ANALYSIS OF PHYTOCHEMICAL IN A MALAYSIAN MEDICINAL PLANT AND THE BIOAVAILABILITY OF DIETARY HYDROXYCINNAMATES

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Abstract

The first study was looking into the phytochemical analysis of herbal medicinal plant from Malaysia. Phenolic compounds in an aqueous infusion from leaves of *Ficus deltoidea* (Moraceae), a well-known herbal tea in Malaysia, were analysed by HPLC coupled to photodiode-array and fluorescence detectors and an electrospray ionization tandem mass spectrometer. Following chromatography of extracts on a reverse phase C_{12} column 25 flavonoids were characterized and/or tentatively identified, with the main constituents being flavan-3-ol monomers, proanthocyanidins and *C*-linked flavone glycosides. The proanthocyanidins were dimers and trimers comprising (epi)catechin and (epi)afzelechin units. No higher molecular weight proanthocyanidin polymers were detected. The antioxidant activity of *F. deltoidea* extract was analysed using HPLC with on-line antioxidant detection. This revealed that 85% of the total antioxidant activity of the aqueous *F. deltoidea* infusion was attributable to the flavan-3-ol monomers and the proanthocyanidins

In terms of the bioavailability of hydroxycinnamates with the high occurrence of *p*-coumaroylquinic acid in *F. deltoidea* extract, further study was carried out using radiolabeled caffeic acid. In the study, male Sprague-Dawley rats ingested 140 x 10^6 dpm of [3-^{14}C]*trans*-caffeic acid and over the ensuing 72 h period body tissues, plasma, urine and faeces were collected and the overall levels of radioactivity determined. Where sufficient radioactivity had accumulated samples were analysed by HPLC with on-line radioactivity and tandem mass spectrometric detection. Nine labeled compounds were identified, the substrate and its *cis* isomer, 3′-*O*- and 4′-*O*-sulphates and glucuronides of caffeic acid, 4′-*O*-sulphates and glucuronides of ferulic acid and isoferulic acid-4′-*O*-sulphate. Four

unidentified metabolites were also detected. After passing down the gastrointestinal tract the majority of the radiolabeled metabolites were excreted in urine with minimal accumulation in plasma. Only relatively small amounts of unidentified ^{14}C-labeled metabolites were expelled in faeces. There was little or no accumulation of radioactivity in body tissues, including the brain. The overall recovery of radioactivity 72 h after ingestion of [3-^{14}C]caffeic acid was ~80% of intake.

The role of colonic microflora in the metabolism of caffeic acid was carried using an in vitro model of the human colonic microflora. Caffeic acid (55 µmoles) was incubated with human faecal materials obtained from five Asian donors, and caffeic acid degradation was monitored from 0-8 h. Faecal samples were analysed by GC-MS, where major phenolic acids identified were dihydrocaffeic acid, 3-(3′-hydroxyphenyl)propionic acid and phenylacetic acid. Caffeic acid was quickly degraded by the colonic microflora, as it disappeared after 4 h of incubation in two of the faecal samples. The degree of degradation of caffeic acid was significantly influenced by the addition of glucose as well as individual variations in the density and the composition of microflora. These findings support extensive metabolism of caffeic acid in the colon, depending on the substrate concentration and the supplement of glucose which resulting the formation of simple phenolics.

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Author′s Declaration

The composition of this thesis and the work described within it was carried out entirely by myself unless otherwise cited or acknowledged. The research for this thesis was carried out between December 2008 and December 2012.

Signed  .....

Maizatul Hasyima Omar

January 2013

Abbreviations

ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
amu	atomic mass unit
APCI	atmospheric pressure chemical ionization
AUC	area under plasma concentration-time curve
CBG	cytosolic β-glucoside
C*max*	maximum plasma concentration
COMT	catechol-*O*-methyltransferase
ESI	electrospray ionization
FRAP	ferric reducing antioxidant power
GI	gastrointestinal tract
HPLC	high performance liquid chromatography
HRF	heterocyclic ring fission
LC-MS^2	liquid chromatography mass spectrometry/mass spectrometry
LPH	lactase hydrolysed phloridzin
λ_{max}	absorbance maxima
MS	mass spectrometry
$[M-H]^-$	negatively charged molecular ion
m/*z*	mass to ratio ion
PDA	photo diode array
QM	quinone methide
RC	Radioactivity counting
RDA	Retro Diels-Alders
ROS	reactive oxygen species
SULT	sulfotransferases
T_{max}	time to reach c_{max}
TPTZ	2,4,6-tri-2-pyridyl-s-triazine
TMCA	2,4,5-trimethoxycinnamic acid
t_R	retention time
UV	ultraviolet
UGT	glucoronosyltransferases
v/v	volume/volume
w/v	weight/volume

1 GENERAL INTRODUCTION

1.1 Herbal medicinal plants

Plants have been used as medicinal remedies long before recorded history. Ancient Chinese and Egyptian writing describe medicinal uses of plants. In China the first ever documented information about the use of herbal remedies was carried out by the legendary Shen Nong, believed to be the father of Chinese medicine, who lived around 2000 B.C (Frederick, 1973). Ibn Sina (known in Europe as Avicenna), a Persian who worked as a pharmacist and physician in Egypt, is well known for his definitive medical work, *Canon Medicine* which was used as a standard medical text up to 1650 in many medieval universities including the universities of Montpellier and Lueven (Britannica, 2012). In *Canon Medicine* or *The Canon*, he describes some 760 medicinal plants and drugs that could be derived from them (Tschanz, 2003). By definition herbal medicines include herbs, herbal material, herbal preparations and commercial herbal products, that contain as active ingredients parts of plants, or other plants material or combinations (WHO, 2000).

In the early 19th century, when chemical analyses first become available, scientists started to extract potential active compounds from herbal plants. Subsequently, developments in synthetic chemistry reduced the use of herbal plants in modern medicine. More recently, however, in a number of countries including Malaysia, there has been a renewed interest in the phytochemicals that occur in herbal medicinal plants which are being used increasingly as alternative treatments in health care, as in many instances modern medicines

and drugs are not only expensive but can also be associated with adverse side effects.

Recently, the World Health Organization estimated that ~80% of the world population is using herbal medicines as a part of their primary care (WHO, 2001). In the USA the increased cost of prescription medications, together with an interest in returning to natural products or organic remedies, has led to approximately 20% of the population using herbal products (Bent, 2008). Worldwide consumption of herbal medicines has increased markedly, and it has been estimated by the Secretariat of the Convention on Biological Diversity, that global sales of herbal products reached $60 billion in 2000 (WHO, 2003).

Natural products are major sources for drug development, with approximately one-third of top-selling drugs have been derived from plant ingredients (Strohl, 2000). Although knowledge of these plant ingredients is increasing, information on their mode of action and clinical pharmacology is still scarce, which limits the efforts for standardization, evaluation and further utilization of herbal remedies (Ma et al., 2009).

1.2 Trend of herbal remedies in Malaysia

Malaysia is among the countries which favour the use of herbal medicines in primary care. Because of the large diversity of plant species, reported to be the twelfth largest in the world, Malaysia has a massive resource of potential medicinal plants (Noridayu et al., 2011). It was reported that about 12,000 flowering plants may to have potential health promoting properties, however only 1,300 species have been identified for medicinal purposes (Jamal et al., 2010). Herbal plants have been the basis of traditional medicines throughout the

world and in Malaysia they have been utilized by three major ethnics groups: the Malay community through shaman and midwives, traditional Chinese herbal medicines, and Ayurveda by Indians (WHO, 2001). The knowledge of the use of herbal and medicinal plants has been passed from one generation to another with some of these medications being self-prescribed for health maintenance, treatment, and prevention of minor ailments and also for chronic illness (Siti et al., 2009).

In Malaysia, the use of herbal and medicinal plants for various applications either for prevention, healing and treatment of certain diseases has been well recognized as alternative medications alongside modern medicines. These traditional medications have come together under Traditional of Complementary Medicine (TCAM) a body which has been formed by Ministry of Health, Malaysia. The Ministry of Health offers TCAM in some of the public hospitals as an alternative medication to the patients, including treatments such as acupuncture and massage. The important of TCAM has been recognized by WHO(2001), as it reported that 50-80% of the population of developed and developing countries use some form of TCAM for prevention and promotion of health, with most of them relying on herbal plants.

Nowadays, herbal uses are not only restricted for healing and treatment purposes, but also extended on a worldwide basis to cosmetics and nutraceuticals. In Malaysia, herbal products have generated substantial income. In the years 2000 to 2005 the annual sales for traditional medicines increased from US$ 385 million (RM 1 billion) to US$ 1.29 billion (RM 4.5 billion) (BERNAMA, 2011). The high demand for the herbal products has encouraged farmers to cultivate selected herbal medicinal plants on a large scale. This kind of

plantation not only supplies the raw materials needed on a large scale but also helps to sustain the supply, since the natural habitats of these plants are now under the threat of deforestation. The Government of Malaysia has also introduced a scheme known as “one district one industry” that encourages people in villages either to invest in the small industry or to grow herbal plants, starting in their own gardens, which indirectly has increased the local people’s knowledge of the uses of these plants and promoted their increased use as local medicines.

