Dietary Flavonoids: Bioavailability and Biosynthesis

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To my husband and daughter

Who have provided me with love, support and encouragement

Abstract

Flavonoids are one of the largest groups of natural plant products and are found in most, if not all, fruits and vegetables. As dietary components, flavonoids have widespread biological properties and have been associated with several health benefits, including reduced risk of cancer and cardiovascular disease. The flavonol, quercetin, one of the most ubiquitous dietary flavonoids, is found principally as glycoside conjugates in plants and quercetin-3-glucosylrhamnoside (rutin) is one of the more common forms. To establish the role of rutin as a protective agent *in vivo*, it is critical to understand the chemical nature of the absorbed forms and their *in vivo* concentrations in the circulatory and excretory system. The bioavailability of rutin is not very well understood as reflected by the varying peak plasma concentrations (C_{max}) and the time taken to reach the peak plasma concentration (T_{max}) reported in the literature. This may, in part, be due to the analytical techniques used which include acid or glucuronidase/sulphatase treatments to release the parent aglycone prior to quantitative analysis by low resolution, isocratic reverse-phase HPLC. In addition, there has been some debate on the types of rutin catabolites produced as a result of colonic breakdown. There is therefore a need for further more detailed information on the absorption, metabolism and bioavailability of rutin.

The underlying objective in Chapter 3 was to investigate the metabolism and absorption of rutin *in vivo* after ingestion of tomato juice supplemented with 164 μmoles (100mg) of rutin. Rutin was found to be extensively metabolised and made bioavailable to humans reflected in the identification of many phase II metabolites and catabolites. Using HPLC-photodiode array (PDA) detection, the phase II metabolites of rutin in the circulatory system were identified with an electronspray ionisation MS^2 as isorhamnetin-3-glucuronide and quercetin-3-glucuronide. The C_{max} of the two plasma metabolites ranged from 5.2-32 nM; with an average accumulation of 15 nM, which corresponds to 0.01-0.06% of the rutin ingested. In contrast, a total of nine rutin metabolites were detected in urine samples. In addition to the plasma metabolites, methylquercetin diglucuronide, three isomers of quercetin glucoside glucuronide, quercetin diglucuronide, quercetin-4'-glucuronide and quercetin-3'-glucuronide were found to accumulate in the urine. There were considerable inter-individual variations in the excretion of the urinary metabolites where a range of 4981 ± 115 to 40 ± 1 nmoles of metabolites were excreted over a 24 h period after the ingestion of the rutin

supplement. Rutin metabolites did not appear in the circulatory system until 4-5 h after ingestion indicating that absorption was occurring in the large rather than in the small intestine. More direct proof was provided in studies with ileostomy volunteers where 92% of the rutin was found to pass through the small intestine and emerged without modification, in the ileal fluid. In the large intestine, rutin appeared to be rapidly catabolised by the colonic microflora leading to ring fission and the appearance in urine of phenolic acids in amounts corresponding to 23% of rutin intake. Utilising both healthy humans and subjects with an ileostomy, three phenolic acids were identified as being potential ring fission products of rutin, namely, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid.

To investigate the role of colonic microflora in the breakdown of rutin, an *in vitro* faecal fermentation study was carried out as discussed in Chapter 4. Accumulation of quercetin in the fermentation vessel as early as 4 h after incubation indicated that deglycosylation was the initial step in the colonic breakdown of rutin. The addition of glucose to the fermentation media enhanced the deglycosylation process by almost 20 h. To identify and quantify further degradation products, which may be produced in smaller concentrations, 55 μmoles of quercetin spiked with 18 x 10^6 dpm [^{14}C]quercetin (60 nmoles) was used as the substrate in *in vitro* fermentation trial. Utilizing the HPLC-MS^2 with atmospheric pressure chemical ionisation (APCI) and PDA detection, a range of intermediate products and catabolites of quercetin were identified. The first intermediate products of quercetin degradation were detected after 2 h of incubation and they were identified as taxifolin, alphitonin and an isomer of taxifolin. Subsequent fermentation products identified were 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 3-(3-hydroxyphenyl)propionic acid. The main catabolite to accumulate during *in vitro* fermentation of faecal samples with quercetin was 3,4-dihydroxyphenylacetic acid (60-97% conversion). Addition of glucose to the fermentation media enhanced both the rate of deglycolysation of rutin and the subsequent catabolism of quercetin, the released aglycone. It was envisaged that the presence of glucose altered the microflora colony, enhancing the growth and the multiplication of bacterial strains responsible for the degradation of quercetin.

The isoflavone, genistein is considered to be one of the most potent flavonoid compounds due to its pharmacological activities as a tyrosine kinase inhibitor and as a phytoestrogen. With limited consumption of genistein-rich food such as soybean, in Western cultures, the idea of genetically directing the synthesis of genistein in plants that do not synthesize isoflavones has gained widespread interest in recent years. The study in Chapter 5 aimed at achieving this objective. An attempt was made to direct the synthesis of genistein in arabidopis and in tomato by introducing isoflavone synthase (IFS), which is the key enzyme for the entry into the isoflavonoid biosynthetic pathway. The full length *IFS* cDNA from soybean roots was isolated and cloned into binary vectors, pGlasglow and pCHF3 habouring the CaMV 35S promoter and into pER8 vector carrying an estrogen inducible G10-90 promoter. Using agrobacterium mediated transformation, the *IFS* gene was introduced in arabidopsis and tomato. Due to the nature of the pER8 construct and its G10-90 promoter, a 3-10 fold higher gene expression level was observed in arabidopsis transgenic plants transformed by pER8 binary vector than those transformed with pGlasglow. High levels of gene silencing was observed when using the CaMV 35S in arabidopsis and total gene silencing was observed when the same promoter was used in tomatoes. HPLC-PDA-MS^2 analysis of leaf extracts in the highly and moderately expressed *IFS* arabidopsis lines failed to detect the presence of genistein. Western blot analysis implied that IFS proteins were synthesized and accumulated in the leaves of arabidopsis and were present 'freely' in the cytoplasm rather than being membrane bound as they would in their natural environment. It was, therefore, hypothesised that the 'free' IFS was not able to access the substrate, naringenin which may be compartmentalized in a pre-existing multienzyme complex, and as a consequence, IFS was unable to synthesize genistein.

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Author's Declaration

The composition of this thesis and the work described within it was carried out entirely by myself unless otherwise cited or acknowledged. The research for this thesis was carried out between December 2001 and May 2003.

Signed.....

Indu Bala Jaganath

August 2005

ABBREVIATIONS

ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
amu	atomic mass unit
APCI	atmospheric pressure chemical ionization
AUC	area under plasma concentration-time curve
BMI	body mass index
CHD	coronary heart disease
Cmax	maximum plasma concentration
Col	Columbia
dH_2O	distilled water
ESI	electrospray ionization
FRAP	ferric reducing antioxidant power
HPLC	high performance liquid chromatography
IFS	isoflavone synthase
LC-MS	liquid chromatography mass spectrometry
λmax	absorbance maxima (wavelength)
MS	mass spectroscopy
$[M-H]^-$	negatively charged molecular ion
$[M+H]^+$	positively charged molecular ion
m/z	mass to charge ratio
PDA	photodiode array
RT-PCR	reverse transcriptase polymerase chain reaction
ROS	reactive oxygen species
RNS	reactive nitrogen species
SE	standard error
Tmax	time to reach *Cmax*
T-DNA	transfer DNA
t_R	retention time
UV	ultraviolet
v/v	volume / volume
w/v	weight / volume
35S	cauliflower mosaic virus 35S promoter

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1: Introduction

1.1 Functional food and health implications

An emerging trend in human healthcare is the focus on the link between diet and disease. Expanding knowledge of the role of physiologically active food components, from both plant and animal sources, has notably changed the role of diet in health. This new dimension of foods called "functional foods" provide an opportunity to combine high compliance food products with biologically active molecules as a strategy to consistently correct metabolic disturbances that lead to chronic disease (Salminen *et al.*, 1998). The beneficial components, which are physiologically active in functional foods have been referred to by various terms such as phytochemicals, functional components, and bioactive components (Pennington, 2002).

Bioactive compounds typically occur in small amounts in foods. Inherently, bioactive compounds have more subtle effects than nutrients as they are more likely to influence cellular activities that modify the risk of disease (Kris-Etherton *et al* 2004). Functional food components have been associated with the prevention and/or treatment of at least four of the leading causes of death in the world: cancer, diabetes, cardiovascular disease, and hypertension. It is also associated with the prevention and/or treatment of other medical ailments including neural tube defects, osteoporosis, abnormal bowel function, and arthritis (Ahmad, 2002). A great deal of epidemiological evidence has indicated that the bioactive ingredients in fruits and vegetables are responsible for the protective effects against numerous forms of cancer and cardiovascular diseases. Epidemiological and observational studies have consistently shown an inverse association between consumption of vegetables and fruits and the risk of human cancers and heart diseases (van't Veer *et al.*, 2000; Jensen *et al.*, 2004). As a result of these studies there has been an active search to identify bioactive compounds particularly from fruit and vegetables to determine their associated health effects and to elucidate their underlying biological mechanism of action.

Through intensive research in past decade, an impressive and growing number of bioactive compounds have been identified that have potentially important health benefits. To date there are approximately 120 naturally occurring foods that have been identified as containing bioactive components and could therefore be designated as functional (Pennington, 2002). These include a range of chemical compounds with

varying structures such as carotenoids, flavonoids, plant sterols, omega-3 fatty acids, allyl and diallyl sulfides, indoles (benzopyrroles), and phenolic acids. These compounds can act as antioxidants, antibacterial, antiviral, enzyme inhibitors and inducers, inhibitors of cholesterol absorption, decreasing platelet aggregation, inhibitors of receptor activities, and inducers and inhibitors of gene expression. (Pennington, 2002 and Kris-Etherton *et al.,* 2004). Due to the chemical and biological diversity of biological active compounds, the range of their molecular targets, and their possible interactions, the research in this field can be immensely complex. One of the more important groups of bioactive components that will be focused on in the present study is flavonoids.

1.2 Flavonoids

Since ancient times, flavonoids appear to have played a major role in the successful medical treatments and their use has persevered up to now (Havsteen, 2004). Nutritionists became interested in flavonoids in the 1930's when it was shown that flavonoids from citrus fruits decreased capillary permeability and had some antioxidant properties (Rusznyák and Szent-Györgi, 1936). In the 1990's flavonoids once again became popular, principally due to a combination of increased concern surrounding high levels of coronary heart disease and a reduction in its incidence by dietary components. Hertog *et al.* (1993) in a study in the Netherlands, reported that flavonol/flavone intake was inversely associated with both mortality and with the incidence of myocardial infarction. Since then, the literature on flavonoids has increased tremendously. At present, flavonoids are one of the major foci of nutritional and therapeutic interest, especially in its involvement as cardioprotection, neuroprotection, and as chemopreventive agents (Havsteen, 2004).

1.3 Distribution and chemical form of flavonoids

The flavonoids are qualitatively and quantitatively one of the largest groups of natural products known (Havsteen, 2004). Favonoids are distributed mainly in plants as they alone possess the ability to synthesize flavonoids. Since many flavonoids are pigments, their colors are undoubtedly associated with some of their important biological functions. As pigments, their electronic properties appear to include not only energy capture and transfer, but also in biological selectivity (Havsteen 2004). The latter is related with the attraction of suitable pollinators, e.g., insects and birds. In

addition to this, flavonoids are also associated with the selective activation of light-sensitive genes, where it is now evident that plants are using light not only as a source of energy, but also for gene regulation (Kirby & Styles, 1970).

Flavonoids are polycyclic structures consisting of 15 carbon atoms based on a C_6-C_3-C_6 skeleton. In a few cases, the six-membered heterocyclic ring C occurs in an isomeric open form or is replaced by a five-membered ring such as in chalcone. According to the cyclization and the degree of unsaturation and oxidation of the three-carbon segment, they can be classified into several groups, the main ones being flavones, flavonols, flavan-3-ols, flavanones, isoflavones, and anthocyanidins (Figure 1.1).

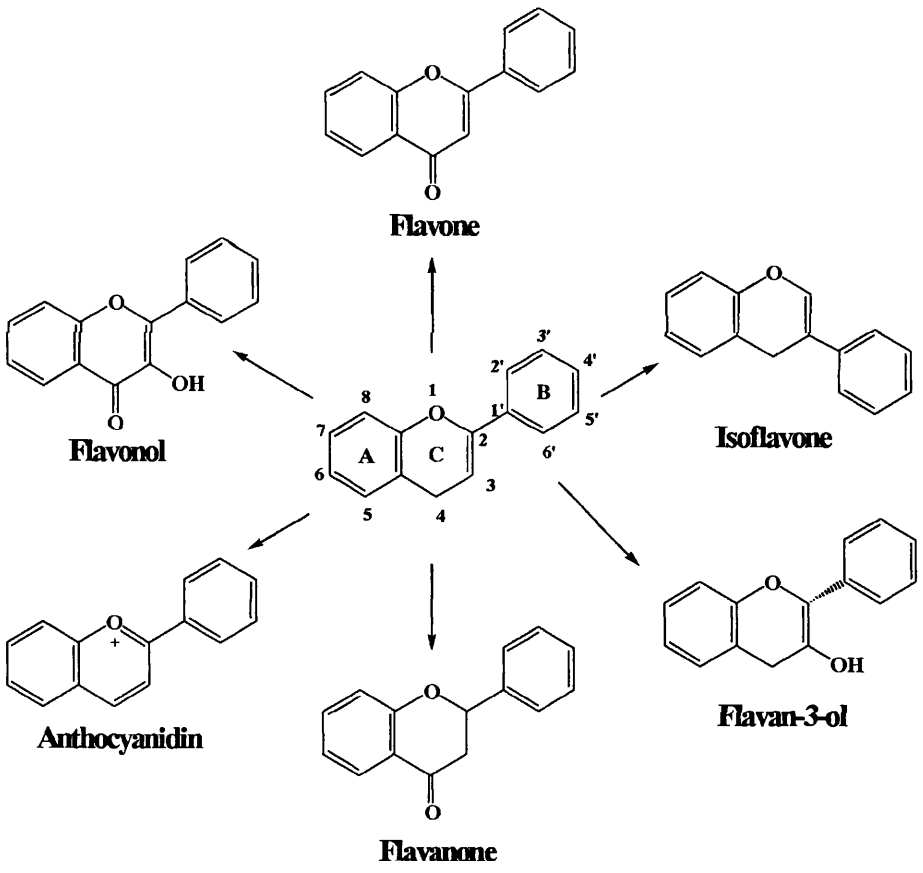
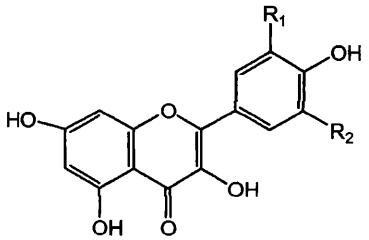


Figure 1.1. The main generic structures of flavonoid

In most cases, the flavonoids are present as glycosides in vacuoles of flowers, leaves, stems or roots ((Iwashina, 2000). At present, about 400 flavone aglycones, 450 flavonol aglycones, 350 flavanone aglycones, 300 isoflavone aglycones, 19 anthocyanidins and 250 chalcone aglycones have been reported (Iwashina, 2000). Flavonoid conjugation occurs in plants with additional substituent groups such as hydroxyl, methoxyl, methyl and/or glycosyl units. Occasionally, aromatic and aliphatic acids, sulphate, prenyl, methylenedioxyl or isoprenyl groups also attach to the flavonoid nucleus and their glycosides. Thus, more than 4,000 kinds of flavonoids have been reported as naturally-occurring compounds (Iwashina, 2000).

1.3.1 Flavonols

Flavonols are widespread in plant foods; the main flavonols are quercetin, kaempferol, isorhamnetin and myricetin (Figure 1.2). Of these the most common flavonol in the diet is quercetin. It is present in various fruits and vegetables, but the highest concentrations are found in onion (Hertog *et al.* 1992). Quercetin is present in plants in many different glycosidic forms and in onions, it is present principally as quercetin-4'-glucoside and quercetin-3,4'-diglucoside. Another widespread form of quercetin is quercetin-3-glucosyl-rhamnoside, more commonly known as rutin. Rutin is found abundantly in crops such as buckweed, tomatoes and asparagus. Other quercetin glycosides present in the diet are, quercetin galactosides (apples) and quercetin arabinosides (berries). Other flavonols in the diet include kaempferol (broccoli), myricetin (berries, maize) and isorhamnetin (onion and pears) (Peterson and Dwyer, 1998).

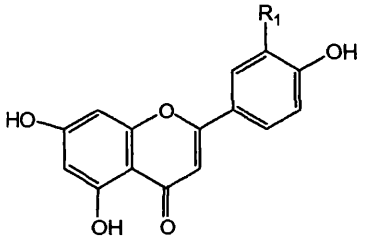


R_1=H R_2=H: kaempferol
R_1=OH R_2=H: quercetin
R_1=OH R_2=OH: myricetin
R_1=OCH_3 R_2=H: isorhamnetin

Figure 1.2. Structure of common flavonols.

1.3.2 Flavones

Flavones are structurally very similar to flavonols (see Figure 1.3) but differ from flavonols only in the absence of hydroxylation at the 3-position in the C-ring. The main flavones in the diet are apigenin and luteolin but since they are not so widely distributed (Crozier, 2003), their dietary intake is rather low. They are most prominent in cereals, herbs and vegetables such as celery, sweet red pepper and parsley (Peterson and Dwyer, 1998).

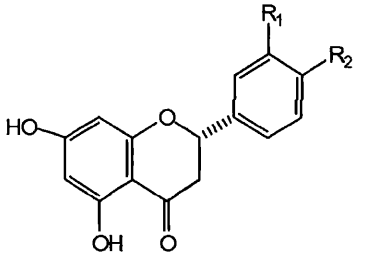


R_1=H; apigenin
R_1=OH luteolin

Figure 1.3. Structure of common flavones.

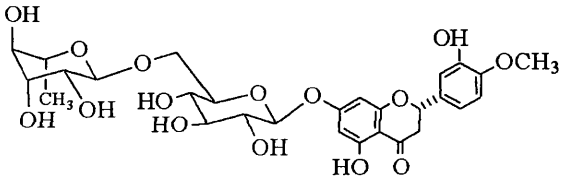
1.3.3. Flavanones

Flavanones are mainly represented by naringenin, hesperitin and eriodictyol. Two structural features, i.e., the absence of a $\Delta^{2,3}$ double bond, and the presence of a chiral center at the carbon-2, characterize flavanones (Iwashina, 2000) (Figure 1.4). The flavanone structure is highly reactive and they have been reported to undergo hydroxylation, glycosylation and *O*-methylation reactions (Crozier, 2003) (Figure 1.5). Flavonones are found principally in citrus fruit mainly as hesperitin and contribute to the flavour of citrus (Peterson and Dwyer, 1998). Naringenin is found in tomatoes and tomato-based products. Fresh tomatoes, especially the skin, also contain naringenin chalcone, which is converted to naringenin during the manufacture of tomato ketchup (Krause & Galensa, 1992).

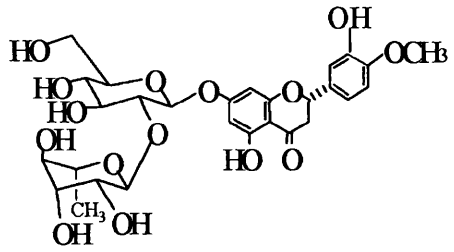


R_1=*H* R_2=*OH:* *naringenin*
R_1=OH R_2=OH: eriodictyol
R_1=OH R_2=OCH_3: hesperetin

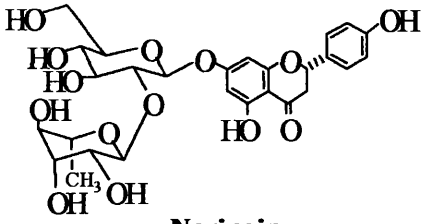
Figure 1.4. Structure of common flavanones



Hesperidin
(hesperitin -7-*O*-rutinoside)



Neohesperidin
(hesperitin-7-*O*-neohesperidoside)

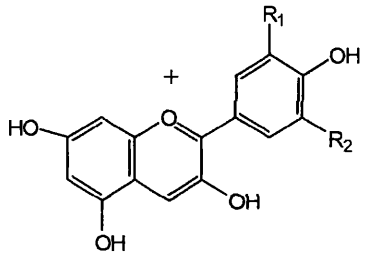


Naringin
(naringenin-7-*O*-neohesperidoside)

Figure 1.5. Glycosides of flavanones

1.3.4 Anthocyanidins

Anthocyanidins are widespread in higher plants (Timberlake, 1988). Nineteen types of naturally-occurring anthocyanidins have been reported (Iwashina, 2000). The common anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Iwashina, 2000) (Figure 1.6). These compounds are found in planta as their sugar-conjugated derivatives, called anthocyanins, a group of water-soluble compounds responsible for most floral, fruit and leaf pigmentation in nature (Clifford, 2000). As pigments, they play an important role in attracting insects to flowers for pollination (Crozier, 2003). Anthocyanidins are also responsible for the red, blue or violet colour of edible fruits including grapes, plums and berries, with levels increasing during fruit maturation (Peterson & Dwyer, 1998).

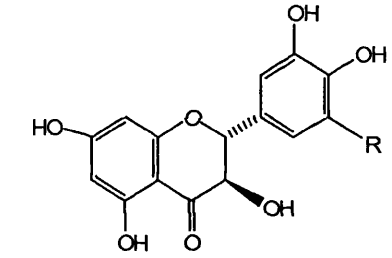


R_1=H	R_2=H:	pelargonidin
R_1=OH	R_2=H:	cyanidin
R_1=OH	R_2=OH:	delphinidin
R_1=OCH_3	R_2=H:	peonidin
R_1=OCH_3	R_2=OH:	petunidin
R_1=OCH_3	R_2= OCH_3:	malvidin

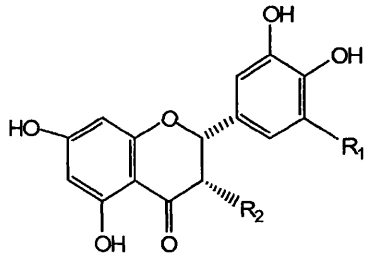
Figure 1.6. Structure of anthocyanidins

1.3.5 Flavan-3-ols

Flavan-3-ols are a complex in nature and range from the simple (+)-catechin and its isomer (-)-epicatechin, to the oligomeric and polymeric proanthocyanidins, also known as condensed tannins (Figure 1.7). Flavan-3-ols are widely distributed in the plant kingdom and are found abundantly in apricots, sour cherries, grapes and blackberries (Porter, 1988). The flavan-3-ols also contribute to the astrigent taste of tea and wine. In addition to forming complexes with other flavan-3-ols to produce proanthocyanidins, they also undergo esterification with gallic acid to form catechin gallates, and hydroxylation reactions to form gallocatechins (Figure 1.7). These forms of flavan-3-ols, particularly (–)-epigallocatechin gallate and (–)-epigallocatechin are abundantly found in green tea (Steward *et al.*, 2005).



R=H: (+)-catechin
R=OH (+)-gallocatechin



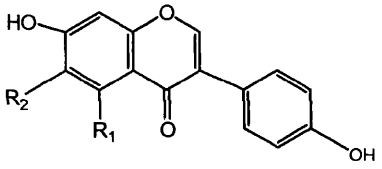
R_1=H R_2=H: (-)-epicatechin
R_1=OH R_2=H: (-)-epigallocatechin
R_1=H R_2=gallate: (-)-epicatechin-3-gallate
R_1=OH R_2=gallate: (-)-epigallocatechin-3-gallate

Figure 1.7. Structure of flavan-3-ols (catechins and epicatechins)

1.3.6. Isoflavones

In contrast to most other flavonoids, isoflavones have a very limited distribution in the plant kingdom. Isoflavones are found primarily in leguminous species (Dixon and Steele, 1999; Graham, 1991). The commonly known isoflavones are genistein, daidzein and glycitein and they are principally found in soybeans, black beans and green split peas. Structurally isoflavones differ from the common flavonoid in the orientation of the B ring. They are characterised by having the B ring attached at the C3

rather than the C2 position (Figure 1.8). Isoflavones undergo various modifications, such as methylation, hydroxylation, or polymerization and these modifications lead to simple isoflavonoids, such as isoflavanones, isoflavans, and isoflavanols, as well as more complex structures, such as rotenoids, pterocarpans, and coumestans (Dewick, 1993). Isoflavones are popularly known for their estrogenic activity and have received much attention due to their putative role in the prevention of breast cancer and osteoporosis (Barnes, 2003).



R_1=OH R_2=H: genistein
R_1=H R_2=H: daidzein
R_1=H R_2= OCH_3: glycitcin

Figure 1.8. Structure of common isoflavones.

1.4 Important dietary flavonoids

Certain dietary flavonoids are particularly bioactive and have pronounced effects on human health. Two of the most potent flavonoids are quercetin and genistein. Both these compounds display a wide range of biological effects (discussed in Section 1.5.2) and epidemiological studies suggest that both these compounds play an important role in disease prevention (discussed in Section 1.5.1). Quercetin, one of the most ubiquitous dietary flavonoids is principally found as glycoside conjugates in plants and one of the more common forms being rutin (Figure 1.9).

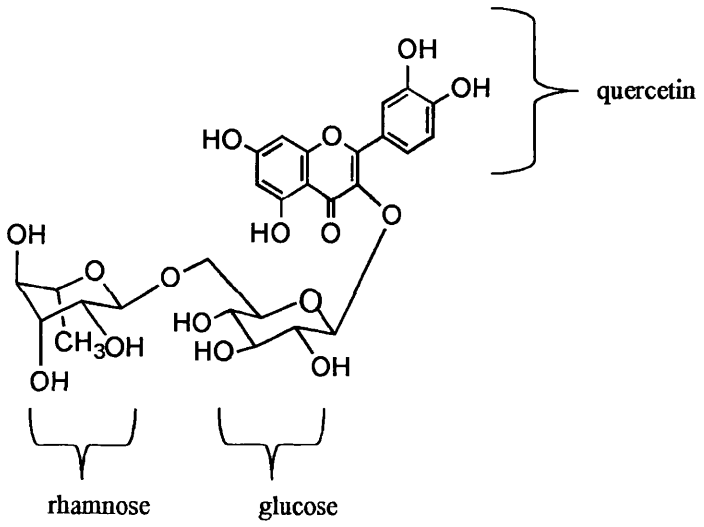


Figure 1.9. Structure of rutin

1.5 Evidence of health effects of flavonoids

1.5.1 Epidemiological studies

Epidemiologic studies suggest an inverse relationship between a high dietary intake of fruits and vegetables with many degenerative diseases such as cancer and cardiovascular disease (Steinmetz and Potter, 1996; Law and Morris, 1998; Riboli and Norat, 2003). It has been hypothesized that vegetables and fruits contain bioactive compounds, such as flavonoids, that have protective effects, independent of those of known nutrients and micronutrients (Arts and Hollman, 2005).

1.5.1.1 Cardiovascular disease

In the past decade a number of epidemiological studies have been carried out attempting to correlate high flavonoid intake with the risk of disease. To date, 12 cohort studies on flavonoid intake and the risk of coronary heart disease (CHD) and 5 cohort studies on the risk of stroke have been published (Arts and Hollman, 2005). Most, but not all, these studies have indicated some degree of inverse association (from borderline to relatively high levels) between flavonoid intake and fatal or nonfatal CHD, and reductions of mortality risk were up to 65%. An inverse association was found in the Zutphen Elderly Study (Hertog *et al.*, 1993), the Finnish Mobile Clinic Study (Knekt *et al.*, 1996), the Iowa Women's Health Study (Yochum *et al.*, 1999), the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study (Hirvonen *et al.*, 2001) and the Rotterdam Study (Geleijnse *et al.*, 2002). In contrast negative or no association was found in three main studies, the Caerphilly Study (Hertog *et al.*, 1997), the Health Professionals Follow-up Study (Rimm *et al.*, 1996) and in the Women's Health Study (Liu *et al.*, 2000 Hennekens, 1992). In addition, case-control studies have also suggested that high flavan-3-ol and flavonol intake through the consumption of tea is beneficial (Sesso *et al.*, 1999; Mukamal *et al.*, 2002). Further support for the benefit of higher flavonoid intake is provided by studies relating to red wine consumption to CHD (Pearson, 1996; Di Castelnuovo *et al.*, 2002). As in the cohort studies, several case-control studies also demonstrated no relationship between flavonoid intake and cardiovascular risk (Vita, 2005).

1.5.1.2 Cancer

Studies relating flavonoid intake to cancer rates have assessed the relationship of food groups rich in these compounds to cancer risk. One of the most widely cited

studies, which provided a strong evidence for a protective role of flavonoids against cancer, was carried out by Knekt *et al.* (1997) in the Finnish Mobile Clinic Health Examination Survey. In another study, Knekt *et al.* (2002) investigated the dietary intakes of flavonols, flavones and flavanones against several chronic diseases. It was revealed that higher quercetin intakes were correlated with lower incidence of lung and breast cancer while the incidence of prostate cancer was lower with higher myricetin intakes. Higher quercetin intake also led to a significant reduction of the incidence of stomach cancer (De Stefani *et al.*, 2000). In a more recent study, six principal classes of flavonoids, namely, flavanones, flavan-3-ols, flavonols, flavones, anthocyanidines, and isoflavones were investigated against the incidence of breast cancer. A reduced risk was obtained with increasing intake of flavones and flavonols while no significant association was found with the other flavonoids (Bosetti *et al.*, 2005).

Lower cancers incidences, including breast, prostate, and colon cancer is associated with Japanese and Chinese diets compared to those in Europe and the United States. Many investigators have suggested that this may be due to the level of consumption of soy foods in Japan and China. It has been proposed that soy foods, which contain high levels of isoflavones, are often associated with reductions in the incidence of cancer, although they do not always appear to be the primary protective component of the Asian diet (Birt *et al.*, 2001). In line with this, consumption of fermented and salted soy paste was positively associated with gastric cancer (Hu *et al.*, 1988) while prostate cancer rates were lowest among Hawaiian men of Japanese descent who consumed soybean curd (Severson *et al.*, 1989).

Although there has been studies that showed inverse correlation between the intake of flavonoids and cancer risk, other investigators had difficulties in assessing the relationship between the level of intake of flavonoids and protection against cancer (Hertog, *et al*, 1994, 1995; Garcia-Closas *et al.*, 1998; Wu *et al.*,1998).

1.5.1.3 Other chronic diseases

Due to the potent biological activities such as their antioxidant and antiinflammatory properties, the influence of high intakes of flavonoids was also investigated on other chronic diseases involving oxidative stress or inflammation, such as rheumatoid arthritis and chronic obstructive pulmonary disease. On studying the relationship between the intakes of different classes of flavonoids and rheumatoid

arthritis, type 2 diabetes mellitus, cataracts, and asthma, Knekt *et al.* (2002) found a significant inverse association between high intakes of quercetin and the incidence of asthma. This finding supported an earlier cross-sectional study, in which intake of flavonoids specifically catechin was beneficially associated with pulmonary function and symptoms of obstructive pulmonary disease (Tabak *et al.*, 2001).

1.5.1.4 Confounding factors of epidemiological studies

Cohort and case control studies in epidemiology investigates the relationship between food intake and disease development and are critically dependent on the accurate measurement of the consumption of specific foods. However in most studies, due to lack of adequate food composition data, it is almost impossible to quantify the daily flavonoid intake. In most of the epidemiological studies mentioned above, only very few flavonoids were assessed and quantified in fruits and vegetables before they were correlated with the development of disease. In addition, inaccuracies in the analytical methods employed, or the small size of the investigations are also factors that may cause confounding results with epidemiological studies (Birt *et al.*, 2001).

Epidemiological studies can never prove causation; that is, it cannot prove that a specific risk factor actually causes the disease being studied. Epidemiological evidence can only show that this risk factor is associated (correlated) with a higher incidence of disease in the population exposed to that risk factor. The higher the correlation the more certain the association, but it cannot prove the causation. In addition, it also cannot prove which compounds in the food consumed were responsible for the observed health benefit. In conclusion, current epidemiological evidence is only suggestive that flavonoids may play a role in disease prevention, particularly in CHD and cancer.

1.5.2 Bioactivity and probable mechanism of action of flavonoids

A considerable body of literature supports a role for oxidative stress in the pathogenesis of age-related human diseases. More and more evidence indicates that a proper balance between oxidants and antioxidants is needed to maintain health and alterations to this balance may result in pathological responses causing functional disorders and diseases. In this context, flavonoids were thought to protect cell constituents against oxidative damage through direct scavenging of free radicals due to their antioxidant properties, which was evident through *in vitro* cell culture or cell free

systems (reviewed in Heim *et al.*, 2002). However, with the recent advances gained through research in the field of metabolism and absorption, it is clear that most flavonoids are modified during absorption from the small intestine, through conjugation and metabolism, and by the large intestine, mainly through the actions of the colonic microflora, and by subsequent hepatic metabolism of the components that are absorbed (Mullen *et al.,* 2004; Day *et al.*, 2003a; Manach *et al.*, 2004; Graefe *et al.,* 2001). Thus, flavonoid metabolites that reach the cells and tissues are chemically, biologically, and in many instances, functionally distinct from the dietary form and such features underlay their bioactivity (Kroon *et al.*, 2004). This, in addition to the fact that very low levels of flavonoids (<10 μM) are actually absorbed, implies that the concept of flavonoids functioning as hydrogen-donating antioxidants *in vivo* appear to be an oversimplified view of their mode of action (reviewed in Williams *et al.*, 2004; Halliwell *et al.*, 2005; Williamson and Manach, 2005). It was, therefore, hypothesised that cells respond to flavonoids mainly through direct interactions with receptors or enzymes involved in signal transduction, or through modifying gene expressions which may result in modification of the redox status of the cell that may trigger a series of redox-dependent reactions (Williams *et al.*, 2004).

However, antioxidant and other protective effects of flavonoids and other phenolic compounds could occur before absorption, that is, within the gastrointestinal (GI) tract itself (Halliwell *et al.*, 2005). This is due mainly to the fact that flavonoids in the GI tract are present at far higher concentrations after consumption of foods and beverages rich in polyphenols than their metabolites in the circulatory system (Watanabe *et al.*, 1998; Gee and Johnson, 2001).

1.5.2.1 Direct and indirect radical scavenging within the GI tract

The GI tract is unique compared to other organs in the body because it represents a critical defence barrier against luminal toxic agents and is constantly challenged by diet-derived oxidants, mutagens, and carcinogens as well as by endogenously generated reactive oxygen species (ROS) (Ames, 1983). To preserve cellular integrity and tissue homeostasis, the GI tract, specifically the large intestine, posses several physiological defense mechanisms such as maintaining high antioxidant concentrations (glutathione, tocopherol, and ascorbic acid), up-regulating antioxidant enzymes systems (glutathione peroxidase, glutathione reductase and superoxide dismutase), and inducing cell death by apoptosis to dispose of injured or spent

enterocytes (Awe, 1999). During oxidative stress, an imbalance in the cellular prooxidant-antioxidant balance in favour of prooxidants occurs, which results in gut pathologies such as inflammation and cancer (Aw, 2003). Flavonoids and their metabolites/catabolites may become concentrated in the intestine due to minimal absorption in the upper GI tract and as such would be well placed to interact with the mucosal cells where they may exert their antioxidant capacity to scavenge free radical cations thereby assisting in the maintainence of a reduced redox state. An indication of the level of antioxidants in human large intestine was provided by Garsetti *et al.* (2000) who reported that the antioxidant levels obtained in faeces at 26.6 ± 10.5 mM Trolox was much greater than in plasma which contained 1.46 ± 0.14 mM Trolox. In addition, the total amount of antioxidants excreted over 24 h was found to be significantly and positively correlated to the consumption of beverages rich in polyphenols (Garsetti *et al.*, 2000).

The antioxidant activity of flavonoids varies considerably among different backbone structures and functional groups. The difference in ROS scavenging between the flavonoids can be accounted for by the variation in the number and kind of functional groups present. Basically, there are three functional groups that are associated with an increase in the ROS scavenging potential among the flavonoids: the dihydroxy structure of the B ring; the C2-C3 double bond in concert with a 4-oxo function of the C ring; and the additional presence of both a 3- and a 5-hydroxyl moiety of the C and A rings, respectively (Bors *et al.*, 1990) (Figure 1.10).

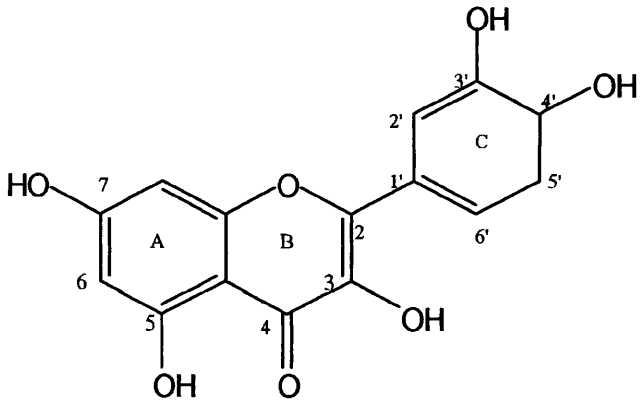


Figure 1.10. Chemical structure of quercetin indicating the number and types of functional groups present which accounts for its antioxidant activity

Free radical scavenging capacity, which is primarily attributed to the high reactivities of hydroxyl substituents can be summarized in the following reaction:

$$F\text{-}OH + R^{\cdot} \rightarrow F\text{-}O^{\cdot} + RH$$

where F – flavonoid; $R^{\cdot}$ - free radical; $F\text{-}O^{\cdot}$ - flavonoid radical

The B-ring hydroxyl configuration is the most significant determinant of scavenging of ROS and reactive nitrogen species (RNS). Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical (reviewed in Heim *et al.*, 2002).

The stomach, small and large intestine are also known to have large amounts of peroxidases, which are capable of inducing lipid peroxidation (Miura *et al.*, 2002). This process causes damage to unsaturated fatty acids, thereby decreasing membrane fluidity, which may lead to many pathological events. However, this process can be quenched effectively by flavonoids. In addition to scavenging of ROS and RNS, the 3′4′-catechol structure in the B-ring of flavonoids strongly enhances inhibition of lipid peroxidation (Dugas *et al.*, 2000; Ratty and Das, 1988). In the large intestine, in addition to flavonoids, the presence of phenolic acids, which are the colonic catabolites of flavonoids can also provide additional protection through their anti-radical scavenging capacity. Phenolic acids such as 3,4,5-trihydroxybenzoic (gallic) acid and 1,2,3-trihydroxybenzene (pyrogallol) with three hydroxyl groups bonded to the aromatic ring in an *ortho* position were found to posses the highest antioxidant activity, followed by those with two hydroxyl groups such as 3,4-dihydroxycinnamic (caffeic), 3,4-dihydroxybenzoic (protocatechuic) and 2,3-dihydroxybenzoic (*o*-pyrocatechuic) acids (Sroka and Cisowski, 2003).

Flavonoids can also act indirectly as antioxidants by forming a "redox inactive complex" with metal ions such as Cu^{2+} and Fe^{2+} (Sugihara *et al.*, 1999; Sahu and Gray, 1997). By removing and neutralizing these metal ions from the intestine, flavonoids inhibit oxidative damage by preventing Fenton type reactions, which is an important generator of ROS *in vivo* (Galey, 1997).

1.5.2.2. Interactions with intracellular signalling cascades

There is now emerging evidence that flavonoids may play an important role as modulators of intracellular signalling cascades, which are vital to cellular function

(Williams *et al.*, 2004). Intracellular signaling cascades are the main routes of communication between the plasma membrane and regulatory targets in various intracellular compartments. These signalling cascades seems to consist of up to five tiers (levels) of protein kinases that sequentially activate each other by phosphorylation, which subsequently affects the activity of transcription factors and thereby gene expression (Seger and Krebs, 1995). The presence of these cascades enables the cells to respond to a variety of different stresses or signals which in turn regulate numerous cell processes, including growth, proliferation and death (apoptosis) (Williams *et al.*, 2004). Flavonoids can exert modulatory effects in cells through selective actions at different components of the signalling cascades (Schroeter *et al.*, 2001; Kong *et al.*, 2000; Matter *et al.*, 1992). Intracellular concentrations of flavonoids required to affect cell signalling pathways are considerably lower than those required to impact on cellular antioxidant capacity, and flavonoid metabolites may still retain their ability to interact with cell signaling proteins, even if their antioxidant activity is diminished (Spencer *et al.*, 2003a and 2003b; Williams *et al.*, 2004). Modulation of the signalling pathway by flavonoids could provide protection against cancer and cardiovascular diseases through a number of mechanisms such as:

- **Regulation of cell cycle**: During the cellular division, DNA becomes more susceptible to damage. To counteract damage, mechanisms within the cell ensure that the cell cycle is transiently arrested at damage checkpoints to allow for DNA repair as defective cell cycle results in the propagation of mutations that contribute to the development of cancer. Chen *et al.* (2004) and Wang *et al.* (2004), in separate experiments demonstrated that apigenin and epigallocatechin gallate could inhibit and arrest growth of tumour and cancer cells through regulating normal cell cycle progression.
- **Apoptosis induction:** Unlike normal cells, cancer cells proliferate rapidly and lose the ability to respond to cell death signals. Sah *et al.* (2004) demonstrated epigallocatechin gallate are able to induce apoptotic cell death in cervical cancer cell lines, thereby providing protective effects.
- **Inhibition of tumor invasion and angiogenesis**: Cancerous cells invade normal tissue and generate new capillaries by sprouting of pre-existing blood vessels via a process known as angiogenesis, to fuel their rapid growth. Luteolin, genistein,

apigenin, and 3-hydroxyflavone were found to posses anti-angiogenesis properties, and the mechanism in which they act are thought to be at least in part via inhibiting the expression and activation of specific components of the signalling cascades (Bagli *et al.*, 2004; Kim, 2003).

- **Decreasing inflammation**: O'Leary *et al.* (2004) and Sakata *et al.* (2003) demonstrated that flavonoids such as quercetin and hesperidin may exert their anti-inflammatory and immunomodulatory properties by suppressing the activation of specific protein kinases, thus inhibiting the production of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) which are related to anti-inflammatory and anti-tumorigenic efficacies.

- **Decreasing vascular cell adhesion molecule expression**: Atherosclerosis is characterized by the accumulation of cholesterol deposits in macrophages in arteries. This deposition leads to a proliferation of certain cell types within the arterial wall that gradually impinge on the vessel lumen and impede blood flow. This process requires the expression of endothelial adhesion molecules (Stocker and Keaney, 2004). Flavones and epigallocatechin-3-gallate were found to reduce the expression of these endothelial adhesion molecules (Choi *et al.*, 2004; Ludwig *et al.*, 2004).

- **Increasing endothelial nitric oxide synthase (eNOS) activity**: eNOS is the enzyme that catalyzes the formation of nitric oxide by vascular endothelial cells. Nitric oxide is needed to maintain arterial relaxation as insufficient levels of it is associated with increased risk of CHD. Endothelium-derived nitric oxide bioactivity appears to be increased by supplementation with a number of polyphenols (Duffy and Vita, 2003). One of the mechanisms suggested is mediated through modulation of the protein kinase signalling pathway which activates production of eNOS (Anter *et al.*, 2005).

- **Decreasing platelet aggregation**: Platelet aggregation contributes to both the development of atherosclerosis and acute platelet thrombus formation. It can therefore result in myocardial infarction or stroke. Inhibiting platelet aggregation is considered an important strategy in the primary and secondary prevention of cardiovascular disease. Hubband *et al.* (2003) and Pignatelli *et al.* (2000) demonstrated that polyphenols such as hydroxycinnamates, catechins and

quercetin are able to inhibit platelet function. The mechanism involved may be mediated, at least partly, through the modulation of the protein kinases signalling pathways (Deana *et al.*, 2003; Bucki *et al.*, 2003).

1.6 Bioavailability, absorption and metabolism of flavonoids

1.6.1 Bioavailability: concepts and methods of detection

The term 'bioavailability' and its underlying concept were initially introduced in the field of pharmacology, where bioavailability was defined by the Food and Drugs Administration (FDA) as 'the rate and extent to which a drug reaches its site of action'. Because of the problems in the quantification of a compound at its 'site of action', this concept was modified to account for the fraction of an oral dose of a substance or its metabolite(s) that reaches the systemic circulation (Stahl *et al.*, 2002). Thus, bioavailability can be determined either in a single or multiple-dose experiment by measuring the peak blood concentration (C_{max}), the time to reach the peak concentration (T_{max}), and the area under the blood concentration time curve (AUC). However the plasma pharmacokinetic parameters to determine bioavailability are limited by several factors. The concentration of the flavonoids in plasma does not represent bioavailability per se, as it actually represents a balance between intestinal absorption, breakdown, tissue uptake, and release from body stores (Yeum & Russell 2002). Flavonoids also tend to have a very low signal-to-noise ratio (Deprez & Scalbert 1999; Heaney 2001) and due to this, relatively large dosages of flavonoids, usually exceeding the typical daily intake, are generally needed for a significant increase in flavonoid concentrations over baseline levels in plasma (Yeum & Russell 2002). The assumption that the blood concentration and the biological activity of a compound are proportional is not always the case as suggested by Hoppe and Krennrich (2000).

Other methods used for the measurement of bioavailability include the balance method (Heaney, 2001). Here the differences between what is ingested and what is found in the faeces are measured. This method, however does not take into consideration bacterial transformation or degradation of the ingested compounds. Another more sensitive and reproducible method of measuring bioavailability is the tracer method using stable or radioactive isotopes. In this technique, the body distribution of labeled compounds is reflected by the absorption of the ingested compound including potentially unknown metabolites and the whole body auto-

radiography can help to identify target tissues (Deprez & Scalbert 1999). Due to radiation hazard, radiolabelled flavonoids can only be fed to animals while stable isotopes can be administered in human intervention studies. However, in using this technique, number and the positioning of the radiolabelled atoms within the flavonoid molecule can very crucial in determining not only the strength of the signal but more importantly in the metabolic fraction that can be identified after metabolism or/and catabolism. The amount of compound excreted into the urine is also sometimes used as a measure of absolute bioavailability. The urine increment method is based on the fact that plasma concentrations of the flavonoids are proportional to the amounts excreted in urine (Heaney, 2001). However, this only describes the minimum amount of compound absorbed since other routes of excretion, such as biliary excretion, are overlooked.

To-date there is no single approach that is recommended for measuring bioavailability of flavonoids. Due to differences in absorption mechanism, metabolism, intestinal enzymes, degradation by intestinal flora, stability of the flavonoid compound, body distribution and excretion pathways, each type of flavonoid may require one or more approach to accurately access its bioavailability (Walle *et al.*, 2003; Scalbert & Williamson, 2000). Other factors including the food matrix, the type of preparation, the individual the mucosal mass, intestinal transit time and rate of gastric emptying can also affect bioavailability of flavonoids (Erlund 2004). To gain a better understanding on the various parameters affecting bioavailability, researchers in this field have employed a number of different approaches. Using human intestinal cell culture, such as Caco-2 cells and enzymatic studies, experimentations on bioavailability were carried out *in vitro* (Walle *et al.*, 2003). In *ex vivo* conditions, investigators mainly employed rodent intestinal preparations while *in vivo* conditions both human and animal intervention trials were used to estimate bioavailability (Spencer *et al.*, 2003).

It is also important to note that flavonoids do not need to enter the systemic circulation to be bioactive (Walle, 2004). Many of them are thought to exert their cancer preventive effects in epithelial cells. One such example is exhibited by the tea flavonoid, epicatechin-3-gallate, where it was demonstrated to be extensively taken up by the Caco-2 cells (Vaidyanathan and Walle, 2003).

1.6.2. Absorption of flavonoids

Food components must, by definition, be made bioavailable in some form to exert biological effects. From recent advances made on flavonoid absorption and metabolism studies, it is apparent that a large number of flavonoids are absorbed. For example, micromolar plasma concentrations of different classes of flavonoids have been detected after consumption of flavonoid-rich onions, red wine and soya. These findings reveal that flavonoids are able to cross the intestinal barrier and reach micromolar concentrations in the bloodstream (Williamson and Manach, 2005).

1.6.2.1 Absorption of flavonoids, small or large intestine?

There are many factors that influence the extent and rate of absorption of ingested flavonoids. These include physiochemical factors of the flavonoid itself which include the molecular size and lingkage, lipophilicity, solubility and, pKa (Spencer 2003). Most polyphenols are present in food in the form of esters, glycosides, or polymers that cannot be absorbed in their native form. These substances must be hydrolyzed by intestinal enzymes or by the colonic microflora before they can be absorbed (reviewed by Manach *et al.,* 2004). Depending on the type of lingkages, the flavonoids are either absorbed in the small or large intestine. Only aglycones and some glucosides can be absorbed in the small intestine, whereas flavonoids linked to a rhamnose moiety must reach the colon and be hydrolyzed by glycosidases of the microflora before absorption. This is supported by human intervention trials carried out by Graefe *et al.* (2001) where absorption from rutin and quercetin glucosides were compared. Peak plasma concentrations of quercetin, following ingestion of either onion or quercetin 4'-glucoside were similar i.e C_{max} 7.0-7.6 μM and maximum absorption occurred 0.5–0.7 h after oral ingestion. Even with double the dose, the peak plasma concentration of quercetin from buckweed tea or rutin was significantly lower with a C_{max} 1.0-2.0 μM and T_{max} was delayed until 6–9 h after ingestion of rutin. Lower absorption rates in the colon than in the small intestine caused by a smaller exchange area and a lower density of transport systems, could result in rutin being absorbed less rapidly and less efficiently in the colon than quercetin glucosides, in the small intestine (Manach *et al.,* 2004). The results of similar human intervention trials carried out by other investigators (Hollman *et al.*, 1997; Hollman *et al.*, 1999; Olthof *et al.*, 2000) are summarized in Figure 1.11. The results obtained imply that although the food matrix may influence absorption, the type of conjugate attached to the flavonoid skeleton, is

the most important factor contributing to the overall bioavailability of the flavonoid moiety. To gain more information on the site of absorption from gastrointestinal tract, studies have been carried out with animals using quercetin and its glycosides (reviewed in Day and Williamson, 2003). All the results revealed similar patterns of absorption to those observed in human intervention trials where quercetin and quercetin glucosides were absorbed much earlier and in higher amounts than rutin.

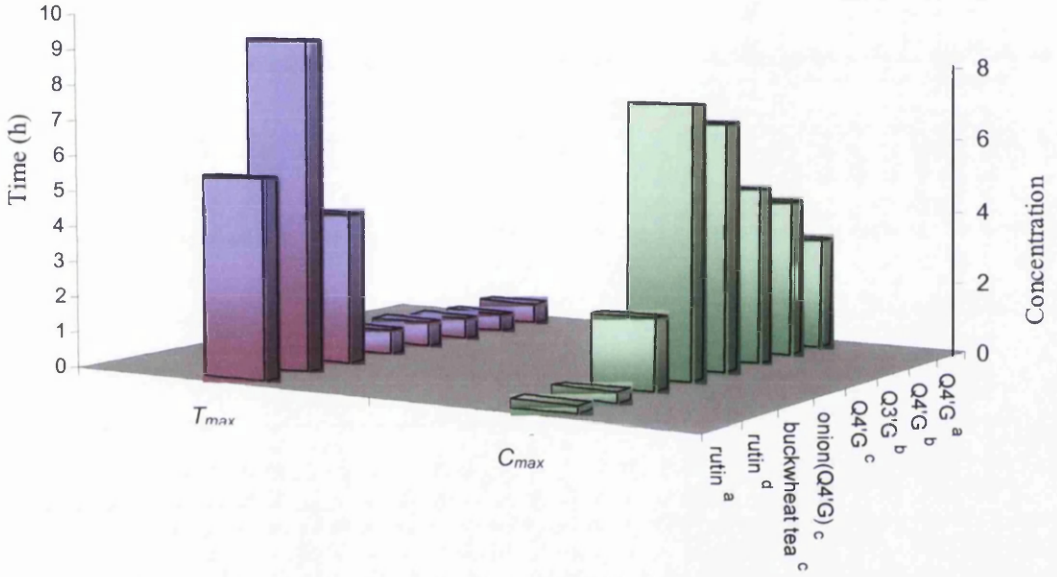


Figure 1.11. Summary of published data. These data reveal the time taken to reach C_{max} and T_{max} in plasma after consumption of rutin and quercetin glucosides. Q3G, quercetin 3-glucoside; Q4'G, quercetin-4'-glucoside. C_{max}: Maximum plasma quercetin concentration (μM); T_{max}: Time taken to reach C_{max} (hours); [a] Hollman *et al.*, 1999; [b] Olthof *et al.*, 2000; [c] Graefe *et al.*, 2001; [d] Hollman *et al.*, 1997

1.6.2.2. Absorption variability between flavonoid aglycones

Absorption also differs greatly from one flavonoid to another. In a recent review by Manach *et al.* (2005) the time to reach C_{max} and the plasma *AUC* were calculated for 18 major polyphenols. The plasma C_{max} of total metabolites ranged from 0.4-4 μM with an intake of 50 mg aglycone equivalents. Gallic acid and isoflavones are the most well-absorbed polyphenols, followed by catechins, flavanones, and quercetin glucosides, but with different kinetics. The least well-absorbed polyphenols are the proanthocyanidins, the galloylated tea catechins, and the anthocyanins.

1.6.2.3. Effect of food matrix on absorption

Although the effects of the food matrix on the absorption of flavonoids have not been thoroughly examined, direct interactions with some components of food, such as binding to proteins and polysaccharides is known to occur, and these interactions may affect absorption. Schramm *et al.* (2003) demonstrated that absorption of flavan-3-ols could be increased significantly by concurrent carbohydrate consumption. In another study, Serafini *et al.* (2003) reported that addition of milk to black tea reduced the antioxidant potential, suggesting interactions of milk proteins with flavonoids. However, this effect was not consistent as subsequent studies showed that addition of milk to either black or green tea had no effect on the absorption of catechins, quercetin, or kaempferol in humans (reviewed by Manach *et al.,* 2004). Similarly, some studies indicate that the presence of alcohol in wine can improve the intestinal absorption of polyphenols by increasing their solubility while other studies indicated otherwise. Donovan *et al.* (1999), for instance, reported that the plasma concentrations of catechin metabolites were similar after consumption of red wine or dealcoholized red wine. Indirect effects of food matrix on the pH, intestinal fermentations, biliary excretion, transit time etc. may also affect the gut physiology and thereby influence absorption of flavonoids (Erlund 2004).

1.6.2.4. Mechanism of absorption.

Due to the complexity of the *in vivo* environment, investigators have used two main approaches to gain information about the mechanisms of the gastrointestinal absorption of flavonoids. These involve the use of rat intestinal perfusion models and human intestinal Caco-2 cell cultures (Walle *et al.*, 2003). Since most dietary flavonoids are probably too hydrophilic to penetrate the gut wall by passive diffusion, membrane carriers have been suggested to be involved in their absorption. To date, the only active transport mechanism that has been described is a Na^+-dependent saturable transport. Hollman *et al.* (1999) proposed that glucosides could be transported into enterocytes by the sodium-dependent glucose transporter SGLT1. This would imply that intact quercetin glucoside can be transported across the enterocyte and possibly could appear in the plasma. However, in human intervention studies, intact quercetin glucosides were absent in plasma after supplementation and this tends to weaken the SGLT1 hypothesis (Day *et al.*, 2001; Mullen *et al.*, 2004). Walgren *et al.* (1998), using Caco-2 cell monolayer, demonstrated that quercetin was reasonably well absorbed but

quercetin glucoside was initially absorbed via the SGLT1 transporter but was later refluxed across the apical membrane. This opposing transporter to the SGLT1 was proposed to be the multidrug resistance-associated protein 2 (MRP2) transporter (Walgren *et al.,* 2000).

The Caco-2 cell and rat intestinal perfusion studies have also pointed to an alternative mechanism of absorption of flavonoids, that is, hydrolysis of quercetin glucosides to release quercetin, which then could then be absorbed (Sesink *et al.*, 2003; Walgren *et al.,* 2000). Additional evidence supporting this pathway was produced by studies carried out in ileostomy volunteers in which the administration of quercetin glucosides resulted in complete hydrolysis within the small intestine, followed by effective absorption of quercetin (Walle *et al.*, 2000). In enzymatic studies, high levels of endogenous β-glucosidase activity have been demonstrated in both human and animal small intestine epithelial cells and in human liver (Day *et al.*, 1998). In rat intestinal cells, two ß-glycosidases are present, cytosolic ß-glucosidase (CBG) and lactase phlorizin hydrolase (LPH) (Sesink *et al.*, 2003; Day *et al.*, 2003). CBG has hydrolytic activity toward several flavonoid glucosides, but not quercetin-3-glucoside while LPH, an extracellular enzyme located at the brush border membrane of rat intestinal cells is able to hydrolyze flavonoid glucosides, including quercetin-3-glucoside (Day *et al.*, 2003). In contrast, quercetin-4'-glucoside is hydrolysed by both LPH and CBG (Day *et al.*, 2003). However, the hydrolytic step *in vivo* has yet to be established.

1.6.3. Metabolism – conjugation and nature of flavonoid metabolites

The term metabolism is used here to describe the typical modifications that occur during or after absorption (Kroon *et al.*, 2004). The gastrointestinal lumen, cells of the intestinal wall, and the liver are important sites of flavonoid metabolism. The metabolism of flavonoids is a matter of interest because metabolism may considerably change the function, activity of a compound and impact on its ability to enter cells (Kroon *et al.*, 2004). The primary defence mechanisms against toxic or xenobiotic compound are the enzymes that catalyse detoxification reactions and are classified as the phase I and phase II metabolising enzymes (Sanchez *et al.*, 2001).

Phase II metabolising enzymes are widely distributed among tissues such as the intestines, liver and the kidneys (Sanchez *et al.*, 2001). After absorption, flavonoids

are metabolized by the phase II drug–metabolising enzymes, the uridine-5'-diphosphate glucuronosyl-transferases (UGTs), cytosolic phenol-sulfotransferases (SULTs), and catechol-*O*-methyltransferases (COMTs) (reviewed in Manach *et al.*, 2004). The resulting molecules are glucuronide and sulfate conjugates with or without methylation across the catechol functional group, and many are multiple conjugates. A proportion of the flavonoid metabolites that resulted from intestinal conjugation are taken up and metabolized by the liver (O'Leary *et al.*, 2003) and then partially secreted in the bile. However, the metabolic fate of the flavonoid conjugates in the liver is not yet known. It has been postulated that after uptake by HepG2 cells, quercetin 7-glucuronide and quercetin 3'-*O*-glucuronide undergo two types of metabolism: methylation of the catechol and deglucuronidation followed by 3'-sulfation (O'Leary *et al.*, 2003). The detection of flavonoid metabolites of quercetin, luteolin, eriodictyol, and catechin in the bile of rodents showed that most flavonoids undergo enterohepatic recycling (Crespy *et al.*, 2003) and are secreted back into the intestinal lumen.

