

Absorption of Dietary Flavonoids

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Abstract

Flavonoids represent a major class of plant polyphenols that are distributed ubiquitously in the human diet. Interest in the possible health-promoting effects of flavonoids has increased due to their various bioactive properties, as demonstrated in several *in vitro* and *in vivo* studies. Knowledge about flavonoid absorption and bioavailability is essential in order to fully understand their potential health benefits. This thesis was concerned with the uptake and absorption of dietary flavonoids in humans, with an emphasis on the effect of the chemical form of the flavonoid (as a product of processing) and the effect of food matrix.

The first study investigated the flavonol content in different varieties of commercially-grown tomatoes and widely consumed tomato-based food products. It was shown that the levels of rutin varied considerably between different cultivars and food-based products, with the highest levels found in cherry tomatoes. Furthermore, cellulase hydrolysis of rutin was shown to be a viable alternative for the release of quercetin aglycone.

The second study was aimed to investigate the absorption and bioavailability of dietary quercetin glycosides and its aglycone from a food-based system (non-supplement) in humans by implementing urinary time-course analysis. Ingested quercetin glycosides resulted in an observed earlier appearance of quercetin conjugates in urine than when quercetin aglycone was ingested (1 h compared to 2 h). Significantly more quercetin was absorbed than the aglycone when present in a similar onion-based food matrix system (1.8% compared to 1.3%).

Finally, a second human study was carried out to investigate the effect of dietary fat, 8.3% (w/w), on the bioavailability of quercetin present in onion-based foods as either quercetin glycosides or its aglycone. Absorption of quercetin in its glycosidic form was shown to be significantly higher than the aglycone form when co-ingested with fat (2.8% compared to 1.7%). The ingestion of quercetin with fat, compared with the study without fat, enhanced the bioavailability of quercetin glycosides and its aglycone by about 50 and 23%, respectively, which was significant for the quercetin glycoside meal only.

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Abbreviations

C β G	Cytosolic β -glucosidase
CHD	Coronary heart disease
C _{max}	Peak plasma concentration
COMT	Catechol-O-methyltransferase
CVD	Cardiovascular disease
DAD	Diode array detection
DMSO	Dimethyl sulfoxide
EGCG	Epigallocatechin gallate
GI tract	Gastrointestinal tract
HPLC	High performance liquid chromatography
MS ²	Tandem mass spectrometry
MRP2	Multi-drug resistance protein 2
LC-MS	Liquid chromatography-mass spectrometry
LPH	Lactase phloridzin hydrolyase
LOD	Limit of detection
Log P	Octanol-water partition coefficient
MAX	Mixed-mode anion-exchange
Q	Quercetin
Q3G	Quercetin-3-glucoside
Q3,4'G	Quercetin-3,4'-diglucoside
Q4'G	Quercetin-4'-glucoside
PTFE	Polytetrafluoroethylene
SE	Standard error
SGLT1	Sodium-dependent glucose transporter 1
SPE	Solid phase extraction
ST	Sulfotransferase
TFA	Trifluoroacetic acid
T _{half}	Half-life of elimination
T _{max}	Time to reach peak plasma concentration
UDP-GT	UDP-glucuronosyltransferase
UV	Ultra violet
XO	Xanthine oxidase

Chapter 1

Introduction

1.1 Chemistry of flavonoids

Flavonoids are a large group of low molecular weight, plant secondary metabolite, polyphenolic compounds with a common diphenylpropane skeleton structure of (C₆-C₃-C₆), consisting of two aromatic rings linked through three carbons that form an oxygenated heterocyclic C-ring (Figure 1.1). The biosynthetic pathway of most flavonoids arises from two main pathways: the shikimate acid pathway and the acetate-malonate pathways (Kuhnau, 1976). The 5, 7-hydroxyl pattern of the A-ring arises from the condensation of three units of malonyl-coenzyme A (CoA) derived from the metabolism of glucose. The B-ring comes from the shikimate acid pathway to yield cinnamic acid. Chalcone synthase catalyses the synthesis of the 15-carbon flavonoid backbone from the condensation of p-coumaroyl CoA and malonyl CoA (Kreuzaler and Hahlbrock, 1975). The immediate product of the reaction is chalcone, which is converted to flavanone by the action of chalcone isomerase. The flavanone is then the main intermediate for the formation of the other flavonoid structures (Figure 1.2).

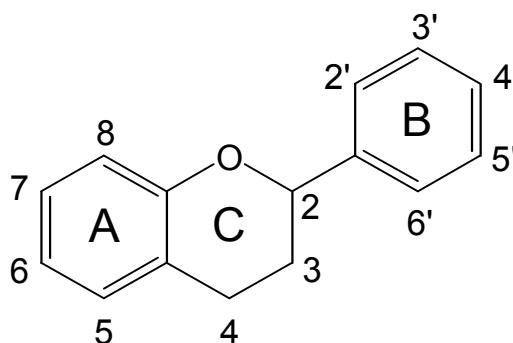


Figure 1.1 Basic flavonoid structure

Flavonoids are most often present in plants as *O*- or *C*-glycosides, which may be bound at one or more positions and so most flavonoids present in foods are present as glycosides with the exception of flavan-3-ols. In addition, fermented foods such as

black tea and wine are a main source of aglycones. Flavonoids are referred to as glycosides when they are conjugated to one or more sugar groups, while flavonoid molecules without the sugar moiety are called aglycones. Glycosylation increases the polarity of the flavonoid molecule, which is necessary for storage in plant cell vacuoles (Aherne and O'Brien, 2002). The preferred position of glycosylation is C-3 and glucose is the most usual sugar residue, but other sugars may also be involved (e.g. galactose, xylose, arabinose), as well as disaccharides such as rutinose (Kuhnau, 1976). Flavonoids may also exist in the form of polymers. For example, catechins may be polymerised to form oligomers and polymers, also called condensed tannins or proanthocyanidins with a high molecular weight.

Flavonoids are classified according to the variations in the heterocyclic C-ring, as well as the oxidation state and functional groups of the C-ring. The major sub-classes of flavonoids include the flavonols, flavones, flavanones, flavan-3-ols (catechins), isoflavones and anthocyanidins (Peterson and Dwyer, 1998). The main flavonoid sub-classes are shown in Figure 1.3. More than 6000 naturally-occurring flavonoids have been identified, many of which are involved in plant growth and development, pigmentation, UV radiation protection and antimicrobial activity (Harborne and Williams, 2000). Over the past two decades, researchers and food manufacturers have become increasingly interested in flavonoids for their potential beneficial role in the prevention of cancers and cardiovascular diseases (CVD) owing to their direct and indirect antioxidant properties observed *in vitro* (Kaur and Kapoor, 2001). In this thesis, the focus will be mainly on the flavonol sub-class, with special emphasis on quercetin.

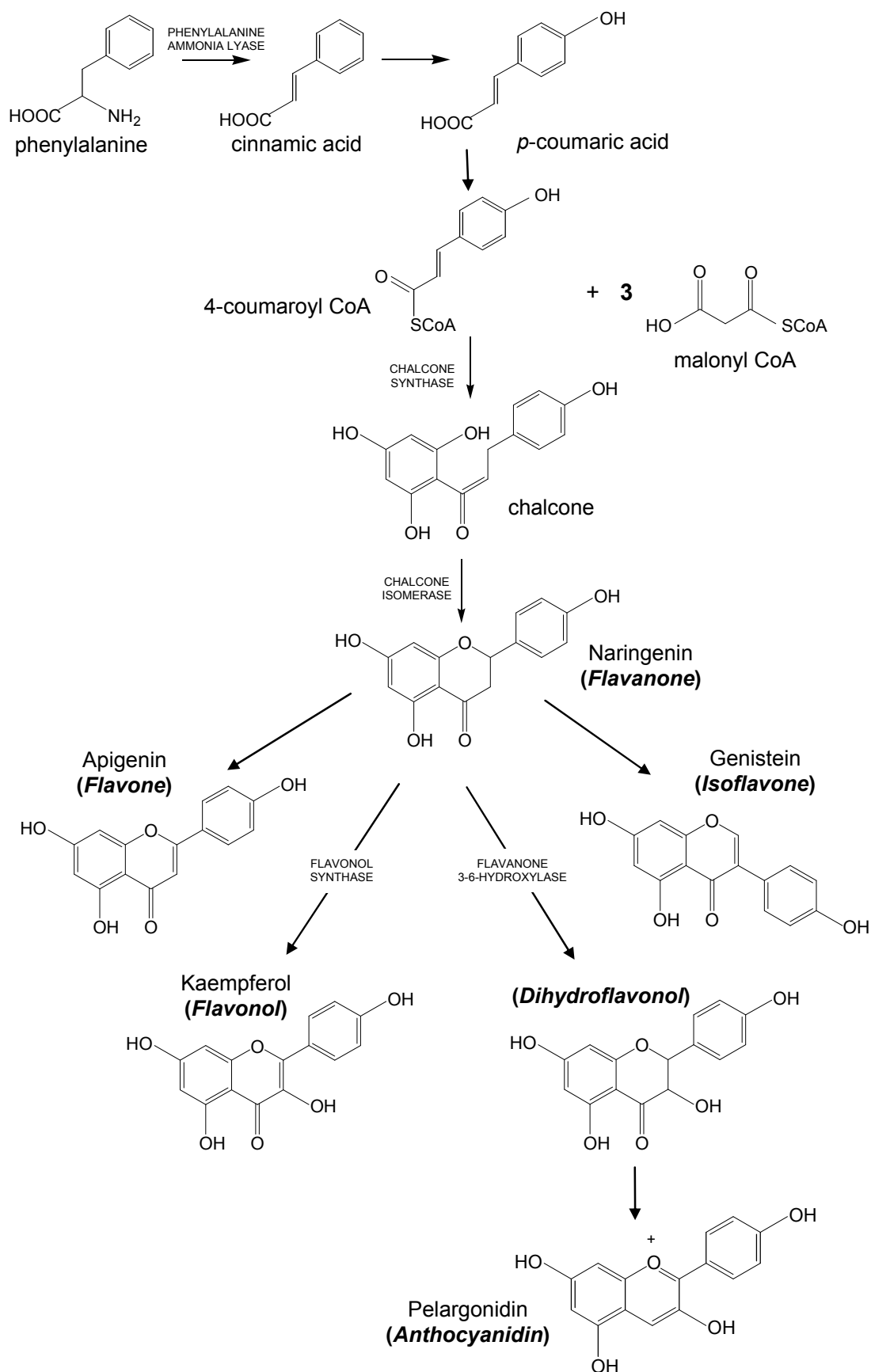
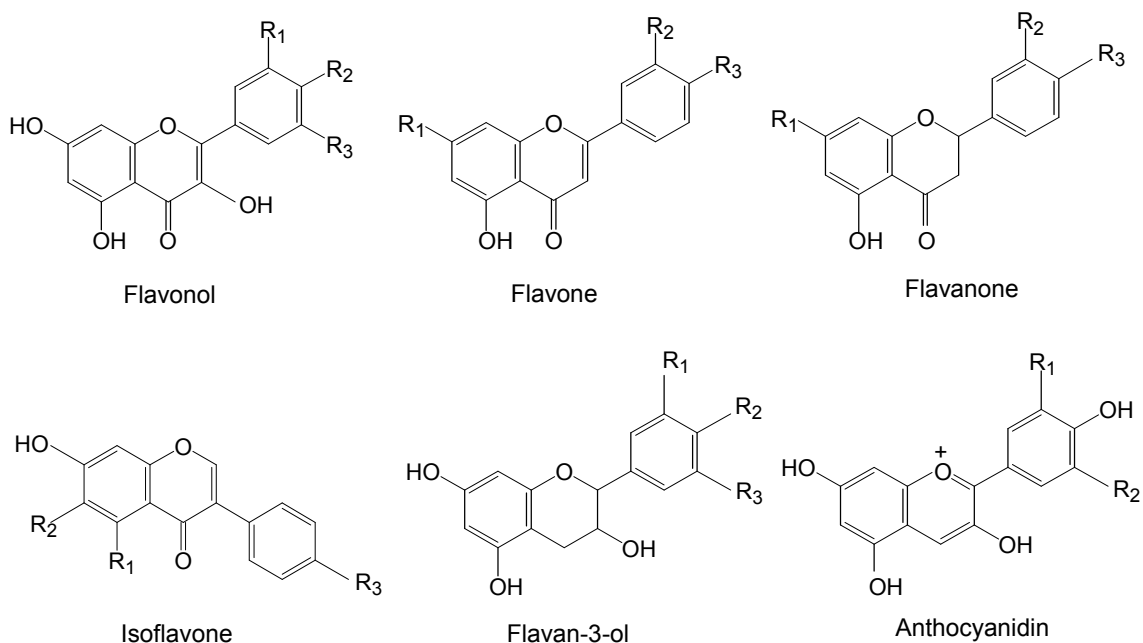


Figure 1.2 The flavonoid biosynthetic pathway



Flavonol	R₁	R₂	R₃
Kaempferol	H	OH	H
Quercetin	OH	OH	H
Myricetin	OH	OH	OH
Isorhamnetin	OCH ₃	OH	OH
Flavone	R₁	R₂	R₃
Chrysin	OH	H	H
Apigenin	OH	H	OH
Luteolin	OH	OH	OH
Flavanone	R₁	R₂	R₃
Naringin	OH	H	OH
Hesperetin	OH	OH	OCH ₃
Eriodyctiol	OH	OH	OH
Isoflavone	R₁	R₂	R₃
Daidzein	H	H	OH
Genistein	OH	H	OH
Glycitein	H	OCH ₃	OH
Flavan-3-ol	R₁	R₂	R₃
Catechin	OH	OH	H
Gallocatechin	OH	OH	OH
Anthocyanidin	R₁	R₂	R₃
Cyanidin	OH	H	—
Peonidin	OCH ₃	H	—
Delphinidin	OH	OH	—
Malvidin	OCH ₃	OCH ₃	—

Figure 1.3 Chemical structures of the main flavonoid sub-classes with examples of common flavonoids.

1.2 Nature and distribution of dietary flavonoids

1.2.1 Flavonols

Flavonoids are widely present in foods of plant origin, such as fruits and vegetables and processed plant-derived foods and beverages (Table 1.1). Flavonols are the most widespread flavonoids in foods, primarily as quercetin. Quercetin is particularly abundant in onions, broccoli and apples. Other notable flavonols include kaempferol in leafy vegetables and myricetin in berries and tea (Hertog *et al.*, 1992a; Hertog *et al.*, 1993a). In plant foods, quercetin is commonly substituted in the 3-position, which include glycosides such as quercetin-3-glucoside (Q3G) and quercetin-3-rutinoside (rutin) and in the 4'-position such as quercetin-4'-glucoside (Q4'G) and quercetin-3,4'-diglucoside (Q3,4'G). The chemical structures of some quercetin glycosides are shown in Figure 1.4.

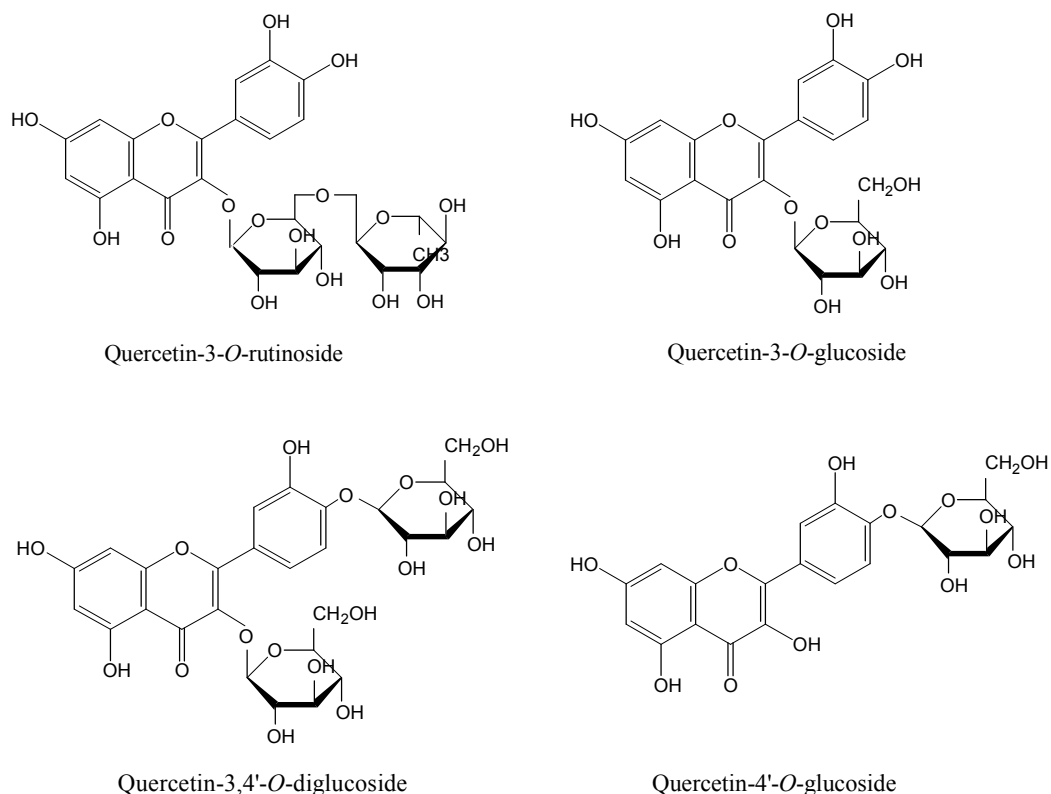


Figure 1.4 Structures of flavonol glycosides

1.2.2 Flavones

Flavones are much less common than flavonols and consist mostly of luteolin and apigenin. Flavones occur mainly as 7-*O*-glycososides although C-glycosides wherein the sugar is directly attached to an aromatic carbon atom are also known (Hollman and Arts, 2000). The primary contributors of the two major flavones in the diet are parsley, celery and thyme.

1.2.3 Flavanones

Citrus fruits are the main dietary source of flavanones, which exist as glycosides, usually as rutinoides and neohesperidosides (glucose – rhamnose diglycosides) attached at the 7-position (Tomas-Barberan and Clifford, 2000). The main aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons with the highest concentrations found in the citrus peel.

1.2.4 Isoflavones

Isoflavones are similar in chemical structure to estrogens and are often referred to as ‘phytoestrogens’. Isoflavones are found predominately in soybeans and processed soy products. The major isoflavones are genistein, daidzein and glycitein which are present in four chemical forms: the aglycones, the glucoside conjugates, and the acetyl and malonylglucosides (Wang and Murphy, 1994). Compared to soybeans, concentrations of isoflavone are relatively insignificant in other leguminous plants.

1.2.5 Flavan-3-ols

In contrast to the other main flavonoid sub-classes, flavan-3-ols (also referred to as catechins), are not found as glycosides in nature. The main monomers are (+)-catechin, (–)-epicatechin (EC), (–)- epigallocatechin (EGC), (–)-epicatechin gallate (ECG) and (–)-epigallocatechin gallate (EGCG). Flavan-3-ols are widely distributed in plant foods with tea, red wine, apples, grapes and chocolate providing the richest food sources (Manach *et al.*, 2004). The condensed tannins or proanthocyanidins formed by the condensation of monomeric units impart astringency in some fruits and beverages and bitterness in cocoa and dark chocolate.

1.2.6 Anthocyanins

Anthocyanins are water-soluble pigments that occur in the vacuoles of epidermal and subepidermal cells of fruits, vegetables and other plant products. Anthocyanins can exist in several chemical forms, both coloured and uncoloured, depending on the surrounding pH. In the human diet, anthocyanins are abundant in wine and fruits such as cherries, grapes, strawberries, cranberries, elderberries, redcurrant and blackcurrant. The six anthocyanidins found in plants are the 3-glycosides of cyanidin, delphinidin, malvidin, petunidin, peonidin and pelargonidin (Beattie *et al.*, 2005). Cyanidin is the most common anthocyanidin in foods.

Table 1.1 Dietary sources of flavonoids in all six flavonoid sub-classes (from Erdman *et al.*, 2007).

Flavonoid sub-class	Prominent dietary flavonoids	Common food source
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin	Onions, curly kale, leeks, broccoli, cherry tomatoes, tea, red wine
Flavones	Apigenin, luteolin	Parsley and celery
Flavanones	Eriodictyol, hesperetin, naringenin	Citrus fruits, citrus juices
Isoflavones	Daidzein, genistein, glycitein	Soybeans, soy products, legumes
Anthocyanins	Cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	Red, purple, and blueberries; black grapes, strawberries
Flavan-3-ols	(+)-Catechin, (–)-gallocatechin, EC, EGC, ECG, EGCG	Tea, red grape, red wine, cocoa, chocolate

1.3 Factors affecting flavonoid content in foods

1.3.1 Genetic variation

The genetic variation between plant species and varieties lead to significant differences in both flavonoid content and composition of plant-derived foods (DuPont *et al.*, 2000). For example, the procyanidin content varied greatly between apples (~20-fold) with the highest amounts found in Red Delicious and Granny Smith apples and the lowest amounts were displayed in the Golden Delicious and McIntosh varieties (Hammerstone *et al.*, 2000). Among the 12 cultivars of onion tested, the yellow onion cultivars had the highest flavonol content (average of 76.5 mg/100 g fresh weight), whereas the white onion varieties had the lowest levels (average of 0.2 mg/100 g fresh weight; Marotti and Piccaglia, 2002). Moreover, the authors reported marked differences in the type of flavonols among the different onion cultivars. For example, quercetin monoglycoside

was the most abundant flavonol in the golden cultivar Dorata Density whereas in the red cultivar Tropea rossa tonda, quercetin and isorhamnetin were at much higher levels. Among a number of samples obtained from six pear cultivars, flavonol content varied by more than 4-fold with Forelle pear having the highest and the Bosc variety having the lowest level at 55.9 and 9.5 mg/100 g fresh weight, respectively (Galvis-Sanchez *et al.*, 2003).

1.3.2 Environmental conditions

The flavonoid content of plant-based foods is affected by growing conditions, including soil type and nutrients, sun exposure, water availability, and pathogen and insect attack (Dixon and Paiva, 1995; Dumas *et al.*, 2003). For example, the flavonol content in field-grown cherry tomatoes originating from countries with warm, sunny climates such as Spain and South Africa are much higher than those grown under greenhouse conditions with lower light intensity and reduced UV exposure in cooler countries such as the United Kingdom (Stewart *et al.*, 2000). Day-night temperature differences have also been shown to be one of the major environmental influences on the synthesis of flavonols. For example in strawberries, high temperature conditions significantly increased their flavonol and anthocyanin content (Wang and Zheng, 2001).

Seasonal variation in the flavonoid content of leafy vegetables such as lettuce, endive and leek have been reported previously by Hertog *et al.* (1992a). The authors found flavonol levels in these vegetables to be three to five times higher during the summer compared to other seasons. Similar seasonal influences on flavonoid content have been

observed in cherry tomatoes (Crozier *et al.*, 1997; Stewart *et al.*, 2000) and blueberries (Harnly *et al.*, 2006).

1.3.3 Food preparation and processing

Food processing also affects flavonoid content. Skins and peels of most fruits and vegetables contain most of the flavonoids, therefore processing these foods by skinning, trimming and peeling may remove and reduce the total amount of flavonoids. In apple, quercetin glycosides are found in the peel whereas the peeled flesh contains no other flavonols (Escarpa and Gonzalez, 1998). Fermentation can affect the levels of flavonoids in several processed plant-derived beverages such as red wine and tea. In red wine, up to 67% of the flavonol content can be present in the aglycone form (McDonald *et al.*, 1998). The authors suggested that the high aglycone content in red wine resulted from the use of thick-skinned grape varieties with a high skin to volume ratio. While green tea is a rich source of catechins, black tea has a much lower content of catechins since they are oxidized during fermentation of tea leaves to the pigments of black tea leaves (Neilson *et al.*, 2006).

Flavonoids are relatively stable molecules; however, domestic preparation procedures, particularly thermal treatments such as cooking, frying and roasting, can have a dramatic impact on flavonoid content of foods. For example, in onions, boiling results in a reduction of 75% of the total quercetin content, and there is a reduction of 65% after cooking in a microwave and of 30% after frying. The great reduction in quercetin content by boiling is probably due to the leaching action of the cooking water (Crozier, *et al.*, 1997). Similarly, an overall loss of up to 25% by leaching into the boiling water

and thermal degradation after frying has been observed (Price *et al.*, 1997). Furthermore, Price *et al.* (1998) showed that significant levels of flavonol glycosides may be leached into the cooking water by boiling of broccoli for 15 min with only 14 to 28% of the flavonol glycoside remaining in the cooked tissue. More recently, Rohn *et al.* (2007) has shown that flavonol glycosides from onion were hydrolysed to flavonol aglycone under roasting conditions (180°C).

1.4 Dietary intake of flavonoids

The structural diversity of dietary flavonoids renders the estimation of the average daily intake of these phytochemicals difficult. Estimation can also be confounded by differences in the flavonoid content for similar foods, because of the influence of several factors such as variety, degree of ripeness, exposure to light, processing, and storage (Manach *et al.*, 2004). Nonetheless assessing flavonoid intake is a key step toward documenting the protective effects of flavonoids against risk of CVD and cancer.

Kuhnau (1976) estimated the daily average intake of total flavonoid glycosides in the United States to be about 1 g, with flavonol and flavone intake (as free flavonoids without sugar groups) at 110 mg/day. Hertog *et al.* (1993a) developed a more advanced method of analysis and estimated the intake of flavonols in Holland to be 22 mg/day, with the flavones providing another 1 mg/day. Quercetin was the main dietary flavonoid (16 mg/day), followed by kaempferol (4 mg/day) and myricetin (1.4 mg/day). Tea was the largest source of dietary flavonoids with 48% of total intake, followed by onions, 29%, and apples, 7%. A similar intake of 26 mg/day has been

estimated for the same flavonoids in the Danish diet (Justesen *et al.*, 1997). In the Seven Countries Study, flavonoid intake was highest in Japan (70 mg/day) and lowest in Finland (6 mg/day). The major dietary sources of flavonols and flavones varied between each cohort. In the Japanese and Dutch cohorts, the major source was tea, while fruits, in particular berries, were the most important source of dietary flavonoids in Finland (Hertog *et al.*, 1995). Another study, however, showed that the intake estimation for flavonols, flavones and flavanones in Finland was higher at 24 mg/day (Knekt *et al.*, 2002). Based on improved analytical methods, data from these studies demonstrate that Kuhnau's estimate of 1 g/day was completely incorrect.

Whilst intake estimations of specific flavonoid groups have been published, very few studies have reported on the intake estimation for a wide range of dietary flavonoids. For example, an estimated average daily intake of total flavonoids in the United States population was shown to be 190 mg/day, mainly from flavan-3-ols, 83.5%, followed by flavanones, 7.6%, flavonols, 6.8%, anthocyanidins, 1.6%, flavones, 0.8%, and isoflavones, 0.6% (Chun *et al.*, 2007). Similarly, a consumption of 216 mg of total flavonoids per day was determined for an average Finnish adult (Ovaskainen *et al.*, 2008). Berries and berry products constituted the main source for anthocyanidins, and proanthocyanidins, and fruits were the main source for flavonols, flavones, and flavanones. These estimations, however, may not represent the flavonoid content of foods consumed in other parts of the world since cultural dietary habits and preferences dictate which foods are most frequently consumed. Therefore, it is necessary to take into account the total flavonoid content of foods consumed in other countries and to apply standardised testing techniques to avoid under- or over-estimating the dietary intake of flavonoids.

1.5 Biological effects of flavonoids

Increased consumption of fruits and vegetables is an important factor in the prevention of major chronic diseases, including CVD, cancers and neurodegenerative disorders (Scalbert *et al.*, 2005). The physiological functions of fruits and vegetables are often attributed to flavonoid compounds rather than known nutrients and micronutrients as they display a wide range of biological activities. It is believed that many of the beneficial effects of flavonoids result from their potent antioxidant properties. According to Halliwell and Gutteridge (1998), the dietary antioxidants can act through several mechanisms such as:

- (i) Inhibiting the formation of radicals and other reactive oxygen species (ROS)
- (ii) Scavenging of ROS
- (iii) Chelating of pro-oxidant metal ions

Several *in vitro* studies have found flavonoids, especially quercetin, to be very potent scavengers of reactive oxygen (Hanasaki *et al.*, 1994) and nitrogen species (Haenen *et al.*, 1997). In addition, flavonoids have the ability to chelate metal ions such as iron and copper, which prevent the ions from acting as pro-oxidants enhancing free radical production in the body (Mira *et al.*, 2002). These activities are the basis of biological effects, such as inhibition of lipid peroxidation and protection of low-density lipoproteins (LDL) against oxidation damage that are thought to be important precursors to atherosclerosis and therefore more advanced stages of CVD (Middleton *et al.*, 2000). Furthermore, data obtained from *in vitro* studies exploring the direct interaction of flavonoids with free radicals and ROS have provided clear evidence that the radical scavenging activity depends on the optimal structural configuration, i.e. the

catechol group in the B-ring and the 3-position hydroxyl group on the C-ring (Haenen *et al.*, 1997).

Flavonoids can inhibit a variety of enzymes indirectly involved in the production of ROS, such as xanthine oxidase (XO; Chang *et al.*, 1993; Dew *et al.*, 2005), which is involved in the oxidation of hypoxanthine and xanthine to uric acid. The reaction yields superoxide radicals that are responsible for damage to living tissues. In addition to XO, flavonoids have also been known to inhibit other enzyme systems including, cyclooxygenase (Hoult *et al.*, 1994), lipoxygenase (Hoult *et al.*, 1994), phase I monooxygenase (Hodek *et al.*, 2002), mitochondrial succinoxidase and NADH oxidase (Bohmont *et al.*, 1987). There are also reports of flavonoids inhibiting enzyme activities affecting cancer cell division and proliferation by triggering the process of apoptosis (Ramos, 2007), platelet aggregation (Landolfi *et al.*, 1984) and stimulate the immune response to cancer (Wei *et al.*, 1993). It should be pointed out, however, that despite the increasing evidence for the *in vitro* antioxidant activity of flavonoids, the existing literature does not provide strong consistent support for the *in vivo* antioxidant potential of flavonoids (Halliwell *et al.*, 2005). Therefore, the capacity of flavonoids to act as antioxidants *in vivo* warrants further study.

1.6 Epidemiological studies

1.6.1 Flavonoids and coronary heart disease

Epidemiological studies supporting the hypothesis for the protective effects of dietary flavonoids against coronary heart disease (CHD) are well reviewed. To date, eight

cohort studies found an association between flavonol, flavone, or flavan-3-ol intake and a reduced risk of fatal or non-fatal CHD by as much as 65% (Erdman *et al.*, 2007).

Hertog *et al.* (1993b) assessed the flavonoid intake of 805 men aged 65-84 years from the Zutphen Elderly Study. The average consumption of flavonoids (flavonols and flavones) was estimated to be 26 mg/day, and the major sources of intake were tea (61%), onions (13%) and apples (10%). Hertog *et al.* (1993b) found a significant inverse relationship between flavonol intake and mortality from coronary heart disease and a borderline significance with the incidence of a first fatal or nonfatal myocardial infarction. The Finland Study also reported a reduction in the risk of CHD in men and women with the increase in flavonol consumption although the significant protective effect was found only among women (Knekt *et al.*, 1996). In the Health Professional Study that followed 34,789 men throughout the United States for six years, no relationship between flavonol intake and risk of CHD mortality was shown, although a possible protective effect of flavonoids among men with previous history of CHD was observed. (Rimm *et al.*, 1996). The Iowa Women's Health Study followed 34,492 women for 10 years in the United States found that flavonoid intake (flavonols and flavones) may reduce risk of mortality from CHD in postmenopausal women (Yochum *et al.*, 1999). A Finnish cohort study of 25,372 male smokers found an association between flavonol and flavone intake and reduction of non-fatal myocardial infarction risk, with a much weaker association with coronary death (Hirvonen *et al.*, 2001). A further cohort study of 4807 men and women in the Netherlands found an inverse association between tea and dietary flavonol intake and the risk of fatal and non-fatal myocardial infarction (Geleijnse *et al.*, 2002).

Interestingly, the Caerphilly Study (Hertog *et al.*, 1997) carried out on a Welsh population of 1900 men aged 45-59 years found increased CHD mortality rates with the consumption of tea (the major source of flavonols in the study). It was postulated that the British habit of adding milk to tea might inhibit flavonol absorption by protein binding. However, it is now known that the absorption of flavonols from the gastrointestinal (GI) tract is not affected by the addition of milk (Hollman *et al.*, 2001). Tea drinking in this group, however, was strongly influenced by socioeconomical factors and associated with an unhealthy lifestyle including heavy smoking, inactivity, poor diet and high alcohol intake.

1.6.2 Flavonoids and cancer

There is increasing evidence for the cancer preventive effects of the consumption of flavonoid-rich fruits and vegetables, although epidemiological investigations on flavonoids and cancer risk still remain scanty. In the cancer component of the Zutphen Elderly Study, tea consumption was not associated with cancer risk, however high intake of flavonoids from fruits and vegetables was inversely associated with the risk of alimentary and respiratory tract cancers (Hertog *et al.*, 1994). The results from the Finnish Mobile study provided convincing evidence for a link between high flavonoid intake (mainly quercetin) and lower rates of lung cancer (Knekt *et al.*, 1997). The protection by flavonoids was greatest in persons under 50 years of age and in non-smokers. Furthermore, the observed inverse association was not due to consumption of antioxidant vitamins C or E or β -carotene that have been assessed for their ability to prevent cancer. In the Finish Mobile Health Examination Survey of 10,050 men and women, Knekt *et al.* (2002) studied the associations between the intake of flavonols,

flavones and flavanones and the incidences of cancer at six organ sites. There was a statistically significant reduction in lung cancer risk for men with higher intakes of flavonoids. A positive protection association between high flavonol (myricetin) consumption and lower prostate cancer risk. In contrast, no significant associations were observed between flavonoid intake and occurrence of cancers of the stomach, colorectum, urinary tract, or breast. More recently, the Multiethnic Cohort Study of 183,518 men and women in Hawaii and California found a significantly inverse association between total flavonol (quercetin, kaempferol and myricetin) intake and pancreatic cancer incidence, especially among smokers (Nothlings *et al.*, 2007). The authors suggested that the direct or indirect antioxidant properties of flavonols may protect against the increase in oxidative stress induced by the carcinogens found in smoke.

1.6.3 Flavonoids and neurodegenerative disease

Some epidemiological studies have examined a possible link between flavonoid intake and the reduced incidence of age-related neurodegenerative diseases including Alzheimer's disease and Parkinson's disease. The Paquid study was used to investigate whether flavonoid consumption was associated with a lower incidence of dementia in 1367 men and women above 65 years of age (Commenges *et al.*, 2000). A significant inverse association was found between high flavonoid consumption and the risk of dementia. The Rotterdam Study which followed 5395 people age 55 years and older in the Netherlands over six years found an association between high flavonoid intake and a lower risk of Alzheimer's disease only among smokers (Engelhart *et al.*, 2002). Furthermore, the consumption of two cups/day or more of tea was shown to reduce the

risk of Parkinson's disease among smokers and ex-smokers (Checkoway *et al.*, 2002). Moreover, animal studies have shown, in the case of aging, that dietary supplementation of blueberry extract for 8 weeks was effective in reversing age-related deficits in neuronal signaling and cognitive behavioral function in 19-month-old Fischer 344 rats (Joseph *et al.*, 1999). There is emerging evidence that consumption of flavonoid-rich foods may help decrease the risk of dementia or Alzheimer's disease in humans, although the link is not as strong as it is for CHD.

1.7 Bioavailability of flavonoids

Bioavailability is broadly defined as the absorption, distribution, metabolism, and elimination of a compound following ingestion. Bioavailability thus quantifies the exposure of the body to the phytochemical in question. Although flavonoids are inevitably present in the omnivorous human diet, the bioavailability of many flavonoids is limited because they are poorly absorbed from the gut, readily metabolised, or rapidly eliminated (Manach *et al.*, 2004). Current evidence from pharmacokinetic studies indicate that the bioavailability varies from 0.3 to 43%, based on urinary excretion, of the dose for the flavonoids studied, reaching plasma concentrations of 0.02 – 2.56 $\mu\text{mol/l}$ after an oral intake of 50 mg aglycone equivalents (Table 1.2; Manach *et al.*, 2005). These estimates, however, do not reflect accurately the amount absorbed since most of the flavonoids have been reported to be eliminated in bile and extensively metabolised by the colonic microflora to phenolic acids (Donovan *et al.*, 2006).

There are a number of factors that govern the bioavailability of flavonoids such as the complexity of the food matrix, chemical structure of the flavonoid (e.g. glycosylation), and the type and amount of co-ingested compounds. Exogenous factors affecting bioavailability are discussed in greater detail in Chapters 4 and 5. Furthermore, endogenous parameters of the gut physiology such as pH, biliary excretion transit time and rate of gastric emptying may also affect bioavailability.

Table 1.2 Pharmacokinetic data for various flavonoids from bioavailability studies in humans (from Manach *et al.*, 2005).

Flavonoid	T _{max} (h)		C _{max} (μmol/l)		Urinary excretion (% of intake)	
	Average	Range	Average	Range	Average	Range
Daidzin	6.3	4.0–9.0	1.92	0.36–3.14	42.3	21.4–62.0
Daidzein	4.9	3.0–6.6	1.57	0.76–3.00	27.5	–
Genistin	6.5	4.4–9.3	1.84	0.46–4.04	15.6	6.80–29.7
Genistein	4.1	3.0–5.2	2.56	1.26–4.50	8.6	–
Glycitin	5.0	–	1.88	1.50–2.26	42.9	19.0–55.3
Hesperidin	5.5	5.4–5.8	0.46	0.21–0.87	8.6	3–22.3
Naringin	5.0	4.6–5.5	0.50	0.13–1.50	8.8	1.1–30.2
Q-glucosides	1.1	0.5–2.9	1.46	0.51–3.80	2.5	0.31–6.4
Rutin	6.5	4.3–9.3	0.20	0.09–0.52	0.7	0.07–1.0
(Epi)catechin	1.8	0.5–2.5	0.40	0.09–1.10	18.5	2.1–55.0
EGC	1.4	0.5–2.0	1.10	0.30–2.70	11.1	4.2–15.6
EGCG	2.3	1.6–3.2	0.12	0.03–0.38	0.06	0.0–0.1
Anthocyanins	1.5	0.7–4.0	0.03	0.001–0.20	0.4	0.004–5.1
Proanthocyanidins	2.0	–	0.02	0.008–0.03	–	–

All data were converted to correspond to a supply of 50 mg aglycone equivalent.