However, despite the huge demand for herbal products there is the drawback of a lack of scientific evidence about their efficacy. The major concern is their safety and actual impact on human health. Vast numbers of commercial herbal medicines have not been thoroughly checked and submitted to rigorous scientific testing and qualities of the ingredients used are not standardized. This is a problem not just in Malaysia but also in Europe and America. Although herbal products are considered natural as they are of plant origin, it does not mean that they are harmless. Certain plants contain toxins as protectants. Compounds such as alkaloids, apiole and certain lignans are toxic to the liver, terpenes and saponins to the kidneys, and sesquiterpene lactones and furanocumarins can adversely affect the skin (Capasso et al., 2000). There is also the possibility that herbal remedies might contain added adulterants and inferior ingredients (Zhang et al., 2012). Therefore precautionary steps must be taken before a plant or a product can be safely being prescribed as a medicinal remedy. The safety of herbal remedies is of particular importance since most of these products are self-prescribed and patients rarely notify their doctor about their use. As a further complication, many herbal products sold as dietary

supplements lack toxicological data and reliable information on their clinical efficacy.

These circumstances have led to more focused research being carry out by research institutes such as MARDI (Malaysia Agricultural Research and Development Institute), FRIM (Forest Research Institute Malaysia), IMR (Institute for Medical Research) and also by local universities, in terms of phytochemical analysis of potential herbal medicinal plants in order to evaluate their safety and effectiveness and define and verify their therapeutic values. Such research will also help to achieve an overall improvement in the standard and quality of herbal medicines. These scientific findings when combined with traditional knowledge from local practitioners will generate information on drug-targeted biochemicals from herbal plants which will be of value to both local traders and the pharmaceutical industry.

1.3 Phenolic compounds from medicinal herbs and dietary plants

Secondary metabolites are chemical compounds produced exclusively by plants.They are synthesized for protection against herbivores, microbial and viral pests, as colouring agents, scent, or attractants and also as hormones (Jaganath et al., 2009). Many of these phytochemicals, including terpenoids, alkaloids and phenolics, have been found to have an impact on human health (Pandey and Rizvi, 2009). In contrast, primary metabolites, such as sugars, fats, and amino acids are the nutrients that are essential for plants growth and development. In recent times plant secondary metabolites have become of increasing scientific interest due to their beneficial effects on human health.

This can be seen from the large number of publications focusing on phytochemicals, their in vitro and in vivo activities and their potential impact on human health (Scalbert et al., 2005).

Phenolics are a class of chemical compounds having at least one aromatic ring with one or more hydroxyl group attached to it. They are synthesized exclusively in plants from L-tyrosine or L-phenylalanine + malonyl-CoA via the shikimate pathway. These important dietary constituents include simple and low molecular weight compounds such as hydroxybenzoic acids, phenylpropanoids, (hydroxycinnamic acids), flavonoids (three-ring hydroxylated polyphenols- which are synthesized by the pathways illustrated in Figure 1.1), complex derivativies such as tannins and derived polyphenols such as theaflavins and thearubigins which are not present in the leaves of *Camellia sinsensis* but form during the fermentation process that converts green tea to black tea (Miller and Ruiz-Larrea, 2002).

Some plant secondary metabolites are believed to be produced as a result of the plant's interaction with the environment, such as their response to stress and defense against bacterial and viral pathogens. In humans they appear to have desirable medicinal effects, with antioxidant and anticancer properties. In nature, phenolics are usually found conjugated to sugars and organic acids and can be classified into two groups: flavonoid and non-flavonoid compounds which consist of hydroxycinnamics, hydrolysable tannins, stilbenes and lignans (Table 1.1).

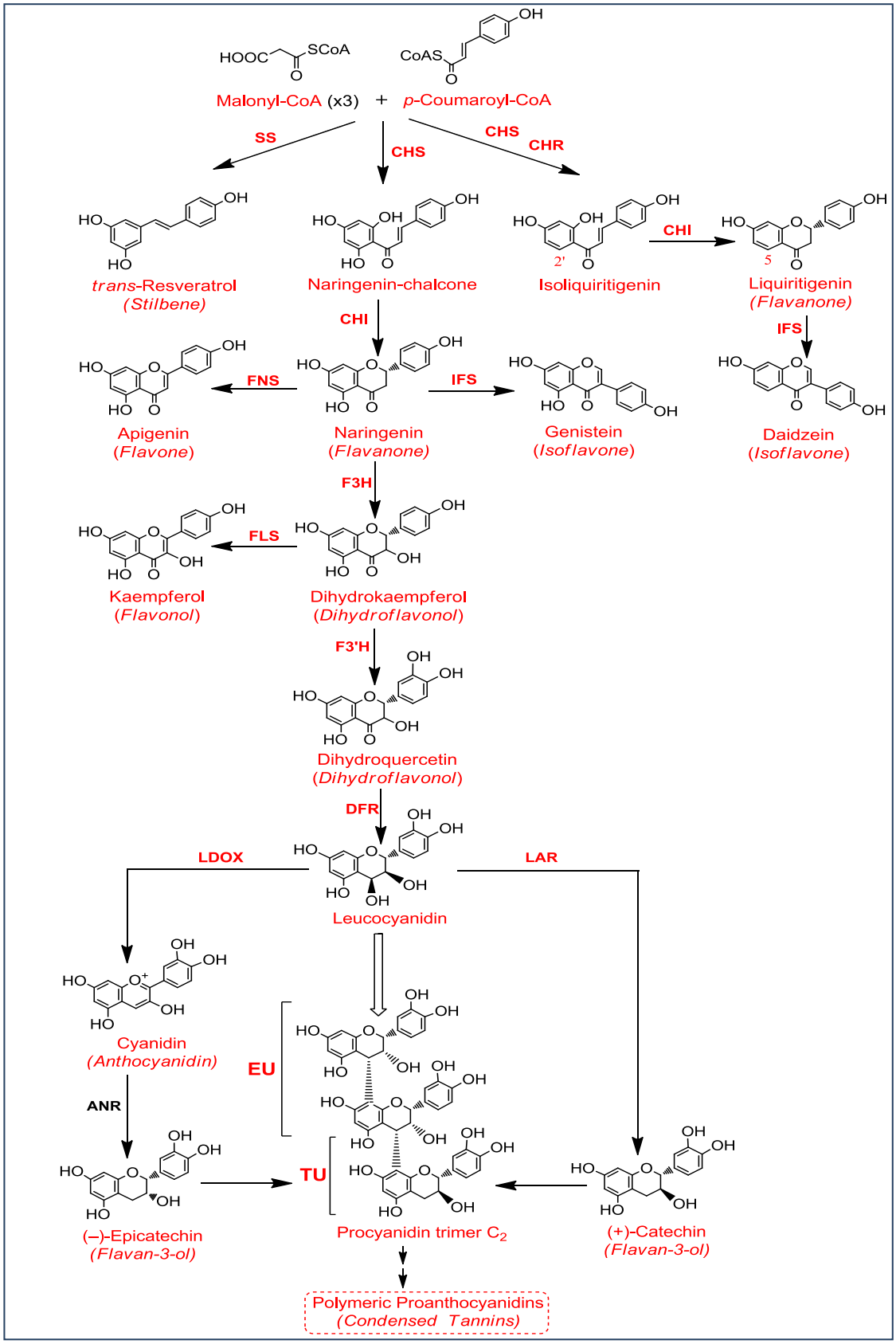


Figure 1. 1 Schematic diagram of the main pathways and enzymes involved for the synthesis of flavonoids and stilbenes. Enzymes abbreviations: SS-stilbenes synthase, CHS-chalcone synthase, CHR-chalcone reductase, CHI-chacole isomerise, IFS-isoflavone synthase, FNS-flavones synthase, FLS-flavonol synthase, DFR-dhydroflavonol 4-reductase, ANS-anthocyanidin 4-reductase, F3H- flavanone 3-hydroxylase, F3′H-flavonol 3′-hydroxylase, LAR-leucocyanidin 4-reductase, LDOX-leucocyanidin deoxygenase, ANR- anthocyanidin reductase, EU-extension units, TU-terminal unit.

Table 1.1 Main classes of phenolic compound

Classes and subclasses	Examples of specific compounds
Flavonoids	
Flavonols	Kaempferol, quercetin, myricetin
Flavones	Apigenin, luteolin
Flavanones	Naringenin, hesperetin
Flavan-3-ols	Catechins, gallocatechin
Anthocyanidins	Pelargonidin, cyanidin, malvidin
Isoflavones	Daidzein, genistein, glycitein
Condensed tannins	Trimeric procyanidin, prodelphinidin
Non-flavonoids	
Phenolic acids	Gallic, protocatechuic, *p*-hydroxybenzoic acids
Hydroxycinnamates	Coumaric, caffeic, ferulic, sinapic acids
Hydrolyzable tannins	Pentagalloylglucose, sanguiin H-6
Stilbenes	Resveratrol
Lignans	Secoisolariresinol, matairesinol

1.3.1 Flavonoids

Flavonoids are a group of more than 4,000 phenolic compounds and are ubiquitous in nature (Iwashina, 2000). They are present in fruits, vegetables, and beverages including tea, fruit juices, wines and herbs. They occur in all parts of plants but are not synthesized by humans or fungi. These compounds play an important role in plant development, defense and pollination and they are responsible for the attractive colours of flowers, fruit, and leaves, (de Groot and Rauen, 1998). It has been estimated 1×10^9 tonnes of flavonoids and related compounds are produced

annually by plants, this is equivalent to about 2% of all photosynthesized carbon (Andrew and John, 2011).

Flavonoids occur primarily conjugated with either one or more sugar residues that are linked to hydroxyl groups, but association with other compounds including carboxylic and organic acids, amines and lipids also takes place. Flavonoids with sugar moieties are referred as the flavonoids glycosides whereas aglycones are flavonoids without a sugar moiety. Flavonoids occur predominantly *in planta* as glycosides. Glycosylation increases their solubility in water facilitating their storage in vacuoles of flowers, leaves, stems and roots (Cuyckens and Claeys, 2004). Flavonoids occur as *O*- or *C*-linked glycosidic conjugates. Although any hydroxyl group can be glycosylated, certain positions are favoured: e.g. the 7-hydroxyl group in flavones, flavonones and isoflavones, the 3- and 7-hydroxyls in flavonols and flavan-3-ols, and the 3- and 5-hydroxyls in anthocyanidins (Cavaliere et al., 2005).