1.6.3.1. Colonic metabolism

Studies have suggested that the extent of absorption of dietary flavonoids in the small intestine is relatively small (10–20%) (Spencer, 2003). Flavonoids that are not absorbed in the small intestine or excreted by the enterohepatic cycling will be carried to the colon where they encounter the colonic microflora. Using animal models, human intervention trials and *in vitro* fermentation of the flavonoid with human faecal samples or anaerobic bacterial strains, investigators have attempted to identify the various catabolites produced in the colon after consumption of dietary flavonoids (Olthof *et al.*, 2003; Aura *et al.*, 2002; Rechner *et al.*, 2004; Labib *et al.*, 2004; Braune *et al*, 2001; Bravo *et al.*, 1994). Results from these various studies indicate that the enormous catalytic and hydrolytic potential of the microflora splits the flavonoid nucleus by ring fission, which results in the production of an array of catabolites (Spencer, 2003). Depending on the chemical structure of the flavonoids, various types of phenolic acids can be produced through the catabolic action of the microflora. The flavonols are catabolised to form hydroxyphenylacetic acids, the flavones and flavanones mainly produce hydroxyphenylpropionic acids, while the flavan-3-ols are known to produce phenylvalerolactones and hydroxyphenylpropionic acids (reviewed in Manach *et al.*, 2004). Other than ring fission, the bacterial enzymes are also known to catalyze many other reactions including hydrolysis, dehydroxylation, demethylation, decarboxylation

and deconjugation (Scheline, 1999). In addition to the phenolic acids mentioned above, formation of benzoic acid, hippuric acid and hydroxyhippuric acids may also be a possible catabolites of flavonoids, in which the colon microflora and the liver were proposed to be active metabolic sites (Scheline, 1999).

The identification and quantification of microbial metabolites constitute an important field of research as some recent studies have revealed higher plasma concentrations and urinary excretion of microbial catabolites in humans compared to conjugated metabolites. (Gonthier *et al.*, 2003; Rechner *et al.*, 2002). In addition, Halliwell *et al.* (2005) reported that concentrations of the colonic catabolites range from 15-410 μM, compared to 0.7μM of flavonoids present in the colon. Halliwell *et al.* (2005) further suggested that as a consequence of these high concentrations, phenolic acids might exert direct protective effects within the large intestine which could include binding of prooxidant iron, scavenging of reactive nitrogen, chlorine, and oxygen species, and perhaps inhibition of cyclooxygenases and lipoxygenases. In addition to this, microbial metabolites may also have physiological effects as indicated by studies carried out by Kim *et al*, 1998 in which hydroxyphenylacetic acids inhibited platelet aggregation.

1.6.4. Tissue uptake

Actual bioavailability of flavonoids occurs when their metabolites reach the site of action, which are the tissues or the organs. Therefore the determination of flavonoid metabolites in tissues or organ may be much more important than their concentrations in the circulatory system as it provides essential information about the potential site of accumulation of flavonoids. However, data in this topic is very limited, even when using animal models. Tissue or organ uptake of flavonoids is normally measured following *in vivo* feeding experiments. One such study that has provided evidence on this aspect was carried out by Abd El Mohsen et al. (2002). After oral ingestion of 100 mg kg^{-1} body weight per day of (-)-epicatechin, two metabolites, i.e., epicatechin glucuronide and 3'-*O*-methyl epicatechin glucuronide were found in the trace quantities in rat brain tissue. Using radiolabelled flavonoids, valuable information have also been obtained which allows the accumulation of flavonoids and their metabolites to be monitored in various organs and tissues following oral consumption. In a study utilising ^{14}C-labelled quercetin-4′-glucoside in rats, only 6.4% of the recovered radioactivity was detected outside the gastrointestinal tract 60 min after

consumption (Mullen *et al.*, 2002) suggesting limited absorption and low levels of metabolites in plasma and peripheral tissues. Flavonoids have also been detected in a wide range of other tissues and this is evident by a feeding trial carried out using [^{3}H](−)-epigallocatechin gallate which led to the detection of radioactivity in various organs, including the liver, lung, pancreas, mammary gland, skin, brain, kidney, uterus and ovary, and testes (Suganuma, 1998). The isoflavone genistein was found to accumulate in the rat brain, liver, mammary, ovary, prostate, testis, thyroid and uterus after ingestion of genistein (Chang *et al.*, 2000).

1.6.5. Elimination of flavonoids

There are three major pathways of flavonoid excretion, i.e, via the biliary and the urinary routes or through the faeces. Large, extensively conjugated metabolites are more likely to be eliminated in the bile, whereas small conjugates such as monosulphates are preferentially excreted in urine (Manach *et al.*, 2004). Using *in situ* perfusion of jejunum and ileum of rats, the relative magnitude of biliary excretion was found to vary from one flavonoid to another. For some flavonoids such as genistein, biliary secretion may be an important excretion route as it encompasses about 30% of the perfused dose while it encompasses only 1% and 6% of the dose in catechins and quercertin, respectively (Crespy *et al.*, 2003). Other than genistein, biliary excretion may also be a major pathway for the elimination of (-)-epigallocatechin gallate and eriodictyol (Kohri *et al.*, 2001). However, biliary excretion of polyphenols in humans may differ greatly from that in rats because of the existence of the gall bladder in humans. To-date this possibility has not been investigated. Since intestinal bacteria possess ß-glucuronidases activity, it is able to release free aglycones from conjugated metabolites secreted from the bile resulting in reabsorption of aglycones produced via enterohepatic cycling. Evidence of enterohepatic cycling has been obtained with rats (Coldham *et al.*, 2000) and with humans as a second C_{max} plasma peak after ingestion of isoflavones (Watanabe *et al.*, 1998; Setchell *et al.*, 2001).

The urinary excretion of flavonoids has been discussed quite thoroughly in a recent review carried out by Manach *et al.* (2005), where data from 97 studies were investigated for the kinetics and extent of polyphenol absorption and excretion among adults. Variation in the urinary excretion profile was obtained depending on the type of flavonoid ingested. The urinary excretion of anthocynanins ranged from 0.004% to 0.1% of the ingested dose while, the excretion of flavonols were slightly higher; the

percentage excreted are 0.9% for rutin and 6.4% quercetin glycosides from onions. The excretion of flavanones was quite high at 4–30% of intake, and is even higher for isoflavones: the percentages excreted are 16–66% for daidzein and 10–24% for genistein. Data on the kinetics of excretion of the catabolites of flavonoids in humans are limited. Olthof *et al.* (2003) reported that after ingestion of rutin by humans, the total urinary excretion of microbial catabolites accounted for as much as 50% of the supplement. Following ingestion of (-)-epigallocatechin and (-)-epicatechin, catabolite excretion equivalent to 6-39% of intake was observed (Meng *et al.*, 2002).

There have been conflicting reports on the excretion of flavonoids in the faeces. Using radiolabelled [^{14}C] quercetin, Ueno *et al.*, 1983, found as much as 45% of the administered dose appeared in the faeces of rats. Coldham & Sauer (2000) found 33% of intake excreted in urine and faeces, following an oral dose of ^{14}C -labelled genistein to rats. In human intervention studies, 5% of the ingested radiolabelled ^{14}C quercetin was excreted in the faeces (Walle *et al.*, 2001). *In vitro* fermentation studies with quercetin glycosides and rutin using human faecal samples, there was a complete degradation to phenolic acids (Aura *et al.*, 2002; Rechner *et al.*, 2003). The excretion of flavonoids in the faeces may differ between individuals, as it may be dependent on the activity of the colonic microflora, the degree of enterohepatic circulation, different intestinal transit time and the amount ingested.

1.7 Value of a varied diet

Not all plant foods contain all the essential nutrients or bioactive compounds needed for human health, nor do they usually contain these compounds in sufficient concentrations or amounts to meet daily dietary requirements in a single serving (Grusak, 1999). In fact, scientific evidence from the various observational and epidemiological studies indicate that consumption of a complex and varied diet that is rich in nutrients and bioactive compounds are essential for achieving good health (Liu, 2004). This observations, however, raises one key question, that is, whether a purified bioactive compound has the same health benefit as the compound when its source is a food or a mixture of foods. Evidences to-date points to the fact that the actions of the dietary supplements alone do not explain the observed health benefits of diets rich in fruits and vegetables, because, taken alone, the individual bioactive compounds do not appear to have consistent preventive effects (Stephens *et al.*, 1996; Yusuf *et al.*, 2000). It is the additive and the synergistic effects of these bioactive compounds found in fruits

and vegetables that are believed to be responsible for the protective effects as against diseases such as CHD and cancer (Liu, 2004).

Evidence of the benefits of food synergy was provided by an experiment carried out by Liu (2000). He demonstrated that a combination of fruits such as orange, apple, grape, and blueberry displayed a synergistic effect in antioxidant activity. The median effective dose (EC_{50}) of each fruit after combination was 5 times lower than the EC_{50} of each fruit alone, suggesting synergistic effects after the combination of the 4 fruits. In another study, Sakamoto (2000) emphasized the importance of consuming black tea together with soybean products as commonly carried out in a typical Japanese diet. In this study, thearubigen (a polyphenol in black tea) did not alter the *in vitro* growth of human prostate cancer cells when administered alone. However, a small amount (0.5 $\mu g\ mL^{-1}$) of thearubigen administered with genistein (20 $\mu g\ mL^{-1}$), the major isoflavone in soybean, synergistically inhibited cell growth and increased the DNA distribution at the G2M phase of the cell division cycle by 34% compared with genistein alone (Sakamoto, 2000). Similar conclusions were derived by Temple and Gladwin (2003) when they reviewed > 200 cohort and case-control studies that provided risk ratios concerning intake of fruits and vegetables and risk of cancer. Their studies showed that the cancer-preventing action of fruits and vegetables are most probably due to the many bioactive compounds that act in concert to prevent cancer rather than due to one or two potent anticarcinogens. It therefore can be concluded that consumption of vegetables and fruits to improve health have more beneficial effects that consuming dietary supplements, which do not contain the balanced combination of bioactive compounds.

1.8 Improving bioactive composition in plants to enhance human health

To ensure an adequate dietary intake of essential bioactive compounds, researchers have been interested in improving the bioactive composition and its concentration in plants. This would enable the increase of essential or potent bioactive components within the complex diverse array of other bioactive components present in the plant. Enhancement of bioactive compounds of plants can be tackled using a variety of approaches. Conventional breeding is one such approach, exemplified by the production of broccoli with an enhanced glucosinolate content (Faulkner *et al.*, 1998).

Glucosinolates are thought to have a role in the prevention of cancer by acting as inducers of the anti-carcinogenic marker enzyme, quinone reductase. Another major approach to bioactive component enhancement involves genetic modification. With the advent of GM technologies, molecular biologists, biochemists, botanists and medical researchers are linking in with plant breeding programmes to develop new varieties of fruit and vegetables that are tailor-made to produce higher levels of health-related bioactive compounds (Verrips *et al.*, 2001). An example of which is the production of 'golden rice', which has enhanced levels of the β-carotene in its grains (Beyer *et al.*, 2002).

1.8.1. Identification of target bioactive compounds for 'biofortification'

Although the ability to manipulate plant metabolism is becoming well established, identifying which target bioactive compound for 'biofortification' can be problematical (Tucker, 2003). In Section 1.5.1.3, on discussing the confounding factors of epidemiological studies it was mentioned that such studies cannot prove causation and thus cannot attribute the beneficial effects of consumption of fruits and vegetables to any particular bioactive compound within these foods. In addition, the total content, or absolute concentration, of a given bioactive compound in a food is not always a good indicator of its useful nutritional quality, because not all of the bioactive compounds in food are absorbed and made bioavailable to the body as discussed in Section 1.6. In addition, most bioactive compounds undergo considerable metabolism upon ingestion, which change their chemical and biological nature and thus are functionally distinct from the dietary form and such features underlie their bioactivity.

Therefore to identify and choose the target bioactive compounds for enhancement, several factors should be taken into consideration, which include the absolute concentration, its bioavailability and the bioactivity of its metabolites. One such compound that excels in these respects is genistein, an isoflavone that is found primarily in leguminous species.

1.8.1.1 Genistein as the target bioactive compound for 'biofortification'

Genistein (4',5,7-trihydroxyisoflavone) is biosynthetically the simplest form of isoflavone (Dixon and Ferreira, 2002). Major dietary sources of genistein for humans are soybean. Soybeans contain between 580 and 3800 mg isoflavones/kg fresh wt, and soymilk contains between 30 and 175 mg/L (Reinli and Block, 1996).

Genistein's many biological activities have made it a widely researched subject with over 3600 published studies in the last 10 years. Genistein, like the other flavonoids have the same biological activities and mode of action as discussed in Section 1.5.2. In addition, genistein is also known to have other pharmacological activities such as being a potent tyrosine kinase inhibitor and possessing phytoestrogen activity (Barnes *et al.*, 2000; Akiyama *et al.*, 1987). Genistein shares structural similarities with the potent estrogen estradiol-17β and tamoxifen, a synthetic anti-estrogen (Barnes *et al.*, 2000). These features confer its ability to bind estrogen receptors and sex hormone binding proteins. Genistein is able to bind to the estrogen receptor with an affinity 20–100 times lower than estradiol, thereby exerting its phytoestrogenic activity (Barnes *et al.*, 2000). Its phytroestrogenic activity may confer its use in postmenopausal symptoms as indicated in a study where an isoflavone-rich diet was also observed to help approximately two thirds of post-menopausal women to better cope with hot flushes, in addition to potentially reducing the risk of CHD, which is elevated at post-menopause (Dixon and Ferreira, 2002). Genistein's anti-cancer property is based on its inhibition of DNA topoisomerase II and protein tyrosine kinase (Akiyama *et al.*, 1987). Unlike other isoflavonoids, genistein exerts toxicity only at concentrations greatly in excess of those at which it first exerts its biological and pharmacological effects, making it a potentially important cancer chemoprevention dietary molecule (Dixon and Ferreira, 2002).

A number of studies have also been carried out on the bioavailability of isoflavones. After reviewing the kinetics and extent of polyphenol absorption from a total of 97 human intervention studies, isoflavones were found to be one of the most well-absorbed polyphenols (Manach *et al.*, 2005). In addition to the conjugated phase II metabolites, about 4-9 % of isoflavone intake is absorbed directly as aglycones without conjugation (Setchell *et al.* 2001) and can therefore exert the biological activities as the aglycone. One of the most important colonic catabolite of isoflavone is equol and many *in vitro* studies and animal models has shown that equol is more estrogenic than its precursor (Setchell *et al.* 2001). Another point worth noting is the evidence that high concentrations of isoflavones were found in breast tissue of premenopausal women and in prostate glands of men, where they are believed to exert their anticancer properties (Maubach *et al.*, 2003, Hong *et al.*, 2002). To date, these are the only available data on polyphenol concentrations in human tissues.

Isoflavones such as genistein are primarily found in leguminous species and their distribution is restricted to crop plants such as soybean. However, soybean is mainly consumed in the eastern and southeastern Asian regions of the world. In contrast, in United States and in Europe, its consumption is limited and restricted to ethic minorities. Due to the traditional limited consumption of soybean in Western cultures, the idea of genetically directing the synthesis of genistein in plants that do not synthesize them has gained widespread interest in recent years (Dixon and Steele, 1999; Winkel-Shirley, 2001).

1.8.1.2. Tomato as the target plant for genetic modification

In order to introduce isoflavone in target plants, certain dietary factors have first to be considered. Various ethnic groups worldwide should frequently consume the target plant in everyday diet as this becomes an excellent way to supply genistein to a vast population. In addition, it becomes more favourable if the target plant itself is a varied source of beneficial bioactive compounds and the addition of genistein will compliment and fortify the beneficial effects. One such plant is tomato. Tomato is one of the most produced and consumed vegetable worldwide, and a rich source of lycopene, β-carotene, folate, potassium, vitamin C, flavonoids, and vitamin E (Metha *et al.*, 2002). The carotenoid lycopene is one of the most researched bioactive compound in tomato. The results of clinical studies indicate that locopene in tomatoes is beneficial for the treatment and protection of prostate cancer (Chen *et al.*, 2001; Kucuk *et al.*, 2001). A number of other clinical studies are also now underway investigating the role of tomatoes or lycopene in prostate cancer prevention or as an adjunct to therapy (Clinton, 2005). In addition to lycopene, the flavonoids in tomato, particular the flavonols, rutin and kaempferol (Stewart *et al.*, 2000), are potentially health-protecting as a result of their high antioxidant capacity (Sugihara *et al.*, 1999; Dugas *et al.*, 2000; Duthie and Crozier, 2000).

Due to the reasons mentioned above, there are commercial interests to metabolically engineer tomatoes to enable them to produce the isoflavone genistein. Studies with soya indicate that substantial health benefits are associated with isoflavone consumption in Asian populations and for these to become available through tomato consumption could have a long-term favourable impact on health in the Western world.

1.9. The challenge: engineering genistein synthesis in tomato.

1.9.1. The pathway leading to isoflavone biosynthesis – the phenylpropanoid pathway

The phenylpropanoid biosynthetic pathway is a complex biological regulatory network that have evolved in vascular plants during their successful evolutions as land plants (Costa *et al.*, 2003). This metabolic pathway is responsible for the synthesis of a wide variety of secondary metabolic compounds, including lignins, salicylates, coumarins, hydroxycinnamic amides, flavonoids, isoflavonoids and various plant pigments (Dixon & Paiva, 1995), all of which play key roles in the growth, development and survival of higher plants.

From the 1970's to the 1990's, there was rapid and substantial progress in research on the phenylpropanoid pathway focusing mainly towards a broad understanding of the metabolic pathway (Hahlbrock & Grisebach, 1975; Ebel & Hahlbrock, 1982; Heller & Forkmann, 1988). However, in more recent years, most research has been on specific metabolic steps mainly to fill the remaining gaps in the phenylpropanoid pathway. Lately, much effort has been directed at elucidating the phenylpropanoid biosynthetic pathway from a biochemical and a molecular genetic point of view by using approaches such as transposon tagging, positional cloning, co-immunoprecipitation, affinity chromatography, and two-hybrid experiments (Winkel-Shirley, 2001). As a result of using these new methodologies, the major steps involved in the phenylpropanoid pathway has been elucidated and nearly all the enzymes have been identified and characterizated (Winkel-Shirley, 2001).

The gateway into the phenylpropanoid pathway requires the sequential action of three enzymes: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL) (Hahlbrock & Grisebach,1979). The enzyme phenylalanine ammonia-lyase (PAL) removes the amine group from the amino acid of phenylalanine and produces cinnamic acid. Cinnamic acid 4-hydroxylase (C4H), adds a hydroxyl group to form *p*-coumarate. The enzyme 4-coumarate:coenzyme A ligase (4CL) further activates the *p*-coumarate by attaching a CoA at the three-carbon side chain. The flavonoid biosynthetic pathway starts with the condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to form the C_{15} flavonoid skeleton. This reaction is catalysed by chalcone synthase (CHS). In most species, the resultant C_{15}

compound is naringenin-chalcone (Forkmann & Heller, 1999). Naringenin-chalcone is converted to the flavanone naringenin by the enzyme chalcone isomerase (CHI). Naringenin is a key central intermediate, as from this point onwards, the flavonoid biosynthetic pathway diverges into several side branches, each resulting in a different class of flavonoids, which include isoflavones, flavanones, flavones, flavonols, proanthocyanidins and anthocyanins. In most plants, a hydroxyl group is added to narigenin at the C3 position to form dihydrokaempferol in a reaction catalysed by flavanone 3-hydroxylase (F3H) (Forkmann & Heller, 1999).

In terms of engineering genistein synthesis in non-legumes, the next step in the phenylpropanoid pathway is very critical as the difference in the biosynthetic pathways of legumes and the non-legumes resides in this step (Yu & McGonigle, 2005). In the leguminous species, the flavanone, naringenin is converted into genistein (Figure 1.12), catalysed by the enzyme, isoflavone synthase (IFS). In addition to this step, chalcone reductase (CHR) and chalcone synthase (CHS) catalyses the conversion of *p*-coumaroyl-CoA to isoliquritigenin which IFS converts to the second isoflavone, daidzein (Dixon & Ferreira, 2002) (Figure 1.12). However in non-leguminous plants isoflavones are not synthesised in the phenylpropanoid pathway due to the absence of the CHR and IFS.

1.9.2. Isoflavone synthase discovery and mode of action

One of the major breakthroughs in our understanding of the isoflavonoid branch pathway was the isolation of the first *IFS* genes. Three laboratories independently reported the identification of the *IFS* gene. Shin-ichi Ayabe's laboratory (Nihon University, Fujisawa, Kanagawa, Japan) isolated *IFS* from a licorice cell lines that produces isoflavonoids upon elicitation (Akashi *et al.*, 1999). At the same time, Richard Dixon's group (The Noble Foundation, Ardmore) identified an *IFS* gene from soybean (*Glycine max*) by functional screening of P450 cDNAs in insect cells (Steele *et al.*, 1999). A group at DuPont Wilmington, identified the same gene using a similar screen in yeast (Jung *et al.*, 2000). Cloning of the *IFS* gene has now provided unprecedented opportunities for metabolic engineering to manipulate and modify the isoflavone synthesis in both legumes and non-legumes.

IFS or 2-hydroxyisoflavanone synthase is an exceptional enzyme because it catalyzes at least two unusual reactions with one protein: a hydroxylation and an

intramolecular aryl migration reaction. It has been proposed that the flavanone first undergoes abstraction of hydrogen radical at C-3 followed by B-ring migration from C-2 to C-3 and subsequent hydroxylation of the resulting C-2 radical (Hashim *et al.*, 1990). IFS comes from the CYP93C subfamily of cytochrome P450s in plants and it is a membrane-bound enzyme attached to the outer surface of the endoplasmic reticulum (ER) by an n-terminal membrane anchor (Kochs & Grisebach, 1986).

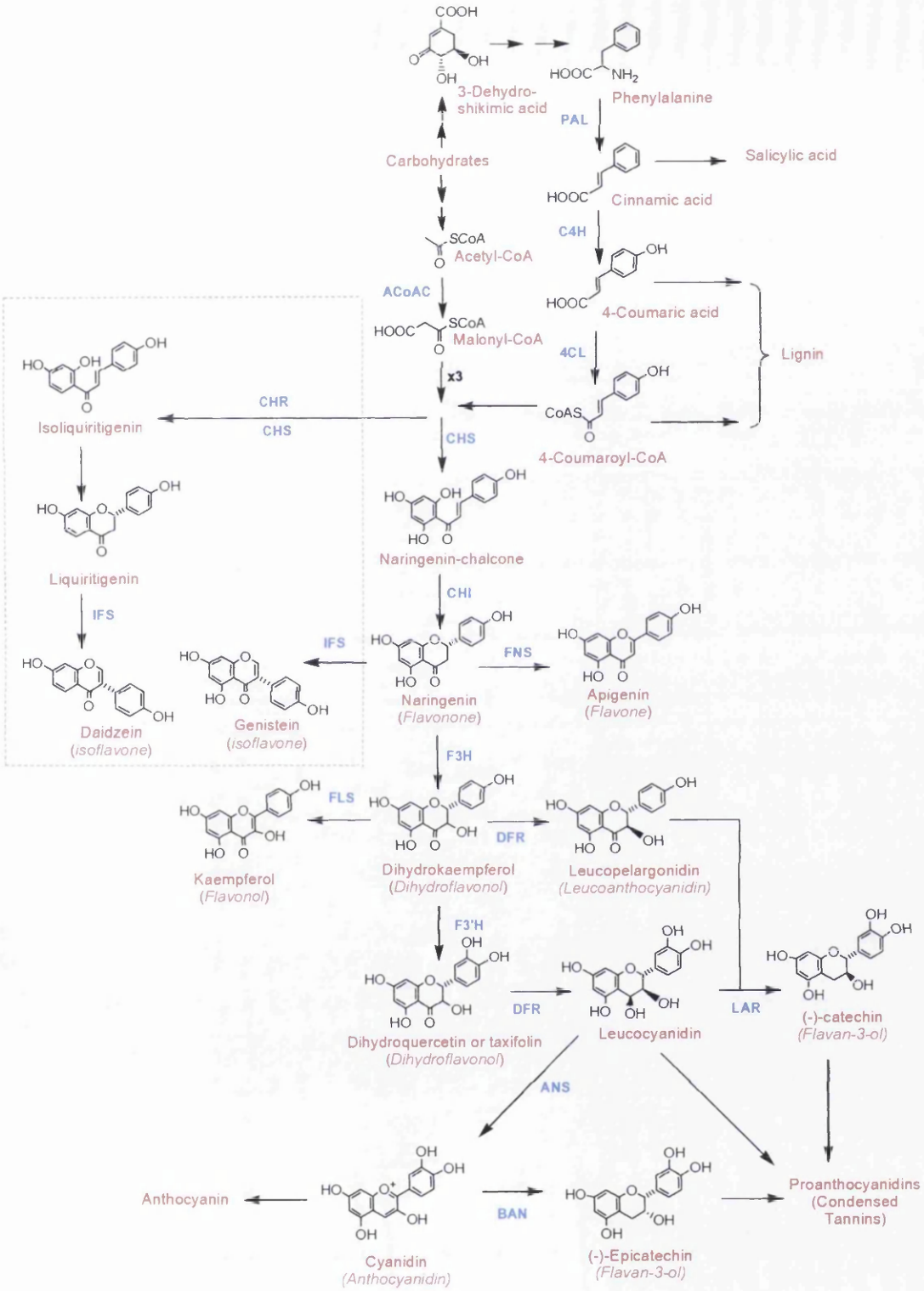


Figure 1.12 A partial diagram of the shikimatic acid, phenylpropanoid & flavonoid biosynthetic pathways. Enzyme abbreviations: ACoAC-acetylCoA carboxylase; PAL-phenylalanine ammonia-lyase; C4H-cinnamate 4-hydroxylase; 4CL-4-coumarate: CoA ligase; CHS-chalcone synthase; CHR- chalcone reductase; CHI-chalcone isomerase; IFS-isoflavone synthase; FNS-flavone synthase; F3H-flavanone 3-hydroxylase; FLS-flavonol synthase; DFR-dihydroflavonol 4-reductase; F3'H- flavonoid 3'-hydroxylase; ANS-anthocyanidin synthase; BAN- banyuls or anthocyanidin reductase; LAR- leucoanthocyanidin 4-reductase. The green dotted lines illustrates the isoflavone branch pathway.

1.9.3. CHI- the other key enzyme in isoflavone synthesis.

The upstream enzymes of isoflavone synthesis in the phenylpropanoid pathway include PAL, C4H, 4CL, CHS, and CHI. Of these, the CHI is thought to be the rate limiting step for metabolically engineering genistein in non-legume plants as it catalyses the reaction which can divert its substrates either towards flavonoid (naringenin) or isoflavonoid production (liquiritigenin) (Yu & McGonigle, 2005).

From recent studies, it has become apparent that there are multiple types of CHIs and their distributions are highly family specific (Shimada *et al.*, 2003). However, there are two major types of CHIs, CHI type 1 which is generally found in non-legumes and type II is found principally in leguminous species. Type 1 CHIs converts only naringenin chalcone to naringenin. In contrast, type II CHIs catalyses the metabolism of both isoliquiritingenin and naringenin chalcone, yielding liquiritigenin and naringenin respectively (Figure 1.13) (Shimada *et al.*, 2003).

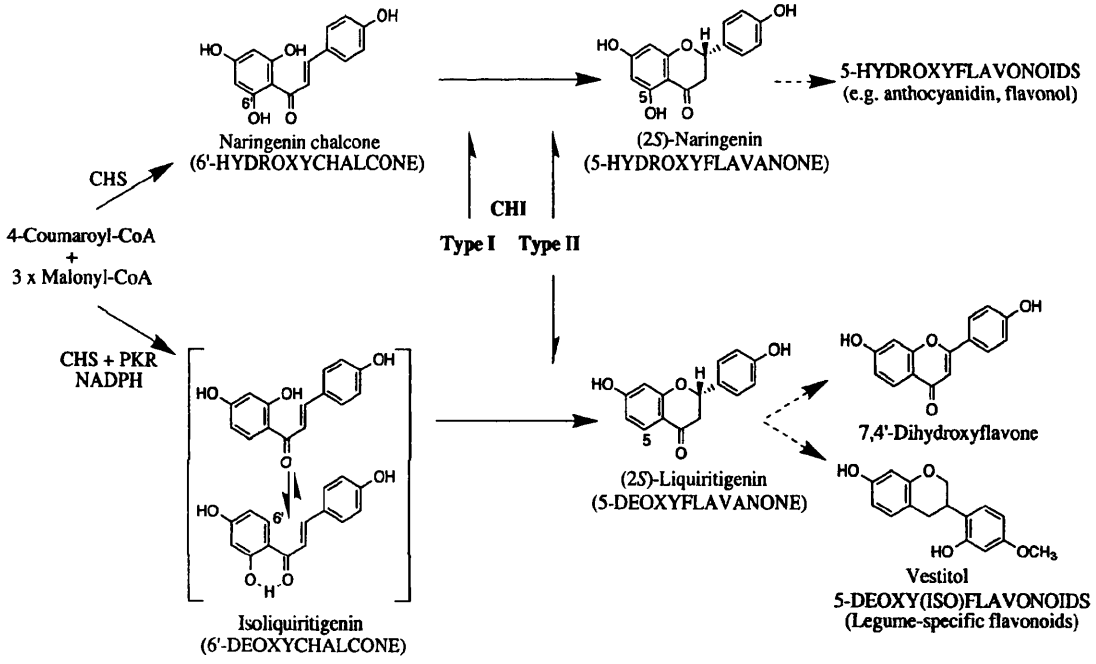


Fig. 1.13: The flavonoid pathway in leguminous plants. Type I CHIs isomerize only naringenin chalcone as the substrate, whereas type II CHIs accept both naringenin chalcone and isoliquiritingenin . Adapted from Shimada *et al.*(2003).

The genes that encode both types of CHIs have been cloned from various plant species (Kimura *et al.*, 2001; Shimada *et al.*, 2003). A high homology of amino acid sequences (>70%) was observed within the same type of CHI while the identity between type I and II CHIs was only about 50% homologous (Kimura *et al.*, 2001; Shimada *et al.*, 2003). This indicates that the different structures of CHI proteins may result in their different substrate specificities. Considering potential enzyme interactions, e.g., the interaction of type I CHI with the flavonoid pathway and type II CHI with the isoflavonoid pathway, it was proposed that introducing specific CHIs may be one of the key factors for metabolic pathway engineering of *IFS* in non-legume plants (Yu & McGonigle, 2005).

1.9.4 Transcriptional regulation

Transcriptional control is believed to play an important role in regulating the overall activity of phenylpropanoid metabolism in response to abiotic and biotic stress (Fahrendorf *et al.*, 1995; Weisshaar and Jenkins, 1998). At least three types of transcriptional genes are known to regulate the flavonoid pathway. These include the MYB gene family, (Paz-Ares *et al.*, 1987), a basic helix-loop-helix (bHLH) type transcriptional factor and a protein containing WD40 repeats (Mol *et al.*, 1998). The formation of transcriptional factor complexes with each other and with the DNA conserved promoter elements is believed to be a fundamental process in fine-tuning gene activity in the metabolic pathways (Wolberger, 1999). Due to their ability to manipulate and regulate the metabolic pathways, transcriptional factors have been used in gene transformation experiments. Since transcriptional factors are able to control multiple steps within a biosynthetic pathway, their use are believed to be more powerful than structural genes in manipulating metabolic pathways in plants (Broun, 2004). The potential benefit of using transcription factors to modify flux through flavonoid pathway was highlighted by a number of studies using the LC and C1 transcriptional factors. One such study was carried out by Bovy *et al.*(2002), where overexpression of the maize transcriptional regulators LC and C1 in tomato, resulted in an increase in flavonols in the flesh of tomato fruit. Total flavonol content of ripe transgenic tomatoes over-expressing LC/C1 was about 20-fold higher than that of the controls where flavonol production occurred only in the skin (Bovy *et al.*, 2002; Le Gall *et al.*, 2003).

1.9.5 Metabolic channeling.

In addition to enzymatic and transcriptional control, there is also a need to balance the activities of the complex branched phenylpropanoid metabolic system, particularly where several branch pathways are active within the cell (Winkel-Shirley 1999). In the phenylpropanoid biosynthetic pathway, phenylalanine can be converted into a lignins, sinapate esters, stilbenes, and flavonoids. Metabolic channeling is envisioned to provide mechanism not only for sequestering unstable or toxic intermediates, but more importantly, for controlling fluxes among the multiple branch pathways that often function concurrently within the same cell (Winkel, 2004).

Metabolic channelling is the spatial organization of cooperating enzymes into macromolecular complexes, which enables the transfer of biosynthetic intermediates between catalytic sites without diffusion into the bulk phase of the cell (Winkel, 2004). The complexes allow for efficient control of metabolic flux, and protect unstable intermediates from non-productive breakdown or access to enzymes from potentially competing pathways. Through metabolic channelling, cellular metabolism may attain high localised substrate concentrations, regulate competition between branch pathways for common metabolites, coordinate the activities of pathways with shared enzymes or intermediates, sequester reactive or toxic intermediates (Winkel, 2004) and protect unstable intermediates from non-productive breakdown (Dixon *et al.*, 2002). Such complexes may involve direct physical interactions between the various enzymes (Winkel-Shirley, 1999), or may be associated with the co-localization of enzymes on membranes or other surfaces (Liu & Dixon, 2001).

1.9.5.1 Metabolic channelling in the flavonoid and isoflavonoid biosynthetic pathway

Evidence for direct interactions between several key flavonoid biosynthetic enzymes, CHS, CHI, and F3H was recently demonstrated in developing Arabidopsis seedlings using yeast two-hybrid assays and was further confirmed by affinity chromatography and immunoprecipitation assays (Burbulis & Winkel-Shirley, 1999) (Figure 1.14). The Dixon group suggest that metabolic channeling also occurs at the entry point into the isoflavonoid pathway which involves CHS, CHR, and CHI. In another experiment, Liu and Dixon (2001) demonstrated through biochemical fractionation and confocal microscopy, that isoflavone *O*-methyltransferase (IOMT)

was localized in the ER membranes and is therefore envisaged to be closely associated with the membrane bound IFS (Figure 1.14). This complex was thought to facilitate rapid B-ring methylation resulting in formononetin (4'-O-methyldaidzein) and biochanin A (4'-O-methylgenistein) from the isoflavone, diadzein and genistein respectively (Akashi *et al.*, 2003). With this concept of metabolic channelling, in leguminous plants, type I and type II CHIs may participate in distinct enzyme complexes devoted to producing, for example, anthocyanin pigments and isoflavonoids at different organs and/or in different subcellular locations (Shimada *et al.*, 2003).

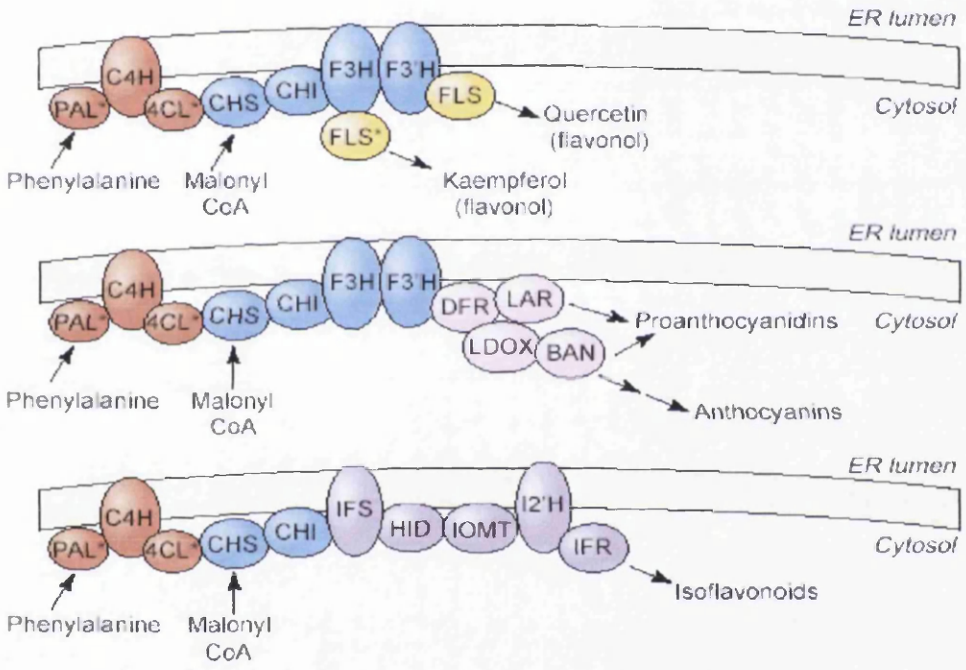


Figure 1.14. Organization of the branched pathways of flavonoid metabolism within separate individual metabolons. Enzymes that participate in multiple branches are shown in red and blue, whereas enzymes that are thought to be unique to specific pathways are shown in other colours. The cytochrome P450 enzymes shown (cinnamate 4-hydroxylase [C4H], flavonoid 3′-hydroxylase [F3′H], flavanone-3β-hydroxylase [F3H], isoflavone synthase [IFS] and isoflavone 2′-hydroxylase [I2′H]) are all membrane-bound proteins. Other enzymes include, anthocyanidin reductase [BAN]; chalcone isomerase [CHI]; chalcone synthase [CHS]; dihydroflavonol 4-reductase [DFR]; flavonol synthase [FLS]; 2-hydroxy-isoflavanone synthase [IFR]; 2-hydroxy-isoflavanone *O*-methyltransferase [IOMT]; leucoanthocyanidin reductase [LAR]; 2-hydroxyisoflavanone dehydratase [HID) and leucoanthocyanidin dioxygenase [LDOX]. Enzymes that are known to be present as multiple isoforms are marked with an asterix.

Adapted from Jorgensen *et al.* (2005)

1.9.6. Manipulating flavonoid biosynthesis

The diverse functions of phenolic compounds in humans together with the increasing knowledge on the regulation of flavonoid biosynthesis by structural enzymes and transcriptional factors have made flavonoid biosynthetic pathway an excellent target for metabolic engineering. The cloning and the characterization of most of the genes in this pathway have opened up possibilities to develop plants with tailor-made optimised flavonoid levels and compositions. The metabolic engineering of flavonoid pathways began in 1987, and has rapidly become an accomplished research area in the past decade (Forkmann & Martens, 2001). A number of metabolic engineering studies within the framework of flavonoid biosynthesis was concentrated on the ornamental plant breeding to generate novel flower colors, in particular blue and yellow flowering cultivars (Forkmann & Martens, 2001; Martens *et al,* 2003). In addition, an attempt was made also to increase the flavonoid content in tomatoes as demonstrated by the research carried out by Muir *et al.* (2001). Introducing the *CHI* gene from *Petunia* resulted in a 78% increase of flavonols, mainly due to the accumulation of rutin. Similarly, expressing the maize transcriptional factors LC and C1 in potatoes also caused a marked increase in the accumulation of kaempferol in the tubers (de Vos *et al.*, 2000).

1.9.6.1 Attempts made in the past to metabolically engineering *IFS* in non-legume plants

In isoflavone biosynthesis, the cloning and characterisation of the *IFS* gene has paved way for the possibilities of introducing isoflavones into widely consumed crops such as potato, tomato and corn, which will ultimately increase their nutritional value and bring the health benefits of isoflavones to more consumers. To engineer the isoflavone pathway in non-legume plants, the heterologous expression of *IFS* is an essential step because it is required to convert the naringenin, which is ubiquitous in higher plants, to isoflavone.

IFS has been successfully introduced into Arabidopsis, maize BMS cell cultures and tobacco (Jung *et al.*, 2000). Transformation of the soybean *IFS* gene regulated by a 35S promoter into Arabidopsis was successful as genistein could be synthesized (Jung *et al.*, 2000). However, in tobacco, which has a tissue specific activation of the phenylpropanoid pathway, genistein was detected in the flowers but not in the leaves of the transformants. This indicates that the production of genistein in

transgenic tobacco is regulated with a positive correlation to the activity of the anthocyanin branch of the phenylpropanoid pathway in only specific tissues (Jung *et al.*, 2000).

1.9.6.2 Constraints in metabolic engineering

Although there has been some success in the field of metabolic engineering in certain crops, due to the complexity of the flavonoid pathway, it nonetheless remains a challenging task to generate desired flavonoids. The final result of the engineering is dependent on a number of factors, including the approach used, the encoded function of the introduced transgene, the type of promoter used and on the activity and the regulation of the endogenous pathway (Broun, 2004; Lessard *et al.*, 2002). The desired results may not always be the case as seen in the stimulation of the flavonoid biosynthesis by the transcription factors LC and C1 in tomato which led to the induction of several flavonoid genes, but was not sufficient to induce F3'5'H activity, which is essential for the production of anthocyanins. This was again repeated in an experiment carried out by Yu *et al.* (2000) where transcriptional regulation by the introduction of the maize C1 and R transcriptional factors in maize BMS cell cultures expressing soybean *IFS* yielded higher levels of flavonols but did not induce production of genistein (Yu *et al.*, 2000). Similarly, no increase in genistein level were detected in transgenic Arabidopsis lines when *CHI* was over-expressed with the aim on increasing the provision of the naringenin substrate for IFS. Nor was genistein production increased in transgenic Arabidopsis lines expressing the soybean *IFS* with a *pap1-D* genetic background in which anthocyanin production was strongly up-regulated (Liu *et al.*, 2002).

1.9.7 The possibility of engineering genistein in tomatoes?

To date no attempt has been made to genetically engineer genistein synthesis in tomato. As with most non-leguminous plants, isoflavones are not synthesised in the tomato phenylpropanoid pathway due to the absence of the *IFS* gene. The pathway is, however, similar to that of Arabidopsis where the main substrate naringenin is present, thus, making it possible to engineer genistein synthesis. In tomato fruits, flavonoids accumulate in a tissue-specific manner (Krause & Galensa, 1992) and analysis at different ripening stages, showed that the accumulation of flavonoids in tomato fruit is not only regulated in a tissue-specific, but also in a development-dependent manner.

Naringenin chalcone, the main flavonoid precursor was found to accumulate almost exclusively in skin and is simultaneously formed with colouring of the fruit, peaking at up to 1% on a dry weight basis (Muir *et al.*, 2001). This may be advantageous in attempting to engineer genistein in tomato as naringenin chalcone is the precursor to the synthesis of naringenin, which is the crucial substrate for IFS to produce genistein. The tissue specific regulation and the development-dependent regulation of the flavonoid pathway in tomatoes may warrant the use of an inducible promoter, which will be utilised in attempting to engineer genistein synthesis in tomato in the present study.

Chapter 2: Aims of Study

Currently, one of the main areas of investigation on flavonoid research is in the field of metabolism and bioavailability of flavonoids. This area demands an urgent examination of identification and quantification of *in vivo* metabolites as they are the key prerequisites to understanding and defining the role of beneficial flavonoids such as rutin in humans. Another area of research which is also gaining momentum is in the field of metabolic engineering where attempts are being made to increase levels of 'novel' flavonoids such as genistein within the native plant or introduce them in crops that normally do not produce them. As such, this thesis is divided into two main parts, Part I (Chapters 3, 4 & 5) deals with the absorption, metabolism and bioavailability of the flavonol, rutin while Part II (Chapter 5) deals with attempts to engineer genistein into tomato and Arabidopsis.

Therefore, the present study was set out to achieve the following objectives:

1. To identify and quantify rutin metabolites in the circulatory and excretory system and to provide quantitative pharmacokinetic data on the individual metabolites after consumption of rutin rich tomato juice
2. To determine the extent and site of absorption of rutin in humans
3. To identify specific degradation products of rutin in the colon by using an *in vitro* faecal fermentation model and to determine their contribution to the total antioxidant activity of the colonic lumen.
4. To engineer into tomato and Arabidopsis, genistein synthesis, the key dietary component of soybean with the aim of ultimately producing plants with novel bioactive compound profiles.

Chapter 3: Absorption and Bioavailability of Rutin

3.1 Introduction

The ubiquitous nature of polyphenolic compounds in plants ensures that they are found in substantial quantities in the diet. However, their potential health benefits are very much dependent upon their absorption and disposition in target tissues and cells. There are many reports of *in vitro* studies designed to elucidate the biological properties of flavonoids, however, these studies may not be readily extrapolated to *in vivo* situations. Therefore, an understanding of the metabolism and bioavailability of these compounds *in vivo* is important. Hollman (2004) proposed that in the metabolism of flavonoids, two compartments should be considered; the first compartment consists of the small intestine, liver and kidneys while the colon constitutes the second compartment. But it is seems more appropriate that a third compartment which encompasses the circulatory system, cell and tissues be also included.

For aglycones and certain flavonoid glycosides such as quercetin glucosides, metabolism occurs in the first compartment where absorption from the gastrointestinal tract generally involves deglycosylation by luminal LPH and/or cleavage within the enterocyte by CBG (Day *et al.*, 2003a; Németh *et al.*, 2003). This is followed by metabolism of the aglycone, which leads to the appearance of quercetin sulphate and glucuronide conjugates in the circulatory system (Day *et al.*, 2003b; Day *et al.*, 2001). Flavonoids such as quercetin 3-rutinoside (rutin) where the conjugating moiety is a rhamnose-glucose disaccharide, are not cleaved by either LPH or CBG and therefore their metabolism occurs in the second compartment as they seemingly pass intact from the small intestine to the large intestine where they are hydrolyzed and possibly degraded by the colonic microflora (Olthof *et al*, 2003; Hollman *et al.*, 1995). At least part of the released quercetin is absorbed (reviewed by Manach *et al.*, 2004). This was indicated, albeit indirectly, by the appearance of rutin metabolites in plasma, 4-8 h after intake, which implies absorption in the colon rather than the small intestines (Graefe *et al.*, 2001, Erlund *et al.*, 2000, Sawai *et al.*, 1987). A more direct approach indicating the colon as the major site of rutin metabolism and absorption involves the use of human subjects with an ileostomy. Using this system, Hollman *et al.* (1995) showed that 83% of ingested rutin was present in the ileal fluid. In addition to the formation of phase II metabolites, a huge array of phenolic acid may also be formed through the degradation

of the flavonoid ring structure by faecal bacteria in the colonic lumen. Thus, information on the site and extent of rutin metabolism can play a crucial role in determining the exact composition of metabolites produced which in turn impacts on its biological activities *in vivo* (DuPont *et al.*, 2004).

Most flavonoids undergo considerable metabolism upon ingestion and are found *in vivo* as conjugates of glucuronate, sulphate, or methyl forms or as phenolic acids, which are ring fission catabolites (Kroon *et al.*, 2004). To establish the role of flavonoids as protective agents *in vivo*, it is critical to understand the chemical nature of the absorbed forms and their *in vivo* concentrations in circulatory system and tissues as this may have a major impact on the contribution of flavonoids as dietary protective agents. This has prompted researchers to make use of more accurate and reliable methods of identification. For instance, it is now known that identification of flavonols based on HPLC elution times and absorbance spectra is prone to produce inaccurate data as it lacks both the selectivity and sensitivity required when analyzing low levels of metabolites in plasma and urine. Traditionally, quantitative analysis of plasma and urine, post ingestion of rutin has involved acid or glucuronidase/sulphatase treatments to release to the parent aglycone prior to quantitative analysis by HPLC (Graefe *et al.*, 2001; Hollman *et al.*, 1997, Erlund *et al.*, 2000). This analytical approach can fail to detect methylated derivatives and, other than a global figure for conjugating forms, provides little or no information on the number and identity of individual metabolites formed during either phase I or phase II metabolism.

With recent advances in analytical instrumentation, more powerful techniques such as the use of mass spectrometry in combination with high-resolution chromatography systems, especially reversed-phase HPLC, now enables structural information to be obtained for individual HPLC peaks without recourse to acid or enzyme treatment. It is possible for instance, to distinguish between metabolites such as quercetin glucuronides and quercetin glucosides without the need for reference compounds and also to differentiate between the different isomers. Some investigators have successfully used HPLC-MS technology to detect a range of quercetin metabolites in plasma and urine post ingestion of quercetin glycosides or onions in human intervention trials (Wittig *et al.*, 2001; Day *et al.*, 2001; and Mullen *et al.*, 2004). Tandem MS (MS^2) and MS^3 offer significant advantages over single stage MS techniques (Mullen *et al.*, 2004). To date these analytical methods have not been used to

unravel the complexities of the metabolism and absorption of rutin in human subjects. Olthof *et al.* (2003) used a GC-MS technique to identify and quantify the phenolic acid metabolites in the urine after ingestion of 440 mg (660 μmoles) of quercetin-3-rutinoside but no attempt was made to detect the conjugated quercetin metabolites in either the circulatory system or urine.

This chapter reports on the use of HPLC with photodiode array (PDA) and MS^2 detection with either an ESI (electrospray ionization) interface or APCI (atmospheric pressure chemical ionization) interface, to analyse human plasma, urine and ileal fluid collected from six healthy human subjects and five volunteers with an ileostomy. The samples were collected at a series of time points over a 24 h period after the consumption of tomato juice fortified with 100 mg (164 μmoles) of rutin. With these procedures, it was possible to identify and quantify rutin metabolites in the circulatory and excretory system, determine the extent of absorption, provide quantitative pharmacokinetic data of the individual metabolites and finally to confirm the colon as the site of absorption of rutin-derived quercetin metabolites. The level of rutin in the tomato juice was chosen so that the amount ingested was the same as an earlier study in which onions rich in quercetin glucosides were fed to healthy human volunteers. This enabled direct comparisons to be made between the fate of quercetin glucosides and the quercetin-3-glucosylrhamnoside (rutin) conjugate.

3.2 Materials and Methods

3.2.1 Study design

Six healthy human subjects, (five females and one male), and five volunteers with an ileostomy, and hence lacking a colon (four males and one female) who were non-smokers and not on any medication, participated in this study and gave their written consent. They were aged between 22 and 48 years and had a mean body mass index of 24.5 ± 1.1. All of the ileostomy volunteers had their operation at least 5 years prior to the study and had minimal resection of the small intestine, establishing that they were at least semi-physiologically normal. Subjects were required to follow a low flavonoid diet for two days prior to the study and this included avoiding most fruits, vegetables and beverages such as tea, coffee, fruit juices, and wine. Food recommended was meat (excluding spices), pasta (without tomatoes), fried or boiled potatoes, rice, milk and unflavored yogurt. On the morning of the study, the volunteers were asked to consume 300 mL of tomato juice fortified with 164 μmoles (100mg) of rutin. Aliquots of the tomato juice were taken for qualitative and quantitative analysis of their flavonol content.

A cannula was inserted into the arm of each volunteer and venous blood samples withdrawn at 0, 2, 3, 4, 7, 8, and 24 h post-ingestion of the tomato juice. In keeping with previous reports (Graefe *et al.,* 2001; Hollman *et al.*, 1997, Erlund *et al.,* 2000) and from preliminary study carried out on feeding trial with rutin, it was clear that metabolism of rutin occurs only after 2 h of ingestion and therefore no blood samples were taken prior to this point. Ten ml of blood was collected in heparinised tubes at each time point and immediately centrifuged at 4000 *g* for 10 min at 4°C. The plasma was decanted from the red blood cells and 1 mL aliquots were acidified to pH 3 with 30 μL of 50% aqueous formic acid and 100 μL of ascorbic acid (10 mM) was added to prevent oxidation. The plasma samples were then stored at -80°C prior to analysis. Urine and ileal fluid was collected prior to supplementation and over 0-2, 2-5, 5-8 and 8-24 h periods after the consumption of rutin-fortified tomato juice. The volume of each sample was recorded prior to storage of aliquots at -80°C. The stability of the stored samples were investigated by spiking the samples with 0.625 μg of catechin (internal standard) and then measuring its concentration before and after

storage. The study protocol was approved by the Glasgow Royal Infirmary Local Research Ethics Committee.

3.2.2 Materials

The Del Monte tomato juice was purchased from a local supermarket (Safeway Stores, Byres Road, Glasgow). HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Scotland). Formic acid was purchased from Riedel-DeHaen (Seeize, Germany) and acetic acid from BDH (Poole, UK). All other chemicals and reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Quercetin-3-glucuronide was extracted from French beans (*Phaseolus vulgaris*) and purified by partitioning against ethyl acetate and fractionation using preparative reversed phase HPLC. Quercetin-3'-glucuronide, quercetin-4'-glucuronide, quercetin-7-glucuronide, quercetin-3'-sulphate and isorhamnetin-3-glucuronide were donated by Dr. Paul Needs and Dr Paul Kroon (Institute of Food Research, Norwich, UK).

3.2.3 Extraction of flavonoids from tomato juice

Aliquots of tomato juice were taken for quantitative analysis of their flavonol content. Triplicate samples of 5 mL of tomato juice were extracted with 5 mL of 50% methanol containing 1% formic acid and 20 mM sodium diethyldiothiocarbamate. (+)-Catechin was used as an internal standard. This mixture was placed on a basic orbital IKA (KS 130) shaker at 350 rpm for 30 min after which it was centrifuged at 4,000 *g* at 4°C for 10 min. The supernatant was collected and the pellet further extracted twice with five mL methanol. The three supernatants were combined and reduced to dryness in a rotary evaporator (Thermo Savant SPFD SpeedVac, Hampshire, UK). The dried extract was dissolved in 125 μL methanol to which 2375 μL of 1% formic acid was added and ten μL aliquots were analysed by HPLC-PDA-MS^2. The extraction recoveries for the internal standard was 94 ± 4.5%.

3.2.4 Extraction of flavonoids from plasma

Triplicate samples of plasma were extracted using the method by Day *et al.* (2001) with slight modifications. Plasma (250 μL) was added dropwise to 600 μL of acetonitrile to precipitate the proteins. The mixture was vortexed for 30 s every 2 min over a 10 min period, before centrifuging at 16,000 *g* at 4°C for 20 min. The supernatant was retained and the pellet re-extracted as described above but with

methanol instead of acetonitrile. The two supernatants were combined and reduced to dryness *in vacuo*. Extracts were then dissolved in 25 µL of methanol plus 225 µL of 1% formic acid in water and centrifuged at 13,000 *g* at 4°C for 2 min prior to the analysis of 250 µL aliquots of the supernatant by HPLC-PDA-MS^2. (+)-Catechin was used as an internal standard with 0.625 µg being added to acetonitrile prior to the addition of the plasma. The extraction recoveries for the internal standard was 91± 5.3% while that of spiked samples was 85 ± 4.9%.

3.2.5 Extraction of flavonoids from ileal fluid

Ileal fluid was collected at the specified time points where all the content of the pouch were emptied. In the 8-24 h time point, the ileal pouch was emptied every 3 h, except when the volunteers were asleep. This is to ensure that there was minimal bacterial growth and contamination. Immediately after collection, the ileal fluid was stored in –80°C. Prior to extraction, the ileal fluid was defrosted and thoroughly mixed. Triplicate 0.5 g samples were extracted with 3 mL of 95% methanol containing 1% formic acid and 20 mM sodium diethyldiothiocarabamate. Five µg (+)-catechin was added to the extraction mixture to act as an internal standard. Samples were homogenised for 5 min (Disruptor Genie, Scientific Industries) and then centrifuged at 16000 *g* for 15 min. The supernatant was collected and the pellet re-extracted twice as described above. The three supernatants were combined and reduced to dryness *in vacuo*. Extracts were then made up to 1 mL with 50 µL methanol and 950 µL 1% formic acid. Aliquots of the centrifuged supernatant 20-100 µL were analysed by HPLC-PDA-MS^2.

3.2.6 Preparation of urine

Urine samples were defrosted, thoroughly mixed, centrifuged at 13,000 *g* at 4°C for 2 min prior to injection of 200 µl aliquots of the supernatant into the HPLC-PDA-MS^2 for analysis of flavonoids and phenolic acids.

3.2.7 HPLC with diode array and MS^2 detection

Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, PDA detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C. (Thermo Finnigan, San Jose, USA). Separation was carried out using a 250 x 4.6 mm I.D. 4 µm Synergi Max-RP column for flavonoids and (Phenomenex, Macclesfield,

UK) eluted with a 65 min gradient of 5-43% acetonitrile in 1% formic acid at a flow rate of 1 mL min^{-1} and maintained at 40°C. Similar conditions were adopted for phenolic acid detection, but being more polar, a 250 x 4.6 mm I.D. 4 μm Synergi Hydro-RP column and a 60 min gradient of 2-20% acetonitrile in 0.25% acetic acid was used. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 mL min^{-1} was directed to a LCQ DecaXP ion trap mass spectrometer fitted with either an ESI interface or an APCI interface (Thermo Finnigan). Analyses utilised the negative ion mode for both flavonoids (ESI) and phenolic compounds (APCI) as this provided the best limits of detection. Analysis was carried out using full scan, data dependant MS^2 scanning from *m/z* 100 to 1000. For the ESI interface, the capillary temperature was 250°C, sheath gas and auxiliary gas were 40 and 5 units respectively, and the source voltage was 5 kV for negative ionisation. For the APCI interface, the capillary temperature was 130°C, vaporizer temperature was 350°C, sheath gas and auxiliary gas were 80 and 60 units respectively, and the source voltage was 6 kV for negative ionisation. Quercetin, rutin, quercetin-3-glucuronide were all quantified by reference to standard calibration curves at 365 nm. Other flavonols were quantified in quercetin-3-glucuronide equivalents. 3-Hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid were both quantified by reference to standard calibration curves at 280 nm. Other phenolic acids were quantified in either 3-hydroxyphenylacetic acid or 3,4-dihydroxyphenylacetic equivalents. In all instances peak identification was confirmed by co-chromatography and MS^2 fragmentation data.

3.2.8 Pharmacokinetic analysis of plasma metabolites

Maximum post-ingestion plasma concentration of rutin metabolites was defined as C_{max}. The time to reach maximum plasma concentration (T_{max}) was defined as the time in hours at which C_{max} was reached. The elimination half-life for the metabolites in hours was computed by using the following formula $T_{1/2} = 0.693/K_e$ where K_e is the slope of the linear regression of the plasma metabolite concentrations.

3.2.9 Statistical analyses

Each sample was analysed in triplicate and data were presented as mean values ± standard error (n=3).

3.3 Results

3.3.1 HPLC-tandem mass spectrometry analysis of tomato juice

The levels of individual flavonoids were determined by HPLC-MS^2 and diode array detection. Samples were analysed on a Surveyor HPLC system using a 60 min, 5-40% gradient of acetonitrile in 1% aqueous formic acid. In total nine compounds were identified in the tomato juice based on MS^2 data and λ_{max} at 365 nm. In keeping with the data of Woeldecke and Herrmann (1974), the major flavonol was rutin (*peak 6* – Figure 3.1). The total amount of rutin in 300 mL tomato juice was 7.3 ± 0.9 mg (12.0 ± 1.4 μmoles). Smaller amounts of quercetin-3-rutinoside-7-glucoside *(peak 4)* and quercetin-rutinoside-pentoside (*peak 5*) were also detected in the juice. The results obtained from the HPLC-PDA-MS^2 are summarised below and presented in Table 3.1 and Figure 3.1

Peak 1 (t_R = 10.8 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 353 with MS^2 yielding a charged fragment ion at *m/z* 191 and relatively intense secondary ion at 179. This peak is, 3-caffeoylquinic acid, according to the fragmentation scheme of Clifford *et al.* (2003).

Peak 2 (t_R = 11.6 , λ_{max} = 290 nm) had a $[M-H]^-$ at *m/z* 341, with MS^2 yielding a charged fragment at *m/z* 179 which corresponds to caffeic acid. Loss of 162 *amu* indicates cleavage of a hexose moiety. This fragmentation, therefore, is in keeping with the presence of a caffeic acid hexose conjugate.

Peak 3 (t_R = 15.9 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 353 with MS^2 yielding a charged fragment ion at *m/z* 191 and weaker secondary ion at *m/z* 179. This peak co-chromatographed with and has the same MS^2 spectrum as 5-caffeoylquinic acid.

