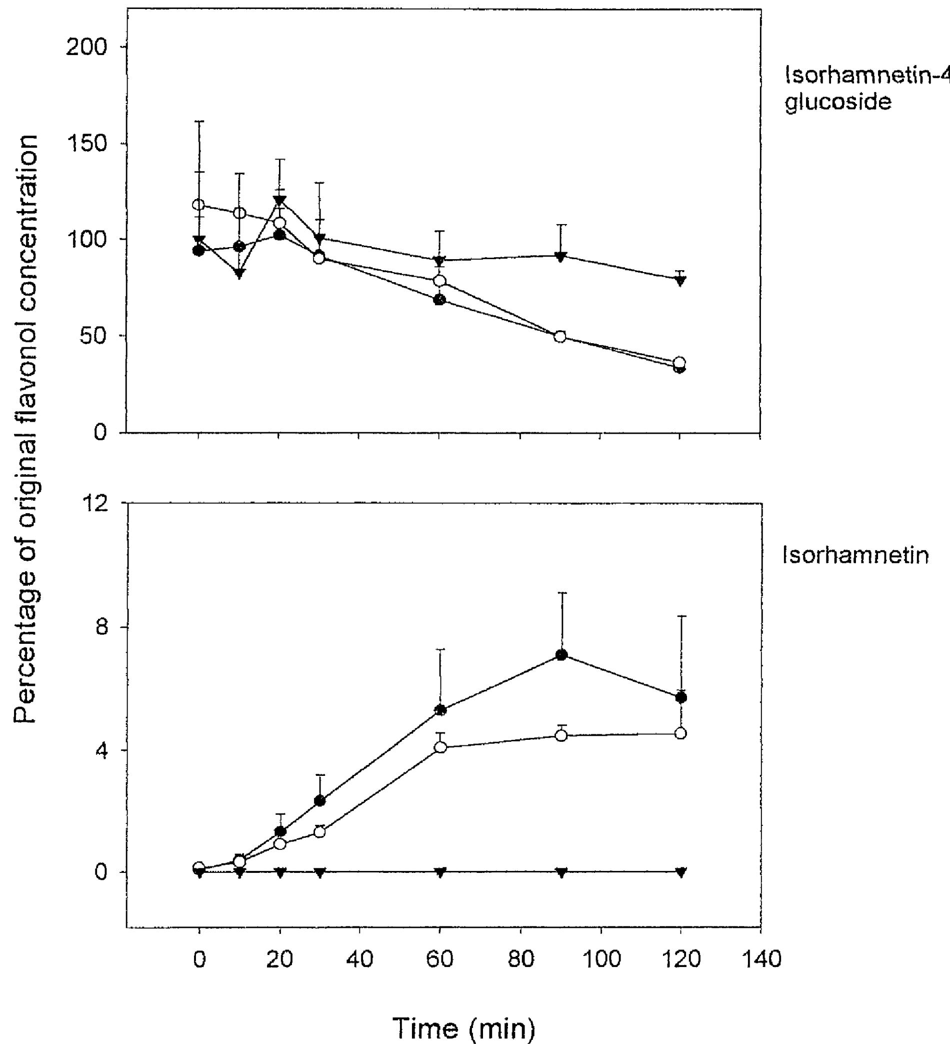

Figure 7.7: The profiles of isorhamnetin-4′-glucoside and its metabolites following incubation with liver homogenates under the conditions stated.

Incubations were performed for 2 h at 37°C and sampling every 10 min for the first 30 min and every half hour thereafter. Following lyophilisation and methanolic extraction, samples were analysed for flavonol content on a reversed phase HPLC system. Flavonol content is expressed as ng/g f.w ± SEM. Each condition is a mean of 3 experiments. Graphs show liver extract profiles of isorhamnetin-4′-glucoside and isorhamnetin.

—•— Contained SAM
—○— Without SAM
—▾— Without liver homogenates

Table 7.1: The extent of flavonol metabolism and the accumulation of metabolites following incubation with rat liver homogenates.

Flavonol	Recovery of substrate after 2 h incubation[a] (%)	Accumulation of metabolites [b] (%)			
		Quercetin-3-glucoside	Isorhamnetin-3-glucoside	Quercetin	Isorhamnetin
Quercetin-3,4'-diglucoside					
With SAM	46.4 ± 6.8	1.2 ± 0.3	-	-	-
Without SAM	68.1 ± 4.1	1.9 ± 0.2	-	-	-
Control	100	-	-	-	-
Quercetin-3-glucoside					
With SAM	8.6 ± 2.2	-	16.7 ± 1.5	0.1 ± 0.05	0.1 ± 0.03
Without SAM	32.4 ± 2.7	-	6.5 ± 1.0	0.2 ± 0.15	0.01 ± 0.01
Control	100	-	-	-	-
Quercetin-4'-glucoside					
With SAM	14.5 ± 2.2	-	-	0.8 ± 0.3	0.4 ± 0.1
Without SAM	10.9 ± 1.7	-	-	0.8 ± 0.2	0.1 ± 0.01
Control	82.9 ± 7.9	-	-	-	-
Isorhamnetin-4'-glucoside					
With SAM	33.6 ± 2.6	-	-	-	5.7 ± 2.6
Without SAM	36.4 ± 0.4	-	-	-	4.6 ± 1.4
Control	79.2 ± 4.9	-	-	-	-

[a] Results are expressed as the percentage of flavonol remaining at the end of the 2 h incubation period relative to the amount at the start of the experiments. (n=3 ± SEM).

[b] Accumulation of the metabolites after 2 h incubation was calculated as a percentage of the initial substrate concentration in the incubation media. (n=3 ± SEM).

accumulation of the metabolites was expressed as percentage of the initial substrate concentration, the figures obtained did not directly correspond to the extent of metabolism of the substrate at the end of the 2 h period. In most instances, accumulation of the metabolites was much lower than we would have expected based on the extent ofmetabolism of the parent compound. It would appear that other types of metabolism are occurring as well as deglycosylation and methylation and this will be further discussed in the next section.

Quercetin-3-glucoside was methylated extensively when SAM was added to the liver homogenates. At the end of a 2 h incubation period, more than 90% of the quercetin-3-glucoside added to the liver preparation had been metabolised. At peak concentration, the accumulation of methylated products, isorhamnetin-3-glucoside and isorhamnetin was 22.6 ± 0.7% and 0.6 ± 0.1%, respectively. In the case where SAM was not present, accumulation of isorhamnetin-3-glucoside and isorhamnetin was only 6.5 ± 1.0% and 0.03 ± 0.02%, respectively, at peak levels. Deglycosylation of quercetin-3-glucoside occurred at a much lower extent than *O*-methylation with the percentage accumulation of quercetin in the range of 0.6-0.8%. The total accumulation of metabolites at 2 h only accounted about 20% of the total quercetin-3-glucoside metabolised.

Deglycosylation of quercetin-3,4′-diglucoside yielded quercetin-3-glucoside (1.2-1.9%) which accounted for only 6% of the decline in the level of the parent compound (32-54%). Quercetin-4′-glucoside was deglycosylated to quercetin (1.0%) and a proportion of the free quercetin was methylated to isorhamnetin, as observed in incubations with the presence of SAM (0.4%). The extent of metabolism of this glucoside was much higher than the metabolites generated implying the involvement of other reactions besides deglycosylation. Some deglycosylation of isorhamnetin-4′-glucoside also occurred, giving rise to approximately 4.6-5.7% of isorhamnetin, representing only about 13% of the metabolised substrate.