The structures of flavonoids are based on the flavonoid nucleus, which consists of three phenolics rings. These (poly)phenolic compounds possess 15 carbon atoms represented as C_6-C_3-C_6 structure. The basic structure allows for a magnitude of substitution patterns in the A, B and C rings. Flavonoids are classified into several sub-classes depending on the degree of unsaturation and oxidation of basic 15-carbon skeleton. The main groups of dietary importance are flavones, flavonols, flavan-3-ols, flavanones, anthocyanidins and isoflavones. The basic chemical structures of these six classes of flavonoids are illustrated in Figure 1.2.

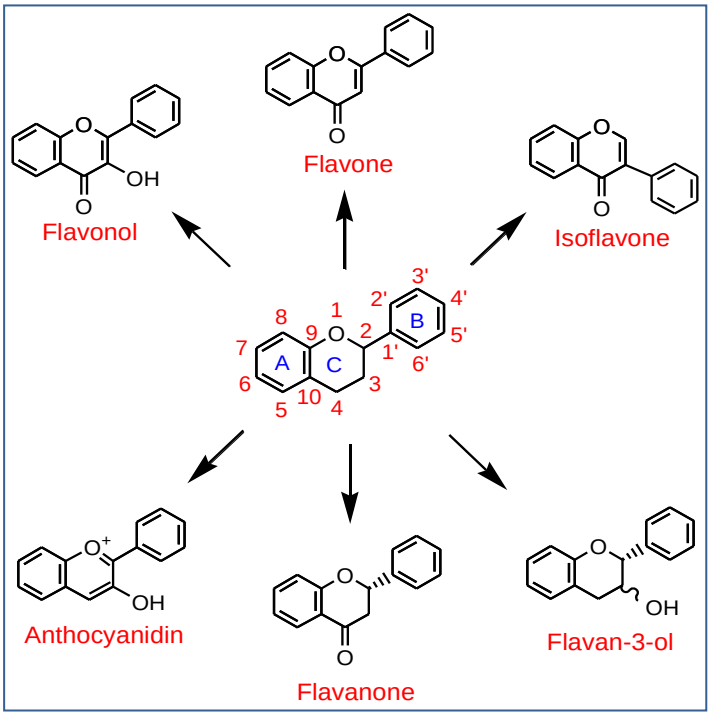


Figure 1. 2 Basic structures of flavonoids.

1.3.1.1 Flavonols

Flavonols are a major class of flavonoids with a hydroxyl group at the 3-position (Figure 1.3). They exist mainly as conjugated glycosides in plants. Glucose and galactose are the most common sugar moieties, while less common are rutinose, arabinose, xylose and rhamnose. Quercetin and kaempferol are flavonols that occur in many fruits, leaves and vegetables, isorhamnetin occurs in onions and pears and myricetin in berries, maize and tea (Hertog et al., 1993). This group of compounds were reported to have an effect on certain type of such viruses such as herpex virus, respiratory syncytical virus, parainfluenza virus and adenovirus. This antiviral activity of quercetin includes antiinfective and antireplicative capabilities (Wang et al., 1998).

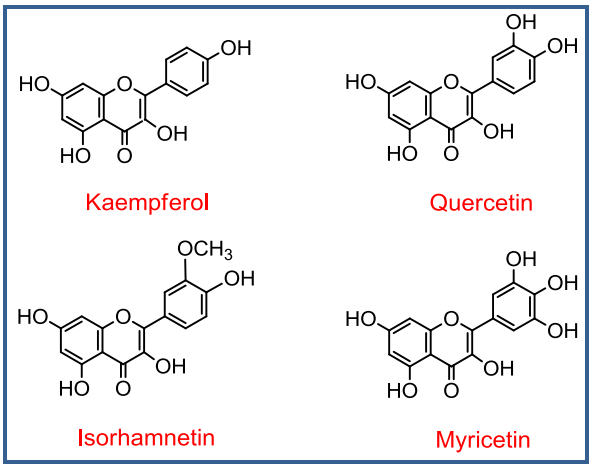


Figure 1. 3 Structures of common flavonols.

1.3.1.2 Flavan-3-ols

Flavan-3-ols are complex subclasses of flavonoids comprising of monomers, oligomers and polymeric proanthocyanidins. One example of monomeric flavan-3-ols is (+)-catechin that can exist together with its isomer (-)-epicatechin, these monomers are abundant in ripe fruits, leaves, tea and red wine (Manach et al., 2004). The beneficial effects of green tea are attributed to the presence of flavan-3-ols which represent 30-40% of the dry weight of the leaf (Wang and Ho, 2009). Predominantly flavan-3-ol monomers found in green tea are (-)-epigallocatechin, (-)-epicatechin-3-*O*-gallate and (-)-epigallocatechin-3-*O*-gallate (Del Rio et al., 2004) (Figure 1.4).

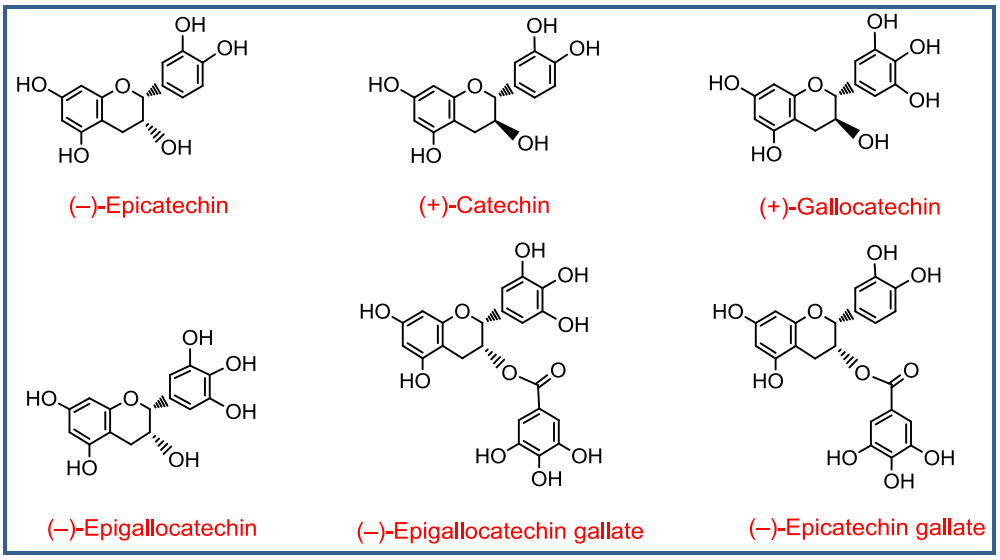


Figure 1. 4 Structures of flavan-3-ols monomers.

Tea is categorized based on the type of fermentation which occurs during the manufacturing process; 'non-fermented' green tea refers to drying and steaming the fresh leaves to inactivate polyphenol oxidase activity. Preparation of 'semi fermented' oolong tea involves subjecting the green leaves a partial fermentation prior to drying. 'Fermented' black and red (*Pu-Erh*) teas, have undergone a post-harvest full fermentation before drying and steaming (Cabrera et al., 2006). With black tea, oxidation is catalyzed by polyphenol oxidase activity, whereas microorganisms are used in the manufacture of red tea (Wu and Wei, 2002). During fermentation the polyphenolic compounds come into contact with polyphenol oxidases and flavan-3-ols are converted to theaflavins and thearubigins. As result, black teas contain high amounts of theaflavins and thearubigins, compounds that do not occur in green tea (Figure 1.5). Thearubigins are brownish in colour, water soluble and have a high molecular weight, and are the major phenolic compound of black tea.

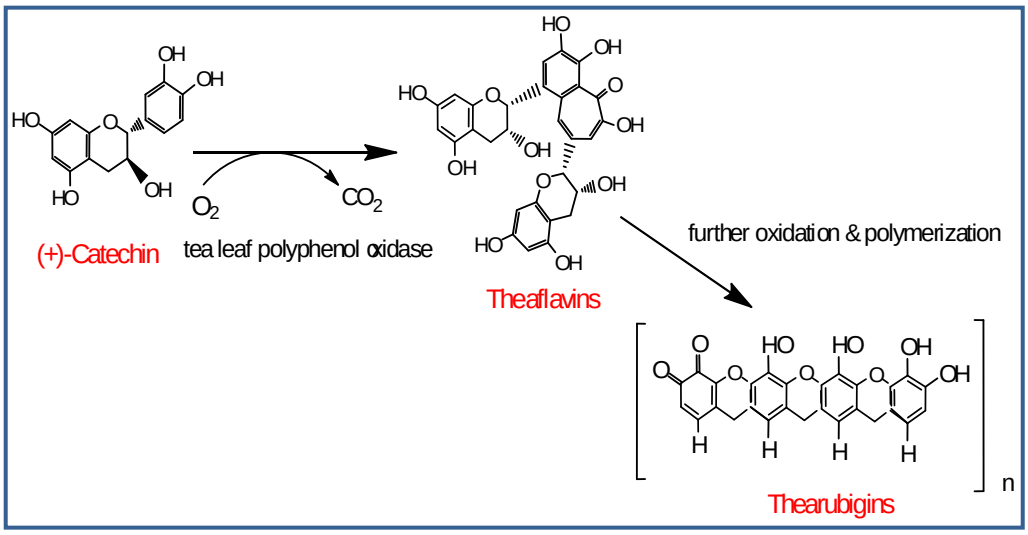


Figure 1. 5 Fermentation to produce black tea results in polyphenol oxidase catalysed reactions that transform flavan-3-ol monomers to dimer-like theaflavins and the polymeric thearubigins**.**

1.3.1.3 Flavanones

Flavanones are abundant in citrus fruits. Hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) is found principally as its rhamnose-glucose conjugate, hesperetin-7-*O*-rutinoside (hesperidin) (Figure 1.6), in oranges and some herbs (Ignat et al., 2011). Other flavanone aglycones include naringenin (Figure 1.6) (4′,5,7-trihydroxyflavanone) and eriodictyol (3′,4′,5,7,-tetrahydroxyflavanone). Flavanone rutinosides are tasteless. In contrast, flavanone neohesperidoside conjugates, such as hesperetin-7-*O*-neohesperidoside (neohesperidin) from bitter orange (*Citrus aurantium*) and naringenin-7-*O*-neohesperidoside (naringen) from grapefruit (*Citrus paradisi*), have an intensely bitter taste (Tomás-Barberán and Clifford, 2000). Flavanones are usually conjugated with a disaccharide at C-7, and their levels vary depending on the part of citrus fruit, with highest concentrations occurring in the peel (Chanet et al., 2012). This sub-class of flavonoids are reported to exert many beneficial health effects on human

possessing antioxidant, cardioprotective and anti-carcinogenic properties (Rice-Evans et al., 1996; Chanet et al., 2012).