Peak 4 (t_R = 16.4 min, λ_{max} = 340 nm) had a $[M-H]^-$ at *m/z* 771, with MS^2 yielding a charged fragment *m/z* 609 and *m/z* 301. These ions were formed by the successive loss of a hexose (162 *amu* cleavage) and a rutinose (308 *amu* cleavage) unit. This compound was tentatively identified as a quercetin-rutinoside-glucoside, possibly quercetin-3-rutinoside-7-glucoside, as reported by Le Gall *et al.* (2003).

Peak 5 (t_R = 25.1 min, λ_{max} = 355 nm) had a $[M-H]^-$ at *m/z* 741, with MS^2 yielding a charged fragment at *m/z* 609 and 301. These ions were formed by the

successive loss of a pentose (152 *amu* cleavage) and a rutinose unit (308 *amu* cleavage). Peak 4 was identified as quercetin-rutinoside-pentoside.

Peak 6 (t_R = 27.3 min, λ_{max} = 360 nm) was the main flavonoid present in the tomato juice. It had a $[M-H]^-$ at *m/z* at 609, which with neutral loss of 308 *amu,* corresponding in this instance to the loss of a glucosyl and a rhamnosyl unit, yielded an MS^2 fragment corresponding to quercetin at *m/z* 301. This compound was identified as quercetin-3-rutinoside (rutin), which was confirmed by co-elution with an authentic standard.

Peak 7 (t_R = 30.9 min, λ_{max} = 360 nm) had a $[M-H]^-$ ion at *m/z* 593, with MS^2 yielding a charged kaempferol fragment at *m/z* 285, produced by cleavage of a 308 *amu* rutinosyl unit. This compound was identified as kaempferol-3-rutinoside.

Peak 8 (t_R = 31.4 min, λ_{max} = 325 nm) had a $[M-H]^-$ ion at *m/z* 515 and produced on MS^2 a main fragment at *m/z* 353 and a minor one at *m/z* 173, in keeping with the presence of 3,4-dicaffeoylquinic acid (Clifford *et al.*, 2003).

Peak 9 (t_R = 49.5 min, λ_{max} = 280 nm) had a $[M-H]^-$ at *m/z* 271 with MS^2 yielding a charged fragment at *m/z* 151 and 177. This compound was identified as the flavanone, naringenin, which was confirmed by co-elution with an authentic standard.

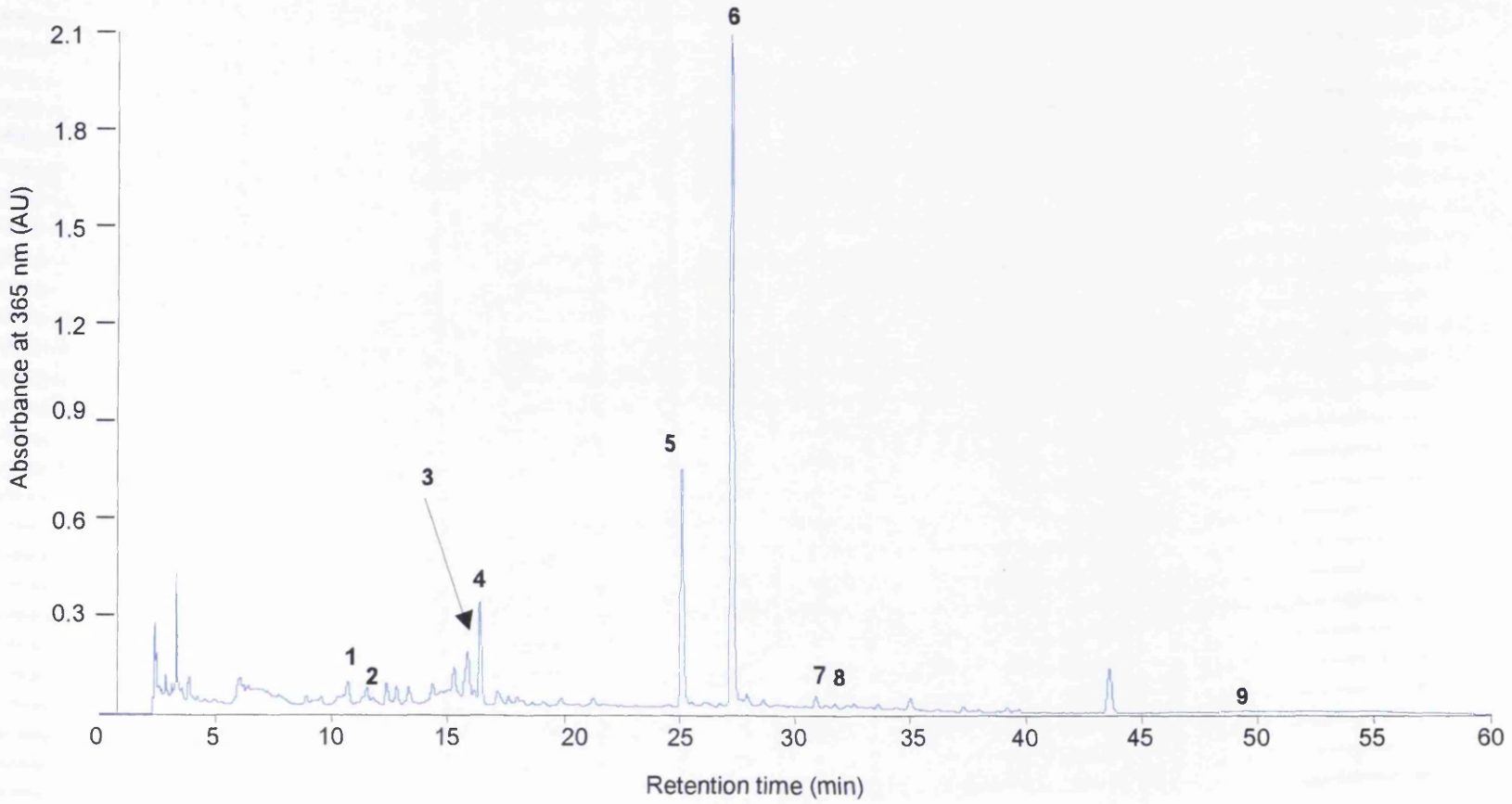


Figure 3.1. Specimen chromatogram of tomato juice extract. For MS^2 data and identification and quantification of peaks 1-4, refer to Table 3.1

Table 3.1. HPLC-MS^2 identification of phenolic compounds in tomato juice

Peak	t_R (min)	Compound	Absorbance	$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)
1	10.8	3-caffeoylquinic acid	325	353	191, 179
2	11.6	caffeic acid hexose conjugate	290	341	179[CafAc]([M-H]$^-$- Hex)
3	15.9	5-caffeoylquinic acid	325	353	191, 179
4	16.4	quercetin-3-rutinoside-7-glucoside	340	771	609([M-H]$^-$-Hex)-301[Q]([M-H]$^-$-Hex-Rut)
5	25.1	quercetin-rutinoside-pentoside	355	741	609([M-H]$^-$-Pent),-301[Q]([M-H]$^-$-Pent-Rut)
6	27.3	quercetin-3-rutinoside	330	609	301([Q]([M-H]$^-$-Glc- Rham)
7	30.9	kaempferol-3-rutinoside	330	593	285[K]([M-H]$^-$-Glc- Rham)
8	31.4	3,4-dicaffeoylquinic acid	325	515	353 ([M-H]$^-$-Caf), 173
9	49.5	naringenin	280	271	151, 177

Peak numbers and HPLC retention times refer to HPLC trace in Figure 3.1 . t_R - retention time; $[M-H]^-$ - negatively charged molecular ion; CafAc- caffeic acid; Hex- hexosyl; Q- quercetin; Pent- pentosyl; Rham- rhamnosyl; Caf- caffeoyl; Rut- rutinosyl

3.3.2 Identification and quantification of conjugated rutin metabolites in plasma and urine of healthy volunteers with a colon

3.3.2.1 Identification of conjugated rutin metabolites

Plasma and urine collected at the different time points over a 24 h period from the healthy volunteers with a colon after ingestion of the rutin-spiked tomato juice were analysed by HPLC-PDA-MS^2 detection. In the 365 nm traces obtained with plasma at 0, 2 and 3 h samples and with urine at 0, 0-2 h samples no peaks that corresponded to any flavonol based compounds were detected. However, in the 4 h plasma samples and the 2-5 h urine samples onwards, small quantities of quercetin-based compounds were detected. A total of nine conjugated quercetin metabolites were detected in urine and two in plasma. Typical HPLC traces obtained at $A_{365\ nm}$ are illustrated in Figure 3.3, their structures in Figure 3.2 and identifications based on MS^2 spectra are summarised in Table 3.2. The different classes of metabolites that were detected can be summarised as follows:

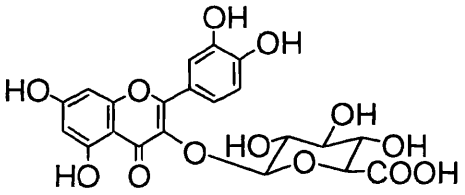
Quercetin monoglucuronides. Three quercetin monoglucuronides were detected, each being characterised by a negatively charged molecular ion $[M-H]^-$ at *m/z* 477, which on MS^2 fragments with a loss of 176 *amu*, corresponding to the cleavage of a glucuronide unit, to produce an ion at *m/z* 301 from quercetin. Co-chromatography with reference compounds facilitated the identification of quercetin-3-glucuronide *(peak 5)*, quercetin-4'-glucuronide *(peak 8) a*nd quercetin-3'-glucuronide *(peak 9)*.

Quercetin diglucuronides. Peak 6 was present only in urine of subjects from 1 and 2. It had a $[M-H]^-$ at *m/z* at 653 which yielded MS^2 fragments at *m/z* 477 ($[M-H]^-$-176) and *m/z* 301 ($[M-H]^-$352), loss of two glucuronide units, indicating the presence of a quercetin diglucuronide.

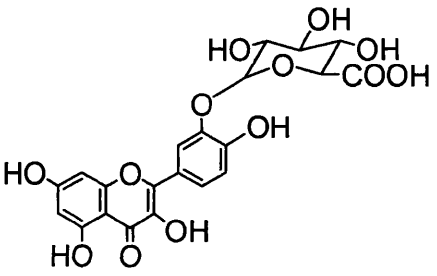
Isorhamnetin-3-glucuronide. This compound was detected in both urine and plasma and corresponded to *peak 7*, which was characterised by a mass spectrum with fragment ions at *m/z* values 14 *amu* higher than obtained with quercetin glucuronides and co-chromatograph with an authentic reference compound.

Methylquercetin diglucuronides. Peak 1, which yielded an MS^2 spectrum with fragment ions 14 *amu* higher than that of *peak 6*, was a methyl quercetin diglucuronide. It was only present only in the urine of volunteer 1 and 2.

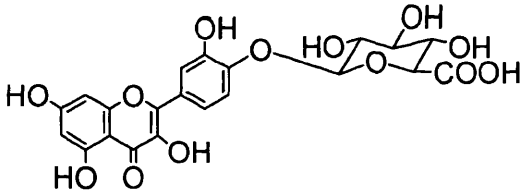
Quercetin glucoside glucuronides. Peaks 2, 3 and 4 which were detected in the urine had a $[M-H]^-$ at *m/z* 639 which on MS^2 yielded ions at *m/z* 477 ($[M-H]^-$-162, loss of glucose), *m/z* 463 ($[M-H]^-$-176, loss of a glucuronide unit) and *m/z* 301 indicating that both compound are quercetin glucoside glucuronides



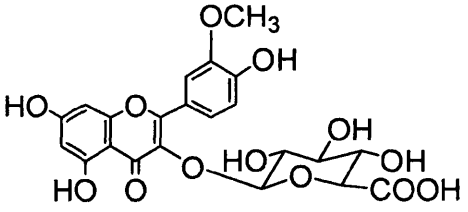
Quercetin-3-glucuronide



Quercetin-3'-*O*-glucuronide



Quercetin-4'-*O*-glucuronide



Isorhamnetin-3-glucuronide

Figure 3.2. Structures of quercetin metabolites in plasma and urine of volunteers with a colon after consumption of rutin spiked tomato juice.

Table 3.2. HPLC-MS^2 identification of quercetin metabolites detected in plasma and urine post-consumption of tomato juice fortified with 164 μmoles rutin in six human volunteers with a colon.

Peak	t_R (min)	Compound	$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)	Location
1	21.5	methylquercetin diglucuronide	667	491([M-H]$^-$-GlcUA), 315([M-H]$^-$-GlcUA-GlcUA)	urine
2	21.9	quercetin glucoside glucuronide	639	477([M-H]$^-$-Glc), 463([M-H]$^-$-GlcUA), 301([M-H]$^-$-GlcUA-Glc)	urine
3	26.8	quercetin glucoside glucuronide	639	477([M-H]$^-$-Glc), 463([M-H]$^-$-GlcUA), 301([M-H]$^-$-GlcUA-Glc)	urine
4	29.1	quercetin glucoside glucuronide	639	477([M-H]$^-$-Glc), 463([M-H]$^-$-GlcUA), 301([M-H]$^-$-Glc-GlcUA)	urine
5	30.5	quercetin-3-glucuronide	477	301 ([M-H]$^-$-GlcUA)	urine, plasma
6	31.9	quercetin diglucuronide	653	477([M-H]$^-$-GlcUA), 301([M-H]$^-$-GlcUA-GlcUA)	urine
7	35.7	isorhamnetin-3-glucuronide	491	315([M-H]$^-$-GlcUA)	urine, plasma
8	36.7	quercetin-4'-glucuronide	477	301([M-H]$^-$-GlcUA)	urine
9	39.8	quercetin-3'-glucuronide	477	301([M-H]$^-$-GlcUA)	urine

Peak numbers and HPLC retention times refer to HPLC trace in Figure 3.3. t_R - retention time; $[M-H]^-$ - negatively charged molecular ion; Glc - glucosyl unit; GlcUA – glucuronyl unit.

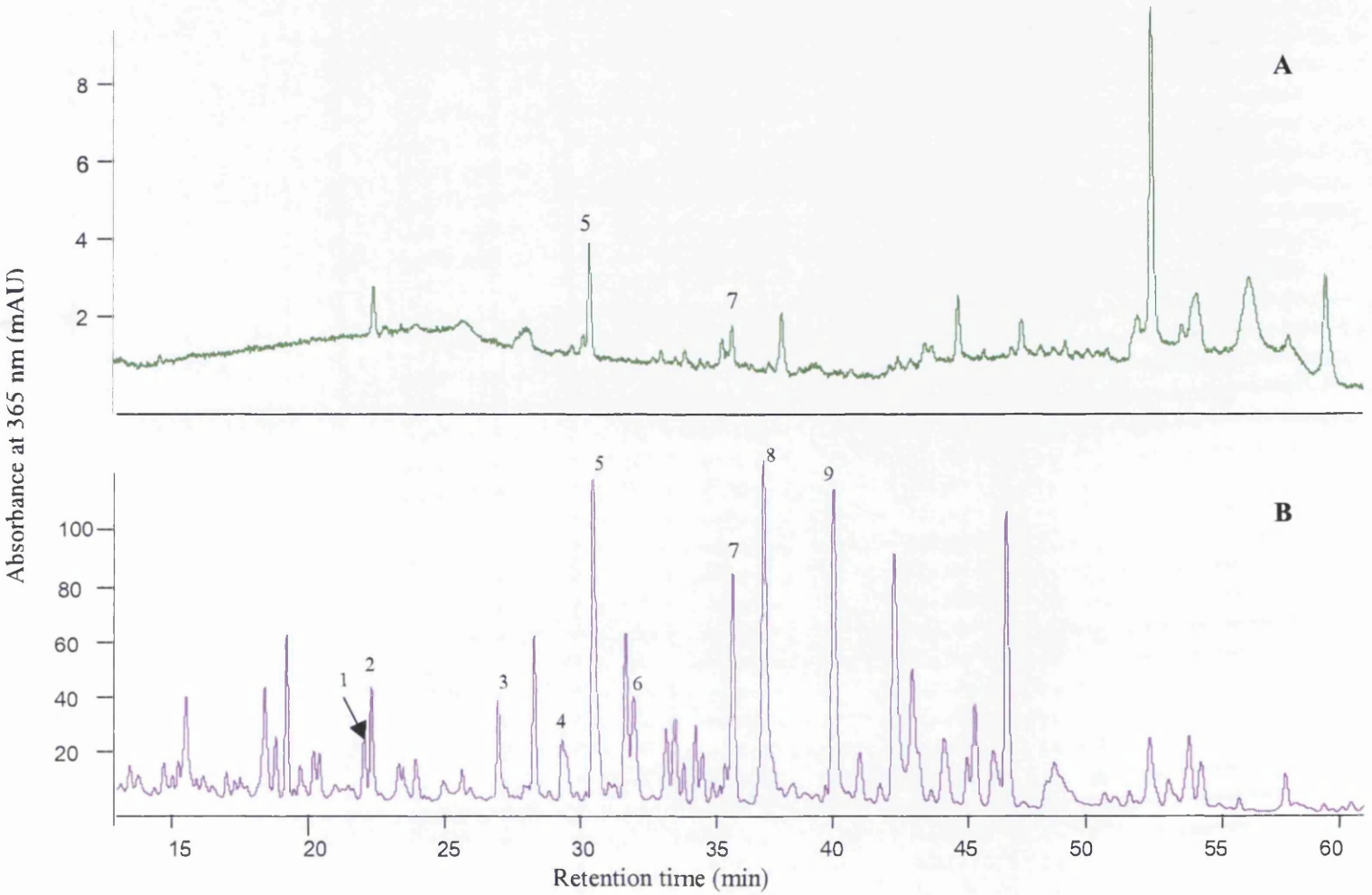


Figure 3.3. Gradient reversed-phase HPLC with detection at 365 nm of quercetin metabolites in (A) a plasma extract and (B) urine obtained from volunteer 1 after consumption of tomato juice fortified with 164 µmoles of rutin. For identity of peaks refer to Table 3.2.

3.3.2.2 Quantitative analysis of rutin metabolite accumulation in plasma

Two quercetin metabolites were detected in the plasma namely, quercetin-3-glucuronide and isorhamnetin-3-glucuronide. Isorhamnetin-3-glucuronide was however not detected in subject 6 (Table 3.3). The 0-24 h pharmacokinetic profiles of these two metabolites are illustrated in Figure 3.4. Neither compound was detected in plasma collected either immediately before ingestion, or after 2, 3 and 24 h. Quercetin-3-glucuronide was also not detected in the 8 h samples. A pharmacokinetic analysis of the data is summarised in Table 3.4. The major component was quercetin-3-glucuronide with a C_{max} of 12 ± 2 nmoles while isorhamnetin-3-glucuronide had a C_{max} of 4.3 ± 1.5 nmoles. In both instances the T_{max} occurred more than 4 h after the ingestion of the rutin supplementation (Table 3.4). The levels of these two metabolites gradually declined after reaching C_{max} (Figure 3.4) and they had a $T_{1/2}$ with values of 5.7 ± 0.6 and 6.9 ± 0.3 h (Table 3.4). The longer $T_{1/2}$ of isorhamnetin-3-glucuronide may be a consequence of it being produced by 3'-methylation of quercetin-3-glucuronide. Due to inter-individual variations, the C_{max} values of flavonols in the plasma is exhibited separately for each individual as presented in Table 3.4. Subject 1 and 2 exhibited similar profiles with a higher C_{max} for quercetin-3-glucuronide than the other volunteers with values of 22 ± 6 and 24 ± 2 nM and with T_{max} of 4 h. The C_{max} of subjects 4, 5 and 6 was much lower ranging from 4.0 ± 1.0 to 5.2 ± 0.4 nM. Due to the low amounts of quercetin-3-glucuronide detected in these volunteers it was difficult to accurately calculate $T_{1/2}$ values.

The pharmacokinetic profile of isorhamnetin-3-glucuronide was slightly different from quercetin-3-glucuronide as there was a slower rate of decline after C_{max} which is reflected in a later T_{max} of 5.4 h and longer $T_{1/2}$ of 6.9 h (Table 3.4). In addition to this the C_{max} values were also lower than those of quercetin-3-glucuronide. Again, subject 1 and 2 exhibited higher C_{max} values for isorhamnetin-3-glucuronide than the others (Table 3.4). The cumulative C_{max} of the two plasma metabolites ranged from 5.2-32 nM; with an average accumulation of 15 nM, which corresponds to 0.01-0.06% of the rutin ingested (Table 3.5). This is broadly similar to the figures of 0.016% and 0.09% obtained by Boyle *et al.* (2000) after an ingestion of 500 mg of rutin. However, the values obtained in the current study is much lower than the figures of 0.18%, 0.15% and 0.09% obtained by Erlund *et al.* (2000), Graefe *et al.* (2001) and Hollman *et al.* (1997) respectively.

Table 3.3. Concentration of two quercetin metabolites in the plasma of six human subjects 0-25 h after consumption of rutin supplemented tomato juice

Metabolite *(Peak number)*	Subject	4 h	5 h	6 h	7 h	8 h
Quercetin-3-glucuronide *(5)*	1	66 ± 17	42 ± 2	42 ± 2	14 ± 1	n.d.
	2	73 ± 7	30 ± 2	30 ± 6	8 ± 2	n.d.
	3	32 ± 0	38 ± 2	23 ± 1	8 ± 2	n.d.
	4	16 ± 5	13 ± 2	6 ± 1	n.d.	n.d.
	5	n.d.	12 ± 3	11 ± 3	9 ± 2	n.d.
	6	n.d.	14 ± 2	16 ± 1	n.d.	n.d.
	Mean	***31 ± 13***	***25 ± 5***	***22 ± 5***	***7 ± 2***	***n.d.***
Isorhamnetin-3-glucuronide *(7)*	1	13 ± 3	25 ± 1	29 ± 1	12 ± 1	n.d
	2	17 ± 2	18 ± 1	23 ± 7	8.8 ± 0.7	10 ± 1
	3	3.6 ± 0.2	11 ± 1	10 ± 0	8.2 ± 1.4	6.0 ± 0.5
	4	6.6 ± 0.6	8.6 ± 0.7	5.1 ± 0.5	3.7 ± 0.5	n.d.
	5	5.4 ± 0.4	6.3 ± 0.1	3.5 ± 0.0	2.6 ± 0.2	n.d.
	6	n.d.	n.d.	n.d.	n.d.	n.d.
	Mean	***7.6 ± 2.5***	***11 ± 4***	***12 ± 5***	***5.9 ± 1.8***	***2.6 ± 1.7***

Data for the individual subjects are expressed as nmoles ± standard error (n = 3). Mean values are expressed as nmoles ± standard error (n = 6). **No metabolites are detected in 0, 2, 3 and 24 h plasma samples**. n.d. – not detected. For MS^2 data and identification of peaks, refer to Table 3.2

Table 3.4. Pharmacokinetic parameters of quercetin metabolites in the plasma of human volunteers after the consumption of rutin supplemented tomato juice

Metabolite	Subject	C_{max}	T_{max}	$T_{1/2}$
Quercetin-3-glucuronide	1	22 ± 6	4	5.5
	2	24 ± 2	4	5.1
	3	13 ± 1	5	5.5
	4	5.2 ± 1.5	4	5.4
	5	4.0 ± 1.0	5	8.6
	6	5.2 ± 0.4	6	4.5
	Mean	***12 ± 2***	***4.7 ± 0.3***	***5.7 ± 0.6***
Isorhamnetin-3-glucuronide	1	9.5 ± 0.1	6	5.98
	2	7.7 ± 0.3	6	7.65
	3	3.8 ± 0.2	5	7.30
	4	2.9 ± 0	5	7.01
	5	2.1 ± 0	5	6.48
	6	n.d	n.d	n.d
	Mean	***4.3 ± 1.5***	***5.4 ± 0.2***	***6.9 ± 0.3***

C_{max} – maximum concentration in plasma expressed in nM. T_{max} – time to reach C_{max} expressed in h. $T_{1/2}$ – the elimination half-life of metabolites in h. Data presented as mean values ± standard error (n = 6). n.d. – not detected.

Table 3.5. Percentage rutin intake based on cumulative maximum concentration of quercetin-3-glucuronide and isorhamnetin-3-glucuronide in the plasma of human volunteers with a colon after consumption of tomato juice fortified with 164 μmoles of rutin.

Subject	Cumulative C_{max}	% of rutin ingested
1	31	0.05
2	32	0.06
3	6.5	0.03
4	8.1	0.01
5	6.0	0.01
6	5.2	0.01
Mean	***14.8 ± 5.2***	***0.03***

C_{max} – maximum concentration in plasma expressed in nM. Data presented as mean values ± standard error (n = 6).

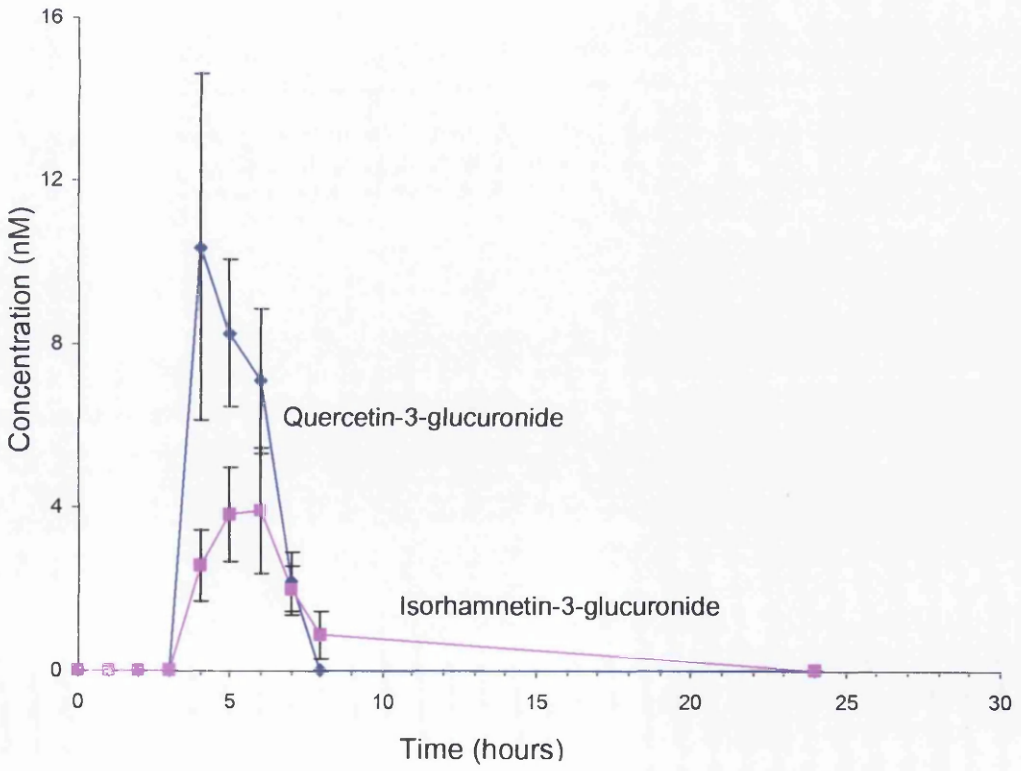


Figure 3.4. Mean concentration of rutin metabolites in the plasma of six healthy human subjects 0-24 h following consumption of tomato juice supplemented with 164 μmoles of rutin. Error bars indicate standard error.

3.3.2.3 Quantitative analysis of rutin metabolite excretion in urine

Nine rutin metabolites were detected in urine samples collected 0-2, 2-5, 5-8 and 8-24 h after the ingestion of tomato juice fortified with 164 μmoles of rutin. All nine metabolites were present in the urine of subject 1 while eight of them were detected in subject 2 (Table 3.6). Due to high inter-individual variation the concentrations of these metabolites are presented separately for each individual. The urinary metabolites detected in this study are methylquercetin diglucuronide, three isomers of quercetin glucoside glucuronide, quercetin-3-glucuronide, quercetin diglucuronide, isorhamnetin-3-glucuronide, quercetin-4'-glucuronide and quercetin-3'-glucuronide. Excretion of rutin metabolites by volunteers 3-6, was much lower and limited to only the quercetin glucuronides and isorhamnetin-3-glucuronide. No methylquercetin diglucuronide, quercetin glucoside glucuronide or quercetin diglucuronide were detected in any of these subjects (Table 3.6).

There were considerable inter-individual variations in the excretion of the urinary metabolites as illustrated in Figure 3.5. A range of 4981 ± 115 to 40 ± 1 nmoles of total metabolites were excreted over a 24 h period after the ingestion of the rutin. The main urinary metabolites present in all the volunteers with the exception of volunteer 4, were the quercetin glucuronides (*peaks 5, 8 and 9*) with 3521 ± 171 to 29 ± 1 nmoles being excreted over the 24 h period. Substantial amounts of isorhamnetin-3-glucuronide was also detected in the urine of the volunteers with 686 ± 5 to 11 ± 0 nmoles being excreted.

In volunteers 1 and 2, the majority of the urinary rutin metabolites were excreted within the first five hours of tomato juice ingestion (Figure 3.6). However, this profile was completely different for volunteers 4, 5 and 6, where the majority of the urinary rutin metabolites were excreted only after 8 hours. An intermediate urinary excretion profile was observed for volunteers 3. Total urinary rutin metabolite excreted during the 0-24 h were 4981, 608, 310, 187, 141 and 40 nmoles respectively for the different volunteers (mean 1045 ± 36) and these corresponded to 2.8% to 0.02% of rutin intake (mean 0.59% intake).

Table 3.6. Concentration of rutin metabolites in urine of six healthy human subjects 0-24 h after consumption of rutin supplemented tomato juice

Subject	Metabolites *(peak number)*	2 - 5 h	5 - 8 h	8 - 24 h	Total
1	Methylquercetin diglucuronide *(1)*	94 ± 5	35 ± 4	n.d	129 ± 9
	Quercetin glucoside glucuronide *(2)*	216 ± 30	n.d	n.d	216 ± 30
	Quercetin glucoside glucuronide *(3)*	131 ± 26	n.d	n.d	131 ± 26
	Quercetin glucoside glucuronide *(4)*	112 ± 10	9 ± 0	n.d	121 ± 9
	Quercetin-3-glucuronide *(5)*	1122 ± 90	70 ± 26	n.d	1192 ± 104
	Quercetin diglucuronide *(6)*	147 ± 27	28 ± 4	n.d	176 ± 23
	Isorhamnetin-3-glucuronide *(7)*	547 ± 5	140 ± 5	n.d	686 ± 5
	Quercetin-4'-glucuronide *(8)*	1198 ± 8	23 ± 3	n.d	1221 ± 10
	Quercetin-3'-glucuronide *(9)*	1108 ± 62	n.d	n.d	1108 ± 62
	Total quercetin glucuronides *(5,8,9)*	3428 ± 149	93 ± 28	n.d	3521 ± 171
	Total metabolites	***4675 ± 90***	***306 ± 30***	***n.d***	***4981 ± 115***
2	Methylquercetin diglucuronide *(1)*	6 ± 0	15 ± 2	n.d	21 ± 2
	Quercetin glucoside glucuronide *(2)*	11 ± 0	5 ± 1	n.d	16 ± 1
	Quercetin glucoside glucuronide *(3)*	2 ± 1	n.d	n.d	2 ± 1
	Quercetin-3-glucuronide *(5)*	71 ± 1	67 ± 2	4 ± 1	142 ± 2
	Quercetin diglucuronide *(6)*	7 ± 1	12 ± 1	n.d	18 ± 2
	Isorhamnetin-3-glucuronide *(7)*	30 ± 3	40 ± 1	60 ± 2	129 ± 3
	Quercetin-4'-glucuronide *(8)*	87 ± 2	35 ± 1	n.d	122 ± 3
	Quercetin-3'-glucuronide *(9)*	92 ± 1	46 ± 0	19 ± 1	157 ± 62
	Total quercetin glucuronides *(5,8,9)*	250 ± 2	147 ± 3	22 ± 2	420 ± 5
	Total metabolites	***305 ± 3***	***219 ± 3***	***82 ± 1***	***608 ± 4***
3	Quercetin-3-glucuronide *(5)*	81 ± 6	43 ± 2	51 ± 19	175 ± 14
	Isorhamnetin-3-glucuronide *(7)*	10 ± 0	20 ± 1	34 ± 1	64 ± 2
	Quercetin-4'-glucuronide *(8)*	18 ± 1	18 ± 1	n.d	36 ± 0
	Quercetin-3'-glucuronide *(9)*	10 ± 1	17 ± 1	10 ± 1	37 ± 1
	Total quercetin glucuronides *(5,8,9)*	108 ± 6	78 ± 1	62 ± 20	248 ± 15
	Total metabolites	***118 ± 6***	***97 ± 1***	***95 ± 19***	***310 ± 14***
4	Quercetin-3-glucuronide *(5)*	16 ± 1	7 ± 0	n.d	23 ± 1
	Isorhamnetin-3-glucuronide *(7)*	21 ± 0	16 ± 0	82 ±1	120 ± 0
	Quercetin-4'-glucuronide *(8)*	41 ± 1	n.d	n.d	41 ± 1
	Quercetin-3'-glucuronide *(9)*	4 ± 0	n.d	n.d	4 ± 0
	Total quercetin glucuronides *(5,8,9)*	61 ± 2	7 ± 0	n.d	68 ± 2
	Total metabolites	***82 ± 2***	***23 ± 1***	***82 ± 1***	***187 ± 2***
5	Quercetin-3-glucuronide *(5)*	6 ± 2	12 ± 2	46 ± 3	64 ± 5
	Isorhamnetin-3-glucuronide *(7)*	21 ± 0	17 ± 1	27 ± 0	65 ± 0
	Quercetin-4'-glucuronide *(8)*	1 ± 0	1 ± 0	n.d	2 ± 0
	Quercetin-3'-glucuronide *(9)*	2 ± 0	6 ± 0	2 ± 0	10 ± 1
	Total quercetin glucuronides *(5,8,9)*	9 ± 1	20 ± 2	49 ± 3	77 ± 5
	Total metabolites	***30 ± 1***	***36 ± 2***	***75 ± 3***	***141 ± 5***
6	Quercetin-3-glucuronide *(5)*	n.d	3 ± 0	24 ± 1	27 ± 1
	Isorhamnetin-3-glucuronide *(7)*	4 ± 0	7 ± 0	n.d	11 ± 0
	Quercetin-3'-glucuronide *(9)*	n.d	n.d	2 ± 0	2 ± 0
	Total quercetin glucuronides *(5,8,9)*	n.d	3 ± 0	26 ± 1	29 ± 1
	Total metabolites	***4 ± 0***	***10 ± 0***	***26 ± 1***	***40 ± 1***

Data for the individual subjects are expressed as nmoles ± standard error (n=3). n.d – not detected

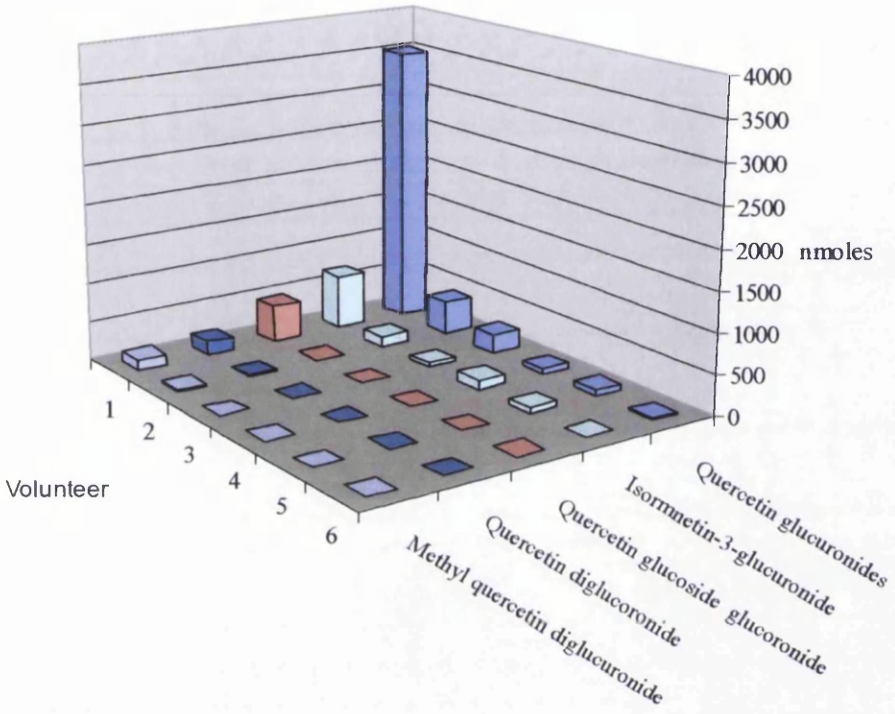


Figure 3.5. Inter-individual variation in the concentration of urinary metabolites in healthy subjects with a colon after ingestion of rutin supplemented tomato juice

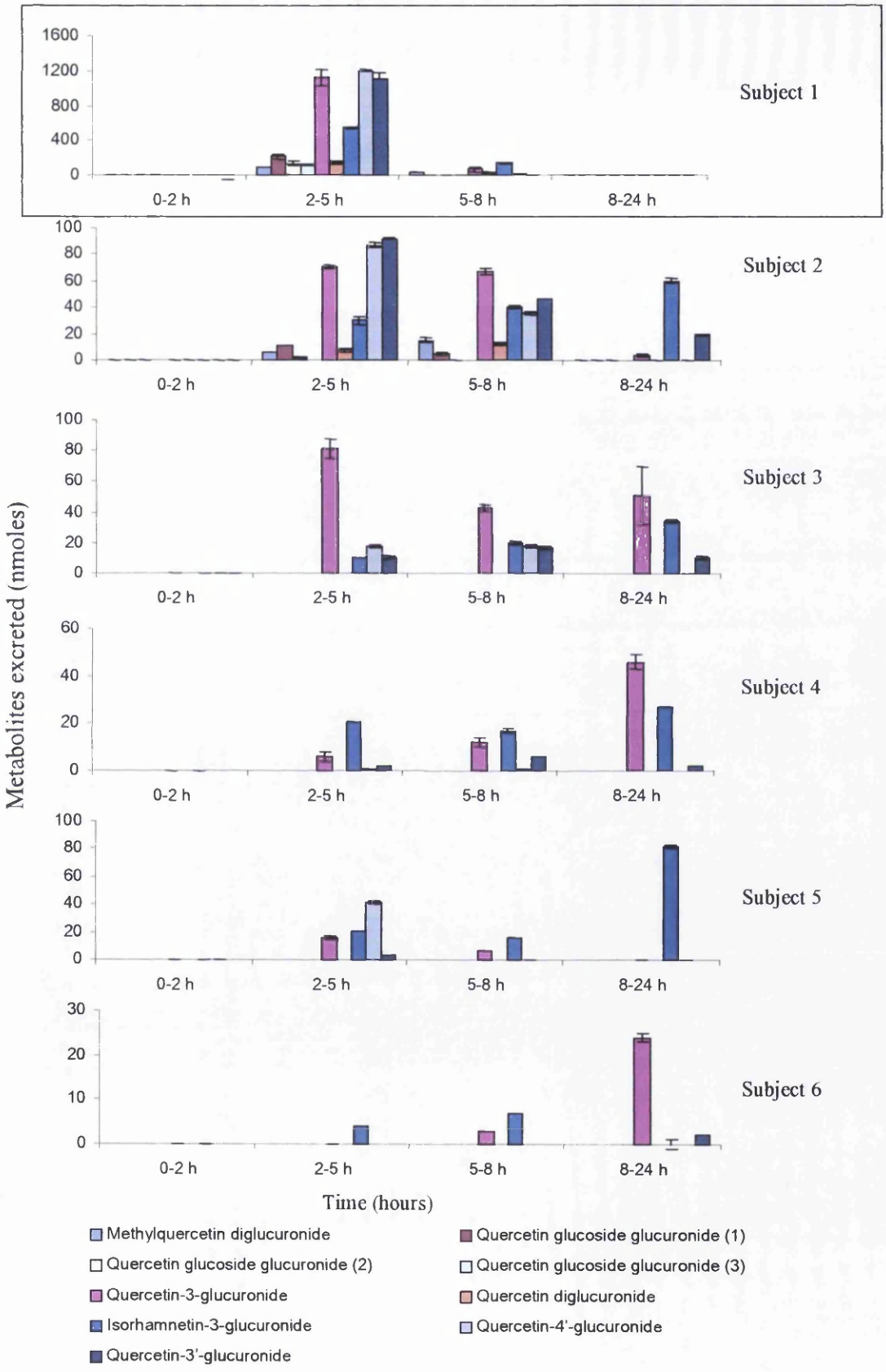


Figure 3.6. Excretion of urinary rutin metabolites in healthy subjects after ingestion of tomato juice fortified with 164 μmoles of rutin.

3.3.3. Identification and quantification of rutin metabolites in the ileal fluid, plasma and urine of ileostomy volunteers

3.3.3.1 Quantitative analysis of ileal fluid

Ileal fluid was collected before consumption of tomato juice supplemented with 164 μmoles rutin and subsequently collected at 0-2, 2-5, 5-24 h. Using the MS^2 and diode array detection, ileal fluid samples were analysed using a 60 min, 5-40% gradient of acetonitrile in 1% aqueous formic acid. In total six phenolic compounds were identified in the ileal fluid based on MS^2 data and λ_{max} at 365 nm (Figure 3.7). Most of these compounds were derived from the consumption of tomato juice as can be seen by comparing the chromatograms of tomato juice and ileal fluid illustrated in Figure 3.7. No conjugated rutin metabolites were detected in any of the ileal fluid samples for all the time points. Rutin, however was detected in an unmodified form and amounted to a total mean of 151 ± 5 μmoles which corresponded to 92 ± 3% of rutin intake (Table 3.7). Most of the rutin (70%) was excreted in the ileal fluid 2-5 h post ingestion of tomato juice.

3.3.3.2. Quantitative analysis of plasma

Plasma was collected before ingestion of rutin and subsequently collected at 2, 3, 4, 5, 6, 8 and 24 h. Using the HPLC-MS^2 and diode array detection, plasma samples were extracted for flavonoids and were analysed at 365 nm using a 60 min, 5-40% gradient of acetonitrile in 1% aqueous formic acid. None of the quercetin metabolites detected in the plasma of healthy volunteers (Table 3.3) were present in detectable quantities in the 0-24 h plasma samples collected from subjects with an ileostomy.

3.3.3.3 Quantitative analysis of urine

Urine was collected before ingestion of rutin and subsequently collected at 0-2, 2-5, 5-8 and 8-24 h. Using the HPLC-MS^2 and diode array detection, urine samples were analysed at 365 nm using a 60 min, 5-40% gradient of acetonitrile in 1% aqueous formic acid. None of the quercetin metabolites detected in the urine of healthy volunteers (Table 3.6) were present in detectable quantities in the 0-24 h urine samples collected from subjects with an ileostomy.

Table 3.7. Amount of rutin in the ileal fluid of five ileostomy subjects 0-24 h after consumption of rutin spiked tomato juice

Subject	0-2 h	2-5 h	5-24 h	Total
1	21 ± 1	132 ± 1	4.7 ± 0.0	158 ± 1
2	6 ± 0	109 ± 7	40 ± 1	155 ± 6
3	11 ± 1	118 ± 3	6 ± 1	135 ± 1
4	1 ± 0	100 ± 6	43 ± 0	149 ± 1
5	n.d	119 ± 2	37 ± 1	156 ± 3
Mean	8 ± 5	115 ± 7	26 ± 11	151 ± 5
% intake	***5 ± 1***	***70 ± 4***	***16 ± 7***	***92 ± 3***

Data for the individual subjects are expressed as μmoles ± standard error (n = 3). Mean values are expressed as μmoles ± standard error (n = 5). No rutin was detected at time 0. n.d - not detected

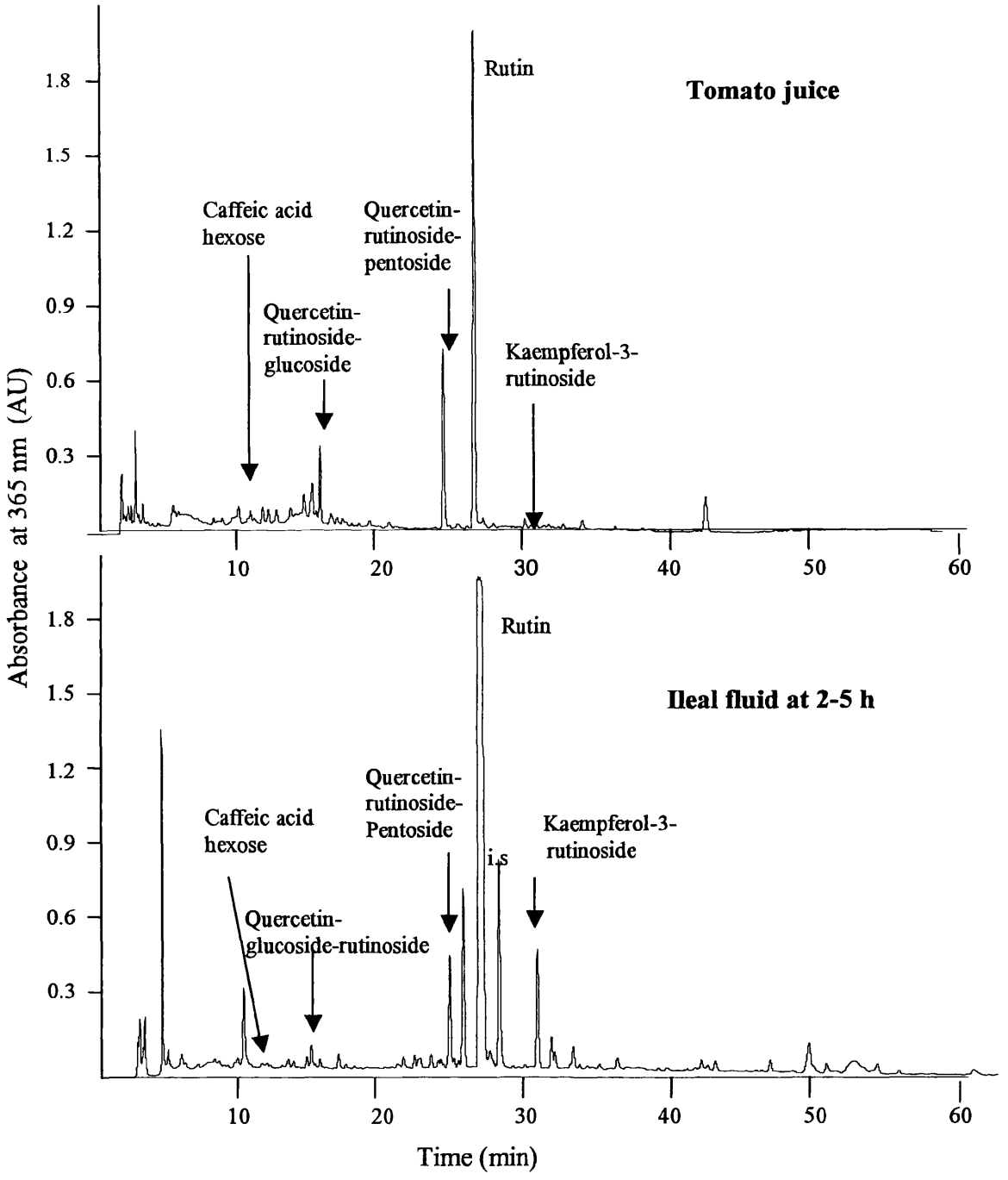


Figure 3.7. Gradient reversed-phase HPLC with detection at 365 nm of phenolics in tomato juice and ileal fluid. The ileal fluid chromatogram is obtained from volunteer 1 after consumption of tomato juice fortified with 164 μmoles of rutin. i.s.-internal standard.

3.3.4 Identification and quantitative analysis of rutin catabolites in urine of volunteers with and without a colon.

3.3.4.1 Identification of urinary phenolic acids

The levels of individual phenolic compounds were determined by HPLC-MS^2 and diode array detection. Samples were analysed on a Surveyor HPLC system using a 60 min, 2-20% gradient of acetonitrile in 0.25% aqueous acetic acid. The column eluate being directed first to a diode array absorbance monitor and then to a mass spectrometer with an APCI interface operating in full scan MS^2 mode. The absorbance at 280 nm trace contained a large number of peaks which were present in the 0 h blank and others which could not be identified (Figure 3.8). However, from the MS^2 data, a total of six phenolic acids were identified in urine of healthy volunteers with a colon and three in volunteers without a colon (Figure 3.8). The major component in the urine was 4-hydroxyhippuric acid. The overall results obtained are presented in Table 3.8 and summarised below. The structures of the compounds are illustrated in Figure 3.9.

Peak 1 (t_R = 13.6 min, λ_{max}= 260 nm) had a $[M-H]^-$ at *m/z* 194 with MS^2 yielding two charged fragments, a main one at *m/z* 100 and a smaller ion at *m/z* 93. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 4-hydroxyhippuric acid.

Peak 2 (t_R = 15.9 min, λ_{max}= 280 nm) had a $[M-H]^-$ at *m/z* 167 with MS^2 yielding two charged fragments, a main one at *m/z* 123 and a smaller ion at *m/z* 108. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 3,4-dihydroxyphenylacetic acid.

Peak 3 (t_R = 26.8 min, λ_{max}= 270 nm) had a $[M-H]^-$ at *m/z* 151 with MS^2 yielding three charged fragments, a main one at *m/z* 107 and two smaller ions at *m/z* 93 and 121. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 3-hydroxyphenylacetic acid.

Peak 4 (t_R = 29.1 min, λ_{max}= 280 nm) had a $[M-H]^-$ at *m/z* 181 with MS^2 yielding one charged fragment at *m/z* 137. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 4-hydroxy-3-methoxyphenylacetic acid.

Peak 5 (t_R = 33.2 min, λ_{max}= 300nm) had a $[M-H]^-$ at *m/z* 194 with MS^2 yielding a charged fragment at *m/z* 150. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 3-hydroxyhippuric acid

Peak 6 (t_R = 39.1 min, λ_{max}= 300nm) had a $[M-H]^-$ at *m/z* 194 with MS^2 yielding a charged fragment at *m/z* 150. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 2-hydroxyhippuric acid

Table 3.8. HPLC-MS^2 identification of phenolic acids detected in urine samples of volunteers with and without colons

Peak	t_R (min)	Compound	$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)
1	13.6	4-hydroxyhippuric acid	194	100, 93
2	15.9	3,4-dihydroxyphenylacetic acid	167	123, 108
3	26.8	3-hydroxyphenylacetic acid	151	107, 93, 121
4	29.5	4-hydroxy 3-methoxyphenylacetic acid	181	137
5	32.2	3-hydroxyhippuric acid	194	150
6	38.1	2-hydroxyhippuric acid	194	150

Peak numbers and HPLC retention times and the peaks refer to HPLC trace in Figure 3.8; t_R - retention time; $[M-H]^-$ - negatively charged molecular ion

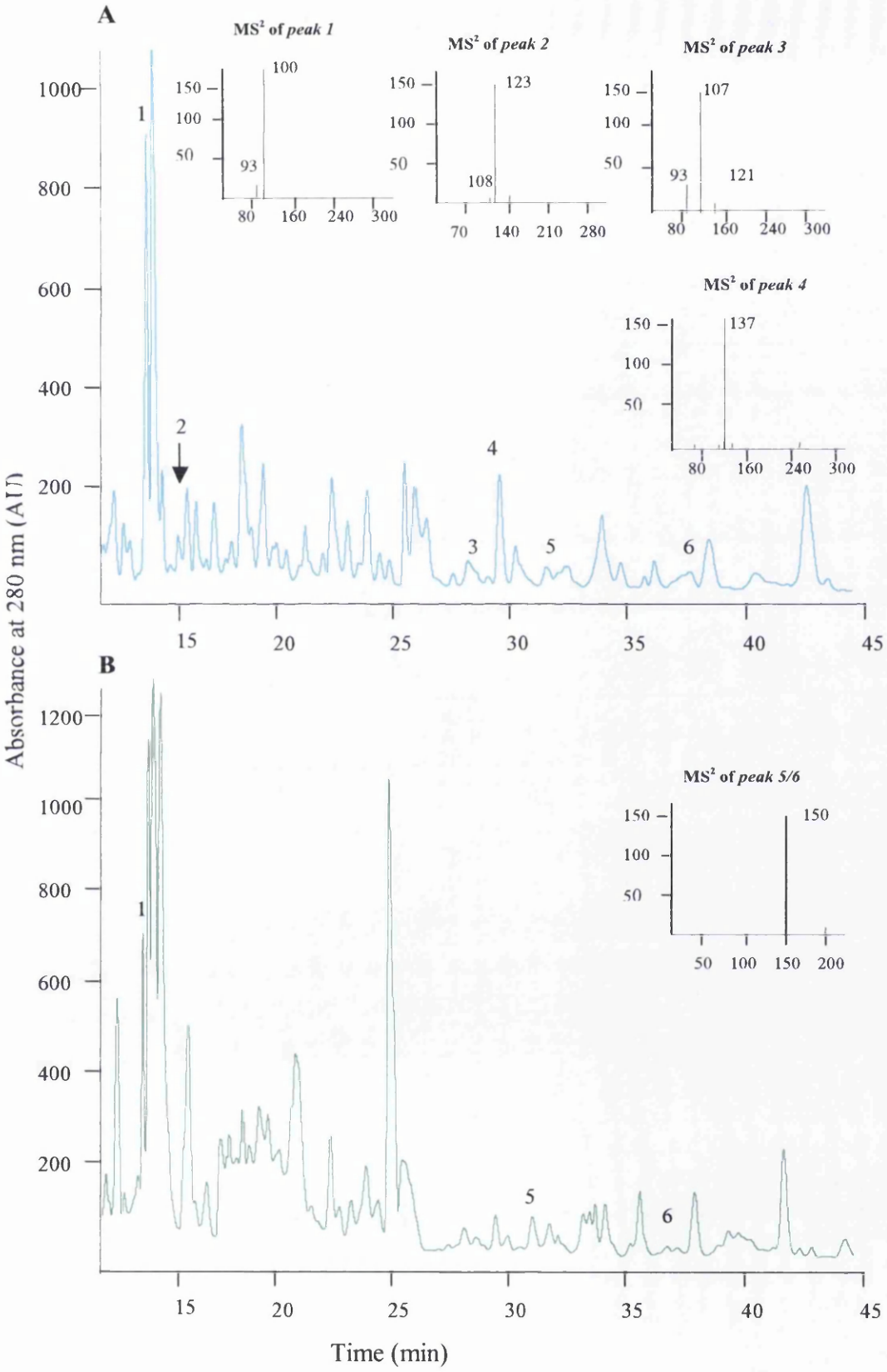
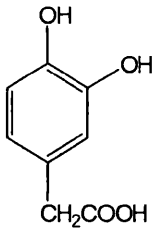
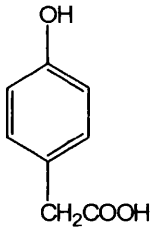


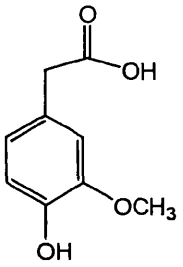
Figure 3.8. 2-20% gradient HPLC chromatogram of urine sample obtained from donor 1 from healthy volunteers with a colon (A) and from an ileostomy volunteer (B), 8-24 h after ingestion of tomato juice fortified with 164 μmoles of rutin. The MS^2 fragmentation profile for each peak is also illustrated. For the identification of peaks, refer to Table 3.8



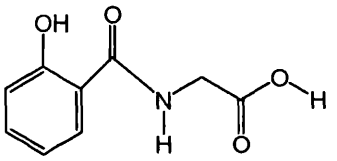
3,4-Dihydroxyphenylacetic acid



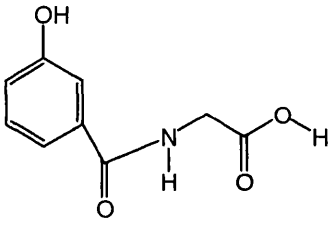
3-Hydroxyphenylacetic acid



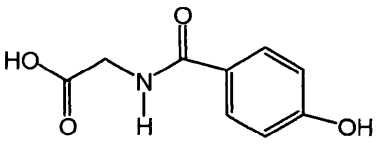
4-Hydroxy-3-methoxyphenylacetic acid



2-Hydroxyhippuric acid



3-Hydroxyhippuric acid



4-Hydroxyhippuric acid

Figure 3.9. Structures of phenolic acids detected in the urine

3.3.4.2 Quantitative analysis of urinary phenolic acids

3.3.4.2.1 Healthy volunteers

Six urinary phenolic acids were detected in healthy human subjects with a colon in amounts that facilitated quantitative analysis (Table 3.9). These catabolites consist of 2-hydroxyhippuric acid, 3-hydroxyhippuric acid, 4-hydroxyhippuric acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxy-phenylacetic acid. The main urinary metabolite excreted was 4-hydroxyhippuric acid *(peak 1)* with a total mean excretion of 65 ± 6 μmoles, 0-24 h after ingestion of rutin.

The excretion profile of all these metabolites were similar where there was a gradual increase in the excretion over the 0-24 h time points (Figure 3.10). The excretion was however, highest 8-24 h after rutin intake (Table 3.9). Total 0-24 h phenolic acid excretion after rutin intake for the individual volunteers were 179, 115, 112, 81, 167 and 126 μmoles and the mean value of 130 ± 15 μmoles corresponded to 74% of rutin intake. The 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxy-phenylacetic acid corresponded to only 23% of intake.

3.3.4.2.2 Ileal volunteers

In contrast to the volunteers with a colon, only three phenolic acid catabolites, that is, 2-hydroxyhippuric acid, 3-hydroxyhippuric acid and 4-hydroxyhippuric acid, were detected in human subjects with an ileostomy (Table 3.10). No phenylacetic acid derivatives were detected in any of the urine samples from these subjects. The excretion of these catabolites had a similar profile to that of the healthy volunteers with 4-hydroxyhippuric acid being the main catabolite excreted in the urine with an average of 30 ± 7 μmoles being excreted over the 24 h period after the ingestion of rutin.