7.4 Discussion

Studies on the antioxidant activities of flavonoids suggest higher activities of flavonoid aglycones compared to the conjugated forms (Laughton *et al.*, 1991, Rice-Evans *et al.*, 1996). However, flavonoids, with the exception of flavan-3-ols, almost always occur in the diet as glycosides and only a very small proportion exists as the aglycone. Thus, metabolic studies of flavonoids are important to provide information on the fate of these compounds following their absorption and the resulting biological activities of their metabolites.

7.4.1 Methylation of flavonols

The ability of the liver catechol-*O*-methyl transferase (COMT) to methylate several flavonoids has been reported (Zhu *et al.*, 1994). In the present study, methylation of quercetin-3-glucoside occurred when SAM was present in the incubation experiments. This was evident from the percentage of the flavonol substrate recovered at the end of the incubation period (8.6%) as well as the amount of metabolites formed. High levels of the metabolite isorhamnetin-3-glucoside could be detected. The presence of a catechol group (C-3′,4′-dihydroxylation) on quercetin-3-glucoside would increase the affinity of the COMT for this flavonol. The additional presence of the aglycone isorhamnetin suggests some deglycosylation of quercetin-3-glucoside to liberate quercetin which subsequently undergoes 3'-O-methylation to isorhamnetin. Alternatively, it could also be a result of deglycosylation of isorhamnetin-3-glucoside. Figure 7.8 illustrates a proposed pathway for the hepatic metabolism of quercetin-3-glucoside.

Methylation of quercetin-3-glucoside was rapid and this was evident in the rapid increase of the *O*-methylated metabolite isorhamnetin-3-glucoside. A higher affinity of the COMT for quercetin, ca. 3 orders of magnitude higher than its natural substrates, catecholamines, has been reported by Zhu *et al.* (1994). This may explain the above observations. As isorhamnetin-3-glucoside was also detected in incubations without SAM, albeit to a lesser extent, some endogenous SAM may already be present in the

Quercetin-3-O-β-glucoside

(3'-O-methylation)

Isorhamnetin-3-O-β-glucoside

Further metabolism?

(Deglycosylation)

(Deglycosylation)

Further metabolism?

Quercetin

(3'-O-methylation)

Isorhamnetin

Figure 7.8: Proposed pathway for the hepatic metabolism of quercetin-3-glucoside

liver homogenates to allow *O*-methylation to occur. *O*-methylation was not observed with quercetin-3,4′-diglucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside, possibly due to the absence of the catechol group on the B ring.

Methylation of flavonols has been demonstrated in rats orally fed quercetin (Manach *et al.*, 1997). In this instance, conjugates of isorhamnetin and tamarixetin (4′-*O*-methyl quercetin) were detected in plasma. Although the 4′-*O*-methylation of quercetin-3-glucoside has not been reported previously, this reaction would appear to be feasible because of the presence of the catechol moiety on the B ring of the quercetin conjugate. Methylation of quercetin and fisetin using porcine liver COMT has also been reported *in vitro* (Zhu *et al.*, 1994). In addition, studies on the tissue distribution of COMT showed that liver had the highest activity, supporting the role of liver in this reaction (Piskula and Terao, 1998).

Of the four flavonol conjugates tested in this study, only quercetin-3-glucoside underwent methylation. This is the only flavonol with a catechol group on the B-ring. Quercetin-3,4′-diglucoside, quercetin-4′-glucoside and isorhamnetin-4′-glucoside do not have a 3′,4′-dihydroxy structure. In addition, the presence of SAM in incubations with quercetin-4′-glucoside and isorhamnetin-4′-glucoside did not result in further metabolism suggesting that methylation of these flavonols did not occur.

When quercetin-4′-glucoside was incubated in the liver homogenates, 3′-*O*-methylation of the flavonol did not occur to yield isorhamnetin-4′-glucoside. This implies that the active site on the enzyme COMT is not able to bind quercetin-4′-glucoside. Thus, the data obtained with the *in vitro* rat liver preparations did not support the hypothesis that in humans, absorbed quercetin-4′-glucoside undergoes 3′-*O*-methylation to form isorhamnetin-4′-glucoside. It is possible that other tissues may play a role in this reaction or that human liver may contain different *O*-methyltransferases to rat liver. Alternatively, the 24 nM SAM concentration used was too little for 3′-O-methylation of quercetin-4′-glucoside to occur.

The enzyme COMT catalyses the inactivation of catecholamines and is also responsible for the detoxification of xenobiotic catechols (Creveling *et al.*, 1970). The presence of the catechol structure is a crucial structural moiety for interaction with

COMT. In addition, the polarity of the compound to be methylated is probably of importance (Piskula and Terao, 1998). Catechols with polar substituents are preferentially methylated at C-3′ whereas the presence of non-polar substituents, e.g. quercetin aglycone leads to random methylation, giving rise to either 3′- or 4′-*O*-methylated metabolites (Creveling *et al.*, 1970). In this study, when the flavonol conjugates were deglycosylated to produce quercetin, COMT will *O*-methylate the aglycone, but not the parent compounds, quercetin-3,4'-diglucoside or quercetin-3-glucoside, to isorhamnetin.

7.4.2 Deglycosylation of flavonols

Deglycosylation was observed with all four flavonols, albeit at seemingly different rates as evident from the concomitant rise in levels of the aglycones (Table 7.1). Variation in the percentage accumulation of deglycosylated products of flavonol glucoside metabolism suggests structural specificity of the liver β–glucosidase. The enzyme appears to have a higher affinity for flavonol glucosides with glucose substitutions at C-4′ than at the C-3 position. This was evident in the conversion of quercetin-3,4′-diglucoside to quercetin-3-glucoside and metabolism of quercetin-4′-glucoside to quercetin compared to the low level of quercetin accumulation in incubations with quercetin-3-glucoside. The apparent lack of conversion of quercetin-3,4′-diglucoside to quercetin-4′-glucoside is in keeping with this proposal.

Day *et al.* (1998) described *in vitro* deglycosylation of several flavonoids by glycosidases of liver cell-free extracts. In particular, they observed 69% deglycosylation of quercetin-4′-glucoside. Interestingly, this group reported that quercetin-3-glucoside and quercetin-3,4′-diglucoside were not hydrolysed by the enzymes from the liver extract. Nevertheless, we detected some metabolism of quercetin-3,4′-diglucoside and possibly quercetin-3-glucoside, with recoveries of ca. 32% and 68% respectively, of the original substrate. However, as mentioned above, the 68% metabolism of quercetin-3-glucoside could be primarily the result of methylation rather than deglycosylation.