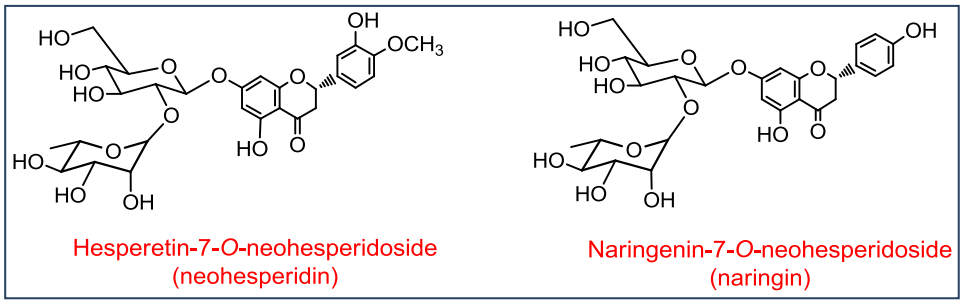


Figure 1. 6 Structures of hesperidin and naringin, flavanone glycosides.

1.3.1.4 Flavones

Flavones occur principally in herbs. They can be found in all parts of the plant, above-and below ground, in vegetative and generative organs: stem, leaves, buds, bark, heartwood, thorns, rhizomes, flower, farina, fruits, seed and also in roots and leaf exudates (Martens and Mithöfer, 2005). The main flavones are apigenin (4′,5,7-trihydroxyflavone) which occurs in vegetables such as celery, and luteolin (3′,4′,5,7-tetrahydroxyflavone) a component of green peppers (Hertog et al., 1992) (see Figure 1.7). Flavones are found as both *O*-or *C*-glycosides, (Wu et al., 2004b). This class of flavonoid has a double bond between carbon 2 and carbon 3 in the C-ring of the flavan skeleton with usually no substituent at C3. They are synthesized via the anthocyanidin/proanthocyanidin pathway with flavanones as the immediate precursor (Fig. 1.1).

Flavones have antioxidant, antimutagenic, anti-cancer and anti-HIV properties (Williams et al., 1999). Epidemiological and animal studies suggest that a high dietary intake of flavonoids, including flavones, has

been reported to have protective effects against several degenerative diseases, such as lung and colon cancer, coronary heart disease, chronic inflammation, and osteoporosis (Elliott et al., 2000).The effects of flavones as antiproliferative agents have been studied extensively using several tumour cell lines. It was reported that flavones such as apigenin have a significant effect in inhibiting the growth of human carcinoma cells (Kathirvel et al., 2009).

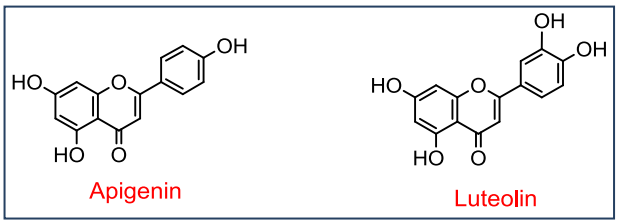
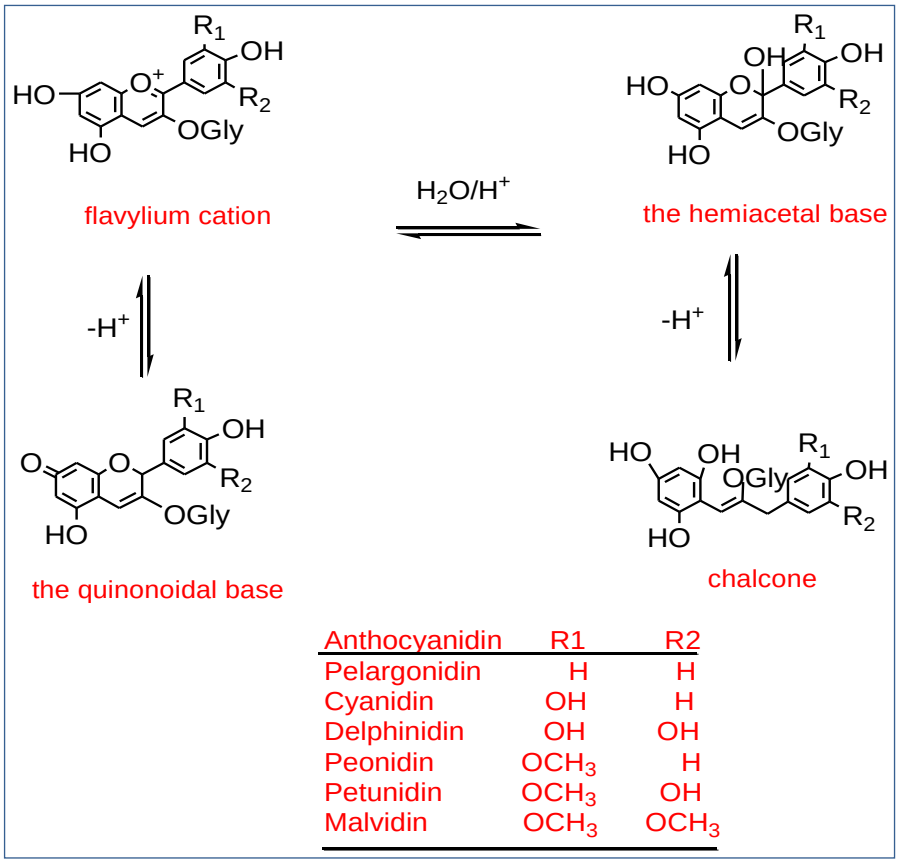


Figure 1. 7 Structures of common flavones.

1.3.1.5 Anthocyanins

Anthocyanins are the glycoside form of anthocyanidins. The word anthocyanin is derived from the Greek *anthos* = flower and *kianos* = blue. Anthocyanins are pigments occurring in vegetables, leaves, flowers and fruits such as grapes, berries, apples, and are responsible for vibrant pink, red, violet and blue colorations (Charron et al., 2009). Anthocyanins are positively charged at acidic pH. In solution anthocyanins occurs in equilibrium with essentially four molecular forms: the flavylium cation, the quinodal base, the hemiacetal, and chalcone (Figure 1.8) (Cooke et al., 2005), and their relative amounts vary with both pH and the structure of the individual anthocyanins. The stable flavylium anthocyanins only exist at pH <2. Their unique structures play an important role in determining their absorption, metabolism, bioavailability, and consequently biological responses to anthocyanins (Prior, 2012).



Anthocyanidin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 1. 8 Anthocyanin structures and pH induced transformations (Prior, 2012).

The aglycones of anthocyanins are unstable and very susceptible to degradation. In plants, anthocyanins are glycosylated, typically with glucose at position 3, and esterified with various organic acids and phenolic acids. This glycosylation can stabilize anthocyanins and protect them from light, pH and oxidation conditions (Manach et al., 2004). A huge variety of anthocyanins occur in nature with the most common being glycosides of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin and peonidin (Andersen and Jordheim, 2006) (Figure 1.9).