Q, quercetin; C_{max}, peak plasma concentration; T_{max}, time to reach C_{max}.

1.7.1 Absorption and uptake

Understanding the absorption of flavonoids after oral uptake is fundamental in determining the relative bioavailability of flavonol glycosides from different food sources and assessing their likely importance to human health. The absorption of the flavonol will depend on its physico-chemical properties such as molecular weight, lipophilicity and pKa.

Most flavonoids, with the exception of catechins, are usually glycosylated in food. The glycosylation influences chemical, physical and biological properties of the flavonoid. For example, quercetin has an octanol-water partition coefficient (Log P) of 1.82; whereas for a glycoside such as quercetin-3-glucoside, the value is lower (0.76), which suggests a greater hydrophilicity (Rothwell *et al.*, 2005). It has long been assumed that quercetin glycosides were too hydrophilic to be absorbed in the small intestine and that bacterial deglycosylation was required for absorption to take place in the large intestine (Griffiths and Barrow, 1972).

In a series of human intervention studies, Hollman *et al.* (1995, 1996, 1997, 1999) studied the absorption and disposition of flavonols and their glycosides. Their results showed that quercetin-4'-glucosides (Q4'G) and quercetin-3,4'-diglucosides (Q3,4'G) from onion were rapidly and efficiently absorbed in the small intestine, whereas rutin absorption was delayed and ultimately less efficient. Taken together, these studies demonstrate that bioavailability of dietary flavonols is greatly affected by the type and binding site of the sugar moieties. The data also suggests that flavonol glycosides are more efficiently absorbed across the wall of the GI tract than their respective aglycones (see Chapter 4 for a more detailed discussion of flavonol pharmacokinetics).

For some years, controversy existed over whether flavonoid glycosides could be absorbed intact (Day and Williamson, 2001). However, several studies have demonstrated that dietary quercetin glucosides are not absorbed intact in humans (Moon *et al.*, 2000; Walle *et al.*, 2000; Graefe *et al.*, 2001). For example, Day *et al.* (2001) could not detect quercetin glucosides in plasma after subjects consumed an onion meal using quercetin metabolites as standards. Similarly, Wittig *et al.* (2001) was unable to detect any quercetin glucosides in plasma samples collected 1 h after the ingestion of a large quantity of stewed onion using HPLC-tandem mass spectrometry (MS) analysis. In another human study, Mullen *et al.* (2004) did not detect quercetin glucoside in the plasma or urine by HPLC with the highly sensitive full scan MS2 of six subjects who had consumed an onion meal.

The results from these human studies clearly emphasize that dietary flavonoid glycosides are deglycosylated during the process of absorption. Non-enzymatic deglycosylation of flavonoids in the human body, such as gastric acid hydrolysis, does not occur (Gee *et al.*, 1998). Two key enzymes are involved in flavonoid absorption. One of them is the broad-specificity cytosolic β -glucosidase (C β G), a soluble enzyme found in human small intestine epithelial cells (Day *et al.*, 1998). The enzyme has a high affinity for Q4'G and genistein-7-glucoside, although the enzyme was not active on rutin. C β G is located intracellularly, so the hydrophilic glucosides would require active transport into the enterocytes. The second enzyme is lactase phloridzin hydrolase (LPH), which is attached to the brush-border of small intestine epithelial cells. LPH is exposed on the luminal side of the brush border, and its action would release the aglycones into the lumen where they would be able to diffuse passively across the membrane (Nemeth *et al.*, 2003). LPH may be responsible for the deglycosylation of

flavonoid glycosides in the intestinal lumen. Day *et al.* (2000b) reported the ability of highly purified LPH from sheep small intestine to hydrolyse isoflavone glycosides, Q3G and Q4'G but not rutin. The authors also noted that LPH is located in close proximity to the active sodium-dependent glucose transporter 1 (SGLT1). They suggested that the cleaved glucoside by the action of LPH is transported into the enterocyte by SGLT1 while the liberated aglycone diffuses passively across the brush border into the enterocyte. The various potential routes of uptake and absorption of flavonoid glycosides are summarised in Figure 1.5.

Transport of flavonoid glycosides into the intestinal enterocytes by SGLT1 has been previously suggested (Hollman *et al.*, 1995). Furthermore, results from a study using everted sacs of rat jejunum showed that Q3G and Q4'G but not rutin interacted with SGLT1 (Gee *et al.*, 1998). On the other hand, Ader *et al.* (2001) demonstrated that Q3G and Q4'G, but not quercetin or rutin, inhibited the uptake of methyl- α -D-glucopyranoside, a glucose analogue, by SGLT1. Likewise, Cermak *et al.* (2004) showed that Q3G and Q4'G inhibited D-glucose uptake into brush-border-membrane vesicles from pig jejunum. However, they noted that other flavonoid glucosides such as quercetin-3-galactoside, quercetin-3-glucorhamnoside, naringenin-7-glucoside, genistein-7-glucoside, and cyanidin-3,5-diglucoside did not inhibit glucose uptake, suggesting a direct interaction of the flavonoid glucosides with SGLT1.

Several studies have sought to identify the relative importance of the SGLT1 and LPH mechanisms of flavonoid glucoside absorption. For example, Sesink *et al.* (2003) incubated a selective LPH inhibitor that does not affect SGLT1 with Q3G in a rat perfused jejunum and ileum. They observed a 67% reduction of intestinal hydrolysis of

Q3G in the perfusion fluid. The authors concluded that LPH was important in the deglycosylation of Q3G, although they did not rule out the possibility that SGLT1 may be involved in Q3G absorption.

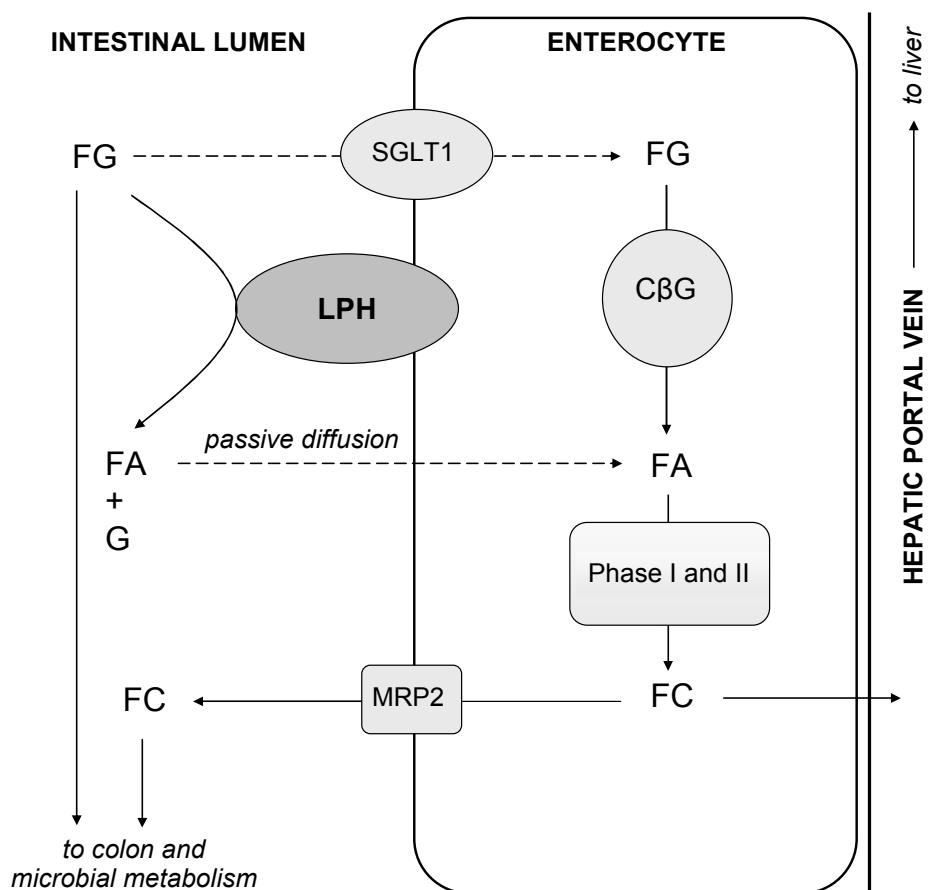


Figure 1.5 Schematic showing the possible routes of absorption of dietary flavonoid glycosides from the small intestine into the hepatic portal vein (after Day *et al.*, 2004). Key: FG = flavonoid glycosides; FC = flavonoid conjugates; FA = flavonoid aglycones; LPH = lactase phlorizin hydrolase; SGLT1 = sodium-dependant glucose transporter 1; CβG = cytosolic-β-glucosidase; MRP2 = multidrug-resistant protein 2.

In another study, Day *et al.* (2003) incubated SGLT1 inhibitor phlorizin and a lactase domain inhibitor of LPH with quercetin glucosides in a rat everted-jejunal sac model. The absorption of Q4'G involved an interaction with SGLT1 and luminal hydrolysis by

LPH, whereas Q3G absorption was observed after hydrolysis by LPH. They concluded that SGLT1-mediated transport of flavonol glycosides was minimal, and that deglycosylation by LPH and passive diffusion of the liberated aglycone was the main route of absorption for quercetin glucosides.

A number of studies have used human colorectal carcinoma (Caco-2) cell lines as an *in vitro* intestinal epithelial model for drug transport and permeability assessment of xenobiotic compounds. These studies suggest that some flavonoid glycosides and their conjugates are transported out of the enterocyte to the intestinal lumen by the multi-drug resistance protein 2 (MRP2), thereby limiting their absorption (Walgren *et al.*, 2000). For a comprehensive evaluation of the role of flavonoids as important modulators or substrates of intestinal ATP binding cassette (ABC) transporters, see the review of Brand *et al.* (2006).

1.7.2 Metabolism and excretion

Once absorbed through the intestinal walls, flavonoids will be metabolised by phase I or phase II enzymes. Hydroxylation and demethylation are examples of phase I cytochrome P450 mono-oxygenase-dependent activities. Much of the research on phase I metabolism has been carried out using cell cultures from animals. For example, liver microsomes prepared from normal and Aroclor-1254-induced rats hydroxylated certain flavonols and flavones (Nielsen *et al.*, 1998). The presence of a single or no hydroxyl groups on the B-ring was required for metabolic activation; however, two or more hydroxyl groups in the B-ring prevented further hydroxylation. In human intervention studies, no evidence for hydroxylation of apigenin to luteolin (Nielsen *et al.*, 1999) or

chrysin to apigenin (Walle *et al.*, 2001a) was found, thus suggesting that phase I metabolism of dietary flavonoids is not a common pathway *in vivo*.

Phase II metabolism of flavonoids consist of conjugative reactions which include glucuronidation, sulfation and methylation. Conjugation of flavonoids is a common detoxification process that reduces the number of reactive hydroxyl groups thus leading to increased water solubility and molecular weight of the metabolites, which are required for urinary and biliary excretion (Gibson and Skett, 2001). In general, the liver is considered to be the main organ involved in the phase II metabolism of flavonoids (Scalbert and Williamson, 2000). However, intestinal perfusion studies in rats have shown that the intestinal mucosa is also capable of phase II conjugation reactions (Crespy *et al.*, 1999; Spencer *et al.*, 1999).

The presence of flavonol conjugates in plasma and urine following the absorption of dietary flavonol glycosides indicates the high efficiency of conjugation reactions in humans. For example, Manach *et al.* (1998) investigated the different forms in which quercetin is present in the plasma of ten volunteers following the consumption of a flavonol-rich meal. The authors did not detect any free flavonols in the plasma samples from 2 hours before the meal and 3, 7, and 20 hours after meal ingestion. Quercetin, however, was detected in human plasma after enzymatic treatment with β -glucuronidase and sulfatase. Isorhamnetin, a 3'-methylated quercetin derivative was also observed in the hydrolysed plasma of three of the volunteers. Similarly, no free quercetin was detected in human plasma prior to β -glucuronidase / sulfatase hydrolysis suggesting that all quercetin metabolites in plasma circulated in the form of conjugates (Moon *et al.*, 2000). In another study, quercetin glucuronides were identified in plasma

as the main quercetin metabolites by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS). Quercetin was detected in plasma and urine samples only after β -glucuronidase / sulfatase hydrolysis (Graefe *et al.*, 2001).

In general, glucuronidation is a major phase II metabolic pathway for flavonoid metabolism. Glucuronidation is catalysed by the UDP-glucuronosyltransferases (UDP-GT; EC 2.4.1.17), a multigene family of membrane-bound enzymes of the endoplasmic reticulum (Figure 1.6a). Glucuronidation occurs as a result of the conjugation reaction of the co-factor UDP-glucuronic acid with the aglycone. UDP-glucuronic acid is readily available in the metabolic tissues of the body and thus does not limit the rate of conjugation (Day *et al.*, 2004).

LC-MS has been widely used for the identification of flavonol glucuronides and patterns of glucuronidation have been studied in detail. For example, Day *et al.* (2000a) demonstrated that UDP-GT from human liver cell-free extracts had the highest affinity for quercetin at the 4'-position, although the maximum rate of glucuronidation was for the 7-position. Glucuronidation, however, did not occur at the 5-position (Figure 1.6a). Likewise, Boersma *et al.* (2002) investigated the regioselectivity of the glucuronidation of quercetin by human liver microsomes. They showed that the hydroxyl groups at the 7-, 3-, 3'-, and 4'-positions were major sites for glucuronidation.

In addition to *in vitro* studies, several human studies have observed the contribution of UDP-GT to the first-pass glucuronidation of flavonols. For example, after consumption of fried onions, five different glucuronides of quercetin at the 3-, 7-, 3'-, and 4'-positions were identified in human plasma samples by means of LC-MS (Witting *et al.*,

2001). It was also found that quercetin-3-glucuronide and isorhamnetin-3-glucuronide were dominant in human plasma 1.5 h after consumption of quercetin glucosides from onion (Day *et al.*, 2001). Similarly, conjugate metabolites, namely quercetin-3-glucuronide, quercetin-3'-glucuronide, and quercetin-4'-glucuronide, were identified in human plasma after ingestion of fried onions by LC-MS (Mullen *et al.*, 2004, 2006).

Sulfation is a major conjugation pathway for flavonoids and other dietary polyphenols. The conjugation reaction is catalysed by sulfotransferases (ST; EC 2.8.2.1), a group of cytosolic enzymes found in the liver and small intestine that conjugate sulfate from the co-factor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to a free hydroxyl group on the flavonoid rings (Figure 1.6b). In contrast to glucuronidation, the levels of sulfation activity may be limited to PAPS availability. Sulfate conjugation is considered to predominate at low substrate concentration, whereas glucuronidation is the main conjugation pathway at high concentration (Gibson and Skett, 2001).

Sulfation of flavonoids and other phenolic dietary compounds by ST has been identified in several human cell culture and human intervention studies. The flavonol chrysin was metabolised to sulfate and glucuronide conjugates in human liver and intestinal cell lines (Galijatovic *et al.*, 1999), and sulfation of quercetin at the 7-position was observed in both rat liver cells and human intestine cell lines (van der Woude *et al.*, 2004). In the *in vivo* study, Day *et al.* (2001) identified several quercetin metabolites, including quercetin-3'-sulfate, in human plasma after consumption of quercetin-rich onions. Mullen *et al.* (2004, 2006) also showed that quercetin-3'-sulfate and other mixed conjugates such as quercetin glucuronide sulfate were the main circulating forms of quercetin in human plasma and in urine following the ingestion of

onion using HPLC-MS². In contrast, Witting *et al.* (2001) did not detect any quercetin sulfates in plasma of human subjects after ingesting an onion meal. However, the failure to detect sulfate metabolites in plasma may be attributed to the nature of the solvent used for extraction. For example, acetone is a poor solvent for highly hydrophilic molecules such as quercetin sulfates (Day and Morgan, 2003).

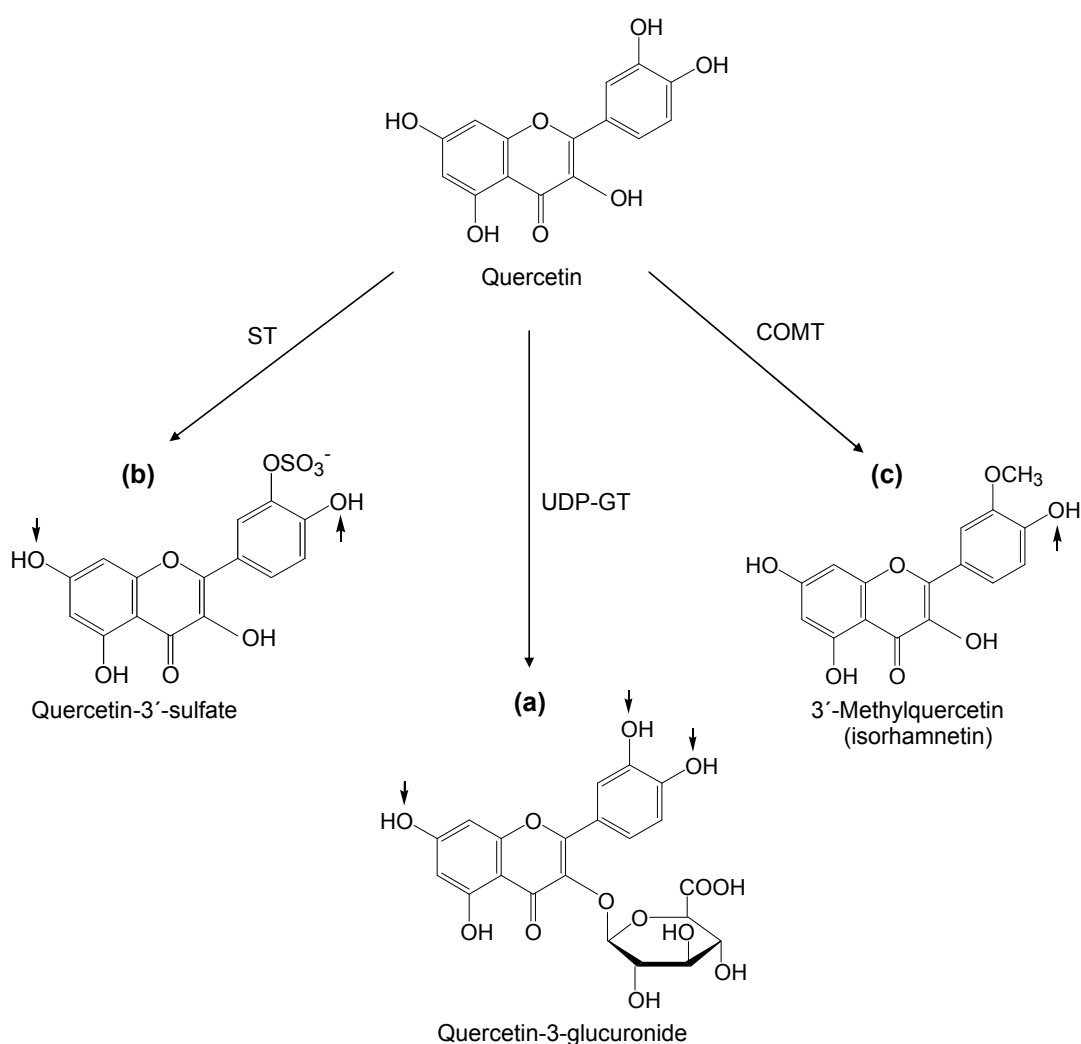


Figure 1.6 Phase II metabolism of flavonols; (a) glucuronidation by UDP-glucuronosyltransferase (UDP-GT), (b) sulfation by sulfotransferase (ST) and (c) methylation by catechol O-methyltransferase (COMT). Small arrows depict other sites of conjugation on the flavonol rings.

Methylation is a common pathway of flavonoid metabolism. Flavonoids with a catechol containing B-ring structure, such as quercetin, are readily methylated at the 3'- or 4'- position in a reaction catalysed by catechol *O*-methyltransferase (COMT; EC 2.1.1.6), resulting in the formation of 3'-methylquercetin or 4'-methylquercetin, also called tamarixetin (Figure 1.6c). Biotransformation reactions catalysed by COMT have been studied particularly well for quercetin in a number of animal models. For example, Zhu *et al.* (1994) demonstrated that quercetin was rapidly methylated in either porcine liver or hamster kidney with 3'-methylquercetin representing most of the excreted metabolites. Interestingly, the rate of methylation of quercetin was much more efficient than that of catechol estrogens and catecholamines. Manach *et al.* (1999) also showed that quercetin was methylated to a much higher degree than catechin following the administration of both flavonoids to rats. In humans, Day *et al.* (2001) reported the presence of 3'-methylquercetin conjugates in human plasma after onion consumption. Another study found that one-tenth of quercetin conjugates circulating in human plasma after the ingestion of a quercetin-rich meal was isorhamnetin (Graefe *et al.*, 2001).

Flavonol glycosides, such as rutin are not deglycosylated by human enzymes and thus not absorbed in the small intestine, are deglycosylated by colonic microflora that express α -rhamnosidase and β -glucosidase activity (Bokkenheuser *et al.*, 1987). The flavonol glucuronides excreted in the bile are also hydrolysed by the microflora and are able to release free aglycones. Aglycones can be re-absorbed, which results in enterohepatic cycling (Figure 1.7). The flavonol aglycones that are not absorbed can be further degraded in the colon by ring fission of the heterocyclic C-ring into phenolic acids, including 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and

3-methoxy-4-hydroxyphenylacetic acid (Olthof *et al.*, 2003; Jaganath *et al.*, 2006). Both the aglycone and the low weight phenolics may passively diffuse through the colon into the blood stream or excreted via the faeces. The absorbed aglycone and other phenolic acids may be glucucronidated in the liver and cycled many times before it is finally excreted through urine. Furthermore, evidence for rapid metabolism of quercetin within the colon has come from a study by Walle *et al.* (2001b) who reported that up to 80% of the administered dose of ^{14}C labelled quercetin was exhaled as carbon dioxide in healthy subjects since the ^{14}C -label was lost and not incorporated into phenolic acids.

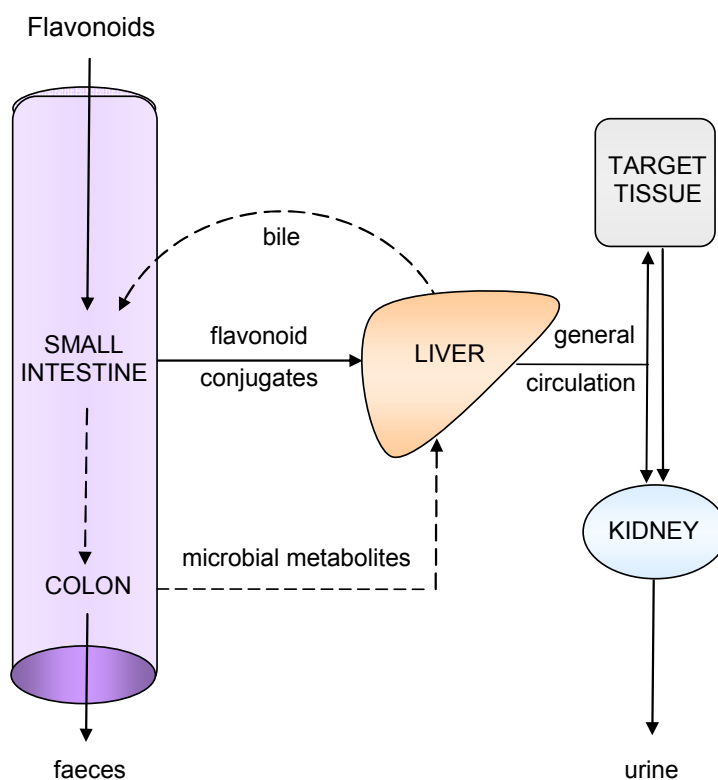


Figure 1.7 Scheme showing the fate of dietary flavonoids, including their glycoside *in vivo*. Dashed lines represent the enterohepatic circulation cycle.

Although extensively studied due to their ubiquity in plants, the absorption and metabolism of flavonoids remains a contentious issue. A solid knowledge of the factors

that effect flavonoid absorption such as the plant food matrix is imperative if metabolic pathways are to be elucidated. Well-designed studies that address this issue should provide new insight for optimising flavonoid bioavailability *in vivo*.

1.8 Aims of thesis

The overall aim of this project was to enhance understanding of the dietary intake and relative bioavailability of flavonols from foods in general, with special emphasis on the flavonol quercetin.

The aim of the first experimental study (Chapter 3) was to identify and quantify flavonol content in different varieties of commercially-grown tomatoes and tomato-based products in order to investigate and optimise flavonol bioavailability from these processed foods through human intervention studies.

Chapters 4 and 5 are concerned with human absorption of flavonols. The aim of Chapter 4 was to investigate the pharmacokinetics and bioavailability of quercetin in various chemical forms from onion-based foods; this was followed up in Chapter 5, with the aim of investigating the effect of dietary fat on the absorption of dietary quercetin glucosides and quercetin in healthy subjects.

Chapter 2

Materials and Methods

The standard materials and general methods described in detail in this chapter are used throughout the thesis. Specific experimental details are described in the relevant methodology section of each chapter.

2.1 Materials

2.1.1 Standards

The following flavonoid standards were purchased from Extrasynthese PLC, Genay, France:

- apigenin
- kaempferol
- quercetin-3-glucoside (isoquercetin)

The following flavonoids were purchased from Sigma Chemicals PLC, Dorset, UK:

- quercetin
- quercetin-3-rhamnoglucoside (rutin)

The following flavonoids were purchased from PlantChem, Sandnes, Norway:

- quercetin-3,4'-diglucoside
- quercetin-4'-glucoside

Flavonoid standards in general were prepared in methanol, a mixture of methanol and water, or a mixture of methanol and dimethyl sulfoxide (DMSO). Stock solutions of flavonoid in methanol were prepared on a regular basis as methanol is easily evaporated under nitrogen in air. Flavonoid standard solutions prepared in a mixture of methanol and water were stored for longer periods at 4°C.

2.1.2 Solvents and acids

The following solvents and acids were purchased from Fisher Chemicals PLC, Loughborough, UK:

- acetone, analytical grade
- acetonitrile, HPLC grade
- DMSO, analytical grade
- ethyl acetate, analytical grade
- methanol, HPLC grade
- acetic acid, glacial
- phosphoric acid, concentrated
- formic acid, 98%

The following solvent was also used:

- acetone, wash grade (VWR, France)

The following chemicals were purchased from Sigma Chemicals PLC, Dorset, UK:

- ammonium hydroxide solution, 28.0-30.0% as NH_3
- L-ascorbic acid, 99%
- trifluoroacetic acid

2.1.3 Enzymes

The following enzymes were purchased from Sigma Chemicals PLC, Dorset, UK:

- Cellulase from *Aspergillus niger* (EC 3.2.1.4); one unit will liberate 1.0 μmole of glucose from cellulose in one hr at pH 5.0 at 37°C (Cat. No. C1184). Enzyme was stored at 4°C.

- β -Glucuronidase from *Escherichia coli* Type IX-A (EC 3.2.1.31); one unit will liberate 1.0 μ g of phenolphthalein from phenolphthalein glucuronide per hr at 37°C (Cat. No. G7396). Enzyme was stored at -20°C.
- Sulfatase from *Aerobacter aerogenes* Type VI (EC 3.1.6.1); one unit will hydrolyze 1.0 μ mole of p-nitrophenyl sulfate per min at pH 7.1 at 37°C (Cat. No. S1629-10UN). Enzyme was stored at -20°C.

2.1.4 Buffers and solutions

The following chemicals were purchased from Sigma Chemicals PLC, Dorset, UK:

- sodium acetate (trihydrate)
- sodium phosphate, monobasic (anhydrous)
- sodium phosphate, dibasic (anhydrous)

The following buffers used in this study were:

- Acetate buffer (0.2 M), pH 5; acetic acid, glacial (0.2 M) was added to sodium acetate (0.2 M) in the ratio 30:70 v:v at pH 7
- Sodium phosphate buffer (0.2 M), pH 7; monobasic sodium phosphate (0.2 M) was added to dibasic sodium phosphate (0.2 M) in the ratio 31:69 v:v at pH 7

Purified water from a Millipore Direct-Q 5 purification system (Millipore, Watford, UK) was used throughout the experiments in this study. The system operates by passing tap water through a Proguard Pretreatment cartridge and a Reverse Osmosis cartridge, which deionises and removes organic contaminants and then stored in a 10 l reservoir. The water is further purified by a Quantum Cartridge and filtered through a

Millipak 0.22 μm sterilizing end filter to produce high resistivity product water (18.2 M Ω .cm at 25°C).

2.2 Food samples

2.2.1 Tomato and tomato-based products

Different cultivars of loose normal-sized and packed cherry tomatoes (*Lycopersicon esculentum*) grown in five European countries were purchased from Sainsbury's supermarket, Manchester, U.K.

The following tomato-based products were purchased from Sainsbury's supermarket, Manchester, UK:

- Sainsbury's tomato puree
- Sainsbury's creamed tomatoes
- Heinz tomato ketchup
- Heinz canned tomato meal

The following tomato-based products were purchased from Tesco supermarket, Leeds, UK:

- Tesco tomato puree
- Tesco tomato juice
- Tesco tomato ketchup

Merchant Gourmet sun dried tomatoes were purchased from Morrisons supermarket, Leeds, UK.

2.2.2 Onions and other foods

Normal-sized Spanish yellow onions (*Allium cepa*) were purchased loose from Sainsbury's supermarket, Manchester, UK.

The following foods were purchased from Morrisons supermarket, Leeds, UK:

- Echalion shallots (*Allium cepa*)
- Morrisons sweet almonds
- Morrisons ground almonds
- Lurpak butter

2.3 Equipment

The following laboratory instruments were used throughout this project:

- Metler PC 2200 digital analytical balance (0.01-1 g)
- Precisa 510 digital analytical balance (0.1-500 g)
- Stanton Instruments Unimatic CL41 analogue analytical balance (0.001-0.1 g)
- Clifton DU-8 ultrasonic waterbath
- Grant OLS 200 shaking waterbath
- Genevac EZ-2 Series personal evaporator
- Büchi rotovapor –EL
- Birchover Instruments Freeze Dryer 3.5
- Hanna Instruments pH 210 bench pH meter with H1131B electrode
- Moulinex Optiblend 2000 blender
- Eppendorf 5415 C desktop micro-centrifuge with maximum force of 16000 g on 24 (1.5-2.0 ml) samples

- Fisherbrand whirlmixers
- Gallenkamp stirrer and hot plate
- Soccorex automatic pipettes (10 μ l – 5 ml)
- IST VacMaster extraction manifold
- Interchangeable Weber Scientific glass syringes (2ml Glass tip Luer centre nozzle)

2.3.1 Solid phase extraction

OasisTM MAX single-use extraction cartridges were purchased from Waters Ltd, Hertfordshire, UK. The 3 cc (60 μ m) cartridges were packed with a mixed-mode, reversed phase/ strong anion-exchange, water-wettable polymer, highly selective for acids and ideal for extraction of metabolites in biofluids and tissue extracts.

2.4 High Performance Liquid Chromatography

2.4.1 HPLC system

The characterisation of flavonoids in foods and biological samples in chapters 3 and 4 was carried out by reversed-phase high performance liquid chromatography using a Merck-Hitachi Lachrom L-series HPLC-DAD, which included the following components:

- L-7200 autosampler (80-vial capacity) allowing automated and precise sample injections with low carryover

- D-7000 quaternary pump providing high pressure pulse-free mixing and delivery of solvents through the system
- C18 column packed with Prodigy 5 μm particle size ODS3 silica (100 Å), of 250 mm length by 4.6 mm internal diameter (Phenomenex, Macclesfield, U.K.)
- L-7450 diode array detector, with a D2 deuterium lamp and a wavelength range from 220 and 600 nm

The system was connected to a data processor by a GPIB connector and operated with the D-7000 HSM software installed on a desktop computer running under Microsoft Windows 2000.

2.4.2 Rapid-resolution HPLC system

Rapid-resolution HPLC offers a far more simple, rapid and economical HPLC method for quantification of flavonoids compared to normal reversed-phase HPLC. The equipment used in chapter 5 was an Agilent 1200 Series Rapid Resolution LC System. The system consisted of the following modules:

- 1200 Series vacuum degasser capable of passing solvent through a membrane tube which is permeable to gas but not liquid to reduce baseline noise and quenching effects
- 1200 Series binary pump SL, optimised to cope with the rapid resolution 1.8 μm particle size column

- 1200 Series high performance autosampler SL (100-vial sample tray) for high speed sample injections with lowest carryover
- 1200 Series diode array detector, a dual-lamp (deuterium and tungsten) design for highest sensitivity with a wavelength range of 190 to 950 nm
- 1200 Series thermostatted column compartment SL, allowing precision heating temperatures up to 100°C for reproducible retention times and peak areas
- SB-C18 rapid resolution 1.8 μm particle size column, of 50 mm length by 3.0 mm internal diameter (Agilent, Cheshire, UK)

Data acquisition and analysis was managed by ChemStation software designed to run on a desktop computer under Microsoft Windows XP which was connected to the module by a GPIB connector.

2.4.3 Solvents and other consumables

HPLC grade solvents (see above) were used in all HPLC analysis in order to achieve accurate, reproducible and high resolution analytical separations. All HPLC consumables were purchased from Chromos Express PLC, Macclesfield, UK, and consisted of the following:

- 2 ml crimp top glass vials (12 x 32 mm), with 11mm aluminium crimp caps with butyl/PTFE septa
- 200 μl tapered bottom inserts to fit 2 ml vials

- PTFE syringe filters (pore size 0.2 μm) of diameter 4 and 13 mm

Glass vials were capped using a vial crimper to achieve perfect crimping as over-crimping may cause coring, while under-crimping may lead to evaporation of sample. Glass inserts were used where sample volumes were less than 1 ml to minimise sample loss and provide excellent injection accuracy. In order to prevent blockage of the autosampler needle by particles or residues, all samples were filtered prior to HPLC injection. Occasionally, samples were centrifuged at 5000 g for 5 min before filtration.

2.4.4 Reversed-phase HPLC analysis

The analysis was performed using a modified method described by Rothwell *et al.* (2005). The mobile phases consisted of solvents A (Milli-Q purified water containing 0.1% TFA) and solvent B (acetonitrile). In the case of the Merck-Hitachi model, mobile phases were degassed in an ultrasonic waterbath for 5 min before use to prevent noisy baseline and minimise differences in retention time. The mobile phases were placed in glass bottles connected to the solvent lines. Air bubbles were purged from the solvent lines by opening the purge valve and pumping the mobile phases at a flow rate of 10 ml/min until the lines were clear of air. The mobile phases were allowed to mix with 83% TFA in water and 17% acetonitrile and the pressure would normally reach 130 bar. When the pressure rose constantly above 170 bar, 100% acetonitrile was pumped through the column to remove any residues. Evidently, column performance decreases with time as signified by increased back-pressure and peak widths, which in case the column would be changed. The mixed solvents were run at a constant flow rate of 1 ml/min, using a 35 min sequence gradient starting at 17% solvent B, increasing to

25% at 7 min, 35% at 15 min, 50% at 20 min and reaching 90% at 25 min and held 3 min, before returning to 17% for a 6 min re-equilibrium post run (Figure 2.1). Separation of flavonoid glycosides and aglycones was sufficient with the glycosides normally eluting typically between 7 and 15 mins, and aglycones usually between 16 and 24 mins. In order to minimise shifts in retention times and peak areas, solvent A (0.1% TFA in water) was changed at least every couple of days.

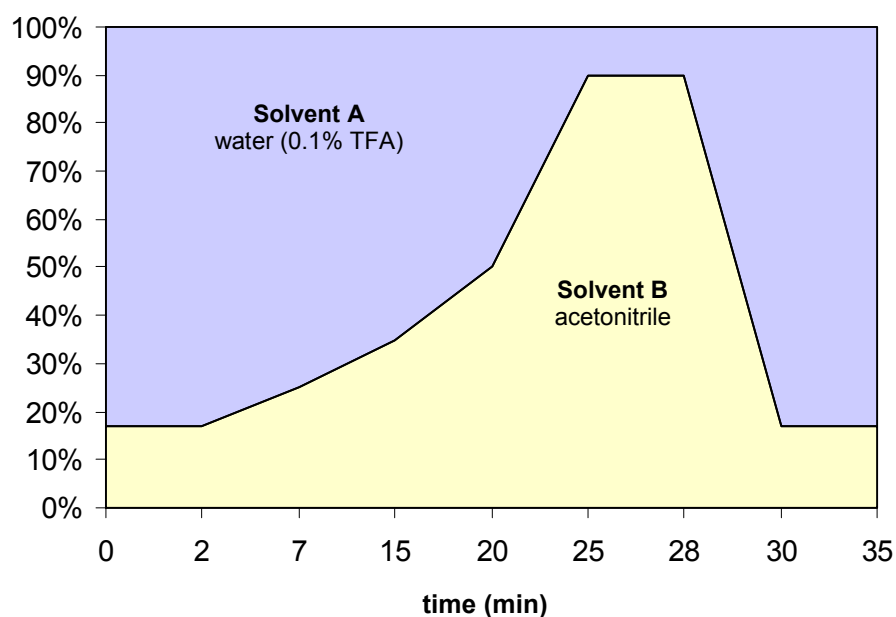


Figure 2.1 Area graph of solvent gradient over the course of a run for the Merck-Hitachi Lachrom L-series model.

The injection needle was set at a draw speed of 200 $\mu\text{l}/\text{min}$. The injection volume for the requirements of each experiment was set at 10 μl . The autosampler needle and injection port were automatically washed with 50% methanol in water five times before every run to prevent retaining any sample from previous injections.

Chromatograms were recorded at 270, 280, 290 and 370 nm. Monitoring wavelength was 270 nm. The detector conditions during the analysis were as follows: acquisition rate of spectra, 800 msec; spectral bandwidth for each channel, 4 nm; wavelength range, 220 to 600 nm. Absorbance was automatically initialised to zero before injection.