7.4.3 Other metabolic reactions

One interesting observation from this study is the possible involvement of other metabolic reactions in addition to methylation and deglycosylation. Based on our results, the accumulation of metabolic products did not correspond to the extent of metabolism of the corresponding flavonol glucoside (see Table 7.1). In most instances, the level of metabolites recovered at the end of the 2 h incubation was equivalent to <6% of the initial substrate concentration. In contrast, there was a >30% fall in the level of substrate, leaving ca. 24% of metabolites unaccounted for. As the flavonol glucosides were relatively stable in the control samples, it would imply that other metabolic reactions are occurring in addition to deglycosylation and methylation. As an example, extensive metabolism of quercetin-3-glucoside was observed from the appearance of methylated metabolites. However, based on the decline in the levels of the glucoside and accumulation of the metabolites, it would appear that a large percentage of the metabolites were not accounted for. As we only detected 0.2% of deglycosylated products, other reactions may have occurred in addition to deglycosylation and methylation. The possibility that other methylated metabolites were produced as well as isorhamnetin-3-glucoside and isorhamnetin should be taken into consideration. In addition to 3′-*O*-methylation of quercetin-3-glucoside, 4′-O-methylation of this glucoside could also have taken place to yield tamarixetin-3-glucoside. Indeed, we detected an unknown HPLC peak, which when analysed by the LC-MS showed similar mass spectrum to isorhamnetin-3-glucoside (peak 3, Figure 7.5 B). However, due to the lack of this standard and limited time, this was not further investigated.

The liver has been reported to be able to glucuronidate, sulphate and hydroxylate flavonoids as well as carry out deglycosylations and methylations (Griffiths, 1982, Hackett, 1986). A study by Day *et al* (1999) demonstrated glucuronidation of free quercetin and isorhamnetin following incubations with liver cell-free extracts. Glucuronidation of these aglycones was extensive and three to four glucuronidation metabolites were detected. Glucuronic acids were added to the flavonol molecule at various sites, mainly C-4′, C-7 and C-3. In addition to *in vitro* experiment, *in vivo* studies with animals also demonstrated glucuronidation and/or sulphation of flavonols following oral administration of the parent compound. Glucuronides of quercetin, isorhamnetin

and tamarixetin as well as sulphates of quercetin were detected in the bile and urine of rats fed [^{14}C]quercetin (Ueno *et al.*, 1983). Sulphation of quercetin was also reported in an isolated perfusion experiment with rat liver (Shali *et al.*, 1991). Zhu *et al* (1994) described extensive metabolism of quercetin following its intraperitoneal administration to hamsters. In addition to extensive *O*-methylation of the parent compound to form isorhamnetin, a portion of the resulting metabolites was also conjugated by glucuronidation or sulphation. In another study where rats were supplemented with 0.2% quercetin, the major circulating metabolites were glucurono-sulpho conjugates of isorhamnetin and quercetin (92%) (Morand *et al.*, 1998). This group also demonstrated *in vitro* sulphation and glucuronidation of quercetin by liver extracts. Although *in vitro* glucuronidation of flavonol glucosides by the liver has not been reported, the likelihood of this happening is high given that the liver is likely to conjugate flavonols rather than deglycosylate.

7.5 Conclusion

This study confirms that the liver is capable of *O*-methylation and deglycosylation of several naturally occurring flavonol glucosides, particularly those that are found in abundance in onions. Quercetin-3-glucoside underwent extensive methylation to yield methylated metabolites of this flavonol. This stresses the importance of the catechol group for methylation of flavonols, catalysed by the enzyme COMT. Deglycosylation of quercetin-4′-glucoside, isorhamnetin-4′-glucoside, and to a less degree quercetin-3,4′-diglucoside, implies structural specificity of the enzyme glycosidases towards the flavonols. Results from this study also suggest that methylation and deglycosylation are not the only metabolic reactions involved in the liver metabolism of flavonol glucosides. The possibility of other metabolic events occurring merits further research, especially the possible formation of glucuronide and sulphate conjugates. Furthermore, the rate and extent of flavonol metabolism appear to be dependent on their structure as well as the position of their sugar moiety. Such study is highly relevant in determining the metabolites formed by flavonols following their absorption. In this way, the potency of the original flavonols ingested can be determined and the benefits of their consumption evaluated. The information will also be useful for any *in vivo* metabolic studies of flavonol glucosides.

8 General Discussion

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CHAPTER 8: GENERAL DISCUSSION

Recent interest in the absorption and metabolism of flavonoids in humans was sparked by reports of their potent antioxidant activities and possible protection against diseases associated with oxidative damage. Evidence from epidemiology studies, although still limited, points to a possible protection of dietary flavonoids against coronary heart disease and possibly cancer (Hertog *et al.*, 1993, Keli *et al.*, 1996, Knekt *et al.*, 1997). This area of research requires further investigation as the information available is limited and several questions remain unanswered.

The main objective of this thesis was to carry out research on the absorption and metabolism of flavonoids particularly from the sub-class flavonols. Attempts were made to identify absorption of individual flavonols prior to acid hydrolyses treatment which breaks the glycoside as well as any glucuronide or sulphate linkages. Most studies have analysed flavonol content after the hydrolyses of conjugates. This gives only a combined estimate of the free flavonols and the aglycones liberated from the conjugates. Specific flavonol glycosides cannot be identified this way. At the same time, the mode and extent of flavonol absorption from the small intestine was also evaluated. The process that occurs in the gut or during transepithelial transport is the first step that determines the bioavailability of flavonol. In addition, we investigated the hepatic metabolism of several flavonols.

To achieve this, first, we performed a feeding study with healthy human volunteers. They consumed a single dose of flavonol-rich fried onions after which the presence of flavonols in the plasma and urine was analysed (Chapter 4). A modified *in vitro* everted rat gut model developed by Wilson and Wiseman (1954) was applied for the investigations of flavonol absorption from the small intestine. This common and widely used method is useful in evaluating intestinal uptake of flavonols as it allows easy sampling from the serosal side as well as maintaining cell viability. In the case of flavonol metabolism, the liver was chosen as it has been widely reported to play a major role in flavonol metabolism. *In vitro* incubations of flavonols commonly found in onions was carried out in the presence of rat liver homogenates. In this way, the route of their

metabolism can be evaluated and will give an indication as to the extent of their metabolism and susceptibility towards biotransformation.

8.1 Discussion of the techniques used in this study

This thesis involved extensive use of HPLC as a means of flavonol identification and quantification. Development of a post-column derivatization procedure involving formation of a flavonol-$Al(NO_3)_3$ fluorescent complex has allowed increased sensitivity and selectivity for flavonol analysis (Hollman *et al.*, 1996). Such method provided ease in analysing flavonol normally present in biological fluids in minute levels. Flavonols with a free hydroxyl group at C3 and a keto group at C4 can form fluorescent complexes with aluminium ion, hence increasing their sensitivity for the purpose of identification (Hollman *et al.*, 1996). The procedures of Hollman *et al* (1996) were made more reliable and suitable for automated HPLC by using 0.1 M rather than the much more viscous 1.0 M methanolic $Al(NO_3)_3$. This resulted in only a 2-3-fold drop in sensitivity and a ten-fold saving in the cost of $Al(NO_3)_3$.

The everted rat gut model adapted for studying intestinal transport of flavonols provided reliable results. It is a convenient way of studying intestinal transport due to its simplicity as well as sensitivity. The large standard error in certain incubations is indicative of the different characteristics (e.g thickness of intestine) and transport behaviour between different segments and also between different animals. The age of the rats used is important as intestine from young rats remain viable for longer periods than those taken from adult animals. However, newborn intestine is not suitable due to their fragility.

The liver is the main organ responsible for the metabolism of absorbed compounds. Development of *in vitro* methods to study hepatic metabolism has enabled the identification of metabolites produced following the metabolism of specific flavonols. This has allowed investigation of the relationship between flavonol structure and the extent of their metabolism. Such study is more complicated in *in vivo* systems

due to interference from other substances and other physiological processes as well as the difficulty of sampling.