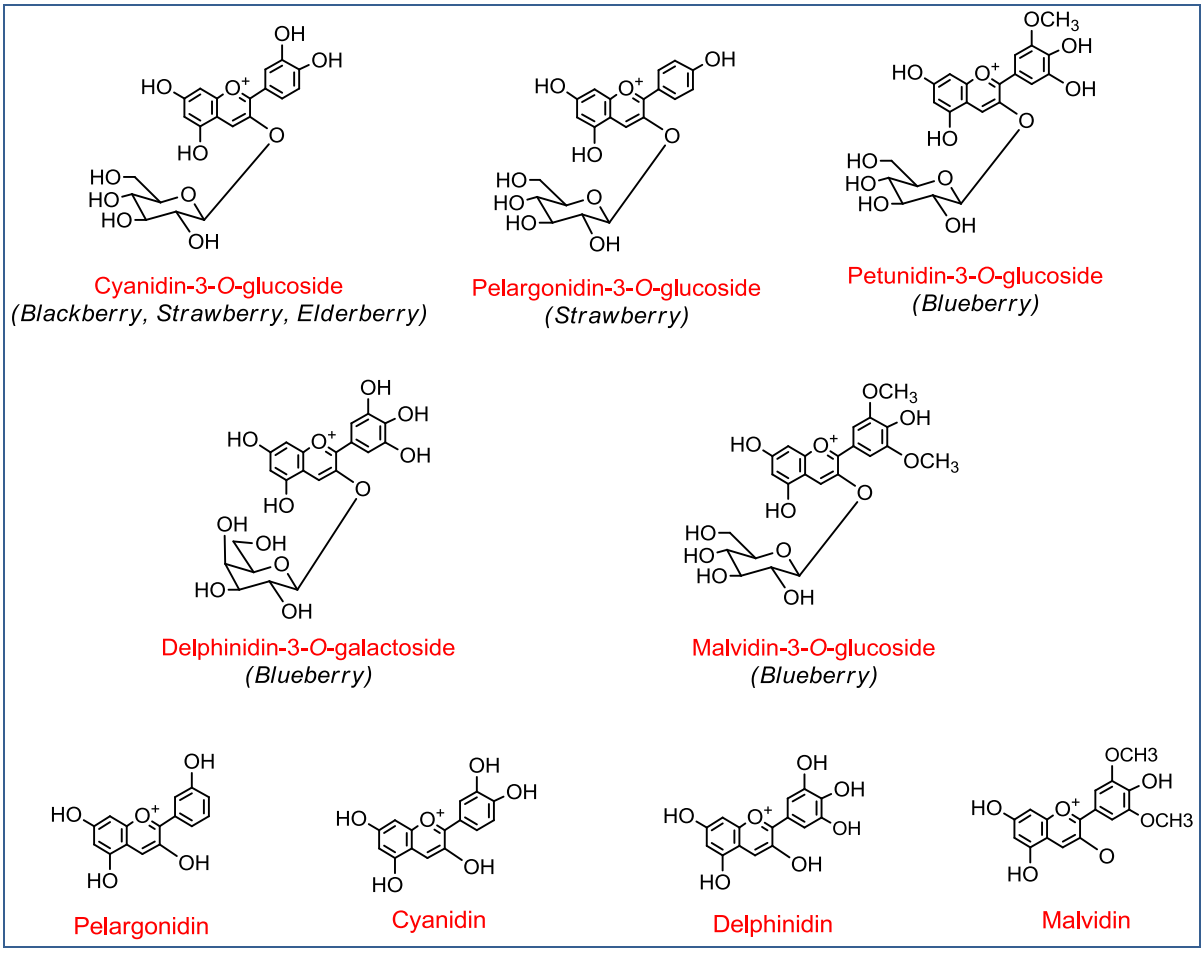


Figure 1. 9 Structures of aglycones and glycoside anthocyanins found in berries.

In plants anthocyanins play an important role in defense against environmental stress factors, such as UV light, in attracting insect pollinators to flowers and animals to fruits which help dispense the seed. Plant colouring properties from anthocyanins are important as natural colorants of food and beverages. Anthocyanins have been reported to possess a variety of health benefits, such as anticancer, protective against cardiovascular disease, and anti-neurodegeneratives, and anti-hyperglycemic effect (Matsui et al., 2002; Faria et al., 2010; Wallace, 2011).

1.3.1.6 Isoflavones

Isoflavones are produced almost exclusively by members of the leguminosae (specifically in the subfamily Papilionoideae) with the best-known source being soybean (Manach et al., 2004). They also exist, albeit in much lower concentrations, in other botanical families such as Compositae, Iridaceae, Myristicaceae and Rosaceae (Dweck, 2006). Isoflavones can acts as steroidal mimics by filling the stereochemical space that is occupied by estrogenic compounds. In plants, isoflavones and their derivatives act as phytoalexins against pathogenic fungi and microbe infection, while in soybean they are involved in nitrogen fixation.

A study has demonstrated that isoflavones have potent antioxidant properties comparable to that of vitamin E. Some of isoflavones possess anti-cancer activity, including certain types of breast cancer and prostate cancer. The isoflavones daidzein and genistein (Figure 1.10) are found mainly in soy, soy products, legumes, and red clover (Peterson and Dwyer, 1998). They are also detected in medicinal herbs of Leguminosae, such as *Astragalus mongholicus* (Cai et al., 2004). Daidzein is also classified as a phytoestrogen as it has estrogen-like activity. Daidzein glycosides, including daidzein-7-*O*-(6′-*O*-malonyl)glucoside, are the second most abundant isoflavones in soybeans after genistein glycosides. Daidzein and genistein glycosides are normally found in nonfermented soy foods (tofu) while the aglycone predominates in fermented soy foods such as miso and tempeh (Zubik and Meydani, 2003).

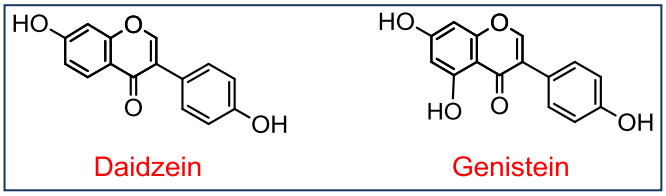


Figure 1. 10 Structures of common isoflavone aglycones.

1.3.2 Non-flavonoids

The C_6-C_1 phenolic acids are important dietary non-flavonoids. The non-flavonoid, gallic acid (3,4,5-trihydroxybenzoic acid) plays a major role in stabilizing and enhancing the colour of red wine, and also adds the flavour of the wine (Moreno-Arribas et al., 2009).

1.3.2.1 Hydroxycinnamic acids

Hydroxycinnamic acids are non-flavonoid compounds widely distributed in plants and are found in foodstuffs and beverages such as coffee, wine and fruit juices (Clifford, 2000). They have a basic C_6-C_3 structure, with one or more hydroxyl groups attached to the phenyl moiety, some of which may be methylated. *p*-Coumaric, caffeic, ferulic, and sinapic acids are the main representatives of dietary hydroxycinnamates (Figure 1.11) (Clifford, 2000; Manach et al., 2004). They occur both as free acids and esterified either with quinic acid, glucose, or carboxylic acids such as shikimic or tartaric acid (Herrmann and Nagel, 1989). Esterification of caffeic acid, ferulic and *p*-coumaric acid with quinic acid produces a family of compounds known as chlorogenic acids (Fig. 1.11). They are abundant in coffee beverage, artichoke, cherries, blueberries, aubergine and apples (Herrmann and Nagel, 1989; Clifford, 2000; Manach et al., 2004).

In coffee, which is a major source of chlorogenic acids, the main sub-classes are caffeoylquinic acids, dicaffeoylquinic acid, and feruloyquinic acids (Monteiro et al., 2007). It is estimated that 5-*O*-caffeoylquinic acid accounts for 35% of total caffeoylquinic acids in roasted coffee, which also contains dicaffeoylquinic acid isomers (Farah and Donangelo, 2006). Caftaric acid (2-*O*-caffeoyltartaric acid) is a caffeic acid-tartaric acid conjugate found in grapes, spinach, lettuce and wine (Clifford, 2000). Glycosylated hydroxycinnamic acids are found in kale, spinach, and tomatoes; malic acid esters occur in radishes and chicory, while rosmarinic acid esters, and are found in rosemary, oregano, thyme, and sage (Herrmann and Nagel, 1989).

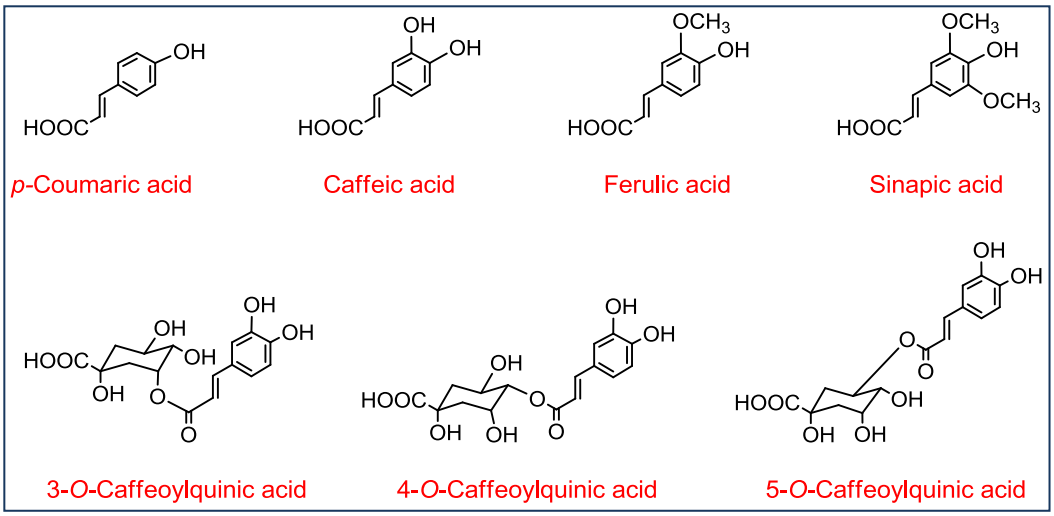


Figure 1. 11 Structure of common hydroxycinnamics and quinic acid conjugates.

Dietary hydroxycinnamates normally occur in the *trans* form although transition to *cis* form is readily induced by UV irradiation (Clifford et al., 2008). The isomers of these hydroxycinnamic acids depend on the position to the ester linkage. 1-, 3-, 4-, or 5-*O*-Caffeoylquinic acid isomers have been identified. These compounds are major components in the human diet with coffee being the principal source (Clifford, 1999). They possess strong antioxidant activity (Williamson and Clifford, 2010) and there is considerable interest in their ability to inhibit various degenerative diseases as they have been reported to exert inhibitory effects against cardiovascular disease, diabetes mellitus and Alzheimer's disease, as well as having antibacterial and anti-inflammatory properties (Lindsay et al., 2002; Federico et al., 2004; Larrosa et al., 2009; Gordon and Wishart, 2010).