2.4.5 Rapid-resolution HPLC analysis

The Agilent 1200 series model was used for rapid-resolution HPLC analysis. The mobile phases consisted of solvent A (Milli-Q purified water containing 0.1% TFA) and solvent B (acetonitrile). The mobile phases were placed in capped glass bottles with the solvent lines immersed and the solvents were degassed in the vacuum degasser before priming the pump by opening the prime valve on the binary pump. The system was allowed to run for 5 min at a flow rate of 5 ml/min until the solvent lines were free of air. Once the pump was properly primed the priming valve was closed. The mobile phase was pumped through the column for 10 min for equilibration. The flow rate of the mixed solvents of 83% TFA in water and 17% acetonitrile was run at 0.85 ml/min with the pressure reaching > 300 bar, using an 8 min sequence gradient starting at 15% solvent B, increasing to 40% at 3 min and finally 90% at 5 min. At 6.5 min the solvent gradient reached the initial conditions again (Figure 2.2). Separation of flavonoid glycosides and aglycones was fast, accurate and efficient with the flavonoid glycosides normally eluting within the first minute, and aglycones usually between 2.5 and 3 mins. The maximum column pressure was set at 600 bar. If the pressure reached 600 bar, an error message was generated in the user interface and the system would shut down. The column temperature was kept at 30°C.

The default draw speed for the autosampler was set at 100 $\mu\text{l}/\text{min}$ and the sample injection volume was 10 μl . The autosampler uses a flow through design to assure washing of the needle outside thus minimising carry-over. Before the start of the injection sequence, and during analysis, the six-port injection valve is in the mainpass position where the solvent flows through the autosampler ensuring that the injection needle is flushed during the run. During the sampling sequence, the injection valve switches to the bypass position and solvent from the pump enters the valve and flows directly to the column. When the needle is lowered into the needle seat, the injection valve switches back to the mainpass position, flushing the sample onto the column.

Absorbances were read at all wavelengths between 190 and 900 nm in a bandwidth of 1 nm. Detection wavelength was 370 nm. Chromatograms were recorded at 270, 280, 320 and 370 nm.

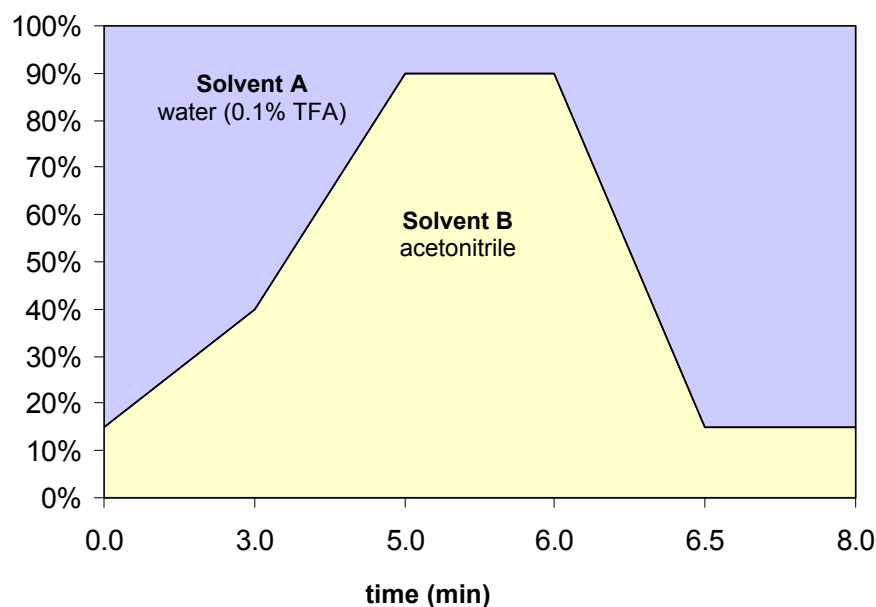


Figure 2.2 Area graph of solvent gradient over the course of a run for the Agilent 1200 series model.

2.5 Characterisation of flavonols

2.5.1 Construction of flavonol calibration curves

Calibration curves were prepared in order to accurately quantify flavonols from the analysis of food and biological samples. Calibration curves for the standard compounds (rutin and apigenin) and quercetin were constructed using the reversed-resolution HPLC method with absorbance measurements at 270 and 370 nm, respectively; calibration curves for the standard compounds quercetin, Q3,4'G, Q3G and Q4'G were constructed from the rapid-resolution HPLC method with absorbance measurements at 370 nm (Figure 2.3). Stock solutions were prepared by dissolving appropriate amounts of flavonoid in methanol to achieve concentrations of 500 µg/ml for each compound. Calibration standard samples of an appropriate range were prepared by serial dilution of the stock solutions. The linearity of the calibration curves for all flavonoid standards was validated by using measurements in duplicate with R^2 values exceeding 0.99.

Calibration curves were prepared by plotting the peak area against the quantity of compound eluted from the column, calculated from the concentration of solution and the volume of injection. A regression analysis was then performed using the equation $y = mx$, in order to calculate the mass of the compound from the peak area. The limit of detection (LOD) of rutin and quercetin using the reversed-resolution HPLC method was less than 10 ng per injection. The LOD of quercetin using the rapid-resolution HPLC method was found to be 5 ng per sample injected.

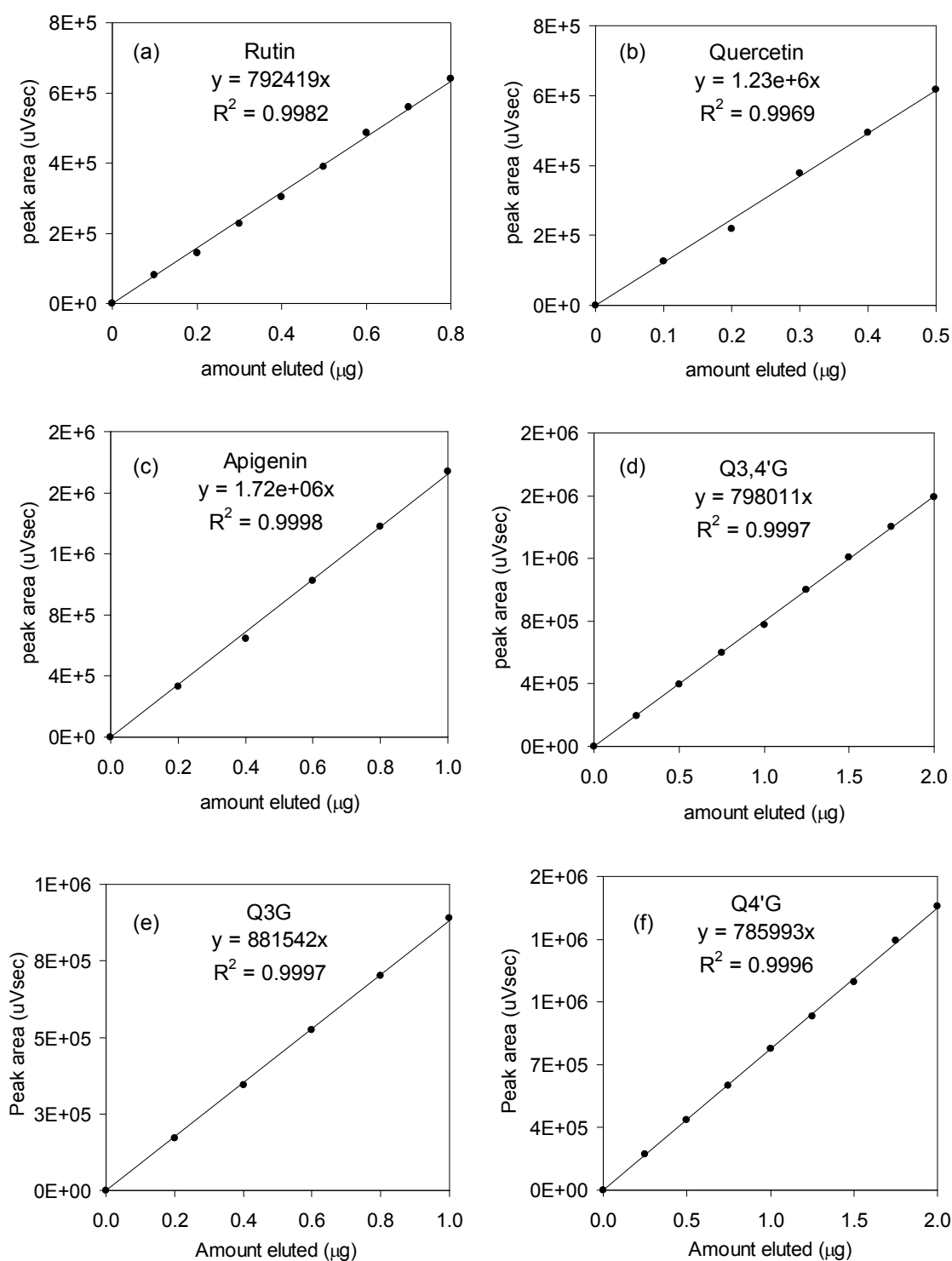


Figure 2.3 Calibration curves for (a) rutin (b) quercetin (c) apigenin (d) Q3,4'G (e) Q3G and (f) Q4'G monitored at 370 nm.

2.5.2 Verification of flavonol peaks

The structure of each eluted flavonoid was determined by one or more of the following methods:

- (i) Absorption spectra. Flavonoids show high and characteristic absorption due to their conjugated polyphenolic ring structure. Flavonoids show two typical absorption bands, one in the range of 300-400 nm (Band I) and the other in the range of 240-280 nm (Band II). The absorption spectra maxima were used as a reference when confirming the identity of the flavonoid. However, identification of flavonoids using spectra alone may be difficult as some flavonoids (e.g. flavonols and flavonones) have very similar spectra. The absorption spectra of four flavonoid standards are shown in Figure 2.4.
- (ii) Comparison of retention times. Flavonol identification was made by comparing the retention time of the eluted sample with that of the standard compound.
- (iii) Spiked input of flavonoid standards. The flavonoid peak in suspicion is verified by spiking the sample with a known amount of the flavonoid standard. An increase in the flavonoid peak area after spiking and the appearance of no other peaks confirm the identity of the flavonoid.

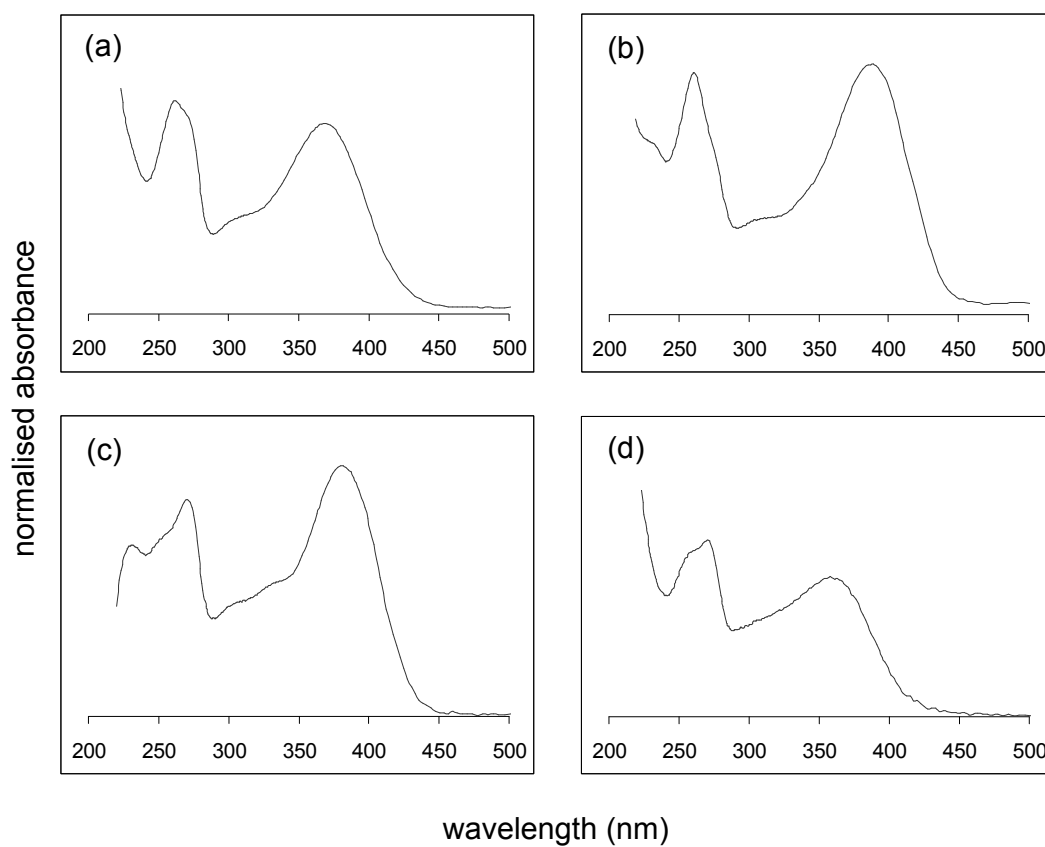


Figure 2.4 Absorption spectra of (a) rutin; (b) quercetin; (c) Q3G and (d) Q3,4'G in methanol using diode array detection.

Chapter 3

Characterisation of Flavonols

CHAPTER SUMMARY

- In this chapter, 12 different varieties of commercially-grown tomato fruits and 10 different tomato-based products were evaluated for their flavonoid content using HPLC with diode array detection.
- The predominant flavonol found in all tomato fruits and tomato-based products was quercetin-3-rutinoside (rutin).
- In general, the highest amounts of rutin in the different varieties of the commercially-grown tomato cultivars analysed were found in the cherry tomatoes.
- The flavonol content varied significantly among the different varieties of cherry tomatoes.
- Tomato puree contained the highest levels of flavonols among the selected range of tomato-based products analysed.
- The present study shows that commonly consumed tomato products, such as tomato puree and tomato juice are generally good sources of dietary flavonols.
- Crude cellulase is capable of hydrolysing the flavonol glycosides in tomatoes and tomato processed products.

3.1 Introduction

Tomato (*Lycopersicon esculentum*) is a major food crop grown commercially worldwide and is available all year round. The popularity of tomatoes comes not only from the fresh fruit market, but also from the processed food industries. Several epidemiological studies have shown that a dietary intake of fresh tomatoes and tomato-based products is associated with reduced incidence of many chronic diseases, such as cardiovascular diseases and some forms of cancer (Giovannucci, 1999; Rao and Agarwal, 1999; Silaste *et al.*, 2007). The nutritional qualities of tomato are characterised by the antioxidant micronutrient properties of lycopene, β -carotene, ascorbic acid, flavonoids, and vitamin E and various phenolic compounds found in the fruit (Willcox *et al.*, 2003).

Flavonoids are present in plants predominately as β -glycosides, i.e., conjugated to one or more sugar molecules. Glycosylation renders the flavonoid molecule more water-soluble, which is necessary for storage in plant cell vacuoles. Although the flavonoid content in tomatoes is moderate compared to other fruits or vegetables, such as onion, the increased consumption of tomatoes and processed tomato products such as sauces, pastes, salsa and juice makes them a good source of various flavonoids. The flavonol rutin, quercetin-3-rutinoside and the flavanone naringenin (as glycosides) are the predominant flavonoids found in the tomato fruit (Stewart *et al.*, 2000; Gall *et al.*, 2003; Bovy *et al.*, 2007). However, most of these flavonoids are concentrated mainly in the peel of the fruit. Table 3.1 shows the different flavonoids found in tomato and tomato products as reported from previous studies.

Flavonoids have drawn considerable attention as a result of epidemiological and *in vitro* studies linking increased consumption of flavonoid-rich fruits and vegetables to decreased incidence of coronary heart disease (Huxley and Neil, 2003) and common human cancers, including cancers of the lung, breast, prostate, and colon (Neuhouser, 2004). It is believed that many of the beneficial effects of flavonoids, result from their potent antioxidative and free radical scavenging properties. In addition, flavonoids have been reported to modulate the activities of several metabolic enzymes which catalyse the oxidation of low-density lipoproteins (e.g., XO, cyclooxygenase and lipoxygenase) and inhibit cellular proliferation (Landolfi *et al.*, 1984; Birt *et al.*, 2001; Dew *et al.*, 2005).

Tomato flavonoid content and antioxidant capacity varies considerably due to a number of factors, such as genetic variations (species and variety), degree of ripeness, environmental and agronomical conditions such as soil type, mineral nutrition, sun exposure and rainfall (Martinez-Valverde *et al.*, 2002; Dorais *et al.*, 2008).

The aim of the present chapter was to determine the flavonol contents of different varieties of commercially grown tomatoes and tomato-based products, chosen on the basis of their morphological feature, availability and popularity, by means of HPLC analysis. Ultimately, characterisation of the dietary flavonoids would help to evaluate the absorption and optimisation of flavonol bioavailability from these foods following consumption in human feeding studies by measuring flavonol levels in urine. It was hoped to investigate the effects of processing on flavonol bioavailability.

Table 3.1 Characterisation of flavonoids found in tomato and tomato-based products (modified from Gomez-Romero, 2007).

Food	Flavonoids detected	Observations	Reference
Commercial varieties of tomatoes	Quercetin, kaempferol, naringenin	Quercetin was the main flavonoid with levels varying in the different tomato varieties.	Martinez-Valverde <i>et al.</i> (2002)
Tomato fruit and processed tomato sauce	Rutin, naringenin	Rutin was the main flavonoid; processing caused a reduction in the concentration of naringenin.	Re <i>et al.</i> (2002)
Cherry tomatoes	Rutin, naringenin, chalconarigenin	Disappearance of chalconarigenin during ripening; rutin content was constant during postharvest ripening.	Slimestad and Verheul (2005)
Cherry tomatoes	Rutin, quercetin, naringenin	Rutin was the main flavonol with low levels of quercetin; declining content of quercetin and naringenin after first ripening stages.	Raffo <i>et al.</i> (2002)
Tomato fruits and tomato-based products	Quercetin, kaempferol and conjugates	Rutin was the main quercetin conjugate; Flavonol levels were found to vary according to fruit variety, size, and country of origin.	Stewart <i>et al.</i> (2000)
Tomato fruits, tomato salsa and tomato juice	Quercetin, kaempferol, myricetin	Quercetin levels varied among different types of fresh tomato, tomato salsa and tomato juice; myricetin was a minor source of aglycone.	Tokusoglu <i>et al.</i> (2003)
Tomato lines and cultivars	Rutin, naringenin, chalconarigenin, rutin-pentoside	Tomato lines with a low carotenoid content produce high levels of polyphenols with high antioxidant activity.	Minoggio <i>et al.</i> (2003)
Tomato varieties	Rutin, quercetin, kaempferol-3-rutinoside, naringenin, chalconarigenin	Increase in rutin levels correlated well with the total antioxidant activity of the tomato variants.	Spencer <i>et al.</i> (2005)

3.2 Methods

3.2.1 Preparation of tomato samples

Tomato cultivars: Different cultivars of tomato fruit (*Lycopersicon esculentum* L.) grown in the Netherlands, England, Spain, Italy and Portugal were purchased from a local supermarket between May 2005 and March 2006.

Tomato-based products: The following selections of processed tomato products were purchased locally from three different supermarkets; tomato puree, tomato juice, canned tomato, canned tomato meal and tomato ketchup.

The fresh tomatoes were washed, sliced and homogenised in a blender containing approximately 200 ml water. Aliquots of homogenised tomato (50 ml) were transferred in a 500 ml round-bottom flask and then submerged in a cold mixture of acetone and dry ice for about 3 min. Once frozen, the samples were freeze-dried overnight. The freeze-dried homogenate was removed from the flask and made into a fine powder using a pestle and mortar and stored in sealed vials at – 20°C until further analysis. Freeze-dried samples from the tomato-based products were prepared in a similar manner.

3.2.2 Extraction and hydrolysis conditions

Aliquots of the lyophilized material (0.5 g) were extracted with 20 ml of 80% aqueous methanol by incubating in a shaking water bath at 37°C for 2 h. The samples were allowed to settle; the extract was removed and reduced in volume under vacuum to 500 µl using a rotary evaporator. An equal volume of methanol was added followed by

filtration through a 0.2 µm PTFE filter prior to HPLC injection. The hydrolysis method used in this chapter was adapted from Garrett *et al.* (1999) as an alternative to acid hydrolysis. The methanolic extract was evaporated to near dryness and 0.2M sodium acetate buffer (5 ml, pH 5.0) containing 250 U of cellulase from *A. niger* was added. Enzymatic hydrolysis of the flavonol glycosides was achieved by overnight incubation at 37°C. Subsequently, 500 µl aliquots were taken and filtered through 0.2 µm PTFE filters prior to HPLC analysis.

3.2.3 Hydrolysis efficiency

The enzymic hydrolysis efficiency of cellulase from *A. niger* was assessed in a preliminary experiment. Pure rutin as a substrate dissolved in 80% methanol (100 µg/ml) was mixed thoroughly with 0.2M acetate buffer solution containing 250 units of cellulase (4 ml) prior to incubating in the shaking water bath at 37°C for 3 h. Aliquots (400 µl) from the enzyme buffer solution were taken during incubation at intervals of 0, 1, 2 and 3 h and mixed with pure methanol (500 µl) to stop the reaction and 0.01M ascorbic acid (100 µl) as stabilizer. The samples were filtered with PTFE filter units before HPLC analysis.

3.3 Results

3.3.1 Flavonoids in tomato fruits

The dominant flavonoid found in all tomato samples was quercetin-*O*-3-rutinoside (rutin). The presence of the flavonol glycoside was confirmed by retention time (9.38 min) and absorption spectra. Furthermore, none of the tomato extracts analysed contained flavonoid aglycones. The quantitative analyses of the flavonol content of 12 different cultivars of fully ripen tomato fruits prior to enzyme hydrolysis are shown in Table 3.2. The levels of rutin present in the fresh tomato cultivars varied, ranging from 4.1 for English Janet (a normal-sized tomato) to 69.5 µg/g fresh weight for Spanish Frunet (a cherry tomato).

Table 3.2 Flavonol content in tomato fruits extracted with 80% aqueous methanol.

Tomato category	Origin	Tomato variety	Date of harvest	Flavonol content ^a
Normal tomatoes	UK	Janet	May 2005	4.1 ± 1.3
Organic tomatoes	UK	Classy	Jun 2005	6.4 ± 0.2
Organic tomatoes	UK	Dometica	Oct 2005	4.3 ± 1.2
Cherry tomatoes	UK	Jennita	May 2005	38.7 ± 0.9
Cherry tomatoes	Holland	Sunita	May 2005	38.1 ± 1.4
Cherry tomatoes	Holland	Claree	May 2005	10.3 ± 1.0
Cherry tomatoes	Holland	Favorita	Oct 2005	22.7 ± 0.3
Cherry tomatoes	Portugal	Conchita	May 2005	14.1 ± 0.7
Cherry tomatoes	Spain	Josefina	Oct 2005	26.1 ± 0.7
Organic cherry tomatoes	Spain	Frunet	Mar 2006	69.5 ± 6.4
Cherry tomatoes	Italy	Shiren	Mar 2006	25.2 ± 2.0
Baby plumb tomatoes	Italy	Santa	Oct 2005	15.1 ± 0.8

^a Values are averages and expressed as µg/g fresh weight ± SE, n= 6

3.3.2 Flavonoids in processed tomato products

Based on their consumption and wide-spread use in different food preparations, a range of tomato-based products were analysed individually. Table 3.3 shows the average values of extractable rutin found in a number of processed tomato products. Sainsbury's tomato puree was identified as being rich in rutin, containing 50.9 $\mu\text{g/g}$ fresh weight. Analysis of Heinz tomato products (i.e. canned meal and ketchup) revealed much lower levels of flavonol compared to fresh cherry tomatoes containing only 10.9 and 8.2 $\mu\text{g/g}$ respectively. Characteristic chromatographic peaks of rutin were identified in the analysed tomato products on the basis of their HPLC retention times (Figure 3.1) and absorption spectra (Figure 2.4a).

Table 3.3 Flavonol content in tomato-based products after extraction with 80% aqueous methanol.

Product	Brand	Date of purchase	Flavonol content ^a
Tomato puree	Tesco	May 2005	29.0 $\pm$ 0.5
Tomato puree	Sainsbury's	May 2005	37.6 $\pm$ 0.9
Tomato puree	Sainsbury's	Aug 2006	50.9 $\pm$ 0.3
Tomato juice	Tesco	May 2005	21.6 $\pm$ 1.0
Canned tomatoes in juice	Tesco	May 2005	16.6 $\pm$ 1.3
Creamed tomatoes	Sainsbury's	May 2005	25.8 $\pm$ 1.5
Creamed tomatoes	Sainsbury's	Aug 2006	35.6 $\pm$ 0.8
Tomato ketchup	Tesco	May 2005	19.0 $\pm$ 3.1
Tomato ketchup	Heinz	May 2005	8.2 $\pm$ 0.3
Canned tomato meal	Heinz	May 2005	10.9 $\pm$ 0.1
Sun dried tomatoes	Merchant Gourmet	Feb 2006	12.0 $\pm$ 0.1

^a Values are averages and expressed as $\mu\text{g/g}$ fresh weight $\pm$ SE, n= 6

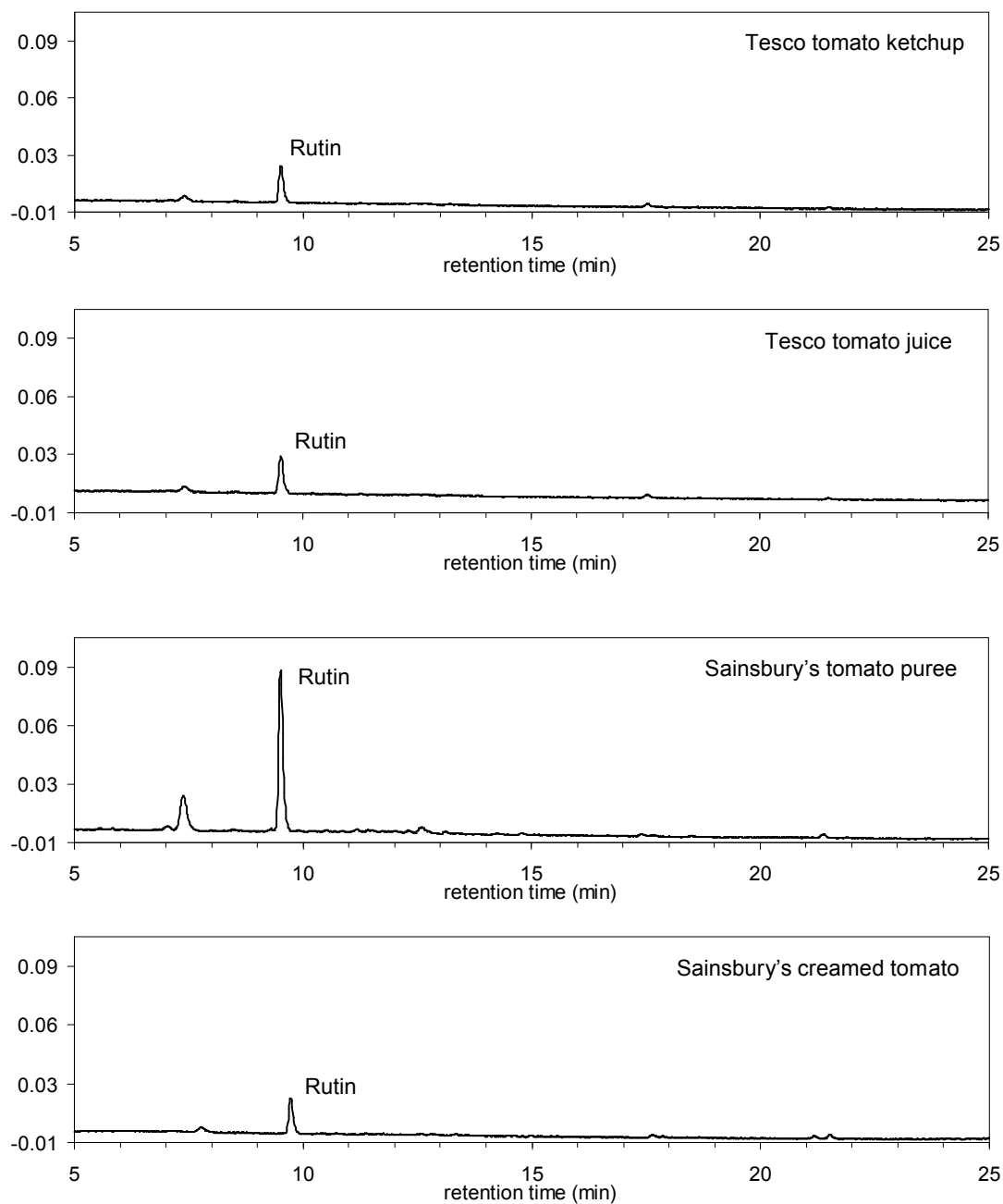


Figure 3.1 HPLC chromatograms, monitoring relative absorbance at 370 nm, of different tomato-based product extracts showing rutin as the main flavonol.

3.3.3 Enzymatic hydrolysis of flavonoids

Enzymatic hydrolysis was used as an alternative to acid hydrolysis for the deglycosylation of the flavonol glycoside rutin in the tomato extracts. In order to determine the effectiveness of the cellulase treatment, a control assay using pure rutin as a substrate was performed. Figure 3.2a and b show chromatograms from 0, 1, 2 and 3 h incubations of pure rutin with cellulase with the quantitative changes in quercetin concentration, demonstrating the conversion of rutin to quercetin by the hydrolytic cleavage of the rutinoside moiety.

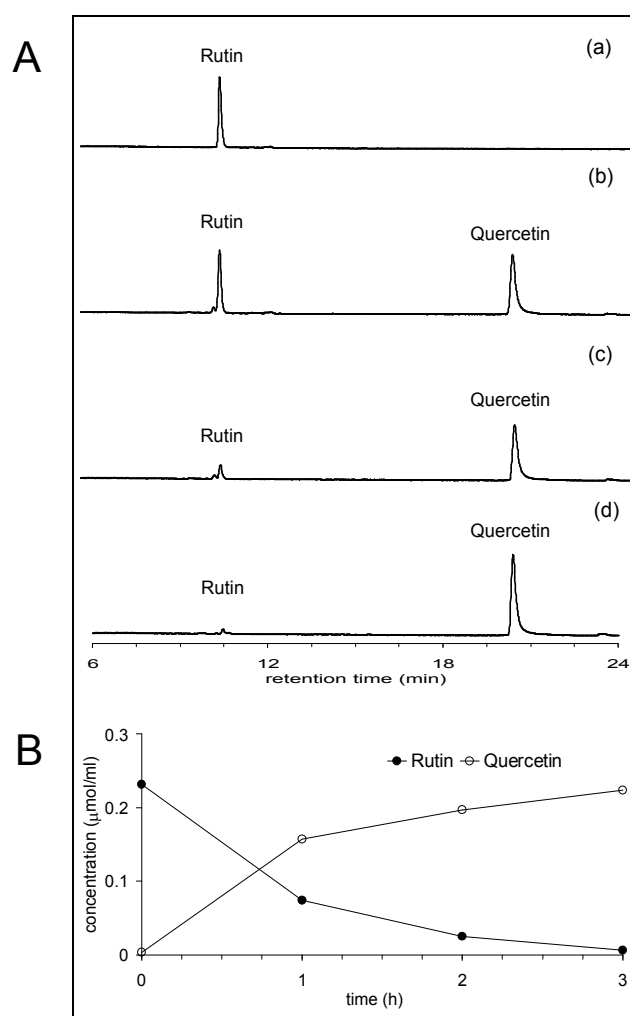


Figure 3.2 HPLC chromatograms (A), monitoring relative absorbance at 370 nm, of pure rutin after incubation with cellulase at 37°C for (a) 0 h (b) 1 h (c) 2 h (d) 3 h and the quantitative changes in quercetin concentration from the hydrolysis of rutin (B).

When the different cultivars of tomatoes were treated with cellulase, the main flavonol, rutin was completely hydrolysed during the 24 h period. A similar pattern of deglycosylation was seen in all the tomato processed products analysed following treatment with cellulase enzyme. However, no peaks corresponding to free quercetin were present (within the limits of detection) in the HPLC chromatograms of the hydrolysed tomato extracts (Figure 3.3).

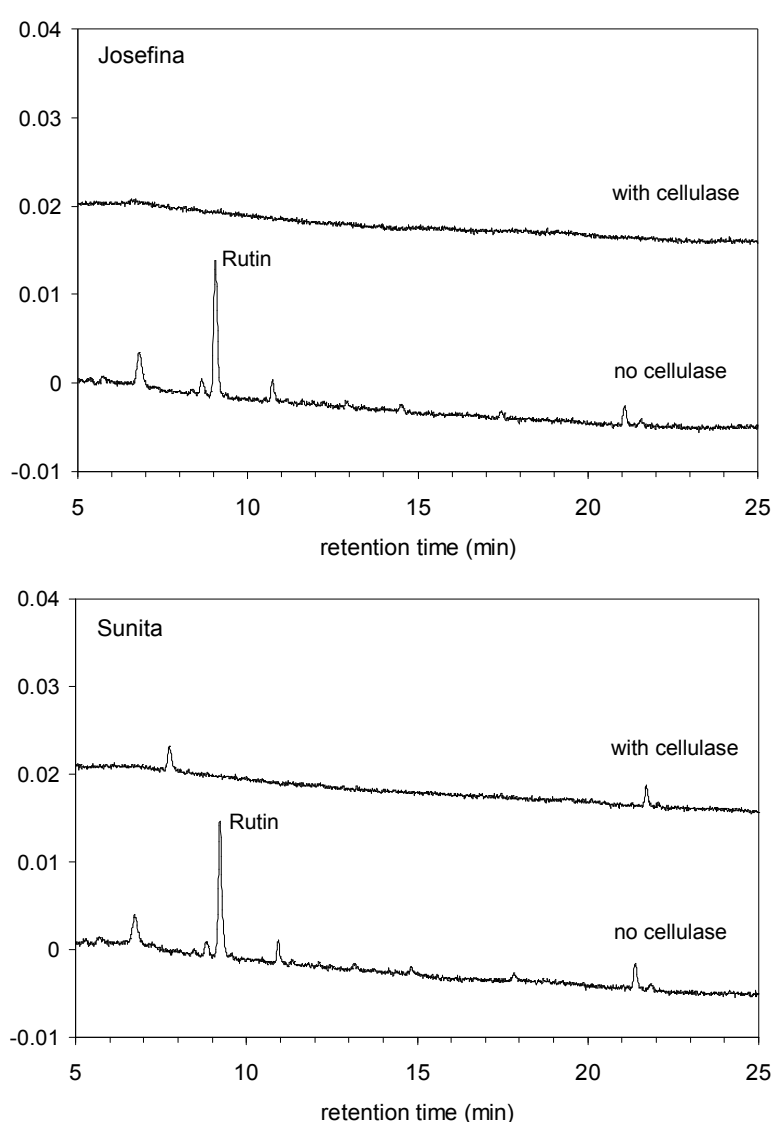


Figure 3.3 HPLC profiles of two different cultivars of cherry tomatoes monitored at 370 nm. Lower traces are chromatograms of tomato extracts showing rutin without cellulase; upper traces are chromatograms 24 h after cellulase treatment.

3.4 Discussion

Reversed-phase HPLC was the main analytical tool used in this study. The combination of HPLC reversed-phase C18 columns with diode array detection offers a reliable and reproducible analytical tool for the quantification of flavonoids from different food sources (Harnly *et al.*, 2006). HPLC has been widely used for the separation and determination of flavonols in tomato and tomato products (Table 3.1). In the present study, a sensitive reversed-phase HPLC method coupled with a suitable choice of mobile phase and an appropriate sequence gradient was used to detect and quantify the flavonoids in different cultivars of tomato and processed tomato products.

Lyophilization of tomato samples prior to extraction and HPLC analysis is thought to be an important procedure as flavonol glycosides are liberated from the food matrix and also better preserved when freeze-dried and stored for long periods. For example, in a recent study on the effects of drying processes on the antioxidant properties of tomatoes, freeze-dried and hot-air-dried tomatoes were found to contain higher amounts of total flavonoids than fresh ones (Chang *et al.*, 2006). The authors proposed that the increase in flavonoid levels may be caused by the release of cell wall-bound polyphenolic compounds during processing.

Determination of dietary flavonoid compounds in plant-derived foods relies on efficient extraction from the sample matrix prior to injection into the HPLC system. The advances in flavonoid detection and quantification using sophisticated instrumental techniques, such as HPLC coupled to UV, tandem-MS or nuclear magnetic resonance (NMR) detectors, cannot compensate for inefficient analyte extraction (Day and Morgan, 2003). The polarity of the solvent used for extraction is critical since, for

example, solvents such as hexane and dichloromethane are poor at extracting the polar flavanone glycosides in citrus juice (Marini and Balestrieri, 1995). In general, aqueous mixtures of methanol, ethanol or acetone are often used in the extraction of dietary flavonoid glycosides from a wide range of samples. For example, a recent study reported the extraction efficiency of rutin from apple peel with methanol being highly efficient compared to that from ethanol and ethyl acetate (Wach *et al.*, 2007). Furthermore, the authors observed a decrease in rutin yield from the apple when the percentage of methanol was reduced from 80%. In this study, methanol was considered to be a suitable choice for the extraction of the flavonol glycosides found in the tomato samples. Moreover, with dried or lyophilized plant materials, low polarity solvents such as ethyl acetate, simply leach the sample whereas alcoholic solvents or water presumably rupture cell membranes and enhance the extraction of endocellular materials (Robards, 2003).

Although it has been demonstrated that the flavonols accumulate mainly in the tomato skin, analysis was performed on whole tomatoes, including the seeds and flesh, since consuming the whole fruit maximises the delivery of the potential health benefits of the tomato (Toor and Savage, 2005). This in turn leads to a better estimation of the bioavailability of these bioactive compounds in human intervention studies.