Table 3.9. Concentration of phenolic acids in the urine of six healthy human subjects after consumption of rutin supplemented tomato juice

Metabolite	Subject	0-2	2-5	5-8	8-24	Total
2-OH-hippuric acid *(peak 6)*	1	0.4 ± 0.2	0.9 ± 0.0	3.7 ± 0.1	7.8 ± 0.4	13 ± 1
	2	0.2 ± 0.0	0.7 ± 0.1	3.2 ± 0.0	4.0 ± 0.6	8.2 ± 0.7
	3	0.6 ± 0.0	0.8 ± 0.1	0.7 ± 0.0	0.9 ± 0.0	2.9 ± 0.9
	4	0.1 ± 0.0	0.1 ± 0.0	1.2 ± 0.2	2.9 ± 0.2	4.3 ± 0.3
	5	0.4 ± 0.1	1.4 ± 0.1	1.7 ± 0.2	0.6 ± 0.1	4.0 ± 0.1
	6	0.2 ± 0.0	0.6 ± 0.0	1.2 ± 0.1	0.2 ± 0.0	2.3 ± 0.1
	mean	***0.3 ± 0.1***	***0.8 ± 0.2***	***2.0 ± 0.5***	***2.7 ± 1.2***	***5.7 ± 1.6***
3-OH-hippuric acid *(peak 5)*	1	0.9 ± 0.0	3.7 ± 0.1	2.0 ± 0.1	6.5 ± 0.2	13 ± 0
	2	1.1 ± 0.0	3.1 ± 0.1	3.1 ± 0.2	5.5 ± 0.3	13 ± 0
	3	2.1 ± 0.1	5.2 ± 0.1	2.8 ± 0.0	9.0 ± 0.1	19 ± 0
	4	0.4 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.9 ± 0.2	4.5 ± 0.1
	5	2.4 ± 0.1	15 ± 0	5.2 ± 0.0	20 ± 1	43 ± 1
	6	0.6 ± 0.0	3.2 ± 0.1	0.8 ± 0.1	9.8 ± 0.5	14 ± 1
	mean	***1.2 ± 0.3***	***5.3 ± 2.1***	***2.5 ± 0.7***	***8.7 ± 2.5***	***18 ± 5***
4-OH-hippuric acid *(peak 1)*	1	3.6 ± 0.1	7.3 ± 0.3	26 ± 1	40 ± 3	78 ± 1
	2	1.9 ± 0.1	4.4 ± 0.5	28 ± 0	47 ± 2	81 ± 3
	3	4.0 ± 0.1	5.1 ± 2.2	14 ± 0	24 ± 1	48 ± 2
	4	4.1 ± 0.2	4.8 ± 0.1	12 ± 0	27 ± 0	48 ± 0
	5	2.9 ± 0.1	12 ± 0	26 ± 1	25 ± 1	66 ± 2
	6	0.1 ± 0.0	0.7 ± 0.0	0.7 ± 0.4	46 ± 1	66 ± 1
	mean	***2.8 ± 0.6***	***5.8 ± 1.6***	***18 ± 4***	***35 ± 4***	***65 ± 6***
3-OHPA acid *(peak 3)*	1	t.a.	0.5 ± 0.1	0.4 ± 0.1	1.1 ± 0.0	2.0 ± 0.2
	2	0.1 ± 0.1	0.4 ± 0.0	2.2 ± 0.0	3.2 ± 0.0	6.0 ± 0.1
	3	0.5 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	1.6 ± 0.1
	4	0.2 ± 0.0	0.4 ± 0.0	0.8 ± 0.0	2.3 ± 0.1	3.7 ± 0.1
	5	0.2 ± 0.0	0.1 ± 0.0	1.4 ± 0.2	2.6 ± 0.2	4.3 ± 0.1
	6	0.1 ± 0.0	0.6 ± 0.0	1.8 ± 0.1	6.5 ± 0.4	9.0 ± 0.2
	mean	***0.2 ± 0.1***	***0.4 ± 0.1***	***1.1 ± 0.3***	***2.7 ± 0.9***	***4.4 ± 1.1***
3,4-DiOHPA acid *(peak 2)*	1	n.d.	2.5 ± 0.0	6.7 ± 0.5	20 ± 1	27 ± 1
	2	n.d.	n.d.	2.2 ± 0.2	n.d.	2.2 ± 0.2
	3	n.d.	2.9 ± 0.1	2.2 ± 0.2	7.8 ± 0.2	13 ± 1
	4	n.d.	n.d.	2.6 ± 0.2	12 ± 0	14 ± 2
	5	n.d.	n.d.	1.8 ± 0.1	14 ± 1	16 ± 0
	6	n.d.	n.d.	3.2 ± 0.3	16 ± 1	20 ± 0
	mean	***n.d.***	***0.9 ± 0.6***	***3.1 ± 0.7***	***12 ± 3***	***15 ± 3***
4-OH-3-methoxy-PA acid *(peak 4)*	1	1.0 ± 0.1	4.2 ± 0.2	13 ± 1	27 ± 2	45 ± 0
	2	n.d.	n.d.	4.3 ± 0.1	n.d.	4.7 ± 0.1
	3	n.d.	3.5 ± 0.1	5.5 ± 0.1	19 ± 1	28 ± 1
	4	n.d.	n.d.	4.7 ± 0.2	1.1 ± 0.0	5.8 ± 0.2
	5	n.d.	3.8 ± 0.1	6.8 ± 0.5	7.9 ± 0.4	21 ± 0
	6	2.1 ± 0.1	2.9 ± 0.3	2.7 ± 0.1	6.7 ± 0.1	14 ± 1
	mean	***0.9 ± 0.5***	***2.4 ± 0.8***	***6.1 ± 1.4***	***10 ± 4***	***20 ± 6***

Data for individual subjects expressed as μmoles ± standard error (n = 3). Mean values expressed as μmoles ± standard error (n = 6). For MS^2 data and identification of peaks, refer to Table 3.8; n.d. – not detected. t.a- trace amounts; n.d. – not detected

Table 3.10. Concentration of phenolic acids in the urine of five human subjects with an ileostomy 0-24 h after the consumption of tomato juice supplemented with 164 μmoles rutin

Metabolite	Subject	0-2	2-5	5-8	8-24	Total
2-OH-hippuric acid	1	0.3 ± 0.0	0.7 ± 0.0	1.2 ± 0.0	5.2 ± 0.0	7.4 ± 0.0
	2	n.d.	n.d.	n.d.	n.d.	n.d.
	3	19 ± 0	6.2 ± 0.2	26 ± 1	9.5 ± 0.5	61 ± 1
	4	1.7 ± 0.1	0.4 ± 0.0	6.1 ± 0.7	6.9 ± 0.3	15 ± 1
	5	1.8 ± 0.1	1.8 ± 0.0	4.5 ± 0.1	12 ± 0	21 ± 0
	mean	***4.5 ± 3.6***	***1.8 ± 1.1***	***7.6 ± 4.7***	***6.8 ± 2.1***	***21 ± 11***
3-OH-hippuric acid	1	2.3 ± 0.0	3.3 ± 0.2	1.6 ± 0.1	9.0 ± 0.4	16 ± 0
	2	3.0 ± 0.2	1.2 ± 0.1	3.7 ± 0.5	9.3 ± 1.9	17 ± 2
	3	0.3 ± 0.0	0.6 ± 0.0	8.1 ± 0.6	1.1 ± 0.5	10 ± 1
	4	3.0 ± 0.1	0.4 ± 0.0	3.7 ± 0.2	n.d.	14 ± 0
	5	n.d.	n.d.	n.d.	n.d.	n.d.
	mean	***1.7 ± 0.7***	***1.1 ± 0.6***	***3.4 ± 1.4***	***3.9 ± 2.2***	***10 ± 3***
4-OH-hippuric acid	1	0.9 ± 0.0	4.1 ± 0.3	8.0 ± 0.4	29 ± 4	42 ± 4
	2	1.7 ± 0.0	0.9 ± 0.1	6.1 ± 0.7	29 ± 3	37 ± 4
	3	5.3 ± 0.1	5.7 ± 0.1	8.9 ± 0.5	22 ± 4	42 ± 4
	4	1.4 ± 0.0	0.3 ± 0.0	3.9 ± 0.5	0.8 ± 0.1	6.4 ± 0.7
	5	4.8 ± 0.1	3.3 ± 0.4	3.3 ± 0.1	13 ± 0	24 ± 1
	mean	***2.8 ± 0.9***	***2.9 ± 1.0***	***6.0 ± 1.1***	***19 ± 5***	***30 ± 7***

Data for individual subjects expressed as μmoles ± standard error (n = 3). Mean values expressed as μmoles ± standard error (n = 6). For MS^2 data and identification of peaks, refer to Table 3.8. n.d. – not detected

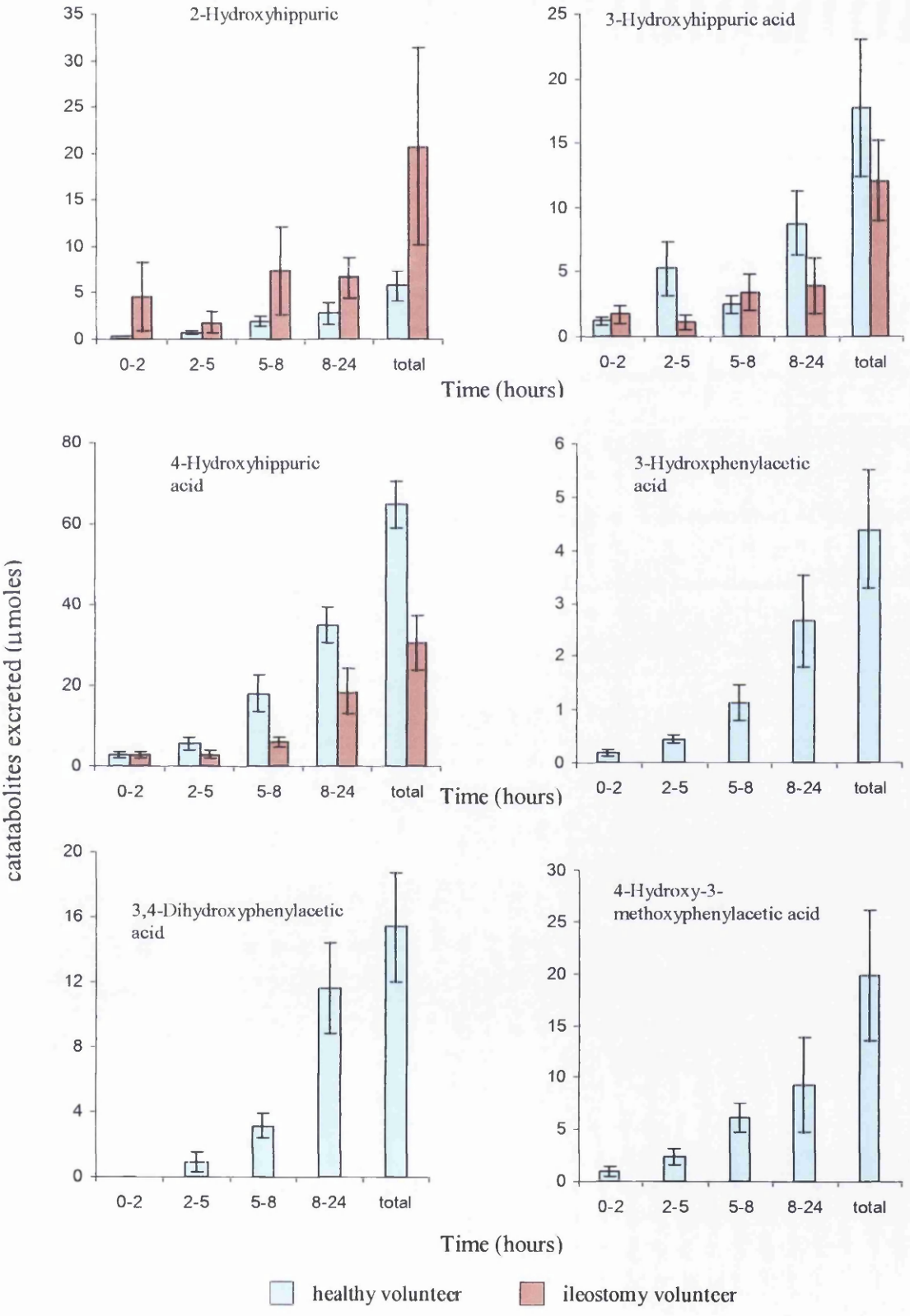


Figure 3.10. Excretion of urinary catabolites over a period of 24 hours after ingestion of rutin supplemented tomato juice in healthy and ileostomy volunteers. Data is expressed as µmoles ± standard error (n=3).

3.4 Discussion

From the metabolite profiles of rutin in the plasma and urine, it is clear that rutin is extensively metabolised in the human body. Following the release of the aglycone, quercetin is subjected to glucuronidation and methylation as these can be found in the circulatory and the excretory system. However, there is no evidence of any sulphation occurring. Glucuronidation seems to be the dominant metabolic pathway in the metabolism of rutin as quercetin glucuronide is the major metabolite appearing in the plasma and excreted in the urine post ingestion of rutin.

3.4.1 Phase II metabolism

The metabolites quercetin-3-glucuronide and isorhamnetin-3-glucuronide appeared in plasma after ingestion of rutin with a cumulative C_{max} value of 15 nM, and a T_{max} value of > 4 h (Table 3.4). These values differ markedly from those reported by Mullen *et al.* (2005) when onions containing quercetin-4'-glucoside and quercetin-3,4'-diglucoside were consumed and a T_{max} value of < 1 h and a C_{max} value of 463 nM were obtained for the same metabolite. The difference in the post-absorption kinetic behaviour of the quercetin glucosides and that of rutin is believed to stem from the sugar moiety attached to quercetin. In contrast to quercetin glucosides, in rutin, the rutinose moiety is not cleaved by either LPH or CBH associated with the wall of the GI tract (Day and Williamson, 2001). As a consequence, most of the rutin passes through the small intestine without modification and enters the colon of healthy subjects or emerges in the ileal fluid in the case of subjects with an ileostomy. The longer time taken to reach the large intestine is reflected in the increased T_{max} value. In the large intestine, rutinose is cleaved by the gut microflora. The microbial enzymes involved in the deglycolysation of the rutinoside moiety from quercetin, such as α-rhamnosidases and β-glucosidase have been detected in the human colon (Aura *et al.*, 2005).

After the initial deglycosylation of rutin, quercetin metabolites that appear in plasma are the result of conversions occurring in the lumen of the large intestine. Relatively low concentrations of quercetin metabolites were detected in the plasma after ingestion of rutin. The peak concentration of quercetin metabolites in plasma was about 30 times lower than when an equivalent dose of quercetin glucosides were ingested (Mullen *et al.*, 2005). The relatively low α-L-rhamnosidase activity compared to β-glucosidase may be partly responsible for the lower C_{max} values observed in the plasma

(Graefe *et al.*, 2001). In contrast to the metabolism in the small intestine, the extent of quercetin absorption and the further breakdown into ring fission products depend largely on the composition of the colonic microflora of individual subjects and their intestinal transit times. The dependency of the metabolism of rutin on the composition of the microflora in the large intestine also explains the inter-individual variations observed both in plasma concentrations and in particular, the urinary excretion of quercetin metabolites. The more quercetin is degraded by the colonic microflora, the less it accumulates with only trace amounts absorbed in the intestinal lumen and hence lower amounts are recovered in plasma and urine as conjugated quercetin metabolites. This phenomenon was most pronounced in volunteers 4, 5 and 6 (Table 3.4 and 3.6). The argument presented here is also in agreement with the findings of other researchers where the absorption of quercetin showed low inter-individual variations compared with that of rutin (Erlund *et al.*, 2000). However, it should be noted that low accumulation of rutin metabolites in the plasma could also be related to reduced breakdown of rutin or differences in the rate of absorption within the colonic lumen of that of individual volunteers.

The plasma pharmacokinetic profile of isorhamnetin-3-glucuronide was slightly different in that it had a longer $T_{1/2}$ and a delayed T_{max} compared to that of quercetin-3-glucuronide. The reason for these two metabolites displaying different pharmacokinetic profiles could be due to differing enzyme specificities and/or varying rates of efflux from the enterocyte into the bloodstream although deposition in body tissues and a slow release in the bloodstream could also be factors of influence. Another possibility is that the major plasma metabolite, quercetin-3-glucuronide is produced in the large intestine, passes into the portal vein and is further converted to other components, including isorhamnetin-3-glucuronide in the liver. This is in line with the investigations carried out by O'Leary *et al.* (2003) where by using the HepG2 hepatic cell model, it was demonstrated that 32% of quercetin-3-glucuronide was methylated to 3'- methylquercetin glucuronide.

No evidence of sulphation of quercetin was detected in this study, which is in contrast with results obtained from studies on bioavailability of quercetin glycosides where quercetin-3'-sulphate was found to be the major plasma metabolite after consumption of onions (Mullen *et al.*, 2004, Day *et al.*, 2001). The lack of sulphation of quercetin in the colonic lumen could be due to the lack of the flavonol-specific

sulphotransferase (SULTs) activitiy. Although high SULTs activities are present in the colon (Harris *et al.*, 2000), no sulphate phase II conjugates of flavonoids, were recovered in the perfusate of the colonic lumen when using a rat model. In fact, the phase II conjugates were found to be region-dependent, with the highest recovery in the jejunum, followed closely by the duodenum, the ileum, and none in the colon (Liu and Hu, 2002).

In contrast to rutin, the absorption of quercetin glycosides (after hydrolysis) from the small intestine into the bloodstream appears to be much more effective (Crespy *et al.*, 1999). In the small intestine enterocytes, quercetin is metabolised to the corresponding glucuronides, sulphates or methylated to isorhamnetin. This assumption is reported by *in situ* experiments with rat intestines, in which quercetin was rapidly and extensively conjugated to quercetin sulphate and quercetin glucuronide (Crespy *et al.*, 1999). However, the activity of UDP-glucuronosyltransferase (UGT), the conjugating enzymes, which transfer the glucuronosyl group from the UDP-glucuronic acid to the flavonoid moiety were found to decrease slightly from proximal to distal small intestine, whereas in the large intestine a sharp fall in activity was observed (Peters *et al.*, 1991). If this also occurs in humans, it could explain the low quercetin glucuronide C_{max} values obtained in the present study.

In summary, the lower bioavailability of rutin and the lack of quercetin sulphate conjugates in the circulatory system and urine could be due to any of following reasons:

- Lack of UGT and SULT activities or possibly the lack of specific isoforms of UGT and SULT for the phase II metabolism of flavonols
- Lack of quercetin intestinal transporters
- Low α-L-rhamnosidase activity
- Enzyme-dose effect. Concentration of quercetin present at any time in the large intestine is dependent on the enzymatic activities of the microflora for both the deglycolysation and also the ring cleavage. Any quercetin formed can be acted upon immediately by the microflora resulting in catabolism to phenolic acids and thus accumulation of quercetin in the large intestine may be insufficient to facilitate significant SULT activity.

Table 3.6 summarises the 24 h urinary excretion of the flavonol metabolites detected after consumption rutin and presents clear evidence of substantial phase II metabolism as only two of the urinary metabolites were detected in plasma (Table 3.3). Quercetin-3'-glucuronide and quercetin-4'-glucuronide were excreted in substantial amounts in the urine but were not detected in plasma. The virtual absence of these urinary metabolites together with other urinary metabolites such as methylquercetin diglucuronide, quercetin glucoside glucuronide and quercetin diglucuronides in plasma could indicate that after metabolism in the liver and/or kidney, they are rapidly removed from the circulatory system via excretion by the kidneys.

3.4.2 Ileostomy model

Five healthy ileostomy volunteers were chosen in this study as analysis of ileal fluid collected after supplementation provides information on the absorption and metabolism occurring in the small intestine. By measuring the level of phenolics in ileal fluid we are able to calculate how much has been absorbed by the small intestine and what amount goes through to the colon in healthy subjects. In addition to this, any conjugated metabolites formed as a result of enterohepatic recycling from Phase II metabolism may also be detected in the ileal fluid. Analysis of plasma and urine data revealed no phase II metabolites of rutin. Further analysis of the ileal fluid showed that no significant absorption had occurred in the small intestines. Ninety-two % of the rutin ingested was excreted in the ileal fluid, implying that the site of the absorption is not the small intestine but the large intestine. The results in this study is in broad agreement with the findings of Hollman *et al.* (1995) who obtained only 17 ± 15% absorption of rutin in ileostomy subjects when supplemented with 164 μmoles of rutin.

The validity of the ileostomy model has been questioned as it has been hypothesized that the distal part of the small bowel in ileostomy subjects has an active microbial population (Norman *et al.*, 2001). Bacterial fermentation was however, minimized with the frequent emptying of ileostomy bags and the rapid freezing of the ileal fluid. In addition, the ileal fluid did not smell or look faecal. The fact that 92% of the rutin ingested was excreted in the ileal fluid, suggest that bacterial fermentation in the ileal fluid was minimal.

3.4.3. Ring fission catabolites

The 0.02-2.8% recovery of the ingested rutin as metabolites in urine clearly does not account for the vast majority of the consumed rutin. The most likely fate of these compounds is that they are broken down to low molecular weight phenolic acids by the colonic microflora (Olthof *et al.*, 2003). Under strictly controlled dietary conditions, a total of six potential rutin catabolites were detected in the urine with significant excretion increases post-supplementation of rutin (Table 3.9).

In healthy subjects with a colon, hydroxyhippuric acids, and 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxy-phenylacetic acid were all detected in urine after ingestion of tomato juice spiked with rutin. This is in agreement with investigations carried out by Olthof *et al.* (2003), Rechner *et al.* (2001) and Gonthier *et al.* (2003). Rechner *et al.* (2001) further proposed that hydroxyhippuric acid is a good indicator for bioavailability and metabolism of phenolic compounds. In subjects without a colon, we recovered hydroxyhippuric acids but none of the hydroxyphenylacetic acids. This difference can be explained by the fact that the hydroxyphenylacetic acid derivatives are most likely the ring fission products of rutin and are, therefore, formed principally in the colon while the presence of hydroxyhippuric acids in urine of ileostomy volunteers suggests their synthesis is not restricted to only the colon. Hydroxyhippuric acid may also originate in the oxidative attack on exogenous benzenoid compounds by detoxifying enzymes system of the endoplasmic reticulum or microsomes (Gelboin *et al.*, 1972). However, bacterial action cannot altogether be excluded as possible source of these compounds (Spencer *et al.*, 2003). This is supported by the higher levels of hydroxyhippuric acids observed in the volunteers with a colon (Tables 3.9 and 3.10). However, when considered with the data obtained from the ileal fluid, it is clear that the hydroxyhippuric acids detected in ileal subjects were not derived from the metabolism of rutin but most probably from other compounds present either in the tomato juice or food consumed during the period of the study. This is in agreement with the investigation carried out by Olthof *et al.*, 2003 where it was shown that hippuric acids, are important catabolites in humans after ingestion of tea, but not after ingestion of rutin.

3.4.4. Extent of metabolism

The plasma concentration of quercetin metabolites accounts for only 0.01-0.016% of the rutin ingested while the hydroxyphenylacetic derivatives corresponded to 24% of intake. The fraction that was not recovered in the urine was likely to be either metabolized to non-phenolic compounds that were not detected, deposited in body tissues, excreted with the faeces or degraded to carbon dioxide (Walle *et al.,* 2001). Distribution and accumulation of metabolites in other tissues may constitute an important part of the unaccounted rutin metabolites as available data, essentially from animal studies, indicate that some polyphenol metabolites may accumulate in certain target tissues rather than just equilibrate between blood and tissues (Manach *et al.*, 2004). Since human subjects were used in this study, the possible deposition of metabolites in body tissues such as the liver, kidneys and brain, is not possible for obvious reasons. Another possibility is that the metabolites of rutin could have been taken into cells or tissues and released back into the bloodstream at concentrations below the limit of detection of the analytical method used in this study or as metabolites or degradation products that were not identified.

3.4.5 Methodology

Most of the earlier studies on the bioavailability of flavonoids either used enzyme or acid hydrolysis of samples to release aglycones, prior to HPLC to determine levels in plasma and urine. However, the current study with human volunteers in which unhydrolysed extracts were analysed by HPLC with PDA and full scan data dependent MS^2 detection, provided a far more detailed and realistic picture of the fate of rutin within the body than it was possible to obtain in earlier investigations. A total of 15 urinary metabolites were detected and quantified. This HPLC-MS^2 technique which employed in these analyses used either an APCI or an ESI interface and such methodology has been available for over a decade and is now firmly established as the primary analytical tool for screening studies related to bioavailability and drug discovery (Ackermann *et al.*, 2002). The ESI, regarded as the softer more versatile method of ionisation, is able to ionise extremely polar/non-volatile molecules such as the flavonols or conjugated metabolites. However, ESI does not ionise phenolic acids. In contrast, APCI provides more robust ionization through a heated nebulizer, which facilitates solvent evaporation and subsequent ionisation of phenolic acids.

3.5 Conclusion

The data obtained in this study revealed that extensive modification of rutin occurs following its ingestion and the appearance of metabolites in the bloodstream and urine. The large intestine or the colon has been verified as the major site for the metabolism of rutin. The absorbance of rutin at this site involves a complex combination of deglycosylation, glucuronidation and methylation processes. Microfloral degradation introduces an additional complexity to the intestinal disposition and biotransformation of rutin *in vivo*. Evidence obtained from this trial suggests that the colonic microflora play a very crucial role in the catabolism of rutin producing large quantities of 3,4-dihydroxyphenylacetic acid, which is the major catabolite in the urine.

However, before any further steps can be taken to study the potential biological effects at the tissue and cellular level, more data is needed on the concentrations and exact metabolic forms produced in the colonic lumen after ingestion of rutin. One way this can be done is though *in vitro* fermentation of rutin with colonic microflora. Detailed study on this aspect is revealed in the following chapter.

Chapter 4: Colonic Metabolism of Rutin

4.1 Introduction

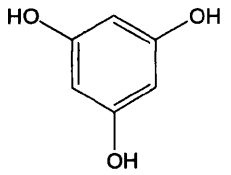
Most flavonoids and polyphenolic compounds are present in plant-derived food as glycosides that undergo hydrolysis before absorption. Depending upon the linkages, they are both hydrolysed and subsequently absorbed either in the small or the large intestines (Murota and Terao, 2003). In recent years, major advances have been made in elucidating structural changes and understanding the mechanism of absorption of flavonoids across the small intestine using both Caco-2 cell models and isolated preparations of rat small intestine (Spencer, 2003). Such studies have however suggested that the extent of absorption of dietary polyphenols in the small intestine may be relatively small (10–20%) (Spencer *et al.*, 1999; Kuhnle *et al.*, 2000) This is partly due to the fact that majority of the absorbed polyphenols in the enterocytes and/or the liver may transport back out into the lumen either directly or via the bile and ultimately will reach the large intestine where they encounter the colonic microflora (Spencer, 2003). Identifying structural changes of polyphenol compounds and the mechanism of absorption in the large intestine is, therefore, the key to not only defining their bioavailability but also their biological activities in humans.

The microflora in the colon, which can amount to ~10^{12} microorganisms/mL has an enormous catalytic and hydrolytic potential that can result in a huge array of new metabolites (Spencer, 2003). The hydrolytic power of the microflora enzymes is able to catalyze the breakdown of the C_6-C_3-C_6 flavonoid skeleton itself to simpler molecules such as phenolic acids. Phenolic compounds, which include unabsorbed compounds from the diet and products of microbial metabolism by the gut microflora; account for the major components of the lower GI tract (Halliwell *et al.*, 2005). Depending on the diet consumed, a huge array of phenolic acids such as phenylacetic acid, 3-phenylpropionic acid, caffeic acid, ferulic acid, 3-hydroxyphenylacetic acid, benzoic acid, 3-(4-hydroxy)phenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 4–hydroxyphenylacetic acid and 3,4-dihydroxyphenylpropionic acid were found in faecal water. Halliwell *et al.* (2005) reported that concentrations of these phenolic acids range from 15 - 410 μM, which was much higher than the 0.7μM of flavonoids present in the colon. Halliwell *et al.* (2005) further suggested that as a consequence of these high concentrations, phenolic acids might exert direct protective effects within the large

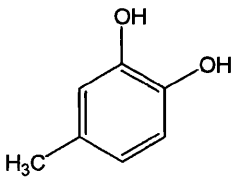
intestines which could include binding of prooxidant iron, scavenging of reactive nitrogen, chlorine, and oxygen species, and perhaps inhibition of cyclooxygenases and lipoxygenases.

The identification and quantification of microbial metabolites is still in its infancy (Manach *et al.*, 2004). To date there has been some debate in the identification of specific degradation products of rutin and also in elucidating the catabolic pathways involved. Aura *et al.* (2002) and Rechner *et al.* (2004) identified quercetin, 3,4-dihydroxyphenylacetic acid, 3–hydroxyphenlyacetic acid and 3-(3-hydroxyphenyl)propionic acid as the colonic catabolites of rutin. In addition to this, Justesen and Arrigoni (2001) also identified 3,4-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzoic acid, 2-(3,4-dihydroxyphenyl)ethanol, 4-hydroxyphenylpropionic acid and 3,4-dihydroxytolene as the possible catabolites of rutin but did not detect quercetin (Justesen *et al.*, 2000 and Justesen and Arrigoni, 2001). Using a pig caecum under anaerobic *ex-vivo* conditions, Labib *et al.* (2004), found that quercetin was metabolized to phloroglucinol, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxytoluene. In studies using the anaerobic bacterial strain, *Eubacterium ramulus,* which is a key organism for flavonoid degradation, Braune *et al.* (2001) demonstrated that alphitonin and taxifolin are intermediates in the conversion of quercetin to 3,4-dihydroxyphenylacetic acid. Structures of the possible catabolites of rutin and quercetin are illustrated in Figure 4.1.

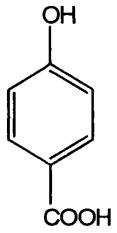
This chapter reports on the use of an *in vitro* faecal fermentation system to investigate the role of colonic microflora in the metabolism of rutin. The aims being i) to identify specific degradation products ii) to obtain information on possible catabolic pathways involved in their formation and iii) to determine their contribution to the total antioxidant activity of the colonic lumen.



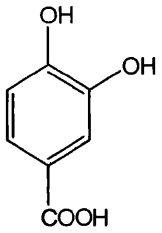
Phloroglucinol



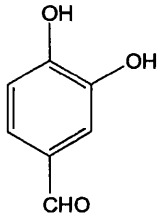
3,4-Dihydroxytoluene



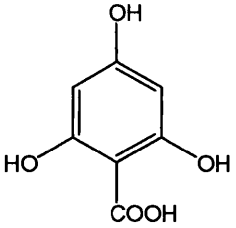
4-Hydroxybenzoic acid



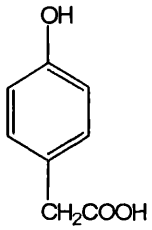
3,4-Dihydroxybenzoic acid



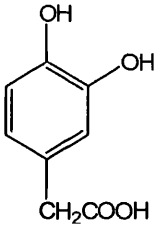
3,4-Dihydroxybenzaldehyde



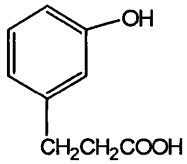
2,4,6-Trihydroxybenzoic acid



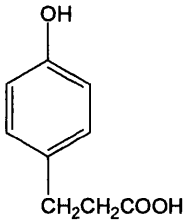
3-Hydroxyphenylacetic acid



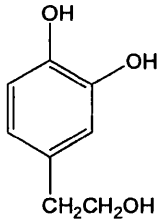
3,4-Dihydroxyphenylacetic acid



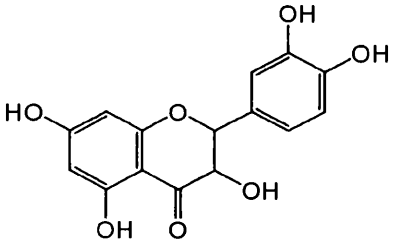
3-(3-Hydroxyphenyl)propionic acid



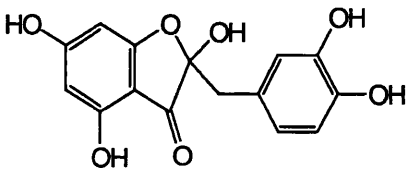
4-Hydroxyphenylpropionic acid



2-(3,4-Dihydroxyphenyl)ethanol



Taxifolin



Alphitonin

Figure 4.1. Structures of possible biodegradation products of rutin and quercetin

4.2 Study model

The *in vitro* fermentation model adopted in this study is based on faecal incubation with conditions designed to simulate events taking place in the human colon (Edwards *et al.*, 1996). *In vitro* studies circumvent some of the problems encountered with *in vivo* studies: the collection of faeces is simple and retention of fermentation products within the system makes their identification and quantification easier (Christian *et al.*, 2003). The fermentations were performed under strictly anaerobic conditions.

4.3 Materials and method

4.3.1 Materials

[4-^{14}C]Quercetin was synthesized by Chemsyn Science Laboratories (Lenexa, KS 66215, USA) (Figure 4.2). The specific activity was 52.9 mCi mmol^{-1}.

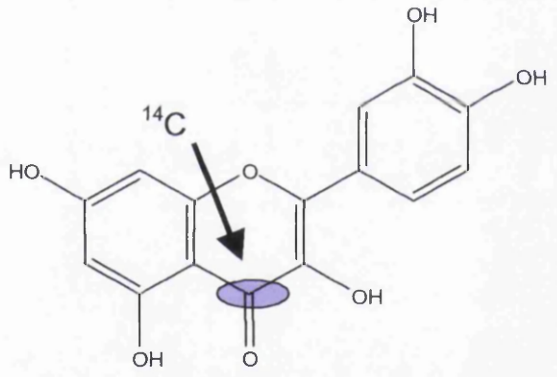


Figure 4.2. Radiolabelled [4-^{14}C] quercetin

Standards of rutin, taxifolin, 2-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,4-hydroxybenzaldehyde, 3,4-dihydroxyphenylpropionic acid, gallic acid and phloroglucinol were purchased from Sigma Aldrich Chemical (Poole, Dorset, UK). 3-(2-Hydroxy)phenylpropionic acid and 3-(3-hydroxy)phenylpropionic acid were purchased from Fluorochem (Derby, UK).

Analytical grade chemicals were used in preparing the buffer, macromineral, micromineral and reducing solutions. Ammonium bicarbonate, sodium bicarbonate, cysteine hydrochloride, sodium sulphide, magnesium sulphate, calcium chloride,

sodium hydrogen phosphate anhydrous, potassium dihydrogen phosphate anhydrous, manganese chloride, cobalt chloride, ferric chloride were obtained from BDH Laboratory Supplies (Poole UK). Resazurin and tryptone were purchased from Sigma-Aldrich while sodium hydroxide was purchased from Riedek-DeHaen (Seelze, Germany).

The HPLC solvents, methanol and acetonitrile (HPLC grade) were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Formic acid was purchased from Riedek-DeHaen) while acetic acid was from BDH. All other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise stated.

4.3.2 Fermentation medium

The fermentation media was prepared by mixing 2 g of tryptone in 400 mL of distilled water and 100 µL of micromineral solution (consisting of 13.2 g of $CaCl_2.2H_2O$, 10.0 g of $MnCl_2.4H_2O$, 1.0 g $CoCl_2.6H_2O$, $FeCl_3.6H_2O$ and distilled water up to 100 mL). This solution was agitated to dissolve the chemicals and then 200 mL of buffer solution (2 g of $NH_3.CO_3$, 17.5 g of $Na_2.2CO_3$, and 500 mL of distilled water), 200 mL of macromineral solution (2.85 g of $Na_2HPO_4.H_2O$, 3.1 g of $KH_2PO_4.H_2O$, 0.3 g of $MgSO_4.7H_2O$ and 500 mL of distilled water) and 1 mL of 0.1% (w/v) of resazurin solution (a redox indicator) were added. This medium was adjusted to pH 7 using HCl and subsequently boiled for a few minutes to sterilize it and remove oxygen. Reducing solution (312.5 mg of cysteine hydrochloride, 2 mL of 1 M NaOH, 312.5 mg of sodium sulphide and 47.5 ml distilled water) was added at 0.5 mL for every 10 mL of medium after which oxygen-free nitrogen (OFN) was admitted until anaerobic conditions (observed through a colour change from pale indigo to colourless) were achieved.

4.3.3 Subjects

Faecal samples were obtained from two males and a female volunteer; A, B and C. As a matter of practicality and time constraints, faecal samples from only three volunteers were used in the present study. Subjects were non-smokers, aged between 26-37 and had not consumed antibiotics for at least three months prior to the study. The volunteers were required to avoid all alcohol and food rich in polyphenols 48 h prior to faecal collection. Faecal samples were collected and processed within 30 min of passage.

4.3.4 *In vitro* fermentation

The *in vitro* fermentation method used in this study was adopted from Edwards *et al.* (1996). For each subject, 6.4 g of freshly voided faecal sample was homogenized with 20 mL phosphate buffer to obtain a 32% faecal slurry. Five mL of the slurry was added to 44 mL of the pre-reduced fermentation media in 100 mL fermentation bottles (Figure 4.3). Each batch culture consist of 50 mL of the above slurry mixture together with one of the following substrates:

a) 28 μmoles of rutin
b) 28 μmoles of rutin plus 0.5 g glucose
c) 55 μmoles of quercetin plus 18 x 10^6 dpm [^{14}C]quercetin (60 nmoles)
d) 55 μmoles of quercetin plus 18 x 10^6 dpm [^{14}C]quercetin (60 nmoles) and 0.5 g glucose
e) Control – no substrate

A single absorbance peak at 365 nm and a single radioactive peak, which corresponded to the retention time of quercetin were obtained when 10^6 dpm of [^{14}C]quercetin was analysed by HPLC. The amount of rutin used in this study corresponds to the results obtained from the previous chapter, i.e after consuming 175 μmoles of rutin, approximately 75% of it was excreted out in the ileal fluid within five hours of ingestion. Assuming that the colon volume content is about 300 mL, the final concentration of rutin in the colon is 560 μM. To obtain this concentration in *in vitro* fermentation studies, 28 μmoles of rutin was dissolved in 50 mL fermentation medium. Glucose was added to the fermentation media so that the test conditions were more representative of the real life situation as rutin is normally consumed through ingestion of rutin containing food or beverages.

The results of the *in vitro* fermentation study with rutin indicated that the first degradation product to be formed was quercetin. This is in agreement with the findings of Aura *et al.* (2002) and Rechner *et al.* (2004). To identify and quantify further degradation products, which may be produced in smaller concentrations, 55 μmoles of quercetin spiked with 18 x 10^6 dpm [^{14}C]quercetin (60 nmoles) was used as the substrate in the *in vitro* fermentation trial.

After adding in the substrate, the fermentation bottles were sealed air-tight and purged with OFN. They were then placed horizontally in a shaking water bath at 60

strokes/min and incubated at 37°C for 48 h, aiming to simulate the conditions in the colonic lumen (Christian *et al.,* 2003). Aliquots of the fermented faecal samples (3-5 mL) were collected after 0, 2, 4, 6, 24, 30 and 48 h and stored immediately at –80°C.

4.3.5 Measurement of fermentation products

4.3.5.1 Extraction of polyphenols

Triplicate 250 µL samples of faecal slurry were extracted twice with 500 µL MeOH in 1% formic acid and 20 mM sodium diethyldithiocarbamate (antioxidant). To calculate the extraction recovery, 250 µg gallic acid was used as an internal standard. For each extraction, samples were homogenised for 5 min with a Disruptor Genie (Scientific Industries, UK) and then centrifuged at 16000 g for 10 min. Supernatants were combined and reduced to dryness in vacuo. Extracts were then re-suspended in 25 µL methanol plus 225 µL 1% formic acid in distilled water and centrifuged at 16000 g at 4°C for 5 min prior to the analysis of 200 µL aliquots of the supernatant by HPLC-PDA-MS^2. Analyte recovery was calculated by comparing the response of samples spiked with the internal standard prior to extraction and its recovery of it after extraction. The extraction recoveries for the internal standard was 93± 3.3%. The precision and accuracy of the assay were evaluated by analyzing standard solutions of rutin and 3,4 dihydroxyphenyl acetic acid, everyday, over the period of the study. Significant changes in the ionization peaks were overcome with the re-tuning of the MS^2. Calibration plots of peak area versus concentration for all the analytes were obtained by linear regression analysis of at least four data points per concentration range of 5 ng to 300 ng using UV absorbance at 365 nm for flavonol and 280 nm for phenolic acids.

4.3.5.2 HPLC with diode array and MS^2 detection with on-line radioactivity detection for metabolite identification

Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, PDA detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C. (Thermo Finnigan, San Jose, USA). Separations were carried out using a 250 x 4.6 mm I.D. 4 µm Synergi Max-RP column (Phenomenex, Macclesfield, UK) for flavonols and eluted with a 60 min gradient of 5-40% acetonitrile in 1% formic acid at a flow rate of 1 mL min^{-1} and maintained at 40°C. Analysis of phenolic acids used a 250 x 4.6 mm I.D. 4 µm Synergi Hydro-RP column (Phenomenex) eluted with a 60 min gradient of 2-20%

acetonitrile in 0.25% acetic acid at a flow rate of 1 mL min^{-1}. After passing through the flow cell of the PDA detector, the column eluate was split and 0.3 mL min^{-1} was directed to a LCQ DecaXP ion trap mass spectrometer fitted with an ESI for the analysis of flavonols or to an APCI interface (Thermo Finnigan) for the analysis of phenolic acids. Analyses utilised the negative ion mode for both flavonols (ESI) and phenolic compounds (APCI) as this provided the best limits of detection. For detection of radioactivity, the column eluate was first directed to a radioactivity monitor (Model 9701, Reeve Analytical, Glasgow, UK) fitted with a 200 µL heterogenous flow cell, before being split to enter the LCQ DecaXP ion trap mass spectrometer. Analysis was carried out using full scan, data dependant MS^2 scanning from *m/z* 100 to 1000. For the ESI interface, the capillary temperature was 350°C, sheath gas and auxiliary gas were 60 and 10 units respectively, and the source voltage was 4 kV for negative ionisation. For the APCI interface, the capillary temperature was 130°C, vaporizer temperature was 350°C, sheath gas and auxiliary gas were 80 and 60 units respectively, and the source voltage was 6 kV for negative ionisation.

Quercetin, rutin, taxifolin, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid were all quantified by reference to standard calibration curves at 365 nm for flavonols and 280 nm for phenolic acids. Other phenolic acids were quantified in either 3-hydroxyphenylacetic acid or 3,4-dihydroxyphenylacetic equivalents. In all instances, peak identification was confirmed by HPLC retention times and MS^2 fragmentation data.

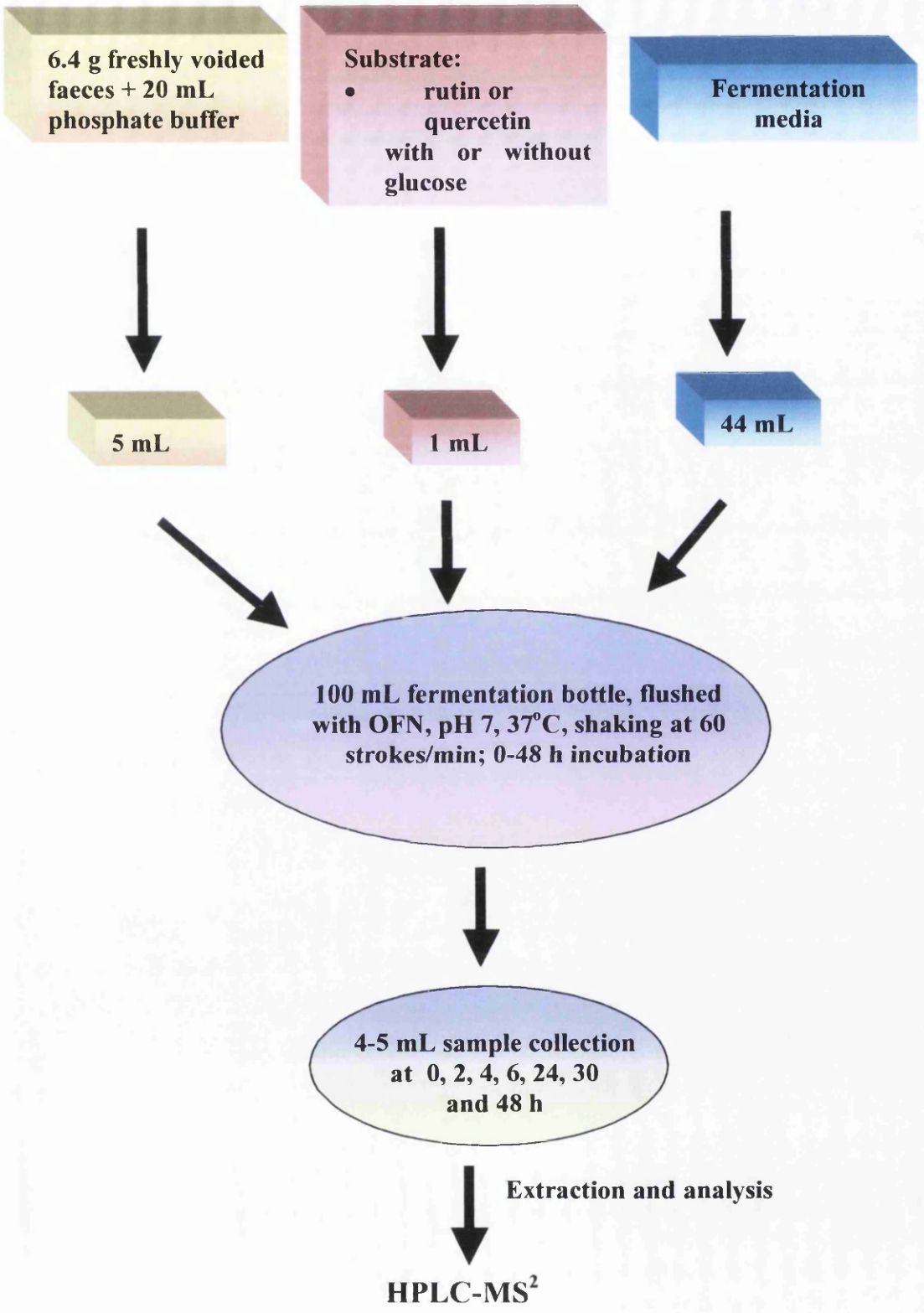


Figure 4.3. Flow chart of the *in vitro* fermentation method

4.3.6 Antioxidant assays

4.3.6.1 Determination of the antioxidant activity using the FRAP assay

The ferric reducing antioxidant power (FRAP) assay, described by Benzie *et al.* (1996), was used to estimate the antioxidant activity of the faecal samples and that of specific flavonol and phenolic acid standards. This method measures the ability of a solution to reduce a ferric-tripyridyl-triazine complex (Fe^{3+}-TPTZ) to the ferrous form, Fe^{2+}, producing a blue colour with absorption at 593 nm. The reaction is non-specific and any half-life reaction, which has a lower positive redox potential under reaction conditions, than the Fe^{3+}/Fe^{2+}-TPTZ half-life reaction will drive the Fe^{3+}-TPTZ reduction. In the FRAP assay excess Fe^{3+} is used and the rate limiting factor of the Fe^{2+}-TPTZ, and hence the colour formation, is the reducing ability of the sample. The absorbance at 593 nm was measured with a Unicam UV500 Spectrophotometer. Thirty µL of sample was added to the freshly prepared FRAP reagent (acetate buffer, pH 3.6; $FeCl_3$, TPTZ in 40 mM HCl). Absorbance was measured at 593 nm after 4 min and compared to a 0-1 mM Fe^{2+} standard curve.

4.3.6.2 Determination of the antioxidant activity using the $ABTS^+$ decolourisation assay

The $ABTS^{\cdot+}$ (2,2'-azinobis-[3-ethylbenzothiazoline-6-sulfonate]) assay is based on the method of Koleva *et al.* (2001) and Dapkevicius *et al.* (2001) and was used in combination with the HPLC to analyse faecal samples in order to identify individual catabolites with antioxidant activity. This on-line HPLC antioxidant detection system is based on procedures developed by Stewart *et al.* (2005). A 2 mM ABTS stock solution containing 3.5 mM potassium persulphate was prepared and incubated at room temperature in darkness overnight to allow for stabilisation of the radical. The next morning, the stock solution was diluted 8-fold with 0.1 M potassium phosphate buffer at pH 8.

The HPLC system comprised of an HPLC pump, an auto-injector with sample cooler, a column oven linked to a photodiode array detector (Thermo Finnegan). Samples, 20 - 30 µL in volume, were analysed with the same column and solvents used for HPLC-PDA-MS^2 analysis. HPLC eluent leaving the PDA arrived at a "T" piece, where the $ABTS^+$ reagent was added. $ABTS^+$ solution was delivered by a LC-10-AD *VP* pump, connected to a GT-154 Vacuum Degasser (Shimadzu, Kyoto, Japan) at a

flow rate of 0.5 mL/min. After mixing by passing through a 1.5 m x 0.4 mm (i.d.) loop, absorbance was measured by a UV-detector at 720 nm (Nemphlar Bioscience, Lanark, UK). Data were analysed using ThermoFinnigan ChromquestTM chromatography software (Version 4.0) (Figure 4.4)

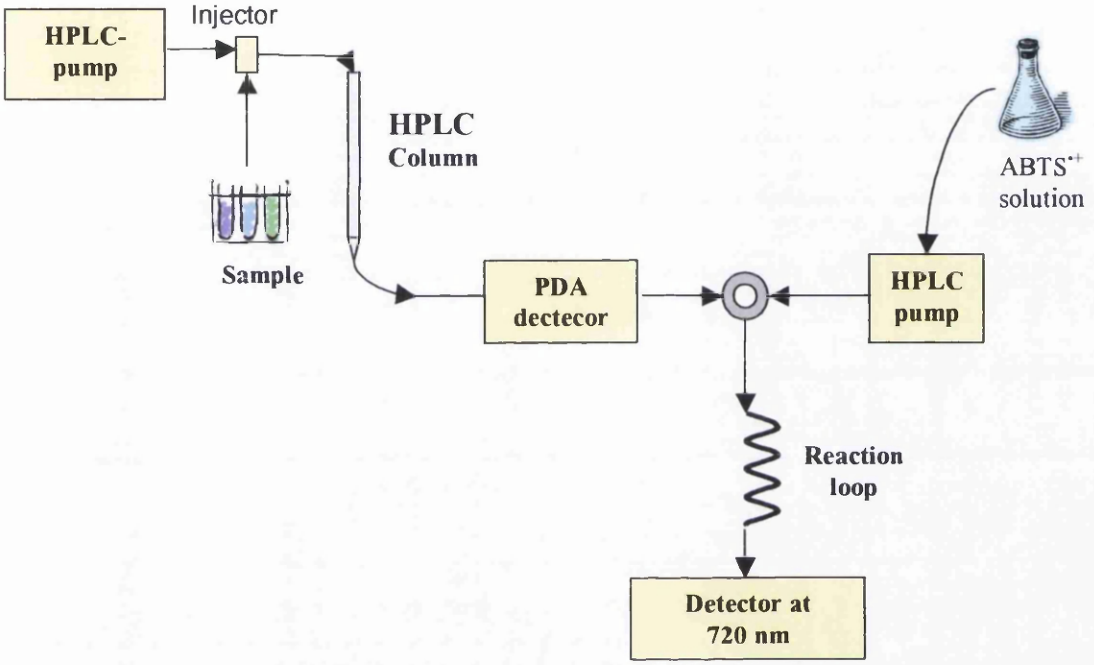


Figure 4.4. Schematic diagram of the on-line antioxidant system used in this study. HPLC separation followed by an on-line post column detection of radical scavenging compounds based on a model oxidation system: $ABTS^{\bullet+}$ bleaching and decreasing absorbance at 720 nm upon its reduction by an antioxidant

4.3.7 Statistics

Each sample was analysed in triplicate and data were presented as mean values ± standard error (n=3). Where appropriate, data were subjected to statistical analysis using analysis of variance (ANOVA) to determine the significance of the observed treatment/response relationship. Multivariate correlation analysis of appropriate data was performed according to Pearson's Correlation. All statistical analyses were performed using Minitab software (Minitab Inc., Addison-Wesley publishing Co, Reading, MA).

4.4 Results

4.4.1 Deglycosylation of rutin

Analysis of the faecal samples of rutin fermentation revealed clear donor-dependent variations in degradation patterns. The data are presented in Figure 4.5 in which seven fermentation time points are presented over a 48 h period following incubations with 28 µmoles of rutin. Identification of metabolites was obtained from HPLC-MS^2 analyses. The first fermentation product of rutin to be detected, was quercetin. Quercetin-3-glucoside was not detected as an intermediate indicating the one step removal of the rutin moiety rather than an initial cleavage of rhamnose followed by the loss of glucose as observed in faecal incubation with cyanidin-3-rutinoside (Aura *et al.,* 2005). The addition of glucose to the fermentation medium enhanced the deglycosylation process by almost 20 h. Quercetin was detected in the faecal samples 4 h after fermentation and complete degradation of rutin occurred within 24 h in the presence of glucose. However, the degradation pattern of rutin and the formation of quercetin differed between the faecal donors when glucose was not present. In the absence of glucose, higher amount of quercetin was observed in samples from donors B and C. This was especially evident in the case of faecal samples from the donor C where 7.5 µmoles of quercetin was present at the 48 h time point. This effect was not observed in the samples from donor A (Figure 4.5). Since quercetin is the first degradation product of rutin, subsequent incubations were carried out using quercetin as the substrate.

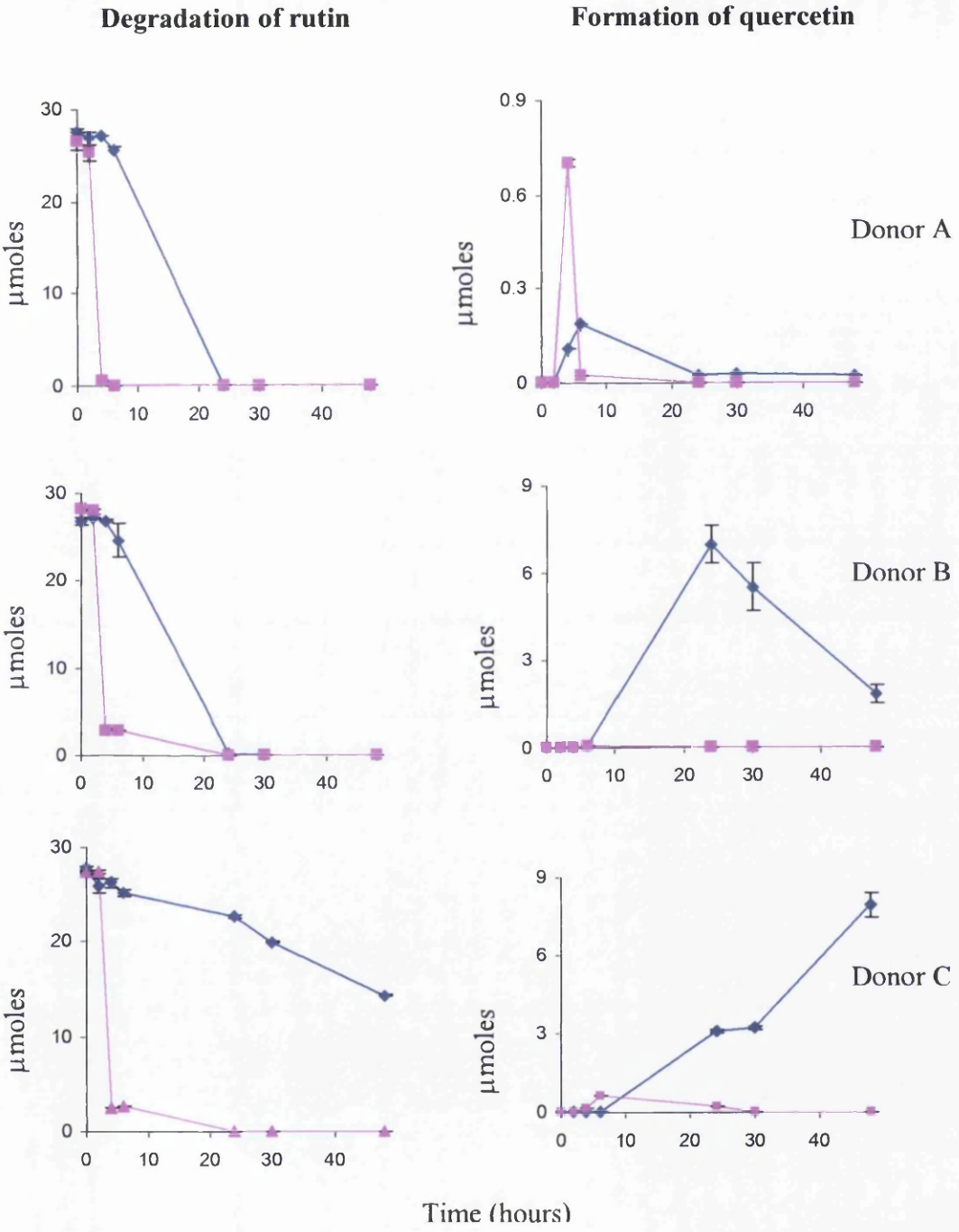


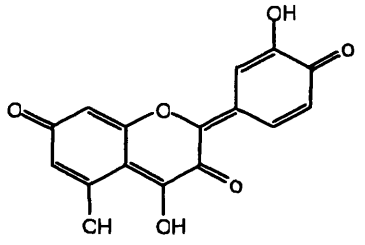
Figure 4.5. Individual fermentation profiles of rutin in faecal samples from three donors, demonstrating the different degradation patterns of rutin (left hand-side graphs) to quercetin (right hand-side graphs)

4.4.2 Radiolabelled intermediate products after deglycosylation of rutin

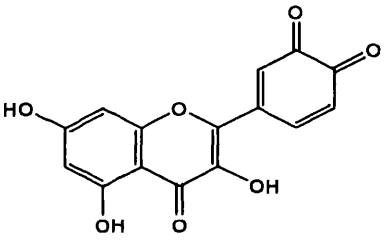
When high a concentration of quercetin amounting to 55 μmoles (1.1 mM) was added directly into the fermentation medium, it was found to be unstable. Within 15 min, only 1.4% remained in the fermentation medium containing glucose and 2.3% in the absence of glucose. This may be due to its pro-oxidant properties as suggested by Awad *et al.* (2002). Quercetin can undergo rapid oxidation especially through reactions with H_2O_2, resulting in its conversion to semiquinone free radicals and to a wide variety of unidentified reaction products (Krishnamachari *et al.*, 2002) (see Figure 4.6). Long *et al.* (2000) demonstrated that at concentrations of 1 mM of quercetin in cell culture media produced substantial quantities of H_2O_2 which increased with concentration and time. Generation of H_2O_2 could explain the rapid oxidation of quercetin in the fermentation media even though oxygen free conditions were maintained throughout the fermentation period. This phenomenon was, however, not so pronounced when quercetin was formed from rutin as indicated in Figure 4.5. The contrasting effect seen here could be due to differing concentrations of quercetin involved. When present in high concentrations, the quantities of H_2O_2 produced would appear to be sufficiently high to cause most of the quercetin to be oxidised while this did not appear to occur to the same extent at lower concentrations of quercetin which was produced from rutin via deglycosylation (see Figure 4.5, donors B & C, without glucose). A number of other studies have also substantiated that pro-oxidant properties of quercetin is only observed when present in high amounts (Cao *et al.*, 1997, Galati, *et* al., 2001)

In the present study, evidence indicates that quercetin may have been oxidised to more polar compounds as indicated by analysis using HPLS-MS^2 with APCI and the on-line radioactivity detector (Table 4.1 & Figure 4.7). Being more polar, these 'oxidised' quercetin compounds were separated by HPLC using a 2-20% acetonitrile gradient. These oxidised forms of quercetin retained their radioactivity where most was associated with peak 3 while only smaller amounts could be detected in peak 1, 2 and 3 (Figure 4.6 and 4.7). The oxidised quercetin compound in peak 2 with a m/z of 299 may be a quinone methide as it is more stable than *O*-Quinone (Awad *et al.*, 2002). The structure of this compound is illustrated in Figure 4.6. However, this compound appeared only transiently in the faecal samples 15 min after incubation and was present in trace and non-quantifiable amounts. The inter-individual degradation profiles of the quercetin and of its other oxidized forms are presented in Table 4.2. As with rutin, the

degradation of quercetin and its oxidised forms were enhanced by the addition of glucose, which resulted in almost all the quercetin being degraded within 6 h of fermentation (faecal samples from donor A and B). However, it must be noted that after 15 min of incubation only 10% of the residual quercetin and its oxidised forms were identifiable while the remaining 90% could not be accounted for. Oxidised quercetin such as quinone methide and *O*-quinone are highly reactive intermediates and have the affinity to form adducts with various compounds (Awad *et al.*, 2000) and also rapidly polymerize (Awad *et al.* 2002). This may explain the appearance of a number of unidentified radioactive peaks particularly in the first 4 h of incubation as observed in Figure 4.9. In addition, its possible attachment to diverse compounds of varying molecular weight present in the fermentation media also makes detecting and identifying these compounds a less than straight forward task.