8.2 Absorption of flavonols

The quercetin glucosides from onions were well absorbed into the plasma of human volunteers and excreted into urine. This was the first study which described the absorption of individual onion flavonols, mainly quercetin-4′-glucoside and isorhamnetin-4′-glucoside. So far, feeding studies with onions reported absorption of only quercetin glycosides, determined after acid or enzymic hydrolyses (Hollman *et al.*, 1995, 1996, McAnlis *et al.*, 1999). Such studies allow estimation of quercetin glycosides only after liberation of the aglycone from the sugar bonds. As they did not analyse the samples before acid/enzyme hydrolysis, it was not clear if the quercetin detected was actually that liberated from sugar bonds or the free quercetin already present before hydrolysis. With this method, the absorption and identification of specific flavonol glucosides is not known. However, the availability of standards as well as sensitive HPLC and post-column derivatization procedure has enabled the identification of the flavonol glucosides. The data obtained in the present study demonstrated absorption of flavonol conjugates, hence contrast and so refutes the hypothesis of Kuhnau (1976) that only flavonol aglycones are absorbed.

In our study, we found high plasma levels of isorhamnetin-4′-glucoside, a minor flavonol in onions whereas quercetin-4′-glucoside, which was present in high concentrations in onions, was present in low levels in plasma. Interestingly, quercetin-3,4′-diglucoside, which was the major onion flavonol was not detected in plasma of the 5 subjects despite being highly absorbed in the everted rat gut model. Our study demonstrated selective absorption of flavonols from the small intestine. On the other hand, their differential metabolism or sequestration into tissues following absorption may explain the results obtained. Several hypotheses are proposed to explain the results obtained based on our work and the existing literature.

The higher accumulation of isorhamnetin-4′-glucoside compared to quercetin-4′-glucoside is in agreement with another recent study where ileostomy volunteers ingested 200 g of fried yellow onions (Aziz *et al.*, 2000). This study reported approximately 40 times greater accumulation of isorhamnetin-4′-glucoside compared to quercetin-4′-glucoside in plasma of the volunteers following a meal of lightly fried onions. An 80 fold difference was obtained in our study (chapter 4). Interestingly, when the content of the ileostomy effluent was analysed for unabsorbed flavonols, more than 80% of the ingested isorhamnetin-4′-glucoside was recovered whereas only about 5% of the quercetin-4′-glucoside remained in the ileostomy effluent. Our *in vitro* incubations of onions with digestive juices to mimic the small intestinal digestion process demonstrated the stability of the flavonol glucosides. Less than 20% degradation was observed despite the presence of 0.1M HCl in the lumen of the small intestine (Chapter 4). Indeed, Hollman *et al* (1995) also reported the relative stability of flavonols to gastric juices *in vitro*. This implies that metabolism of flavonols did not occur in the lumen of the small intestine, leaving the flavonols unchanged for absorption. Thus, the flavonols recovered in the ileostomy effluent were the unabsorbed flavonols remaining after passing through the small intestine. The high recovery of isorhamnetin-4′-glucoside in the ileostomy fluid implies that intestinal absorption of this flavonol glucoside was not very efficient despite its presence in high levels in plasma. Certainly our *in vitro* everted rat gut model showed less efficient uptake of isorhamnetin-4′-glucoside compared to the other flavonol glucosides (Chapter 5). One explanation for the high levels of isorhamnetin-4′-glucoside in plasma is its poor metabolism and/or less efficient removal from the bloodstream. Longer retention of isorhamnetin in plasma compared to quercetin has been reported, possibly as a result of more effective release of isorhamnetin into the hepatic venous blood (Manach *et al.*, 1997). An alternative explanation is that a proportion of the absorbed isorhamnetin-4′-glucoside is excreted into bile and is reabsorbed via the enterohepatic circulation, thus maintaining its presence in plasma. Biliary excretion of conjugated isorhamnetin (Manach *et al.*, 1996) as well as conjugated forms of quercetin and tamarixetin (Crespy *et al.*, 1999) has been described in rats orally fed quercetin. It has also been proposed that some of the isorhamnetin-4′-glucoside present in plasma may have been formed by 3′-O-methylation of the absorbed quercetin-4′-glucoside (Aziz *et al.*, 1998), hence explaining higher levels of the former and lower levels of the latter. However, our *in vitro* experiments with liver homogenates did not show this. The

absence of the catechol structure on ring B, required for *O*-methylation by COMT (Creveling *et al.*, 1970) could have prevented this reaction. The possibility that tissues other than the liver playing a role in this reaction should also be considered. The kidney has been reported to have the ability to methylate the anthocyanin cyanidin-3-glucoside (Tsuda *et al.*, 1999). Alternatively, rat liver and human liver may have different metabolic properties.

Unlike isorhamnetin-4′-glucoside, the low recovery of quercetin-4′-glucoside in the ileostomy effluent implies their efficient absorption from the small intestine. Thus the low levels of this glucoside in plasma is likely due to their effective metabolism and/or removal from the bloodstream. One possible pathway for their metabolism is by deglycosylation of the sugar bonds, catalysed by the liver β-glycosidase. Our incubation study with liver extracts demonstrated almost 90% metabolism of quercetin-4′-glucoside (Chapter 7). Another study reported 69% deglycosylation of quercetin-4′-glucoside following incubations with cell-free preparations from human liver (Day *et al.*, 1998). This group also found extensive deglycosylation of quercetin-4′-glucoside following incubations with cell-free preparations from small intestine which may further explain their low levels in plasma. They proposed that deglycosylation occurs during the transport of quercetin-4′-glucoside across the intestinal membrane, releasing free quercetin. If this was the case, large amounts of free quercetin should be present in plasma and urine of the volunteers following the fried onion meal. However, we did not detect the presence of high levels of this aglycone in plasma and urine. This implies that if deglycosylation of quercetin-4′-glucoside occurred during their passage across the small intestine and in the liver, there is another pathway which metabolises or effectively removes the free quercetin from the bloodstream.

Our feeding study did not detect the presence of quercetin-3,4′-diglucoside in plasma or urine despite their efficient absorption in the everted rat gut model (chapter 5) and also in the study of Gee *et al* (1998). The ileostomy fluid of volunteers fed the fried onion meal was also devoid of this diglucoside (Aziz *et al.*, 2000). This suggests the efficient absorption of quercetin-3,4′-diglucoside from the small intestine. The diglucoside did not accumulate in plasma in detectable amounts despite its absence in the ileostomy fluid (Aziz *et al.*, 2000). This indicates that if quercetin-3,4′-diglucoside is

absorbed from the small intestine, there exists an efficient pathway that metabolises this compound or effectively removes it from the bloodstream. The liver has been proposed as the organ responsible for metabolism of quercetin-3,4′-diglucoside. Our *in vitro* hepatic metabolism of quercetin-3,4′-diglucoside showed approximately 50% metabolism (chapter 7). However, we only detected 2% of deglycosylation products, indicating the possible involvement of other reactions, possibly glucuronidation and/or sulphation. From our feeding study with the volunteers, estimation of levels of conjugated quercetin after the fried onion meal in the acid hydrolysed samples gave a percentage of intake of 0.97% at peak plasma concentration (chapter 4). Compared to the 0.13% accumulation of quercetin-4′-glucoside in the non-hydrolysed plasma, a percentage of the conjugated quercetin could be contributed by metabolites of this aglycone as well as its glucosides and diglucosides, possibly glucuronide or sulpho-conjugates. Certainly, more *in vitro* experiments are needed to confirm the metabolic pathway for flavonol glucosides as well as the metabolites formed as this is the most common ingested form of flavonols. Glucuronidation and sulphation of quercetin by enzymes of the liver is widely reported (Ueno *et al.*, 1983, Shali *et al.*, 1991).