HPLC analysis of the different varieties of tomato fruits confirmed that the dominant flavonoid glycoside was rutin, with maximum levels in Spanish cherry tomato, Frunet ($69.5 \pm 6.4 \mu\text{g/g}$), English cherry tomato, Jennita ($38.7 \pm 0.9 \mu\text{g/g}$) and Dutch cherry tomato, Sunita ($38.1 \pm 1.4 \mu\text{g/g}$). This is comparable to the findings of Crozier *et al.* (1997), who showed that quercetin content in hydrolysed extracts of English and

Spanish cherry tomatoes ranged from 17 to 203 $\mu\text{g/g}$. Similar variations in rutin content, ranging from 5.4 to 44.5 $\mu\text{g/g}$ of fresh weight, have been recently reported in different types of tomato cultivars using 2D-NMR techniques and high resolution LC-MS (Slimestad *et al.*, 2008). In another study, Stewart *et al.* (2000) demonstrated that tomatoes were a rich source of conjugated quercetin and that the majority of the flavonols (98%) were found in the skin with only negligible quantities (1%) in the seeds and flesh. In contrast to the flavonol glycosides, flavonoid aglycones such as quercetin, kaempferol and naringenin were not found in the tomato extracts. This is in agreement with Crozier *et al.* (1997), who showed that prior to acid hydrolysis of the tomato samples, quercetin and kaempferol were present primarily as conjugates. Similarly, Raffo *et al.* (2002) found that naringenin in Naomi cherry tomatoes was present as chalconaringenin or in a conjugated form, and its content before hydrolysis (in the free state) was negligible.

The results obtained from the analysed tomato samples indicate large variational differences in the levels of rutin, the highest concentrations being found in cherry tomato cultivars. For example, the flavonol content of English Jennita, a red cherry tomato, was 38.7 $\mu\text{g/g}$, compared to the normal-sized fruit variety, Janet, which contained 4.1 $\mu\text{g/g}$ fresh weight (Table 3.2). This is in agreement with Duthie and Crozier (2000), who reported the flavonol content in cherry tomatoes per g fresh weight being six times higher than that of normal size varieties of tomatoes. This may be explained by the fact that during exposure to high levels of sunlight flavonols are synthesized and stored in the skin of tomato fruit, thus the higher skin to volume ratio in cherry tomatoes will further enhance their flavonol content compared to that of normal size cultivars. For example, when flavonol levels in Spanish Favorita and

English Spectra fruit were estimated based on square centimeter of skin, the cherry tomato was found to contain 12.5 $\mu\text{g}/\text{cm}^2$ compared to 2.4 $\mu\text{g}/\text{cm}^2$ in the skin of the Spectra fruit (Stewart *et al.*, 2000).

In addition, varietal differences in flavonol levels among the different cultivars of cherry tomatoes existed. For example, rutin concentration in Spanish Frunet was approximately 7-fold higher than that of the Dutch Claree fruit (Figure 3.4). Similar variations in flavonol content, ranging from 3.4 to 22.2 $\mu\text{g}/\text{g}$ of fresh weight, have been reported in different varieties of cherry tomatoes (Stewart *et al.*, 2000). More recently, Lenucci *et al.* (2006) reported a content of flavonoid (equivalent to rutin) ranging between 622 and 134 $\mu\text{g}/\text{g}$ of fresh weight in southern Italian cherry tomato cultivars. The large variation in flavonol content of the different tomato cultivars may be attributed to differences in genotype and degree of ripeness (George *et al.*, 2004; Raffo *et al.*, 2002). Furthermore, environmental factors such as the amount of sun exposure and ambient temperature, and agronomic practices including mineral nutrition and water availability will also affect final levels of flavonoids (Dumas *et al.*, 2003). Organic agricultural practices have been shown to produce tomato varieties with high levels of flavonoids compared to conventionally produced tomatoes. For example, Mitchell *et al.* (2007) recently reported higher mean levels of quercetin and kaempferol in organic tomatoes as compared to their conventional counterparts grown in model plots over a ten-year period. This may provide some explanation to the high flavonol concentration found in the Spanish organic cherry tomato cultivar Frunet.

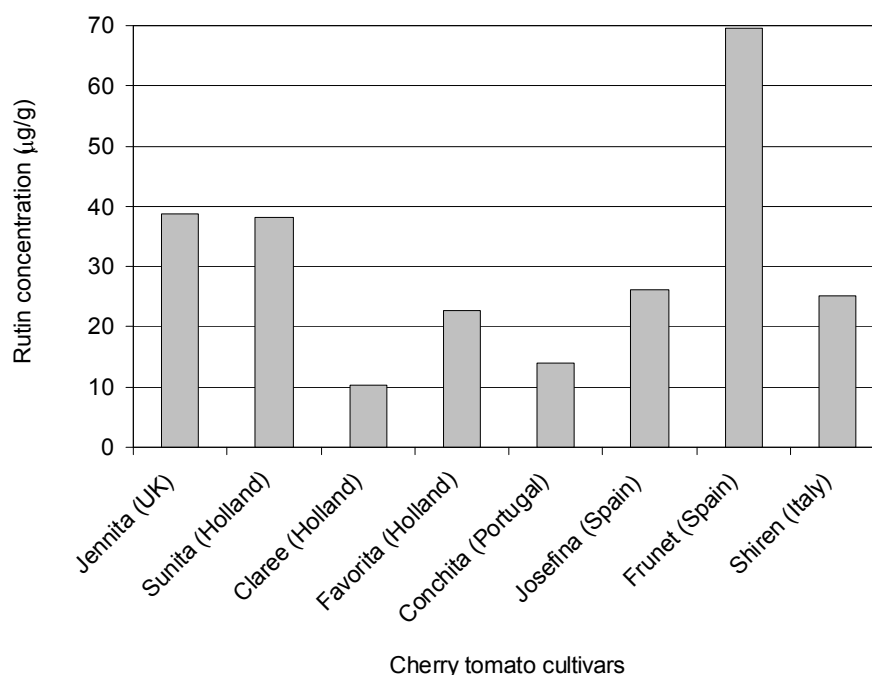


Figure 3.4 Flavonol content of eight different cultivars of cherry tomatoes produced in various countries.

Understanding the flavonoid composition in processed tomatoes, such as tomato puree, tomato ketchup and tomato juice is critical for assessing the activity and bioavailability of these compounds. Among nine different categories of tomato-based products analysed, tomato puree and creamed tomato were found to be a rich source of flavonol, with Sainsbury's tomato puree containing 50.9 µg/g rutin compared to that of creamed tomato with 35.6 µg/g rutin (Table 3.3). The flavonol glycoside (i.e. rutin) content was relatively lower than that previously reported for Sainsbury's double concentrate tomato puree in human intervention studies (Mauri *et al.*, 1999; Simonetti *et al.*, 2005). Differences in rutin concentration were also seen in the two samples of creamed tomato analysed. Such variations may be due to seasonal differences as previously observed in tomatoes (Crozier *et al.*, 1997).

According to the label on the packaging, the manufacturer claims that 100 g of the creamed tomato contains the equivalent of 150 g of fresh tomato. Processing typically involves thermal treatment with homogenisation followed by evaporation to produce tomato sauce and puree from fresh tomatoes. This may explain the high flavonol content found in tomato puree and also in creamed tomato relative to the other processed tomato products studied. Several studies have reported increased levels of flavonols in heat-treated tomato products. For example, Re *et al.* (2002) studied the effects of three different methods of food processing on the concentration of flavonoids in the Italian tomato variety Sorrento. The authors showed that under a cold break process at 65°C after evaporation, the rutin concentration increased considerably from 154.5 to 239.8 µg/g dry weight. In a more recent study, Chang *et al.* (2006) reported that high temperature drying treatments in two different varieties of tomatoes had significantly increased their total flavonoid content compared to that in the fresh ones.

Analysis of canned tomatoes showed high levels of flavonol glycoside compared to normal-sized fresh fruit (Table 3.3). In contrast, Stewart *et al.* (2000) reported considerably lower values of flavonols in canned tomatoes. The authors explained this finding in terms of flavonoid loss through leaching in the cooking water during the boiling process. For example, Crozier *et al.* (1997) observed an 80% loss in flavonol glycosides due to boiling; however, they did not provide information about the amount of flavonol leached into the cooking water. It is suggested that the higher values from the present study resulted because the canned tomatoes in juice were analysed as whole without discarding the juice. Tesco tomato juice was also identified as being a good source of rutin, containing about 22 µg/g. This might be due to the extraction of flavonols from the crushed tomatoes (mainly the skins) into the juice. Previously, an

increase in flavonol levels during fruit juice processing has been reported for citrus fruits (Pierpoint, 1986) and apples (van der Sluis *et al.*, 1997).

The qualitative and quantitative determination of individual flavonoid glycosides in foods is difficult, due to their large number. Acid hydrolysis is commonly used to remove sugars from the flavonoids, which reduces the number of standards required for identification and quantification (Hertog *et al.*, 1992b). Since evidence suggests that acid hydrolysis destroys some of the flavonoids, enzymatic hydrolysis was used (Dwyer and Peterson, 2002). Furthermore, the specificity and efficiency of the deglycosylating enzyme allows the release of larger amounts of aglycone from their sugar moieties than acid hydrolysis and elevated temperatures combined (Torres *et al.*, 2005).

In the present study, cellulase from *A. niger* was used to hydrolyse the flavonol glycosides in tomato extracts, using a protocol based on that of Garrett *et al.* (1999). This enzyme was selected because it has been predominately used in hydrolysing naturally-occurring flavonoid and isoflavone glycosides (Liggins *et al.*, 1998; Garrett *et al.*, 1999), and the relatively impure enzyme was inexpensive. In a preliminary experiment, the hydrolytic capacity of the cellulase enzyme was evaluated using pure rutin as a substrate. Hydrolysis with cellulase deglycosylated rutin well as the disappearance of the flavonol glycoside was associated with an increase in its hydrolytic product, quercetin (Figure 3.2a and b). This suggests that crude cellulase from *Aspergillus* sp. contains rutinase activity, which can cleave the rutinose group to release quercetin aglycone (Figure 3.5). Some species of *Aspergillus* fungus have demonstrated the capability of degrading rutin by secreting a wide range of

extracellular enzymes (Hund *et al.*, 1999). Interestingly, Sun *et al.* (2005) investigated rutinase activity in fungal enzyme preparations. The authors found rutinase activity in six of the eight crude pectolytic enzyme preparations. Furthermore, when asparagus juice was treated with pectinase from *A. niger*, the rutin content significantly decreased, reaching to zero within the first 2 h.

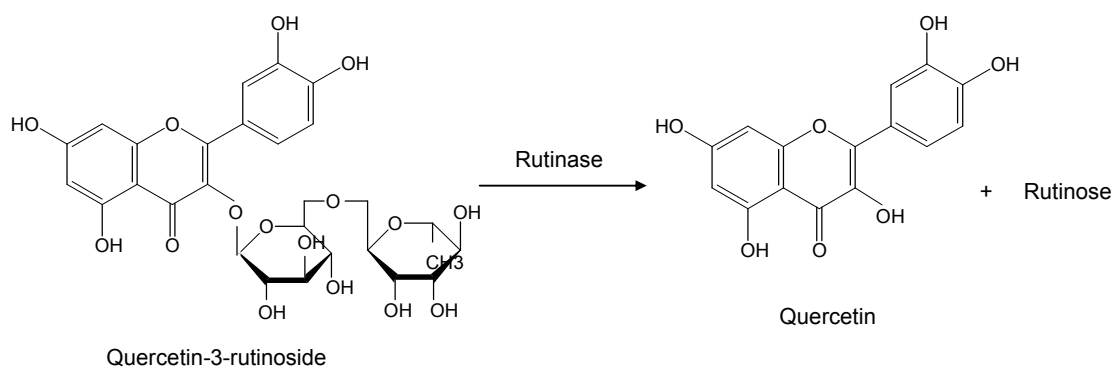


Figure 3.5 Hydrolytic cleavage of the glycoside group from rutin to form quercetin by rutinase.

According to the results for both the enzyme-hydrolysed extracts of tomato and tomato-based products no rutin was found. Thus, all the flavonol glycosides were completely hydrolysed by the cellulase enzyme. This complements and confirms the data observed from the control assay experiment. However, quantification of the free quercetin aglycone has been problematic. With only trace amounts of quercetin aglycone detected in the English cherry tomato Jennita, no substantial amounts of quercetin were detected in vast majority of the tomato samples analysed as evident from the chromatograms shown in Figure 3.3. A possible explanation could be the low solubility of quercetin in aqueous medium (i.e. water). It is pertinent to note that while flavonol glycosides are soluble in the acetate buffer, the possibility of quercetin precipitation in the aqueous solution after enzyme treatment cannot be discounted. Indeed, quercetin

solubility tests showed a decrease in the amount of quercetin solubilised in 25% propylene glycol in water by almost 3 orders of magnitude and in water by 5 orders of magnitude compared to pure propylene glycol (Piskula and Terao, 1998). Also, recent work by Rothwell *et al.* (2005) on Log P showed unusual behavior of quercetin among the flavonoids studied. The authors reported that quercetin was partially soluble in both solvents, thereby enabling irreversible precipitation to occur. Therefore, it may be prudent to utilise a more suitable solvent for the extraction of quercetin to allow better quantification of hydrolysed products of enzyme treatment.

Besides solubility in a buffer solution, the failure to detect any peaks corresponding to the aglycone in the HPLC chromatograms of the hydrolysed tomato extracts could also be due to chemical instability of quercetin. The effect of pH on the oxidation of quercetin in aqueous buffer solutions has been previously reported. For example, Takahama and Hirota (2000) observed the aggregation of quercetin after prolonged incubation in 0.1M sodium phosphate between pH 5 and 7. Another study investigating the optimal *in vitro* conditions for quercetin stability in human cell lines also reported similar results (van der Woude *et al.*, 2003). The researchers demonstrated that quercetin was unstable and easily decomposed at neutral buffer solutions with a half-life of 10 h. They also found that addition of ascorbic acid prevented quercetin degradation. Whilst the presence of ascorbic acid may serve as an antioxidant, at high concentrations, antioxidants start acting as pro-oxidants (Nuutila *et al.*, 2002). It is worth noting that the technical choice to perform the enzyme hydrolysis under sub-optimal conditions (i.e. no addition of antioxidants) was indeed a compromise between efficient production of aglycones and degradation of aglycones. The outcome of the enzyme hydrolysis experiments can be explained by considering the effect of

antioxidants such as ascorbic acid on quercetin stability. Further work is certainly needed to overcome the problems realised in the methodology used for the enzymatic hydrolysis of flavonol glycosides.

The results of the present study provide useful preliminary data on the flavonol glycoside levels in different cultivars of tomatoes and that of commonly consumed processed tomato products. In addition, the enzyme hydrolysis experiments have provided an insight into the problems encountered when working with flavonols such as quercetin. It is however important to note that despite these difficulties, enzyme hydrolysis is considered to be a viable alternative to acid hydrolysis. The possibility of maximising the increase in flavonol absorption and thus increasing the potential health benefits within different individuals by consuming particular fresh and/or processed foods is worthy of further study.

Chapter 4

Bioavailability of Flavonols

CHAPTER SUMMARY

- In this chapter, two methods for extraction of quercetin in biological fluids have been tested and compared.
- A human intervention study was carried out to compare the bioavailability of quercetin present in onion-based foods as either quercetin glycosides or quercetin by implementing time-course analysis of urinary excretion.
- A novel onion-based food was developed by incorporating almond extract to utilise endogenous enzymes to convert quercetin glycosides to quercetin.
- The main quercetin glycosides present in the control onion meal were Q4'G and Q3,4'G, with lower levels of quercetin and Q3G.
- There was inter-individual variability in patterns and amounts of excreted quercetin after consumption of the control and hydrolysed onion-based meals.
- The two onion-based meal studies showed dominant patterns with a major first peak of urinary quercetin (around 2 h) and a second peak (around 5-6 h).
- The ingested quercetin glycosides observed as quercetin conjugates in urine were excreted earlier than when quercetin aglycone was ingested (1 h compared to 2 h).
- Absorption of quercetin in its glycosidic form was significantly higher than the aglycone form (1.8% compared to 1.3%; $p<0.05$).

4.1 Introduction

4.1.1 Overview of flavonol bioavailability studies

Over the past 30 years, quercetin, a major representative of the flavonol subclass of flavonoids, has been extensively investigated due to its biological activities and wide distribution in plant-derived foods and beverages, mainly as glycosides, such as onions, apples and tea.

In an earlier study, Gugler *et al.* (1975) investigated the pharmacokinetics of quercetin in six human subjects after intake of 4 g pure quercetin aglycone. The authors failed to detect measurable concentrations of quercetin in plasma or urine samples. Thus, it was thought that the bioavailability of quercetin was poor (<1% of the original dose). However, this assumption was based on the limitation of the analytical techniques for detection of quercetin conjugates.

In 1995 Hollman *et al.* employed healthy ileostomy subjects (who had their lower bowel surgically removed) to investigate quercetin absorption at dietary concentrations. Ileostomy subjects were selected to avoid complications of microbial degradation in the colon. The flavonol in this study was provided by either an onion meal rich in quercetin glucosides, rutin (quercetin-3-rhamnoglucoside) supplement, or quercetin aglycone supplement. Flavonol content was measured using a sensitive fluorescence detection technique by post-column chelation with aluminium. The analysis of the hydrolysed ileostomy fluids revealed that the quercetin glycosides from the fried onions were highly retained in the body (52%) compared to the aglycone supplement (24%). In contrast, the amount of quercetin (as aglycone equivalents) absorbed from the rutin

supplement was much lower (17%). The data suggested that quercetin glucosides from onion were more efficiently absorbed than quercetin aglycone itself and that rhamnoglucoside, a common glycoside in tea, from the rutin supplement was even less rapidly absorbed. The study also suggested that absorption of quercetin glucoside occurs mainly in the small intestine. It was concluded that the sugar-conjugate was important for absorption and that the type of sugar moiety was the major determinant of the pharmacokinetics and bioavailability of quercetin glycosides. However, the researchers could not establish whether the quercetin glycosides were absorbed from the small intestine intact or deglycosylated before absorption of the aglycone, since they measured total quercetin after acid hydrolysis of the ileal fluid conjugates. More detailed analysis using LC-MS has facilitated the analysis of quercetin conjugates directly without resorting to acid or enzyme treatment. In another study with ileostomy subjects (Walle *et al.*, 2000) reported substantial amounts of quercetin aglycone present in the ileostomy fluid after consumption of onions despite trace amounts of free quercetin was found in the onion meal. They concluded that the quercetin glycosides were enzymatically hydrolysed in the small intestine, with most of the free quercetin then absorbed.

Hollman *et al.* (1997) studied the plasma pharmacokinetics of flavonol glycosides in healthy human subjects following the ingestion of onions, apples, or quercetin rutinoside. The authors showed that the maximum concentration of total quercetin in plasma ($C_{\max}$) appeared 0.7 h after the ingestion of onions, 2.5 h after apples and 9 h after rutinoside. It was concluded that quercetin glucosides from onions are absorbed rapidly, whereas the rutinoside, a quercetin glycoside found in apples, is absorbed slowly as they are hydrolysed by colonic microflora. In the same study, urinary

excretion of quercetin from the onions was 1.39% of the administered dose compared to 0.44% from the apples and 0.35% from rutinoides over a 24 h period. Hollman *et al.* (1999) in a similar experiment compared the plasma pharmacokinetics of quercetin after the ingestion of pure quercetin-4'-glucoside or pure quercetin-3-rutinoside in healthy volunteers. The time taken for total quercetin to reach peak plasma concentration ($T_{\max}$) was 0.5 h and 6 h after the intake of the glucoside and rutinoside respectively. Moreover, $C_{\max}$ of quercetin was 20 times higher after the intake of Q4'G than after quercetin-3-rutinoside. The pharmacokinetic data suggests that the sugar moiety determines the extent of quercetin absorption and that quercetin glucoside is absorbed rapidly from the small intestine, whereas the quercetin rutinoside is absorbed later on in the colon after deglycosylation. Similarly, Erlund *et al.* (2000) compared the absorption and pharmacokinetics of quercetin from supplements of quercetin aglycone and rutin in humans. They showed that $T_{\max}$ was considerably longer for rutin (6.5 h) than it was for quercetin aglycone (1.9 h).

Olthof *et al.* (2000) compared the absorption of orally administered pure Q3G and Q4'G in nine human subjects. The authors showed that the kinetic variables of quercetin absorption and elimination in the plasma for each glucoside were similar. They concluded that the bioavailability of Q3G and Q4'G were similar and that both forms of quercetin glucoside were rapidly absorbed irrespective of the position of the glucose moiety. Similarly, Graefe *et al.* (2001) found no significant difference in $C_{\max}$, $T_{\max}$ and the elimination half-life (T_{half}) between the onion-derived supplement and pure Q4'G. In a study on rats, Morand *et al.* (2000) reported a higher absorption of Q3G from the small intestine than quercetin aglycone resulting in a 3-fold increase in plasma levels of quercetin metabolites when fed with a diet containing Q3G than a diet

containing the quercetin aglycone. The authors concluded that the nature of the glycosylation markedly influenced the efficiency of quercetin absorption in the small intestine. This was confirmed several years later by Arts *et al.* (2004) who used an *in situ* rat intestinal perfusion model to study the absorption of five naturally occurring quercetin glycosides. After perfusing the rat small intestine with Q3G, Q4'G, quercetin-3-galactoside, quercetin-3-rhamnoside and quercetin-3-arabinopyranoside for 30 min, significant amounts of Q3G and Q4'G disappeared from the perfusate indicating their uptake and absorption. Taken together, these studies suggest that quercetin glycosides are more efficiently absorbed than quercetin aglycone.

4.1.2 Bioavailability of other flavonoids

The pharmacokinetics of other flavonoid compounds has also been explored. Absorption and distribution of isoflavones after soy consumption have been reported to be high compared to other flavonoid sub-classes; however results from these human studies were contradicting. For example, Izumi *et al.* (2000) reported greater bioavailability of isoflavones aglycones with $T_{\max}$ values of 2 h and 4 h after ingestion of aglycones and glucosides, respectively, whereas Zubik and Meydani (2003) found no difference between aglycone and glucoside on the $T_{\max}$ for plasma daidzein and genestein. They concluded that there were no significant differences in the absorption efficiency of genistein and daidzein when consumed as either aglycone or glucoside.

Few human studies have been performed concerning the bioavailability of flavones. Apigenin has been shown to have relatively low bioavailability in humans with 0.58% of the ingested dose excreted in urine and a short half-life (T_{half}) of about 12 h (Nielsen

et al., 1999). More recently, Meyer *et al.* (2006) reported the pharmacokinetics of apigenin plasma in 11 subjects after the consumption of 2 g of blanched parsley. The authors observed an average $T_{\max}$ value of 7.2 h. The urinary excretion of apigenin after 24 h was only 0.22% of the ingested dose of parsley.

A number of studies have reported on flavanone bioavailability in humans after the intake of pure compounds or citrus juices (Ameer *et al.*, 1996; Ishii *et al.*, 2000). In one study, the average urinary recovery of naringenin was 30.2% and 1.1% for five subjects ingesting the grapefruit juice and eight subjects ingesting the orange juice, respectively. For hesperetin, the urinary recovery was 2.2% after ingesting the orange juice (Erlund *et al.*, 2001). Kanaze *et al.* (2007) recently compared the pharmacokinetics of hesperetin and naringenin in plasma and urine, after healthy subjects were administered with a dose of 135 mg of pure hesperetin and naringenin. The authors reported that the flavanone aglycones, hesperetin and naringenin were both rapidly absorbed; $C_{\max}$ was reached within 4 h and the amounts of the total dose recovered in urine were 3.1% and 2.3% respectively.

The bioavailability of catechins after tea and cocoa consumption has been reviewed (Manach *et al.*, 2005). Evidence suggests that bioavailability is variable as urinary recovery has been found to be 0.5 – 6.0% for green tea epicatechin and EGC (Lee *et al.*, 1995; Yang *et al.*, 2000), 2.0 – 10% for red wine catechin (Donovan *et al.*, 2002), and as much as 30% for chocolate and cocoa epicatechin (Baba *et al.*, 2000). Catechins are rapidly absorbed in plasma and eliminated with T_{half} values of less than 7 h from black tea (van het Hof *et al.*, 1998) and as little as 1.9 h from pure EGCG (Chow *et al.*, 2001).

The majority of investigation into the bioavailability of anthocyanins in human suggests that they are poorly absorbed with reports of relatively very low urinary excretions ranging between 0.004% and 0.1% of the ingested dose (Murkovic *et al.*, 2001; Mulleder *et al.*, 2002; Wu *et al.*, 2002). However, the possibility that all of the metabolites might not have been identified in these studies emphasises the need to optimise more suitable methods for the analysis of anthocyanins (Manach and Donovan, 2004; Manach *et al.*, 2005). In a more recent study of 11 volunteers, Ohnishi *et al.* (2006) reported elevated levels of anthocyanin excretion (5%) after cranberry juice consumption.

Table 4.1 summarises the results of 14 selected human studies which reflect wide variation in the bioavailability of the dietary flavonoids, based on urinary recovery expressed as percent of the ingested dose.

4.1.3 Aim of chapter

The aim of this study was to (a) develop a novel, onion-based food incorporating almond extract to utilise endogenous enzymes to convert the naturally occurring quercetin glycosides to quercetin, (b) compare the bioavailability of quercetin present in onion-based foods as either quercetin glycosides or quercetin aglycone by implementing a 12 h time-course analysis of urinary excretion, and (c) carry out the human intervention study without the use of supplements as a high source of quercetin aglycone and to use experimental foods instead to avoid the matrix effects of food components.

Table 4.1 Examples of human intervention studies showing the recovery of various flavonoids from urine after dietary intake.

<i>Flavonoid sub-class</i>	<i>Flavonoid compound</i>	<i>Food source</i>	<i>Recovery in urine (%)</i>	<i>Reference</i>
Flavonols	quercetin	onion	1.4	Hollman <i>et al.</i> (1997)
	kaempferol	endive	1.9	DuPont <i>et al.</i> (2004)
		bean	5.4 – 6.1	Bonetti <i>et al.</i> (2007)
Flavanones	hesperetin	orange juice	5.3	Erlund <i>et al.</i> (2001)
	naringin	grapefruit juice	5.2 – 58.5	Fuhr and Kummert (1995)
Anthocyanins	peonidin glycosides	cranberry juice	5	Ohnishi <i>et al.</i> (2006)
	pelargonidin glucosides	strawberries	1.8	Felgines <i>et al.</i> (2003)
	cyanidin glycosides	elderberry	0.01 – 0.04	Murkovic <i>et al.</i> (2001)
Isoflavones	daidzein	soybean	62	King and Bursill (1998)
	genistein	soymilk	9 – 16	Xu <i>et al.</i> (2000)
Catechins	(-)-epigallocatechin (-)-epicatechin catechin metabolites	green tea	4.2 & 6.5	Pietta <i>et al.</i> (1998)
		red wine	3 – 10.3	Donovan <i>et al.</i> (2002)
Flavones	apigenin	parsley	0.6	Nielson <i>et al.</i> (1999)
	luteolin	supplement	2.3	Li and Jiang (2006)

4.2 Methods

4.2.1 Hydrolysis of onion samples

To determine the extent of β -glucosidase hydrolysis, two preliminary experiments were conducted. In the first experiment, yellow onion samples (approximately 200 g) were

skinned, chopped and homogenised in water (100 ml) containing ascorbic acid (0.01M) using a food processor. The onion homogenate was transferred to a glass beaker, covered with aluminum foil and stored at room temperature for 24 h. A sample (1ml) was taken at 0, 1, 2, 3, 4, 5 and 24 h and added to methanol (3 ml), to stop enzymatic reaction and vortexed for 1 min. An aliquot (1 ml) was taken and centrifuged (5000 g for 2 min) and then filtered using PTFE units (0.22 μ m) prior to HPLC analysis. In the second experiment, yellow onion samples (200 g) were peeled, chopped and homogenised in a food processor containing either 10 g of either assorted almond powder or freshly ground almond with skin in 100 ml of water with ascorbic acid (0.01M). The homogenised onion mixture was stored in a glass beaker and covered with foil for 5 h at room temperature. A sample (1ml) was taken at 0, 1, 2, 3, 4 and 5 h and treated as above.

Further studies on the effects of temperature and almond extract on the rate of hydrolysis of quercetin glycosides, both shallots and yellow onion samples were used. The shallots (110 - 180 g) were peeled, cut into small pieces and added into a food processor containing 10 g of ground almond in 100 ml of water with ascorbic acid. The chopped onions were homogenised and incubated for 3 h in a shaking water bath at 37°C, 50°C, 60°C and 70°C on separate occasions. At time zero, a baseline sample of the homogenised shallot (1 ml) was added to 3 ml of methanol and vortexed for 1 min to inactivate the β -glucosidases activity. An aliquot (1 ml) was centrifuged at 5000 g for 2 min, filtered using PTFE units (0.22 μ m) and subjected to HPLC analysis. The procedure was repeated after 0.5 h, 1 h, 2 h and 3 h in duplicates.

4.2.2 Onion meal preparation

Loose yellow onions (*Allium cepa*) used to prepare the two onion meals were purchased from a local supermarket. Both meals were prepared on the same day in the Food Technology Laboratory using the same batch of onions. For the control onion meal, 2000 g of medium-sized onions were peeled, chopped and homogenised with water (1500 ml) in a blender for 2 min. Sweet almond (10 g) purchased from a local supermarket was freshly ground and added to boiling water (500 ml). The almond was left at 100°C for 5 min to stop enzymatic activity before adding it to the homogenised onion in a large cooking pot. The pot was covered and left to simmer for 20 min before cooling. The same ingredients were used to prepare the hydrolysed onion meal but the preparation was carried out in a different manner. Freshly ground almond (10 g) was added in 500 ml cold water and then to the homogenised onion. The cooking pot was covered and left to incubate in a water bath (3 h at 50°C) stirring every 20 to 30 min. After incubation, the meal was brought to a simmer for 20 min. Both meals were left to cool and portions (200 ml) were frozen at – 20°C until use.

4.2.3 Quantification of flavonols in onion meal

Triplicate samples of each onion meal (1 ml) were extracted with 2 ml of ethyl acetate and vortexed for 2 min at room temperature. The mixture was allowed to separate before removing the ethyl acetate solvent phase. Ethyl acetate extraction was repeated three times. The combined solvent phase extractions (6 ml) were evaporated to dryness under vacuum using a rotary evaporator at 50 – 60°C. The dried yellow extract was dissolved in 50% aqueous methanol (400 µl) and filtered through 0.2 µm syringe filters prior to HPLC analysis. Aliquots of triplicate portions (1 ml) were taken from both

onion meals 24 and 48 h post-defrosting at 4°C, extracted with ethyl acetate and analysed.

4.2.4 Subjects

Ethical approval for the study protocol was obtained from the University of Leeds Local Ethics Committee. Ten healthy, non-smoking subjects (5 male and 5 female) were recruited among students and employees from the university. Subjects were not on prescription drugs or any other medication. The details of the study were fully explained to the participants, who gave informed and written consent after carefully reading the protocol. Subjects were aged between 26 and 56 years with a mean age of 34.3 years and a mean body mass index of 25 kg/m² (range: 21.6 – 27.7 kg/m²).

4.2.5 Study design

The two part study was carried out as follows (see Figure 4.1). Subjects were required to follow a diet low in flavonoid-rich foods for two days prior to the day of study. They were provided with a list of food items allowed and a list of flavonoid-rich foods to avoid (see Table 4.2). Subjects were requested to maintain a food diary to ensure they complied with the dietary requirements of the study. Each subject was given a measured portion of frozen control onion meal (200 ml) and a set of 13 plastic urine collection bottles containing 0.1 g of ascorbic acid labelled in sequence of time from 0 to 12 h to take home before day 3 of the study. The feeding study was carried out in two phases.

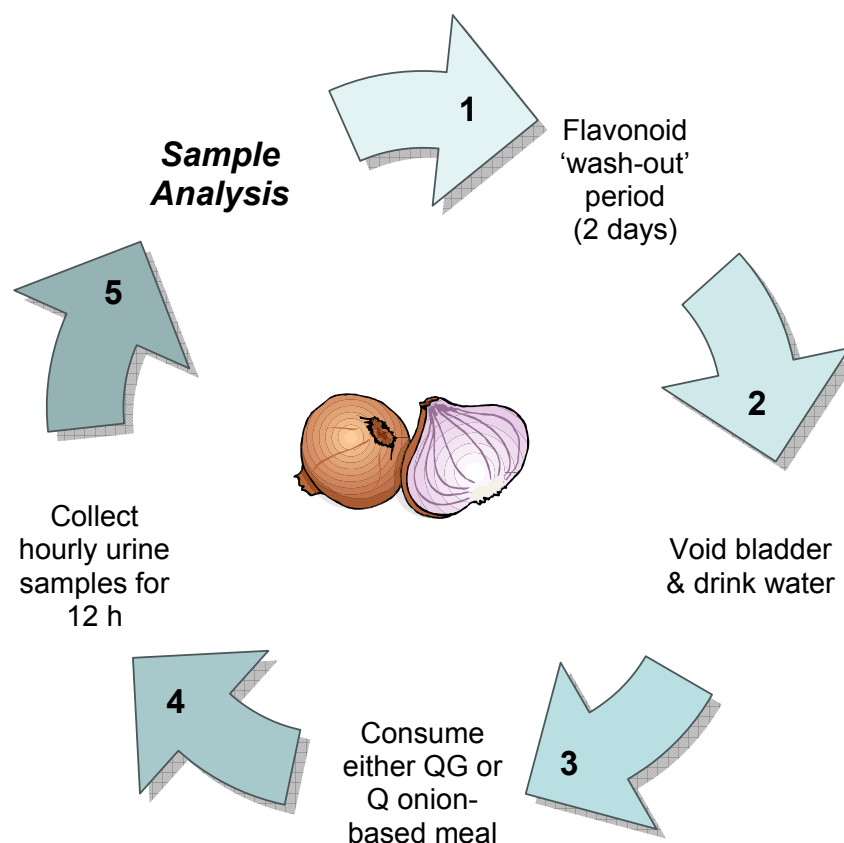


Figure 4.1 Diagram showing human intervention study design with the control onion meal containing quercetin glycosides (QG) and the hydrolysed onion meal containing quercetin (Q).

Phase 1: On day 3, subjects were instructed to void the bladder and immediately drink water (approximately 200 ml). After an hour, subjects would void the bladder and collect the urine sample in the bottle labelled 0 h as a baseline sample. The subjects then consumed the defrosted control onion meal within 10 min and were instructed not to have any other food or drink, except water, during the following 4 h. Every successive hour for 12 h, subjects were required to collect urine in the plastic bottle and immediately drink water. During the 12 h period of the study, subjects were instructed to consume a flavonoid-low meal for lunch and dinner. Subjects returned the 13 bottles

of collected urine to the Department on the following day after completing the first phase of the study. The urine samples were either analysed on the same day or stored at -20°C .

Table 4.2 Dietary intake advice given during the 3-day intervention study.

<i>Food items allowed</i>	<i>Food items not allowed</i>	<i>Food items allowed in moderation</i>
Meat, fish, poultry & eggs	All fruits (fresh, dried or canned)	Coffee
Dairy products	Fruit juices & jams	Chocolate
Vegetable oil, fats & butter	Onions, spring onions, shallots, leeks, chives and garlic	Green peas
White bread, pasta & pastry	Tea (black & green)	Herbs & spices
White rice & cereals	Tomato & tomato-based products	Dried nuts (all varieties)
Potato, carrot, & corn	Alcoholic beverages & vinegar	Cucumber with peel
Mushroom	Legumes	Honey
	Vegetables (broccoli, parsley celery, lettuce, pepper, fennel, spinach, kale & watercress)	

Phase 2: After at least a one week ‘washout period’, subjects were given a measured portion of frozen hydrolysed onion meal (200 ml) to consume and collect 12 h urine samples in a similar manner to the first part of the study.

The volume of each urine sample collected from individual subjects was measured and recorded. An aliquot (40 ml) of each urine sample was transferred to 50 ml centrifuge

tubes and stored at -20°C prior to analysis for total quercetin content. The remainder of each urine sample was discarded.

4.2.6 Solid-phase extraction of urine samples

Trial experiments to optimise the conditions for SPE of quercetin from urine samples were carried out using Oasis MAX SPE method for the analysis of acidic analytes, which include flavonoids (Figure 4.2). Urine samples (1 ml) were pretreated prior to solid-phase extraction by diluting with an equal volume of acidified water containing concentrated phosphoric acid (40 μl) and apigenin (0.1 $\mu\text{g/ml}$) as an internal standard. The cartridge was placed on the vacuum manifold and then conditioned by washing with methanol (5 ml) and purified water (5 ml). The diluted urine sample was loaded onto the cartridge. The cartridge was first washed with 5% ammonium hydroxide in water (2 ml) and then with methanol (2 ml). In another experiment, the ammonium hydroxide wash step was omitted. After purging with air, the quercetin was eluted with 2 ml of 2% formic acid in methanol (v/v). The flow rate of elution from the cartridge was 1 ml/min. The methanolic extract was evaporated to dryness under vacuum using a rotary evaporator at 45°C . The dried extract was reconstituted with 50% aqueous methanol (400 μl) and the solution was filtered before HPLC analysis. The 5% ammonium hydroxide in water solution and 2% formic acid in methanol wash solutions were always freshly prepared.

To determine the efficiency of quercetin extraction by the SPE method used, hydrolysed onion extract (1 ml) containing 204 μg of quercetin was added to urine (1:1) and extracted using the above procedure.