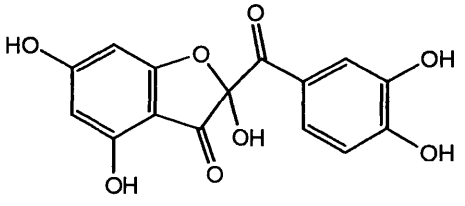


Quinone methide
Molecular weight:300
$C_{15}H_8O_7$

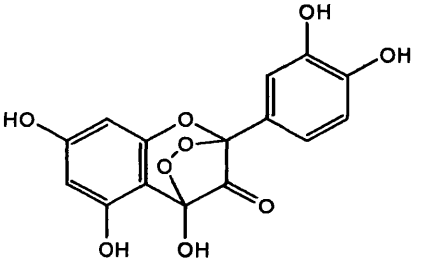


O-Quinone
Molecular weight:300
$C_{15}H_8O_7$

Adopted from Awad *et al.* (2002)



Unidentified 'oxidised'quercetin
Molecular weight:318
$C_{15}H_{10}O_8$



Unidentified 'oxidised'quercetin
Molecular weight:334
$C_{15}H_{10}O_9$

Adopted from Krishnamachari *et al.* (2002)

Figure 4.6. Possible oxidised intermediate products formed during the *in vitro* fermentation of quercetin.

Table 4.1. HPLC-MS^2 identification of 'oxidised' quercetin metabolites detected in faecal samples after 15 min of fermentation

Peak	t_R (min)	Oxidised Quercetin	$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)
1	9.3	Quercetin + [H] + [O]	318	274, 244, 199, 200
2	14.8	Quinone methide	299	271, 277, 255
3	37.1	Quercetin + [O]	317	231, 255, 187, 191, 302
4	50.0	Quercetin + 2[O]	333	315, 300, 181, 151

Peak numbers and HPLC retention times refer to HPLC trace in Fig. 4.5. t_R - retention time; $[M-H]^-$ - negatively charged molecular ion. APCI.

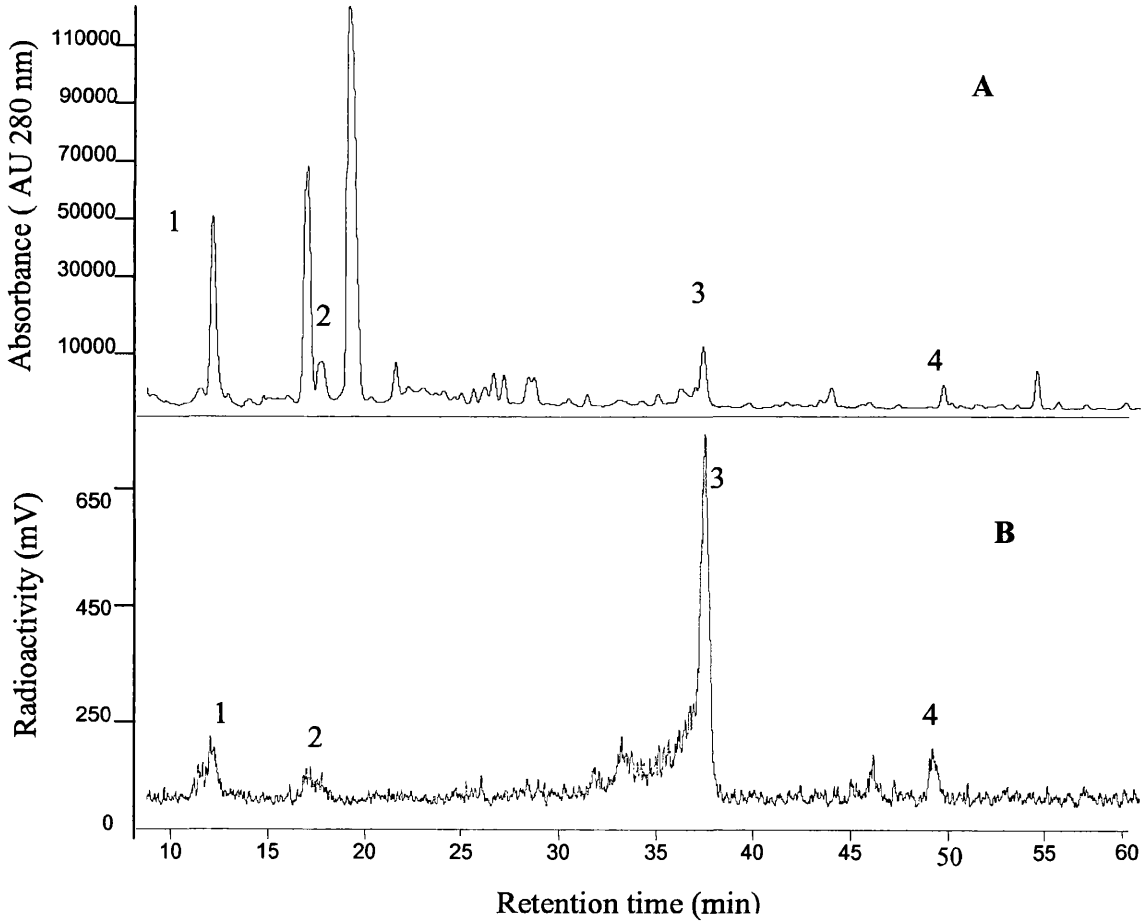


Figure 4.7. HPLC chromatogram (A) and radioactivity (B) of faecal samples from donor A at time 0.15 h as analysed by HPLC-MS-radiodetector. Radiolabelled compounds are numbered according to the order of elution. (For MS^2 data and identification and quantification of peaks 1-4, refer to Table 4.1)

Table 4.2. Accumulation of quercetin and 'oxidized quercetin' structures 0.15-6 h following *in vitro* fermentation of faecal samples from three donors with 55 μmoles of quercetin

Donor	Metabolite	Glucose	0.15 h	2 h	4 h	6 h
A	Quercetin	-	1.4 ± 0.1	1.3 ± 0.1	0.7 ± 0.0	n.d.
		+	0.8 ± 0.0	0.7 ± 0.0	0.5 ± 0.0	n.d.
	Quercetin+[H]+[O]	-	2.6 ± 0.0	3.9 ± 0.1	4.1 ± 0.1	3.1 ± 0.1
		+	2.6 ± 0.1	n.d.	n.d	n.d.
	Quercetin+[O]	-	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.5 ± 0.1
		+	0.8 ± 0.0	0.3 ± 0.0	n.d.	n.d.
	Quercetin+2[O]	-	0.2 ± 0.0	0.2 ± 0.0	n.d.	n.d.
		+	0.2 ± 0.0	n.d.	n.d.	n.d.
B	Quercetin	-	1.1 ± 0.0	0.9 ± 0.0	0.5 ± 0.0	n.d.
		+	0.8 ± 0.0	0.9 ± 0.1	n.d.	n.d.
	Quercetin+[H]+[O]	-	3.1 ± 0.1	3.9 ± 0.2	4.1 ± 0.1	3.5 ± 0.1
		+	3.4 ± 0.1	n.d.	n.d.	n.d.
	Quercetin+[O]	-	1.0 ± 0.0	5.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.1
		+	0.8 ± 0.0	0.4 ± 0.0	n.d.	n.d.
	Quercetin+2[O]	-	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	n.d.
		+	0.3 ± 0.0	n.d.	n.d.	n.d.
C	Quercetin	-	1.1 ± 0.1	1.6 ± 0.1	1.5 ± 0.0	0.5 ± 0.1
		+	0.8 ± 0.0	1.6 ± 0.1	0.5 ± 0.0	n.d.
	Quercetin+[H]+[O]	-	4.1 ± 0.3	2.7 ± 0.0	n.d.	n.d.
		+	4.5 ± 0.1	4.2 ± 0.3	1.4 ± 0.0	n.d.
	Quercetin+[O]	-	0.7 ± 0.0	1.6 ± 0.1	1.1 ± 0.0	1.8 ± 0.1
		+	0.7 ± 0.0	0.9 ± 0.0	0.3 ± 0.0	n.d.
	Quercetin+2[O]	-	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	n.d.
		+	3.7 ± 0.1	3.2 ± 1.0	0.9 ± 0.0	0.3 ± 0.0

Data for individual donors are expressed as μmoles ± standard error (n=3); n.d.-not detected; + : incubated with 500 mg glucose; - : incubated without glucose. No quercetin or its oxidized compounds were detected at 24 h, 30h and 48 h after fermentation.

4.4.3 Intermediate metabolites and phenolic acids formed after degradation of quercetin

4.4.3.1 Identification of intermediate metabolites and phenolic acids

Analysis by HPLC- MS^2 with APCI and PDA detection revealed the presence of intermediate products and catabolites of quercetin resulting from fission of the C_6-C_3-C_6 structure. The results obtained are illustrated in Figure 4.8 & Table 4.3 and summarised below. The structures of the compounds are illustrated in Figure 4.1.

Peak 1 (t_R = 14.7 min, λ_{max}= 255nm) had a $[M-H]^-$ at *m/z* 153 with MS^2 yielding a charged fragment at *m/z* 109. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 3,4-dihydroxybenzoic acid

Peak 2 (t_R = 15.5 min, λ_{max}= 280 nm) had a $[M-H]^-$ at *m/z* 167 which on MS^2 yielded a major ion at *m/z* 123 and two smaller fragments at *m/z* 108 and 164. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 3,4-dihydroxyphenylacetic acid

Peak 3 (t_R = 21.0 min, λ_{max}= 245nm) had a $[M-H]^-$ at *m/z* 137 with MS^2 yielding a charged fragment at *m/z* 93. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 4-hydroxybenzoic acid

Peak 4 (t_R = 26.6 min, λ_{max}= 270 nm) had a $[M-H]^-$ at *m/z* 151 with MS^2 yielding a major ion at *m/z* 107 and two smaller ions at *m/z* 93 and 121. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 3-hydroxyphenylacetic acid.

Peak 5 (t_R = 28.2 min, λ_{max}= 295 nm) had a $[M-H]^-$ at *m/z* 303 with MS^2 yielding three charged fragments, a main one at *m/z* 285 and two smaller ones at *m/z* 117 and 241. From the mass spectrometric data, this compound may be an isomer of taxifolin, alphitonin, previously identified as a quercetin degradation product by Braune *et al.* (2001).

Peak 6 (t_R = 38.2 min, λ_{max}= 270 nm) had a $[M-H]^-$ at *m/z* 165 with MS^2 yielding a charged fragment at *m/z* 121. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is 3-(3-hydroxyphenyl)propionic acid.

Peak 7 (t_R = 47.7 min, λ_{max}= 295 nm) had a $[M-H]^-$ at *m/z* 303 with MS^2 yielding three charged fragments, a main one at *m/z* 285 and two smaller ones at *m/z* 117 and 241. The mass spectrometric data and co-chromatography with an authentic standard confirmed that this compound is taxifolin.

Peak 8 (t_R = 50.3 min, λ_{max}= 295 nm) had a $[M-H]^-$ at *m/z* 303 with MS^2 yielding three charged fragments, a main one at *m/z* 285 and two smaller ones at *m/z* 117 and 241. From the mass spectrometric data, this compound appears to be an isomer of taxifolin.

In addition to these catabolites, trace amounts of phloroglucinol were detected in the faecal samples from donor B at 4 h (with glucose) and 6 h (without glucose). This catabolite was also detected in small amounts in faecal samples of donor C but was only observed at 24 h (with glucose). This catabolite eluted at 7.2 min with a $[M-H]^-$ at *m/z* 125 with MS^2 yielding a major ion at *m/z* 57 and a smaller fragment at *m/z* 81. Analysis of a phloroglucinol standard confirmed the identity of this peak as it yielded the same mass spectrum and also co-chromatographed with the catabolite peak.

Table 4.3. HPLC-MS^2-APCI identification of phenolic acids detected in faecal samples after fermentation with 55 μmoles of quercetin

Peak	t_R (min)	Compound	$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)
1	14.7	3,4-dihydroxybenzoic acid	153	109
2	15.5	3,4-dihydroxyphenylacetic acid	167	123, 108, 164
3	21.0	4-hydroxybenzoic acid	137	93
4	26.2	3-hydroxyphenylacetic acid	151	107, 93, 121
5	28.2	alphitonin	303	285, 117, 241
6	38.2	3-(3-hydroxypheny)propionic acid	165	121
7	47.7	taxifolin	303	285, 117, 241
8	50.3	isomer of taxifolin	303	285, 117, 241

Peak numbers and HPLC retention times and the peaks refer to HPLC trace in Figure 4.8; t_R - retention time; $[M-H]^-$ - negatively charged molecular ion.

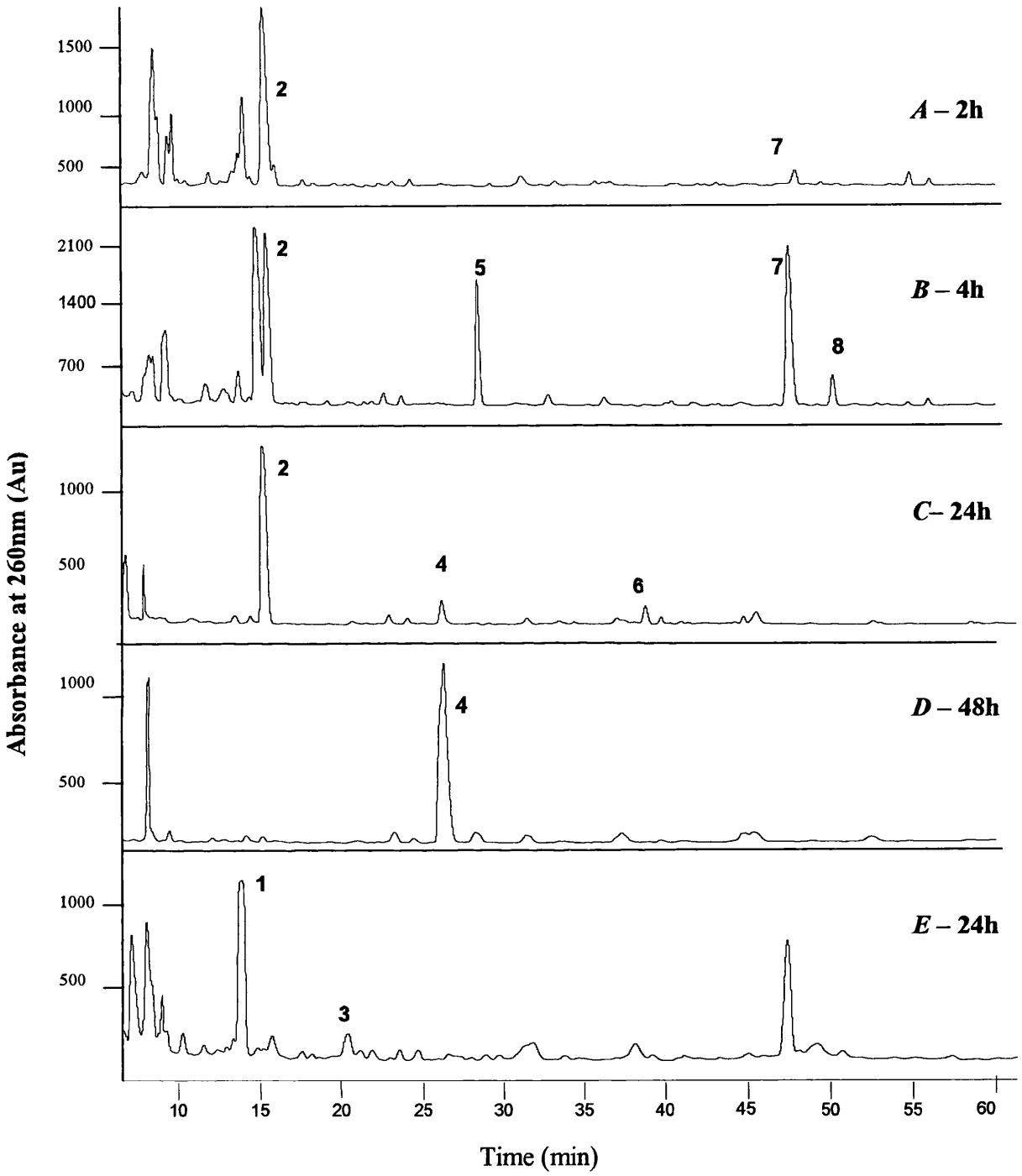


Figure 4.8. 2-20% gradient HPLC chromatogram of fermented faecal samples (with glucose) at time points indicated in the respective chromatograms obtained from donor B (chromatogram *A-D*) and donor C (without glucose; chromatogram *E*), with diode array detector operating at absorbance 260 nm. The absorbance at 280 nm was saturated for most of the peaks, and therefore absorbance at 260 nm was used both for quantification and illustration. For MS^2 data and identification of peaks, refer to Table 4.3 for the detection of phenolic acids.

4.4.3.2 Quantitative estimates of quercetin catabolites

4.4.3.2.1 Taxifolin and related compounds

Taxifolin, the first intermediate metabolite formed during the degradation of quercetin was observed in faecal samples after 2 h of fermentation and peaked in the 4 h sample. The amount produced varied between the individual donors. It was significantly higher in faecal samples of donor B and only appearing transiently in faecal samples from donor A and C (Table 4.4). Three isomers of taxifolin were identified in the fecal samples (Table 4.3, Figure 4.8). Taxifolin *(peak 8)* and its isomer *(peak 9)* retained their radioactivity whereas no radioactivity was detected on alphitonin *(peak 6)* (Figure 4.9). The synthesis of alphitonin involves a ring opening and recyclization mechanism (Schoefer *et al.*, 2003) and during this process it could have lost its radioactivity.

Table 4.4. Accumulation of taxifolin and its isomers 0-48 h following *in-vitro* fermentation of faecal samples from three donors with 55 µmoles of quercetin

Donor	Metabolite	Glucose	2 h	4 h	6 h	24 h
A	alphitonin	-	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	t.a.
		+	t.a.	0.1 ± 0.0	n.d.	n.d.
	taxifolin	-	n.d.	t.a.	t.a.	0.1 ± 0.0
		+	0.1 ± 0.0	0.2 ± 0.0	n.d.	n.d.
	Isomer of taxifolin	-	n.d.	n.d.	0.01 ± 0.00	n.d.
		+	n.d.	t.a.	n.d.	n.d.
B	alphitonin	-	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	n.d.
		+	t.a.	4.7 ± 0.0	n.d.	n.d.
	taxifolin	-	0.1 ± 0.0	0.3 ± 0.0	0.7 ± 0.0	t.a.
		+	0.4 ± 0.0	5.6 ± 0.2	n.d.	n.d.
	Isomer of taxifolin	-	n.d.	t.a.	0.1 ± 0.0	n.d.
		+	t.a.	0.8 ± 0.0	n.d.	n.d.
C	alphitonin	-	t.a.	n.d.	n.d.	n.d.
		+	0.1 ± 0.0	n.d.	n.d.	n.d.
	taxifolin	-	n.d.	n.d.	n.d.	n.d.
		+	n.d.	n.d.	n.d.	n.d.
	Isomer of taxifolin	-	n.d.	n.d.	n.d.	n.d.
		+	n.d.	n.d.	n.d.	n.d.

Data for individual donors are expressed as µmoles ± standard error (n=3); n.d.- not detected; + : with 500 mg glucose; - : without glucose; t.a. - trace amounts. No taxifolin or its isomers were detected at 0, 30 and 48 h after fermentation.

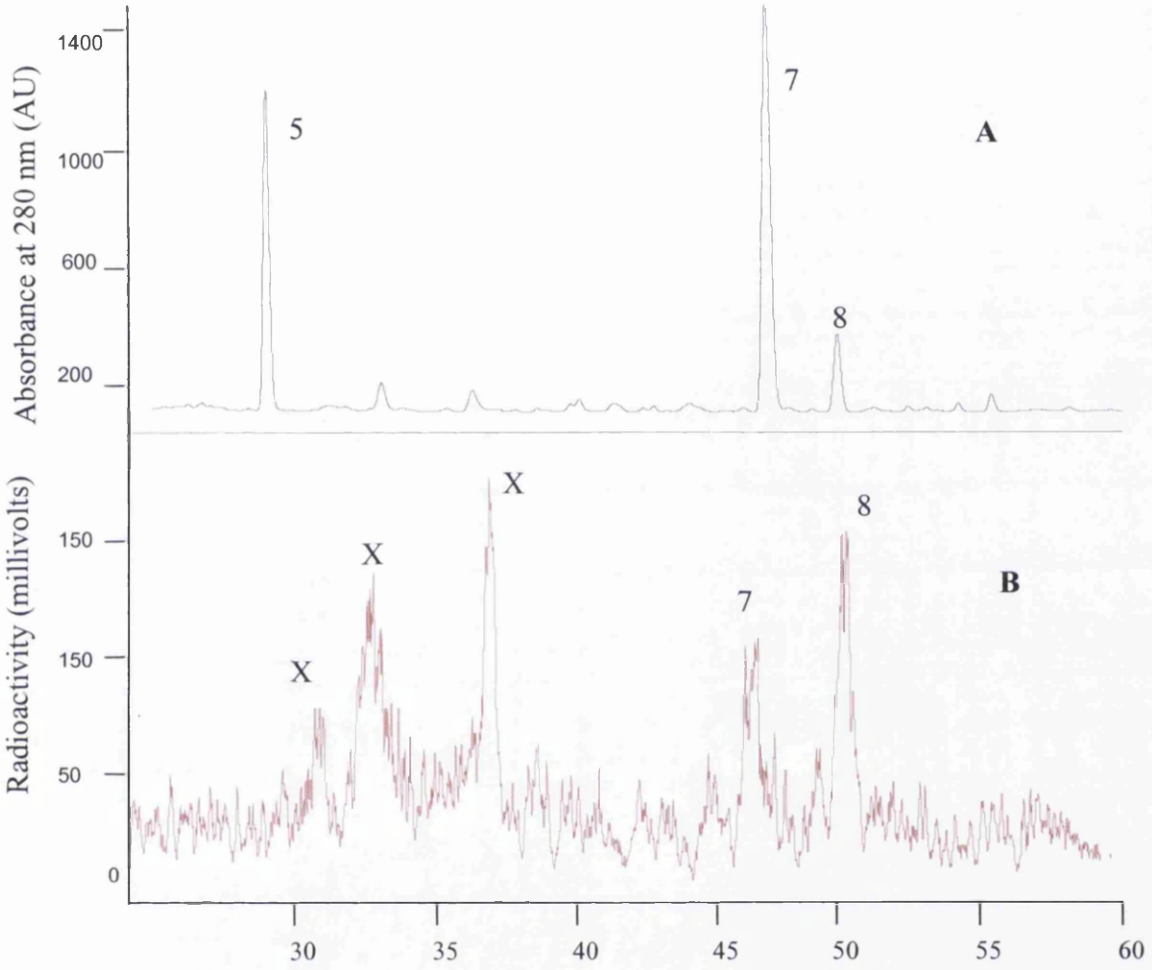


Figure 4.9. HPLC chromatogram (A) and radioactivity (B) of taxifolin and its isomers obtained from the faecal samples of donor B at time 4 h as analysed by HPLC-MS^2-radiodetector. Radiolabelled compounds are numbered according to the order of elution. (For MS^2 data and identification and quantification of peaks 5, 7 and 8, refer to Table 4.3). X- appearance of unidentified peaks

4.4.3.2.2 Phenolic acids

Five phenolic acids were identified and quantified in the faecal samples. A sixth, phlorogucinol appeared only in trace amounts and, therefore, was not quantified. Generally, the rate of catabolism was dependent on the individual differences in colonic microflora and the presence of glucose, which enhanced the rate of breakdown. The bio-degradation to the phenolic acids was slower in the faecal samples from donor C compared to those from donors A and B. The data obtained are presented in Table 4.5.

3,4-Dihydroxyphenylacetic acid appeared within 2 h of fermentation (faecal samples from donor A & B) and gradually increased in quantity in the fermentation media without glucose. The rate of increase was more rapid in the presence of glucose (Figure 4.10). 3,4-Dihydroxyphenylacetic acid was the most abundant metabolite formed during the degradation of quercetin and accounted for 60-97% of the breakdown products when glucose was present. Its maximum amount ranged between 33 to 53 μmoles (Table 4.5). This metabolite was however not present in the faecal samples from donor C (without glucose) and only appeared in the 24th hour of fermentation when glucose was present (Table 4.5).

3-Hydroxyphenylacetic acid, a dehydroxylation product of 3,4-dihydroxyphenylacetic acid first appeared after 24 h (donor B) and 48 h (donor C) (Table 4.5). This metabolite accounted for 15-20% of the breakdown products of quercetin in the presence of glucose.

3,4-Dihydroxybenzoic acid was observed transiently in the faecal samples of donors A and B (Figure 4.10) but more abundantly, up to 37 μmoles, in samples from donor C (Table 4.5). This metabolite started appearing in the faecal samples as early as in the second hour of fermentation.

4-Hydroxybenzoic acid, a dehydroxylation product of 3,4-dihydroxybenzoic acid, appeared in the faecal samples 2-4 h later than its parent compound (Figure 4.10) and as detected in low amounts ranging from 1.1 μmoles (donor A) to 2.2 μmoles (donor C) (Table 4.5).

3-(3-Hydroxyphenyl)propionic acid, appeared in the faecal samples in varying amounts. This catabolite appeared after 2-4 h of fermentation in the faecal samples of donor A but was not present until 6–24 h in the faecal samples of donor B and C (Table 4.5). It was detected in relatively low quantities with the highest amount, 3.3 μmoles, found in faecal samples from donor C after 48 h fermentation (Table 4.5).

In summary, in the absence of glucose, a high percentage of quercetin was degraded to 3,4-dihydroxyphenylacetic acid in faecal samples from donors A and B and appears to be further catabolised to 3-hydroxyphenylacetic acid by donor B. However in faecal samples from donor C, these catabolites did not accumulate and most of the quercetin was catabolised to 3,4-dihydroxybenzoic acid and subsequently to 4-hydroxybenzoic acid. The rate of breakdown of quercetin and accumulation of 3,4-dihydroxyphenylacetic acid was enhanced in the presence of glucose. But glucose did not have much effect on the levels of other catabolites such as 3,4 dihydroxybenzoic acid, 4-hydroxybenzoic acid and to 3-(3-hydroxyphenyl)propionic acid. Without the addition of the flavonol, quercetin or rutin, none of the catabolites were detected in any of the faecal samples fermented over a 0-48 h period.

Results from section 4.4.2 implies that upon dissolving 55 μmoles of quercetin in the fermentation media, most of it was oxidised or converted to unidentified quercetin adducts. The 60-97% conversion of the substrate to 3,4-dihydroxyphenylacetic acid in the presence of glucose, therefore, strongly suggests that in addition to the residual quercetin remaining in the fermentation media, the oxidised forms of quercetin together with the unidentified quercetin adducts must have also been catabolised by the colonic microflora to produce 3,4-dihydroxyphenylacetic acid and other phenolic acids.

Table 4.5. Accumulation of five phenolic acid catabolites formed 0-48 h following *in vitro* fermentation of faecal samples from three donors with 55 μmoles of quercetin

Donor	Metabolite	Glucose	2 h	4 h	6 h	24 h	30 h	48 h
A	3,4-dihydroxyphenylacetic acid	-	n.d.	2.0 ± 0.0	3.1 ± 0.1	38 ± 1	33 ± 2	41 ± 1
		+	0.9 ± 0.1	7.1 ± 0.3	34 ± 2	43 ± 1	48 ± 1	45 ± 1
	3-hydroxyphenylacetic acid	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d
		+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	3,4-dihydroxybenzoic acid	-	n.d.	n.d.	0.6 ± 0.0	0.7 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
		+	n.d.	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	n.d
	4-hydroxybenzoic acid	-	n.d.	n.d.	n.d.	0.6 ± 0.0	0.4 ± 0.0	0.6 ± 0.0
		+	n.d.	n.d.	1.1 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
	3(3-hydroxy)phenylpropionic acid	-	n.d.	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1± 0.0	0.9 ± 0.1
		+	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.9 ± 0.0
	Total metabolites	-	**n.d.**	**2.1 ± 0.0**	**3.8 ± 0.1**	**39 ± 1**	**34 ± 2**	**43 ± 2**
		+	**1.0 ± 0.0**	**7.6 ± 0.2**	**36 ± 2**	**44 ± 2**	**49 ± 1**	**46 ± 1**
B	3,4-dihydroxyphenylacetic acid	-	0.6 ± 0.0	3.0 ± 0.1	4.6 ± 0.1	32 ± 1	30 ± 2	0.9 ± 0.0
		+	1.1 ± 0.1	18 ± 2	53 ± 0	30 ± 2	32 ± 2	n.d
	3-hydroxyphenylacetic acid	-	n.d.	n.d.	n.d.	0.8 ± 0.2	3.8 ± 0.2	15 ± 0
		+	n.d.	n.d.	n.d.	1.9 ± 0.1	4.5 ± 0.1	15 ± 1
	3,4-dihydroxybenzoic acid	-	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.8 ± 0.1
		+	1.0 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	4-hydroxybenzoic acid	-	n.d.	n.d.	n.d.	0.2 ± 0.0	n.d.	n.d
		+	n.d.	n.d.	0.5 ± 0.0	n.d	n.d.	n.d
	3(3-hydroxy)phenylpropionic acid	-	n.d.	n.d.	n.d.	0.2 ± 0.0	0.4 ± 0.0	0.9 ± 0.0
		+	n.d.	n.d.	0.1 ± 0.0	1.0 ± 0.0	0.4 ± 0.0	n.d
	Total metabolites	-	**0.8 ± 0.0**	**3.4 ± 0.1**	**4.9 ± 0.1**	**34 ± 1**	**35 ± 2**	**18 ± 1**
		+	**2.1 ± 0.2**	**18 ± 2**	**54 ± 2**	**33 ± 2**	**37 ± 2**	**15.2 ± 1**
C	3,4-dihydroxyphenylacetic acid	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		+	n.d.	n.d.	n.d.	32 ± 2	23 ± 1	33 ± 1
	3-hydroxyphenylacetic acid	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d
		+	n.d.	n.d.	n.d.	n.d.	n.d.	9.0 ± 0.3
	3,4-dihydroxybenzoic acid	-	1.7 ± 0.1	2.0 ± 0.0	5.5 ± 0.1	37 ± 0	36 ± 0	37 ± 2
		+	1.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	n.d
	4-hydroxybenzoic acid	-	n.d.	n.d.	n.d.	2.2 ± 0.0	0.6 ± 0.0	0.3 ± 0.0
		+	n.d.	n.d.	n.d.	0.8 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
	3-(3-hydroxy)phenylpropionic acid	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d
		+	n.d.	n.d.	n.d.	0.1 ± 0.0	0.6 ± 0.0	3.3 ± 0.1
	Total metabolites	-	**1.7 ± 0.1**	**2.0 ± 0.0**	**5.5 ± 0.1**	**39 ± 1**	**36 ± 0**	**37 ± 2**
		+	**1.2 ± 0.1**	**0.2 ± 0.0**	**0.2 ± 0.0**	**33 ± 2**	**24 ± 1**	**46 ± 1**

Data for individual donors are expressed as μmoles ± standard error (n=3) ; n.d.- not detected; + : with 500 mg glucose; - : without glucose

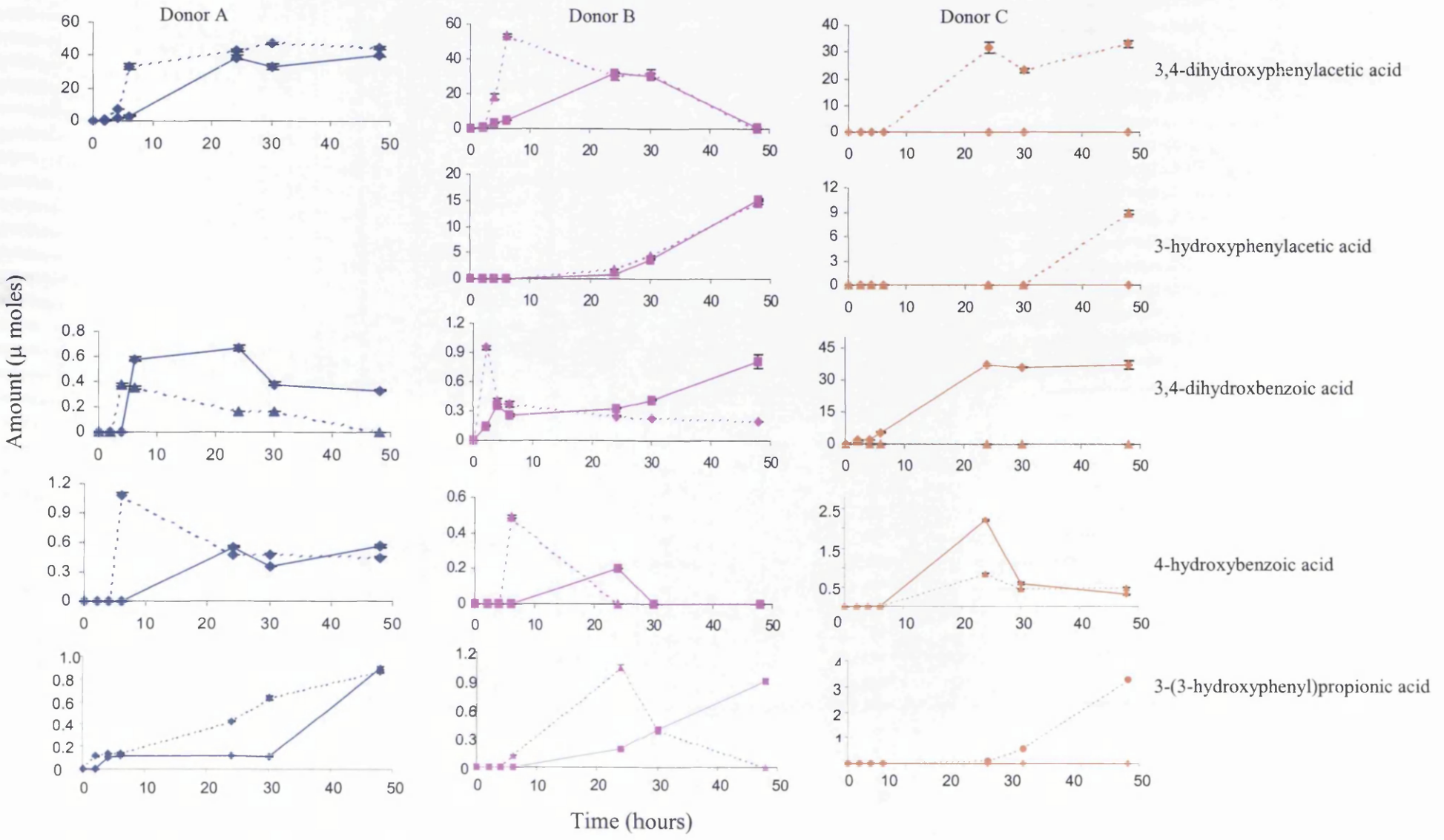


Figure 4.10. Formation of phenolic acid catabolites 0-48 h after fermentation of quercetin in faecal samples from donor A B and C.

donor A (without glucose) donor A (with glucose) donor B (without glucose) donor B (with glucose)
donor C (without glucose) donor C (with glucose)

4.4.4. Recovery of radioactivity after 48 hours of fermentation

The recovery of the radioactivity of the fermentation medium at the different time points was monitored using a scintillation counter. A faster rate of decline of radioactivity was observed in the first six hours of the faecal fermentation (Table 4.6) where about 25% of the radioactivity was lost. This is in keeping with the rapid biodegradation of quercetin occurring within the first few hours of fermentation. Depending on the absence or presence of glucose, a total of 40-44% of total radioactivity was lost after 48 h of fermentation (Table 4.6). The conversion to gaseous fermentation products may account for the loss of radioactivity as suggested by Walle *et al.* (2001) who found that 52% of oral dose of [^{14}C]quercetin was lost as [^{14}C]carbon dioxide in exhaled gases. Since no radioactivity was present in any of the phenolic acids formed as a result of the biodegradation of quercetin, the residual radioactivity detected (56-60%) most probably came from other unidentifiable compounds, illustrated as peak A in Figure 4.11. These small molecular weight compounds were present in all the faecal samples and could not be identified using the HPLC-MS^2 system. They may be the end products of the biodegradation of ring A of the quercetin structure such as the acetates and butyrates as indicated by Griffiths and Smith (1972).

Table 4.6. Recovery of radioactivity in faecal samples after fermentation with 55 μmoles of quercetin (18 x10^6 dpm)

Donors	glucose	% recovery of [^{14}C]					
		2 h	4 h	6 h	24 h	30 h	48 h
1	-	96 ± 0	84 ± 2	73 ± 0	70 ± 3	62 ± 1	56 ± 1
	+	84 ± 0	80 ± 0	68 ± 1	69 ± 0	67 ± 1	58 ± 1
2	-	94 ± 1	84 ±0	71 ± 2	68 ± 1	67 ± 0	64 ± 0
	+	86 ± 2	81 ± 0	76 ± 1	67 ± 1	63 ± 0	46 ± 0
3	-	95 ± 1	85 ± 1	84 ± 2	64 ± 1	65 ± 0	61 ± 0
	+	91 ± 0	85 ± 0	78 ± 0	67 ± 0	68 ± 1	63 ± 0
Mean	-	**95 ± 1**	**84 ± 1**	**76 ± 4**	**67 ± 2**	**65 ± 1**	**60 ± 2**
	+	**87 ± 2**	**82 ± 2**	**74 ± 3**	**68 ± 1**	**66 ± 2**	**56 ± 9**

Data for individual donors are expressed as % [^{14}C] remaining in the fermentation media ± standard error (n = 3).

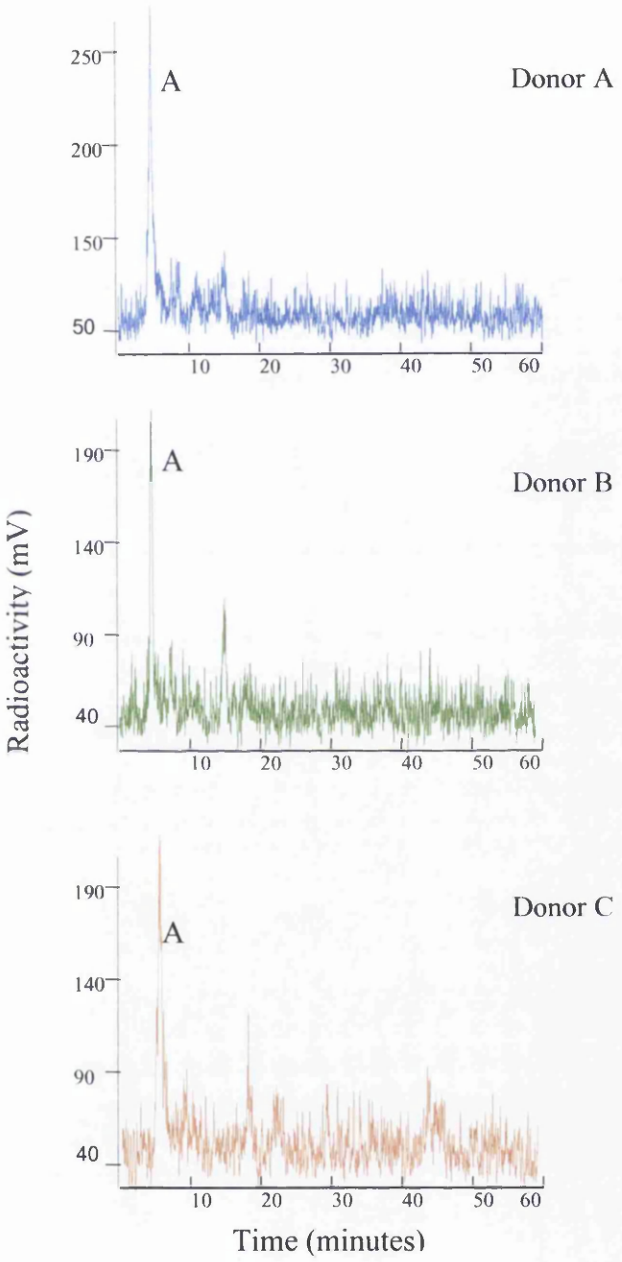


Figure 4.11. Radioactivity (millivolts) of faecal samples from 3 donors 48 h after fermentation with 55 µmoles of quercetin as analysed by LC-MS^2-Radiodetector. Unidentified radiolabelled compound (A) is eluting at 5.6 mins.

4.4.5 Relationship between the colonic catabolites of rutin and antioxidant activity

4.4.5.1 Total antioxidant activity

The antioxidant activity of the 0-48 h fermentations in the absence of substrate (*A*) and with the addition of 55 μmoles quercetin at 0 h (***B***), was measured with the FRAP assay. To obtain the FRAP values of the residual quercetin and its catabolites produced as a result of fermentation, ***B*** was subtracted from ***A*** for the corresponding time points. On addition of quercetin, the FRAP value was 2.2 but this value rapidly declined as the quercetin became oxidised in the fermentation media. The resultant FRAP values ranging from 0–3.3 mM Fe^{2+} measured from 0.15–48 h are illustrated in Table 4.7. The relationship between the concentrations of total colonic metabolite products with the reducing ability in the FRAP-derived antioxidant assay of individual faecal samples from the three donors were analysed using Pearson's correlations. FRAP-derived antioxidant activity was highly and significantly correlated ($P<0.001$) with the total colonic catabolite products produced as a result of *in vitro f*ermentation of quercetin in all the faecal samples except for the one derived from donor C (without glucose) (Table 4.7). This could be due to the absence of 3,4- dihydroxyphenylacetic acid, in faecal samples from donor C incubated without glucose.

Table 4.7. Total colonic metabolite content and antioxidant capacity of fermented faecal samples after incubation with 55 μmoles of quercetin

Donor	Glucose	Time (h)	Total Metabolite[a]	FRAP[b]	Pearson's correlations r	p
A	w/o	2	6.3 ± 0.3	0.5 ± 0.0		
		4	7.9 ± 0.1	0.8 ± 0.1		
		6	7.8 ± 0.1	0.6 ± 0.0		
		24	40 ± 1	2.4 ± 0.2		
		30	34 ± 2	1.0 ± 0.1		
		48	47 ± 2	2.0 ± 0.0	0.885	0.000
	w	0.15	4.6 ± 0.0	0.3 ± 0.0		
		2	2.6 ± 0.0	0.3 ± 0.0		
		4	8.6 ± 0.3	0.8 ± 0.1		
		6	35 ± 2	1.9 ± 0.0		
		24	44 ± 1	3.3 ± 0.1		
		30	49 ± 1	3.2 ± 0.1		
		48	46 ± 1	2.8 ± 0.1	0.979	0.000
B	w/o	2	6.5 ± 0.2	0.2 ± 0.0		
		4	8.9 ± 0.1	0.1 ± 0.0		
		6	9.8 ± 0.2	0.2 ± 0.0		
		24	33 ± 1	2.7 ± 0.1		
		30	35 ± 2	2.5 ± 0.1		
		48	18 ± 0	0.3 ± 0.0	0.936	0.000
	w	0.15	5.5 ± 0.0	0.5 ± 0.1		
		2	3.9 ± 0.0	0.1 ± 0.0		
		4	30 ± 2	1.7 ± 0.1		
		6	54 ± 1	2.1 ± 0.1		
		24	33 ± 2	2.6 ± 0.1		
		30	37 ± 2	2.2 ± 0.1		
		48	15 ± 0	0.0 ± 0.0	0.760	0.000
C	w/o	2	8.0 ± 0.1	0.8 ± 0.0		
		4	4.8 ± 0.0	0.4 ± 0.0		
		6	8.1 ± 0.1	0.1 ± 0.0		
		24	39 ± 0	0.7 ± 0.0		
		30	37 ± 0	0.6± 0.0		
		48	37 ± 2	2.1 ± 0.1	0.552	0.009
	w	0.15	10 ± 0	0.8 ± 0.0		
		2	11 ± 0	0.7 ± 0.0		
		4	3.2 ± 0.0	0.0 ± 0.0		
		6	0.5 ± 0.0	0.0 ± 0.0		
		24	33 ± 2	2.2 ± 0.1		
		30	25 ± 1	1.4 ± 0.0		
		48	46 ± 2	2.3 ± 0.0	0.969	0.000

[a]Amount of residual quercetin plus total metabolites produced at the time intervals specified above, expressed as μmoles; [b]FRAP antioxidant capacity of fermentated faecal samples expressed as mM Fe^{2+}. **On addition of quercetin, the antioxidant capacity of faecal samples at time 0 is 2.2.** All data expressed as mean values ± standard error, where n = 3. w – incubated with glucose; w/o – incubated without glucose

4.4.5.2 Antioxidant activity of individual catabolites

In order to investigate the antioxidant capacity of the individual catabolites, the standards of these compounds at 1 mM were compared with each other and with their parent compounds (rutin and quercetin). Of the compounds analysed, only quercetin, rutin, 3,4-dihydroxyphenyacetic acid, 3,4-dihydoxybenzoic acid and taxifolin had antioxidant activity in the FRAP assay. No reaction was observed with 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, phloroglucinol and 3-(3-hydroxyphenyl)propionic acid. Quercetin and 3,4-dihydroxyphenylacetic exhibited the highest reducing ability in the FRAP antioxidant assay with values ranging from 1.7 - 1.9 mM Fe^{2+} (Table 4.8).

Table 4.8. FRAP values of 1 mM flavonols and phenolic acid standards

Flavonol/phenolic acid standard	FRAP
Rutin	1.0 ± 0.1^{b}
Quercetin	1.9 ± 0.1^{a}
Taxifolin	0.9 ± 0.1^{b}
3,4-Dihydroxyphenylacetic acid	1.8 ± 0.1^{a}
3-Hydroxyphenylacetic acid	0.0
3,4-Dihydroxybenzoic acid	1.0 ± 0.1^{b}
4-Hydroxybenzoic acid	0.0
3-(3-Hydroxyphenyl)propionic acid	0.0
Phloroglucinol	0.0

FRAP values are expressed as mM Fe^{2+}. Data expressed as mean values ± standard error, where n = 3. Means followed by different superscript letters are significantly different at $p < 0.01$.

4.4.5.3 Contribution of individual catabolites to the total antioxidant capacity

The concentrations of each of the catabolites, 3,4- dihydroxyphenylacetic acid, taxifolin and 3,4-dihydroxybenzoic acid, during the *in vitro* fermentation at various time intervals was used to calculate FRAP values from the standard calibration curves of each individual compound. From this, the contribution of each of these catabolites to the total antioxidant capacity of the faecal samples was then determined.

Taxifolin

Taxifolin did not contribute significantly to the total antioxidant capacity of the faecal samples. This is due to the fact that very low amounts of antioxidant activity was observed in most of the faecal samples except for that from donor B (with glucose) at the 4 h time point which gave a FRAP value of 0.2. This value is equivalent to a total of 11 μmoles (5.6 + 4.7 + 0.1 μmoles of taxifolin, alphitonin and isomer of taxifolin respectively) as revealed in Table 4.4 (Donor B, 4 h + glucose).

3,4-Dihydroxybenzoic acid

3,4-Dihydroxybenzoic acid did not contribute to the total antioxidant capacity of the faecal samples of donor A and B, due to low level accumulation (0.15–0.95 μmoles) in these samples. In donor C, only those samples incubated without glucose accumulated higher amounts of 3,4-dihydroxybenzoic acid (up to 37 μmoles) and produced FRAP values of up to 0.7 which in turn was responsible for most of the antioxidant activity observed in the faecal sample (Table 4.9).

3,4-Dihydroxyphenylacetic acid

3,4-Dihydroxyphenylacetic acid is the main metabolite which plays a major role in the contribution of total antioxidant activity in the faecal samples (Table 4.9). This is evident in all the faecal samples except for faecal samples from donor C (without glucose). The high antioxidant activity observed in the faecal samples is attributed to the large amounts 3,4-dihydroxyphenylacetic acid present. In faecal samples from donor A, high concentrations of 3,4-dihydroxyphenylacetic acid amounting to 38-48 μmoles corresponded to high FRAP value of 2.4-3.0 and this in turn was responsible for most of the antioxidant activity observed in the faecal sample (Table 4.9) Similarly in donor B, a high FRAP value of 2.5 corresponded to 53 μmoles of 3,4-dihydroxyphenylacetic acid which again contributed to most of the antioxidant capacity exhibited in the faecal sample (Table 4.9).

Table 4.9. FRAP values of 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid and their contribution to the total antioxidant activity of faecal samples after fermentation with 55 μmoles of quercetin

Donor	Metabolite	Variables	Glucose	2 h	4 h	6 h	24 h	30 h	48 h
C	3,4-diOHBA	Catabolite content	w/o	1.7 ± 0.1	2.0 ± 0.0	5.5 ± 0.1	37 ± 0	36 ± 0	37 ± 2
		Theoretical FRAP	w/o	0.0	0.0	0.1 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
		Actual Total FRAP	w/o	0.8 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	2.1 ± 0.0
		% contribution to total FRAP	w/o	0.0	0.0	**100**	**100**	**100**	31
A	3,4-diOHPAA	Catabolite content	w/o	n.d	2.0 ± 0.0	3.1 ± 0.1	38 ± 1	33 ± 2	41 ± 1
		Theoretical FRAP	w/o	0.0	0.1 ± 0.0	0.1 ± 0.0	2.4 ± 0.0	1.2 ± 0.1	1.9 ± 0.1
		Actual Total FRAP	w/o	0.5 ± 0.0	0.8 ± 0.1	0.6 ± 0.0	2.4 ± 0.2	1.0 ± 0.1	2.0 ± 0.0
		% contribution to total FRAP	w/o	0.0	13	17	**100**	**120**	**95**
		Catabolite content	w	0.9 ± 0.1	7.1 ± 0.3	34 ± 2	43 ± 1	48 ± 1	45 ± 1
		Theoretical FRAP	w	0.0	0.2 ± 0.0	1.4 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	2.6 ± 0.0
		Actual Total FRAP	w	0.3 ± 0.0	0.8 ± 0.1	1.9 ± 0.0	3.3 ± 0.2	3.2 ± 0.1	2.8 ± 0.1
		% contribution to total FRAP	w	0.0	25	74	**85**	**94**	**93**
B	3,4-diOHPAA	Catabolite content	w/o	0.6 ± 0.0	3.0 ± 0.1	4.6 ± 0.1	32 ± 1	30 ± 2	0.9 ± 0.0
		Theoretical FRAP	w/o	0.0	0.1 ± 0.0	0.2 ± 0.0	1.8 ± 0.1	2.0 ± 0.1	0.0
		Actual Total FRAP	w/o	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	2.7 ± 0.2	2.5 ± 0.1	0.3 ± 0.0
		% contribution to total FRAP	w/o	0.0	**100**	**100**	**67**	**80**	0.0
		Catabolite content	w	1.1 ± 0.1	18 ± 2	53 ± 0	30 ± 2	32 ± 2	n.d.
		Theoretical FRAP	w	0.0	0.6 ± 0.0	2.2 ± 0.1	2.5 ± 0.1	1.3 ± 0.1	0.0
		Actual Total FRAP	w	0.1 ± 0.0	1.7 ± 0.1	2.1 ± 0.0	2.6 ± 0.2	2.2 ± 0.1	n.d.
		% contribution to total FRAP	w	0.0	25	**105**	**96**	**59**	0.0
C	3,4-diOHPAA	Catabolite content	w	n.d.	n.d.	n.d.	32 ± 2	23 ± 1	33 ± 1
		Theoretical FRAP	w	0.0	0.0	0.0	1.5 ± 0.1	0.8 ± 0.1	1.2 ± 0.0
		Actual Total FRAP	w	0.7 ± 0.0	n.d.	n.d.	2.2 ± 0.1	1.4 ± 0.0	2.3 ± 0.0
		% contribution to total FRAP	w	0.0	0.0	0.0	**68**	**57**	52

The catabolite content expressed as μmoles was used to calculate the FRAP values from standard calibration curves to obtain theoretical FRAP values. Actual total FRAP is the antioxidant capacity of fermentated faecal samples and is expressed as mM Fe^{2+}. All data expressed as mean values ± standard error, where n = 3. w – incubated with glucose; w/o – incubated without glucose; 3,4-diOHBA - 3,4-dihydroxybenzoic acid and 3,4-diOHPAA – 3,4-dihydroxyphenylacetic acid

4.4.5.4 Relationship between antioxidant activity and the content of 3,4-dihydroxyphenylacetic acid in the faecal samples

Since 3,4-dihydroxyphenylacetic is the main contributor to the total antioxidant activity of the faecal samples, the relationship between concentration of this metabolite with the reducing ability in the FRAP-derived antioxidant assay of individual faecal samples from the three donors were analysed using Pearson's correlations. FRAP-derived antioxidant activity was highly significantly correlated with the concentration of 3,4-dihydroxyphenylacetic produced as a result of *in vitro* fermentation of quercetin (Table 4.10). This is also illustrated graphically in Figure 4.12.

Table 4.10. Correlation of 3,4-dihydroxyphenylacetic acid content with its antioxidant capacity of individual faecal samples from the three donors.

Donor	Glucose	Pearson's correlations	
		r	p
1	-	0.814	0.000
	+	0.975	0.000
2	-	0.974	0.000
	+	0.872	0.000
3	+	0.868	0.002

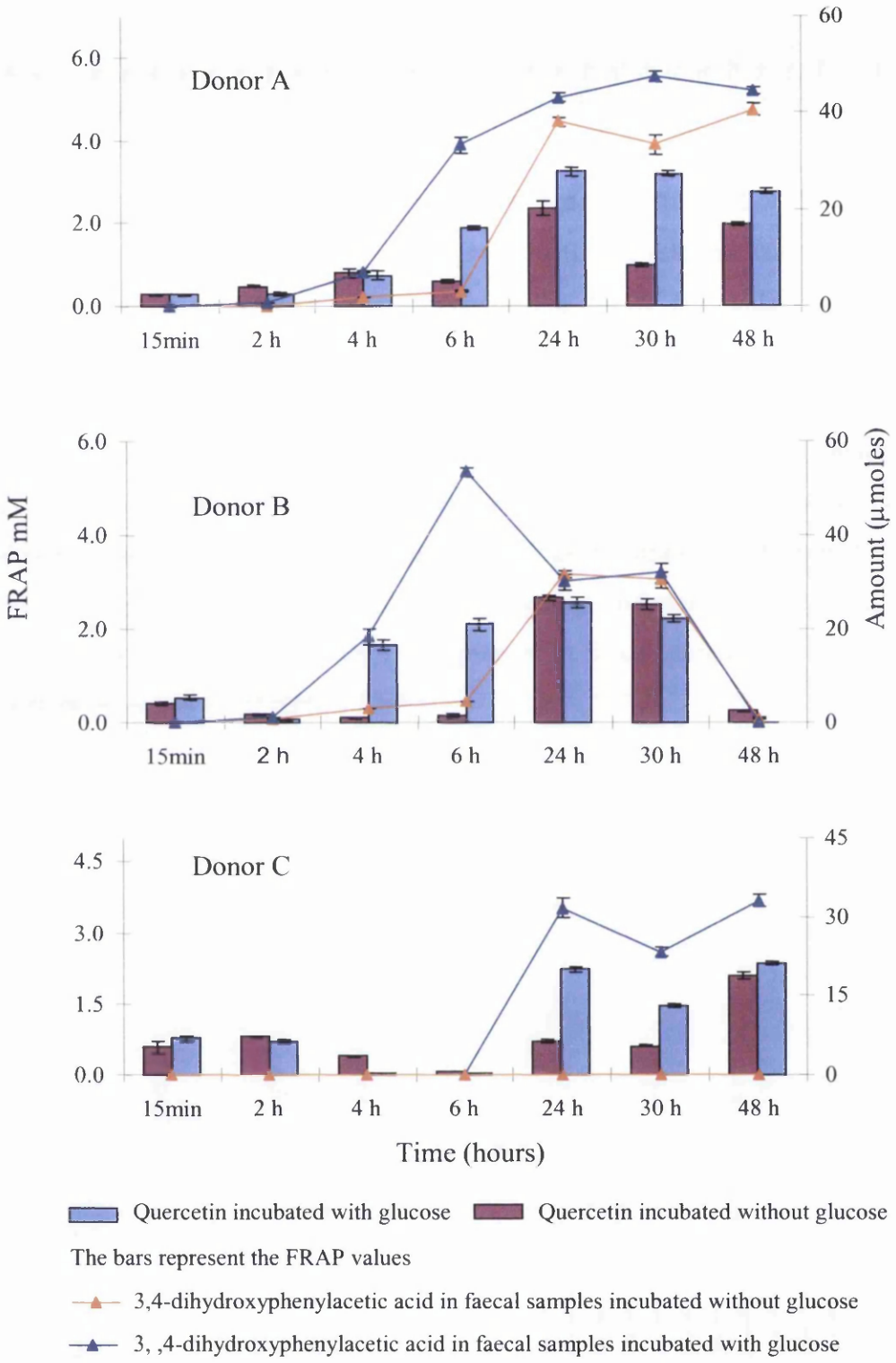


Figure 4.12. Relationship between antioxidant activity (expressed as FRAP in mM Fe^{2+}) and 3,4-dihydroxyphenylacetic acid (expressed as µmoles) at specific time points after *in vitro* fermentation of faecal samples obtained from three donors with 55 µmoles of quercetin. All data expressed as mean values ± standard error, where n = 3.

4.4.5.5 Illustration of the contribution of individual catabolites to the antioxidant capacity of the faecal sample using the $ABTS^{+}$ on-line decolorisation assay.

To illustrate and confirm the contribution of the antioxidant capacity of individual catabolites in the faecal samples, 20 μL of selected faecal samples were analysed by gradient HPLC system with the on-line $ABTS^{+}$ antioxidant detector system. The major antioxidants in most of the samples were associated with the presence of 3,4-dihydroxyphenyacetic acid. However, in the faecal sample of donor B (with glucose), 4 h after fermentation, taxifolin and its isomers were observed to reach its maximum level (11 μmoles) and the antioxidant capacity of this compound together with that of 3,4-dihydroxyphenyacetic acid (18.5 μmoles) is as illustrated in Figure 4.13-B. The antioxidant peaks observed in the ABTS chromatogram before 10 min corresponded to unidentified compounds that were also present in faecal samples when no substrates (quercetin) was added (Figure 4.13-A).