In recent studies, the ability of several flavonoid glycosides as well as free quercetin and isorhamnetin to undergo glucuronidation during their passage across the small intestine has been described (Day *et al.*, 1999, Spencer *et al.*, 1999). This raises the possibility that the liver is not the only organ capable of this reaction. Although the authors did not investigate the glucuronidation of quercetin-3,4′-diglucoside and quercetin-4′-glucoside, the possibility of these two flavonols following this reaction should be considered. Spencer *et al* (1999) suggested that glucuronidation favours the presence of the catechol structure (3′,4′-*O*-dihydroxylation) on ring B. However, neither quercetin-3-glucoside nor rutin have this structure and both were absorbed mainly as glycosides. It should be noted that a higher concentration of the perfused compound was employed by Spencer *et al.* (> 50 μM) than was used in our study (10 μM). It is unlikely that such a high concentration of flavonols will be present in the small intestine at one time given that the average daily intake of flavonols is only 23 mg/d (Hertog *et al.*, 1993) spread throughout the day. Based on these results, it is highly unlikely that a large proportion of the quercetin-3,4′-diglucoside and quercetin-4′-glucoside pools are subjected to intestinal glucuronidation. Furthermore, these two

flavonol glucosides did not possess the catechol structure on ring B which have been proposed as a structural requirement for intestinal glucuronidation (Spencer *et al.*, 1999). Perhaps the liver plays a more prominent role in glucuronidation of flavonols. The characteristic of the liver glucuronyltransferase and sulphotransferase is not known and the requirements for binding to the flavonols are not known. It is possible that a different glucuronyltransferase and sulphotransferase from that found in the intestinal membrane is reacting in the liver.

8.3 Accumulation of flavonols in plasma

We estimated the relative area under the curve (AUC) of plasma flavonol glucosides and the aglycone in the onion study. In keeping with their presence in plasma, the bioavailability of quercetin-4′-glucoside was one eighth that of isorhamnetin-4′-glucoside. When AUC was estimated for conjugated quercetin after acid hydrolysis, a much higher value was obtained, approximately 2- fold and 16-fold higher than isorhamnetin-4′-glucoside and quercetin-4′-glucoside, respectively. Conjugated quercetin in the hydrolysed plasma may contain other glycosides, glucuronides or sulphates in addition to quercetin-4′-glucoside. Their bioavailability may be different from quercetin glucoside, depending on the rate and extent of their absorption from the intestine, hence the high AUC observed (Chapter 5).

Bioavailability most likely differs with different flavonols. Tomatoes for example are rich in quercetin rutinoside (Stewart, personal communication), apples contain high levels of quercetin galactoside (Lister, 1994) whereas tea contains mainly conjugated forms of quercetin and kaempferol as well as low levels of myricetin (Hertog, 1994). The bioavailability of dietary rutin in humans is not widely studied. A pilot study investigating the absorption of flavonols in human volunteers who ingested cherry tomatoes only detected very low levels of conjugated quercetin in plasma (<2%) (Crozier *et al.*, 2000). Unchanged rutin was not detected in samples of unhydrolysed plasma, probably because it was present at levels below the limits of detection. Bioavailability of pure rutin was one third (Hollman, 1997) that of quercetin glycosides from onions (Chapter 4). In addition, the bioavailability of quercetin glycosides from apples was also

lower than onions (Hollman, 1997). The fact that apples contain different sugar substitution attached to quercetin compared to onions may explain the difference in bioavailability (Lister, 1994). On the other hand, the bioavailability of tea flavonols has not been described, possibly because flavonol concentration in tea is lower than other dietary source and coupled with extensive metabolism will preclude their identification in plasma. A pilot study detected less than 2% accumulation of conjugated quercetin at peak plasma level following ingestion of 400 ml of black tea (Crozier *et al.*, 2000). The clear difference in bioavailability of quercetin glycosides described above suggests that eating tomatoes and apples or drinking tea may not increase plasma flavonol levels as much as onions.

Determination of the bioavailability of flavonols from various dietary sources can provide useful data on foods containing highly bioavailable flavonoids. The information is highly useful in epidemiology studies whereby the major source of dietary flavonols can be compared with their bioavailability. In this way, a more precise association between flavonol intake and disease occurrence can be made. Several epidemiology studies reported tea as the main source of flavonols, but tea flavonols did not appear to be highly absorbable (Crozier *et al.*, 2000). This may indicate that the inverse association may have been contributed by other compounds in tea such as catechins instead of flavonols.

8.4 Mechanisms of absorption of flavonols

The mode and extent of flavonol absorption from the small intestine is one of the determinants of their bioavailability. Results from both the onions feeding study and uptake of individual flavonols from the everted rat gut model suggest differences in the extent of their absorption. A carrier protein is the most likely vehicle for the transfer of flavonol glycosides across the intestinal membrane due to the polar properties of flavonol conjugates. Existing literature also seems to point to the involvement of a carrier protein for the transport of flavonols across the small intestine (Gee *et al.*, 1998). Attempts to identify the carrier involved have provided conflicting results. Our study

indicated that SGLT1 was unlikely to play a major role in mediating flavonol glucoside uptake across the small intestine (Chapter 6).

Clearly, more studies are required to determine the exact mechanism. Other transport pathways and carrier protein exist in the membrane of the small intestine responsible for the uptake of a variety of compounds. The prospect of these carriers being involved in mediating flavonol uptake should not be ruled out. We proposed several possible mechanisms for intestinal transport of flavonols (Figure 8.1). GLUT5, a fructose transporter is a potential carrier. It has a 39-65% sequence identity with other glucose transporters (Mueckler, 1994), suggesting a possible involvement in mediating the uptake of flavonols. In addition to SGLT1, the presence of a second Na^+-dependent glucose transporter in the brush border membrane has been suggested based on kinetic studies (Ferraris and Diamond, 1986). The ability of phloridzin to inhibit this transporter has not been determined and its involvement in flavonol uptake is possible. In addition to the known transporters, there maybe other carrier proteins with the ability to transport flavonols which to-date have not been identified. As mentioned previously (Chapter 6), several compounds including glucose are capable of being transported via the tight junction which can be regulated by SGLT1. However, the likelihood of flavonols following this pathway is dependent on their structure particularly their polarity.