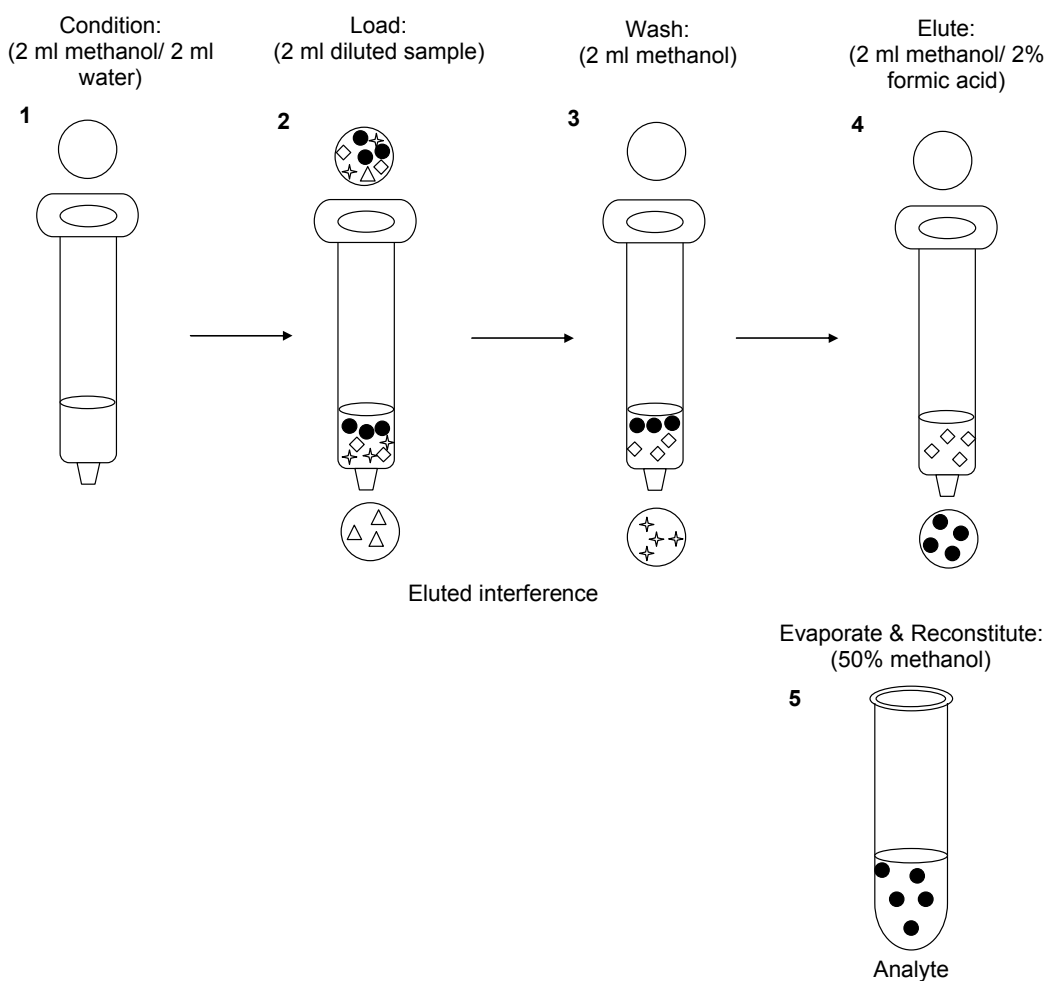


Figure 4.2 Process of solid-phase extraction of quercetin from urine sample using Oasis MAX 3cc cartridges.

4.2.7 Extraction and hydrolysis of urine samples

Total quercetin in urine was quantified using a modified version of the method previously described by DuPont *et al.* (2002). A sample (10 ml) of urine from each collection taken from the subjects was added to 1.5 ml of sodium phosphate buffer (0.1M; pH 7) containing 50 units β -glucuronidase and 0.30 units sulfatase (see Chapter 2 for units definition) in a 50 ml glass test tube. The tubes were sealed with parafilm and hydrolysis of the quercetin conjugates was achieved by incubation in a shaking

water bath (100 rpm; 37°C for 2.5 h). After enzymatic hydrolysis, solvent extraction was performed. Ethyl acetate (10 ml) was added and the sample vortexed for 2 min. The ethyl acetate fraction was collected and the buffer fraction further extracted twice with 10 ml ethyl acetate. The combined solvent extract (30 ml) was reduced to dryness under vacuum using a rotary evaporator and reconstituted in 50% methanol (400 µl). The sample was taken and filtered through a 0.2 µm PTFE filter unit prior to HPLC analysis. Non-hydrolysed urine samples were extracted directly with ethyl acetate.

To determine the efficiency of quercetin extraction with ethyl acetate, hydrolysed onion extract (1 ml) containing 204 µg of quercetin was added to urine (1:1) and extracted using the above method. In another experiment, apigenin (0.1 µg/ml) was added to urine (1 ml) as an internal standard and extracted with ethyl acetate.

4.2.8 Statistical Analysis

The results are presented as the average and standard error (SE). Statistical differences between the bioavailability from the control onion meal and the hydrolysed onion meal as well as gender differences were assessed by performing a Student *t* test. Differences with $P < 0.05$ were considered statistically significant.

4.3 Results

4.3.1 Determination of extraction conditions

The extraction recoveries of the flavonoids in urine using SPE were determined by comparing the areas of extracted samples with the non-extracted samples. Prior to the

elimination of the 5% ammonium hydroxide wash step from the original SPE method, recovery of quercetin from the spiked urine sample was $45.2 \pm 3.2\%$ (n=6). After modifying the SPE method, a considerable increase in quercetin recovery from urine was observed ($60.3 \pm 1.3\%$; n=6). On the other hand, the amount of extracted quercetin was much higher after extracting with ethyl acetate with an average recovery of $90.7 \pm 3.6\%$ (n=10).

Preliminary experiments showed that the recovery of apigenin from urine with the Oasis MAX cartridges was slightly higher at $69.6 \pm 9.8\%$ (n=6) in comparison with quercetin. On the other hand, recovery of the internal standard from urine after extraction with ethyl acetate was higher at $83.3 \pm 4.0\%$ (n=6). Moreover, the recoveries of both quercetin and apigenin from the urine samples after ethyl acetate extraction were similar.

4.3.2 Effect of homogenisation on flavonoid composition

Autolysis by endogenous enzymes of the flavonol β -glucosides in the onion tissue following homogenisation was studied over a 24 h period. During this extended period there were significant changes in the flavonoid profile (Figure 4.3). However, no detectable loss was found in total quercetin during the 24 h period. The HPLC analysis of the homogenised onion meal showed a gradual loss of Q3,4'G with a steady increase in the levels of quercetin aglycone. Also, the loss of Q3,4'G was accompanied by an initial increase in Q4'G and then a decrease after 5 h. By the end of 24 h no Q3,4'G could be detected. However, large amounts of Q4'G (62% of original amount) were not hydrolysed.

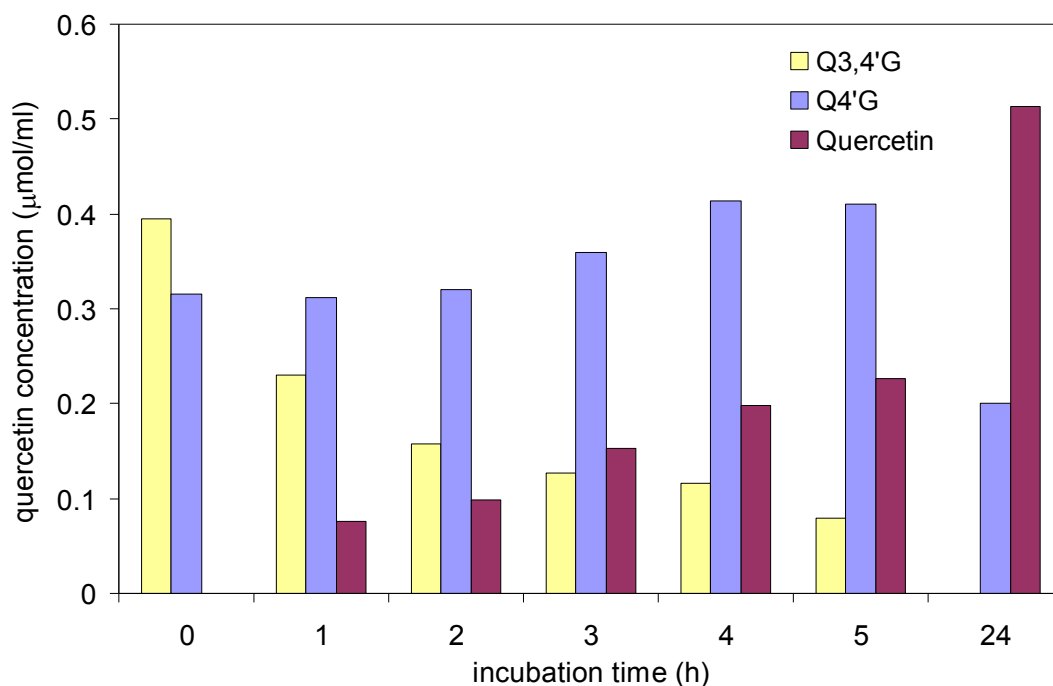


Figure 4.3 The effect of homogenisation on the composition of quercetin glycosides in yellow onion incubated at room temperature. (n= 2)

4.3.3 Effect of almond on hydrolysis of quercetin glycosides

Compositional changes in the flavonol glycoside profile of the homogenised shallot are shown in Figure 4.4. During the first 3 h there was a 75% loss of Q3,4'G accompanied by increases in both Q4'G and quercetin aglycone. In trial experiments, an additional source of β -glucosidases from almond was used to increase the efficiency of the deglycosylation of Q4'G and increase the levels of free quercetin. Analysis of the homogenised shallot extracts which had been incubated with the freshly ground almond for 3 h showed a much higher increase in free quercetin which was associated with a substantial decrease in Q4'G and Q3,4'G.

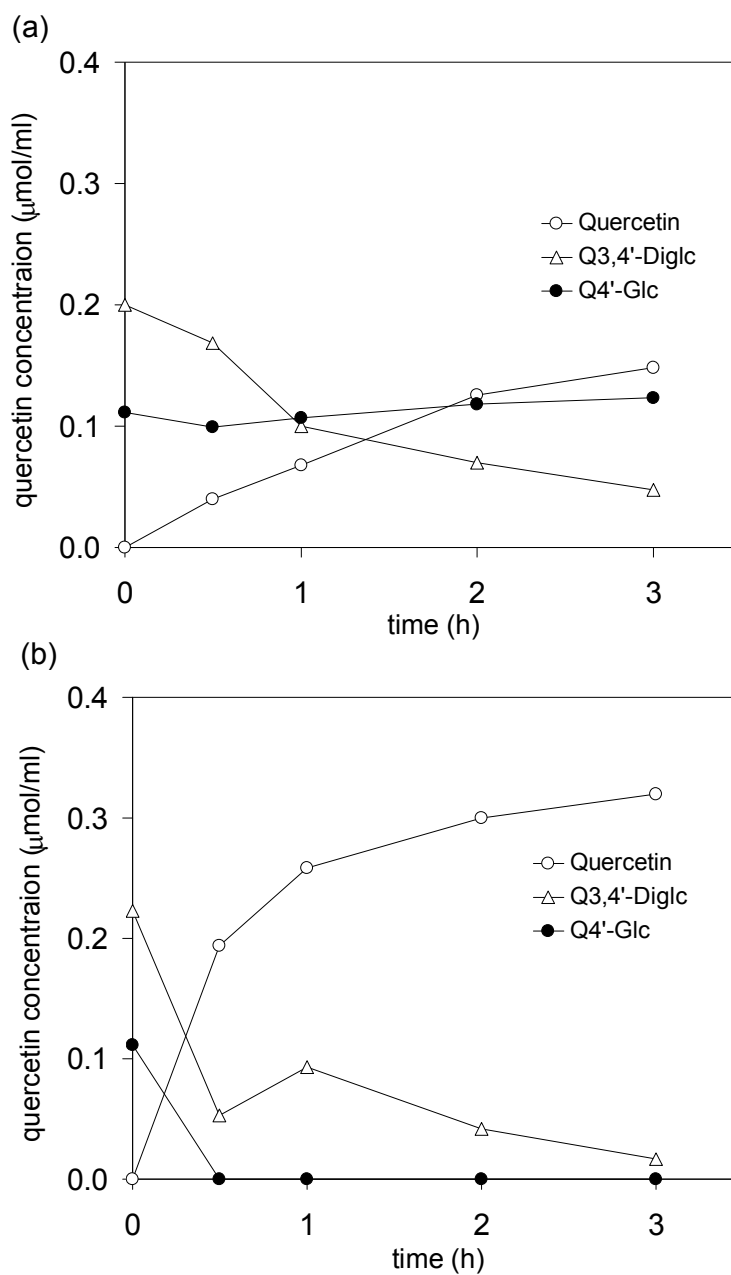


Figure 4.4 Changes in quercetin glycoside profile of homogenised shallot of the control (a) and with freshly ground almond (b) incubated at 37°C for 3 h. (n= 2)

Addition of freshly ground almond (10 g) to the homogenised shallot was associated with a 1.7 fold increase in the levels of free quercetin after 3 h at 37°C compared with the almond powder (Figure 4.5).

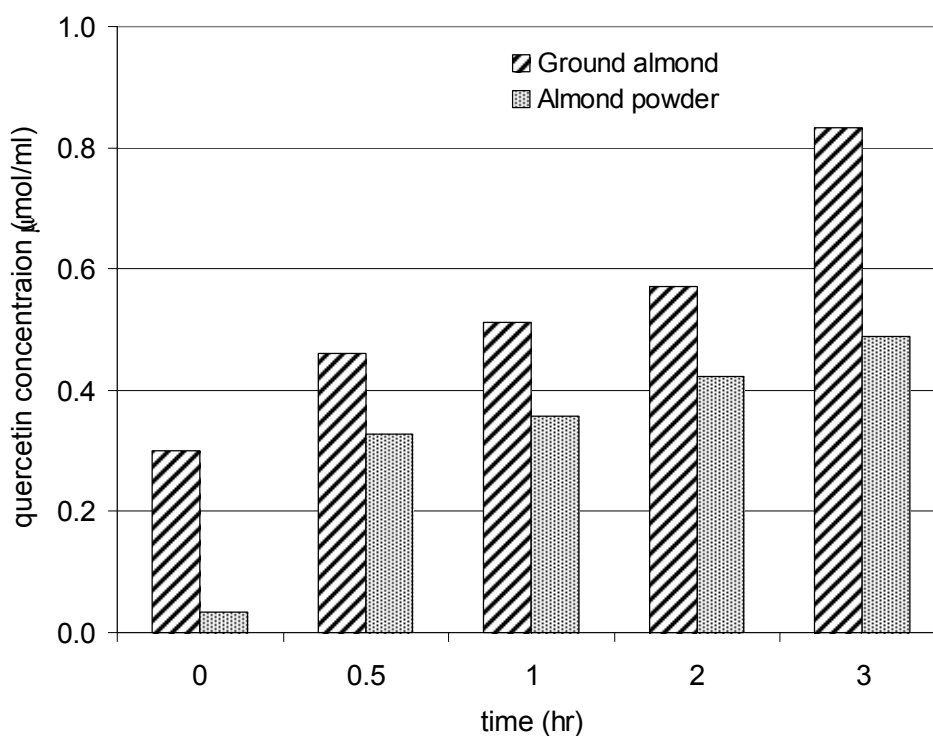


Figure 4.5 Hydrolysis of flavonol β -glucosides in homogenised shallot using two different forms of almond at 37°C. (n=2)

4.3.4 Effect of temperature on hydrolysis of quercetin glycosides

To determine the optimal temperature for hydrolysis of the quercetin glycosides in the homogenised onion meal containing the almond extract, a range of temperatures were tested. Complete hydrolysis of Q4'G and Q3,4'G was observed after 3 h of incubation at 50°C (Figure 4.6).

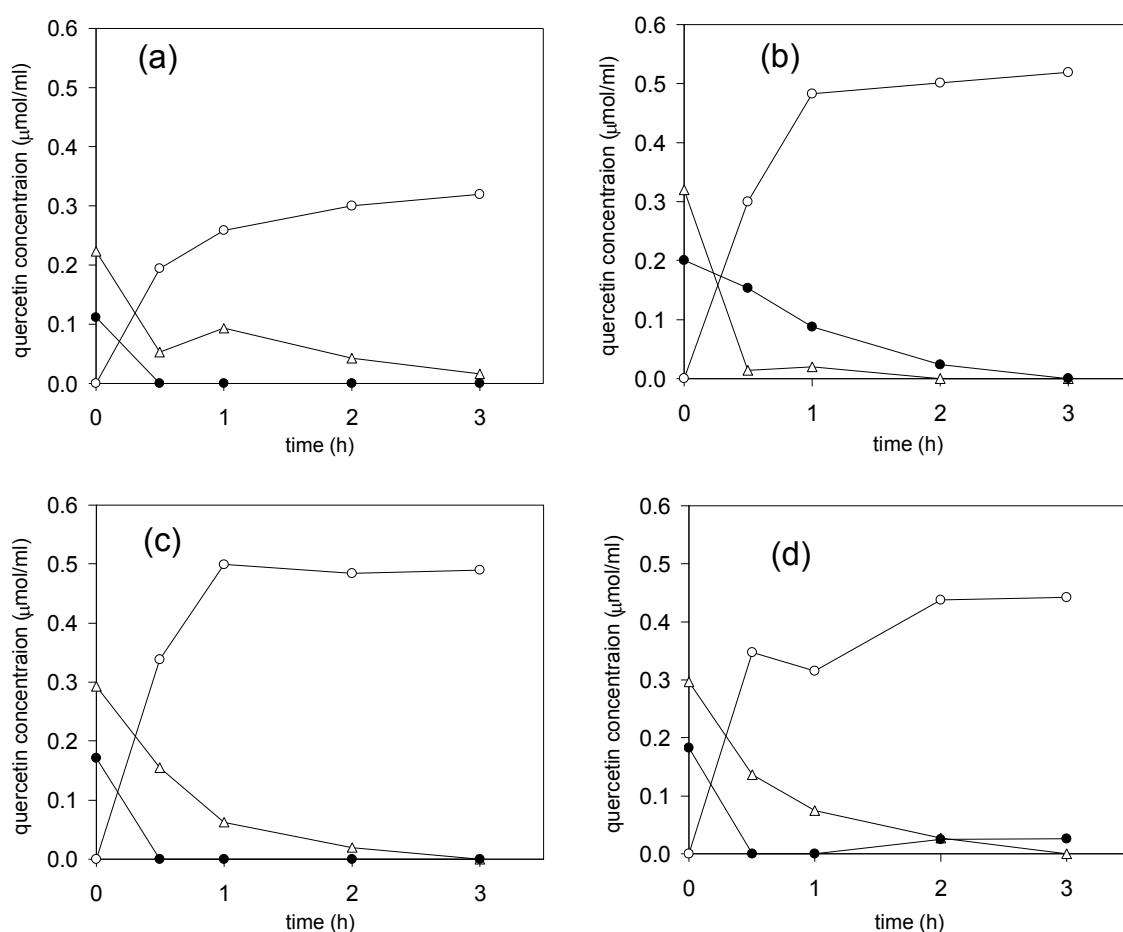


Figure 4.6 Hydrolysis of quercetin glycosides to quercetin aglycone in homogenised shallot with ground almond incubated at (a) 37°C, (b) 50°C, (c) 60°C and (d) 70°C.

—○— quercetin —△— quercetin-3,4'-diglucoside —●— quercetin-4'-glucoside

4.3.5 Analysis of the onion-based meals

Flavonoid quantification of the control and hydrolysed onion meals was determined by using reversed-phase HPLC with diode array detection. The only flavonoid found in the onion meal extracts was quercetin. The control onion meal contained 36 mg quercetin (expressed as quercetin aglycone equivalent). The concentration of the quercetin aglycone in the 200 ml portion of hydrolysed onion meal ingested was 32 mg. No quercetin glycosides were detected in the hydrolysed onion meal. The main quercetin

glycosides present in the control onion meal were quercetin Q4'G and Q3,4'G, with lower levels of quercetin (< 3%) and quercetin-3-glucoside (7%). Figure 4.7 shows typical HPLC chromatograms of the two types of onion meal in methanol at 370 nm.

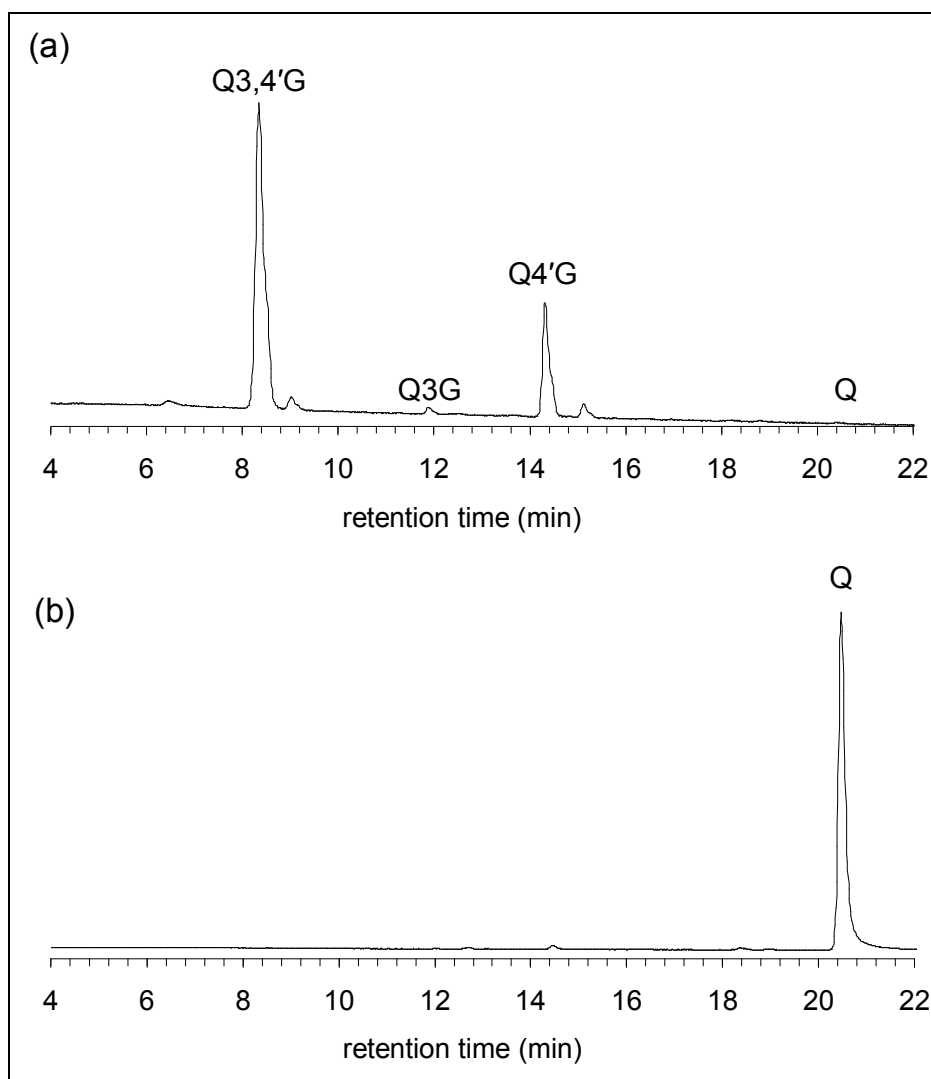


Figure 4.7 Chromatogram showing HPLC analysis of (a) control onion meal with almond; Q3,4'G, quercetin-3,4'-diglucoside; Q3G, quercetin-3-glucoside; Q4'G, quercetin-4'-glucoside; Q, quercetin and (b) hydrolysed onion meal with almond β -glucosidase; Q, quercetin aglycone. Absorbance measured at 370 nm.

4.3.6 Stability of flavonoids in onion meal

The impact of freeze-thaw on stability of the quercetin aglycones and quercetin glycosides was assessed prior to the feeding study. HPLC analysis of onion meal extracts showed a 0% and 8% loss of the total amount of quercetin glycosides from the frozen control onion meal 24 and 48 h post-defrosting, respectively. Total quercetin aglycone loss from the frozen hydrolysed onion was (12.5%) after 24 h and (17%) after 48 h post-defrosting at 4°C.

4.3.7 Efficiency of enzyme hydrolysis

Urine samples collected from each subject were analysed for quercetin prior to consumption of the onion-based meal (time 0). Quercetin was not detected in the hydrolysed urine baseline samples of any subjects. Quercetin appeared rapidly in conjugated form in the urine samples after consumption of the onion-based. However quercetin aglycone was not detected in the urine samples before enzyme hydrolysis. Quantification of quercetin in 0-12 h urine was determined after hydrolysis of quercetin metabolites with β -glucuronidase and sulphatase. Figure 4.8 shows the peak corresponding to quercetin in the enzymatically treated urine extract from one subject after consumption of the onion-based meal. In some cases, quercetin was detected in very low concentrations which resulted in poorly defined peaks. The identity of such peaks was confirmed by reference to absorption spectra and / or retention time of quercetin standard.

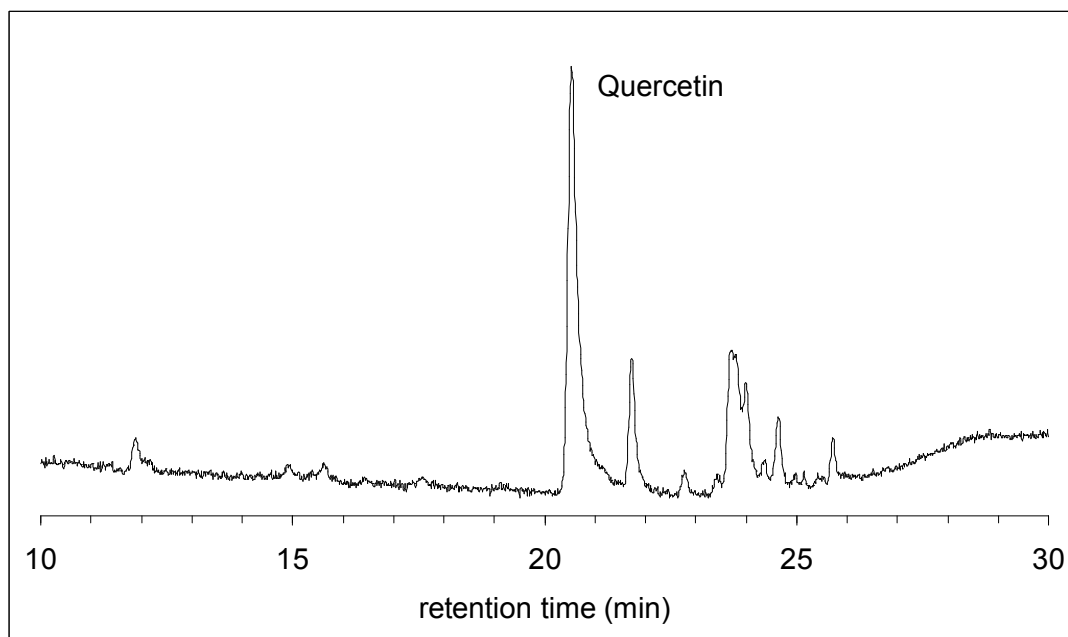


Figure 4.8 HPLC chromatogram showing quercetin in hydrolysed urine sample 1 h after ingestion of onion meal. Absorbance measured at 370 nm.

4.3.8 Time-course of excretion: quercetin glucosides from control onion

The time-course of total quercetin excretion (averaged for all ten subjects) following the consumption of the control onion meal is shown in Figure 4.9. A large inter-individual variability in 12 h-excreted quercetin after the consumption of the control onion meal was observed ranging between 231 μg and 850 μg . In nine out of ten subjects, the peak excretion after ingestion of the control onion meal was seen after 1 h followed by a subsequent smaller peak after 6 to 7 h. This was followed by a decline in quercetin excretion over the successive hours up until 12 h. The second peak of quercetin excretion was more prominent in the urinary time-courses of some of the subjects than others. A summary of the time-studies from the control onion meal in all ten subjects is shown in Table 4.3.

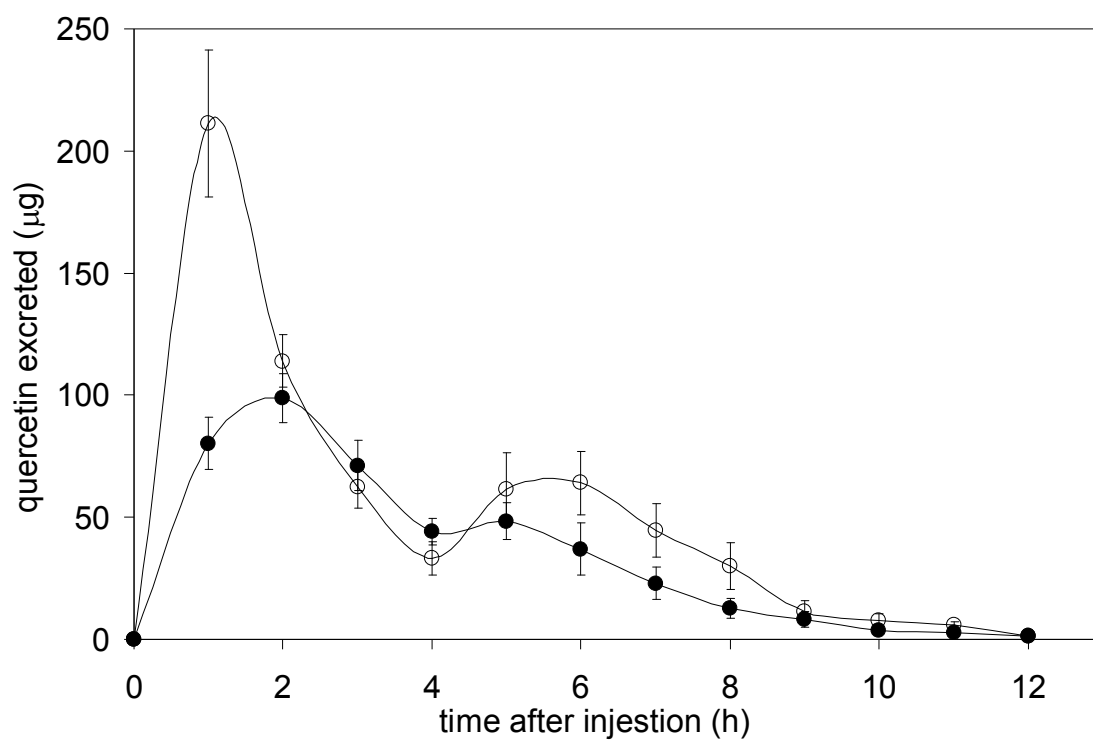


Figure 4.9 Time-courses of quercetin urinary excretion 0-12 h after ingestion of the onion-based meals. Values are averages for ten subjects for each type of onion meal. Error bars indicate SE. (○) control onion meal containing 36 mg Q; (●) hydrolysed onion meal containing 32 mg Q equivalent as quercetin glucosides.

Table 4.3 Table showing the hourly volumes of urine collected and quantities of quercetin detected from each subject after ingestion of the control onion meal.

♂	Subject 1		Subject 2		Subject 4		Subject 5		Subject 6	
Time (h)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)
0	155	ND	73	ND	62	ND	34	ND	37	ND
1	480	142	259	244	286	341	13	10	110	248
2	381	111	391	104	513	122	465	77	380	131
3	208	60	273	62	458	78	256	46	244	37
4	392	ND	318	23	228	35	187	29	244	19
5	137	79	73	35	102	4	430	54	173	60
6	264	73	224	73	30	11	19	3	56	37
7	242	62	192	107	97	20	276	9	207	10
8	216	21	158	68	318	14	217	3	203	7
9	260	20	325	46	379	7	248	ND	322	4
10	148	11	221	23	242	7	226	ND	236	4
11	120	11	99	13	360	10	266	ND	60	ND
12	212	ND	110	5	227	7	250	ND	106	ND
Totals	3215	590	2716	803	3302	655	2887	231	2378	556
♀	Subject 3		Subject 7		Subject 8		Subject 9		Subject 10	
Time (h)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)
0	56	ND	123	ND	69	ND	375	ND	244	ND
1	375	235	534	328	425	177	385	213	378	176
2	204	172	527	161	555	110	434	70	239	82
3	327	68	542	58	542	49	390	35	257	129
4	169	33	364	39	471	69	297	20	220	65
5	77	30	43	114	124	49	65	25	179	165
6	329	94	307	104	153	39	364	133	238	74
7	77	81	375	21	150	14	392	74	175	49
8	302	77	377	16	123	7	395	73	140	16
9	532	20	379	ND	205	ND	193	10	76	9
10	194	ND	541	ND	172	5	259	21	258	7
11	138	ND	267	8	143	4	323	6	342	6
12	193	ND	200	ND	186	ND	468	ND	174	3
Totals	2973	810	4579	850	3318	524	4340	681	2920	781

ND = not detected. Q = total amount of hydrolysed quercetin conjugates.

4.3.9 Time-course of excretion: quercetin aglycone from hydrolysed onion meal

The time-course of quercetin excretion following the ingestion of the hydrolysed onion meal showed a slightly different pattern to the control onion meal (Figure 4.9). The first peak appeared 1 to 2 h after ingestion of the onion meal in all subjects followed by a smaller second peak between 5 to 6 h, which was non-existent in some of the subjects. For example, the urinary quercetin excretion profile from subject 2 showed a gradual decline in the amount of quercetin excreted after 2 h of ingestion of quercetin aglycone from the hydrolysed onion meal (Figure 4.10b). Inter-individual variability in excreted quercetin after the consumption of the hydrolysed onion meal was clearly observed ranging between 84 μg and 850 μg . A summary of the time-studies from the hydrolysed onion meal in all ten subjects is shown in Table 4.4.

Gender-related differences in urinary excretion of quercetin from the original dose of control onion meal were not significant, being $1.57 \pm 0.26\%$ and $1.99 \pm 0.20\%$ (average $\pm$ SE) for males and females, respectively (Figure 4.11a). Similarly, the average urinary excretion of quercetin after ingestion of the hydrolysed onion meal for male ($1.27 \pm 0.35\%$) and female ($1.51 \pm 0.03\%$) subjects showed no statistically significant gender difference (Figure 4.11b; $P > 0.05$).

Table 4.4 Table showing the hourly volumes of urine collected and quantities of quercetin detected from each subject after ingestion of the hydrolysed onion meal.

♂	Subject 1		Subject 2		Subject 4		Subject 5		Subject 6	
Time (h)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)
0	66	ND	74	ND	35	ND	44	ND	233	ND
1	98	116	285	114	144	47	144	33	255	74
2	388	109	327	138	490	107	500	27	329	104
3	400	64	295	55	227	71	509	ND	325	77
4	310	63	337	45	331	39	438	14	227	26
5	74	95	83	60	185	20	290	8	100	46
6	88	113	137	46	163	9	482	ND	99	4
7	277	68	244	43	94	7	358	3	139	ND
8	217	24	232	42	225	6	358	ND	312	ND
9	146	24	261	28	104	3	556	ND	232	ND
10	62	12	152	8	333	ND	306	ND	242	ND
11	69	16	213	10	219	ND	323	ND	85	ND
12	70	ND	130	10	276	ND	486	ND	167	ND
Totals	2265	705	2770	599	2826	309	4794	84	2745	331
♀	Subject 3		Subject 7		Subject 8		Subject 9		Subject 10	
Time (h)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)
0	42	ND	173	ND	78	ND	252	ND	290	ND
1	445	123	513	61	690	117	473	72	438	47
2	382	108	417	71	610	120	430	85	409	121
3	168	80	340	124	428	77	340	104	310	59
4	88	51	258	61	352	69	141	40	278	35
5	106	49	67	57	104	47	63	62	130	40
6	128	59	335	46	95	34	289	37	372	23
7	402	31	202	25	179	18	442	17	168	18
8	420	ND	174	17	315	14	337	12	303	12
9	231	ND	275	6	294	ND	275	14	186	6
10	106	ND	320	4	165	6	384	8	309	ND
11	319	ND	391	ND	288	ND	278	4	338	ND
12	308	ND	318	ND	295	ND	149	2	307	ND
Totals	3145	501	3783	473	3893	500	3853	457	3838	358

ND = not detected. Q = total amount of hydrolysed quercetin conjugates.

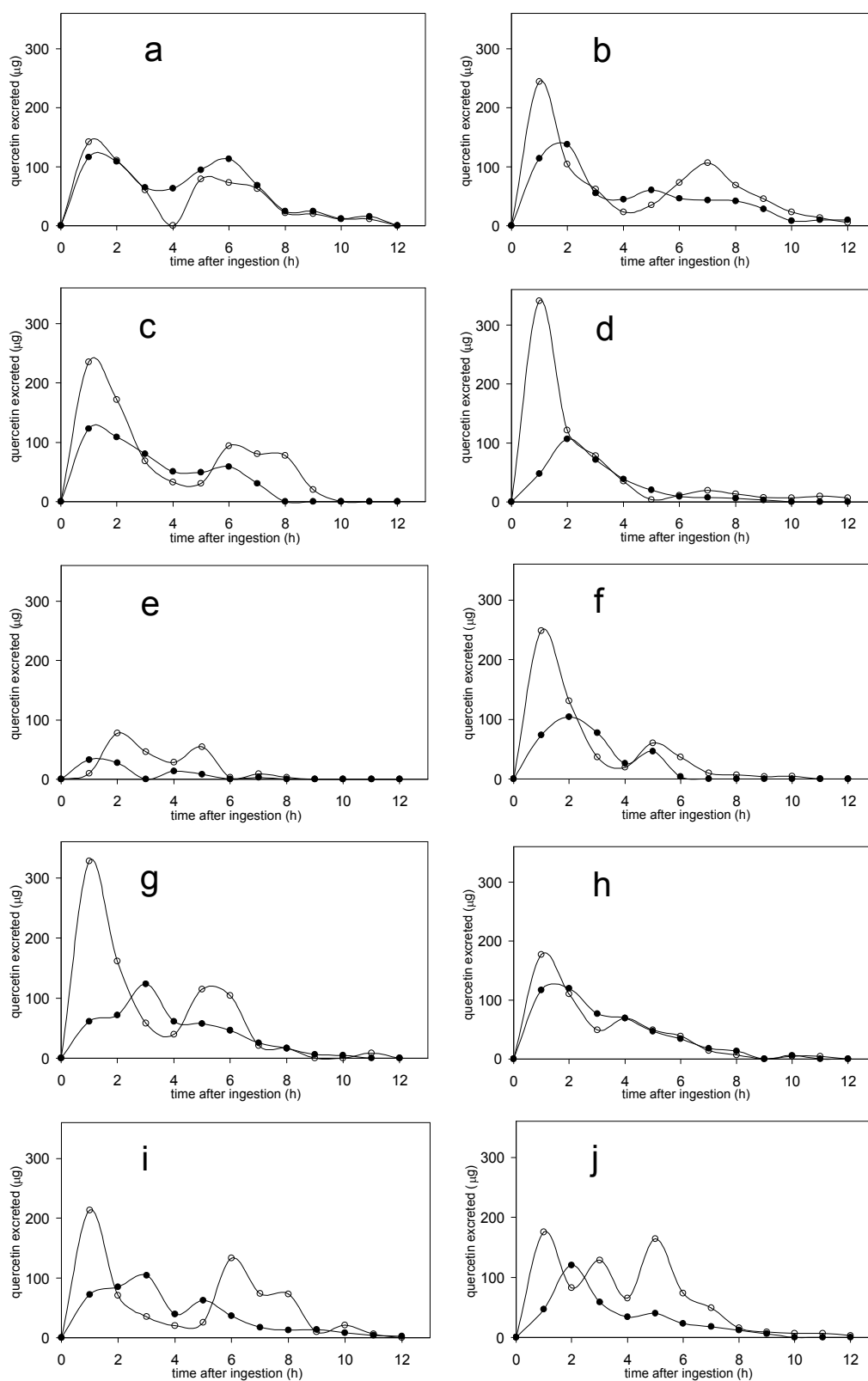


Figure 4.10 Time-courses of quercetin excretion after ingestion of the control onion meal (○), and the hydrolysed onion meal (●), in subjects 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 6 (f), 7(g), 8 (h), 9 (i) and 10 (j).