1.3.2.2 Lignans

Lignans are one of the major classes of phytoestrogens, formed by two phenylpropane units (Fresco et al., 2006). Linseed is the main source of lignans, in the form of the lignanolides, secoisolariciresinol and matairesinol. Much lower levels of lignans are found in cereal, grains, fruit and certain vegetables (Milder et al., 2005). Plant lignans, such secoiolariciresinol diglucoside, which is found in flaxseed (*Linum usitatissimum*), is a precursor of the mammalian lignans, enterodiol and enterolactone, metabolites produced by the colonic microflora (Figure 1.12). These metabolites have been reported to exert phytoestrogenic and antioxidant effects both in vivo and in vitro (Kitts et al., 1999; Prasad, 2000). Cyclolignanolide, first identified in *Podophyllum peltatum,* is also

found in the Chinese medical plant *Sinopodophyllum emodi* which is well known as the source of the tumor therapy drug podophyllotoxin (Huang et al., 2009).

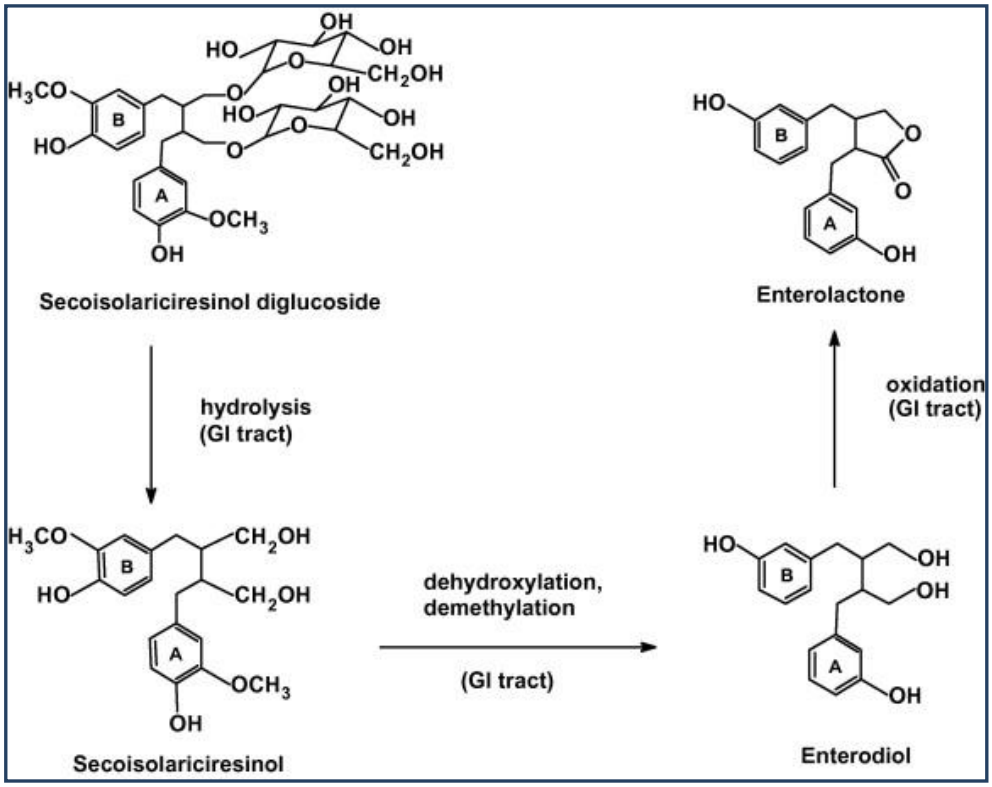


Figure 1. 12 Colonic microflora metabolism of plant lignin secoisolariciresinol diglucoside into the aglycones enterodiol and enterolactone (Hu et al., 2007).

1.3.2.3 Stilbenes

Stilbenes are small group of phenylpropanoids which have C_6-C_2-C_6 structure. They can be either constitutive components, or induced in response to environmental stresses and are therefore known as phytoalexins, a class of antimicrobial compounds produced by plants as a defense against pathogens. They are very minor constituents in the human diet. Plants naturally contain stilbenes in both free and glycosylated forms (Figure 1.13). The most well known and well characterized stilbene is

resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) which is synthesized by condensation of 4-coumaryl CoA with three units of malonyl-CoA via shikimate pathway (Fig. 1.1). Resveratrol exists as *trans* and *cis* isomers, is commonly found in grapes, mulberries, and peanuts. It exerts a wide variety of pharmacological activities with anticancer and cardiovascular protective effects, prevention of ischemic injuries and extension the life expectancy of various organisms (Baur and Sinclair, 2006). Resveratrol was first isolated in 1963 from *Polygonum cuspidatam*, known as Ko-jo-kon in Japan, which is used by Asian traditional practitioners for treating suppurative dermatitis, gonorrhoea, favus and hyperlipemia (Saiko et al., 2008). In addition to resveratrol, other stilbene structures include piceatannol, pinosylvin, rhapontigenin and ptestilbene (Roupe et al., 2006).

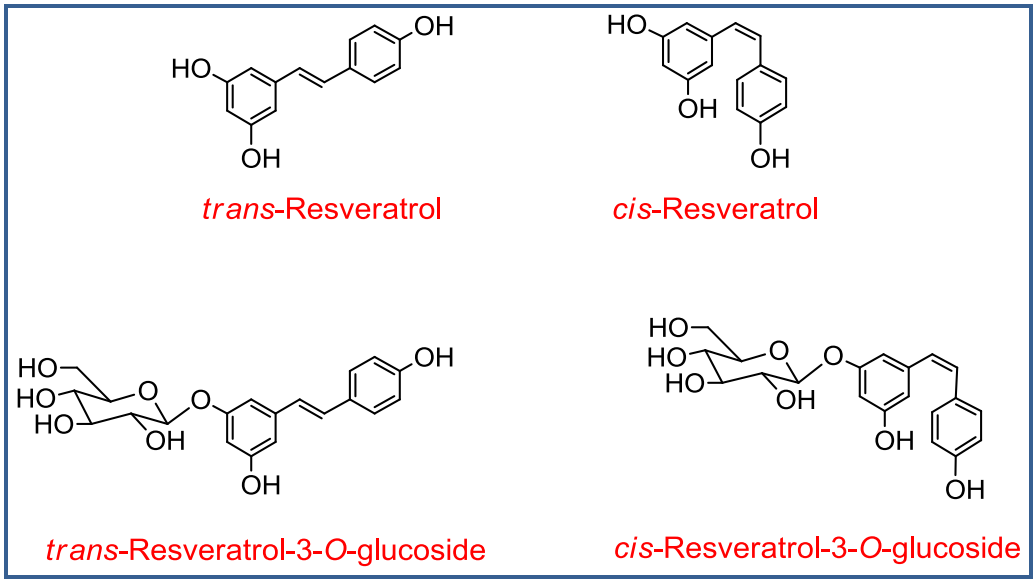


Figure 1. 13 Structures of free and glycosylated resveratrol.

1.3.2.4 Tannins

Tannins are water soluble, high molecular weight (ranging from 500-4000 Da) polyphenolic compounds. They are the second most abundant group of natural phenolics after lignin and can be categorized as hydrolysable tannins (gallo and ellagitannins) (Aron and Kennedy, 2008) and condensed tannins (proanthocyanidins)(Cai et al., 2004)(see Figure 1.14). Ellagitannins differ from proanthocyanidins in their chemical structures and their occurrence. Hydrolysable tannins are derivatives of gallic acid and ellagic acid which are the main precursors of the more complex gallotannins and ellagitannins (Gross, 1992). Proanthocyanidins are oligomers and polymers of flavan-3-ols and are more and wide spread in the plant kingdom than hydrolysable tannins. Proanthocyanidins are major components in grapes, especially seeds, and cocoa while ellagitannins occur in fruits like strawberries, raspberries, blackberries, apples, and pomegranate (Clifford and Scalbert, 2000). Ellagitannins are esters of hexahydroxydiphenic acid and a polyol (glucose of quinic acid), and are converted to ellagic acid by acid or base hydrolysis. Tannin constituents with anticancer properties have been detected in many traditional Chinese medicinal plants (Cai et al., 2004).