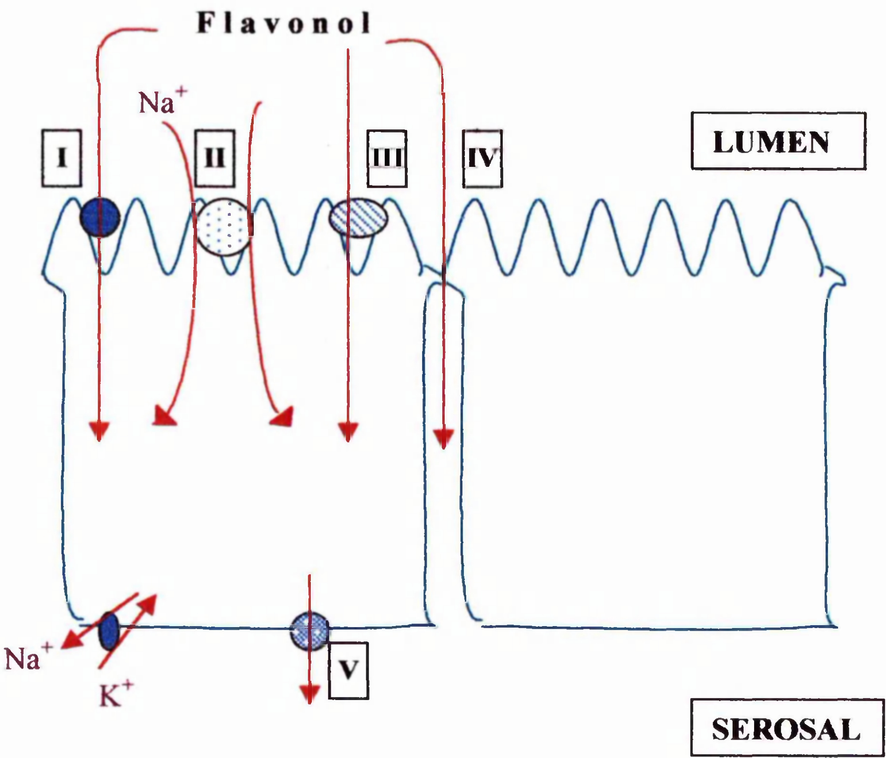

Figure 8.1: Possible mechanisms of intestinal flavonol transport.

Four possible routes are hypothesised for the transport of flavonols on the apical membrane and one possible route for transport across the basolateral membrane: (**I**) sodium-independent fructose transporter (GLUT5); (**II**) the sodium-dependent glucose transporter; (**III**) an unidentified transporter; (**IV**) the paracellular pathway and (**V**) the sodium-independent glucose transporter.

8.5 Metabolism of flavonols

8.5.1 Liver metabolism

Differences in the degree of metabolism of various flavonols by liver extracts imply structural-dependence on their metabolism. This was observed in the feeding study with onions (chapter 4). Excretion of onion flavonols in urine following ingestion of the fried onions ranged from as low as 0.2 % (quercetin-4′-glucoside) to 17.4 % (isorhamnetin-4′-glucoside), expressed as a percentage of the total intake (Chapter 4). Their accumulation in plasma showed a similar trend. Differences in excretion of the onion flavonols imply differences in the extent of their metabolism. Hollman *et al* (1995) reported 52% absorption of quercetin glucosides from onions whereas we detected less than 1% at peak levels in plasma and 0.8% in urine. This suggests extensive metabolism of quercetin glucosides following their absorption. Indeed, substantial metabolism of flavonol glucosides was seen with experiments using rat liver homogenates with percentage metabolism ranging from 32-91% (Chapter 7). Although we could detect only methylation and deglycosylation of flavonol glucosides, the possible involvement of other reactions should not be discounted.

In addition to methylation, liver is also capable of forming flavonol conjugates with glucuronic acid and sulphate. The enzyme glucuronosyltransferase and phenolsulfotransferase are responsible for glucuronidation and sulphation, respectively. Formation of glucuronides and sulphates of quercetin has been demonstrated using an isolated perfused rat liver system (Shali *et al.*, 1991). The types of metabolites formed appear to be dependent on the parent compound. In rats fed a quercetin diet, 92% of the circulating metabolites were glucurono-sulfo derivatives of isorhamnetin and quercetin (Morand *et al.*, 1998). The remaining 8% was contributed by the glucuronides of methoxylated forms of quercetin (e.g isorhamnetin or tamarixetin). The glucurono-sulpho derivative of isorhamnetin is 6 times higher than the glucurono-sulpho derivatives of quercetin. This implies extensive methylation of the parent compound. The extent of the same reaction occurring following ingestion of quercetin glucosides is not known. *In vivo* glucuronidation of quercetin glucosides following their ingestion has not been

reported. This is because analysis of quercetin conjugates from biological samples has always been done based on the free quercetin liberated after acid or enzymic treatment. Nevertheless, the prospect of this happening is likely and merits further investigation.

Our *in vitro* incubation of quercetin-3,4′-diglucoside with liver extracts showed about 32-54% metabolism of this diglucoside with 2% accumulation of quercetin-3-glucoside. If the same occurred *in vivo*, we should have detected some quercetin-3-glucoside in plasma or urine after the onion supplement. However, there was no evidence of this. This is probably a result of the extensive methylation of the liberated quercetin-3-glucoside to isorhamnetin-3-glucoside or possibly tamarixetin-3-glucoside. As these two methylated metabolites do not form fluorescent complexes with $Al(NO_3)_3$, which facilitates detection with enhanced sensitivity, their possible presence in plasma or urine could not be confirmed using an absorbance monitor operating at 365 nm. Conjugation is possibly the most common final step in the metabolic pathway of flavonoids to generate hydrophilic molecules to assist in their excretion.

8.5.2 Small intestinal metabolism

In addition to liver, recent findings suggest that the small intestine may play a substantial role in the initial metabolism of flavonoids. *In vitro* incubations of fried onions with digestive juices demonstrated the stability of onion flavonols towards enzymes in the juices as well as the acidic environment. However, deglycosylation and glucuronidation of flavonols has been reported in the small intestine (Noteborn *et al.*, 1997, Day *et al.*, 1998, Shimoi *et al.*, 1998, Spencer *et al.*, 1999). It was proposed that these reactions occur in the brush border membrane during flavonol transport across the intestinal membrane rather than in the lumen of the intestine (Day *et al.*, 1998). Quercetin was mainly glucuronidated whereas quercetin-3-glucoside was deglycosylated more extensively than being subjected to glucuronidation during their movement across the intestinal wall (Spencer *et al.*, 1999). If deglycosylation of quercetin-3-glucoside occurred during their uptake, we should have detected quercetin aglycone in our everted segment incubations. However, this was not apparent and several reasons could have caused this. The 10 μM concentration of quercetin-3-glucoside was probably too low to

see any measurable amount of free quercetin in the serosal side, given that the percentage absorption of the parent compound was low in the first place. Furthermore, Spencer *et al.*, 1999, in their study used a higher concentration of quercetin-3-glucoside and longer incubation periods and such high concentration may not be achieved at normal dietary intake.

8.5.3 Colonic metabolism of flavonols

An *in vitro* fermentation of digested and undigested fried onions was carried out to investigate colonic bacterial metabolism of flavonols which escaped absorption. Extensive degradation of flavonols occurred with neither flavonol glycosides nor their aglycones being present in significant amounts at the end of a 24 h incubation period. The liberated through hydrolysis were not detected. Thus, absorption of unchanged flavonols in the large intestine is improbable, leaving the small intestine responsible for flavonol absorption. Ring fission of flavonols appears to be the main step in colonic bacterial metabolism of flavonoids. This reaction yields metabolites such as phenolic acids which possess considerable antioxidant activities and can be absorbed (Kim *et al.*, 1998). Hence, a small part of the ingested onion flavonols which escaped absorption in the small intestine could still provide beneficial health properties through their action with colonic microorganisms. Studies investigating the extent of phenolic acid absorption following ingestion of flavonol-rich food and their antioxidant activities can provide more information on the potential of these metabolites.