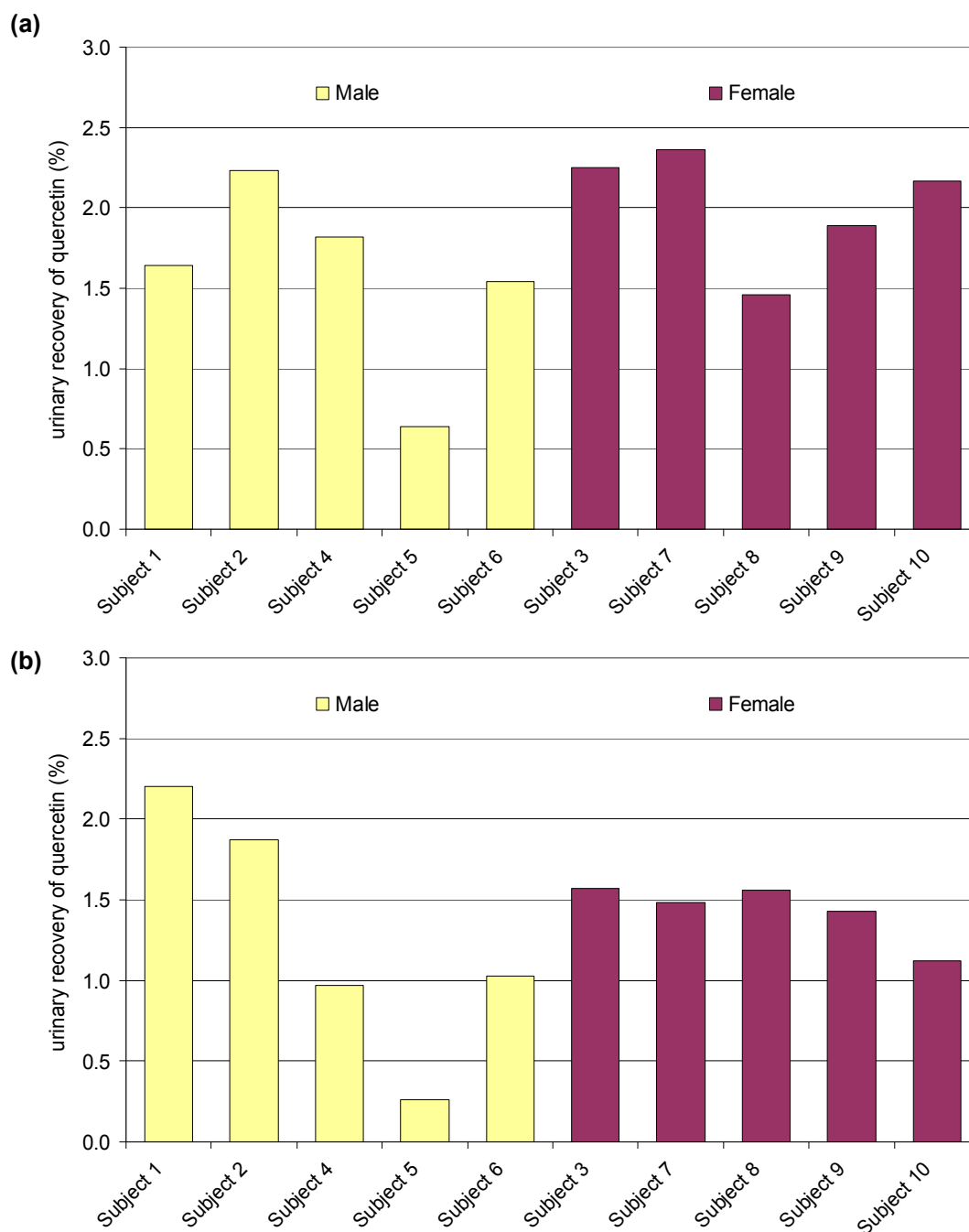


Figure 4.11 Variation in excretion recoveries of quercetin for male and female subjects 12 h after ingestion of (a) quercetin glycosides in control onion meal and (b) quercetin in hydrolysed onion meal.

4.3.10 Differences in time-courses of excretion

Quercetin excretion after the intake of the control onion meal was much higher than after the hydrolysed onion meal as demonstrated by the areas under the curve for quercetin excretion time curve profiles of the control onion meals in all ten subjects compared to the areas under the curves for the hydrolysed onion meal (see Figure 4.10). The amount of quercetin excreted over 12 h was higher after consumption of the control onion meal than after consumption of the hydrolysed onion meal. The average total amount of quercetin recovered in urine after 12 h of consumption of the control onion meal was $1.76 \pm 0.18\%$ of the ingested dose, whereas it was $1.37 \pm 0.19\%$ after the consumption of the hydrolysed onion meal. However, subject 1 had more quercetin in urine after consumption of the hydrolysed onion meal than did the other nine subjects (Figure 4.11b). For subject 8, the percentage of quercetin uptake was slightly higher after the ingestion of the hydrolysed onion meal (1.56%) from that obtained after the ingestion of the control onion meal (1.46%). Comparison of the amount of excreted quercetin from both types of onion meals in all the subjects over a 12 h recovery in urine are presented in Table 4.5. The calculated t value showed that there was a significant difference between the absorption of quercetin (as glycosides) from the control onion and the absorption of the quercetin from the hydrolysed onion with $P < 0.05$.

Table 4.5 Comparison of total amount of quercetin excreted in urine over 12 h after the ingestion of an onion-based meal.

Subject	Control onion^a		Hydrolysed onion^b	
	Quercetin excreted (µg)	Proportion of intake (%)	Quercetin excreted (µg)	Proportion of intake (%)
1	590	1.64	705	2.20
2	803	2.23	599	1.87
3	810	2.25	501	1.57
4	655	1.82	309	0.97
5	231	0.64	84	0.26
6	556	1.54	331	1.03
7	850	2.36	473	1.48
8	524	1.46	500	1.56
9	681	1.89	457	1.43
10	781	2.17	358	1.12

^a 36 mg quercetin equivalent as quercetin glycoside consumed.

^b 32 mg quercetin aglycone consumed

4.3.11 Dose-response study of quercetin aglycone

On a separate occasion, a double dose of quercetin aglycone from the hydrolysed onion meal (420 ml containing the equivalent of 67 mg aglycone) was given to subject 4 to consume. The pattern of quercetin excretion of the hydrolysed onion was similar to that of the lower dose (32 mg). The total amount of quercetin excreted from the low and high dose of deglycosylated quercetin were 0.97% and 1.11%, respectively. Figure 4.12 shows the time-courses of urinary quercetin excretion in subject 4 after ingesting the control onion meal and the two different doses of quercetin from the hydrolysed onion meal.

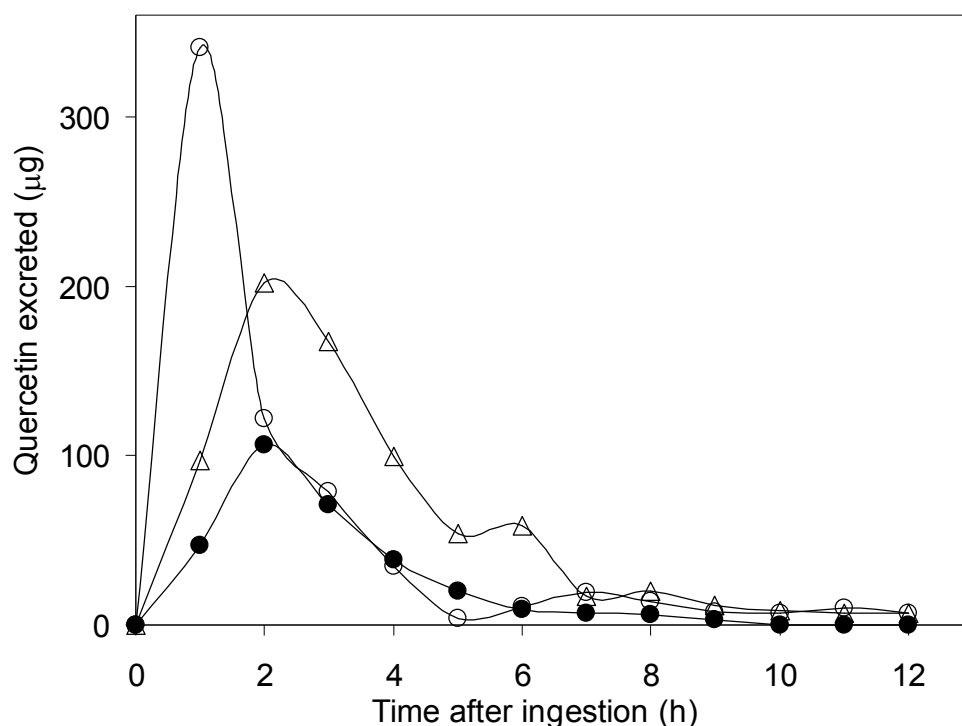


Figure 4.12 Quercetin excretion in subject 4 after ingestion of 36 µg quercetin from control onion meal (○), 32 µg quercetin from hydrolysed onion meal (●), and 67 µg quercetin from hydrolysed onion meal (Δ).

4.4 Discussion

4.4.1 Experimental design

Two methods for extraction of quercetin were tested in the present study, solvent extraction and solid phase extraction. Solvent extraction is one of the most commonly used methods to extract flavonoids from biological fluids mainly because of its ease of use, efficiency, and wide-ranging applicability. The nature of the extraction solvent plays an important role in the extraction of flavonoid conjugates from biological material. For example, ethyl acetate is poor at extracting hydrophilic metabolites such

as quercetin sulfates (Day and Morgan, 2003). In the present study, ethyl acetate was considered suitable due to the interest in extracting the less polar aglycones.

Solid-phase extraction (SPE) is a widely used technique which allows rapid purification and concentration of biological samples prior to HPLC analysis. Various stationary phases are used in the extraction of flavonoids, including ion exchange, functionalised and C₁₈ sorbents. In the present study, Oasis MAX cartridge was tested for the recovery of quercetin aglycones from urine. Oasis MAX cartridge was chosen because the manufacturer claimed that the mixed-mode polymeric sorbent containing strong anion-exchange quaternary amine groups on its surface gives high and reproducible recoveries for acidic compounds such as flavonoids (Figure 4.13). Preliminary experiments showed the recovery of quercetin from urine to be unsatisfactory. The recovery of quercetin using the original method was greatly affected by the first washing step. Prior to the methanol wash, 5% ammonium hydroxide solution (pH 11.65) was added to the Oasis column to remove proteins and bind the flavonoids to the sorbent by means of ion exchange mechanism. Consequently, the low recovery of quercetin under basic conditions is most likely a result of oxidation or other degradation (Moon *et al.*, 2008). The modified SPE method using only methanol solution to wash the Oasis MAX column showed a slightly better recovery of quercetin, although complete elimination of interfering substances was not possible. Table 4.6 shows the recoveries observed for the extraction of several flavonoids from urine using SPE method obtained by other investigators.

In order to achieve quantitative recovery of quercetin whilst avoiding any chemical degradation, ethyl acetate was selected to extract quercetin aglycone from the

hydrolysed urine samples. Ethyl acetate extraction of quercetin is simple, rapid and efficient. The less polar solvent separates easily from the enzymatically hydrolysed aqueous phase resulting in efficient extraction recoveries with minimal interferences from biological matrices. Overall recovery of quercetin after three separate extractions was over 90%, which was found to be satisfactory and in agreement with previous studies (Goldberg *et al.*, 2003).

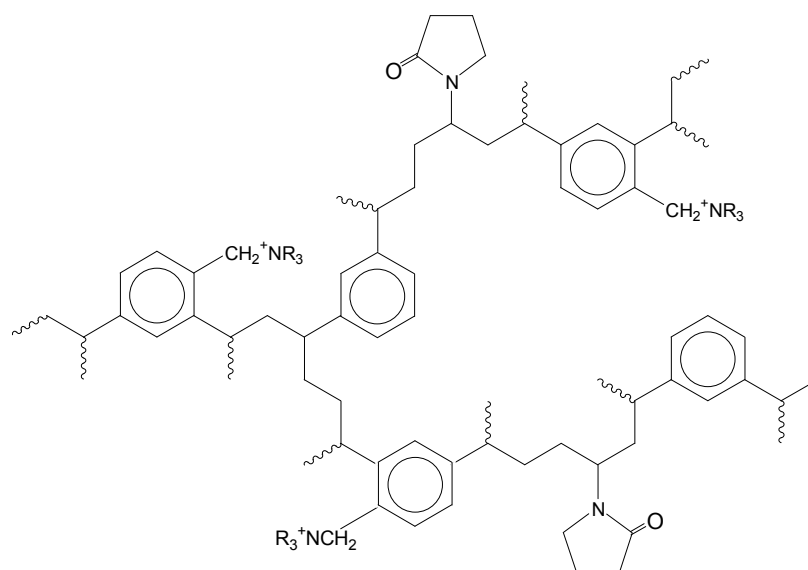


Figure 4.13 Chemical structure of Oasis MAX sorbent

Urinary excretion of flavonoids has been previously recognised as a useful biomarker for fruit and vegetable intake; analysis of urine is a relatively simple and non-invasive tool in determining the bioavailability of dietary flavonoids (de Vries *et al.*, 1998; Nielsen *et al.*, 2002). A urinary time-course study for quercetin excretion was chosen since it correlates well with quercetin absorption and was therefore a good marker of its bioavailability (Hollman *et al.*, 1997). In addition, urine is much safer and easier to collect, handle and store than blood and flavonoids are extracted relatively easily with less polar solvents without the need for centrifugation.

Table 4.6 Data from a number of studies showing the recovery efficiency of flavonoid solid-phase extraction from urine.

<i>Flavonoid sub-class</i>	<i>Flavonoid compound</i>	<i>Column-solvent</i>	<i>Recovery (%)</i>	<i>Reference</i>
Flavonols	quercetin	Oasis MAX cartridge, eluted with 2% formic acid in methanol	60	Present study
		ENV ⁺ cartridge, eluted with aqueous acetonitrile	73	Watson & Oliveira (1999)
		Oasis HLB cartridge, eluted with methanol	> 95	Ishii <i>et al.</i> (2003)
Flavanones	naringin	Sep-Pak Accell QMA cartridge, eluted with formic acid in methanol	> 92	Ishii <i>et al.</i> (1997)
Anthocyanins	cyanidin-3-glucoside	Sep-Pak C18 Plus cartridge, eluted with HCl in methanol	80 – 90	Felgines <i>et al.</i> (2003)
	glycosides of cyanidin	Sep-Pak C18 cartridge, eluted with 5% formic acid or Na acetate in methanol	> 90	Cao <i>et al.</i> (2001)
Isoflavones	daidzein genistein glycitein	Strata C18-E cartridge, eluted with 1:1 ethyl acetate:acetonitrile	92 – 99	Grace <i>et al.</i> (2003)
Catechins	catechin gallate compounds	Oasis HLB cartridge, eluted with 2:1 methanol/ethyl acetate	> 85	Chu <i>et al.</i> (2004)
Flavones	apigenin	Bond Elut C18 cartridge, eluted with methanol	> 90	Nielsen <i>et al.</i> (1999)
		Oasis MAX cartridge, eluted with 2% formic acid in methanol	70	Present study

HLB, hydrophilic-lipophilic balance; QMA, quaternary methyl ammonium

Previous intervention studies have used supplements as sources of flavonoid aglycones or have looked at quercetin glycosides in single foods without the capability of comparing the different chemical forms in comparable foods (Manach *et al.*, 2005). Since dietary quercetin is almost always present in glycoside forms which are essentially stable to processing and cooking conditions (though not fermentation), such

as boiling and frying (Price *et al.*, 1997; Takenaka *et al.*, 2004), it is difficult to study absorption of different chemical forms of quercetin in food matrices. In order, therefore, to test the idea that enhanced levels of aglycone in a food would lead to enhanced absorption, a novel approach was adopted in order to generate aglycone at the expense of glycosides but without the significant losses that often occur concomitantly during fermentation. The major quercetin glycosides in onion are Q3,4'G and Q4'G (Price and Rhodes, 1997). Disruption of the onion tissue releases the endogenous β -glucosidases that can lead to release of quercetin (Tsushida and Suzuki, 1996); however, Q4'G remains intact, as described previously (Rhodes and Price, 1996; Price and Rhodes, 1997). In order to obtain significant levels of free quercetin, an alternative source of β -glucosidases of wider specificity was required. Freshly ground almond contains the appropriate β -glucosidases capable of hydrolysing the Q3,4'G and Q4'G to quercetin aglycone. Optimal temperatures of around 50°C for maximal enzyme activity of β -glucosidases have previously been reported (Hosel and Barz, 1975; Lecas *et al.*, 1991). It was, therefore, possible to generate an onion-based food through hydrolysis by the endogenous enzymes of almond in which quercetin glycosides were absent, and in which levels of quercetin were comparable to the levels of quercetin equivalents in a 'normal' onion-based food that was not subjected to hydrolysis. The levels in the two foods as consumed were in accordance with previous quantitative estimates of quercetin in onion ranging from 18 – 63 mg/100 g fresh weight (Crozier *et al.*, 1997).

The impact of freeze-thaw on stability of the aglycones and glycosides was assessed prior to the feeding study. The levels of quercetin glycosides in the frozen control onion meal and, to a lesser extent, the quercetin aglycone levels in the frozen hydrolysed onion meal did not decline considerably following thawing and storage at 4°C for 24 h.

This is in broad agreement with Berger *et al.* (2007), who studied the flavonol content in green beans during storage at -25°C and 4°C and found no significant difference in the flavonol content of green beans after 12 months of deep-frozen storage compared to 14 days at 4°C. Consequently, transport of frozen foods by volunteers prior to consumption was not regarded as an issue.

Enzymatic hydrolysis of conjugates in urine samples was achieved using a combination of two enzymes; β -glucuronidase from *E. coli* and sulfatase from *A. aerogenes*. The analysed samples of urine showed the absence of quercetin aglycone prior to hydrolysis with β -glucuronidase/sulphatase enzymes. This is in agreement with previous studies reporting the absence of free quercetin in human plasma and urine following the consumption of an onion meal (Moon *et al.*, 2000; Wittig *et al.*, 2001). Hydrolysis of urine with deconjugating enzymes yielded free quercetin.

4.4.2 Absorption of quercetin from onion-based meals

Ingested quercetin appeared to be absorbed slightly quicker from the control onion meal than from the hydrolysed onion meal with a maximum amount of the absorbed quercetin being excreted at 1 or 2 h, respectively. The short times clearly indicate that quercetin is absorbed from the small intestine. Several intervention studies have demonstrated that quercetin (from glycosides) was absorbed earlier with a substantially higher bioavailability compared with quercetin (as aglycone) from a food supplement and indicated the small intestine as being the primary site of absorption of quercetin (Hollman *et al.*, 1996; Graefe *et al.*, 2001). For example, Hollman *et al.* (1995) showed in ileostomy subjects that 52% of quercetin glycosides from onion were absorbed

compared to only 24% of the aglycone supplement. As the preparation of the onion-based meals in the present study was carried out using the same ingredients and processed in a similar manner, the effect of the cellular food matrix from both foods on the release of quercetin in the (GI) tract should be minimal. Thus, the ability of quercetin (as glycosides) to be absorbed more rapidly than quercetin (as aglycone) provides further evidence of the predominant role of the sugar moiety on the rate of absorption.

A critical aspect that is often overlooked in the study of quercetin pharmacokinetics is the solubility of quercetin upon oral administration. In view of the poor solubility of quercetin in water (Piskula and Terao, 1998), an increase in quercetin aglycone absorption was expected when dissolved in a food matrix compared to the absorption of quercetin glycosides. However, the results of the study clearly showed that quercetin aglycone in a food matrix was less bioavailable than quercetin glycosides. This is in agreement with previous non-food studies in which plasma and urine levels of quercetin after an oral dose of pure aglycone were shown to be less than after the ingestion of quercetin glycosides (Hollman *et al.*, 1995; Graefe *et al.*, 2001).

It has been suggested that flavonol glucosides might be transported via the active intestinal SGLT1, resulting in an elevated intestinal uptake (Hollman *et al.*, 1995; Aziz *et al.*, 1998). Previous studies indicate that deglycosylation by LPH at the brush border of the small intestine and subsequent diffusion of the liberated quercetin aglycone into the epithelial cells is the most likely route in the absorption flavonoid glycosides (Day *et al.*, 2000b; Nemeth *et al.*, 2003; Wilkinson *et al.*, 2003). Apparently, the higher concentration of released quercetin at the apical membrane would result in a higher

diffusion rate than aglycone from the gut lumen, and potentially increase the rate of absorption (Day *et al.*, 2003). Thus current understanding of quercetin absorption, as reviewed by Donovan *et al.* (2006) has emphasised the critical role of hydrolysis of quercetin glycosides by LPH located at the surface of the wall of the small intestine (Day *et al.*, 2000b), followed by rapid diffusion across the cell membrane and subsequent conjugation (Nemeth *et al.*, 2003).

Although the amounts of quercetin and its conjugates found in urine following ingestion of control onion meal were higher than those found from the hydrolysed onion meal in all subjects, the time-courses of excreted quercetin for both foods were similar in pattern. Most of the quercetin ingested appeared rapidly as a large peak between 1-2 h after the ingestion of onion meal with a smaller one after 5-6 h. Hollman *et al.* (1997) described a similar biphasic profile of plasma quercetin elimination consisting of a rapid distribution and elimination followed by a small elimination. One possible explanation may be the involvement of enterohepatic circulation, with the second peak corresponding to bile-duct release and re-absorption of the quercetin metabolites (Manach *et al.*, 2004). The appearance of a second excretion peak 5-6 h after ingesting the onion meal corresponds with the consumption of a flavonoid-low meal for lunch following an initial 4 h period of fasting. Eating a meal results in emptying of the gallbladder into the intestinal tract thus quercetin that is excreted into the bile would be re-absorbed creating a second excretion peak. Sfakianos *et al.* (1997) demonstrated in rats the excretion of genistein metabolites in the bile after 4 h infusions of the isoflavone genistein into the intestine. Another explanation is absorption from the large intestine following microbial hydrolysis of the flavonoid glycosides

(Bokkenheuser *et al.*, 1987). The lipophilic quercetin aglycone diffuses passively through the biological membrane of the intestinal wall.

To determine whether a dose-response relationship existed between the intake and the excretion of quercetin in urine when given a higher dietary dose, the time-courses of excreted quercetin in subject 4 were studied after the administration of 32 and 67 mg of deglycosylated quercetin in the form of 200 and 420 ml of hydrolysed onion meal, respectively on separate occasions. A clear dose response was observed for these two levels of quercetin ingested, which is in agreement with Erlund *et al.* (2000) who reported a similar response in healthy volunteers being fed with different levels of quercetin aglycone supplements. There was, however, no significant difference in level of total quercetin excreted as a percentage of the amount ingested. The result confirms that the original doses of quercetin given by the two onion-based foods were close enough to make direct comparisons in terms of bioavailability.

Cumulative amounts of quercetin excreted after 12 h ranged between 231 µg and 850 µg for the control onion meal, and 84 µg and 705 µg for the hydrolysed onion meal. From these results, the proportion of ingested quercetin recovered in 0-12 h urine collection were between 0.64% and 2.36% for the control onion meal, but 0.26% and 2.20% for the hydrolysed onion meal. The figures for quercetin excretion from onion are in agreement with previous studies, ranging from 0.50% to 4.7% (Hollman *et al.*, 1997; Aziz *et al.*, 1998; Young *et al.*, 1999; Olthof *et al.*, 2000; Mullen *et al.*, 2004; Table 4.7). In particular, de Vries *et al.* (1998) reported a similar value of 1.1% recovery from fried onions, and Aziz *et al.* (1998) calculated an average recovery of 0.8% from 5 volunteers. It should be noted that these estimates of bioavailability were

based on 24 h urine collections. However, the 12 h time-course of urinary quercetin was considered suitable for the present study due to the peak elimination profile of quercetin occurring well within the 12 h period and the relatively small amounts of quercetin (3% of total quercetin excretion) eliminated during the 12-24 h period (Aziz *et al.*, 1998). Recently, Mullen *et al.* (2006) showed that 92% of the urinary flavonol metabolites were excreted within the first 8 h after ingestion of onions in human subjects.

Table 4.7 Results from selected bioavailability studies with quercetin based on urinary recovery from administered dose. Values are averages from several subjects.

Quercetin form	Source	Recovery in urine (%)	Reference
Glucosides	Onion	4.7	Mullen <i>et al.</i> (2006)
Glucosides	Onion	4.4	Graefe <i>et al.</i> (2001)
Q4'G	Supplement	3.3	
Q3G	Supplement	3.0	Olthof <i>et al.</i> (2000)
Q4'G	Supplement	2.6	
Glucosides	Fruits and vegetables	2.1	Kim <i>et al.</i> (2003)
Glucosides	Onion	2.0	Mullen <i>et al.</i> (2004)
Glucosides	Onion	1.8	Present study
Glucosides	Onion	1.4	Hollman <i>et al.</i> (1997)
Aglycone	Onion	1.3	Present study
Glucosides	Onion	1.1	de Vries <i>et al.</i> (1998)
Glucosides	Onion	0.8	Aziz <i>et al.</i> (1998)
Glucosides	Conventionally produced food	0.6	Grinder-Pedersen <i>et al.</i> (2003)
	Organically produced food	0.5	

Inter-individual variation was considerable, which has also been reported in previous human studies with quercetin (Erlund *et al.*, 2000; Graefe *et al.*, 2001; DuPont *et al.*, 2002). There were differences in the shapes of the urinary time-course within the two types of onion meal. The differences in relative bioavailability (calculated from total quercetin concentration) between the subjects were prominent. For example, the difference between the subject with the lowest amount of excreted quercetin and the subject who excreted the highest amount of quercetin was about 4-fold in the control onion meal and about 9-fold in the hydrolysed onion meal. The variation in the time-courses of quercetin excretion of the subjects may be attributed to the differences in intestinal physiology, microflora populations and/or changes in enzyme activities, which in turn are influenced by the diet.

The data presented in the present study provide clear evidence that the chemical form of quercetin affects the rate of absorption, and hence determines the bioavailability of quercetin from quercetin glycosides. Moreover, the results suggest that the physical location of quercetin inside the small intestine, which is influenced by the nature of the food matrix, appears to play a significant role in its release and subsequent absorption. Therefore, understanding the effects of food matrix is important in maximising the rate of quercetin absorption from the GI tract and ultimately increasing the bioavailability. Future work should explore the factors influencing the extent of quercetin absorption, which may be related to other dietary components in which quercetin is ingested with such as different fat or non-digestible carbohydrates levels.

In conclusion, the present human intervention study is the first to investigate the relative bioavailability of quercetin present in comparable onion-based systems as

either quercetin glycosides or quercetin aglycone. The absorption of quercetin from its glycosidic form was significantly higher than from the aglycone form as determined by urinary excretion. Furthermore, this study has demonstrated, by using time-course analysis, that urine patterns mimic plasma patterns observed in pharmacokinetic studies of quercetin absorption even though at a lower level. Consequently, urinary excretion profile of flavonoids can be used as an important marker for the intake of fruit and vegetables and as a non-invasive tool for comparative measurement of absorption of flavonoids.

Chapter 5

Influence of Dietary Fat on Flavonol Bioavailability

CHAPTER SUMMARY

- A human intervention study was carried out to determine the effect of dietary fat on the bioavailability of quercetin present in onion-based foods as either quercetin glycosides or quercetin by implementing time-course analysis of urinary excretion.
- Q4'G and Q3,4'G, were the main quercetin glycosides in the control onion meal (92%) with low levels of quercetin aglycone (7%).
- Quercetin appeared rapidly in conjugated form in urine 1h after the administration of the control and hydrolysed onion-based meals with 8.3% (w/w) fat.
- Inter-individual variability was observed in patterns and amounts of excreted quercetin after the co-ingestion of fat with the control and hydrolysed onion-based meals.
- The ingested quercetin glycosides observed as quercetin conjugates in urine were excreted earlier (around 1 – 2 h) then when quercetin aglycone was ingested (around 2 – 4 h).
- Absorption of quercetin in its glycosidic form (n=6) was significantly higher than the aglycone form (n=5) when co-ingested with fat (2.8% compared to 1.7%; $p<0.05$).
- Co-ingested fat significantly increased the absorption of quercetin glycosides in the control onion meal from 1.9 to 2.8% (n=6, $p<0.05$) and slightly increased the absorption of the aglycone in the hydrolysed onion meal from 1.5 to 1.7% (n=5).

5.1 Introduction

A number of factors can influence the oral absorption and bioavailability of dietary flavonoids such as the nature of the attached sugar moiety, the vehicle of delivery, and solubility and food matrix effects (Hollman *et al.*, 1999; Piskula, 2000; Manach *et al.*, 2004; Scholz and Williamson, 2007). The factors that affect the absorption and bioavailability of flavonols, notably quercetin, are reviewed in this chapter.

5.1.1 Sugar moiety

The flavonol quercetin is found predominantly in most plant-derived foods as glycosides. It was originally believed that absorption of flavonol glycosides were negligible as only the aglycones were capable of passing through the small intestinal wall. A growing number of studies support the view that intestinal absorption of quercetin is largely determined by the chemical composition of the attached sugar moiety. For example, in the study of Gee *et al.* (2000), an everted sac preparation of rat small intestine was used to compare the uptake of radiolabeled quercetin aglycone with that of three similarly labeled quercetin glycosides. They reported that Q3G and Q4'G, but not Q3,4'G, were transported into the everted sacs significantly more quickly than quercetin aglycone. The authors suggested that quercetin glucosides are the preferred form for uptake in the human intestine. Similarly, Crespy *et al.* (2001) reported in a study of *in situ* perfusion in rats an increase in the net absorption of quercetin when Q3G was perfused instead of its aglycone form. Another study investigated the oral absorption of two similar flavonoid glycosides, Q3G and hyperoside (quercetin-3-*O*-galactoside) in rats (Chang *et al.*, 2005). The researchers found that while quercetin conjugates, mainly glucuronides, were present in the plasma after oral administration of

Q3G, hyperoside could not be detected in plasma neither as unchanged form nor its aglycone or conjugated aglycone form. They also demonstrated that hyperoside was more stable than Q3G in the GI tract, thus rendering it more difficult to hydrolyse and less readily absorbed in comparison to Q3G. For a more detailed review on the role of the sugar moiety on the absorption of dietary flavonoids in humans, see Chapter 4.

5.1.2 Solubility

The effect of solubilisation on the efficiency of intestinal absorption of quercetin has been questioned (see Chapter 4). Piskula and Terao (1998) investigated the solvent effects of propylene glycol, 25% propylene glycol in water or water on the absorption of pure quercetin when administered orally in rats. The authors reported that quercetin was rapidly absorbed (during the first 2 h) when soluble in propylene glycol or 25% propylene glycol in water, but only slowly absorbed (at a constant rate for 8 h) when in water. Piskula and Terao (1998) suggested that the decrease in the quercetin absorption rate may be due to its low solubility since quercetin solubility tests showed decreases in solubilised quercetin in 25% propylene glycol in water by almost three orders and in water by five orders compared to pure propylene glycol. However, the authors could not establish a direct correlation between the extent of quercetin absorption and quercetin solubility. In another experiment, Azuma *et al.* (2002) demonstrated the role of ethanol as a vehicle for delivery of quercetin in rats. They reported an absorption-enhancing effect at concentrations of 30 and 50% ethanol. The intestinal absorption of quercetin was directly correlated with the increase in quercetin solubility at higher concentrations of ethanol. This conclusion is supported by the findings of Goldberg *et al.* (2003), who reported significantly higher quercetin plasma levels in human subjects

when a single dose of quercetin was administered dissolved in white wine compared to grape juice or vegetable juice.

It should be noted that the integrity of the gut with solvents such as propylene glycol and ethanol is, however, questionable. For example, Worthington *et al.* (1978) studied the effects of ethanol ingestion on the permeability of rat small intestine using the tracer macromolecule, horseradish peroxidase. Both light and electron microscopy observations showed that mucosal uptake of horseradish peroxidase was significantly increased in alcohol-treated animals. The authors concluded that chronic alcohol ingestion alters the morphological characteristics of the gut and increases intestinal mucosal permeability to 'undigested' macromolecules. The findings of Azuma *et al.* (2002) confirmed the potential effect of alcohol concentration on intestinal integrity, as they observed a significant increase in intestinal absorption of quercetin in rats when co-administered with 30% or more ethanol.

The influence of dietary fat on the extent of quercetin absorption has been previously investigated. In general, lipids have been considered to increase the solubility of quercetin and thus enhance their uptake and absorption. For example, Azuma *et al.* (2002) studied the effects of lipids and emulsifiers on the absorption of orally administered quercetin in rats. They found a significant increase in plasma concentration of quercetin metabolites when 3% emulsifiers, sucrose fatty acid ester, polyglycerol fatty acid ester, or sodium taurocholate was combined with either 10% lecithin or 20% soybean oil. However, co-administration of lipids or emulsifiers did not significantly enhance quercetin absorption. They attributed the weak absorption-enhancing effects to partial solubilisation of quercetin in the vehicles. The authors

concluded that the co-administration of lipids and emulsifiers effectively enhance the intestinal absorption of quercetin. On the other hand, Azuma *et al.* (2003) demonstrated in rats fed with onion that the addition of more than 4.6% soybean oil to the diet significantly enhanced the accumulation of quercetin metabolites in the plasma. Similarly, Lesser *et al.* (2004) showed that a 17% fat diet enhances the absorption of quercetin in pigs compared to the 3% fat control diet. Another study showed that the bioavailability of sea buckthorn flavonols, namely isorhamnetin, quercetin and kaempferol (78 mg) in humans had increased by ~21% when an oatmeal porridge containing the flavonol extract was co-ingested with a relatively small amount of sea buckthorn oil (Suomela *et al.*, 2006). Furthermore, the composition of dietary fat may also have an influence on the extent of quercetin absorption. Recently, it was shown that bioavailability of quercetin had increased by 38% and 12% after the intake of medium and long-chain fatty acid triacylglycerols enriched diets, respectively, compared to the standard diet of wheat, barley and de-fatted soybean in pigs (Lesser *et al.*, 2006).

5.1.3 Food matrix

In addition to the nature of the sugar moiety and the solubility effects, the composition of the diet may have a considerable affect on the absorption and bioavailability of quercetin. For example, Graefe *et al.* (2001) investigated the influence of food matrix on the absorption of quercetin. In the four-way cross over study, 12 healthy subjects received an onion meal (containing Q4'G and Q3,4'G) or Q4'G, as well as rutin supplement and buckwheat tea (containing rutin). The bioavailability and pharmacokinetic parameters between the onion and Q4'G supplement showed no

significant difference. The $C_{\max}$ of quercetin from buckwheat tea and rutin were 2.0 and 1.0 μM , respectively and $T_{\max}$ were reached after 4.3 and 7 h, respectively. It was suggested that the increase in quercetin absorption from the buckwheat preparation may be dependent on the cofactors in the plant matrix, which increase the solubility of rutin and decrease the viscosity of the mucus layer. The authors concluded that, to a minor extent, the food matrix influences both the rate and extent of absorption in the case of buckwheat tea administration compared with the isolated rutin compound.

The effect of meat as a food matrix on the absorption of quercetin in pigs has also been investigated. Bioavailability of Q3G was significantly higher from the test meal (i.e. ground beef) compared to the standard diet of crude protein, crude fat and neutral detergent fibre (Cermak *et al.*, 2003). It was suggested that the preferential absorption of Q3G from the test meal was due to the low amount of glucose and the fat content of the meat in addition to the more favorable Q3G-glucose ratio present in the meat matrix. The study confirmed that the bioavailability of quercetin glycosides is, to a large extent, dependent on food composition. The various interactions that have been reported to influence the absorption and bioavailability of quercetin and its glycosides from animal and human studies are illustrated and summarised in Table 5.1.

Table 5.1 Summary of the different interactions affecting the bioavailability of flavonols (from Scholz and Williamson, 2007).

Type of interaction	Flavonol compound	Relative effect on bioavailability	Influencing factor	Reference
Pure rutin vs. buckwheat tea	Rutin	100% increase in $C_{\max}$ and 52% increase in AUC from tea compared to rutin (only a trend)	Food matrix	Graefe <i>et al.</i> (2001)
Red wine, onion meal or black tea	Quercetin	Increase in plasma concentration of total flavonols by 138% from wine, 288% from onions and 169% from tea compared to baseline, 16 nM	Sugar moiety	de Vries <i>et al.</i> (2001)
White wine, grape juice or vegetable juice	Quercetin	Plasma quercetin concentration AUC (4h) was significantly higher from white wine	Food matrix/ Solubility	Goldberg <i>et al.</i> (2003)
Meat	Q3G	Plasma total AUC increased significantly from ground beef compared to standard diet	Food matrix	Cermak <i>et al.</i> (2003)
Fat (17%)	Quercetin and Q3G	Plasma total AUC increased significantly by 57% from quercetin and 32% from Q3G compared to 3% fat diet (control)	Applied vehicle/ Solubility	Lesser <i>et al.</i> (2004)
Combination of lipids and emulsifiers	Quercetin and metabolites	Plasma $C_{\max}$ increased significantly from the combination of lipids and emulsifiers compared to control (quercetin in water)	Applied vehicle/ Solubility	Azuma <i>et al.</i> (2002)
Single pure compounds or a mixture	Quercetin, rutin and α G-rutin or mixture of rutin and α G-rutin	Plasma AUC for α G-rutin increased by 390% and 103% compared to quercetin and rutin and was similar to the mixture	Solubility	Shimoi <i>et al.</i> (2003)
Propylene glycol (PG)	Quercetin	Plasma total quercetin $C_{\max}$ 70.3 μ M (0.5 h) from PG and 15.5 μ M (0.25 h) from PG with water compared to 5.5 μ M from water (8 h)	Solubility	Piskula and Terao (1998)

AUC, area under the concentration-time curve; $C_{\max}$, peak plasma concentration; α G-rutin, a water soluble flavonol.