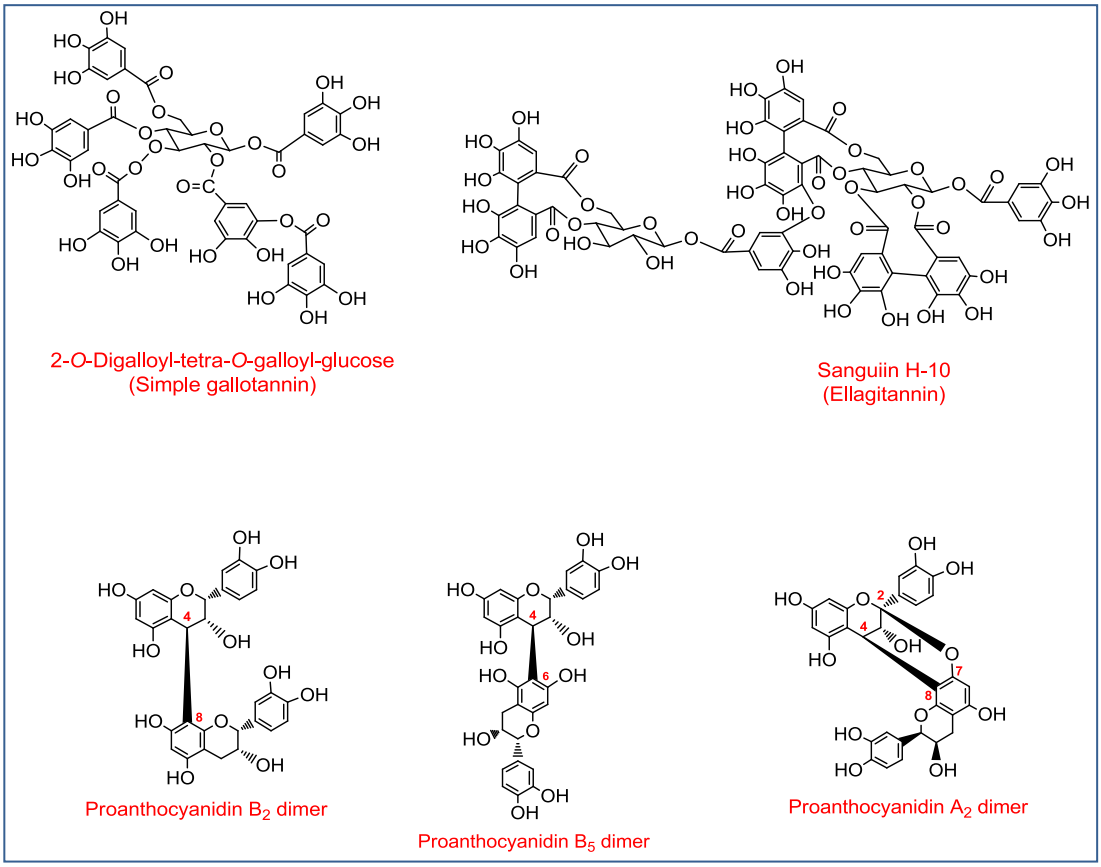
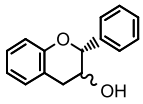
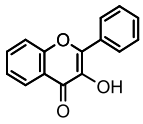
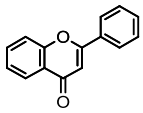
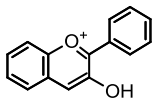
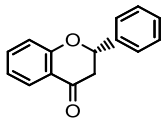
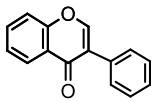
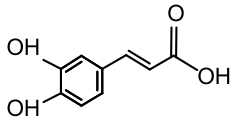
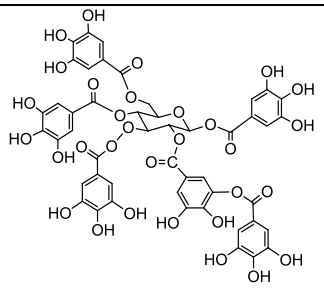


Figure 1. 14 Structures of hydrolysable and condensed tannins.

1.4 Dietary (poly)phenols

Flavonoids and cinnamic acids are two important groups of dietary phenolics. The most reported sources of these dietary compounds are beverages such as tea (flavan-3-ols) and coffee (cinnamic acids) along with red wine and cocoa-based products (see Table 1.2). Tea, which is consumed in amounts only exceeded by water, is prepared from young leaves of *Camellia* spp. Annually ~3.2 million metric tons of dried leaves were processed into black tea (78%), green tea (20%) and the rest of oolong tea (Crozier et al., 2009). One litre of strong black tea can provide ~0.5 g or more of phenolics, with flavanols and the derived flavan-3-ols theaflavins and thearubigins being the main constituents (Balentine et al., 1997).

Table 1.2 Typical sources of dietary phenolic compounds

Phenolic compounds	Basic molecular structure	Typical sources
Flavan-3-ols Catechin Epicatechin		Tea, red wine, and chocolate (Del Rio et al., 2004)
Flavanols Quercetin Myricetin		Onion, broccoli, beans, apple and tea (Hertog et al., 1993)
Flavones Apigenin Luteolin		Vegetables (red pepper and celery) (Hertog et al., 1992)
Anthocyanidins Pelargonidin Cyanidin Delphinidin Peonidin Malvidin		Red fruits (plum and grape) and berries (strawberry, raspberry, blueberry, blackberry, red and black currant) (Borges et al., 2010)
Flavanones Naringin Hesperetin		Citrus fruits (orange, lemon, limes and tangerines) (Peterson et al., 2006)
Isoflavones Daidzein Genistein Glycitein		Soyabeans and soy products (Peterson and Dwyer, 1998)
Hydroxycinnamates Chlorogenic acids Ferulic acid		Coffee, blueberry, apple and cider (Clifford, 1999)
Tannins Ellagitannins Proanthocyanidins		Pomegranate, raspberries, walnut and wine (Crozier et al., 2006) .

Quercetin conjugates, the principal dietary flavonol, are particularly abundant in onions (0.3 mg/g fresh weight) and tea (10-25 mg/L) which are the main sources of flavonols in the Dutch diet (Hertog et al., 1993). Quercetin glycosides are also common in a wide variety of plants products (fruits, vegetables, cereals, legumes). It was reported that in the USA human dietary intake of flavones and flavonols is estimated to be ~20 to 22 mg/day (Sampson et al., 2002). Unlike quercetin, the isoflavones, daidzein and genistein occur almost exclusively in soya and soya products. The isoflavone content of soya and its derived products varies as a reflection of the geographic zone and growing conditions as well as processing methods (Manach et al., 2004). Soybeans contain between 580-3800 mg of isoflavones/kg fresh weight, and soymilk ~30 to 175 mg/L (Reinli and Block, 1996). It has been estimated that ~10% of Asian population consumes 25 g of soy protein per day which corresponds to an isoflavone intake of 100 mg isoflavones (Messina et al., 2006). In Japan, the average dietary intake of isoflavones is 30-40 mg/day, which is higher than the amounts consumed in Western countries (Wakai et al., 1999).

Anthocyanins are major dietary components for those who eat berries and drink red wine on the regular basis. One hundred gram of berries might contain up to 500 mg of anthocyanins, while serving 200 g of aubergine or black grapes can provide up to 1500 mg of athocyanins (Manach et al., 2005). In Finland, the mean dietary intake of anthocyanins has been estimated to be 82 mg/day, with berries, red wine and juices, and the colouring agent E163 as the main sources (Manach et al., 2005).

Citrus fruits are main dietary source of flavanones and their concentrations vary depending on the part of the fruit that is consumed. Citrus peels (albedo and flavedo), are richest in flavanones compared to the juices (Tomás-Barberán and Clifford, 2000). It has been estimated that the mean flavanone content in orange juice (hesperidin and narirutin) range between 14-77 mg/100 mL, whereas with grapefruit juices containing naringin intake was estimated to be 17- 76 mg/100 mL (Ross et al., 2000; Tomás-Barberán and Clifford, 2000). The daily flavanone intake in French and German adults has been estimated to vary from 2.7 to 78 mg of aglycone equivalents (Erdman et al., 2007; Pérez-Jiménez et al., 2011).

Flavones, mainly glycosides of luteolin and apigenin, have a limited distribution in fruits and vegetables, and as a consequence, their daily intake in different countries was estimated <1-2 mg/d, which is low compared to other flavonoids (Beecher, 2003).

As noted previously, coffee is a very rich source of chlorogenic acids and as such ~70% of the world's intake is through the consumption of coffee (Grigg, 2002). 5-*O*-Caffeoylquinic acid is the main chlorogenic acid found in green coffee beans (Clifford, 1999). It was reported that one cup of instant coffee (200 mL) contains 50-150 mg of chlorogenic acid (Clifford, 1999). It has been reported that coffee contributes 92% of caffeic acid and 82% of the chlorogenic acid intake in Germany (Radtke et al., 1998). Hydroxycinnamic acids intake varies widely. Coffee drinkers can consume up to 500-800 mg/d while for people who did not drink coffee the amount is >25 mg/d (Clifford, 2000). A recent study has shown that the caffeoylquinic acid content of a single serving of espresso coffee from high street coffee shops in Glasgow ranged from 24 to 322 mg (Crozier et

al., 2012b). This implies that for regular coffee drinkers may have a caffeoylquinic acid intake well in excess of 1 g/day.

It was reported that two thirds of the (poly)phenols consumed in the diets are flavonoids. The daily flavonoids intake in the USA population based on 5 classes of flavonoids excluding isoflavones was reported as 1 g/d (Kühnau, 1976), whereas for a Dutch population the total flavonoids intake might be at least 75 mg/d (Arts et al., 2001). In the Spanish adult population, the total flavonoid intake was reported 313 mg/day, with proanthocyanidins as the main contributor and isoflavones as the lowest (Zamora-Ros et al., 2010).This indicates that the total (poly)phenol intake depends to a great degree on dietary habits and preferences.

1.5 Beneficial health effects of dietary (poly)phenols

Research on dietary flavonoids was awakened with the discovery of French Paradox (Renaud and de Lorgeril, 1992). The French Paradox is defined as a low incidence of coronary heart disease associated with a high fat diet and red wine consumption. It has been well established that (poly)phenol-rich foods and beverages may increase plasma antioxidant activity. Antioxidants have been associated with reduced levels of oxidative stress to lymphocytic DNA. Antioxidants will protect cell against oxidative damage and prevent the risk of various degenerative diseases. Numerous studies have shown that intake of (poly)phenols can prevent or reduce the deleterious effects of oxygen-derived free radicals. Elevated levels of reactive oxygen species can lead to clinical disorder such as arteriosclerosis, ischemia-reperfusion injury, cancer, stroke and neurodegenerative disorder (Gotz et al., 1994; Halliwell, 2001)

1.5.1 Cardiovascular disease

Epidemiological studies have revealed that high consumption of fruits and vegetables is associated with a lowered risk of cardiovascular disease (CVD) (Dauchet et al., 2006). Such a diet contains high levels of (poly)phenolic compounds that inhibit the oxidation of low density lipoprotein (LDL) which initiates is associated with the development of atherosclerosis (Halliwell, 1989). (Poly)phenols act as antioxidants, have anti-platelet and anti-inflammatory effects, increase HDL and improve endothelial function.