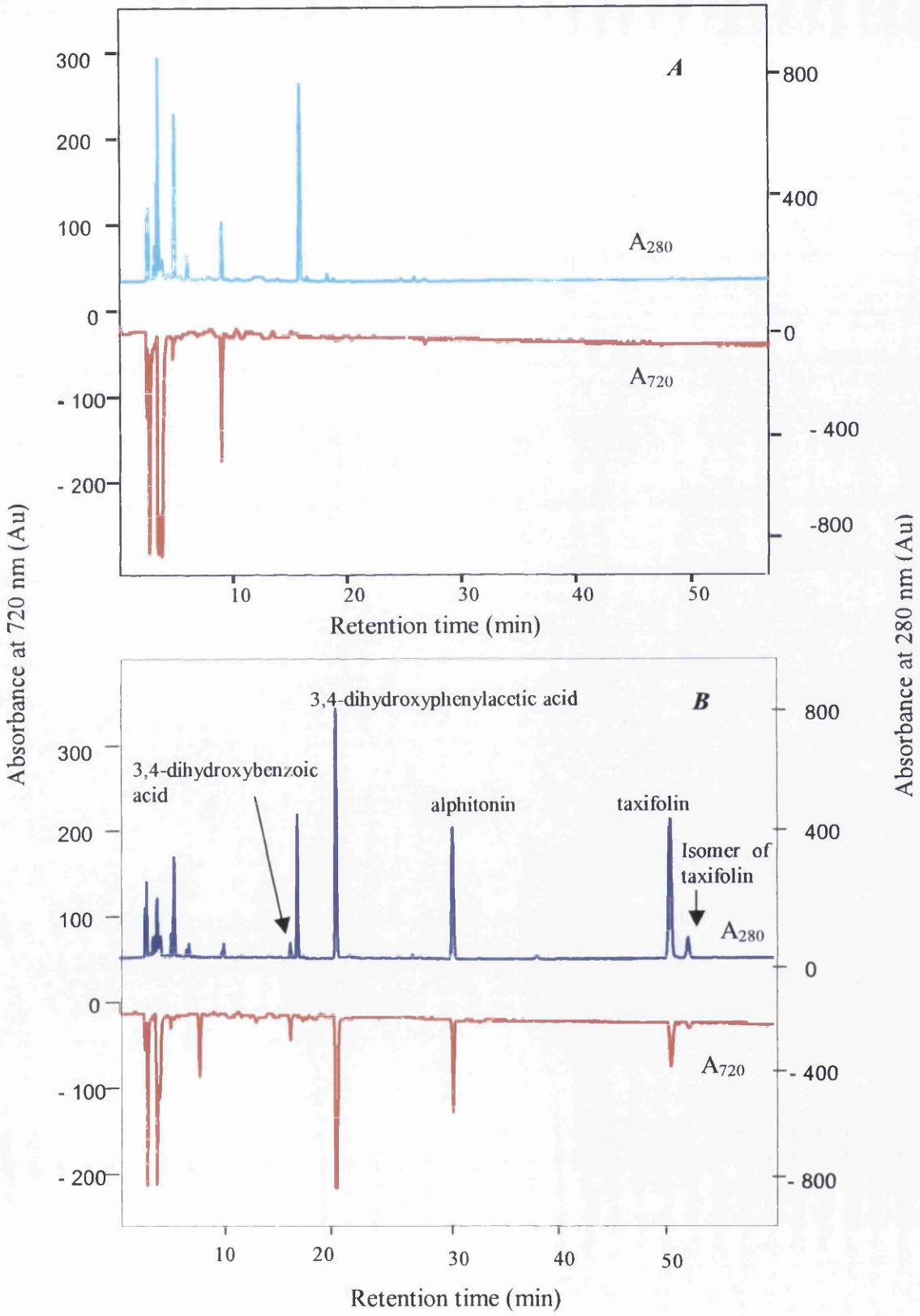


Figure 4.13 . On-line $ABTS^{+}$ analysis faecal samples from donor B, 4 h after *in vitro* fermentation with 55 µmoles quercetin (***B***) and with no substrate (***A***), showing UV absorbance at 280 nm and at 720 nm ($ABTS^{+}$ assay).

4.5 Discussion

4.5.1 Methods, material and model used

To assess the colonic metabolism of rutin, an *in vitro* fermentation model was adopted. This method involves faecal incubation with a substrate, aiming to simulate events occurring in the human colon. *In vitro* fermentation has been used by other researchers to study colonic catabolism of flavonoids (Aura *et al.*, 2002, 2005; Rechner *et al.*, 2004, and Justesen *et al.*, 2000) but in most cases, the experimental approach used varied with respect to the inoculum size, type of media, anaerobic conditions, choice of buffer and mixing rate. All these factors can affect the bacterial ecotypes, which in turn affects the catabolism of the flavonoid (Edwards *et al.*, 1996). In this study, a slight modification of the method derived by Edwards *et al.* (1996) was adopted. The mixing rate chosen was 60 strokes min^{-1} which was considered to be gentle and not vigorous and was important to disperse the rutin/quercetin substrate throughout the fermentation media. In contrast with the other researchers who used 100-167 gL^{-1} of faecal inocula (Aura *et al.*, 2002; Rechner *et al.*, 2004), in this study a relatively large amount of inoculum, 320 gL^{-1}, was used to provide a large initial bacterial population which ensured a better survival rate of the bacterial species (Edward *et al.*, 1996). In addition to this, the effect of glucose as a carbon source on flavonoid degradation was also investigated.

The main limitations of the *in vitro* method is that it may not fully depict the *in vivo* conditions. The removal of faecal material may alter the bacterial composition and thus may not represent the actual colonic microflora, which in turn may not depict the catabolism process that is occurring in the colon. The accumulation and retention of the degradation products in the fermentation vessel makes collection, identification and quantification of the metabolites easier but is not representative of the events that occur *in vivo*. The actual concentration of a metabolite at any time interval is dependent on the combined rates of catabolism and absorption and this cannot be simulated *in vitro*. However, through this *in vitro* model, the elucidation of the pathway of the degradation process, types of breakdown products, rate of catabolism can be determined in a quick, reliable and an affordable manner (Edwards *et al.*, 1996).

The most commonly applied ionization methods for the LC-MS are ESI and APCI. In this study, the ESI method was adopted for the detection of the flavonols, rutin

and quercetin while the APCI method was used for the detection of all the phenolic acids and taxifolin. ESI lacks sensitivity for the detection of phenolic acids as they are low molecular weight compounds and are not readily ionized by the ESI interface. The APCI interface, on the other hand has the ability to evaporate the samples by using temperatures up to 600°C after which they are ionised with a corona discharge. To optimize the detection of phenolic acids, the level of acid modifier in the mobile phase had to be reduced, as this suppresses ionization. However, at this level, the flavonols do not chromatograph, therefore two systems were used.

The results in this study were illustrated separately for each of the three faecal donors as they exhibited differences in both degree of catabolism and the resultant catabolite profile. Presumably this reflects variations in the gut microflora of the three faecal donors.

4.5.2 Deglycoslyation of rutin

In the human colonic microflora, the presence of bacterial enzymes such as α-rhamnosidases and β-glucosidase, result in hydrolysis of rutin to release the rutinoside moiety from quercetin (Aura *et al.,* 2005). α-Rhamnosidases split off the rhamnose group from the rutinosides leaving behind quercetin-3-glucoside which is then acted upon by β-glucosidase which cleaves off the glucoside moiety. Alternatively, the rutinose sugar moiety may undergo a one step cleavage releasing quercetin.

In this study, although there was significant individual differences in the deglycoslation pattern of rutin, quercetin was detected in all the faecal samples. In contrast, similar studies carried out by other researchers have not always detected quercetin as indicated by Justeen *et al.* (2000). In other studies, the flavonol aglycone has appeared only transiently in the fermentation process (Aura *et al.,* 2002). At the other extreme, more substantial quantities of quercetin were detected by Rechner *et al.* (2004). The most plausible explanation for these discrepancies is that they are due to the use of different concentrations of the rutin substrate (Table 4.11). When lower doses of rutin are incubated with faecal preparations, quercetin tends not to accumulate. Highest levels of quercetin accumulation appear to be associated with the use of high doses of rutin. This indicates that deglycoslyation of rutin may occur more rapidly than catabolism of quercetin. This did not occur in the present study when glucose was added to the fermentation media. Although a high concentration of rutin was used in the

present study, the addition of glucose enhanced not only the rate of deglycoslyation of rutin but also hastened the rate of quercetin catabolism such that quercetin only appeared transiently in the faecal samples. Without the addition of glucose, the degradation process appeared to be much slower and quercetin was found to accumulate in higher amounts in the case of donors B and C (Figure 4.5).

The addition of glucose in the fermentation media probably altered the microflora colony, encouraging the growth and the multiplication of bacterial strains that are responsible for both the deglycoslyation of rutin and the catabolism of the resultant quercetin. Other than having a direct effect via stimulation of the fermentation process, glucose could have also caused an alternation in the pH of the fermentation media, which in turn could have an effect on the ecosystem of the colonic microflora. The addition of glucose in the media was considered important in the present study as it was carried out to mimic *in vivo* conditions. The addition of glucose *in vitro* translates to dietary fibre intake *in vivo* where fermentation is stimulated by non-digested carbohydrate.

The degradation profile of rutin in faecal samples obtained from donor C was distinctly different from the other two donors; about 48% of the rutin still remained in the fermentation vessel 48 h after the onset of fermentation. A possible explanation could be that the bacteria responsible for the production of α-rhamnosidase were initially present at a low concentration which resulted in insufficient enzyme activity to completely degrade all the rutin added into the medium. Addition of glucose to the medium enhanced bacterial growth and seemed to overcome this effect as rutin disappeared more rapidly (Figure 4.5).

4.5.3 Radioactivity

The use of radioactive quercetin is advantageous in the identification the breakdown products and is particularly useful in the *in vitro* conditions as used in the current study. However, the correct positioning of the radiolabelled compound can be crucial in trying to optimize the identification of breakdown products. In this study, the carbon was radiolabelled at the C4 position and the only catabolites that retained radioactivity were the oxidised forms of quercetin and taxifolin. The rest of the breakdown products, which either originated from ring A or C of the catechol structure lost their radioactivity due to the pattern of ring cleavage by the colonic microflora

(Figure 4.14). Although the C4 position was not an ideal position for radiolabelling, position 2 would be better according to the ring cleavage pattern, but it did reveal the following:

- Instability of quercetin in the fermentation media – quercetin was found to be oxidised into various forms which the ^{14}C-label helped detected and facilitate MS^2 identification.
- The pattern of ring fission as suggested by earlier investigations can be confirmed, as explained below.

According to Justesen and Arrigoni (2001) and Rechner *et al.*, 2004, the possible cleavage positions are

- between C2-ring oxygen and C3-C4 (blue dotted)
- between C2-ring oxygen and the C4 (red dotted)

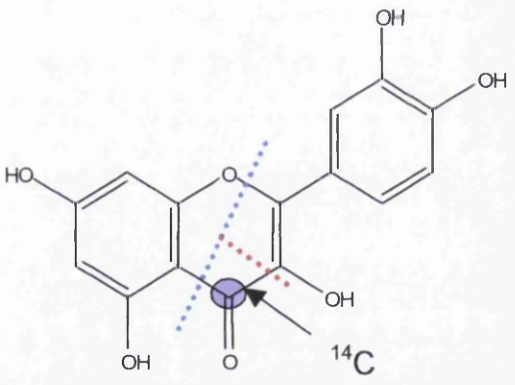


Figure 4.14 [4-^{14}C]quercetin and possible types of ring fission by colonic catabolism. Adapted from Justesen *et al* 2001

If the carbon is labelled at the C4 position, during the degradation process, both types ring fission will result in the loss of radioactivity as observed in this study.

4.5.4 Intermediate products of the degradation of quercetin

When [^{14}C]quercetin was used as a substrate, the main intermediate radiolabelled product detected was taxifolin (Figure 4.8). They appeared in highest amounts in faecal sample of donor B when incubated with glucose but were observed only transiently in all the other faecal samples (Table 4.4, Figure 4.9). Phloroglucinol,

another intermediate was only detected in trace amounts in faecal samples of donor B and C, suggesting that both these intermediates were either produced by minor pathways or catabolised to other compounds very rapidly and therefore could not be detected within the 2 hour interval of sampling. Similar results have been obtained in other studies where phloroglucinol was detected only transiently in *in vitro* fermented human faecal samples (Schneider *et al.*, 1999) and also in pig caecum (Labib *et al.*, 2004). Griffiths and Smith (1972) could not detect the presence of this metabolite in faecal samples and proposed that it may be rapidly metabolized to acetate and butyrate, which may correspond to the unidentified radioactive compounds detected in the current study (Figure 4.11). The low levels of taxifolin and phloroglucinol in *in vitro* fermented human faecal samples seemingly prevented their detection by Aura *et al.* (2002); Rechner *et al.* (2004); Justesen *et al.* (2000) and Justesen & Arragoni (2001).

In the present study, three isomers of taxifolin were detected, all of which fragmented when analysed by HPLC-MS^2, yielding identical MS^2 spectra (Table 4.3). The first isomer, which had a retention time of 28.2 min is most probably alphitonin as suggested by Schoefer *et al.* (2003) and Braune *et al.* (2001) while the second isomer was identified as taxifolin. The third isomer, which has a slightly longer retention time could not be positively identified. Only taxifolin and the third isomer retained ^{14}C-label while no radioactivity was detected with alphitonin. Alphitonin could have lost its ^{14}C at the 4 position during the ring opening and recyclization involved in its formation. Experiments were also carried out by other researchers to show that taxifolin is the initial product of quercetin degradation. In separate experiments, taxifolin was shown to be transformed to 3,4-dihydroxyphenylacetic acid by the human faecal bacteria *Clostridium orbiscindens* (Schoefer *et al.*, 2003) and *Eubacterium ramulus* (Braune *et al.*, 2001). In addition, alphitonin was also identified as an intermediate of the conversion of taxifolin to 3,4-dihydroxyphenylacetic acid (Braune *et al.*, 2001).

4.5.5 Ring fission products

Five ring fission catabolites were identified in this study, namely; 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid and 3-(3-hydroxyphenyl)propionic acid. The composition of the phenolic acids present in the faecal samples of the three donors were found to be dependent on two main factors; the individual microflora profiles and the presence of glucose.

The ring fission products obtained as a result of fermentation of quercetin were found to differ considerably in the faecal samples of donor C compared to those of the other two donors. This is probably due to differences in the profiles of the microflora in the individual faecal samples. In the samples of donor A & B, most of the quercetin (>60%) was converted to 3,4-dihydroxyphenyacetic acid which was subsequently degraded into 3-hydroxyphenylacetic acid. Only small amounts of 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid and 3-(3-hydroxyphenyl)propionic acid was detected here. However, in the faecal samples of donor C, the main ring fission product was 3,4-dihydroxybenzoic acid which was further degraded to 4-hydroxybenzoic acid. 3,4-Dihydroxyphenyacetic acid and its the subsequent degradation product, 3-hydroxyphenylacetic acid, were not detected. However, with the addition of glucose, the profile of the breakdown products were altered as both 3,4-dihydroxyphenyacetic acid and 3-hydroxyphenylacetic acid accumulated in the faecal samples of donor C. Glucose could have altered the composition of the microbial flora where it enhanced the growth and the multiplication of the bacterial strains responsible for the degradation of quercetin to 3,4-dihydroxyphenyacetic acid.

All other *in vitro* fermentation studies with human faecal samples were able to detect the major ring fission products identified in the current study but in most of the cases the percentage conversion of rutin to its catabolites after degradation was very low as indicated in Table 4.11. It was postulated that the remainder of the metabolite was either converted to other metabolites, which were below the limit of detection or were bound to faecal material and were thus not extractable by solvents (Aura *et al.*, 2002), converted to gaseous fermentation products (Walle *et al.*, 2001) or converted to smaller molecular weight compounds such as butyrate and acetate (Schnieder *et al.*, 1999). The results of the current study give a more detailed picture of the mechanism of degradation of rutin/quercetin by colonic microflora. The loss of about 40 % of the radioactivity implies that the cleavage of the C ring could have resulted in the formation of the C-ring derived [^{14}C] carbon dioxide while the 60 % retention of radioactivity could be due to the formation of low molecular weight compounds such as butyrate and acetate. Phloroglucinol, a phenolic fragment of the A-ring of quercetin was detected only transiently. B-ring cleavage results in the production of phenolic acids and in the present study, the main product to accumulate was 3,4-dihydroxyphenyacetic acid. In the presence of glucose there was 60-97% conversion of quercetin to 3,4-

dihydroxyphenyacetic in the faecal samples. This is in agreement with the report of Braune *et al.*(2001), where the human faecal bacteria *Eubacterium ramulus,* brought about an 80% conversion of quercetin to 3,4-dihydroxyphenyacetic acid. In the current study, in addition to 3,4-dihydroxyphenyacetic acid, significant amounts of 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid was also detected especially from the faecal samples of donor C suggesting that these compounds can also be important biodegradation products of the B-ring of quercetin based compounds.

Table 4.11. Metabolites recovered after *in vitro* fermentation of quercetin and its glycosides with colonic bacteria

Reference	Substrate	Catabolites recovered	% conversion	Other products
Aura *et al.* (2002)	human faecal fermentation with 100 μM/L rutin	3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid 3-(3-hydroxyphenyl)propionic acid	60 %	-
Rechner *et al.* (2004)	human faecal fermentation with 573 μM/L rutin	3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid 3-(3-hydroxyphenyl)propionic acid	About 35 %	-
Schneider *et al.* (1999)	5 mM/L quercetin-3-glucoside in growing culture of *Eubacterium ramulus*	3,4-dihydroxyphenylacetic acid	34 %	phloroglucinol, acetate, butyrate
Schoefer *et al.* (2003)	0.5 mM/L quercetin in growing culture of *Clostridium orbiscindens*	3,4-dihydroxyphenylacetic acid	Not indicated	taxifolin, alphitonin
Braune *et al.* (2001)	1mM Quercetin in growing cultures of *Eubacterium ramulus*	3,4-dihydroxyphenylacetic acid, 3-(3-hydroxyphenyl)propionic acid	80 %	taxifolin, alphitonin
Justesen *et al.* (2000)	human faecal fermentation with 20 mM /L rutin	3,4-dihydroxytoluene 3,4-dihydroxybenzaldehyde 3 or 4-hydroxyphenylacetic acid 3 or 4-hydroxyphenylpropionic acid 3,4-dihydroxyphenylacetic acid	Not indicated	phloroglucinol
Justesen and Arrigoni (2001)	human faecal fermentation with 20 mM /L rutin, quercetin, quercetin-3 glucoside	3,4-dihydroxybenzoic acid 3,4-dihydroxyphenylacetic acid 3,4-dihydroxybenzaldehyde 2,4,6-trihydroxybenzoic acid 2-(3,4-dihydroxyphenyl)ethanol 4-hydroxyphenylpropionic acid 3,4-dihydroxytoluol	Not indicated	-

4.5.6. Antioxidant activity

Catabolites of rutin/quercetin were analysed for antioxidant activity using the FRAP assay. Quercetin and 3,4-dihydroxyphenylacetic acid exhibited the highest antioxidant activity followed by rutin, 3,4-dihydroxybenzoic acid and taxifolin. No antioxidant activity was observed for phloroglucinol, 3-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl)propionic acid. This indicates that the antioxidant activity of these compounds is correlated to their structure, specifically the presence of the 3', 4'–dihydroxy structure in a catechol group. Compounds without a 3'-hydroxyl group such as phloroglucinol, 3-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl)propionic acid, have considerably reduced scavenging capacity. Flavonoids with both a 3-hydroxyl group and a 3',4'–dihydroxy structure are reported to be 10-fold more potent that ebselen (Figure 4.15), a known reactive nitrogen species (RNS) scavenger, against peroxynitrite (Heim *et al.*, 2002). In addition to the 3-hydroxy and 3',4'–dihydroxy structure, the 2,3 double bond in conjugation with a 4-oxo function, and the presence of both 5- and 7-hydroxyl groups also contribute significantly to the antioxidant capacity of the molecule (Russo *et al.*, 2000).

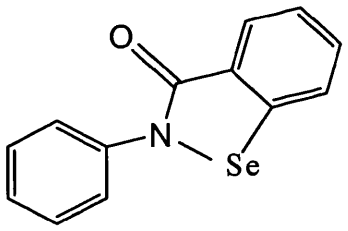


Figure 4.15. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)) is a seleno-organic compound with high radical scavenging capacity (Heim *et al.*, 2002).

Quercetin added at 55 μmoles to the 50 mL (1 mM/L) faecal slurry gave a FRAP value of 2.2 mM Fe^{2+} but within just 15 min, the FRAP reading was reduced to an average of 0.5 mM Fe^{2+} due to the fact that quercetin was rapidly oxidised into quinine-type structures which are pro-oxidants. This is in accordance with the report of Babbs (1990) who demonstrated that dilution of faeces could produce radicals, as a result of bacterial oxidative metabolism and the catalytic action of available Fe^{3+}. However, this was soon counteracted by the production of phenolic acids. With the degradation of quercetin and the production of colonic metabolites, the FRAP value

increased to about 3.3 mM Fe^{2+}, suggesting that the colonic catabolites produced had significant antioxidant power (Table 4.7). The total colonic metabolites formed were found to be highly significantly correlated with the total antioxidant capacity of the fermented faecal samples with exception to that derived from donor C in the absence of glucose. 3,4-Dihydroxyphenylacetic acid, the major catabolite formed was the main contributor to the total antioxidant activity of the faecal samples with its levels being strongly correlated with the total antioxidant activity of the faecal samples. The only exception to this was faecal samples derived from donor C where the absence of the 3,4-dihydroxyphenylacetic acid accounted for low FRAP values during the first 24 h of the fermentation process after which a higher FRAP reading corresponded to the appearance of 3,4-dihydroxybenzoic acid (Table 4.9).

The intestine is unique among all fully differentiated organs as it sits at the interface between the organism and its luminal environment, it represents a critical defense barrier against luminal toxic agents. Thus, in addition to being exposed to luminal nutrients, the intestinal mucosa is constantly challenged by diet-derived oxidants, mutagens, and carcinogens as well as by endogenously generated ROS (Ames, 1983). In addition to this, the intestinal epithelial cells are amongst the most highly proliferative tissues of the human body and there is a continuous need to preserve cellular integrity and tissue homeostasis through maintaining a reduced redox state. An oxidative shift in this potential can elicit growth arrest and cell transition to a differentiated or apoptotic phenotype. Tissue oxidative stress, which results from a disturbance in the cellular prooxidant-antioxidant balance in favour of prooxidants, is an important contributor to the genesis of gut pathologies such as inflammation and cancer (Aw, 2003). A change in the redox status of mucosal glutathione has been shown to be associated with inflammation and disease activity in ulcerative colitis in humans (Holmes *et al.*, 1998). In a more recent study, the differences in the redox potential in the colon and the small intestine was used to explain the variation in cancer occurrences at these two sites (Sanders *et al.*, 2004). Basal reactive oxygen species and antioxidant enzymes activity in the colon were found to be higher than the small intestine, which in turn was suggested to create an environment for more extensive oxidative damage. In addition to this Sanders *et al*, (2004) also suggested that the colon responds to oxidative stress less effectively than the small intestine and, thus, is more prone to cancer occurrences. This may relate to the fact that the lumen of the colon is normally highly

reducing and anaerobic (-400mV) and that oxygen may be present only at the mucosal surface. The facultative bacteria mop up available oxygen and antioxidants molecules may have an important role in maintaining a healthy gut microflora and mucosa.

The feeding studies outlined in Chapter 3 indicate that rutin is not absorbed extensively in the small intestine and that sizable amounts pass into the colon. Rutin and its colonic catabolites, 3,4–dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid may become concentrated in the colorectal lumen and, as such, would be well placed to interact with the mucosal cells of the distal gut. These compounds, therefore, may exert their antioxidant capacity to scavenge free radical cations in the colonic lumen thereby assisting in the maintainence of a reduced redox state especially in the mucosal cells. Other researchers such as Garsetti *et al.*, (2000), have shown that antioxidant levels in faeces can be much greater than in plasma (faeces 26.6 ± 10.5 (SD) and plasma 1.46 ± 0.14 mM Trolox). In addition, the total amount of antioxidants excreted over 24 h was found to be significantly and positively correlated to the consumption of beverages rich in phenols (Garsetti *et al.*, 2000). In addition to the antioxidant activity, the presence of rutin and quercetin in the gut suggests these compounds may also exert other protective effects. Both quercetin and rutin has also been suggested to be a potent inhibitors of inflammatory enzymes such as epidermal cyclooxygenase-2 and lipo-oxygenase (Lipkin *et al.*, 1999; Kwan *et al.*, 2005). In addition, quercetin has a broad range of other activities as it can inhibit Na+K+-ATPase, and various tyrosine kinases, all of which are elevated during carcinogen-induced colon neoplasias (Lipkin *et al.*, 1999).

4.5.7 Proposed scheme of the colonic degradation of rutin and conclusion

As schematized in Figure 4.16, rutin is first deglycoslyated to produce quercetin by the β-glycosidase activity of the gut microflora, releasing quercetin for subsequent degradation (Aura *et al.*, 2002 and Rechner *et al.*, 2004). Quercetin being unstable in the faecal slurry, is oxidised to produce quinones and subsequently get attached to water molecules (Dangles *et al.*, 1999). These 'oxidised' quercetin molecules act as the substrates for gut microflora which cleave the flavonoid structure at the C ring as shown in Figure 4.14, resulting in the production of 3,4-dihydroxybenzoic acid and 3,4-dihydroxyphenylacetic acid from the B ring. In an alternative route, quercetin may undergo reduction of the double bond in the 2-3-position yielding taxifolin. This is most probably followed by a ring opening recyclization mechanism, forming an isomer of taxifolin, and a subsequent ring contraction to produce alphitonin

(Braune *et al.*, 2001). Taxifolin, and their isomers are then transformed to either of 3,4-dihydroxyphenylacetic acid or/and 3 (3-hydroxyphenyl)propionoc acid via fissions of the C-ring. Further dehydroxylation of 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid by dehydroxylases produced by the colonic microflora result in the formation of 3-hydroxyphenylacetic acid and 4-dihydroxybenzoic acid respectively. Phloroglucinol, on the other hand is derived from ring A of the flavonol structure.

The results presented in this study also indicate that modification of the structural properties of dietary polyphenols, rutin and quercetin by the colonic microflora can have a substantial effect on their subsequent biological activity *in vivo*. The structural modifications derived from the C-ring cleavage may either increase or decrease the cation radical scavenging capacity depending on the retention of 3',4'-hydroxyl groups. Of the catabolites formed, only 3,4-dihydroxyphenylacetic acid and 3,4 dihydroxybenzoic acid retained this catechol structure and thus exhibit antioxidant activities. 3,4-Dihydroxyphenylacetic acid which accumulated in large amounts in the faecal samples was found to possess significant antioxidant activity and it may play a crucial role in the overall contribution of the antioxidant capacity of the colonic lumen.

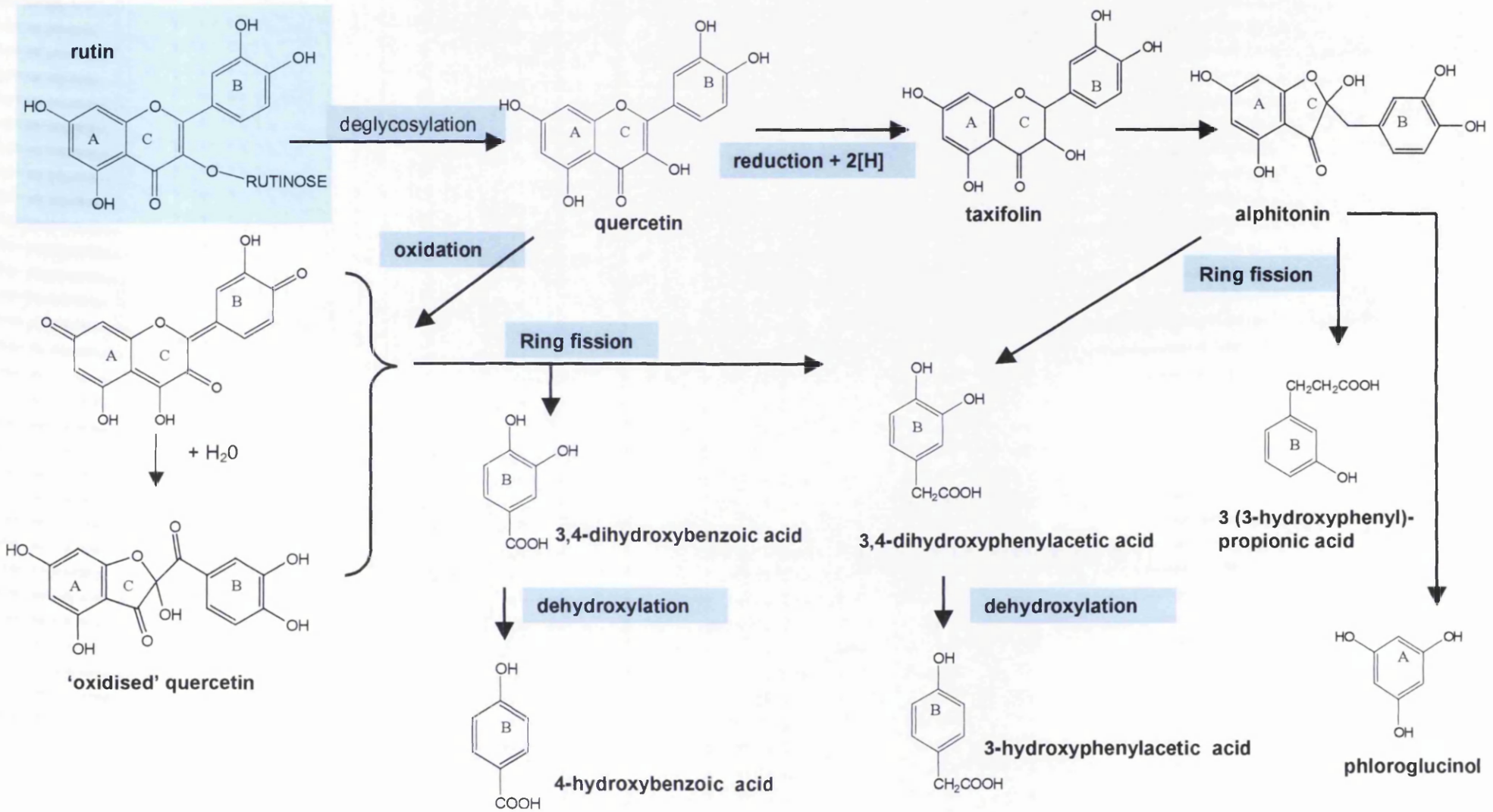


Figure 4.16. Proposed scheme of the colonic degradation of rutin

Chapter 5: Summary and Conclusion on Part I

The research presented in Chapters 3 & 4 concerns a series of studies undertaken in an effort to determine the absorption, metabolism and bioavailability of rutin. The aim of this chapter is to discuss and draw together the main conclusions arising from these two chapters, to compare the results obtained with that of an earlier feeding study carried out with quercetin glucoside-rich onions and finally to summarise the prospects for future research.

5.1 Unveiling the fate of rutin in humans

The underlying objective in Chapter 3 was to investigate the metabolism and absorption of rutin *in vivo*. As discussed in Chapter 3, rutin was found to be extensively metabolised and made bioavailable to humans following consumption of tomato juice spiked with 164 μmoles of rutin. The targets of this study were met by the identification of a huge array of phase II metabolites and catabolites of rutin. Isorhamnetin-3-glucuronide and quercetin-3-glucuronide are absorbed into the bloodstream from the large intestine following deglycosylation of rutin molecule presumably by glycosidase activity of the colonic microflora and/or the wall of the large intestine. Further metabolism of isorhamnetin-3-glucuronide and quercetin-3-glucuronide, probably in the liver and kidney, results in the formation of quercetin glucoside glucuronides, a quercetin diglucuronide, a methylquercetin diglucuronide, quercetin-4'-glucuronide and quercetin-3'-glucuronide, which although not detected in the circulatory system were excreted in the urine after the consumption of rutin. Indirect evidence of metabolism occurring in the large intestine, rather than the small intestine was the delayed appearance of rutin metabolites in plasma 4-5 h after intake and more direct proof was provided by ileostomy volunteers where 92% of the rutin was found to pass through the small intestine and emerged without modification in the ileal fluid.

Results from Chapter 3 also suggested that in the large intestine, the flux of the rutin metabolic pathway is directed towards catabolism rather than the production of conjugated quercetin metabolites. Following deglycosylation, only a small percentage of quercetin (0.01-0.06% of rutin intake) appears to be absorbed and proceeds to the production of phase II metabolites. A substantially larger percentage of quercetin produced is presumably rapidly acted upon by the colonic microflora leading to ring fission and production of phenolic catabolites. This is substantiated by a significant

increase in the excretion of the catabolites in the urine of healthy volunteers post-consumption of rutin. However, when considered with the data obtained from the ileostomy model, only three of the six phenolic acids excreted in the urine of healthy volunteers were postulated to be derived from the breakdown of rutin, namely, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid.

5.2 Role of colonic microflora in the catabolism of rutin

Further evidence of the role of colonic microflora in the breakdown of rutin is provided in Chapter 4. The accumulation of quercetin during *in vitro* fermentation provides direct proof that the initial step in the breakdown of rutin is deglycosylation. In Chapter 3, large individual variations were observed particularly in the production of the phase II metabolites and it was hypothesised to be due to the differences in the composition of colonic microflora. This was also reflected in the data presented in Chapter 4 where variations in deglycosylation profiles of rutin in the fermented faecal samples of the three individuals were observed, presumably because differences in microflora composition, resulted in quercetin accumulating in varying quantities.

The metabolic/catabolic pathways that dictate the fate of rutin in the colon is very much dependent on two main factors as revealed in the results of Chapter 4. The first is the composition of the colonic microflora. Inter-individual composition of the bacterial microflora can either direct the catabolic pathway towards the production of hydroxyphenylacetic acid derivatives or towards the production of hydroxybenzoic acids. The composition of the colonic microflora of donor C directed the catabolism of quercetin towards the production of hydroxybenzoic acid while that of donors A and B, was towards the production of hydroxyphenylacetic acid derivatives. The latter pathway seemed to be a more common route *in vivo* as indicated by the results in Chapter 3 where all the six volunteers excreted substantial levels of 3,4-dihydroxyphenyacetic acid, 3-hydroxyphenylacetic and 4-hydroxy-3-methoxyphenylacetic acid but not hydroxybenzoic acids. The second factor that plays an important role in influencing the rutin catabolism pathway is the presence of glucose. The results in Chapter 4 imply that glucose stimulated the growth and the production of bacterial microflora responsible for both the deglycolysation of rutin and catabolism of quercetin, which was indicated by a more rapid accumulation of quercetin and subsequently phenolic acids in the fermentation medium. The presence of glucose appeared to have a more pronounced

effect on the bacterial strains responsible for the production of hydroxyphenylacetic acid derivatives rather than hydroxybenzoic acids. The pathway which was originally directed towards hydroxybenzoic acid in volunteer C was redirected towards the production of hydroxyphenyacetic acid derivatives in the presence of glucose. When this is translated to *in vivo* conditions, the food matrix associated with the production of glucose in the large intestine could have significant effects on the fate of rutin as it preferentially directs the catabolism towards the production of hydroxyphenylacetic acid deivatives. The presence of glucose in the large intestine, is the most probable cause as to why no hydroxybenzoic acids were excreted in the urine of all six volunteers after ingestion of rutin as reported in Chapter 3.

The *in vitro* study in Chapter 4 indicated a very high percentage of quercetin (60-97%) was converted to 3,4-dihydroxyphenylacetic acid. This was, however not reflected in the *in vivo* study where only 23 % of the ingested rutin was excreted as hydroxyphenylacetic acid derivatives. It is, therefore, feasible to assume that *in vivo,* a high percentage of rutin may have been catabolised to 3,4-dihydroxyphenylacetic acid in the large intestine but depending on a number of internal factors (such as mechanism of transport, intestinal transitional time, blood flow etc) which dictates bioavailability, only a proportion of it is absorbed and transported across the colonic lumen into the circulatory system. A clearer relationship between rutin catabolism and rutin uptake in the large intestinal may be resolved if the levels of phenolic acids in the plasma were analysed. As a result of time limitations, this was, however, not carried out in the present study because the HPLC-MS-APCI technique for the analysis of phenolic acids was only developed and put into routine use towards the end of this study. Further metabolism post absorption of phenolic acid in the liver may also alter the chemical structure either through methylation as indicated by the presence of 4-hydroxy-3-methoxyphenylacetic acid in the urine of the six volunteers (Chapter 3). This phenolic acid was not detected in the *in vitro* fermentation study. In addition, further structural breakdown may occur *in vivo* to form other smaller molecular weight compounds such as acetates and butyrates.

5.3 Comparison of metabolism and bioavailability of rutin with that of quercetin glucoside.

The level of rutin used in Chapter 3 was chosen so that the μmoles ingested were similar to the intake of quercetin-4'-glucoside and quercetin-3,4'-diglucoside in an earlier study carried out by Mullen *et al.* (2005) when onions were ingested by healthy human volunteers. This enables direct comparisons to be made between the fate of the quercetin glucosides and rutin (quercetin-3-glucosylrhamnoside) in humans. Distinct differences in the plasma pharmacokinetic profiles of subjects after ingestion of onion and rutin were observed as indicated in Table 5.1. The longer T_{max} about 5 h observed after ingestion of rutin implies that quercetin glucosides from onions were absorbed in the small intestine while the absorption of rutin takes place in the more distal part of the GI, that is, the large intestine. The C_{max} value of quercetin-3-glucuronide and isorhamnetin-3-glucuronide were about 30-fold higher when onions were consumed compared to the values obtained in the rutin study. The higher C_{max} values indicate that absorption is much more effective in the small intestine compared than the large intestine. Two factors may complicate and reduce absorption of quercetin in the large intestine. Deglycosylation of quercetin glucoside occurs in the presence of β-glucosidase, which is present in relatively high amounts in the GI (Graefe *et al*, 2001). Rutin, on the other hand is dependent on two enzymes, β-glucosidase and α-rhamnosidases to release the quercetin moiety. α-Rhamnosidases, in contrast to β-glucosidase is not produced by the human body but by the colonic microflora and is produced in relatively small amounts (Graefe *et al*, 2001). In addition to this, the fact that the released quercetin can be catabolised by the colonic microflora restricts the amount of quercetin available for absorption which results in a lower C_{max} value (Table 5.1).

In addition to the low level of quercetin absorption in volunteers fed with rutin, reduced number of the phase II metabolites were produced compared to the metabolites obtained when onions were consumed (Table 5.2). In contrast to the seven metabolites detected in plasma of volunteers fed with onions, only two were found to accumulate in detectable quantities in plasma following rutin ingestion. Similarly, out of 18 metabolites excreted in urine post onion ingestion, only nine were detected in urine collected after rutin supplement. When rutin derived metabolites were compared as percentage of the level obtained from onion, a range of 1.4 – 30%

was obtained (Table 5.2) which clearly demonstrates that phase II metabolism of quercetin is much more extensive when quercetin is ingested as quercetin glucosides as opposed to rutin. The higher recoveries of quercetin-3'-glucuronide (30%) and quercetin-4'-glucuronide in the rutin-fed subjects could be due to skewed data resulting from relatively high excretion of these metabolites in the urine of volunteer one as indicated in Chapter 3 (Table 3.6).

When comparing the metabolite profiles in Table 5.2, it is quite clear that the inter-individual variation, reflected in the standard errors, is much higher in subjects fed with rutin than it is after onion supplementation. This difference appears to be dependent of rutin metabolism, specifically the deglycosylation step, on the colonic microflora that differs considerably between individuals. Another striking difference that can be seen is the lack of quercetin sulphate conjugates in plasma and urine of rutin fed subjects. Quercetin-3'-sulfate is the major plasma metabolite in onion fed subjects. Its appearance in the plasma 0.75 h after ingestion of onions suggest that deglycosylation and sulphation occurs in small intestine. Absence of sulphated quercetin metabolites in rutin fed volunteers could be due to a lack of flavonol-specific sulphotransferase in the colonic lumen as suggested by Harris *et al.* (2000) and Liu and Hu (2002). In addition to this, the results obtained with the onion and rutin feeds imply that the site of quercetin sulphation is the enterocyte of the small intestine and the liver, contrary to the proposal of O'Leary *et al.* (2003), does not play an important role.

Table 5.1. Pharmacokinetic parameters of quercetin metabolites in the plasma of human volunteers after the consumption of onions and rutin supplemented tomato juice

Source	Metabolite	C_{max}	T_{max}	$T_{1/2}$
Onions	Quercetin-3'-sulfate	665 ± 82	0.75 ± 0.12	1.7
	Quercetin-3-glucuronide	351 ± 27	0.60 ± 0.10	2.3
	Isorhamnetin-3-glucuronide	112 ± 18	0.60 ± 0.10	5.3
	Quercetin diglucuronide	62 ± 12	0.80 ± 0.12	1.8
	Quercetin glucuronide sulfate	123 ± 26	2.5 ± 0.22	4.5
Tomato juice spiked with rutin	Quercetin-3-glucuronide	12 ± 2	4.7 ± 0.3	5.7
	Isorhamnetin-3-glucuronide	4.3 ± 1.5	5.4 ± 0.2	6.9

C_{max} – maximum concentration in plasma expressed in nM. T_{max} – time to reach C_{max} expressed in h. $T_{1/2}$ – the elimination half-life of metabolites in h. Data presented as mean values ± standard error (n = 6). Pharmacokinetic data on onion metabolites from Mullen *et al.* (2005).

Table 5.2. Quercetin metabolites detected in plasma and urine after the consumption of fried onions and rutin supplemented tomato juice by six human volunteers.

Compound	plasma from onion fed subjects	plasma from rutin fed subjects	urine from onion fed subjects	urine from rutin fed subjects
Quercetin diglucuronide	n.d.	n.d.	trace	n.d.
Methylquercetin diglucuronide	n.d.	n.d.	1003 ± 156	25 ± 21 *(2.5%)*
Quercetin glucoside glucuronide	n.d.	n.d.	163 ± 23	39 ± 36 *(24%)*
Methylquercetin diglucuronide	n.d.	n.d.	426 ± 99	n.d.
Quercetin diglucuronide	n.d.	n.d.	trace	n.d.
Quercetin glucoside glucuronide	n.d.	n.d.	trace	22 ± 21
Quercetin glucoside glucuronide	n.d.	n.d.	trace	20 ± 20
Quercetin diglucuronide	62 ± 12	n.d.	2223 ± 417	32 ± 28 *(1.4%)*
Quercetin-3-glucuronide	351 ± 27	12 ± 2 *(3.4%)*	912 ± 149	270 ± 186 *(30%)*
Quercetin glucoside sulfate	n.d.	n.d.	393 ± 60	n.d.
Quercetin glucuronide sulfate	n.d.	n.d.	1384 ± 163	n.d.
Quercetin glucuronide sulfate	123 ± 26	n.d.	1229 ± 190	n.d.
Quercetin glucoside sulfate	n.d.	n.d.	821 ± 156	n.d.
Isorhamnetin-3-glucuronide	112 ± 18	4.3 ± 1.5 *(3.8%)*	1789 ± 27	179 ± 101 *(10%)*
Quercetin-4'-glucuronide	n.d.	n.d.	trace	237 ± 198
Quercetin-3'-glucuronide	trace	n.d.	1845 ± 193	220 ± 179 *(12%)*
Isorhamnetin-4'-glucuronide	trace	n.d.	700 ± 11	n.d.
Quercetin-3'-sulfate	665 ± 82	n.d.	trace	n.d.

Estimates of levels in plasma expressed as nM ± S.E. (n =6) at peak plasma concentration. Amounts in urine expressed as total amount excreted in nmoles ± S.E. over a 24 h period post-ingestion of onions and rutin spiked tomato juice. Italicised figures in parenthesis represent rutin derived metabolites as percentage of the level obtained from quercetin glucosides in onions. Data on onion metabolites from Mullen *et al.* (2005).

5.4 Health implications

Many of the biological actions of flavonoids have been attributed to their antioxidant properties through *in vitro* evaluation of aglycones or the glycosides that are present in food. However, recent studies have speculated that their classical hydrogen-donating antioxidant activity is unlikely to be the sole explanation for cellular effects of flavonoids (Williams *et al.,* 2004). Flavonoids such as quercetin and rutin are extensively metabolised *in vivo* as indicated in Table 5.2, which will almost certainly result in a significant alteration in their redox potential. Further catabolism in the colon by the gut microflora to phenolic acids also adds on to their structural alterations. The *in vivo* results indicate that the phase II metabolites are present in very low amount and accounts for only 0.01-0.06% of rutin intake, which is probably too low to exert any biological effects. However, phenolic acids are present in more substantial amounts, as indicated by higher levels excreted in the urine, corresponding to 24% of rutin intake. These compounds may therefore be more bioavailable to the cells and tissues than the quercetin derivatives from which they originate and therefore, may have important physiological roles. In keeping with this possibility, 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid possesses more effective anti-platelet aggregation activity than their parent compounds, rutin and quercetin (Kim *et al.*,1998). In a more recent study, 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid were found to exhibit anti-proliferative and apoptotic effects on T47D human breast cancer cell lines, indicating their potential protective role as anticancer agents (Kampa *et al.*, 2004).

The antioxidant and other protective effects of flavonols and phenolic acids could also occur before absorption, that is, within the gastrointestinal tract itself (Halliwell *et al.*, 2005). Results in Chapter 4 indicated that rutin, quercetin and those phenolic acids that retained a 3',4'-dihydroxyl structures, such as 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid, possess substantial antioxidant activity. Of these, quercetin and 3,4-dihydroxyphenylacetic are the strongest antioxidants. Substantial protective effects can be produced if these compounds are present in high amounts as indicated in the results from Chapter 4. The feeding studies outlined in Chapter 3 indicated that most of rutin pass into the large intestine 2–5 h after ingestion, implying that they were present in their natural intact form in the small intestines for the approximately 2 h during which they may have a favourable impact on the health of the small intestine before they enter the colon. A high proportion of the

rutin was found to be catabolised to 3,4-dihydroxyphenylacetic acid, indicating that 3,4-dihydroxyphenylacetic acid is present in sizable amounts in the large intestines, prior to absorption and excretion in the urine, therefore, may play a crucial role in its contribution to the overall antioxidant capacity of the colonic lumen.

5.5 Conclusion

Figure 5.1 summarizes the possible metabolic fate of rutin in the human body after ingestion. It is clear that the GI, particularly the large intestine, plays a vital role in the metabolism and catabolism of rutin. Rutin passes into the large intestine, in substantial quantities and the first metabolic reaction begins with the deglycosylation step which involves β-glucosidase and α-rhamnosidases. On the release of the quercetin moiety, quercetin either can be absorbed directly into the colonic lumen or be subjected to further catabolism by the colonic microflora. On absorption into the lumen, glucuronidation and methylation occur due to the presence of glucuronyltransferase (UGT) and methyltransferase (MT). This yields quercetin-3-glucuronide and isorhamnetin-3-glucuronide which then pass into the portal splanchnic vein into the liver. In the liver and possibly also other sites such as the kidneys, further metabolism of these two metabolites are believed to occur resulting in the production of a range of metabolites, including quercetin glucoside glucuronide, quercetin diglucuronide, methylquercetin diglucuronide, quercetin-4'-glucuronide and quercetin-3'-glucuronide. A large portion of the unabsorbed quercetin in the large intestine are catabolised, undergoing ring fission to produce 3,4-dihydroxyphenyacetic acid, 3-hydroxyphenylacetic and hydroxybenzoic acids. 3,4-Dihydroxyphenyacetic acid can either be methylated by the MT in the lumen of the colon or in the liver to produce 4-hydroxy-3-methoxyphenylacetic acid. Quercetin derived metabolites and catabolites which are not sequestered in cells and body tissues are excreted in the urine.

5.6 Future research

Improved knowledge of the consumption and bioavailability of dietary polyphenols is essential as many questions remain to be answered. Extending the work in this thesis on the identification and quantification of phenolic acids would provide valuable information on the extent of absorption and modification occurring in the large intestine following rutin consumption. Intravenous injection of metabolites compared to

oral consumption of their parent compounds can also provide key answers on the extent of metabolism occurring in the gastrointestinal tract versus that in the liver and kidneys and the rate of turnover of these compounds in the circulatory system. Determination of the actual accumulation of rutin metabolites and catabolites in tissues may be just as more important, if not more so, than knowledge of their plasma concentrations. Data on this subject is very scarce, even in animals. Detailed information could be obtained by feeding [2-^{14}C-]rutin to rats housed in metabolic cages. This methodology will enable valuable information to be generated by monitoring accumulation of all the metabolites and possible catabolites in the various organs and tissues following administration. By counting radioactivity in body tissues and fluids, using liquid scintillation counters, sites of accumulation of metabolites and catabolites can be readily detected. The identity of the individual compounds at each site can then be determined by HPLC with an on-line radioactivity detection coupled with MS^2. Preliminary studies in which [2-^{14}C] quercetin-4-glucoside was fed to rats have demonstrated the value of this approach (Mullen *et al.*, 2002 and Graefe e*t al*, 2005). This approach is certainly more valuable than a recent study in which high doses of quercetin were fed to rats for 11 weeks and pigs for 3 days after which tissues and fluids were extracted and hydrolysed to release aglycones prior to the analysis by HPLC (de Boer *et al.*, 2005).

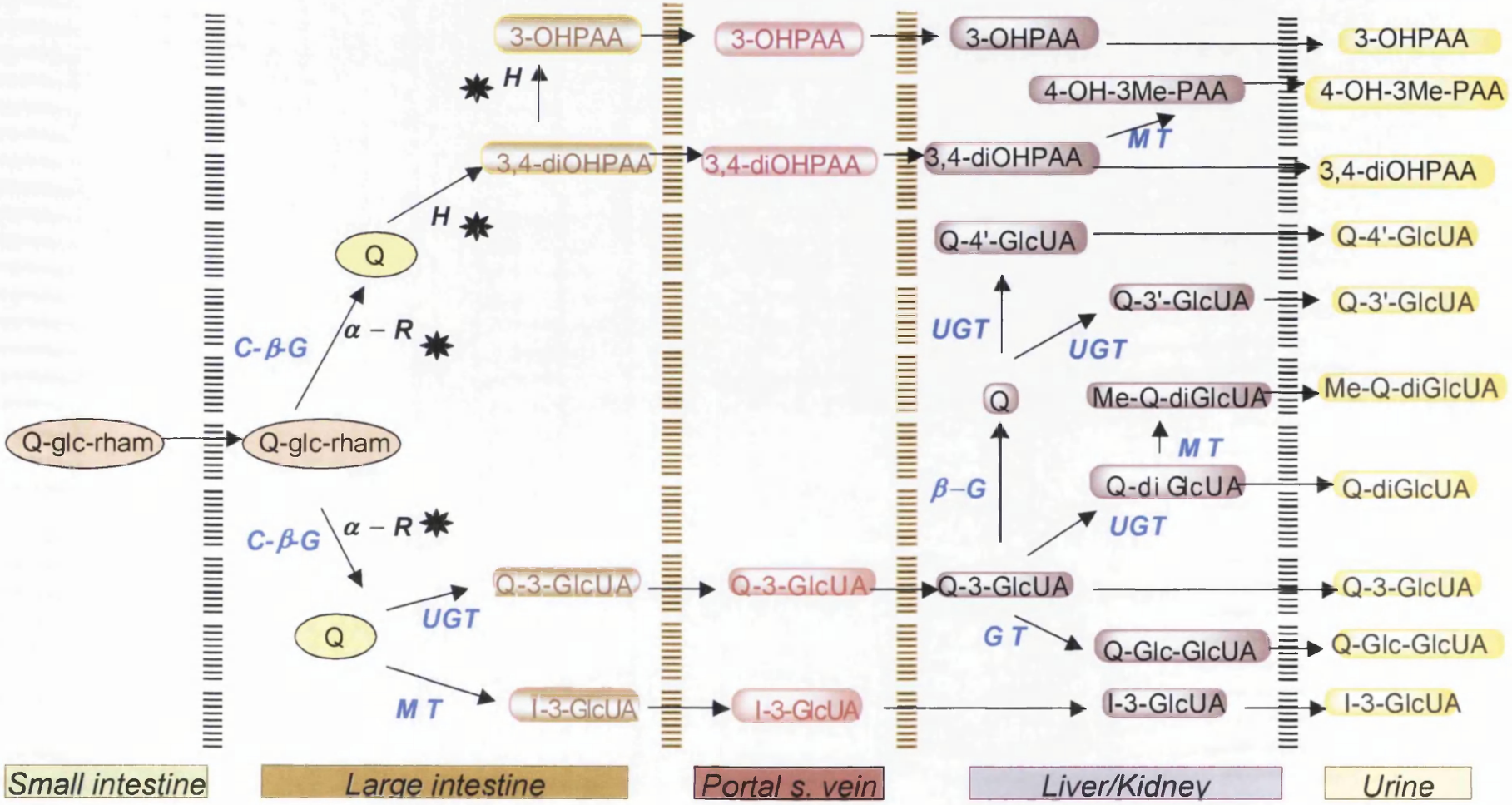


Figure 5.1. Schematic of the possible metabolic fate of rutin as they are transported from the small intestine to the liver and kidneys where they are further metabolised before being excreted in urine. Q-glc-rham- quercetin-3-glyucosylrhamnoside; Q – quercetin; I – isorhamnetin; glc – glucoside; glcUA - glucuronide; diglcUA – diglucuronide; c-β–G – cytosolic-β-glucosidase; α-R-α- Rhamnosidase; H- hydrolysase;UGT - glucuronyltransferase; MT – methyltransferase; GT – glucosyltransferase; 3-OHPAA – 3-hydroxyphenylacetic acid; 3,4-diOHPAA – 3,4-dihydroxyphenylacetic acid; 4OH-3Me-PAA – 4-hydroxy-3-methoxyphenylacetic acid ; ✸– colonic bacteria

Chapter 6: Engineering Genistein Synthesis in Arabidopsis and Tomato

6.1 Introduction

In the past decade there has been a major shift towards food-for-health especially with the identification of bioactive ingredients in food. Since then, numerous investigations were carried out linking the bioactive compound with the prevention and/or treatment of degenerative diseases such as cancer, cardiovascular diseases and diabetes (Premier, 2002). Until recently, research has concentrated on producing new varieties that have a longer shelf life, higher production, better cosmetic value and higher resistance to pest and disease. However, with the advent of GM technologies together with the new focus on bioactive ingredients in food, there is pressure to reconsider plants for economic purposes. In line with this, molecular biologists, biochemists, botanists and medical researchers are linking in with plant breeding programmes to develop new varieties of fruit and vegetables that are tailor-made to produce higher levels of health-related bioactive compounds (Verrips *et al.*, 2001). Research activities in producing functional agrifood is becoming well established as new phytochemical-enhanced products such as broccoli, berries and tomato are being evaluated for commercial exploitation (Premier, 2002).

With respect to bioactive compounds, flavonoids especially the sub-class isoflavones are important since there is evidence that they protect against oxidative stress, coronary heart disease and other age-related diseases (Kuo, 1997, Yang *et al.*, 2001 and Ross and Kasum, 2002). In addition, isoflavone, as a phytoestrogen, has also been associated with reduced menopausal symptoms and reduced risk of some hormone-related cancers (Davis *et al.*, 1999; Messina, 1999; Watanabe *et al.*, 2002). The isoflavone genistein has been the subject of over 3,600 published studies in the last ten years. Since isoflavones are found primarily in leguminous species, there is an interest to direct synthesis of beneficial isoflavones through genetic manipulations in plants that do not synthesize them (Winkel-Shirley, 2001).

Many of the structural and some of the regulatory genes of the flavonoid pathway have been cloned from several model plants, including maize, *Antirrhinum*, tobacco, *Petunia* and Arabidopsis (Holton and Cornisch, 1995) and have been expressed in genetically modified model plants and micro-organisms (Dixon and Steele, 1999, Forkmann and Martens, 2001, Winkel-Shirley, 2001 and Hwang *et al.*, 2003). The

knowledge and availability of these genes provides the tools to genetically up-regulate the overall flavonoid biosynthesis or to engineer new pathways towards production of elite flavonoid species within crop plants.

In this study, we attempted to direct isoflavonoid synthesis towards branches that are normally not present in the host plant by introducing a foreign gene, isoflavone synthase (*IFS*) that branches off from the existing pathway towards the synthesis of the bioactive isoflavone, genistein. To date, some attempts have been made to produce isoflavonoids in non-legume plants such as Arabidopsis, tobacco and maize cell lines by over-expressing the soybean *IFS* gene (Liu *et al.*, 2002, Yu *et al.*, 2000). In the current study, a different crop model, tomato, was used. Tomato is an excellent candidate as it is not only one of the most-consumed foods but is also a reservoir of diverse antioxidant molecules, such as ascorbic acid, vitamin E, carotenoids and phenolic acids (Gahler *et al.*, 2003). In addition to this, the skin from the tomato fruit contains high levels of the flavonol, rutin, which also possesses potential health benefits and is the subject of research in Chapter 3 & 4.

Therefore, the main aim of this study was to engineer genistein synthesis into tomato, with the aim of ultimately producing a crop with enhanced bioactive components. In order to develop methodologies and better understand the requirements and regulation of genistein production in the flavonoid biosynthetic pathway, pilot studies were first conducted on Arabidopsis. Arabidopsis was chosen as it has a simpler genome that has been completely characterised and sequenced and it is therefore an easier model system for the introduction of genes than tomato.

6.2 Experimental approach

Isoflavonoids are synthesized from a branch of the phenylpropanoid pathway. IFS is the first committed enzyme in the isoflavone pathway and converts the flavanone naringenin to genistein or liquiritigenin to daidzein (illustrated by the green dotted lines in Figure 1.12). However, in non-leguminous plants, isoflavones are not among the complex array of secondary metabolites synthesized by the phenylpropanoid pathway due to the absence of IFS. Therefore to synthesize isoflavonoids in non-legume plants that lack the pathway, the introduction of IFS is crucial as it is the key entry point for isoflavonoid biosynthesis. With the recent identification of the *IFS* gene (Akashi *et al.*, 1999; Steele *et al.*, 1999; Jung *et al.*, 2000), we were able to clone the *IFS* gene using RT-PCR from soybean and direct it in Arabidopsis and tomato

6.3 Materials and Method

6.3.1 Materials

6.3.1.1 Plant seed stocks

Wild-type *Arabidopsis thaliana* cv Columbia (Col 0) seeds were originally obtained from Nottingham Arabidopsis Stock Centre (ie. NASC, Nottingham, UK) (http://nasc.nott.ac.uk). *Glycine max* cv Envy (soybean) and *Lycopersicum esculentum* cv. Micro Tom (tomato) seeds were obtained from Nicky's nursery (http://www.nickys-nursery.co.uk/seeds).

6.3.1.2 Chemicals

The chemicals used in this study were obtained from Sigma (Poole, Dorset) and Fisher Scientific (Loughborough, Leicestershire). Specialist chemicals and reagents were supplied as indicated.

6.3.1.3 DNA modifying enzymes

DNA restriction, synthesis and modification enzymes were purchased from Promega (Southampton, U.K.), New England Biolabs (Hitchin, Hertfordshire), and Roche Diagnostics (Mannheim, Germany) or as indicated. Enzymes were used in accordance with the manufacturer's instructions.

6.3.1.4 Plasmids and bacterial strains

The plasmids used in this study are

- 35S-pGlasglow. p-Glaslgow was derived from pJO530 binary vector [derivative of pBIN19 (D.Becker, NAR 18 203 (1991)], with the insertion of Luciferase and the Basta resistance gene (BAR) (A. Aakkha and J. Milner- unpublished) (Figure 6.8)
- pER8 plasmid (Zuo et al., 2000), genebank no. AF309825, an estradiol induced plasmid (Figure 6.9) was obtained from F.Auseabel, Harvard University.
- pCHF3 plasmid with a CaMV 35S full promoter and the RBCS terminator from pea (Figure 6.11).

All these plasmids were modified with the insertion of the c-DNA and then transformed into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986). The *Escherichia coli* strain used was DH5α.