5.1.4 Aim of chapter

The aim of this study was to investigate the influence of dietary fat on the bioavailability of quercetin present in onion-based foods as either quercetin glycosides or quercetin aglycone by implementing a 12 h time-course analysis of urinary excretion. The bioavailability of quercetin aglycone was significantly different to that of its glycosides when present in a similar food-based system (as determined in Chapter 4). Therefore, it is speculated that the lipophilic aglycone will show different patterns of absorption, excretion and bioavailability compared to the more hydrophilic glycosides in the presence of dietary fat.

5.2 Methods

5.2.1 Onion meal preparation

A batch of yellow onions (*Allium cepa*) purchased loose from a local supermarket was used to prepare the onion-based meals. Both control and hydrolysed onion meals were prepared as previously described in Chapter 4. Portions (300 ml) of both onion meals were stored at – 20°C until further use. In addition, foil-wrapped butter was purchased from a local supermarket as a source of dietary fat. Slices of butter (25 g) were cut, weighed and wrapped in heavy duty kitchen aluminium foil prior to storage by refrigeration at 4°C.

5.2.2 Analysis of flavonols in onion meals

Liquid-liquid extraction with ethyl acetate was used to quantify the amount of quercetin glycosides and quercetin aglycone from the control and hydrolysed onion meals. The

detailed procedure for flavonoid identification and quantification can be found in section 4.2.3 of the methodology in Chapter 4.

5.2.3 Subjects

Ethical approval for the study protocol was obtained from the University of Leeds Local Ethics Committee. Six healthy, non-smoking subjects (4 male and 2 female) were recruited among students and employees from the university. Subjects were not on prescription drugs or any other medication. The details of the study were fully explained to the participants, who gave informed and written consent after carefully reading the protocol. Subjects were aged between 28 and 56 years with a mean age of 36.5 years and a mean body mass index of 26 kg/m² (range: 21.7 – 27.7 kg/m²). All six volunteers completed the first phase of the intervention study. One subject withdrew after completing the first phase of study, due to pregnancy.

5.2.4 Study design

The study was carried out in two phases, consisting of a control onion meal with butter and a hydrolysed onion meal with butter (see Figure 5.1). Subjects were required to follow a diet low in flavonoid-rich foods for two days prior to the study. They were provided with a list of food items allowed with low-flavonoid content and a list of restricted foods to avoid (i.e. fruits and vegetables). For a detailed list of food items, refer to Table 4.2. Subjects were requested to maintain a food diary to ensure they complied with the dietary requirements of the study. Each subject was given a measured portion of frozen control onion meal (300 ml), a foil-wrapped slice of butter (25 g) and a set of 13 plastic urine collection bottles containing ascorbic acid (0.1 g)

labelled in sequence of time from 0 to 12 h to take home prior to the study. On the third morning, after an overnight fast, subjects were instructed to void the bladder and immediately drink water (~ 200 ml). An hour later, subjects would collect the urine sample in the (0 h) bottle as a baseline sample. Each subject was instructed to prepare the onion meal by gently melting the slice of butter in a sauce pan and mixing it thoroughly with the defrosted control onion meal prior to consumption. No other foods or beverages, apart from drinking water, were allowed during the first four hours. Every successive hour for 12 h, subjects would collect urine in the labelled bottles and immediately drink water. During the 12 h period of the study, subjects were allowed to have a flavonoid-low meal for lunch and dinner (after at least 4 h). Subjects returned the 13 bottles of collected urine to the Department on the following day after completing the first phase of the study. The urine samples were either analysed on the same day or stored at -20°C .

After a one week 'washout period', subjects were given a measured portion of frozen hydrolysed onion meal (300 ml) with a slice of butter (25 g) to consume and collect 12 h urine samples in a similar manner to the first part of the study.

The volume of each urine sample collected from individual subjects was measured and recorded. An aliquot (40 ml) of each urine sample was transferred to 50 ml centrifuge tubes and stored at -20°C prior to analysis for total quercetin content. The remainder of each urine sample was discarded.

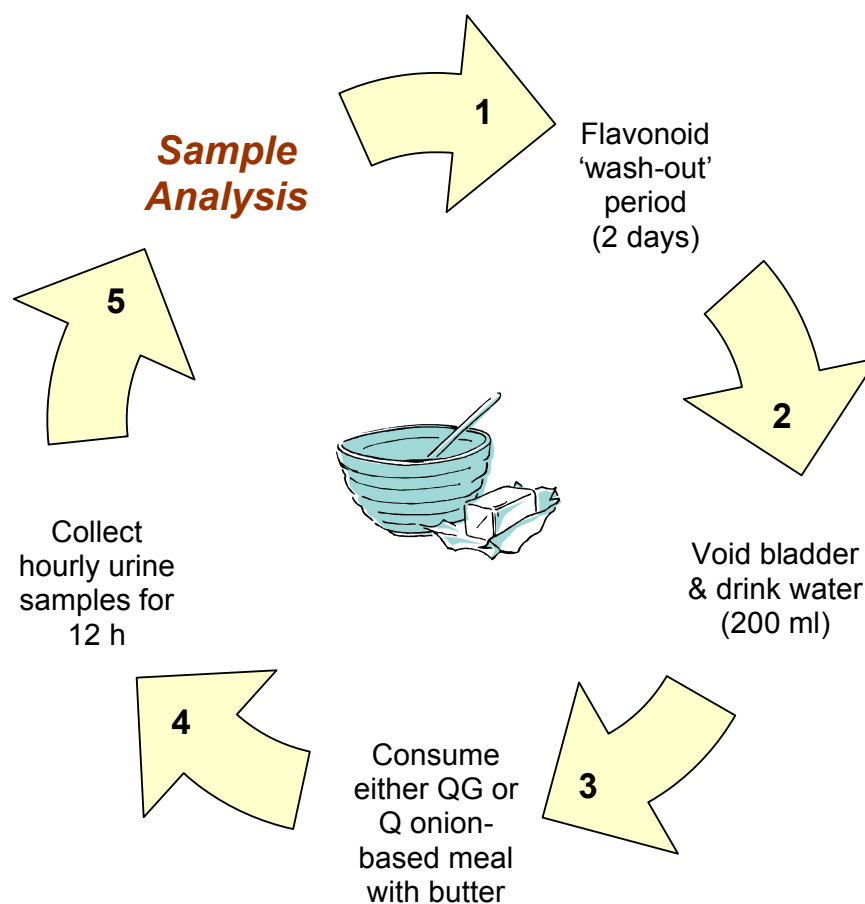


Figure 5.1 Diagram showing human intervention study design with the control onion meal containing quercetin glycosides (QG) and the hydrolysed onion meal containing quercetin (Q) consumed with 25 g of butter.

5.2.5 Extraction and hydrolysis of urine samples

Urine samples were enzymatically hydrolysed using a combination of β -glucuronidase and sulfatase enzymes. Liquid-liquid extraction using ethyl acetate was followed to quantify the total amount of quercetin from urine. For a more detailed description of the hydrolysis and extraction procedures, refer to section 4.2.7 in Chapter 4.

5.2.6 Data Analysis

The values are presented as the average $\pm$ SE. As a result of the small number of male and female subjects in each time-course study, gender differences were not assessed in this human study. Statistical differences between the bioavailability from the control onion meal and the hydrolysed onion meal from both human studies was assessed by performing a Student *t* test. Differences with $P < 0.05$ were considered statistically significant.

5.3 Results

5.3.1 HPLC analysis of the onion-based meals

Flavonoid quantification of the control and hydrolysed onion meals was determined by using rapid-resolution HPLC with diode array detection. The main flavonoid found in the onion meal extracts was quercetin. The control onion meal (300 ml) contained 26 mg quercetin (expressed as quercetin aglycone equivalent). The concentration of quercetin aglycone in the 300 ml portion of hydrolysed onion meal ingested was 30 mg. No significant levels of quercetin glycosides were detected in the hydrolysed onion meal. The main quercetin glycosides present in the control onion meal were Q4'G and Q3,4'G, which combined accounted for 92% of the total flavonol with lower levels of quercetin aglycone (7%) and Q3G (< 1%). In the hydrolysed onion meal, 96.5% of total quercetin was deglycosylated. Figure 5.2 shows typical HPLC chromatograms of the two types of onion with quercetin and its glycosides eluted between 0.5 – 3.0 min at 370 nm.

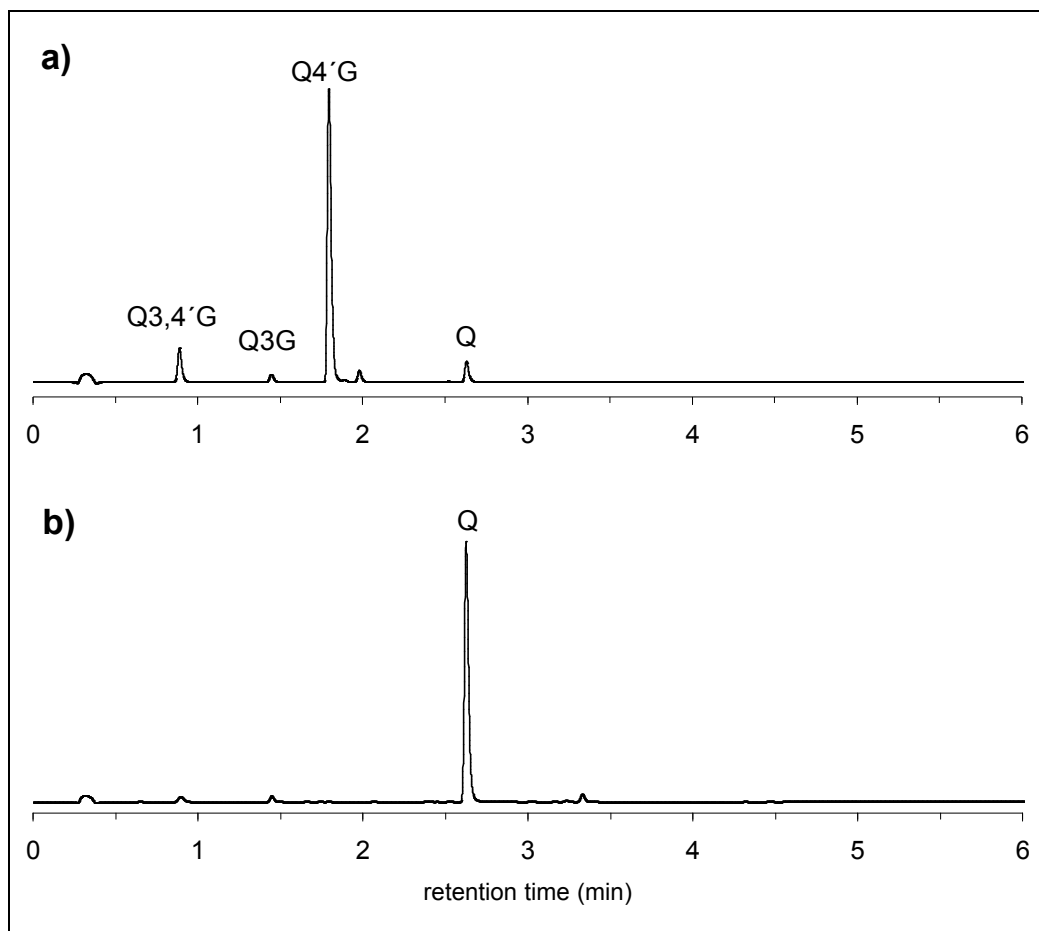


Figure 5.2 Chromatogram of a methanolic extract of (a) control onion meal and (b) hydrolysed onion meal at 370 nm. (Q3,4'G) quercetin-3,4'-diglucoside, (Q3G) quercetin-3-glucoside, (Q4'G) quercetin-4'-glucoside, (Q) quercetin aglycone.

5.3.2 Detection and quantification of urinary quercetin

Urine samples collected from each subject were analysed for quercetin prior to consumption of the onion-based meal with butter (time 0). Hydrolysis of urine baseline samples from all subjects revealed no detectable quercetin. Quercetin appeared rapidly in conjugated form in the urine samples after 1h of ingesting both the onion-based meals with butter. Quercetin aglycone, however, was not detected in the urine samples of volunteers prior to enzymatic hydrolysis. Quantification of total quercetin in 12 h urine was detected after hydrolysis of quercetin conjugates with β -glucuronidase and

sulfatase. Quercetin was detected by absorption spectra at 370 nm with a retention time of 2.6 min. Figure 5.3 shows a typical chromatogram of urine collected 1 h after ingestion of onion meal with butter from a subject after enzymatic treatment.

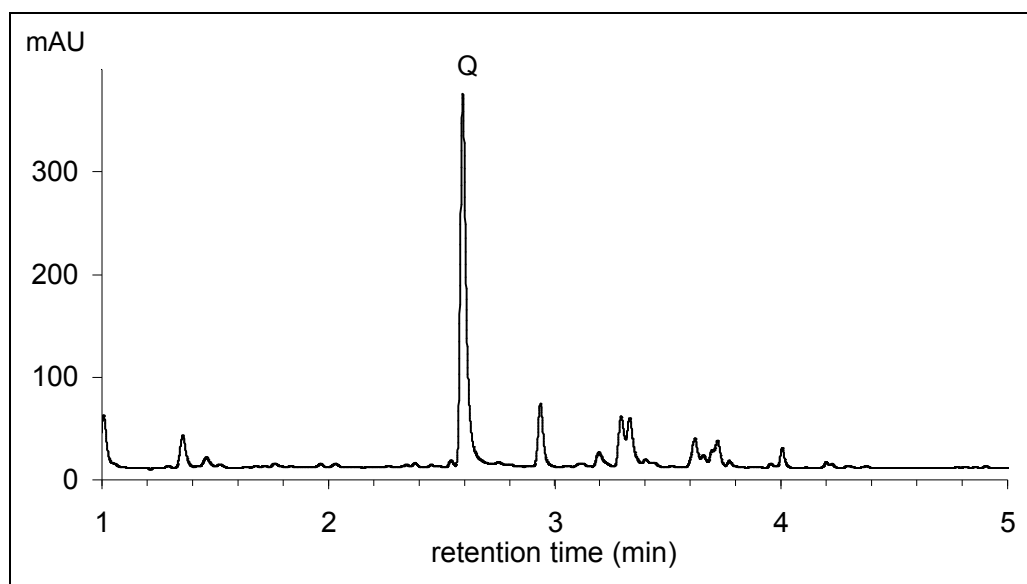


Figure 5.3 Chromatogram showing the methanolic extract of urine collected 1 h after ingestion of the onion-based meal with butter after hydrolysis with sulfatase and β -glucuronidase (370 nm). Q is quercetin.

5.3.3 Time-course of excretion: quercetin glucosides from control onion with butter

The average time-course of total quercetin excretion (measured as quercetin aglycone) following the consumption of the control onion meal with 8.3 % (w/w) butter in all six subjects is shown in Figure 5.4. The inter-individual variability in excreted quercetin after the consumption the control onion with butter was visible, ranging between 459 and 953 μg . In five out of six subjects, the peak excretions were observed after 1 to 2 h followed by a subsequent smaller peak after 5 to 6 h. This was followed by a decline in quercetin excretion over the successive hours up until 12 h. The second peak

of quercetin excretion was more prominent in the urinary time-courses of some of the subjects than others. Measurable amounts of quercetin was detected in several subjects after 12 h of consuming the control meal with butter (11 μg and 37 μg excreted in subjects 6 and 9, respectively). Table 5.2 provides a summary of the time-studies from the consumption of the control onion meal with butter for all six subjects.

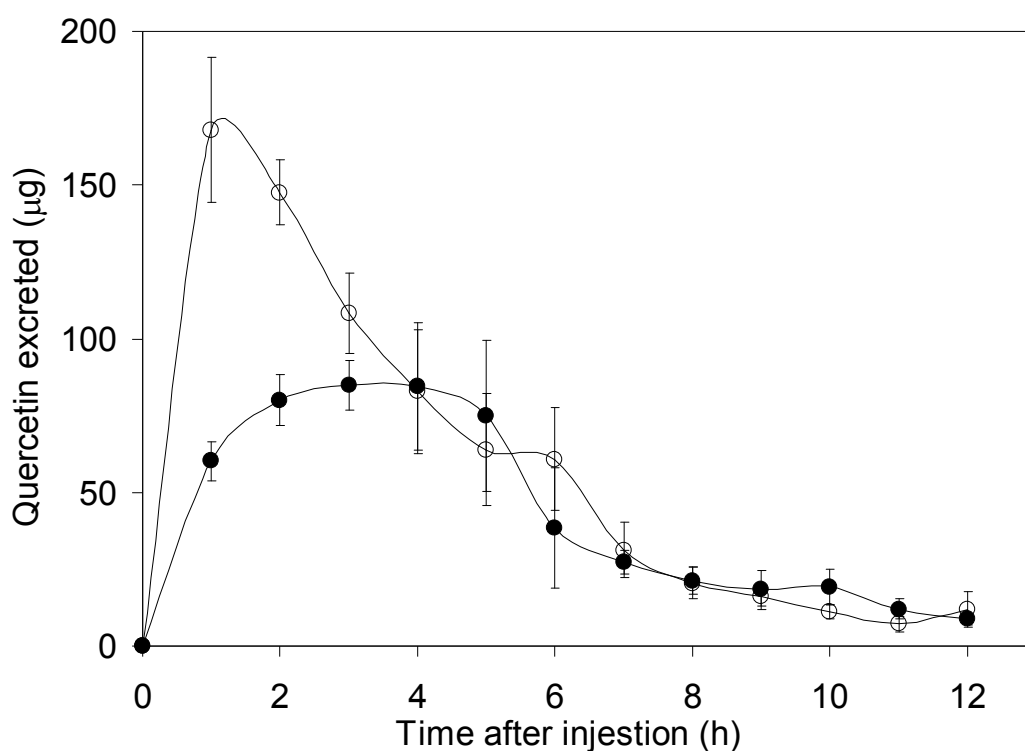


Figure 5.4 Time-courses of quercetin urinary excretion 0-12 h after ingestion of the onion-based meals with 25 g of butter. (○) control onion meal containing 26 mg Q (n=6); (●) hydrolysed onion meal containing 30 mg Q equivalent as quercetin glucosides (n=5). Error bars indicate SE.

Table 5.2 Table showing hourly volumes of urine collected and quantities of quercetin detected from each subject after ingestion of the control onion meal with butter.

♂	Subject 1		Subject 2		Subject 4		Subject 6	
Time (h)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)	urine (ml)	Q (µg)
0	130	ND	178	ND	47	ND	30	ND
1	328	116	430	210	343	210	75	103
2	392	126	380	125	382	185	360	154
3	88	115	403	74	428	130	301	71
4	232	153	325	87	397	42	277	37
5	120	120	314	70	0	0	122	42
6	122	105	281	57	141	19	222	22
7	369	53	205	58	80	11	112	9
8	242	31	350	25	51	6	199	7
9	318	22	338	21	462	15	285	5
10	44	15	372	13	238	6	206	4
11	75	12	238	12	170	2	123	3
12	160	9	232	9	72	3	168	3
Totals	2620	876	4046	760	2811	627	2480	459
♀	Subject 3				Subject 9			
Time (h)	urine (ml)		Q (µg)		urine (ml)		Q (µg)	
0	104		ND		295		ND	
1	304		137		528		233	
2	512		170		418		126	
3	189		104		397		157	
4	56		67		209		112	
5	165		79		72		71	
6	156		65		295		98	
7	160		10		244		47	
8	138		20		223		34	
9	305		11		170		22	
10	194		12		130		17	
11	265		13		0		0	
12	320		11		526		37	
Totals	2868		699		3507		953	

ND = not detected. Q = total amount of hydrolysed quercetin conjugates

5.3.4 Time-course of excretion: quercetin aglycone from hydrolysed onion with butter

The time-course of quercetin excretion following the ingestion of the hydrolysed onion meal with 8.3% (w/w) fat showed a different pattern to the control onion meal (Figure 5.4). The first peak of excretion appeared between 2 and 4 h after ingestion of the onion meal with butter in four of the five subjects. A much smaller second peak was observed after 10 h in some of the subjects, while in others it was almost non-existent. For example, the urinary quercetin excretion profile from subject 2 showed an overall decrease in the amount of quercetin excreted after 3 h of ingestion of quercetin aglycone with butter followed by a subtle increase at 10 h (Figure 5.5b).

Urinary quercetin excreted, in some subjects, was still above baseline levels even after 12 h of consuming the hydrolysed onion with butter. This was clearly evident in subject 9, in which the amount of quercetin detected was 26 µg. Inter-individual variability in the amounts of excreted quercetin following the consumption of the hydrolysed onion with butter was considerable ranging between 411 µg (subject 6) and 759 µg (subject 2). Table 5.3 provides an average of the time-course studies from the consumption of the hydrolysed onion meal with butter for all five subjects.

Table 5.3 Table showing hourly volumes of urine collected and quantities of quercetin detected from each subject after ingestion of the hydrolysed onion meal with butter.

 	Subject 1		Subject 2		Subject 4		Subject 6		Subject 9	
Time (h)	urine (ml)	Q (μ g)	urine (ml)	Q (μ g)	urine (ml)	Q (μ g)	urine (ml)	Q (μ g)	urine (ml)	Q (μ g)
0	48	ND	127	ND	152	ND	24	ND	329	ND
1	149	29	252	111	360	53	65	48	431	49
2	347	43	158	137	271	64	466	76	334	44
3	365	46	445	149	507	79	304	65	286	74
4	446	55	240	86	358	131	150	65	209	54
5	246	126	173	80	364	29	173	64	64	44
6	250	89	161	20	168	14	57	30	0	0
7	214	34	324	33	282	22	117	20	125	35
8	253	26	278	37	205	9	324	13	323	52
9	225	30	167	28	415	8	339	8	180	24
10	142	25	186	39	61	3	308	9	48	24
11	165	15	313	25	54	2	208	6	173	19
12	78	11	254	13	160	5	184	5	338	26
Totals	2928	530	3078	759	3357	419	2719	411	2840	446

ND = not detected. Q = total amount of hydrolysed quercetin conjugates

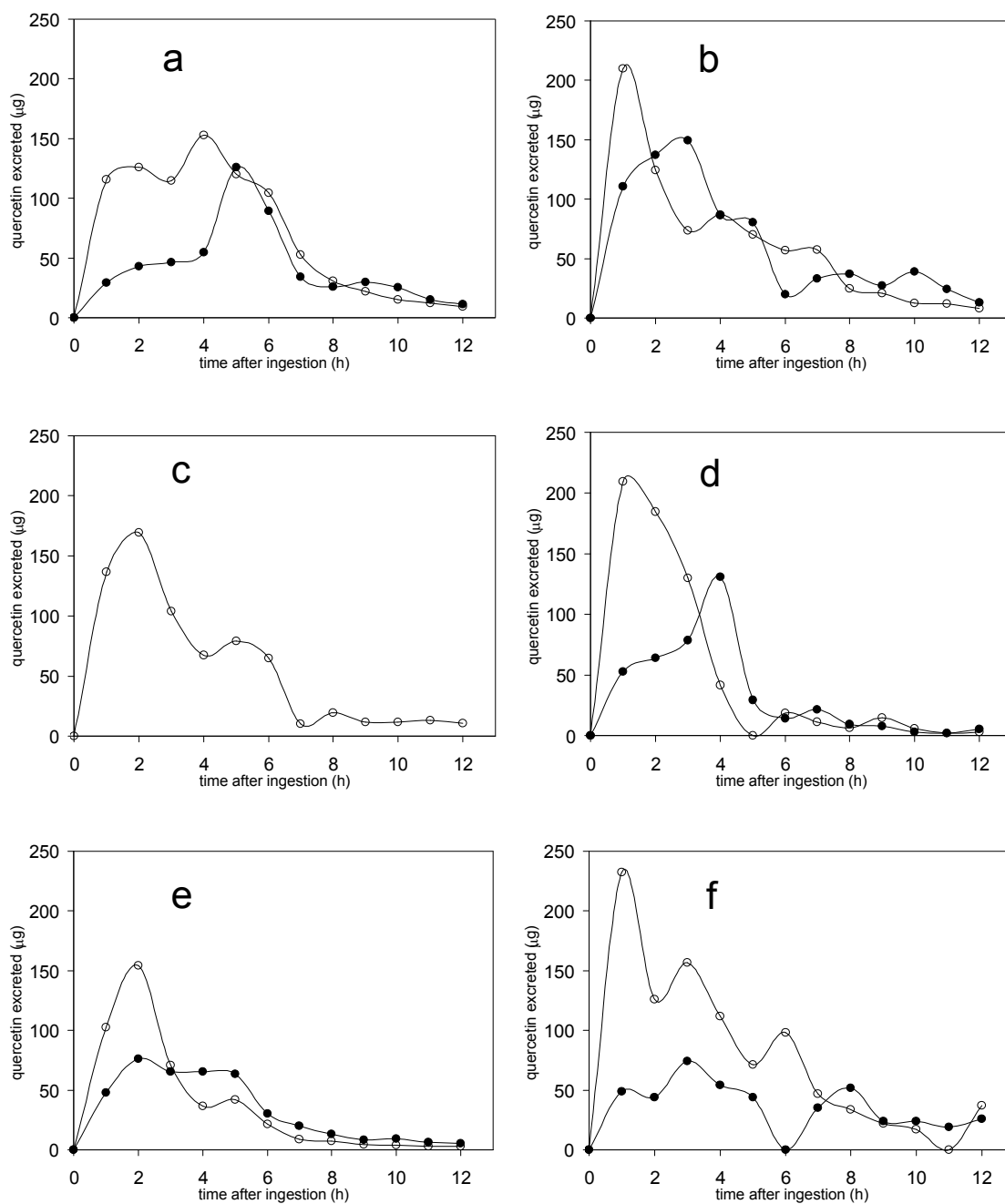


Figure 5.5 Time-courses of quercetin excretion after ingestion of the control onion meal ($\circ$), and the hydrolysed onion meal ($\bullet$), with 25 g of butter in subjects 1 (a), 2 (b), 3 (c), 4 (d), 6 (e) and 9 (f).

5.3.5 Differences in time-courses of excretion

Urinary quercetin excretion after consumption of the control onion meal with 8.3% (w/w) fat was much higher than after the hydrolysed onion meal as demonstrated by the areas under the curve for the average quercetin excretion time curve profile from the control onion meal compared to the areas under the curves from the hydrolysed onion meal with butter (Figure 5.4). The average amount of quercetin recovered in urine after 12 h of consuming the control onion meal with butter was $2.81 \pm 0.28\%$ of the ingested dose ($n=6$), whereas it was $1.71 \pm 0.22\%$ after consuming the hydrolysed onion meal with butter ($n=5$). The average time-course of urinary quercetin showed an overall sharp decline in the amount of quercetin excreted after 1 h from the consumption of control onion with butter. On the other hand, the average time-course of urinary quercetin showed a steady increase in quercetin excretion up to 4 h before gradually decreasing without reaching baseline level within 12 h. Comparison of the amount of excreted quercetin from both types of onion in all the subjects over a 12 h recovery in urine are presented in Table 5.4. The calculated t value showed that there was a statistically significant difference between the absorption of quercetin (as glycosides) from the control meal with butter and the absorption of the quercetin aglycone from the hydrolysed onion meal with butter ($P < 0.05$).

Table 5.4 Comparison of total amount of quercetin excreted in urine over 12 h after the ingestion of an onion-based meal with butter.

Subject	Control onion ^a		Hydrolysed onion ^b	
	Quercetin excreted (µg)	Proportion of intake (%)	Quercetin excreted (µg)	Proportion of intake (%)
1	876	3.37	530	1.77
2	760	2.92	759	2.53
3	699	2.69	NA	NA
4	627	2.41	419	1.4
6	459	1.77	411	1.37
9	953	3.67	446	1.49

^a 26 mg quercetin equivalent as quercetin glycoside consumed.

^b 30 mg quercetin aglycone consumed.

NA = not available.

5.3.6 Effect of co-ingested fat on the bioavailability of quercetin aglycone and its glycosides

The amount of urinary quercetin excreted, expressed as a percentage of the ingested dose of quercetin glycosides from the control onion meal containing 8.3% fat was significantly higher compared to the same onion meal without the dietary fat (Table 5.5; $P < 0.05$). When the control onion meal was consumed together with the 8.3% fat, urinary quercetin excretion peaked after 1 h, which was similar to that observed from the control onion without fat. A smaller peak was observed after 6 h with the control onion and fat. However, quercetin levels from the control onion with the 8.3% fat between 1 and 6 h were dramatically higher than the corresponding value attained after intake of the ‘fat-free’ control onion meal despite the lower dose of quercetin glycoside consumed (36 mg quercetin equivalent from the fat-free onion meal vs. 26 mg quercetin

equivalent from the onion meal with 8.3% fat; Figure 5.6a). Moreover, the elimination of quercetin from the control onion meal was clearly delayed after co-ingestion with fat as relatively higher levels of quercetin (12 μg , $n=6$) were detected after 12 h compared to near baseline levels from same subjects (2 μg , $n=6$) with the fat-free onion meal.

After the intake of quercetin aglycone from the hydrolysed onion meal with 8.3% fat, the peak of quercetin excretion reached a plateau after 2 h before decreasing gradually after 4 h. This was in sharp contrast to the aglycone when consumed without dietary fat since urinary levels of excreted quercetin rose more sharply and reached their peak levels relatively earlier between 1 to 2 h (Figure 5.6b). The elimination of quercetin was delayed in both cases with no significant difference in amount of quercetin detected after 12 h (11 and 12 μg with fat-free and 8.3% fat onion meal respectively).

Table 5.5 Comparison of relative bioavailability of quercetin in human subjects after the ingestion onion-based meals with or without dietary fat.

Subject	Control onion ^a			Hydrolysed onion ^b		
	Q absorption without fat (%)	Q absorption with fat (%)	Increase in bioavailability (%)	Q absorption without fat (%)	Q absorption with fat (%)	Increase in bioavailability (%)
1	1.64	3.37	105	2.20	1.77	0
2	2.23	2.92	31	1.87	2.53	35
3	2.25	2.69	20	1.57	NA	NA
4	1.82	2.41	32	0.97	1.4	44
6	1.54	1.77	15	1.03	1.37	33
9	1.89	3.67	94	1.43	1.49	4

^a Onion meal rich in quercetin glycosides

^b Onion meal rich in quercetin aglycone; NA = not available

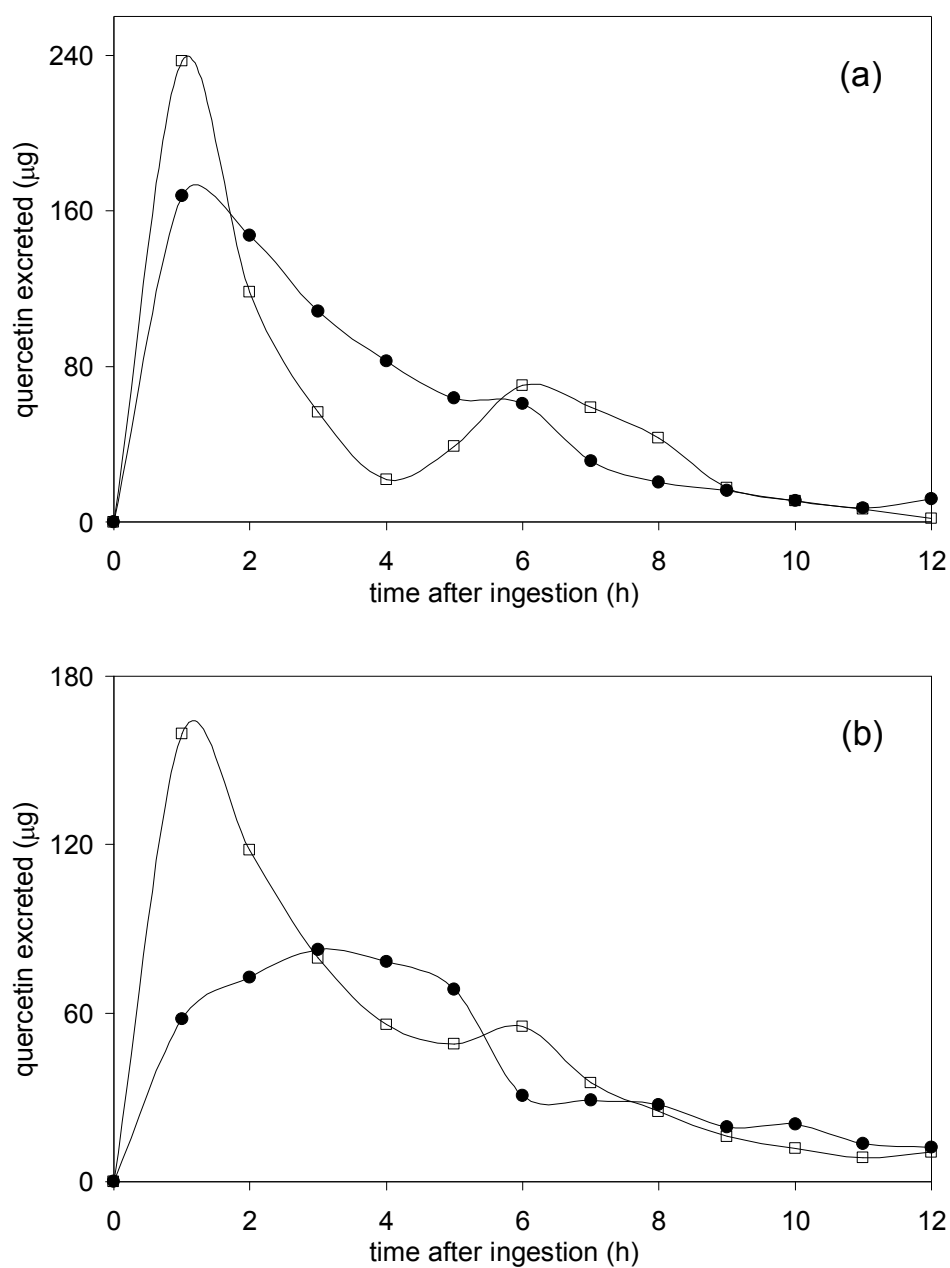


Figure 5.6 Comparison of time-courses of quercetin excretion following the intake of (a) control onion meal (n=6) and (b) hydrolysed onion meal (n=5) before (Study 1) and after the co-ingestion of 8.3% fat (Study 2). Control onion meal contained 36 and 26 mg quercetin equivalent as quercetin glycosides and hydrolysed onion meal contained 32 and 30 mg quercetin aglycone in study 1 and 2 respectively. $\square$ Study 1 $\bullet$ Study 2

Compared with the consumption of the hydrolysed onion meal without dietary fat, bioavailability was increased with the co-ingestion of fat; however the increase was non-significant (Table 5.5). The only exception was subject 1, in which the interaction of fat with the quercetin aglycone from the hydrolysed onion meal had the opposite effect. There was a considerable decrease in bioavailability with 1.77% of the ingested dose being bioavailable compared to 2.20% initially observed after the intake of the hydrolysed onion meal without fat.

5.4 Discussion

The aim of this present study was to investigate the influence of dietary fat in an onion-based meal on the absorption and bioavailability of quercetin either applied as aglycone or as quercetin glycosides. Rapid-resolution HPLC coupled with diode array detection at 370 nm was used in this study for the determination of quercetin. The method offers several significant advantages in terms of greater sensitivity; good peak resolution and a much shorter analysis time. This enables the development of a rapid and reproducible method for the separation and detection of flavonoids from food as well as biological samples. Moreover this method consumes much smaller volumes of solvent compared to the traditional reversed-phase HPLC because the run time was reduced dramatically from 35 to only 8 min (see Chapter 2). Compared with the traditional reversed-phase HPLC method, which needs more than 35 minutes to separate the flavonoid compounds, the chromatograms in Figure 5.2 demonstrate that the separation of the flavonol glycosides in the onion extract can be achieved within 3 min. In addition, the rapid-resolution HPLC method allowed the separation of the same number of quercetin derivatives in the same elution order. For example, quercetin glycosides, Q3,4'G and

Q4'G were eluted after 0.9 and 1.8 min whereas quercetin had a retention time of 2.6 min. There have been previous attempts to optimise a rapid gradient elution method for the separation of flavonoids (glycosides and the aglycones) in fruits and vegetables. In one study for example, a fast HPLC method was developed for the analysis of flavonols and flavan-3-ols in vegetables, including white onion (Escarpa *et al.*, 2000). The authors reported that gradient elution with phosphoric acid-acetonitrile mixtures and phosphoric acid-methanol mixtures resulted in fast and complete separation of the flavonoid compounds within analysis times of less than 10 min. In another study, quercetin quantified from human plasma and urine samples was recently showed to have a retention time of 2.8 min (Moon *et al.*, 2008).

In order to test the reliability of the rapid-resolution HPLC method, the 12 h hydrolysed urine samples from subject 9 following the ingestion of the control onion meal from the previous study (see Chapter 4) were reanalysed using the Agilent 1200 series model with the shorter C18 rapid resolution 1.8 μm particle size column. The pattern of quercetin excretion was almost identical to the one observed from the traditional reversed-phase HPLC method (Figure 5.7). Despite the small increase (about 8%) in the total amount of urinary quercetin quantified in the 12 h urine samples using the rapid resolution HPLC method, the differences in urinary quercetin concentrations were non-significant ($P > 0.05$). Moreover, the agreement with the previous analysis provides evidence that the present method was both effective and reliable in estimating urinary recovery, and thus bioavailability. The relatively small difference in the time-courses of urinary quercetin can be attributed to loose vial crimp caps that may have caused the methanolic samples to change concentration over time.