8.6 Proposed pathway for the metabolism of flavonols

Based on the results obtained in this study as well as published reports, we proposed a hypothetical pathway for the metabolism of flavonols. Absorption of flavonols occurred in the small intestine, most likely in the jejunal segments. Following absorption, flavonols enter the portal vein primarily as the unchanged compound. Glucuronidation and deglycosylation may occur in the liver as well as sulphation and

methylation before appearing in the general circulation (see Figure 7.8, chapter 7). Again, the extent of these reactions occurring on the flavonol molecule is highly dependent on their structure. The kidney may be involved in methylation of flavonols (Piskula and Terao, 1998, Tsuda *et al.*, 1999) although the extent of their contribution is not known. Deglycosylation by the liver may only be a minor pathway as the metabolism of flavonols tend to go in the direction of producing more polar molecules for the purpose of excretion. This was observed in rats fed a diet of quercetin whereby conjugated forms of quercetin including glucuronides and sulphates were detected in plasma instead of aglycone (Ueno *et al.*, 1983; Manach *et al.*, 1997). The order in which these reactions occurred and the metabolites that accumulate will be dependent on the flavonols present, their structure and sugar substitution. In a study where rats were fed a quercetin diet, conjugated forms of isorhamnetin accumulated in levels ca. 4.5 fold higher than quercetin conjugates (Manach *et al.*, 1995). This implies methylation as a more prominent reaction in the metabolism of quercetin than conjugation.

Following the above reactions, the metabolites generated can be sequestered in various tissues or excreted either via urine or bile. Metabolites excreted in bile are capable of further metabolism and absorption through the enterohepatic circulation.

8.7 Absorption of flavonols and the impact of their biological properties.

Based on the onion feeding study, flavonols particularly quercetin conjugates persist in the blood for a number of hours following consumption and had a long elimination period. Levels above baseline could be detected in samples of several volunteers collected 24 h after they first ate the onions. Subjects on a normal diet had quercetin levels of 25 ng/ml. This implies that continuous consumption of high flavonol food can lead to an elevated steady state level of flavonols in plasma. Owing to the low bioavailability and extensive metabolism of flavonoids, consumption and identification of highly bioavailable flavonoids is crucial.

Results from this present study, together with published reports show very low levels of the unchanged administered flavonol excreted in urine (< 20%). The relevance of the absorption and metabolism of flavonols in the human body is ultimately related to their biological properties particularly as antioxidants. Flavonol aglycones are more potent antioxidants than their glycosides owing to the higher numbers of free OH groups for hydrogen donation (Rice-Evans *et al.*, 1996). However, flavonols in the diet exist almost exclusively in the glycoside form. Although some flavonol glycosides may undergo deglycosylation during their absorption, the likelihood that the resulting aglycone will circulate in the blood circulation is minimal. Most likely, the flavonol aglycone will be further metabolised by the liver or kidney to conjugated flavonol to give a more polar molecule.

Thus, epidemiological studies describing association between flavonol intake and reduced risk of heart disease is probably not caused by the unchanged flavonols due to their extensive metabolism. Rather, the protective effect is probably a result of the biological properties of the metabolites of flavonoids or the involvement of other compounds with antioxidant properties. Several phenolic acids, arising from ring fission of quercetin glycosides showed considerable antioxidant activities (Merfort *et al.*, 1996). Phenolic acids for instance 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid were more effective in anti-platelet aggregation activity than quercetin (Kim *et al.*, 1998). Thus, the potential of the metabolites of flavonoids acting as antioxidants cannot be discounted. Alternatively, the association observed with increased flavonoid intake may only mean that high flavonoid intake can be associated with a healthier lifestyle, consumption of high amounts of fruit and vegetable which are rich in other antioxidants and regular exercise.

8.8 *In vivo* antioxidant activity of flavonoids

Several flavonols showed higher antioxidant activity than vitamins C and E. Quercetin, for instance, is more potent than the 2 vitamins when their antioxidant activity was measured as the Trolox equivalent antioxidant activity (TEAC) (Rice-Evans *et al.*, 1997). The TEAC technique measures the relative ability of flavonoids to scavenge the

ABTS radical cation (2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) generated in the aqueous phase, compared with standard amounts of Trolox, a synthetic water-soluble vitamin E analogue. Using a different antioxidant assay, flavonoids were again shown to have higher antioxidant activities than vitamin C, E and β-carotene. In this instance, antioxidant activity was measured as the concentration of flavonoids needed for 50% inhibition of LDL oxidation (Vinson *et al.*, 1995).

In *in vitro* experiments, metabolites of quercetin including quercetin glucuronides were shown to significantly delay copper-induced lipoprotein oxidation at concentrations of 0.5 μM (Morand *et al.*, 1998). Furthermore, in another recently published article, several quercetin glucosides found in onions, mainly quercetin-3-glucoside and quercetin-4′-glucoside were capable of inhibiting lipoxygenase-induced LDL oxidation *in vitro* (Da Silva *et al.*, 1998). Quercetin and quercetin-3-glucoside exhibited a higher inhibitory effect than quercetin-4′-glucoside with IC_{50} values of 0.4, 0.5 and 1.2 μM respectively. Glucose substitution at C-4′ significantly reduced antioxidant activity, due to absence of C-3′,4′ *O*-dihydroxylation, which is one of the requirements for strong antioxidant activities (van Acker *et al.*, 1996). It is highly likely that isorhamnetin-4′-glucoside will exhibit similar antioxidant properties as quercetin-4'-glucoside due to sugar binding at C-4′.

One way to determine the effectiveness of flavonol consumption is to evaluate their *in vivo* antioxidant activity. This can be done by measuring the antioxidant activity of a sample of plasma following ingestion of a high-flavonoid food. One recent study measured plasma antioxidant activity of human volunteers following ingestion of 225g fried onions (McAnlis *et al.*, 1999). Although the antioxidant activities of plasma increased after the onion meal, it was not enough to cause a significant change in the susceptibility of plasma to LDL oxidation. This study gave a peak plasma concentration of 284.4 ng/ml (0.82 μM) for quercetin. Comparing this value with the IC_{50} values of quercetin metabolites obtained by Da Silva *et al* (1998) (0.4 – 1.2 μM) and the concentration for inhibiting copper-induced lipoprotein oxidation (Morand *et al.*, 1998) (0.5 μM), some inhibition should have been observed in LDL oxidation. However, the possibility of quercetin binding to protein in plasma may explain the lack of inhibition of LDL. Binding of quercetin to plasma albumin following their oral administration to rats

has been described (Manach *et al.*, 1995). Furthermore, an *in vitro* study also demonstrated extensive binding of quercetin to plasma proteins with more than 90% attached to serum albumin (Boulton *et al.*, 1998). The extent to which plasma binding inhibits the cellular association of quercetin is of utmost importance as this may affect their antioxidative capability.