6.3.1.5 Antibiotics/pesticide

For bacterial or plant selection, the following antibiotics/pesticide were used:

Plasmid	Bacteria	Plant
pER-8	Spectinomycin	Hygromycin
p-Glasglow	Kanamycin	Basta
pCHF3	Spectinomycin	Kanamycin

The selection marker for *A. tumefaciens* strain GV3101 was gentamycin. Kanamycin and spectinomycin stocks were at 50 mg mL^{-1} and used at a final concentration of 50 μg mL^{-1}. Hygromycin and gentamycin stocks were at 25 mg mL^{-1} and used at a final concentration of 25 μg mL^{-1}. In cultured media, Basta (ammonium glufusinate) stock was 10 mg mL^{-1} and used at a final concentration of 10 μg mL^{-1}. To soil grown plants, basta was sprayed at 100 μg mL^{-1} in 0.005% Silvet L-77.

6.3.2 General Laboratory Procedures

6.3.2.1 pH measurement

The pH of solutions was measured using a Jenway 3320 pH meter and glass electrode (Jenway, Felsted, Essex).

6.3.2.2 Autoclave sterilization

Equipment and solutions were sterilized at 15 psi for approximately 30 min using benchtop (eg. Prestige Medical, Model 220140) or free-standing (eg. Laboratory Thermal Equipment Autoclave 225E) autoclaves.

6.3.2.3 Filter sterilization

Heat labile solutions were sterilized by filtration through a Nalgene filter (pore diameter 0.2 μM) and collected in a sterile container.

6.3.3 Growth media

6.3.3.1 Liquid and solid bacterial growth

LB medium consisting of 1% tryptone, 0.5% yeast extract and 0.5% sodium chloride at pH 7.0 was used to grow *E. coli.* For plates, 15 g L^{-1} microagar was added to the media prior to autoclaving. Antibiotics were added after sterilisation, when the solutions have cooled to about 50°C. In growing agrobacterium, YEP medium, consisting of 1% tryptone, 1% yeast extract and 0.5% sodium chloride was used.

6.3.3.2 Solid plant media

Solid plant media for growth of Arabidopsis plants consist of 1 x Murashage and Skoog (MS) salts + Gamborg's Vitamin, 0.05% morpholinoethanesulfonic acid organic buffer (MES), 0.8% agar and 2% sucrose. The medium was sterilised and once cooled to 50°C, it was poured under sterilised conditions into 90 mm Petri dishes and allowed to set. For selection of transformants, 0.5 x Murashage and Skoog (MS) salts + Gamborg's Vitamin was instead used. The appropriate antibiotic was added to the solution once it was cooled to 50°C.

6.3.3.3 Tissue culture media for tomato

a. Seed Germination media for tomato

The seed germination media of tomato consist 1 x MS salts, 1.5% sucrose, 0.01% vitamin mix (consisting of thiamine, glycine, nicotinic acid, pyridoxine, folic acid, biotin and myo-inosital) at pH 5.7 and 0.7% agar.

b. Pre-culture media for ex-plants

The pre-culture media consist of 1 x MS salts, 3% sucrose, 0.01% vitamin mix, 1 mg L^{-1} benzylamino purine (BAP), 0.5 mg L^{-1} naphthalene acetic acid

(NAA), and 0.8% agar. pH was adjusted to 5.7 with KOH (before adding agar) and the medium was sterilized by autoclaving .

c. Shoot induction medium

The shoot induction media was prepared as above but instead of BAP and NAA, zeatin at 1 mg L^{-1} was added. The appropriate antibiotic was added to the solution once it was cooled to 50°C.

d. Shoot elongation medium

This media was similar to the shoot induction media but the quantity of sucrose and zeatin was reduced to 1.5% and 0.5 mg L^{-1} respectively. In addition to this, gibberellic acid (GA_3) at 1 mg L^{-1} was added to the mixture. The appropriate antibiotic was added to the solution once it was cooled to 50°C.

e. Rooting medium

The rooting media was similar to the shoot elongation media but instead of zeatin and GA_3, indole butyric acid (IBA) at 1 mg L^{-1} was added.

6.3.4 Plant growth and maintenance

6.3.4.1 Soil grown plants

Arabidopsis seeds were gown in autoclaved peat-based potting compost (Levington Horticulture Ltd.) that was pre-autoclaved and placed in trays soaked in 0.15 g L^{-1} of a solution of the insecticide Intercept® (Scotts U.K., Bramford, Ipswich) for the control of aphids. The pots were then covered with cling film and vernalized for 2-3 days at 4°C before transfer to growth conditions at 20°C under 16 hours daylength. Trays were maintained for three weeks at 100 μE m^{-2} s^{-1} white light. Similar conditions were maintained for tomato but without vernalization and they were grown in pots.

Soybean seeds were pre-soaked in water for 5 h before placed on moist vermiculite in trays and placed in the growth chamber with similar conditions as above. The seedlings were harvested after seven days of sowing.

6.3.4.2 Surface sterilization of seeds

Seeds which were to be sown on sterile growth media or selection plates were surface sterilised.

A. Arabidopsis

One CONCLOR 1000 tablet (Coventry Chemicals Ltd.) was dissolved in 35 mL dH_2O, to which 2 drops of 1% (v/v) Tween 20 was added. Five mL of this stock solution was added to 95% ethanol. One mL of this sterilising solution was added to an appendorf tube containing 10-15 mg of Arabidopsis seeds. The tube sealed and inverted several times and left for 6.5 min. Further manipulations were carried out inside a sterile flow cabinet using sterile equipment and solutions, where the liquid was removed and washed twice in absolute ethanol. All traces of ethanol were removed and the seeds were allowed to dry for a further 30 min. Finally, the seeds were resuspended and washed five times in sterile water. The seeds were then distributed evenly on the agar plates. Agar plates were sealed with nescofilm and vernalized at 4°C for 2-4 days before being transferred to a growth chamber. The conditions of the growth chamber were similar to that of the soil grown plants. Seedlings were harvested and packaged into aluminium foil and frozen in liquid nitrogen before storage at –80°C.

B. Tomato

Tomato seeds were placed in a tea strainer and placed in a beaker with 70% ethanol and left for 1 min. Inside a sterile flow cabinet, the seeds were then sterilised with 5% sodium hypochlorite to which two drops of 1% (v/v) Tween 20 was added. The mixture was swirled occasionally for left for 20 min. The seeds were then transferred to agar plates using sterile forceps. The seeds were allowed to germinate at 20°C with 16 h photoperiod and at a light intensity of 100 $\mu E\ m^{-2}\ s^{-1}$.

6.3.5 Plant treatments

6.3.5.1 UV-B illumination of plant material

UVB illuminations were carried out in controlled environment rooms at 20°C and the spectra were measured using a Macam Spectroradiometer SR9910 (Macam Photometrics Ltd., Scotland). UV-B light was derived from Q-Panel UV-B 313 tubes (Q-Panel Co., U.S.A.). A cellulose acetate filter (Catalogue No. FLM400110/2925, West Design Products, Nathan Way, London) was used as standard and changed every 24 h to eliminate any UV-C (wavelengths <280 nm) from the UV-B tubes (UV-C is therefore absent from the UV-B spectrum). Fluence of about 3 $\mu E\ m^{-2}\ s^{-1}$ was achieved by varying both the number of fluorescent tubes used and the distance between the tubes and the plant material being illuminated. No white light was present. In addition

to the Macam Spectroradiometer, a hand held RS232 meter, Skye Instruments (Powys, Wales) was also used to measure the UV-B (280-315 nm) with an SKU 430 sensor.

6.3.5.2 Basta selection

Selection of transgenics plants carrying the basta resistant genes was performed by sowing approximately 0.5 mL of seeds from infiltrated plants in soil trays (260 mm x 310 mm). Germination was synchronised by treatment at 4°C for 3-5 days. Trays were placed under long-day light at 21°C and seedlings sprayed first upon emergence and twice afterwards at 3-day intervals with 20% glufosinate ammonium. Basta-resistant plants were transferred to pots and grown to maturity. Plants were observed during growth for the presence of visible phenotypes.

6.3.6 Genomic DNA

6.3.6.1 Isolation of total DNA from plant material

(A) Arabidopsis

Approximately 100 mg of leaf tissue was harvested and DNA extracted from tissue using a Nucleospin kit (Clontech, Oxford, England). Leaves were ground in liquid nitrogen prior to addition to lysis buffer. DNA was then extracted and purified according to the manufacturer's instructions.

(B) Tomato / soybean

For plants with higher tannin content, such as tomato and soybean, the GenElute Plant Genomic DNA miniprep Kit (Sigma-Aldrich, Poole, Dorset, UK) was used to extract the DNA. As above, 100 mg of the plant material were ground in liquid nitrogen before adding the lysis buffer. DNA was then extracted and purified according to the manufacturer's protocol.

DNA was assayed spectrophotometrically on a Perkin Elmer Lambda 800 spectrophotometer. The absorbance was measured between 260 nm and 280 nm against a sterile distilled H_2O blank. The following formula was used to quantify the amount of DNA in the sample:

$$A_{260} \times (\text{dilution factor}) \times 50 \text{ (conversion factor)} / \mu L = \mu g/\mu L$$

The integrity of the total DNA was determined by running 2 µL aliquots on agarose gel

6.3.6.2 Agarose gel electrophoresis

Electrophoresis grade agarose (0.8-1.2 % (w/v) was added to 0.5 x TBE (44 mM Tris-borate, 1 mM disodium EDTA, pH 8.0) for the gel cast. The suspension was heated in a microwave until the agarose had completely dissolved. Once cooled to about 60°C, ethidium bromide was added to a final concentration of approximately 0.3 $\mu g\ mL^{-1}$ and the gel solution poured into a casting tray. After the gel had set it was placed in the electrophoresis apparatus and submerged in 0.5 x TBE running buffer. DNA samples to be loaded were mixed with 1/10 volume of loading buffer (25% (v/v) glycerol, 5 mM disodium EDTA pH 8, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol, 50mM Tris base) and applied to the gel with a pipette. Electrophoresis was allowed to proceed at 80-120 mA until the DNA molecules had been clearly separated. The gel was then visualized under UV light (transilluminator model TFM-20; Ultra-Violet Products, Cambridge, U.K.) and photographed using a Bio-Rad (Hemel Hempstead, Hertfordshire, U.K.) Gel Documentation System with Quantity One® (Version 4.3.0) Software. DNA Molecular Weight Marker X (catalogue No. 1498037, Roche Diagnostics, Mannheim, Germany) was used to calibrate DNA fragment sizes.

6.3.6.3 Oligonucleotide primers

The oligonucleotide primers used in this work were synthesized by MWG Biotech. The nested *IFS* primers were obtained from a published article (Jung et al., 2000).

The nested primers were synthesised by MWG and their sequence are:

Forward: 5'- TGT TTC TGC ACT TGC GTC CCA C – 3'

Reverse: 5'- CCG ATC CTT GCA AGT GGA ACA A – 3'

Primer 3 (http://www.basic.nwu.edu/biotools/Primer3.html) was used to design the full length *IFS* cDNA primers. Their sequences are

Forward: 5'- GTT ACC TCG GGA TCA CAG AAA CCA – 3'

Reverse: 5'– TCT AGA CCA GTA ACA CCT TAT TGA AAT GAT-3'

6.3.6.4 PCR

HotStarTaq master mix kit (Qiagen) was used with appropriate primers according to the protocol outlined by the manufacturers in Polymerase Chain Reaction to amplify cDNA fragments. The Polymerase Chain Reaction was carried out using DNA Engine PTC-200 Peltier Thermal Cycler (Genetic Research Instrumentation,

Braintree, Essex, U.K.) machine. cDNA sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) provided by The National Center for Biotechnology Information (NCBI) online (http://www.ncbi.nlm.nih.gov/blast/)

6.3.7 Genomic DNA

6.3.7.1 Isolation of RNA from plant material

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, West Sussex, UK). Young leaves were harvested and then quickly frozen with liquid nitrogen and ground to a fine powder using an RNase-free, chilled mortar and pestle on an ice bucket. Fine powder (approximately 100 mg) was transferred into a previously tared 1.5 mL microfuge tube and the nitrogen liquid was allowed to evaporate, and then 450 µL RNeasy Lysis buffer (RLT) containing 1% 2-mercaptoethanol (BME, Fisher) was added. RNA was then extracted and purified according to the manufacturer's protocol.

The absorbance (A) of the collected RNA was determined at 260 and 280 nm wavelengths using a Perkin Elmer Lambda 800 spectrophotometer and the following formula was used to quantify the amount of RNA in the sample:

A_{260} x (dilution factor) x 40 (conversion factor)/ µL = µg/µL

The integrity and size distribution of the total RNA was determined by running 1 µL and 2 µL aliquots of RNA on formaldehyde agarose (FA) gel. Two sharp RNA bands representing ribosomal RNA appearing on the stained gel indicate good RNA.

6.3.7.2 Formaldehyde agarose (FA) gel for RNA analysis

This method was used for gels that were to be blotted for Northern hybridization and to check the integrity of RNA extraction. Electrophoresis grade agarose at 1.2 % (w/v) was added to 72 mL of dH_2O. The agrose suspension was heated in a microwave until the agarose had completely dissolved. Once cooled to about 60°C, 10 mL of 10 x MOPS (0.2M MOPS, 20 mM sodium acetate, 10 mM EDTA, pH 8.0) and 18 mL of formaldehyde (37% (v/v), Sigma) were added and the gel solution poured into a casting tray. After the gel had set it was placed in the electrophoresis apparatus and submerged in 1 x MOPS running buffer.

Total RNA, 5-10 µg per lane was added to equal volume of RNA loading buffer (100 µL of 5 x MOPS, 175 µL formaldehyde, 160 µL Ficoll dye, 10 µL ethidium bromide) and applied to the gel with a pipette. Electrophoresis was allowed to proceed

in a fume hood at 50-70 mA until the RNA molecules have migrated to at least two-thirds down the gel. The gel was then visualized under UV light (transilluminator model TFM-20; Ultra-Violet Products, Cambridge, U.K.) and photographed using a Bio-Rad (Hemel Hempstead, Hertfordshire, U.K.) Gel Documentation System with Quantity One® (Version 4.3.0) Software.

6.3.7.3 RNA analysis by northern blot

A. RNA blotting

From the formaldehyde agarose gel, RNA was transferred to Hybond N membrane (Amersham, Bucks, England) according to the protocols outlined in Sambrook and Russell (2001) and fixed under UV-crosslink (Syngene Synoptics Ltd.).

B. Hybridisation, probes and phosphorimaging

(i) Pre-hybridisation

Membranes were placed in Techne roller hybridisation tubes (Techne, Stakks, UK) and covered with 15 ml of Northern Max hybridisation solution (Ambion, Huntingdon, Cambridgeshire, UK) containing denatured sheared salmon sperm (Ambion, Huntingdon, Cambridgeshire, UK) to a final concentration of 100 μg mL^{-1}. Prehybridisation was carried out at 42°C for 18 h.

(ii) Probe

Denatured purified DNA fragments were labelled using using the Rediprime II DNA labeling system (Amersham Biosciences, UK), according to the manufacturer's guidelines. The Rediprime II DNA labeling mixture consists of buffered solution of dATP, dGTP, dTTP, exonuclease free Klenow polymerase and random primers. Five μL of Rediprime mixture was added to a tube containing about 25 ng of denatured DNA and 50 μCi of α[32P]-dCTP (3000 Ci $mmol^{-1}$; Amersham,UK). The labelling reaction progressed as outlined in the manufacturer's protocol. The probe was then purified with Sephadex G50 Nick columns (Amersham, UK), which retains the unincorporated radiolabelled dCTP, but allows newly synthesised labelled DNA of greater than 50bp to pass through.

(iii) Hybridisation

The radiolabelled probe was boiled for 10 min, added to the filters and allowed to hybridise for 18 h at 42°C. Subsequently, the filters were subjected to SSPE washes

of different stringencies according to Sambrook and Russell (2001). Filters were then wrapped in cling film, placed in a cassette for phosphorimaging.

(iv) Phosphorimaging and quantification

Hybridization signals detected using a FLA 5000 phosphorimager (Raytek Scientific, Sheffield, United Kingdom). The total amount of RNA loaded onto each lane was quantified by pixel analysis of digitally reversed images of fluorescence, using Quantiscan for Windows (Biosoft, Cambridge, UK). The phosphorimages were scanned on a flatbed scanner (Hewlett Packard Scanjet 4470c), digitised and hybridisation was quantified by pixel analysis using Quantiscan for Windows.

6.3.7.4. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Following extraction of total RNA, a DNase treatment (DNA-free, Ambion, Huntingdon, UK) was used to eliminate contamination with genomic DNA according to the manufacturer's protocol. First-Strand cDNA Synthesis was carried out using SuperScript™ II reverse transcriptase (Invitrogen) according to the manufacture's protocol. (http://www.invitrogen.com/content/sfs/manuals/superscriptII_pps.pdf.). A 20-μL reaction volume was used for 3-5 ug of total RNA and the reaction was allowed to proceed for 50 min at 42°C. Finally, the enzyme was inactivated by heating at 70°C for 15 min. The cDNA can now be used as a template for amplification in PCR.

6.3.8 Plasmid DNA

6.3.8.1 Isolation of DNA fragment from agarose gel

The DNA fragment of interest was initially separated from the residual DNA by agarose gel electrophoresis (section 6.3.6.2) and identified by comparing against 1 Kbp ladders (FMC, Surrey, UK). The appropriate bands were excised and the fragments were purified using a Quantum Prep Freeze-N-Squeeze Spin Column kit (Biorad, herts, UK).

6.3.8.2 Cloning of PCR products in plasmids

After purification the DNA fragment was inserted into pGlasglow and pER-8 binary vectors by a series of digestion with restriction endonucleases followed by DNA ligations.

(i) Digestion of DNA with restriction endonucleases

DNA restriction enzymes were used in accordance with the manufacturer's instructions. DNA was prepared in a 1 x solution of the appropriate buffer (as recommended by the supplier) and approximately 5-20 units of restriction enzyme added to a concentration not exceeding 10 % (v/v). Reactions were incubated at the appropriate temperature overnight or for the required duration.

(ii) DNA ligations

After restriction endonuclease digestion and gel purification of DNA, the ligation reaction was as follows: The amount of insert needed = vector (ng) x kb size of insert/kb size of vector x molar ratio of insert/vector. Rapid DNA Ligation Kit (Roche Diagnostics, UK) consisting of T_4 DNA ligase, T_4 DNA Buffer and DNA dilution buffer was used to ligate the cDNA inserts in the pGlasglow, pCHF3 and pER-8 vectors according to the manufacturer's protocol. The plasmids were then checked by PCR using appropriate primers and by restriction digestion and gel electrophoresis, measuring the resultant fragments against a 1 Kb ladder.

6.3.8.3 Cloning of PCR products in Topo-vector

For the purpose of sequencing, PCR products were directly cloned into a plasmid vector, pCR®II-TOPO and subsequently transferred to *E. coli* competent cells by One Shot® Chemical Transformation with the TOPO TA Cloning Kit (Invitrogen) as described in the manufacture's manual .

6.3.8.4. Transformation of plasmids in *E. Coli*

For the purpose of amplification of the plasmid DNA, the pGlasglow, pCHF3 and pER-8 binary vector with the cDNA insert was transformed into *E. coli* DH5α cells using the methods described in Sections 6.3.8.5 – 6.3.5.6.

6.3.8.5 Preparation of competent *E. Coli* cells

Chemical transformation was used to transform *E. coli* DH5α cells. DH5α cells were streaked onto a minimal plate (LB without antibiotics) and incubated at 37°C for 18 h. A five ml LB culture was inoculated with a single colony of the bacterial culture and incubated in a 37°C shaking incubator (200 rpm) overnight. Two mL of this starter culture was then added to 100 ml of LB and allowed to grow until the absorbance at 650 nm reached about 0.5. The culture was centrifuged at 1000 *g* for 10 min at 4°C

and the pelleted cells resuspended in 10 mL of pre-chilled Transfer Buffer 1 (consisting of 30 mM, potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride and 15% glycerol at pH 5.8. After chilling on ice for 5 min, the culture was re-centrifuged and the pellet resuspended in 4 ml of Transfer Buffer II (consisting of 10 mM MOPS buffer, 10 mM rubidium chloride, 75 mM calcium chloride and 15% glycerol at pH 6.5). The cells were chilled on ice for a further 15 min and aliquots of it stored in –80°C until further use.

6.3.8.6 Transformation of competent *E. coli* cells

Approximately 10-30 ng of binary vector plasmid DNA was added to thawed *E. coli* competent cells and left on ice for 20 min. The cells were then heat-shocked at 42°C for 90 sec and returned to ice for a further 5 min. The cell suspension was then added to 5 mL of LB medium and incubated in a shaking incubator set at 200 rpm and at 37°C for 2 hours to allow expression of the antibiotic resistance encoded on the plasmids. Cells were then pelleted by centrifugation at 10,000 *g* for 1 min and the pellet resuspended in 150 µL of LB medium. 50-150 µL of the cell suspension was spread onto selective agar plates. The agar plates were incubated at 37°C and colonies appeared after 24 h.

6.3.8.7 Bacterial culture and isolation of plasmids

The *E. coli* stocks containing the vectors were grown under appropriate antibiotics according to Sambrook and Russell (2001). Bacteria was pelleted twice by centrifugation at 3000 *g* and resuspended in buffer P1 of the QIAprep Spin Miniprep Kit (Qiagen, West Sussex,UK). The protocol outlined in the manufacturer's manual of the QIAprep Spin Miniprep Kit was used to extract the plasmids.

6.3.8.8 Transformation of plasmids in agrobacterium cells

Since agrobacterium-mediated transformation will be adopted to transfer the cDNA of interest in tomato and Arabidopsis plants, the plasmids carrying the cDNA of interest was transformed into *A. tumefaciens* strain GV3101 using the methods described in Sections 6.3.8.8.1-6.3.8.8.2.

6.3.8.8.1 Preparation of competent agrobacterium cells

Five mL of YEP starter culture, containing 25 µg mL^{-1} of gentamycin was inoculated with a single colony of *A. tumefaciens* strain GV3101 and left to grow

overnight at 28°C, shaking at 200 rpm. Two mL of this culture was added to 50 mL of YEP with 25 μgmL^{-1} of gentamycin in a 250 mL flask and left to grow at 28°C, and shaking at 200 rpm. Once the cultures reached an OD_{600} of 0.5–1.0, they were chilled prior to centrifugation at 3000 *g* for 5 minutes at 4°C. The pellets were resuspended in one mL of pre-chilled 20 mM calcium chloride. Aliquots of the agrobacterium cells were frozen in liquid nitrogen or dried-ice before storing at –80°C until further use.

6.3.8.8.2 Transformation of competent agrobacterium cells

Approximately 1 µg of binary vector plasmid DNA was added to thawed agrobacterium competent cells. The cells were frozen in liquid nitrogen and then thawed by incubating at 37°C for 3-5 min. One mL of YEP was added and the cells further incubated at 28°C for 2-4 h with gentle shaking to allow the cells to express the antibiotic resistance. The cells were centrifuged at 10,000 *g* for 2 min and the pellet resuspended in 0.1 mL LB media. 50-100 µL of the cell suspension was spread onto selective agar plates. The agar plates were incubated at 28°C and colonies appeared after 48 h.

6.3.9. Agrobacterium-mediated transformation into plants

A. Arabidopsis

The floral dip method was employed to produce Arabidopsis transgenic plants as outlined by Clough and Bent (1998). *A. tumefaciens* strain GV3101 was transformed with the plasmid pGlasglow, pCHF3 and pER-8 carrying the *c DNA* insert

B. Tomato

The tissue culture method as described by Oktem *et al.* (1999) was adopted to produce transgenic tomato plants. Once the tomato shoots have regenerated from the calli, they were transferred to the rooting media, which lacks the hormone zeatin, but contains Indol Butyric Acid (IBA) and the appropriate antibiotic for selection of transformants. Rooted plantlets were then transferred to boxes containing soil and kept covered with cling film in the growth chamber under low light intensity to acclimatise or "hardened off" for at least a week.

6.3.10 Protein analysis

6.3.10.1 Total protein isolation

One g of freeze-dried plant tissue was homogenized in 5 mL of 1 x PBS solution to which protease inhibitor cocktail tablet (Roche Diagnostics, UK) was added. The homogenate was filtered through a 70 μm nylon mesh cloth and centrifuged at 13,000 rpm for 10 min. The supernatant was collected and used for western blotting

6.3.10.2. Membrane protein isolation

One g of freeze-dried plant tissue was homogenized in chilled mortar on ice with 5 mL of homogenization buffer (consist of 25 mM MOPS, 0.25 M sucrose, 0.1 mM magnesium chloride at pH 7.8). The homogenate was filtered through a 70 μm nylon mesh cloth and centrifuged at 8,000 g for 10 min at 4°C. The supernatant was removed to an ultra-centrifuge tube and centrifuged at 100,000 g for 1 h 15 min at 4°C. The pellet was then resuspended in 200 μL of resuspension buffer (250 mM sucrose, 4 mM potassium nitrate and 5 mM potassium phosphate). This was then stored at –80°C in 20 μL aliquots.

6.3.10.3. Western blotting

An IFS antibody was prepared using a peptide sequence derived from the *IFS* coding region. The peptide NH2-DPKYWDRPSEFRPER (Yu *et al.*, 2000) conjugated with KLH was produced by Davids Biotechnologie (Regensburg, Germany) and injected into a rabbit using their suggested immunization schedule. Serum from a bleed 60 days after initial immunization, together with the extracted proteins from the transgenic plants was used to carry out western-blot analysis according to Sambrook and Russell (2001). The membranes were wrapped in clingfilm, placed in a cassette and overlaid with Kodak MXB autoradiograph film in a dark room. The autoradiographs were exposed for an appropriate period and the film developed in an X-OMT Kodak developer.

6.3.11. Isoflavone analysis

Five hundred mg of frozen plant tissue samples were ground in liquid nitrogen, extracted with 80% (v/v) methanol. To hydrolyze any possible isoflavone conjugates, 1 mL of 6 M HCl were added to 5 mL of the extract and the sample was incubated at 90°C for 2 h. This was followed by partitionising into ethyl acetate. The

ethyl acetate extract was then reduced to dryness in *vacuo* (Thermo Savant SPFD SpeedVac, Hampshire, UK). The dried extract was dissolved in 50 µL of 80% methanol. Ten µL aliquots of the centrifuged supernatant were analysed by HPLC-PDA-MS^2. Separation was carried out using a 250 x 4.6 mm I.D. 4 µm Synergi Max-RP column and eluted with a 30 min gradient of 15-40% acetonitrile in 1% formic acid at a flow rate of 1 mL min^{-1} and maintained at 40°C. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 mL min^{-1} was directed to a LCQ DecaXP ion trap mass spectrometer fitted with either an ESI interface. Analyses utilised the negative ion mode for flavonoids. Analysis was carried out using full scan, data dependant MS^2 scanning from *m/z* 100 to 1000. The capillary temperature was 250°C, sheath gas and auxiliary gas were 40 and 5 units respectively, and the source voltage was 5 kV for negative ionisation.

6.4 Results

6.4.1 Detection of the *IFS* gene in Micro Tom tomato

The purpose of this study was to confirm earlier reports that the *IFS* gene is only found in the Leguminosae family and is not present in tomatoes. For this purpose, DNA of tomato was tested against the published nested *IFS* primers using PCR. Using the technique described in Section 6.3.6.1 tomato DNA was isolated and quantified at 260 nm absorbance.

The nested primers were obtained from a published article (Jung *et al.*, 2000) and synthesised by MWG. The nested primers are:

Forward: 5'- TGT TTC TGC ACT TGC GTC CCA C – 3'

Reverse: 5'- CCG ATC CTT GCA AGT GGA ACA A – 3'

DNA ranging from 74 – 300 ng μL^{-1} was used. The thermal profile started with 15 min of denaturation at 95 °C, followed by 30 cycles at 94 °C for 45 min, 50-54 °C for 1 min, 72 °C for 1min 40 sec, and ended with an elongation step at 72 °C for 10 min. The gel electrophoresis indicated that PCR products were present with sizes of 700 bp and 1.5 kb (Figure 6.1).

DNA was then extracted and purified from these two bands following the protocol described in Section 6.3.8.1 and cloned into the pCR 2.1 Topo vector using the TOPO TA Cloning® Kit for Sequencing (Section 6.3.8.3). Recombinant clones containing an insert of the correct size were selected and confirmed through PCR using colonies as template (Figure 6.2) and by restriction analysis (*Eco* R1) on purified plasmids (Figure 6.3). The plasmids were then isolated and purified using the Q1prep Spin miniprep Kit as discussed in Section 6.3.8.7

The pCR4 TOPO vector-plasmids with the 1.5 kb and 700 bp fragments were sequenced. Blast was carried out on the above sequences using the pairwise and the standard nucleotide blast from NCBI (with the published IFS gene sequence Genebank no: AF195819). The pairwise blast revealed no homology to the published *IFS* sequence, suggesting that the *IFS* gene was not detected in tomato using the above nested primers. With the standard nucleotide blast, an 80% match was found between this sequence and the EST of tomato from cell culture (genbank no:BI209400) and an 85% match with that of tomato radicle (genbank no: AW626375). This basically showed that the sequence identified is not *IFS* but another tomato gene.

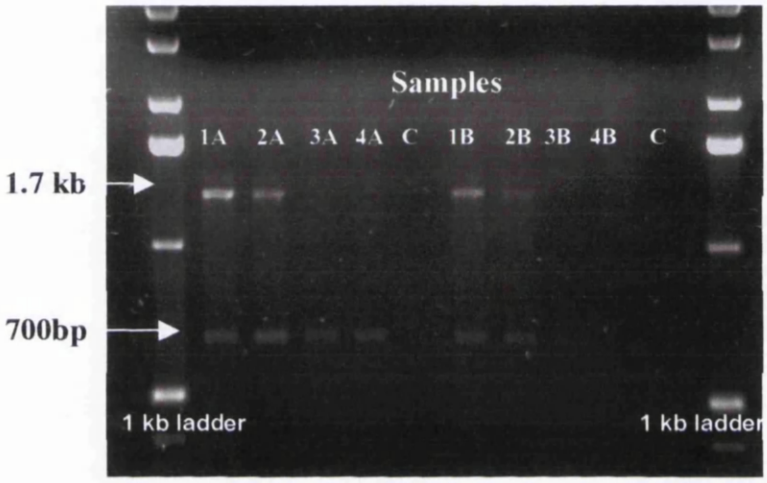


Figure 6.1: Gel electrophoresis of PCR product after amplification of tomato genomic DNA with nested *IFS* primers. 5 µL of the PCR product was used with 7 µL H_2O and 3 µL loading buffer for gel electrophoresis with 1% agarose. C denotes negative controls, i.e., samples with distilled H_2O instead of genomic DNA as template. A & B are duplicate samples of tomato genomic DNA at varying concentration as indicated below:

Sample	**1**	**2**	**3**	**4**	**C**
Temperature	50.0 °C	51 °C	52°C	54 °C	53 °C
DNA A	75 ng uL^{-1}	150 ng uL^{-1}	210 ng uL^{-1}	300 ng uL^{-1}	0
DNA B	110 ng uL^{-1}	220 ng uL^{-1}	330 ng uL^{-1}	440 ng uL^{-1}	0

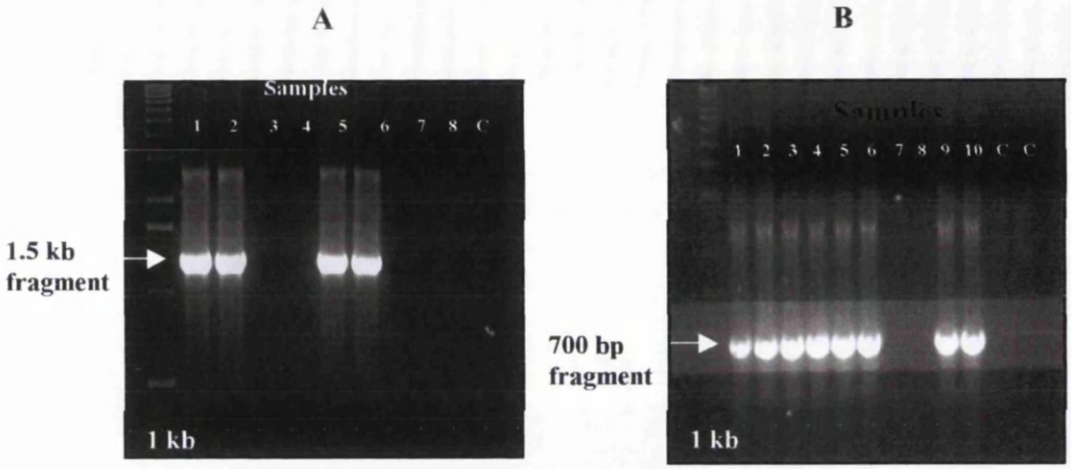


Figure 6.2: Gel electrophoresis of PCR product to confirm recombinant clones containing the 1.5 kb (A) and 700 bp (B) DNA fragment. 16 µL of the PCR product with 4 µL loading buffer was used for gel electrophoresis with 1% agarose. C denotes negative controls, i.e., samples with distilled H_2O instead of genomic DNA as template.

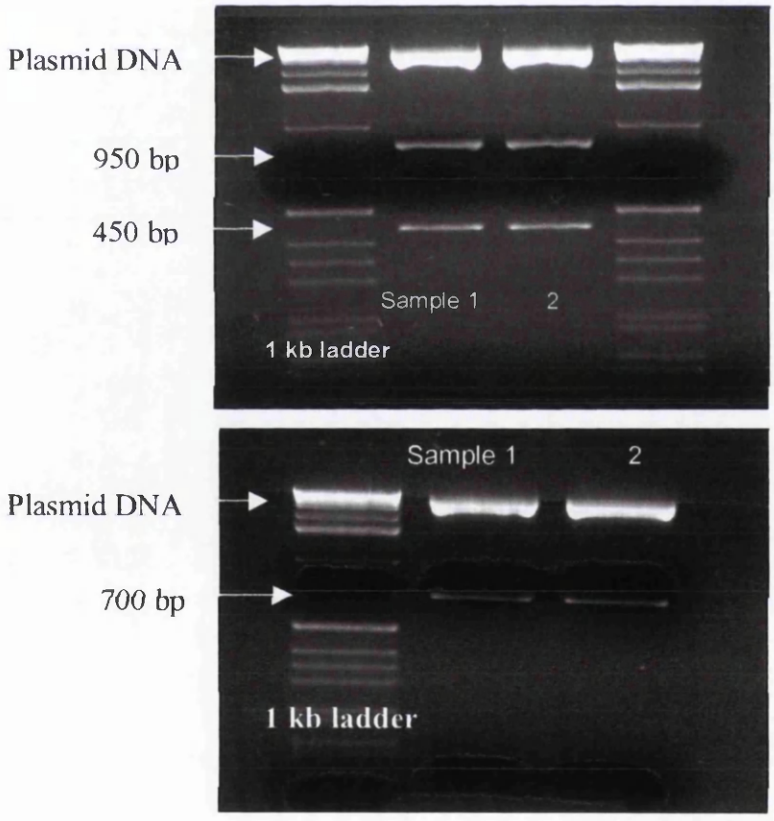


Figure 6.3: Gel electrophoresis of plasmid DNA containing the 1.5 kb (A) and 700 bp (B) fragment inserts after digestion with *Eco* R1. 20 µL reaction mixture was added to 5 µL of loading buffer for gel electrophoresis with 1% Agarose. There is an *Eco* R1 digestion site in the 1.5 kb insert causing it to digest further producing a 950 bp and 450 bp fragment.

6.4.2 Designing primers for the full length sequence of the *IFS* gene

Primer 3 (http://www.basic.nwu.edu/biotools/Primer3.html) was used to design primers to clone the full length *IFS* cDNA based on the published *IFS* cDNA sequence from *Glycine max* (soybean), Genebank No:AF195798. Since efficacy and sensitivity of PCR largely depend on the efficiency of primers (He *et al.*, 1994), precaution was taken to ensure the following: primers were selected to have a G+C content of approximately 50% and had a melting temperature (Tm) of 60°C. The difference in Tm between two primers within a primer pair was less than 3°C. Mis-priming was also minimized. Primers were synthesized by MWG Biotech. Since the purpose of the study is to clone *IFS* cDNA into a given vector for expression, short sequences with restriction sites were added prior to the 5' end of primer segments. The *Hpa I* restriction site was added to the forward primer and the *Xba I* was added to the reverse primer.

Their sequences are 5'-GTT ACC TCG GGA TCA CAG AAA CCA-3' for the forward primer and 5'-TCT AGA CCA GTA ACA CCT TAT TGA AAT GAT-3' for the reverse primer, respectively (The red letters denote the restriction sites). The primers were located 35 bp before the start codon and 65 bp after the stop codon on cDNA sequence (Appendix 1.1). The total length of the sequence is thus 1678 bp, of which 1566 bp is the coding region (Appendix 1.2).

6.4.3 Detection and cloning of *IFS* cDNA from *Glycine max* cv. Envy (soybean)

The purpose of this study was to detect the expression of the *IFS* gene in the various plant parts and then to subsequently clone the soybean *IFS* gene using the full length *IFS* primers designed in section 6.4.2.

Using the technique described in Section 6.3.6.1, soybean RNA was isolated from the roots, stem, cotyledons and leaves of one week old soybean seedlings and subsequently quantified at 260 nm absorbance. The integrity of the RNA was checked by running the RNA on a formaldehyde (FA) agarose gel as indicated in Section 6.3.7.2 (Figure 6.4). DNA was removed from RNA samples by using the DNA-free kit (Ambion) according to the manufacturer's instructions.

RT-PCR (as indicated in Section 6.3.7.4) was carried out to clone the *IFS* cDNA using oligo dT to prime the reaction and to generate a cDNA fragment containing the full coding region from the RNA. Following this, PCR of the cDNA was

carried out using the following thermal profile: 15 min of denaturation at 95 °C, followed by 30 cycles of elongation at 94 °C for 45 min, 59-64 °C for 1 min, 72 °C for 1 min 40 sec, and ended with an elongation step at 72 °C for 10 min. To detect the presence of *IFS* cDNA in the PCR products, gel electrophoresis was carried out. Only the roots had detectable *IFS* expression (Figure 6.5). The expression of *IFS* in the other plant parts may either be absent or too low for detection.

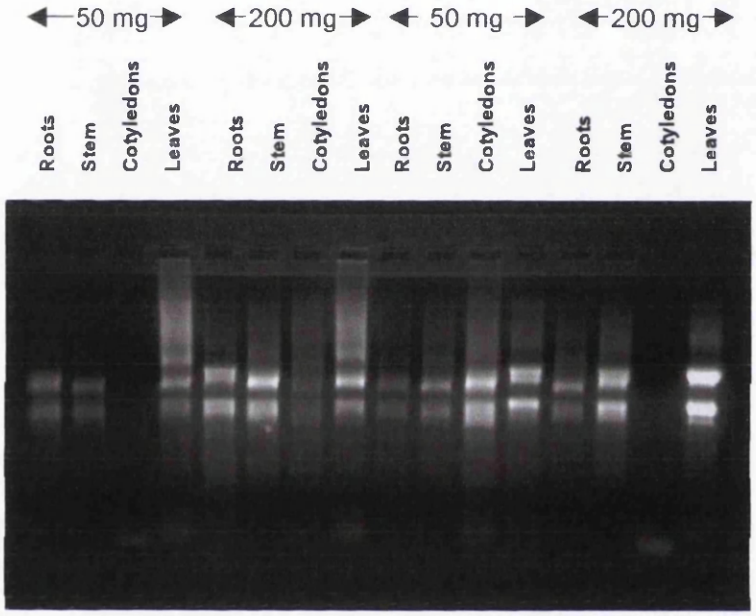


Figure 6.4. FA agarose gel of RNA extracted from the roots, stem, cotyledons and leaves of one-week old soybean seedlings. Varying amounts of plant tissue was used as indicated in the diagram.

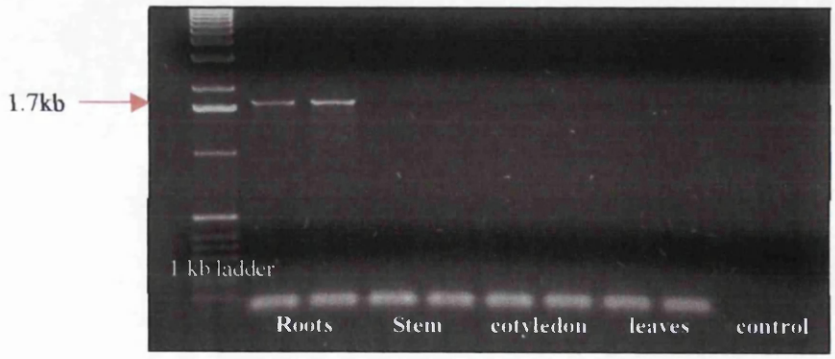


Fig 6.5. Gel electrophoresis of RT-PCR product after amplification of cDNA from various tissue parts of soybean using 'full-length' *IFS* primers. 8 µL reaction mixture was added to 7 µL of water and 7 µL of loading buffer in 1% agarose.

DNA was then extracted and purified from PCR products of the root following the protocol described in Section 6.3.8.1 and cloned into the pCR 2.1 Topo vector using the TOPO TA Cloning® Kit for Sequencing (Section 6.3.8.3). Recombinant clones containing the *IFS* insert were selected and confirmed by PCR using positive colonies as template (Figure 6.6) and by restriction analysis (*Ec0* RI) on purified plasmids. The plasmids were multiplied and then isolated and purified using the Q1prep Spin miniprep Kit as discussed in Section 6.3.8.7. The pCR4 TOPO vector -plasmids with the *IFS* insert were sequenced with oligonucleotides M13Uni (−21) and M13 Rev (−29). Sequence verification was performed using the NCBI Blast 2 Sequences engine (Tatusova and Madden, 1999) and compared with the GenBank database of published *IFS* cDNAsequence Genebank no:AF195819. We sequenced three independent clones, all were 99% identical to the published *IFS* sequence (Appendix 1.2) and showed differences in three nucleotides. However none of these changes altered the derived amino acid sequence. These differences may have resulted either from a PCR error or more likely from polymorphism differences between soybean cultivars.

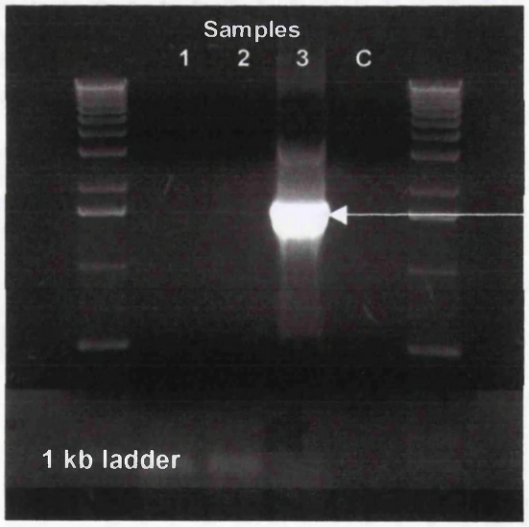


Figure 6.6. Gel electrophoresis of PCR product to confirm recombinant clones containing *IFS* insert. 16 µL of the PCR product with 4 µL loading buffer was used for gel electrophoresis with 1% agarose. C denotes negative controls, i.e., samples with dH_2O instead of genomic DNA as template. Only cDNA of sample 3 had the *IFS* insert.

6.4.4 Construction of *IFS* plant expression vector

6.4.4.1 35S::*IFS* from pGlasglow

A schematic diagram of the construction of the plant expression vector 35S::*IFS* from pGlasglow is illustrated in Figure 6.8. In pGlasglow, the *Luciferase* gene was removed by restriction digestion at the *Hpa* I and *Xba* I site (Figure 6.7) according to the protocol outlined in Section 6.3.8.2. The full length *IFS* cDNA, was isolated from pCR 2.1 Topo vector using the same restriction sites. These digest were then run on an agrose gel and the *IFS* cDNA and the linearized vector was removed. T_4 DNA ligase was used to join the compatible sticky ends of these two DNA fragments, and then transform the resulting recombinant plasmid into bacteria (Sections 6.3.8.2 and 6.3.8.6). The 35S::*IFS* binary vector derived from the pGlasglow contains a kanamycin resistance marker gene for bacterial selection and a *Bar* (BASTA) resistance marker for plant selection.

6.4.4.2 G10-90::*IFS* from pER8

A schematic diagram of the plant expression pER8 vector is illustrated in Figure 6.9. *Xho* I and *Spe* I were used to cut Topo-*IFS* vector and the pER8 binary vector at the multiple cloning site. These digest were then run on an agrose gel and the *IFS* cDNA and the linearized vector was removed (Figure 6.10). T_4 DNA ligase was used to join the compatible sticky ends of these two DNA fragments, and then transform the resulting recombinant plasmid into bacteria (sections 6.3.8.2 and 6.3.8.6). The G10-90::*IFS* from pER8 contains a spectinomycin resistance marker gene for bacterial selection and a hygromycin resistant marker for plant selection. In addition, G10-90::*IFS* also adopted the estrogen receptor-based chemical-inducible system (Zuo *et al.*, 2000).

6.4.4.3 35S::*IFS* from pCHF3

The binary vector pCHF3, which contains a CaMV 35S full promoter, the RBCS terminator from pea, and the kanamycin and spectinomycin resistance gene for plant selection and bacterial selection respectively was also used in this study. This vector was used to transform tomato as BASTA resistance is not a suitable selectable marker in this host. The pCHF3 plasmid was cut at the restriction digestion sites with *Sma* I and *Xba* I and the resultant linearized plasmid was ligated with the *IFS* cDNA, which was isolated from pCR 2.1 Topo vector (Figure 6.11).

All these chimeric plasmids, containing the *IFS* cDNA insert, were confirmed by PCR using the *IFS* primers and by restriction analysis on purified plasmids. To confirm the correct orientation of the *IFS* cDNA insert, restriction fragment mapping analysis by further digestions with appropriate restriction sites were preformed as indicated in Figure 6.10. The plasmids were transformed into *E. coli* DH5α cells (as outlined in Section 6.3.8.6), multiplied and then isolated and purified using the Q1prep Spin miniprep Kit as discussed in Section 6.3.8.7

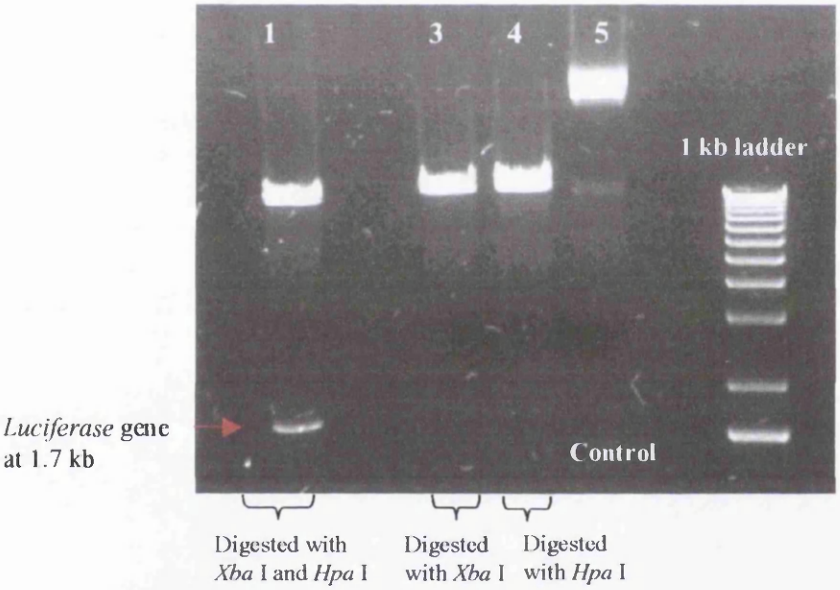


Figure 6.7 Gel electrophoresis after digestion of pGlasglow with *Xba* I and *Hpa* I (lane 1) to remove the 1.7 kb *Luciferase* gene. Digestion with a single restriction enzyme (lane 3 and 4) resulted in no release of the luciferase gene. Lane 5 is the undigested plasmid. 20 μL reaction mixture was added to 5 μL of loading buffer in 1% agarose.

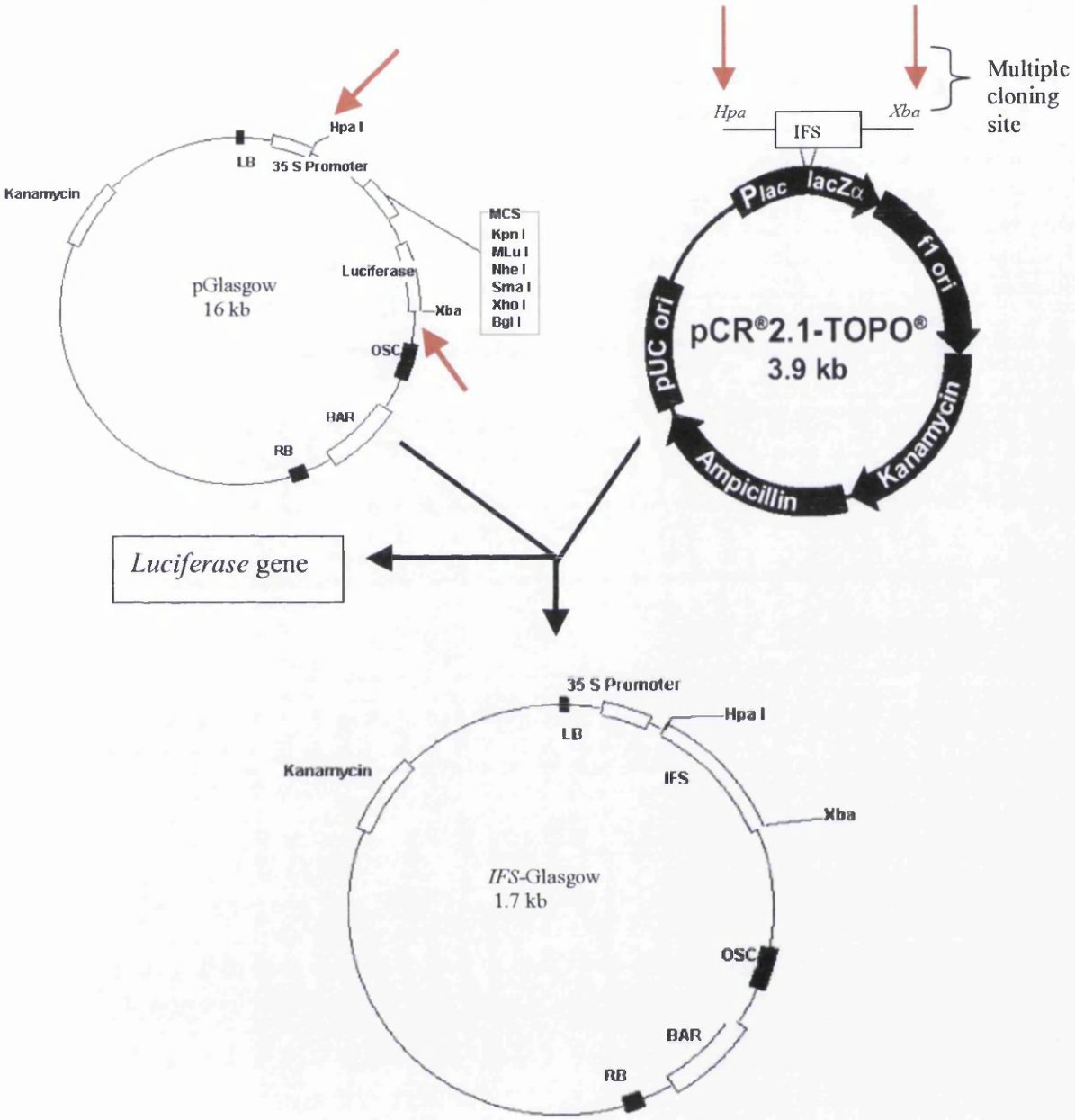


Figure 6.8. Construction of the 35S::*IFS* from pGlasglow. The *Luciferase* gene was removed by restriction digestion at the *Hpa* I and *Xba* I site and the resultant linearized plasmid was ligated with the *IFS* cDNA, which was isolated from pCR 2.1 Topo vector. LB: left border; RB: right border; MCS; multiple cloning site; OSC: operator sequence

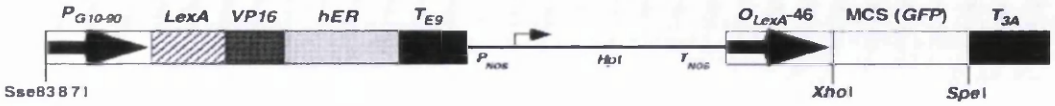


Figure 6.9. A schematic diagram of the pER8 vector.
Only the region between the right and left borders is shown (not to scale). P_{G10-90}, a synthetic promoter controlling XVE; XVE, DNA sequences encoding a chimeric transcription factor containing the DNA-binding domain of LexA (residues 1−87), the transcription activation domain of VP16 (residues 403−479) and the regulatory region of the human estrogen receptor (residues 282−595); T_{E9}, rbcS E9 poly(A) addition sequence; P_{nos}, nopaline synthase promoter; HPT, hygromycin phosphotransferase II coding sequence; T_{nos}, nopaline synthase poly(A) addition sequence; O_{LexA}, eight copies of the LexA operator sequence; −46, the −46 35S minimal promoter; MCS, multiple cloning sites for target genes; T_{3A}, rbcsS3A poly(A) addition sequence. Arrows indicate the direction of transcription. (adapted from Zuo *et al,* 2000)

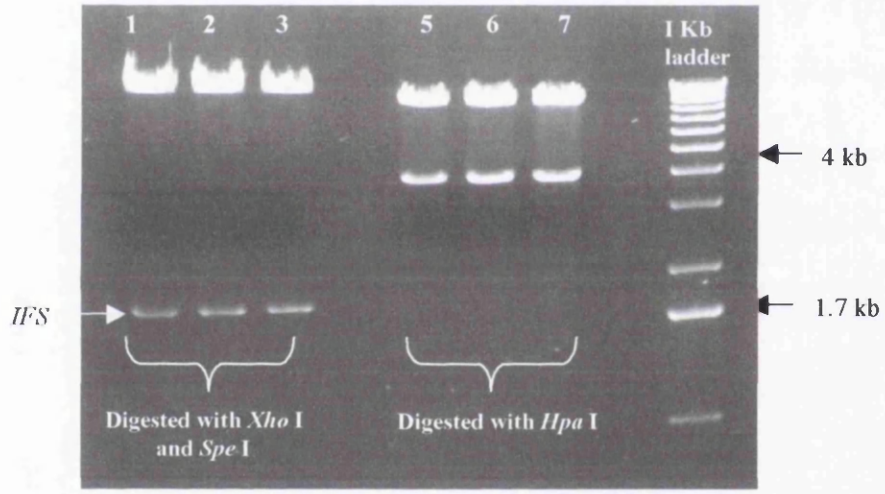


Figure 6.10. Gel electrophoresis after digestion of G10-90::*IFS* from pER8 with *Xho* I and *Spe* I (lane 1-3) to remove the 1.7 kb *IFS* gene. Digestion with *Hpa* I (lane 5-7) was to confirm the orientation of the *IFS* gene. With correct orientation a 4 kb DNA fragment is released from the vector as observed above. 20 µL reaction mixture was added to 5 µL of loading buffer in 1% agarose.

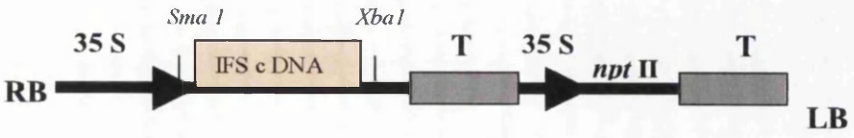


Figure 6.11. A schematic diagram of the 35S::*IFS* from pCHF3.
The pCHF3 plasmid was cut at the restriction digestion sites with *Sma* I and *Xba* I site and the resultant linearized plasmid was ligated with the *IFS* cDNA, which was isolated from pCR 2.1 Topo vector. LB: left border; RB: right border; 35S, cauliflower mosaic virus 35S promoter, T, pea rubisco 3' terminator.

6.4.5 Introduction of recombinant plasmids into agrobacterium

The plant expression vectors 35S::*IFS* from pGlasgow and pCHF3 and G10-90::*IFS* from pER8 were transformed into competent *A. tumefaciens*, strain GV3101 as outlined in Section 6.3.9. After transformation, the agrobacterium were plated out on gentamycin and kanamycin plates for 35S::*IFS* from pGlasgow and on gentamycin and spectinomycin for 35S::*IFS* from pCHF3 and G10-90::*IFS* from pER8. Colonies were picked to inoculate LB medium, which was left to grow overnight. Confirmation of the *IFS* inserts in the agrobacterium cultures were carried out by PCR using *IFS* primers (Figure 6.12). Positive cultures were used to proceed with the transformation of the *IFS* cDNA into plants.

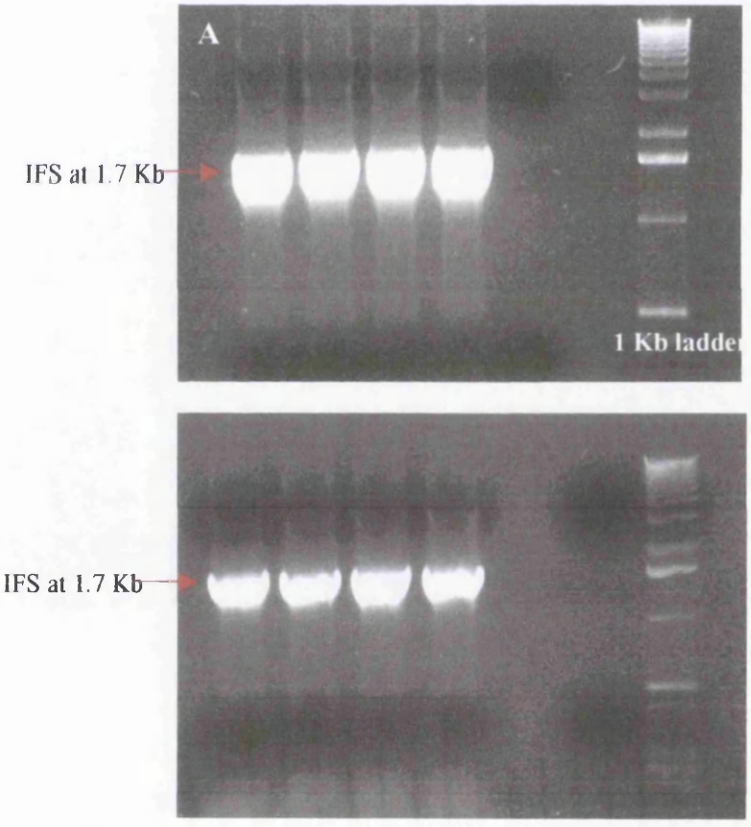


Figure 6.12. Gel electrophoresis of PCR product to confirm recombinant plasmids clones containing *IFS* insert in agrobacterium. A: agrobacterium-G10-90::*IFS* from pER8 ; B: agrobacterium-35S::*IFS* from pGlasglow. 16 μL of the PCR product with 4 μL loading buffer was used for gel electrophoresis with 1% agarose.

6.4.6. Agrobacterium-mediated transformation into Arabidopsis and tomato

6.4.6.1 Arabidopsis tansformation

The agrobacterium cultures with 35S::*IFS* (from pGlasglow) and G10-90::*IFS* (from pER8) were used to transform Arabidopsis by floral dip method as outlined in Section 6.3.9. The Arabidopsis plants that were infiltrated with the agrobacterium were returned to the growth room and left to set seeds. After several weeks, the plants were left to dry and the seeds collected and surface sterilised as indicated in Section 6.3.4.2. The 35S::*IFS* (from pGlasglow) seeds from agrobacterium-infiltrated plants were sown on soil and the transgenic plants were selected for basta resistance as outlined in Section 6.3.5.2. In contrast, G10-90::*IFS* (from pER8) seeds from agrobacterium-infiltrated plants were sown on selection plates containing hygromycin. For both selections, a control, Col 0, the wild-type untransformed lines were also sown. After 2 weeks the robust-growing G10-90::*IFS* transformants were transplanted from the selection media onto soil.