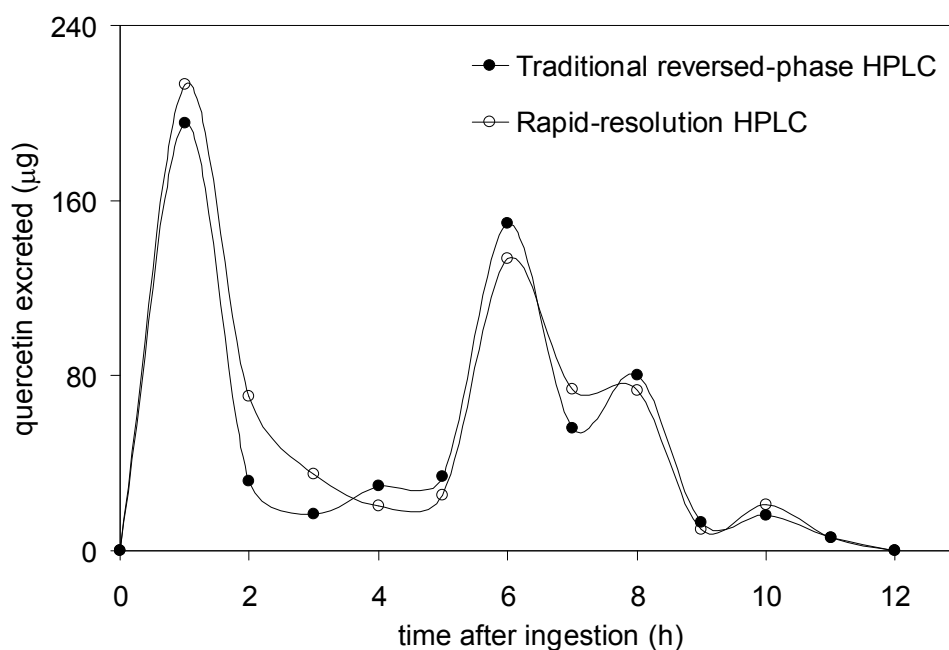


Figure 5.7 Comparison of time-courses of quercetin excretion in subject 9 after ingestion of control onion meal using the traditional reversed-phase HPLC method on the Merck-Hitachi Lachrom L-series model, and the rapid-resolution HPLC method on the Agilent 1200 Series Rapid Resolution LC System.

The yellow onions used to prepare the onion-based meals contained the same profile of quercetin glycosides to those previously reported in onions (Price and Rhodes, 1997). HPLC analysis of the control onion meal revealed two major peaks representing the glucosides, Q3,4'G and Q4'G, which accounted for most (about 92%) of the total flavonol compounds in the onion. Small quantities of free quercetin and Q3G were also present. Lombard *et al.* (2002) reported very similar results with Q3,4'G and Q4'G accounting for 84 to 93% of total flavonol content detected in four different varieties of yellow onion. In the present study, Q4'G was the main quercetin glucoside component, agreeing with Lombard *et al.* (2002) and Mogren *et al.* (2007), contrary to the first human study (Chapter 4) and other previous studies who reported higher levels of Q3,4'G (Price and Rhodes, 1997; Caridi *et al.*, 2007).

The concentrations of total quercetin from the HPLC analysis of the onion-based meals fell within a range of previous determinations for onion. For example, the quercetin content of different varieties of onions varied from 5.4 to 28.6 mg/100g fresh weight (Patil *et al.*, 1995). In another study, total quercetin content was reported to be 11-fold higher in yellow onions than white varieties (Yang *et al.*, 2004). The variability in flavonoid content is mainly due to cultivar differences (Marotti and Piccaglia, 2002). Besides genetic factors, environmental and postharvest factors play an important role in determining the flavonoid composition of fruits and vegetables (Tomas-Barberan and Espin, 2001). In the present study, the homogenised control onion meal contained 8.7 mg/100g quercetin equivalents while the hydrolysed onion meal contained 10 mg/100g quercetin aglycone. The total amount of quercetin consumed in the 200 g meal of control and hydrolysed onion was 36 and 32 mg respectively (see Chapter 4). Therefore, it was desirable though not essential to increase the quantity of quercetin by giving each subject a higher portion of the onion-based meals (300 g) to consume in order to make valid comparisons with the onion meal study without dietary fat.

The initial difference in the amount of quercetin ingested from the control and hydrolysed onion meals (26 and 30 mg quercetin aglycone respectively) were close enough to make a valid comparison in terms of bioavailability as previously determined (see Chapter 4). In another study, the difference between the bioavailability of quercetin from Q4'G in humans when administered with 150 and 300 mg of Q4'G supplement appeared to be minimal (Hubbard *et al.*, 2004).

No trace amounts of free quercetin could be detected in the non-hydrolysed urine samples. This agrees well with a recent study by Mullen *et al.* (2006), who did not detect free quercetin in the urine of human subjects after the ingestion of onions. Once absorbed, quercetin is subjected to phase II metabolism by the intestinal enzymes. Conjugation of quercetin with glucuronide and sulphate are the most likely metabolic routes as previously described (Day *et al.*, 2001). Therefore, a mixture of sulfatase from *A. aerogenes* and β -glucuronidase from *E. coli* was used to hydrolyse the quercetin conjugates in the urine samples and liberate the quercetin aglycone.

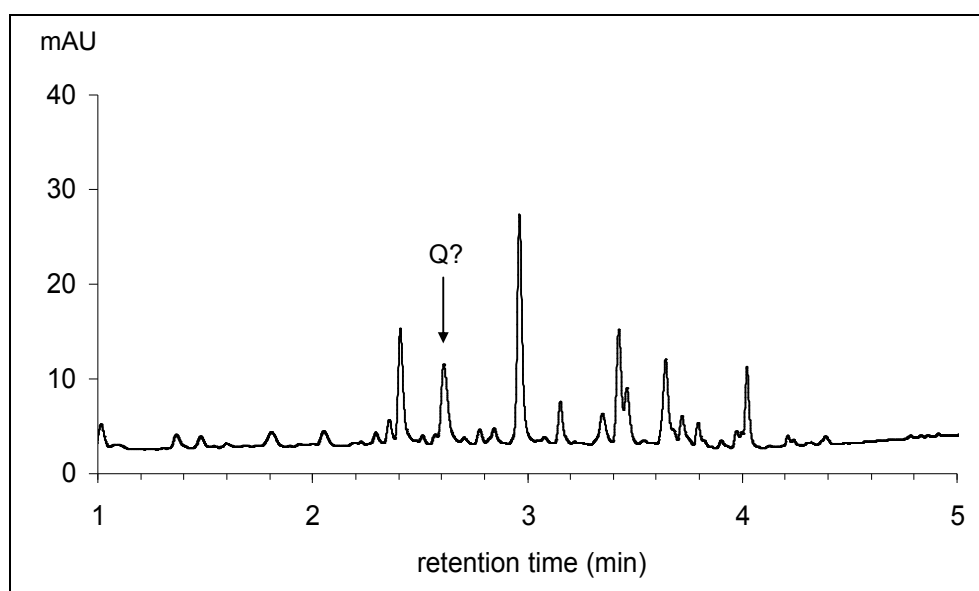


Figure 5.8 Chromatogram showing the methanolic extract of urine collected 1 h after ingestion of the onion-based meal with butter before hydrolysis with sulfatase and β -glucuronidase (370 nm). Q is quercetin.

A small peak with a retention time of 2.59 min and almost identical to that of quercetin was observed in a non-hydrolysed urine sample from one of the subjects (Figure 5.8). In addition, the peak disappeared and was replaced by one large peak at 2.62 min after treatment with the mixture of β -glucuronidase/sulfatase enzymes (Figure 5.3),

suggesting that it might have corresponded to a quercetin metabolite. Evidence based on absorption spectra confirmed that the peak in question was not quercetin (Figure 5.9).

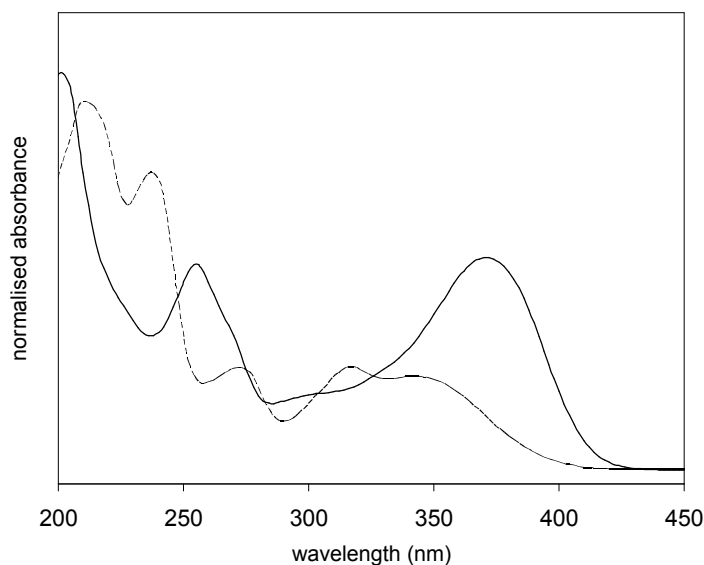


Figure 5.9 Absorption spectra of quercetin and quercetin metabolite in urine. Quercetin, — ; quercetin metabolite, -----.

In the first human study, quercetin excretion (equivalent to quercetin aglycone) was measured with peak concentrations reached within 1 to 2 h after the ingestion of the control onion meal. This indicates that quercetin from Q3,4'G and Q4'G is absorbed in the upper part of the small intestine. The co-administration of 8.3% dietary fat enhanced the relative bioavailability of the quercetin glycosides from the control onion meal with an average increase of ~50% (n=6). This effect was more prominent in some subjects than in others. For example, the presence of dietary fat in the control onion meal had increased quercetin absorption by 105% in subject 1, whereas bioavailability was enhanced by only 15% in subject 6 (Figure 5.10a; Table 5.5). These results are comparable to those found by Lesser *et al.* (2004) who reported that the

co-administration of 17% fat enhanced the bioavailability of Q3G in pigs by 32% in comparison to the lower fat (3%) control. Similarly, Azuma *et al.* (2003) observed a significant increase in the accumulation of quercetin metabolites in the plasma of rats after the addition of 4.6% soybean oil to their onion-based diet.

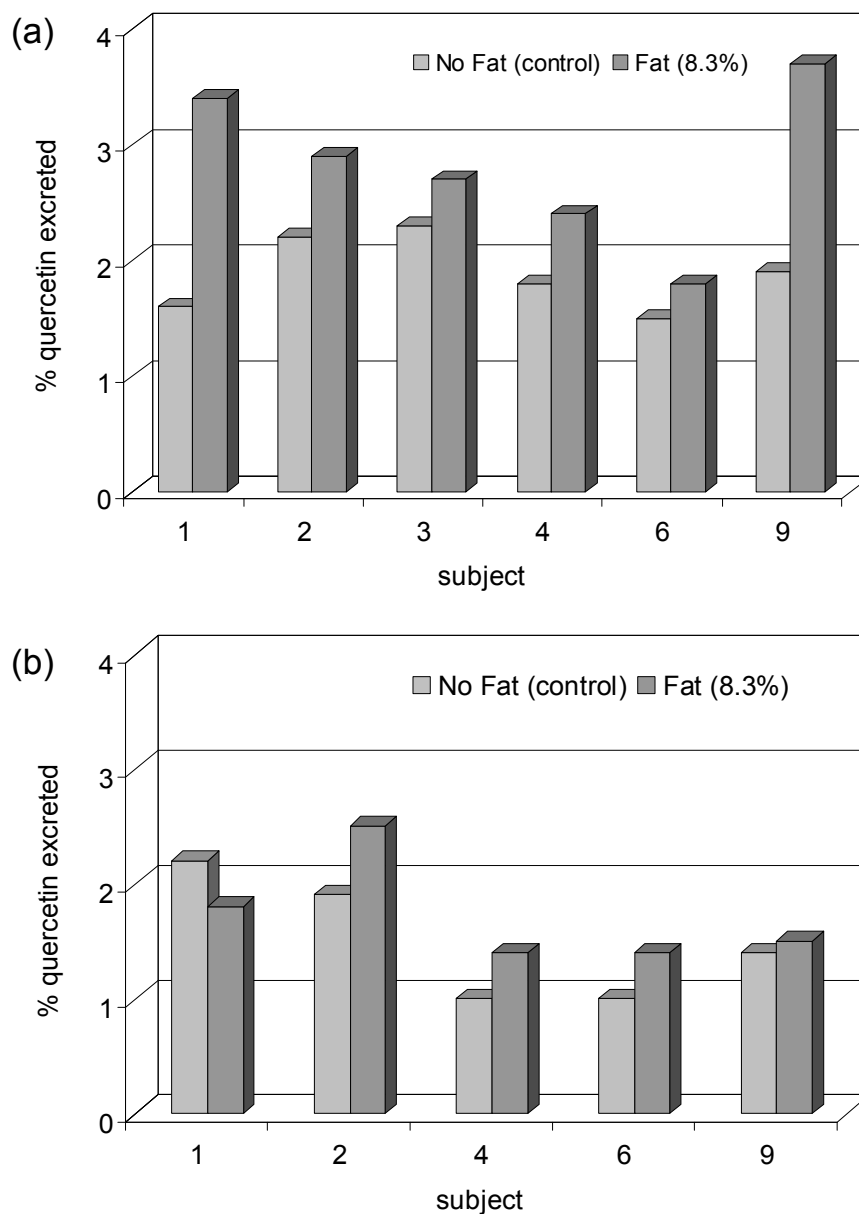


Figure 5.10 Effect of dietary fat on the total amount of quercetin excreted as a percentage of the ingested dose of (a) quercetin glycosides from the control onion meal, and (b) quercetin aglycone from the hydrolysed onion meal.

The food matrix of the present study was composed from the homogenised onion tissue. The polysaccharide composition of the onion cell wall is made up of solid cellulose, xyloglucans and galactin-rich pectins (Ha *et al.*, 1997). One possible explanation for the enhancing effect of dietary fat on quercetin glycoside absorption from the control onion meal is the binding of fat molecules to the cell wall debris of the homogenised onion meal thus displacing the quercetin glycosides near the border brush membrane of the small intestine and to the LPH enzyme system. Molecular interactions leading to significant binding of lipids to pectin have previously been shown to occur using C^{13} NMR spectroscopy and gel exclusion chromatography (Falk and Nagyvary, 1982).

Previous reports have shown that hydrolysis of quercetin glycoside by LPH at the brush border of the small intestine and absorption by passive diffusion of the released aglycone into the enterocytes is the major route of absorption for flavonol glycosides (Day *et al.*, 2003; Sesink *et al.*, 2003). The close proximity of the released aglycone to the apical membrane could result in a higher diffusion rate than aglycone from the intestinal lumen, and potentially increase the rate of absorption (Day *et al.*, 2003). A more comprehensive review by Day *et al.* (2004) has emphasised the critical role of LPH on the hydrolysis and subsequent absorption of quercetin glycosides *in vivo*. Alternatively the quercetin glycoside may be actively transported via the intestinal SGLT1, resulting in an elevated intestinal uptake (Wolffram *et al.*, 2002).

Differences in the quercetin glucoside profiles of the control onion meals, Q3,4'G being the main glucoside in the first study (Chapter 4) while Q4'G being the primary quercetin glucoside in the second study (Figures 4.7a and 5.2a), did not appear to have

an effect on total bioavailability of quercetin (Table 5.5). Based on the high specificity of the C β G and LPH towards quercetin glucosides, namely Q3G and Q4'G (Day *et al.*, 2000b), it is possible that these glycosides enhance the absorption and increase quercetin bioavailability. As for the increase in quercetin bioavailability from the control onion meal without fat, LPH would be required to hydrolyse Q3G from Q3,4'G as hypothesised by Nemeth *et al.* (2003).

The addition of 8.3% fat enhanced the total bioavailability of quercetin (quercetin and its conjugates) from the quercetin aglycone containing meal by only ~23%. However, there was no statistically significant difference in the total bioavailability of quercetin between the present study and the previous (control) study due to the low number of subjects (Figure 5.10b; Table 5.5). The 12 h time-course of quercetin excretion following the co-ingestion of fat with the hydrolysed onion meal showed a relatively slow increase in urinary quercetin levels reaching a maximum after 2 – 4 h, which was followed by a gradual decline. The slow appearance of the quercetin indicates that the absorption and excretion of quercetin aglycone is substantially delayed by the presence of dietary fat. Thus, the effect of dietary fat appears to slow transit of quercetin through the GI tract and result in delayed gastric emptying after intake of quercetin aglycone. The slowing of gastric emptying induced by the incorporation of fat into the meal may be attributed to the higher energy density, around 9 kcal/g while sugars and proteins have about 4 kcal/g, and possibly due to its relatively slow rate of absorption in the intestine (Gentilcore *et al.*, 2006). In agreement with this finding, Mullen *et al.* (2008) recently observed significantly lower amounts of anthocyanins excreted 0-2 h after eight human subjects had consumed strawberries with double cream (422 nmol), in comparison to the amount of anthocyanins excreted when consumed without cream

(718 nmol). The authors also reported that the $T_{\max}$ for the main anthocyanin metabolite, pelargonidin-*O*-glucuronide at 2.4 h was delayed by more than 1 h and with a lower $C_{\max}$, although the difference was not significant when subjects consumed the strawberries with cream.

Interestingly, the enhancing effect of dietary fat on the bioavailability of total quercetin from the aglycone in the hydrolysed onion meal was less pronounced compared to the glycosides from control onion meal. Because quercetin aglycone is relatively lipophilic, it is expected that the presence of dietary fat would enhance its solubility and increase the absorption of the aglycone by passive diffusion through the border brush membrane into the enterocyte. The presence of lipids (lecithin and soybean oil) and emulsifiers such as sodium taurocholate have been demonstrated to increase the intestinal absorption of quercetin in rats by enhancing its solubility in lipid micelles (Azuma *et al.*, 2002). Similarly, green tea catechins were absorbed more extensively when administered as a phospholipid complex rather than as free catechins (Pietta *et al.*, 1998). It has been proposed that the presence of dietary fat could have led to the incorporation of quercetin into mixed bile micelles in the duodenum thus promoting its solubility and transport through the brush border membrane (Lesser *et al.*, 2004).

The enhancing effect of dietary fat on the re-absorption of excreted quercetin for both meals can be explained by enterohepatic circulation. The role of bile in lipid digestion and absorption is well documented. Hence, stimulation of biliary secretion by dietary fat excretes the flavonoid metabolites with the bile into the upper small intestine. Deconjugation of the excreted metabolites by the microflora in the colon occurs, and the released aglycone is then absorbed or subjected to further metabolism.

In agreement with previous studies, wide inter-individual variability in the urinary recovery of quercetin was observed (DuPont *et al.*, 2002; Moon *et al.*, 2008). In the present human study, the urinary recovery of quercetin from quercetin aglycone ranged from 1.77% to 3.67% of the ingested dose of hydrolysed onion with fat (n=6), and the urinary recovery of quercetin glycosides ranged from 1.37% to 2.53% of the ingested dose of control onion with dietary fat (n=5). Such large variation may be attributed to the different composition of the microflora or differences in intestinal physiology. It has been established that quercetin excretion from the ingestion of quercetin glycosides was consistently higher than that from the aglycone (see Chapter 4). Given the fact that the sugar moiety is a major determinant of quercetin absorption in the (GI) tract (Hollman *et al.*, 1997), it was thus not surprising that urinary quercetin levels from the consumption of the control onion were consistently higher than those from the ingestion of the hydrolysed onion meal even though both meals contained 25 g of fat. This is in contrast to observations of Lesser *et al.* (2004) who found the bioavailability of quercetin from the quercetin aglycone diet had increased by 57% when 17% fat was added to the diet compared with an increase of only 30% with the Q3G diet in pigs.

In conclusion, the results in this study indicate that dietary fat has a considerable effect on the bioavailability of quercetin. The bioavailability of quercetin aglycone and its glycosides are enhanced by the addition of fat to the onion-based meals. This indicates that quercetin absorption is, to a large extent, affected by solubility. The presence of dietary fat may enhance absorption through the incorporation of the lipophilic aglycone in mixed micelles. The sugar moiety however, remains the most important factor governing the absorption of quercetin. Results from the two intervention studies repeatedly show that quercetin is more bioavailable from quercetin glycosides when

consumed from the control onion meal compared to the aglycone when consumed from the hydrolysed onion meal regardless of whether fat is present in the meals or not. Furthermore, the nature of the food matrix may possibly influence the bioavailability of quercetin through binding with plant materials.

Chapter 6

Discussion

6.1 Flavonol absorption and bioavailability

6.1.1 Tomato studies (Chapter 3)

The first experimental chapter (Chapter 3) used HPLC analysis to examine the flavonol content in different varieties of commercially-grown tomatoes and commonly consumed tomato-based food products. With this knowledge a better understanding of the bioavailability of dietary flavonols from these foods would emerge. Chapter 3, concerning the characterisation of flavonols, aimed to select a suitable tomato-based product(s) that would enhance the absorption and increase flavonol bioavailability in long-term feeding trials. Data obtained in Chapter 3 showed a large variation in the levels of flavonoid, namely rutin, among the different varieties of tomato and tomato-based food products analysed, with the highest flavonol levels recorded for cherry tomatoes. Identification and characterisation of the flavonoids was achieved by HPLC-DAD analysis and comparison of retention times to standards. The determination of flavonoid content served to investigate the effects of processing on flavonoid bioavailability. Previous research has shown that lycopene, a naturally occurring carotenoid from processed tomato products, is more bioavailable in humans than from fresh tomatoes (Gartner *et al.*, 1997). The release of lycopene from the food matrix due to processing may enhance the absorption and bioavailability of lycopene (Rao and Agarwal, 1999). Thus the question arises of whether the bioavailability of flavonoids in tomato is affected by processing. Although a few studies have reported on the bioavailability of flavonoids in humans from fresh tomato (Bourne and Rice-Evans, 1998) and tomato-based products (Mauri *et al.*, 1999; Bugianesi *et al.*, 2002; Simonetti *et al.*, 2005), no comparative studies for the bioavailability of flavonoids (fresh tomatoes vs. processed tomato products) exist.

Furthermore, some of these studies have claimed to detect flavonol glycosides (rutin) in human plasma (Mauri *et al.*, 1999; Simonetti *et al.*, 2005). Some doubt remains over the identification of glucosides in these studies (Day and Williamson, 2001), particularly when recent studies reported the extensive metabolism of rutin following the release of the aglycone in the colon with the subsequent appearance of the conjugates in the circulatory and excretory systems (Jaganath *et al.*, 2006).

The purpose of the experimental studies in Chapter 3 was to eventually investigate the effects of food processing on flavonol bioavailability by conducting comparative human trials using different sources of tomato and tomato-based products. Cellulase-catalysed hydrolysis of rutin was utilised in this study as an alternative to acid hydrolysis in order to produce free quercetin aglycone. However, progress was constrained by several problems. The differences in the chemical properties of flavonoids in terms of solubility were problematic due to the fact that quercetin aglycone is lipophilic whereas its glycosides are not (Rothwell *et al.*, 2005). Consequently, different experimental conditions may be required in the same experiment as illustrated by the difficulty in detecting the enzyme-hydrolysed flavonoid glycosides. Moreover, given that the amount of quercetin excreted in urine is less than 4.7% and the generally low levels of quercetin found in tomatoes and tomato-based products, experimental work would require collecting and concentrating large volumes of urine from subjects in order to compensate for the lack (at the time) of a more sensitive analytical method such as LC-MS/MS to measure the trace concentrations of quercetin aglycone.

6.1.2 Human intervention study (Chapter 4)

Previous intervention studies have used supplements as sources of flavonoid aglycones or have looked at quercetin glycosides in single foods without the capability of comparing the different chemical forms in similar foods (Manach *et al.*, 2005). The intervention study in Chapter 4 was intended to assess the extent of quercetin absorption and bioavailability from a food-based system in humans. There were two main aims: (a) to compare the bioavailability of dietary quercetin glycosides and its aglycone by using experimental foods rich in deglycosylated quercetin aglycone rather than relying on supplements, and (b) to carry out the human study by implementing time-course analysis of urinary excretion as a relatively simple and non-invasive tool for comparative measurement of flavonoid absorption. With regard to the first aim, a significant difference was found (based on urinary excretion) between bioavailability of quercetin glucoside and its aglycone when present in a similar food matrix system. Although quercetin glucosides are considered to be better absorbed than quercetin aglycone (Hollman *et al.*, 1995; 1997), the outcome of the experiments in Chapter 4 indicated that the sugar moiety was the major determinant of the absorption of flavonoids in humans when present in comparable food-based meals. With regard to the time-course studies, the patterns of urinary quercetin excretion were shown to mimic plasma patterns generally observed in pharmacokinetic studies of quercetin absorption.

The outcome from this study is not consistent with the proposed hypothesis that quercetin aglycone is probably more bioavailable than its glycosides when they are dispersed in a food matrix. Moreover, these results contrast with the findings of Wiczkowski *et al.* (2008) who reported that quercetin aglycone from pulverized shallot

skin was more bioavailable than quercetin glucosides from shallot flesh. Dry shallot skin was used as an alternative to quercetin aglycone supplements since around 83% of the total quercetin content was in the aglycone form. Although the authors aimed to compare the bioavailability of quercetin aglycone and its glucosides from dietary sources, one could argue that dry shallot skin does not make the most meaningful source for dietary quercetin in humans. As discussed in Chapters 1 and 5, flavonoid bioavailability depends, amongst other factors, on the food matrix and the type and extent of processing the food has undergone. Thus, it may be speculated that differences in food matrices existed between the pulverized shallot skin and homogenised shallot flesh. This could explain the differences found in the extent of absorption and bioavailability between the quercetin aglycone and its glucosides. Furthermore, because of the differences between the food matrices of dry shallot skin and that of shallot flesh, the study did not provide a control group for each shallot preparation. For these reasons, the claim in Wiczowski *et al.* (2008) that the bioavailability of quercetin aglycone, when consumed as part of a diet, is better than that of quercetin glucosides was not accurate.

Unlike dry shallot skin, a novel onion-based food was developed in this study as an alternative source of dietary quercetin aglycone with a food matrix structure similar to that of an onion-based preparation containing quercetin glycosides. Previous studies have suggested that absorption of quercetin was determined primarily by the nature of the attached sugar moiety (Hollman *et al.*, 1997; Erlund *et al.*, 2000; Morand *et al.*, 2000). The outcome from this research provides conclusive evidence that when quercetin and its glycosides are consumed from dietary sources, the bioavailability of quercetin glycosides in humans is better than that of quercetin aglycone. Among the

many factors that may contribute to bioavailability of flavonoids (see Chapters 4 and 5), one possibility that may account for this finding is the interaction of flavonoids with the various components and structure of the food by binding to macromolecules, membranes or cell walls. For example, the observed low levels of deglycosylated quercetin in the supernatant of the centrifuged hydrolysed onion homogenate provide indirect evidence of such interaction (Liddle C., personal communication). The possible mechanisms relating to food microstructure that influence the bioavailability of flavonoids *in vivo* are represented in Figure 6.1.

6.1.3 Effect of food matrix (Chapter 5)

The aim of the third experimental chapter (Chapter 5) was to complement the second experimental chapter by investigating the influence of dietary fat on the bioavailability of quercetin present in a similar food matrix system as either quercetin glucosides or quercetin aglycone. The study showed that bioavailability of both quercetin glucoside and quercetin aglycone was enhanced considerably in the presence of fat. However, as with the previous experimental chapter (Chapter 4), quercetin glucoside was more bioavailable than quercetin aglycone. Although the extent of flavonoid absorption is affected by its solubility (Piskula and Terao, 1998; Piskula, 2000), the outcome of the experiments in Chapter 5 implied that the sugar moiety in quercetin glycosides was the most important determinant governing the intestinal absorption of dietary quercetin.

As in the first human study (Chapter 4), quercetin aglycone present in a food system (non-supplement) was used to evaluate the effects of co-ingested fat on the bioavailability of quercetin. Thus, this approach takes into account possible effects of

the food matrix and therefore truly reflects bioavailability of quercetin from natural food sources.

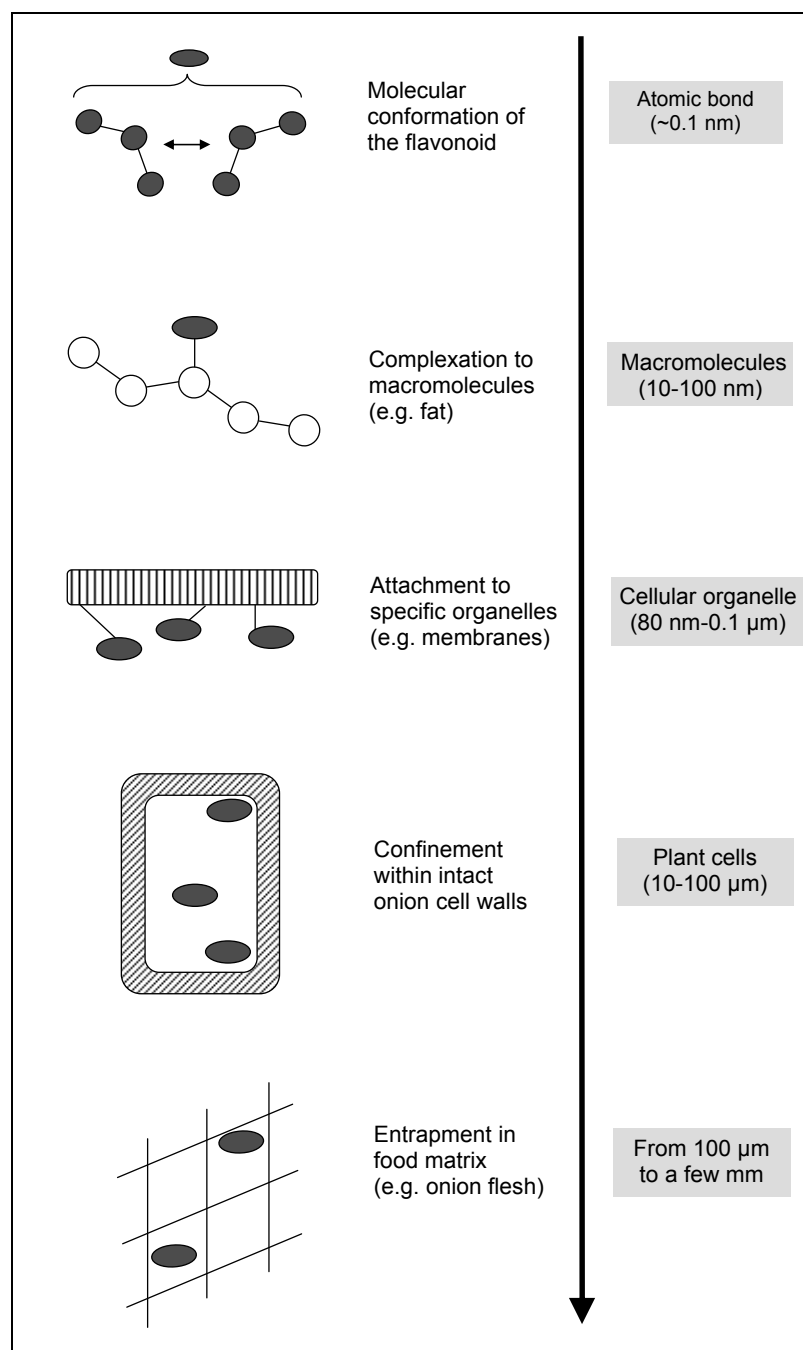


Figure 6.1 Microstructural mechanisms that affect the bioavailability of flavonoids (●) in a food matrix (adapted from Parada and Aguilera, 2007).

In the first phase of the experimental study, a significant enhancing effect of dietary fat (~50%) on the bioavailability of quercetin from the control onion meal was observed. However, the increase in quercetin bioavailability from the hydrolysed onion meal (~23%) failed to reach the level of significance (Chapter 5). Two reasons were theoretically considered to explain this finding. First, the displacement of quercetin glycosides from the cellular debris to the LPH enzyme system in the presence of fat would result in quercetin deglycosylation and subsequent diffusion of the aglycone. The other possibility is that some of the quercetin aglycone dissolved in fat may have entered the lymphatic system in the GI tract and escaping first pass metabolism. This in turn would result in delaying the excretion of quercetin as observed in the quercetin urinary profile from the hydrolysed onion meal (Figure 5.4). The uptake and transport of flavonoids in the lymph has previously been investigated. For example, in the study of Murota and Terao (2005), quercetin was detected in the lymph of rats 30 min after the administration of quercetin in propylene glycol.

A substantial change in the average profile of urinary quercetin from the hydrolysed meal in comparison to the first study (Chapter 4) was documented. The incorporation of fat to meals is known to slow gastric emptying rates (Stacher *et al.*, 1990). Thus, a possible explanation for the difference in profile between the two human studies is that the fat component in the second study (Chapter 5) delayed gastric emptying of the hydrolysed onion meal. However, decreased gut motility was not the only reason for the difference in the urinary quercetin profile, because the peak of urinary quercetin from the quercetin glycoside was similar (1 h after ingestion of meal) to that observed in the first human study (Chapter 4).

6.2 Directions for future research

There are a number of future directions for this present work. To date, very few intervention studies with flavonoids from tomatoes and tomato-based products have been reported in the literature. An obvious direction would be to extend the work in Chapter 3 by determining the bioavailability and excretion characteristics of flavonoids in long-term human feeding trials following the consumption of tomato-based meals. This could be achieved by (a) consuming a variety of flavonol-rich tomatoes and/ or tomato-based products, (b) collecting and concentrating urine at specified intervals over a 24 h period and (c) providing a more sensitive detection method for quantifying quercetin in urine such as LC-MS/MS. Another approach would be to evaluate the effects of food processing on flavonol bioavailability by comparing the absorption of quercetin from tomato-based products such as tomato puree and tomato juice with that from high-flavonol containing varieties fresh tomatoes (cherry tomatoes) in a 24 h feeding study. Initially, the study could be designed to determine the extent of absorption in one subject. The intervention study would be performed several times using the same volunteer thus allowing for the determination of absorption parameters, and consequently manipulating the diet in order to achieve maximum absorption without the complications of inter-individual variations. Eventually, the study could be extended to include more subjects and different tomatoes and tomato-based food products. Finally, it might be useful to develop and improve the method currently used for cellulase hydrolysis of flavonoids from plant-derived foods. Technical considerations for maximising extraction efficiency and accuracy of flavonoids, such as quercetin, should be revised as a result of its low solubility and stability in various solvent systems (Chapters 3 and 4).

The work in Chapter 4 showed that absorption of quercetin glucosides from a food system was considerably higher than quercetin aglycone from the same food system (non-supplement). The methodology used to investigate the bioavailability of flavonols in human involved the treatment of urine samples obtained from volunteers following ingestion of the onion-based meals with deconjugating enzymes (β -glucuronidase and sulfatase). To extend this research further, more advanced analytical methods such as HPLC coupled with MS could be used in order to provide a full account of the flavonoid concentration and metabolic composition of urine after the consumption of both meals. The study could be extended to assess to effects of other dietary components such as fat (Chapter 5) and fibre intakes on the extent of flavonoid absorption. Of lesser importance but still relevant would be to shorten the low-flavonoid diet period before consuming the flavonoid-rich meal and the washout period between the two phases of the intervention study. To investigate this possibility, subject(s) would be allowed to consume their normal diet throughout the day before starting an overnight fast of 12-16 h. Urine samples would be collected at specific intervals during the overnight fast period and analysed to evaluate whether adequate baseline urine samples could be provided. For the purification and extraction of flavonoids from urine samples, SPE offers considerable advantages over solvent extraction. Suitability testing of other SPE columns such as Waters OasisTM hydrophilic-lipophilic-balanced (HLB) cartridges would be feasible in order to obtain a flavonoid recovery higher than 90%.

The influence of dietary fat on the absorption of both quercetin glycosides and its aglycone from a similar food system was investigated in Chapter 5. Although the experiment studies (quercetin glycosides and deglycosylated quercetin) showed an

increase in flavonoid absorption with the co-administration of fat, the results were strikingly different in terms of amount of quercetin absorbed. Thus, a possible explanation is the binding of the deglycosylated flavonol to the plant cell wall material (Figure 6.1). The use of microscopy and imaging techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) or confocal laser microscope for studying food microstructure would undoubtedly help to determine the extent of binding in the presence of fat. Several studies have investigated the effect of solubility on the efficiency of intestinal absorption of flavonoids (Piskula and Terao, 1998; Lesser *et al.*, 2004). Another obvious and useful extension would be to investigate whether deglycosylated quercetin present in a food system is absorbed to the same extent as quercetin from a supplement in the presence of fat.

Finally, it would be interesting to investigate the influence of dietary fat (matrix) on flavonoid phase II metabolism and flavonoid metabolite excretion profile in urine. Urine samples collected from the two intervention studies (Chapters 4 and 5) should be re-extracted and analysed to determine and compare the excreted metabolite profile from each study. Such work would require the use of a more sensitive detection method for positive identification of metabolites such as LC-MS in the presence of appropriate metabolic standards.

6.3 Further questions

Is there any chance of increasing flavonoid absorption? To answer that question it is important to investigate the factors affecting absorption, transport and metabolism of flavonoids. Specifically, the role of matrix in preventing or slowing the release of

quercetin glycosides or its aglycone and reaching the LPH system or the mucosal membrane needs to be further assessed. This is particularly important when considering the fact that glycosylated soluble quercetin cannot cross the cell wall or that cell wall-bound quercetin may inhibit its intestinal absorption. Furthermore, the role of butter (fat) in quercetin's solubility needs to be assessed in order to establish whether a maximum amount of quercetin absorption can be achieved.

6.4 Concluding statement

Flavonoids represent an important class of bioactive plant phenolics that are known for their widespread occurrence in the human diet, e.g. in fruits, vegetables, and beverages such as tea. The possible beneficial effects of dietary flavonoids on human health are widely discussed. Studying the absorption and bioavailability of flavonoids within the diet is essential in determining any associated health-promoting effects. Considering the data generated as part of this thesis, flavonoid absorption in humans can be manipulated within the diet. However, a great deal of research is needed to better understand the various conditions that influence the absorption and bioavailability of flavonoids *in vivo*, which can provide significant insight into methods for maximising the utilisation of these health-promoting phytochemicals.

Chapter 7

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