In our study, we obtained a peak plasma concentration of 45 ng/ml (0.1 μM) for quercetin-4′-glucoside after the fried onion meal. This concentration was much lower than the reported IC_{50} of 1.2 μM for quercetin-4′-glucoside (Da Silva *et al.*, 1998). This implies that the presence of quercetin-4′-glucoside at this concentration was probably insufficient to inhibit LDL oxidation. In contrast to this, the 452 ng/ml (1.5 μM) peak plasma concentration of conjugated quercetin was significantly higher than the *in vitro* quercetin conjugates concentration of 0.5 μM required to delay copper-induced lipoprotein oxidation (Morand *et al.*, 1998). This is assuming that a large proportion of the quercetin conjugates is present as glucuronides. Thus, there is a high possibility of plasma inhibiting lipoprotein oxidation at this concentration and this clearly merits further investigation. In theory, we probably do not consume 300 g of fried onions in a single meal. However, consumption of flavonol-rich foods spread throughout the day may lead to build-up of flavonols in the circulation. Although the onions used in our feeding studies did not contain quercetin-3-glucoside, which showed substantial antioxidant activity (Da Silva *et al.*, 1998), other varieties of onions contain substantial levels of this monoglucoside (Tsushida and Suzuki, 1996, Price and Rhodes, 1997). Since their *in vitro* antioxidant activity is higher than that of quercetin-4′-glucoside (Da Silva *et al.*, 1998), further investigation on the absorption of quercetin-3-glucoside is interesting in view of its potential role as an *in vivo* antioxidant.

Based on the IC_{50} values for quercetin and the concentration of quercetin glucuronides required to inhibit lipoprotein oxidation, the reported daily quercetin intake of 16 mg (Hertog *et al.*, 1994) will be insufficient to cause any beneficial antioxidant activities. Assuming 1% of the total intake was present at peak plasma concentration and approximately 3 L volume of plasma, a circulating quercetin concentration of 0.17 μM is obtained at peak levels. Thus, the presence of quercetin at this concentration is likely insufficient to prevent LDL oxidation. Nevertheless, the bioavailability of quercetin

from different dietary sources may vary, hence influencing antioxidant capacity of plasma. Therefore, studies evaluating the absorption of flavonols following ingestion of different flavonol-rich foods can provide more information on their bioavailability. At the same time, encouraging the public to increase intakes of high-flavonol foods above the present reported levels may lead to increase in plasma flavonol concentration.

8.9 Prospects for future research

8.9.1 Bioavailability of flavonoids

Clearly, there is still limited information on the bioavailability of flavonoids. Our main interest is of the bioavailability of flavonoids from dietary sources. Although extensive studies have been performed on the absorption of flavonols from onions, not much is known regarding the bioavailability of flavonols from other main sources such as tea, tomatoes, apples, red wine and fruit juices. In addition, estimation of the absorption of specific flavonol glycosides will provide us with information regarding the bioavailability of these flavonols. Identification of the absorption of specific flavonol glycosides is desirable rather than their estimation based on the aglycone liberated following acidic or enzymic hydrolyses. This is because similar flavonol conjugates, but with variations in the nature/position of the sugar moiety, may be absorbed at different rates. Therefore, hydrolysis of the sugar bonds or any glucuronide or sulphate bonds will only give estimates of the overall bioavailability of the flavonoid conjugates and not the individual compounds. Ultimately, such studies are useful in determining the relationship between the structure of flavonoids including sugar substitution and the extent of their absorption.

8.9.2 Metabolism of flavonoids

In addition to bioavailability, knowledge on the metabolism of flavonoids is also vital to determine the end products and the route of their metabolism. In view of the extensive metabolism of flavonols particularly quercetin glucosides, perhaps research should focus instead on identifying the metabolites produced. The prospect of tissues

other than the liver playing a role in flavonol metabolism should also be investigated. Metabolic studies with radiolabelled flavonols are the best approach as metabolites produced can be easily tracked and identified. The availability of complex instrumentation, for instance mass spectrometry analysis has opened up a wider area of research and enabled the identification and confirmation of various structures of flavonols which could not be done on a normal HPLC system. Identification of metabolites is important towards evaluating the biological properties of dietary flavonoids. It is not sufficient from epidemiology evidence to conclude that high intakes of flavonoids are protective against diseases if in truth, they are metabolised to a non-reactive form.

8.9.3 The mode and extent of flavonoid absorption

The mechanisms of flavonoid absorption from the intestine warrants further research. *In vitro* methods are ideal as they allow easy access to the serosal side of the intestine. Thus, flavonols absorbed can be immediately sampled prior to metabolism as would have probably occurred with *in vivo* system. Determination of the carrier protein requires a more specific method. Experiments involving isolation of brush border vesicles for instance can be used to investigate the involvement of SGLT1 in flavonoid uptake. In addition, the expression of specific transporters for instance SGLT1 or GLUT5 in the oocytes of *Xaenopus laevis* offers a powerful tool for identification of the carrier involved for intestinal transport of specific compounds (Hediger and Rhoads, 1994). Such method can be adapted for investigating the transport system of flavonols. Furthermore, this technique can also be used to identify the actual flavonol glucoside transporter if any of the transporters mentioned before did not play a role in their uptake. As current data are limited and showed contrasting results, clearly more studies are needed, comparing various techniques for intestinal absorption, before any conclusion can be drawn.

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CHAPTER 9: CONCLUSION AND RECOMMENDATIONS

9.1 Conclusion

9.1.1 Absorption of flavonoids

Absorption of quercetin occurred in the small intestine. Following a single dose of fried onions, there was higher accumulation of isorhamnetin-4′-glucoside than quercetin conjugates, including quercetin-3,4′-diglucoside and quercetin-4′-glucoside in plasma and urine of humans. Glycosylation of flavonoids enhanced their absorption. A carrier protein is most likely involved in the uptake of flavonoid glycoside across the intestinal membrane. The study described in this thesis provided no evidence that the sodium-glucose co-transport protein (SGLT1) mediates the uptake of flavonol glucosides. In addition, quercetin-3-glucoside did not inhibit the glucose transport pathway. Structural modification in the flavonoid molecule, particularly the nature/position of sugar substitution determines the extent of their absorption.

9.1.2 Metabolism of flavonoids

Based on the low levels of flavonols appearing in plasma and excreted in urine after the onions feeding study, flavonols especially quercetin glycoside are extensively metabolised. Methylation is one route for metabolism of flavonols with a catechol moiety, for example quercetin and quercetin-3-glucoside. Deglycosylation of flavonol glycosides may occur to a certain extent although reactions favouring the formation of more polar molecules appear to predominate. The extent of flavonol metabolism by the liver is dependent on their structure as well as sugar substitution. Changes in stereospecificity will affect binding to the active site of the relevant metabolic enzymes. It remains to be determined whether metabolites arising from flavonol metabolism have the potential to act as antioxidants and provide protection against oxidative damage.

9.1.3 Flavonols in plasma and antioxidant activity

The reported average quercetin intake of 16 mg/d is probably insufficient to provide significant quercetin concentration in the circulating blood to prevent LDL oxidation. Consumption of foods containing highly bioavailable flavonoids, spread throughout the day can potentially lead to build up of flavonols in plasma. Determination of the concentration of quercetin metabolites in plasma and their antioxidant ability is required to fully evaluate the beneficial effects of flavonoids.

9.2 Application of findings

Knowledge on the absorption and metabolism of flavonoids can be used to evaluate their biological role, particularly as antioxidants, and their ability to prevent LDL oxidation. Determination of the structural modification of the ingested flavonols is essential to obtain further understanding of their beneficial health properties *in vivo*. Identification of flavonoids that are highly bioavailable can be used as a public health message to encourage increased consumption of foods containing high concentration of these flavonols, which will accumulate in high levels in the blood stream.

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