6.4.6.2 Tomato transformation

In tomato, the tissue culture technique was adopted whereby cotyledons produced from sterile seeds were excised and used as explants following the protocols outlined in Section 6.3.9. The explants were cocultivated in agrobacterium containing the 35S::*IFS* (from pCHF3) and G10-90::*IFS* plasmids. These explants were then placed on selection plates containing hygromycin or kanamycin for G10-90::*IFS* and 35S::*IFS* transformants respectively. Carbencillin was also added onto the media to control the agrobacterium growth. Regenerated plants with shoot length of approximately 2-4 cm were excised from the callus and transferred onto a rooting medium in sterilized magenta boxes. The various stages of plant regeneration are illustrated in Figure 6.13. Within four weeks, transformed shoots which developed good rooting system were transferred to soil. These plantlets were placed in trays and covered to increase humidity and reduce light intensity for at least a week before exposed to normal conditions.

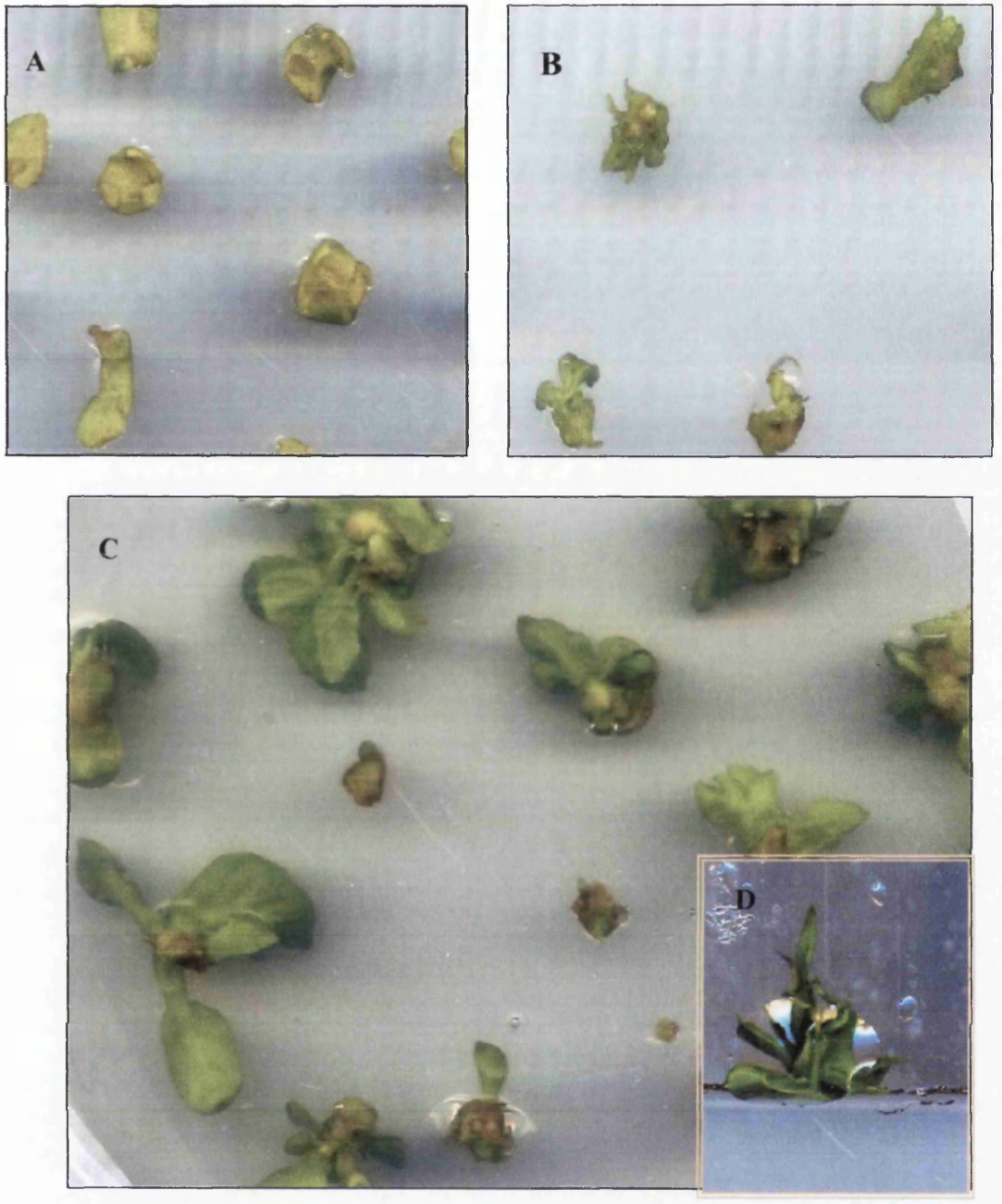


Figure 6.13. Various stages of tomato plant regeneration after infiltration with agrobacterium containing 35S::IFS plasmids. A- one week old explants showing signs of callus induction; B- shoot formation on 2.5-week old explants ; C- shoot elongation and leaf production in 4-week old explants; D- root formation on 5.5-week old explants in majenta boxes

6.4.7 Confirmation of *IFS* transgenic plants

Genomic DNA was isolated from the leaves of the primary (T1) antibiotic-resistant plants using the protocol specified in Section 6.3.6.1. To detect the transgenic *IFS* lines, PCR was carried out using the *IFS* primers.

A total of 98 PCR-positive *IFS* transformants lines were obtained from Arabidopsis and 4 PCR positive lines were obtained from tomato (Figure 6.14). No transformants were obtained in tomatoes when the pER8 binary vector was used. This may be due to the sensitivity of growth of tomato callus to the selection marker, hygromycin.

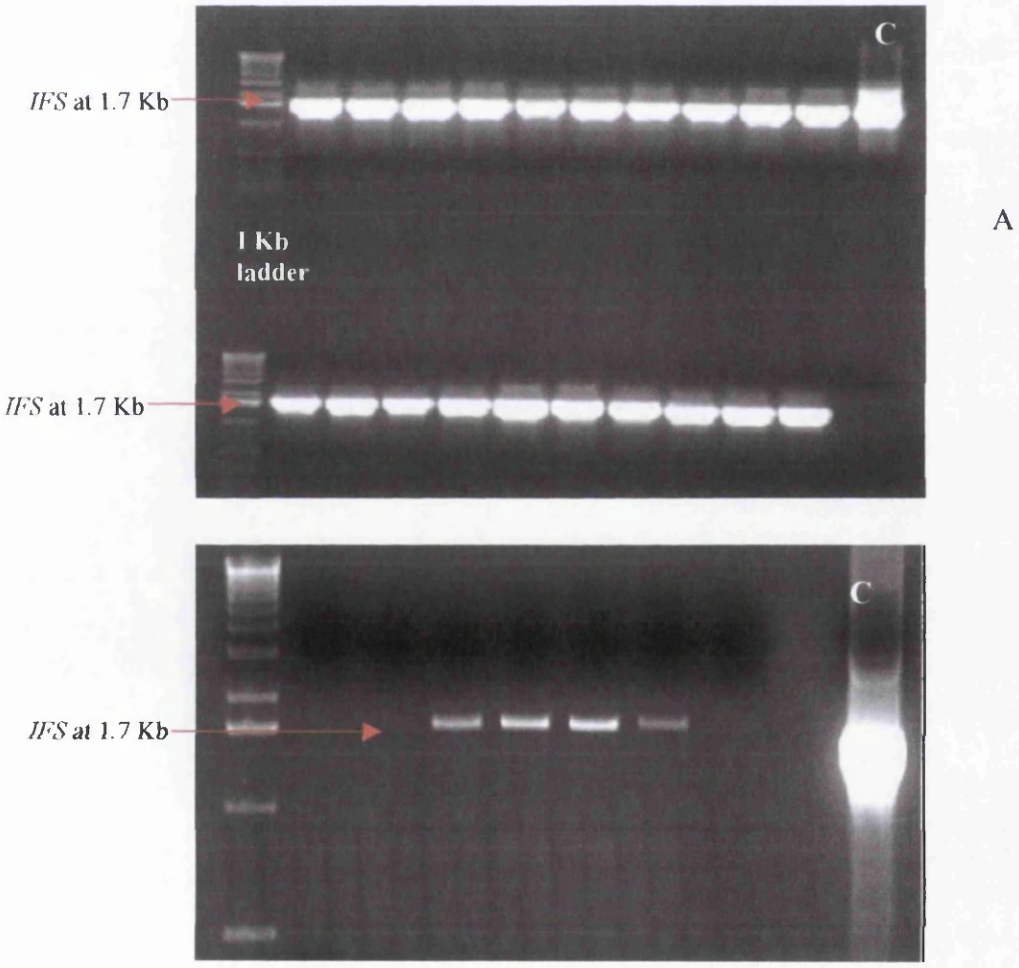


Figure 6.14. Gel electrophoresis of PCR products using *IFS* primers of (A) antibiotic-resistant T1 Arabidopsis plants (B) antibiotic resistant tomato plants. 16 µL of the PCR product with 4 µL loading buffer was used for gel electrophoresis with 1% agarose. C denotes positive control, i.e., samples with 35S::*IFS* plasmid DNA as template

6.4.8 *IFS* transgene expression

Independent PCR positive Arabidopsis transformants containing *IFS* were selfed, segregation ratios were determined based on their respective antibiotic/basta resistance, and then the process was repeated to identify lines homozygous for a single transgene locus. Seedlings of a homozygous line were germinated on the appropriate selective plates and were then transferred after 2 weeks to soil. 60 μM estradiol was sprayed to induce *IFS* transgene expression in pER8 Arabidopsis transformants. After 24 h, leaves were harvested to investigate the *IFS* transgene expression. Total RNA, extracted from leaves of the PCR-positive T2 /T3 transformants in Arabidopsis and T1 transformants in tomato (protocol outlined in Section 6.3.7.1) were used for Northern Blot analysis (protocol outlined in Section 6.3.7.3). In the Northern blots, full length *IFS* cDNA amounting to 50 ng μl^{-1} isolated from the 35S::*IFS* plasmid was used as the probe to hybridized the RNAs. RNA extracted from soybean was used as the positive control.

6.4.8.1. Quantification of *IFS* transgene expression

To offset the variation of the quantity of RNA loaded from lane to lane, for each lane, the quantity of hybridizing RNA was divided by the value of the total RNA. *IFS* transcript expression levels (arbitrary units) relative to the amounts of total RNA were then presented.

A. Arabidopsis

Out of the 20 PCR positive T2 homozygous 35S::*IFS* lines (from pGlasglow), only one hybridised the *IFS* probe in the RNA leaf samples (Figure 6.15). In contrast, nineteen T2 homozygous lines out of thirty G10-90::*IFS* (from pER8) line exhibited transcripts levels of the *IFS* transgene as illustrated in the Northern Blot analysis (Figure 6.17). No hybridisation was observed in any of the G10-90::*IFS* lines when no induction with estradiol was carried out (Figure 6.16), suggesting that the G10-90 promoter from pER8 activation system is tightly regulated and highly inducible in Arabidopsis (Zuo, *et al.*, 2000). When the *IFS* transcript level of 35S::*IFS* or G10-90::*IFS* were compared (in a single filter), a 3-10 fold higher expression level was observed in Arabidopsis transformed by pER-8 vector as indicated in Figure 6.18. In addition to this, the expression observed with the G10-90::*IFS* was also about 10-fold higher than that of soybean. These results indicate that in Arabidopsis, *IFS* transgene is

effectively transcribed and pER8 is a better plant expression vector compared to pGlasglow. The moderate to highly expressed lines, that is, lines 13, 14, 15, 16 and 19 were selected for the detection of the isoflavone, genistein.

B. Tomato

No hybridization was observed in all the four PCR positive tomato lines indicating very low *IFS* gene expression in tomato.

6.4.9 Phenotypical characterization of transgenic plants

Transgenic Arabidopsis plants overexpressing the *IFS* gene displayed no characteristic phenotype and they resembled the wild-type plants thoughout their life cycle.

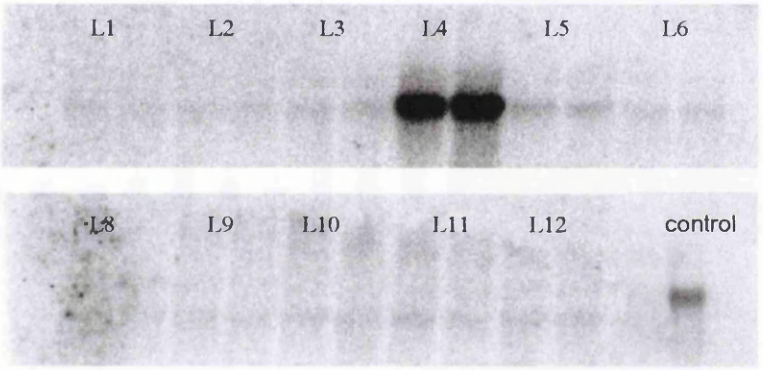


Figure 6.15. Northern blot of T2 Arabidopsis 35S::*IFS* lines (from pGlasglow). Sample are applied in duplicates. Only line four (L4) exhibited transcript levels of the *IFS* transgene. Control - positive control from soybean.

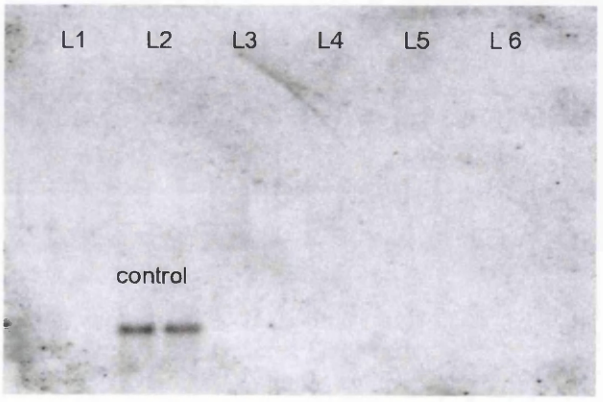


Figure 6.16. Northern blot of Arabidopsis T2 G10-90::*IFS* lines (from pER8) before induction with estradiol. Sample are applied in duplicates. None of the line exhibited transcripts levels of the *IFS* transgene. Control - positive control from soybean.

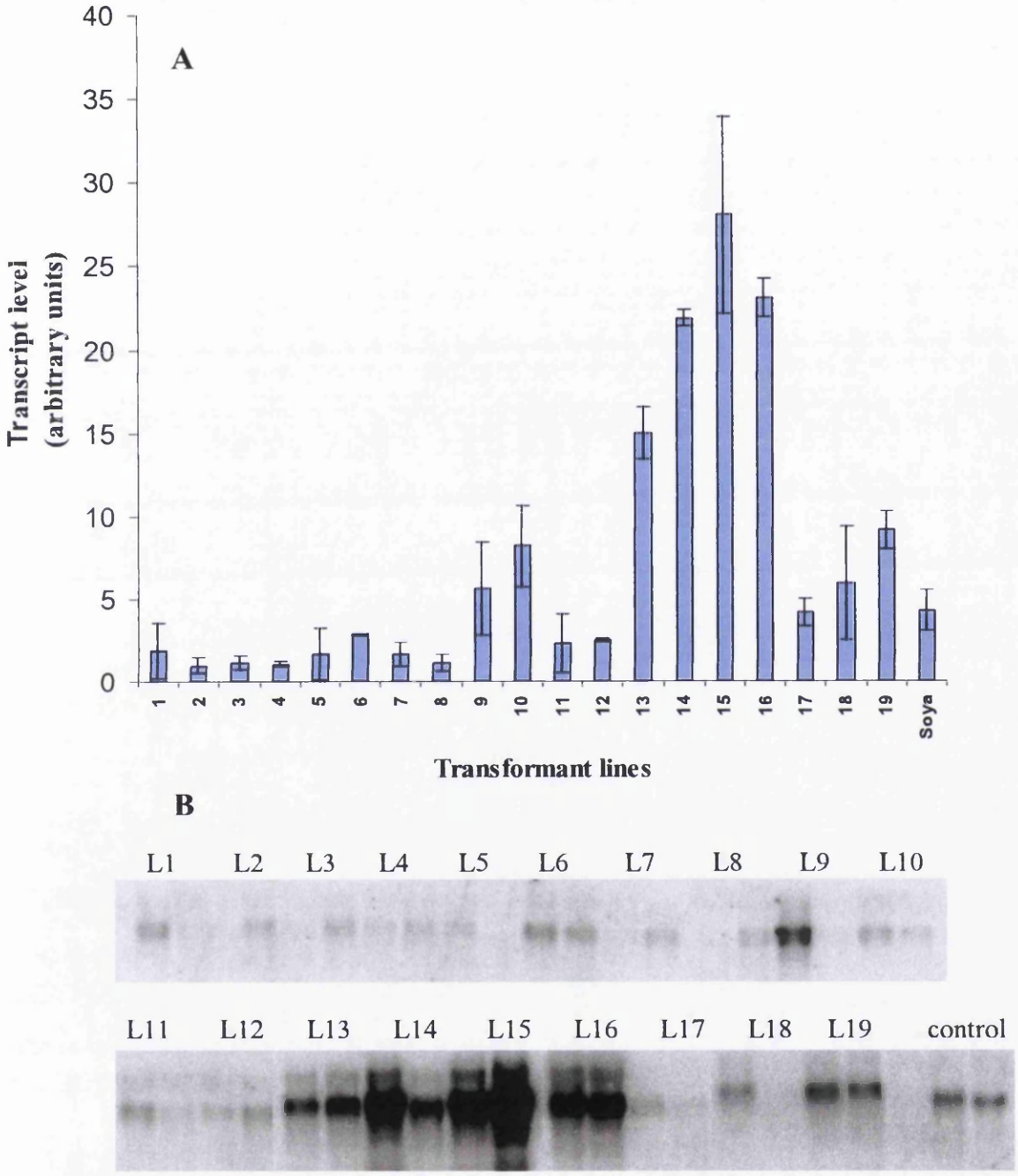


Figure 6.17. A: *IFS* transcript expression levels (arbitrary units) relative to the amounts of total RNA are presented for all the independent transgenic lines. Error bars indicate standard deviation.
B: Northern blot of Arabidopsis T2 G10-90::*IFS* lines. Samples are applied in duplicates. A total of 19 lines exhibited transcript levels of the *IFS* transgene. C is a positive control from soybean.

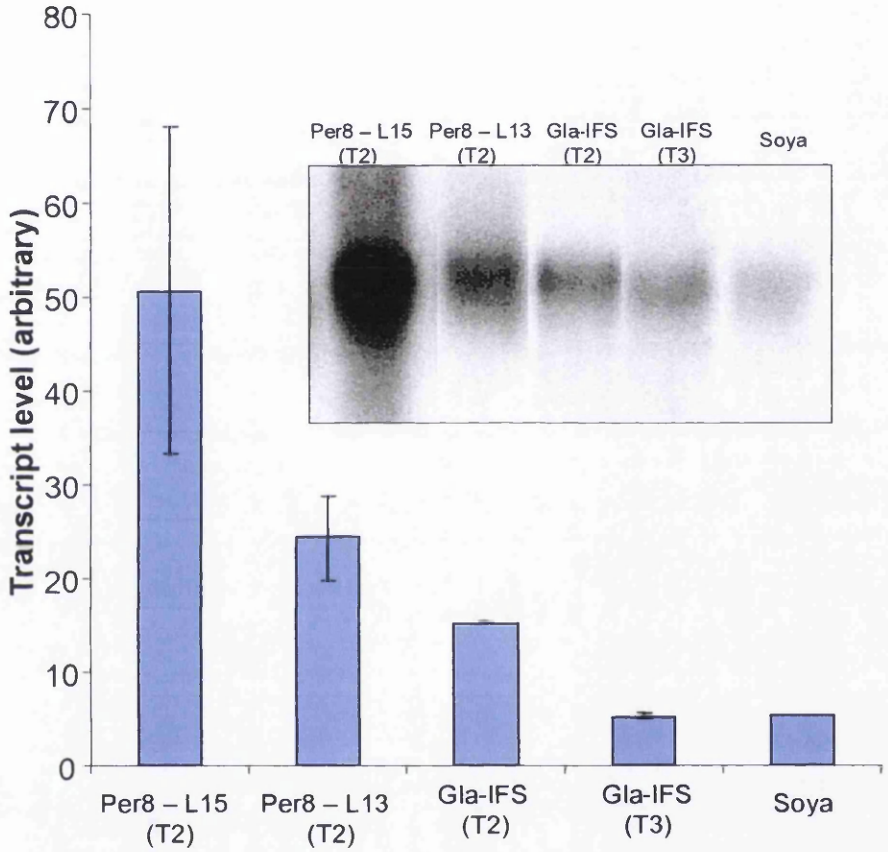


Figure 6.18. Comparison of the *IFS* transcript expression levels (arbitrary units) relative to the amounts of total RNA are presented for soybean and the highly expressed Arabidopsis lines transformed by pER8 and pGlasgow binary vectors. Northern blot indicating the expression levels of these transformants on the same filter membrane is also presented. Error bars indicate standard deviation.

6.4.10 Flavonoid analysis

6.4.10.1. Detection of genistein in transgenic plants

G10-90::*IFS* (from pER8) lines, 13, 14, 15, 16 and 19 and the single 35S::*IFS* (from pGlasglow) that expressed the *IFS* transgene in Arabidopsis were ground to a fine powder in liquid nitrogen as outlined in Section 6.3.11 and analyzed for genistein accumulation by using the HPLC-MS^2 and diode array detection. No fragmentation pattern or peak corresponding to the genistein standard was observed in any of these six independent transformant lines (Figure 6.19).

6.4.10.2. Induction of phenypropanoid pathway by UV-B

Availability of the naringenin substrate for *IFS* may be a factor limiting the synthesis of genistein in Arabidopsis as suggested by Jung *et al.* (2000). To further investigate the relationship of genistein production and phenylpropanoid pathway activity in Arabidopsis plants expressing *IFS*, the effect of stress induction of the pathway on the genistein production was investigated. As in other plant species, treatment of Arabidopsis plants with UV-B light has been shown to cause an increase in flavonoid accumulation in the leaves (Li *et al.*, 1993), indicating an increase in flux in the phenylpropanoid pathway, which in turn activates the production of the substrate naringenin.

The selected Arabidopsis transformants were placed directly under UV-B light for 6 hours following the protocol outlined in Section 6.3.5.1. Immediately following the UV-B treatment, the above-ground tissues of each UV-treated plant, as well as of untreated control plants, were ground to a fine powder in liquid nitrogen as outlined in Section 6.3.11, prior to analysis for genistein and naringenin accumulation by HPLC-MS^2. No naringenin or genistein was detected in the control plants or in the *IFS* transformants after UV-B treatment. These results prompted us to investigate whether the *IFS* gene was functioning *in vivo* and synthesising the IFS protein.

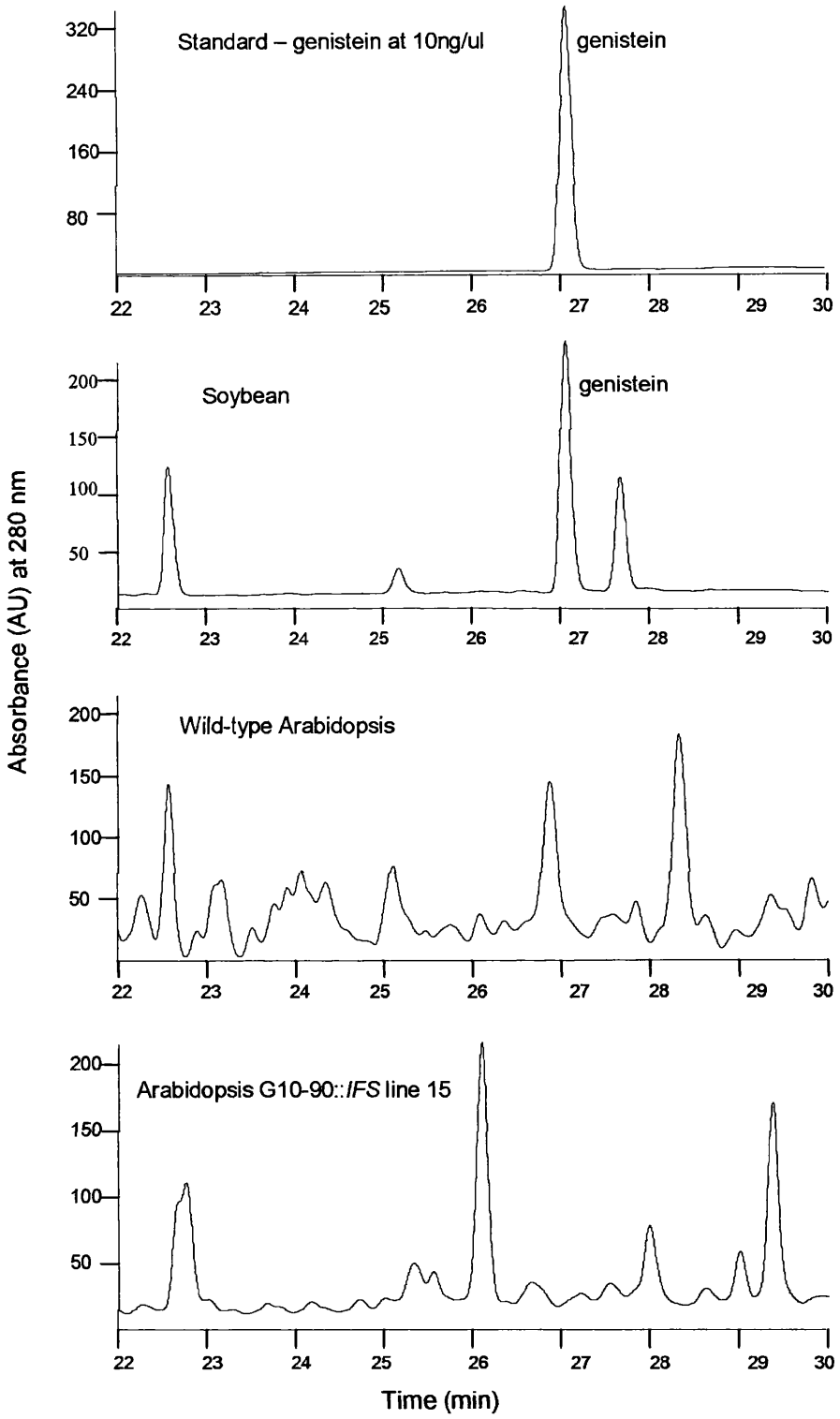


Figure 6.19. Comparison of a 15-40% gradient HPLC chromatogram of wild-type and transgenic Arabidopsis to a genistein standard and to soybean root samples. Genistein is only detected in soybean.

6.4.11. IFS protein analysis

Presence of the IFS protein was assessed in leaves of the highly and moderately expressed transgenic Arabidopsis lines using an antibody raised to a synthetic peptide derived from the amino acid sequence of the IFS protein (Section 6.3.10.3). Because the cytochrome P450 IFS is a membrane bound enzyme, both total protein and membrane protein isolation were carried out as outlined in Section 6.3.10.1 and 6.3.10.2 on transformed Arabidopsis leaves. These were then used for western-blot analysis.

The IFS antibody cross-reacted to a number of proteins of variable molecular sizes in both wild-type and transformed Arabidopsis as indicated in Figure 6.20. A very strong cross-reaction of IFS antibody with an unknown protein of about 250 kDa was observed in both the wild-type and transformed Arabidopsis (marked by a yellow arrow in Figure 6.20). The IFS antibody bound consistently to a band of 59 kDa in soybean, which is equivalent to the size of an IFS protein (Yu *et al.*, 2000) in both the total protein and membrane protein extracts. A slightly larger protein of approximately 62 kDa in two independent Arabidopsis transformed *IFS* lines (G10-90::*IFS*) cross-reacted with the IFS antibodies which was absent from extracts of wild-type Arabidopsis (Figure 6.20-A, marked with a violet arrow). A corresponding higher *IFS* transcript of transformed lines was also observed in the Northern blots compared to that of soybean (Figure 6.20-C). The larger transcript seen here is presumably due to the extra addition base pairs added by the T-DNA of the vector pER8. This may have translated to a larger sized protein as observed in Figure 6.22-A. Similar size bands were however not detected in the transgenic lines when membrane protein isolation was carried out (Figure 6.20-B). These results tend to suggest that IFS protein did accumulate in the leaves of transgenic Arabidopsis but were not membrane-bound as it would be in its natural environment.

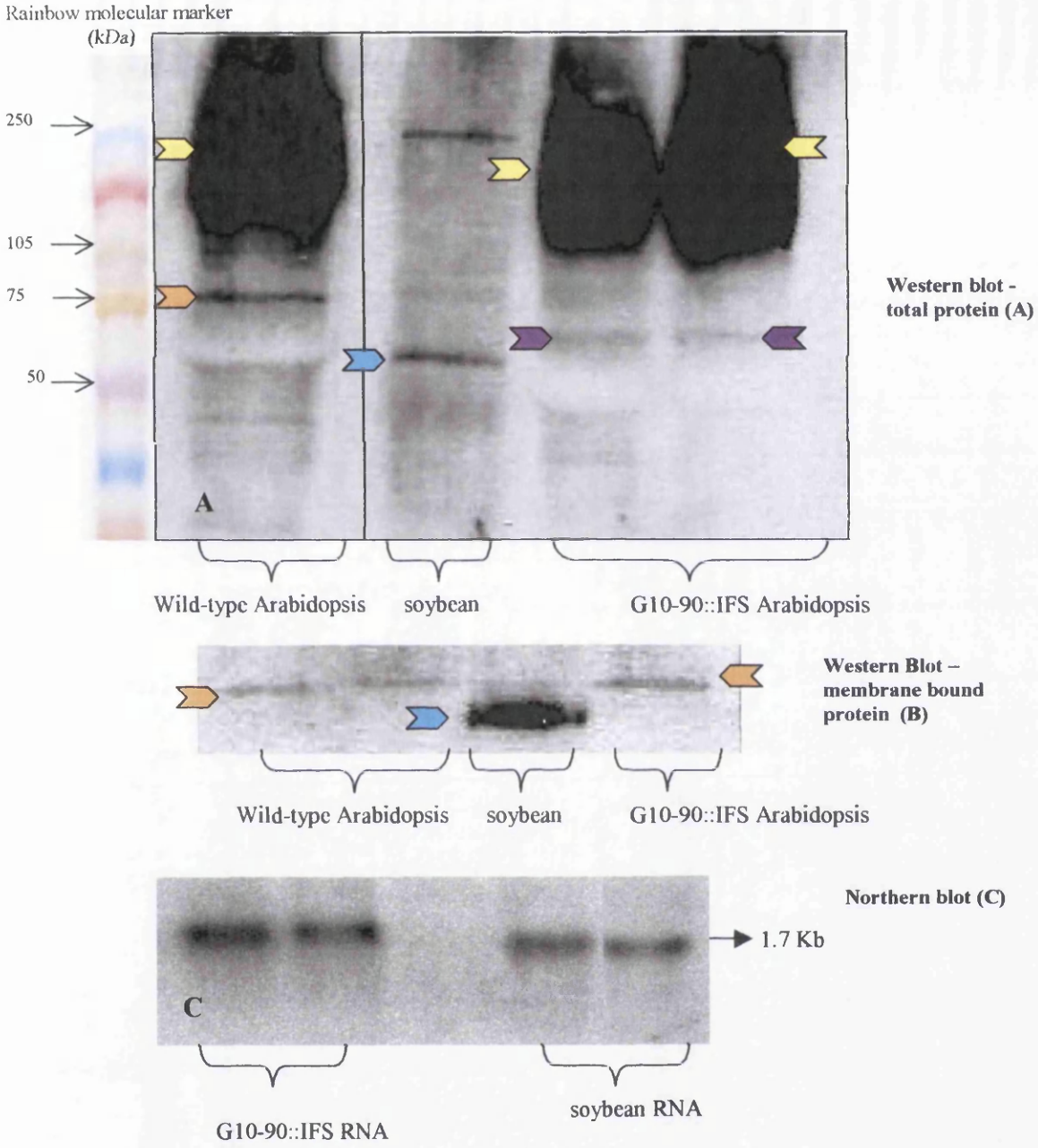


Figure 6.20. Western (A, and B) and northern (C) blots of Arabidopsis transformants compared to its wild-type and soybean. A was blotted with total proteins while B was blotted with membrane bound proteins.

- In Blot A, an unknown protein of band size of approximately 250 kDa cross-reacted strongly with the antibody in Arabidopsis but very lightly in soybean, marked by the yellow coloured arrow.
- In Blot A & B, there was a modest but consistent binding to a band of 59 kDa indicated by the blue coloured arrow in soybean (which is equivalent to the size of the IFS protein).
- In Blot A, in the two independent pER8 transformant lines there was also reaction with the band of about 62 kDa which was absent from extracts of wild-type Arabidopsis, marked by a violet arrow.
- In wild-type Arabidopsis, the antibody reacted with a protein which was also present in the transgenic lines (Blot B), indicated by an orange coloured arrow.
- The *IFS* transcripts in the northern blot (C) are slightly larger that the *IFS* transcript from soybean due to the extra base pairs added by the T-DNA of the pER8 vector. This may have translated to a larger protein size as seen in blot A indicated by the violet coloured arrow.

6.5 Discussion

Two classes of genes can be distinguished within the flavonoid pathway: (a) the structural genes encoding enzymes that directly participate in the formation of flavonoids, and (b) regulatory genes that control the expression of the structural genes. Manipulation of the flavonoid pathway can be carried out by either down regulating or over-expressing these genes. In this study, manipulations were only carried out on the structural genes. A foreign structural gene from soybean, that is *IFS* was introduced and over-expressed in Arabidopsis and tomato to metabolically engineer a new isoflavonoid pathway that branches off from the existing phenylpropanoid pathway with the aim of leading to the production of isoflavones such as genistein.

6.5.1 *IFS* is not detected in tomato

Results from this study reveal that the *IFS* gene is not present in tomatoes. This is in accordance to the many reports that indicate isoflavonoids branch pathway is only prevalent in the Papilonoideae subfamily of the Leguminosae. (Dixon and Steele, 1999; Graham, 1991). The absence of this branch pathway in most crops is due to the absence of the *IFS* gene. Although isoflavones such as genistein are formed from the flavanone, naringenin, which are ubiquitously present in plants, their formation involves an unusual aryl migration reaction, which is only catalyzed by the cytochrome P450 enzyme, IFS enzyme (Steele *et al.*, 1999; Jung *et al.*, 2000).

6.5.2 Tissue specific localization of *IFS* gene expression

In this study, the full length *IFS* cDNA from soybean roots was cloned. IFS mRNA was not detected in the other soybean plant parts. This is consistent with the physiological roles of isoflavonoids as defense compounds against pathogens and signal molecules to symbiotic bacteria in soybean (Dixon & Steele, 1999). Recent investigation carried out by Subramanian *et al.* (2004a) provided direct genetic evidence that isoflavones are essential for nodule formation in soybean. In addition to this, the *IFS* expression was also found to be tissue-specific in response to both nodulation and defence signals. This was found to be due to the root-specific activity of the endogenous *IFS* promoter in soybean (Subramanium *et al.*, 2004b).

6.5.3 Inducible versus a constitutive promoter

The availability of a broad spectrum of promoters that differ in their ability to regulate the temporal and spatial expression patterns of the transgene can dramatically increase the successful application of transgenic technology (Potenza *et al.*, 2003). Due to this, two types of promoters were used, the CaMV 35S and an estrogen-inducible promoter G10-90. The CaMV 35S promoter is haboured in the pGlasglow and the pCHF3 plant expression vectors while the G10-90 promoter is in pER8 inducible vector.

The CaMV 35S promoter is valuable not only because it delivers high expression in virtually all regions of the transgenic plant, but also because it is readily obtainable in research and academic settings, and available in plant transformation vector cassettes that allow for easy subcloning of the transgene of interest (Potenza *et al.*, 2003). However, there are also a number of limitations in the use of virally derived promoter. When multiple copies of the transgene integrate into the genome, the probablity of transcriptional gene silencing is higher when viral promoters are used compared to constitutive promoters of plant origin (Meyer & Saedler, 1996; Potenza *et al.*, 2003).

In some instances localized and targeted gene expression may also be more appropriate especially where the constitutive overexpression of transgenes may interfere with normal processes in a plant. This may be particularly important in the synthesis of genistein in non-legume crops. In their host plant, as metioned earlier *IFS* promoter is tissue-specific and is expressed mainly during root nodulation and pathogenic attack (Subramanium *et al*, 2004b). This together with the fact that genistein exhibits antimicrobial, anti-insecticidal and is a tyrosine kinase inhibitor (Dixon, 1999) indicate that the localization or spatial distribution of genistein is important. Exposure to genistein in all plant tissues over the life cycle of the plant may therefore have undesirable effects on the plant. Due to this an estrogen inducible G10-90 promoter haboured in the pER8 vector was used.

6.5.4 Agrobacterium mediated transformation

This method takes advantage of the 'natural' plant genetic transformation system that evolved in agrobacterium. The T-DNA is removed from agrobacterium and replaced with multicloning sites where genes of interest as well as dominant selectable

markers are integrated. The agrobacterium harboring recombinant Ti plasmids can then be introduced onto wounded tissues (e.g.,leaf explants in culture) or even directly onto mature plant organs and in doing so, the bacterium will transfer the modified T-DNA to some of the cells of the host plant (Lessard *et al.*, 2001). This system is relatively straightforward using the floral dip method as carried out in Arabidopsis. Using this method more than 90 PCR positive transformants were obtained, out of which 50 were selected for further investigation. However if complete regenerated plants are required as in tomatoes, then the process is complicated by the need for tissue culture-mediated plant regeneration. Despite considerable effort over the years, plant regeneration through tissue culture remains difficult, problematic, and time consuming. In some cases, unwanted somaclonal variation has been introduced through the tissue culture regeneration system (Lessard *et al.,* 2001). In this study only 4 PCR positive independent tomato plants were regenerated through tissue culture. Most of the plants that regenerated on kanamycin (selection marker) were found to be false positives. When a more stringent marker, hygromycin, was used none of the callus regenerated into plantlets as observed with explants transformed with agrobacterium containing G10-90::*IFS* derived from pER8 vector.

6.5.5 pER8 a better plant expression vector

Due to the nature of the pER8 construct and its promoter (Figure 6.9), a 3-10 fold higher gene expression level was observed in Arabidopsis transgenic plants transformed by pER8 binary vector as compared to those transformed with pGlasglow (Figure 6.18). This is in keeping with the investigations carried out by Zoa *et al.* (2000) where upon induction, the pER8 promoter is capable of stimulating expression of a *GFP* transgene by eight folds higher than that obtained with a 35S::*GFP* transgene. In the present study, 63% of the PCR positive lines exhibited *IFS* transgene expression with the G10-90 promoter derived from pER8 vector. In contrast, only one line out of the 20 PCR positive lines exhibited *IFS* transgene expression when a 35S promoter was used. Similarly, when pCHF3 plasmid habouring the CaMV 35S promoter was used in tomatoes, very low gene expression was observed. A plausible explanation could be that due to the constitutive nature of the promoter, there may have been a natural plant defense mechanism by the host plant towards the introduced foreign DNA sequences and DNA modification such as:

- methylation of the promoter and/or coding region (Scheid *et al.*, 2002)

- chromatin remodeling and excision of foreign DNA (reviewed in Kumpatla *et al.*, 1998),
- homologous or ectopic pairing when multiple copies of the transgene integrate into the genome (Meyer & Saedler, 1996).

All these possibilities could have rendered inactivation of the transgene.

This study clearly indicates that the exogenous inducible promoter in the pER8 plant expression vector was far more superior in inducing the *IFS* gene expression. In addition to reduced silencing, this system can also be applied at the discretion of the researcher to turn the transgene(s) on or off. This study also revealed that there was no basal level of gene expression in the absence of estrogen suggesting that the inducible promoter system in pER8 is a tightly controlled system.

6.5.6 Gene expressed but genistein not produced

Although very high levels of transcripts were obtained from the Arabidopsis transformants (Figure 6.17), no genistein was produced. This is in contradiction with the results obtained by other investigators (Yu *et al.*, 2000; Jung *et al.*, 2000 and Liu *et al.*, 2002) where trace amounts of genistein was found to accumulate in transformed Arabidopsis.

The lack of genistein obtained in this study could be due to a number of reasons. Varietal or ecotype differences may explain the contradicting results obtained as variation between plant species and cultivars have been demonstrated to lead to complications or unexpected results in pathway engineering (Lessard *et al.*, 2002). Genistein may have been produced in the *IFS* transgenic Arabidopsis with Col 0 ecotype background but in very low amounts that were below the limit of detection. Other investigators used ecotype WS (Yu *et al.*, 2000; Jung *et al.*, 2000) and Landsberg Erecta (Liu *et al.*, 2002). Another plausible explanation is that genistein could be a transient intermediate, which were either rapidly metabolised or broken down to compounds that were not identified in this study.

The introduction of a new branch point within the existing pathway could interfere with endogenous flavonoid biosynthesis and/or fail to compete with the endogenous pathway for common substrates such as narigenin, which may result in limited or no genistein production. Naringenin is the common substrate, which is shared

between flavonoid and the isoflavonoid pathways. There are several other enzymes that utilize naringenin as a substrate. There are four known enzymes that use naringenin. In addition to F3H, flavone synthase (FNS), flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H), all have been known to use naringenin to produce flavanones, flavones and precursors of flavones (Yu and McGonigle, 2005). Dihydroflavone reductase (DFR) can also directly use naringenin as a substrate to initiate the tissue-specific production of phlobaphenes (Grotewold *et al.*, 1994). In the present study, absence of naringenin in untreated control or *IFS* transgenic plants suggests that the endogenous amount of naringenin present may be insufficient for IFS to synthesise genistein. Alternatively, naringenin could also be a transient intermediate, which is rapidly metabolised and therefore does not accumulate in plant tissues. Direct evidence that naringenin was the rate-limiting step in transformed Arabidopsis lines were provided by Liu *et al.* (2002). Since F3H directly competes with IFS for the naringenin substrate, silencing of the F3H gene by the introduction of *tt6/tt3* double mutant blocked a major portion of the flavonoid pathway and redirected the flow of substrate toward isoflavone biosynthesis and resulted in high levels of genistein (Liu *et al.*, 2002).

Stress condition is known to activate the phenylpropanoid pathway, which could provide increased levels of the naringenin substrate. Therefore Arabidopsis transformants expressing the soybean *IFS* gene were exposed to UV-B irradiation for a specified length of time. However, no naringenin or genistein were detected in these treated Arabidopsis transgenic lines. This is in agreement with the investigations carried out by Yu *et al*, (2000) where UV-light treatment of *IFS*-transformed tobacco leaves to increase naringenin levels resulted in elevated flavonol levels but failed to enhance naringenin or isoflavone levels. It was, therefore, postulated that the flux through the phenylpropanoid pathway was tightly channeled to flavonol production, which in turn results in limited flux to the introduced isoflavone pathway (Yu & McGonigle, 2005).

6.5.7 IFS protein is formed but is not membrane bound

The evidence discussed earlier on the tightly regulated channeling towards the flavonol production tends to suggest that there is a closely regulated system of enzymes in the phenylpropanoid pathway. Winkel-Shirley, (1999) hypothesized that a physical complex exists between the following enzymes, CHS, CHI and F3H in non-legume crops. In addition, 2 main types of CHIs have recently been discovered that is, type I

and type II, and their distributions are highly family-specific (Shimada *et al.*, 2003). Type 1 is present ubiquitously in most plants within the phenylproponoid pathway and isomerize only naringenin chalcone to naringenin while type II, is specifically distributed to only leguminous plants (Heller and Forkmann, 1993) and catalyses both naringenin chalcone to naringenin and isoliquiritigenin to liquiritigenin. Therefore, it is envisaged that, in leguminous plants both type I and type II CHIs participate in distinct enzyme complexes devoted to producing, for example, anthocyanin pigments and isoflavonoids in different organs and/or in different subcellular locations (Shimada *et al.*, 2003). In the context of metabolic engineering, the utilization of the correct CHI in the production of isoflavonoids in transgenic plants can therefore be crucial. This was substantiated by the investigations carried out by Liu *et al.* (2002) where when *IFS* and type II *CHI* were introduced into a *tt6/tt3* double mutant background in Arabidopsis, genistein accumulation was enhanced by up to 30-fold as compared to plants expressing *IFS* alone. In total, these evidences tend to suggest that multienzyme complexes and protein-protein interaction between the key enzyme exist and they closely regulate the flux towards the isoflavonoid and flavonoid pathway. According to Dixon & Steele (1999), these multienzymes complexes sometimes maintain specific interactions and are even colocalized to defined regions in the cell to form dynamic complexes called "metabolons". Metabolons facilitates rapid conversion of the product of one enzyme by the subsequent enzyme of the channel, which may overcome kinetic constraints imposed by the diffusion of metabolites within the cytosol. In addition, they can protect unstable intermediates from spontaneous breakdown or from potentially competing enzymatic reactions (Srere, 1987). However, metabolons can also confound attempts to engineer pathways by the introduction of transgenes that encode enzymes acting on intermediates that are channeled (Dixon, 2000).

In the present study, there is evidence indicating that the IFS protein is synthesized but the results also suggest that the protein is more likely to be free in the cytoplasm rather than membrane bound (Figure 6.20). In its natural environment, that is, in soybean, IFS is a membrane-bound protein and has been shown to be localized in the microsomes. In addition, IFS is closely associated with CHI II and isoflavone-*O*-methyltransferase (IOMT) and is postulated to form a mutienzyme complex as illustrated in Figure 6.21 (Liu & Dixon, 2001). The 'free' IFS detected in this study implies that IFS is not able to access the pre-existing multienzyme complex between

CHS, CHI and F3H due to compartmentalisation and therefore is not able to synthesise genistein. Another possible explanation for the lack of genistein production in the transgenic plants is the absence of the legume CHI II, which may be important in forming enzyme complexes with IFS for the production of isoflavonols. Alternatively, failure to obtain genistein could also be due to the transport and accumulation processes rather than of biosynthesis *per se* (Dixon, 2005). This is also in keeping with the model of metabolons suggested by Liu and Dixon (2001) and Yu and McGonigle (2005). In this model the transient ER localization of IFS is important for the function of IOMT so that intermediates of the isoflavonoid pathway could flow rapidly from one enzyme center to the next, which may eventually fuse with vacuoles (Figure 6.21). IOMT methylates the A-ring hydroxyl group, rendering the isoflavone more soluble for storage in vacuoles.

It is evident from the suggestion given above that the correct localization and transportation of IFS is crucial for the synthesis of genistein. The IFS protein has to migrate from its site of synthesis and orientate itself in the correct membrane. IFS being a membrane bound protein carries signals embedded within its structures that specify its subcellular distribution and endow it with the capacity to participate in regulated membrane trafficking processes. However, a matching sequence has to be present in the targeted membranes. A lack of recognition in the targeted membrane can result in the IFS being loose and not membrane-bound which could explain the results obtained in the present study.

Finally, there is also a possibility that some finer details in this pathway have not been unravelled. For entry into the isoflavone pathway, naringenin first undergoes abstraction of a hydrogen radical at C-3 followed by B-ring migration from C-2 to C-3 and subsequent hydroxylation of the resulting C-2 radical, all of which is believed to be catalysed by IFS (Dixon, 2004). Before the production of genistein, an intermediate, 2-hydroxy-isoflavanone is formed. 2-Hydroxy-isoflavanone is unstable and readily undergoes dehydration to yield genistein at acidic pH. It has been suggested that a specific dehydrase enzyme catalyzes the 2-hydroxy-isoflavanone to isoflavone conversion in *planta*, and such an enzyme has been purified from *Pueraria lobata*, although it has not been characterized at the molecular level (Dixon, 2004). The absence of this enzyme in Arabidopsis may further reason for the failure to obtain genistein even though the introduced IFS enzyme is present.

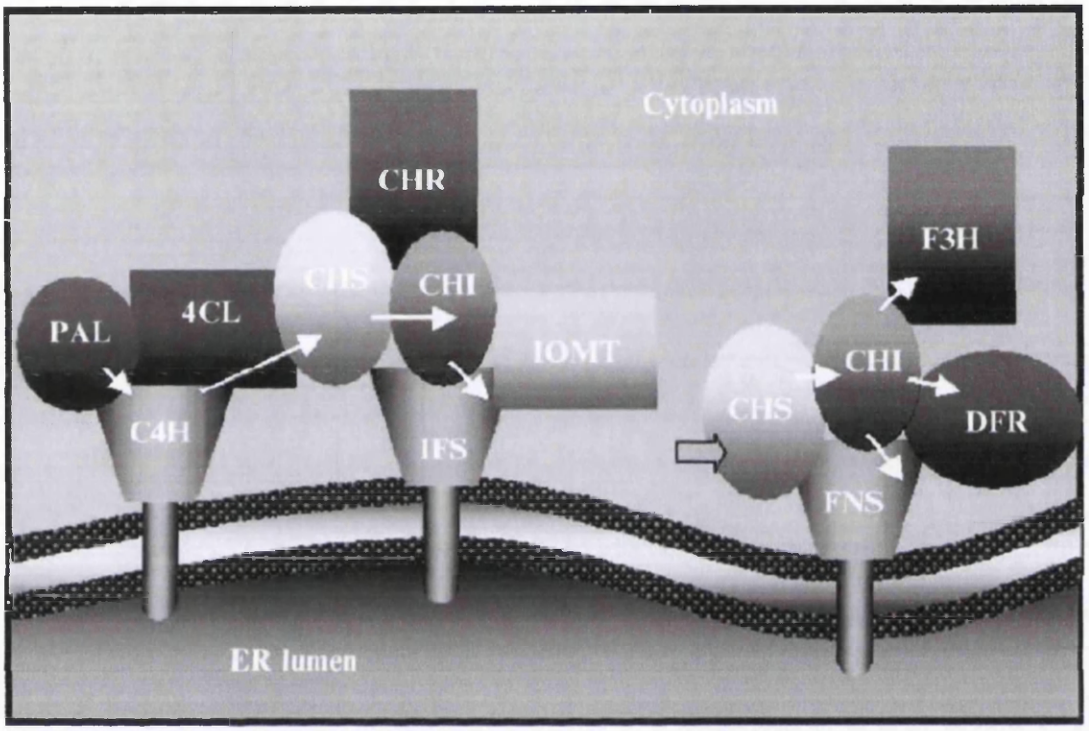


Figure 6.21. Proposed macromolecular complexes in the phenylpropanoid pathway. The isoflavonoid metabolon is shown on the left. The flavonoid metabolon after CHS is on the right. Arrows indicate metabolite flow (Adapted from Yu and McGonigle, 2005).

6.6. Conclusion and further research

The success of metabolic engineering not only depends on the relative activities of all the enzymes that they comprise but also on the hidden more complex, non-linear, and largely unknown interactions of enzymes, regulators, and metabolites (Jung & Stephanopoulos, 2004). In addition, the level, timing, subcellular location, and tissue, organ and cultivar specificity that will be required from a transgene to ensure successful manipulation is also crucial (Lessard *et al.*, 2002). Results from the present study indicates that correct choreographing of these factors is crucial in successfully engineering a foreign gene into plants.

Future research should be geared towards understanding these factors particularly of endogenous enzyme pathways which competes with introduced metabolically engineered enzymes. This can be an important factor when designing strategies for metabolic engineering. Beside this, not much is known about modifying enzymes and the corresponding genes that are responsible for glycosylation, methylation and prenylation reactions that are important for flavonoid stability, cellular distribution, bioactivity and bioavailability. Many 'failures' in pathway engineering might also reflect a lack of understanding of transport and accumulation by these processes rather than of biosynthesis *per se*.

Alternative strategies for marker selection that will not have deleterious effect on the host plant especially during plant regeneration in the course of tissue culture should also be carried out. It will be even better, if resistance markers can be removed as this would also ease public concerns relating to the transfer of resistance markers to non-target species such as weeds or microbes (Lessard *et al.*, 2002). Preliminary work on this topic involving the simultaneous but independent 'co-transformation' of plant cells with the marker gene on one DNA molecule and the desired second trait on a separate DNA molecule has been carried out by DeBlock and DeBrouwer (1991). Selectable markers were then removed from the whole plants via normal chromosomal segregation. However this technique is still at its infancy and needs to be fine-tuned before it can be used for plant transformation.

Chapter 7: References

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Appendix 1.1 : Isoflane synthase c DNA from soybean *Glycine max* (Soybean)-AF195798.

```
1    gtaattaacc tcactcaaac tcgggatcac agaaaccaac aacagttctt gcactgaggt
61   ttcacgatgt tgctggaact tgcacttggt ttgtttgtgt tagctttgtt tctgcacttg
121  cgtcccacac caagtgcaaa atcaaaagca cttcgccacc tcccaaaccc tccaagccca
181  aagcctcgtc ttcccttcat tggccacctt cacctcttaa aagataaact tctccactat
241  gcactcatcg atctctccaa aaagcatggc cccttattct ctctctcctt cggctccatg
301  ccaaccgtcg ttgcctccac ccctgagttg ttcaagctct tcctccaaac ccacgaggca
361  acttccttca acacaaggtt ccaaacctct gccataagac gcctcactta cgacaactct
421  gtggccatgg ttccattcgg accttactgg aagttcgtga ggaagctcat catgaacgac
481  cttctcaacg ccaccaccgt caacaagctc aggcctttga ggacccaaca gatccgcaag
541  ttccttaggg ttatggccca aagcgcagag gcccagaagc cccttgacgt caccgaggag
601  cttctcaaat ggaccaacag caccatctcc atgatgatgc tcggcgaggc tgaggagatc
661  agagacatcg ctcgcgaggt tcttaagatc ttcggcgaat acagcctcac tgacttcatc
721  tggcctttga agtatctcaa ggttggaaag tatgagaaga ggattgatga catcttgaac
781  aagttcgacc ctgtcgttga aagggtcatc aagaagcgcc gtgagatcgt cagaaggaga
841  aagaacggag aagttgttga gggcgaggcc agcggcgtct tcctcgacac tttgcttgaa
901  ttcgctgagg acgagaccat ggagatcaaa attaccaagg agcaaatcaa gggccttgtt
961  gtcgactttt tctctgcagg gacagattcc acagcggtgg caacagagtg ggcattggca
1021 gagctcatca acaatcccag ggtgttgcaa aaggctcgtg aggaggtcta cagtgttgtg
1081 ggcaaagata gactcgttga cgaagttgac actcaaaacc ttccttacat tagggccatt
1141 gtgaaggaga cattccgaat gcacccacca ctcccagtgg tcaaaagaaa gtgcacagaa
1201 gagtgtgaga ttaatgggta tgtgatccca gagggagcat tggttctttt caatgtttgg
1261 caagtaggaa gggaccccaa atactgggac agaccatcag aattccgtcc cgagaggttc
1321 ttagaaactg gtgctgaagg ggaagcaggg cctcttgatc ttaggggcca gcatttccaa
1381 ctcctcccat ttgggtctgg gaggagaatg tgccctggtg tcaatttggc tacttcagga
1441 atggcaacac ttcttgcatc tcttatccaa tgctttgacc tgcaagtgct gggccctcaa
1501 ggacaaatat tgaaaggtga tgatgccaaa gttagcatgg aagagagagc tggcctcaca
1561 gttccaaggg cacatagtct cgtttgtgtt ccacttgcaa ggatcggcgt tgcatctaaa
1621 ctccttttctt aattaagata atcatcatat acaatagtag tgtcttgcca tcgcagttgc
```

1681 tttttatgta ttcataatca tcatttcaat aaggtgtgac tggtacttaa tcaagtaatt

1741 aaggttacat acatgcaaaa aaaaaaaaaa aaaa

The red letters denote the location of the primers while the blue letters denote the start and the ends of the full length cDNA sequence

Appendix 1.2. IFS cDNA cloned from soybean

1 gtaattaacc tcactcaaac tcgggatcac agaaaccaac aacagttctt gcactgaggt

61 ttcacgatgt tgctggaact tgcacttggt ttgtttgtgt tagctttgtt tctgcacttg

121 cgtcccacac caagtgcaaa atcaaaagca cttcgccacc tcccaaaccc tccaagccca

181 aagcctcgtc ttcccttcat tggccacctt cacctcttaa aagataaact tctccactat

241 gcactcatcg atctctccaa aaagcatggc cccttattct ctctctcctt cggctccatg

301 ccaaccgtcg ttgcctccac ccctgagttg ttcaagctct tcctccaaac ccacgaggca

361 acttccttca acacaaggtt ccaaacctct gccataagac gcctcactta cgacaactct

421 gtggccatgg ttccattcgg accttactgg aagttcgtga ggaagctcat catgaacgac

481 cttctcaacg ccaccaccgt caacaagctc aggcctttga ggacccaaca gatccgcaag

541 ttccttaggg ttatggccca aagcgcagag gcccagaagc cccttgacgt caccgaggag

601 cttctcaaat ggaccaacag caccatctcc atgatgatgc tcggcgaggc tgaggagatc

661 agagacatcg ctcgcgaggt tcttaagatc ttcggcgaat acagcctcac tgacttcatc

721 tggcctttga agtatctcaa ggttggaaag tatgagaaga ggattgatga catcttgaac

781 aagttcgacc ctgtcgttga aagggtcatc aagaagcgcc gtgagatcgt cagaaggaga

841 aagaacggag aagttgttga gggcgaggcc agcggcgtct tcctcgacac tttgcttgaa

901 ttcgctgagg acgagaccat ggagatcaaa attaccaagg agcaaatcaa gggccttgtt

961 gtcgactttt tctctgcagg gacagattcc acagcggtgg caacagagtg ggcattggca

1021 gagctcatca acaatcccag ggtgttgcaa aaggctcgtg aggaggtcta cagtgttgtg

1081 ggcaaagata gactcgttga cgaagttgac actcaaaacc ttccttacat tagggccatt

1141 gtgaaggaga cattccgaat gcacccacca ctcccagtgg tcaaaagaaa gtgcacagaa

1201 gagtgtgaga ttaatgggta tgtgatccca gagggagcat tggttctttt caatgtttgg

1261 caagtaggaa gggaccccaa atactgggac agaccatcag aattccgtcc cgagaggttc

1321 ttagaaactg gtgctgaagg ggaagcaggg cctcttgatc ttaggggcca gcatttccaa

1381 ctcctcccat ttgggtctgg gaggagaatg tgccctggtg tcaatttggc tacttcagga

1441 atggcaacac ttcttgcatc tcttatccaa tgctttgacc tgcaagtgct gggccctcaa

```
1501 ggacaaatat tgaaaggtga tgatgccaaa gttagcatgg aagagagagc tggcctcaca

1561 gttccaaggg cacatagtct cgtttgtgtt ccacttgcaa ggatcggcgt tgcatctaaa

1621 ctcctttctt aattaagata atcatcatat acaatagtag tgtcttgcca tcgcagttgc

1681 tttttatgta ttcataatca tcatttcaat aaggtgtgac tggtacttaa tcaagtaatt

1741 aaggttacat acatgcaaaa aaaaaaaaaa aaaa
```

The red letters denote the location of the primers while the blue letters denote the start and the ends of the full length cDNA sequence. The pink letters denote the slight changes in the sequence when compared to the published sequence of soybean