Consumption of flavanone-rich citrus fruit has been reported to have a strong inverse relationship with CVD incidence and reduced mortality risk from coronary heart disease (Mink et al., 2007; Yamada et al., 2011). Quercetin glycosides from onion have an inverse association with mortality from coronary heart disease through inhibiting the expression of metalloproteinase 1(MMP1), and atherosclerotic plaques (Song et al., 2001). Diets rich in (poly)phenols have been reported to help reverse hyperlipidemia and reduced the oxidation of LDL (Yang et al., 2001).

1.5.2 Anti-cancer properties

Hollman et al.(1996) have reported that consumption of fruits, vegetables, spices, cereals and beverages, rich in flavonoids (in reality flavonols and flavones), helps prevent many degenerative diseases including cancer. Epidemiological studies on (poly)phenol intake by different populations have established an inverse association between the high intake and the risk of cancer (Le Marchand, 2002).

The mechanism by which flavonoids exert their effects is through their interaction with phase I metabolising enzymes (such as cytochrome P_{450}) and phase II metabolising enzymes (GST, quinone reductase, and UDP-GT). These enzymes have a protective role against cellular damage and are involved in detoxification and elimination of carcinogens from the body (Maheep et al., 2011). Flavonoids from citrus fruits and tea have been widely studied in cancer prevention and induction of apoptosis. Flavonols and flavones have been shown to inhibit the action of a cytochrome P_{450} that activates several human carcinogens (Lautraite et al., 2002) while catechins increase the activity of several detoxifying and antioxidant enzymes (Khan et al., 1992). Tea flavonols and flavan-3-ols are reported to have protective effects against stomach cancer (Inoue et al., 1998). Isoflavones have protective effect against mammary tumours and inhibit tumorigenesis in several animal models for prostate cancer (Messina et al., 1994). High intake of isoflavone-rich soy was associated with a reduced risk of premenopausal breast cancer in case-control studies conducted in Singapore and Japan (Lee et al., 1991; Hirose et al., 1995). Although inverse correlation between high (poly)phenol intake with low cancer risk has been reported, a number of studies have found no association (Zheng et al., 1996; Chow et al., 1999; Julie et al., 2005). The discrepancies observed could be due to other constituents in the diet, lifestyle factors such as smoking, and early life exposure with specific diets (Le Marchand, 2002).

1.5.3 Anti-diabetic effect

Tea, contains high levels of flavonols and flavan-3-ols. Based on the epidemiological studies it was reported that green tea has an effect on glucose tolerance. When green tea was fed to rats, they acquired lower levels of glucose, insulin, triglycerides and free fatty acids than control rats (Wu et al., 2004a). (-)-Epicatechin-3-*O*-gallate was shown to inhibit glucose uptake in Caco-2 cells and the sodium-dependent glucose transporter (SGLT), and is the principal compound that enhances insulin activity (Anderson and Polansky, 2002). The high intake of flavan-3-ols (tea and cocoa) and isoflavones (soy and soy products) have been reported to improve insulin sensitivity and reduce type II-diabetes in medicated postmenopausal women (Curtis et al., 2012). Red fruits such as berries (strawberries and raspberries) contain anthocyanins that can inhibit α-glucosidase activity, and ellagitannins that inhibit α-amylase activity. The inhibition of these digestive enzymes has potential synergism in reducing the blood glucose levels for controlling type II diabetes and starch degradation after ingestion of starch-rich meals (McDougall and Stewart, 2005).

1.5.4 Neuroprotective effects

Neurodegenerative diseases developed through oxidative stress and damage to brain macromolecules and defined as hereditary and sporadic conditions which lead into dysfunction of nervous system. Alzheimer's disease is very common, affecting up to 18 million people worldwide (Pandey and Rizvi, 2009). It was reported that people who follow a

Mediterranean diet have lower mortality from Alzheimer disease (Scarmeas et al., 2007).The Mediterranean diet is characterized with the high intake of vegetables, legumes, fruits, cereals and unsaturated fatty acids with low intake of meat and poultry (Trichopoulou et al., 2003). Epigallocatechin-3-*O*-gallate exert protective effect against the neurotoxin MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), an inducer of Parkinson-like disease symptoms (Rossi et al., 2008).

1.5.5 Other diseases

Consumption of dietary (poly)phenols has also been linked with several other health benefits. Intake of apple and soy has a reported positive association with lung function in asthma patients (Pandey and Rizvi, 2009). Diets containing the soy isoflavones genistein and daizdein have a significant bone-sparing effect (Setchell and Lydeking-Olsen, 2003). Flavonols and flavan-3-ols found in tea have protective effects against the adverse reaction of skin following UV exposure in animal models (Kim et al., 2005). Theaflavins present in black tea has reported to have anti HIV-I activity (Pandey and Rizvi, 2009).

1.6 Bioavailability of dietary (poly)phenols

Bioaccessibility is defined as the amount of a food component present in the gut, released of its constituent food matrix and passing through the intestinal barrier. Only (poly)phenols that are accessible in the gut are potentially bioavailable. The amount of the (poly)phenols consumed in the diet and its bioavailability is important to evaluate the correlation of (poly)phenols

intake with human health. Information on bioavailability of dietary (poly)phenols has been obtained by measuring their concentrations in plasma and urine after the intake of a specific compound or a food with known content of the compound interest. Parameters which are used to measure their bioavailability are; area under the curve (AUC), which provides a gauge of the metabolite concentration in blood versus time; $T_{max,}$ time at which the concentration of the compound in blood has reached a maximum, and $C_{max,}$ referring to the maximum plasma concentration (Piskula et al., 2012).

Dietary (poly)phenols have received much attention due to their potential beneficial effects on health, which are based largely on epidemiological evidence. It has been estimated that the (poly)phenols dietary intake can range between 0.15-1.0 g/day of total phenolics (Scalbert and Williamson, 2000). However, most of these compounds have limited bioavailability with only low amounts being transiently detected in plasma after ingestion.

Dietary (poly)phenols, such as flavonoids and hydroxycinnamates, with the notable exception of flavan-3-ols, occur principally in foods as glycoside conjugates that are hydrophilic and poorly absorbed. The absorption of some of these compounds into the circulatory system occurs in the small intestine with the hydrolysis of glycoside conjugates and release of the aglycone through the action of lactase phloridzin hydrolase (LPH). LPH has a high affinity for flavonoid-*O*-β-glucosides, and this results in the released of aglycones which are more lipophilic and can permeate the epithelial cell by passive diffusion (Day et al., 2000). Glycosides which have been transported into the epithelial cell possibly by sodium-dependent glucose transport (SGLT) can be hydrolysed by cytosolic β-glucosidase (CBG) (al-Swayeh et al., 2000).

These two routes hydrolyses of glucoside conjugates are known as "LPH/diffusion" and "transport /CBG". Released aglycones will undergo sulfation, glucuronidation and/or methylation by sulfotransferases (SULT), uridine-5′-phosphate glucuronosyltransferases (UGT) and catechol-O-methyltransferases (COMT). This concept of (poly)phenols absorption is simplified as illustrated in Figure 1.15.

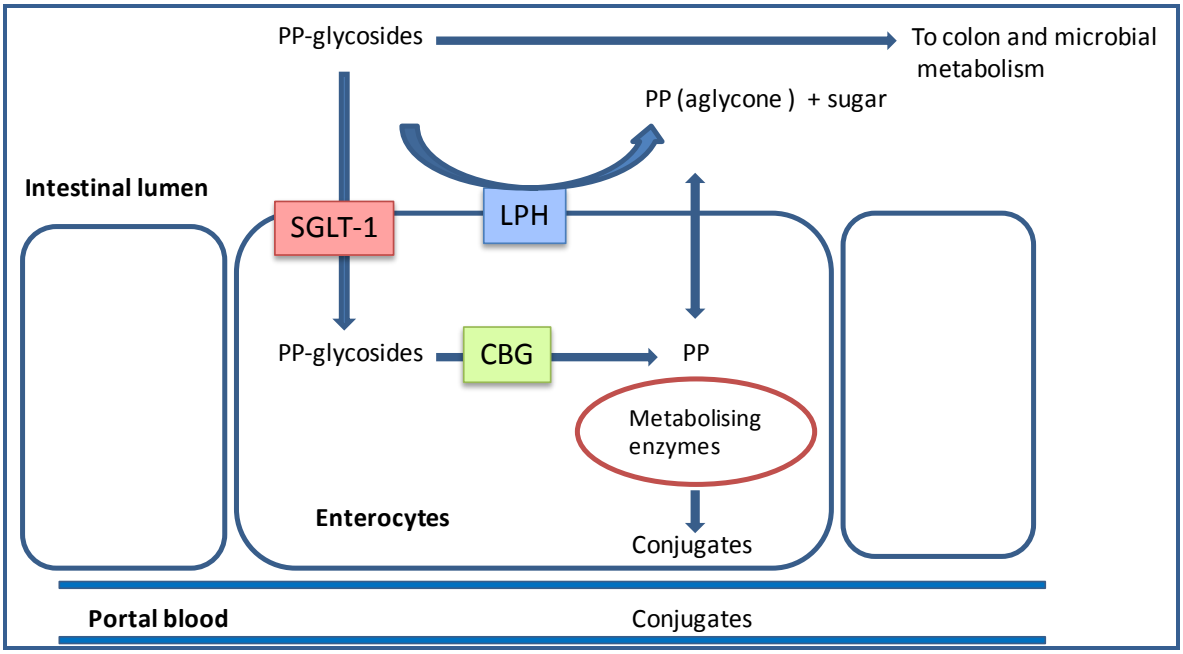


Figure 1. 15 Schematic diagram metabolism of dietary (poly)phenols in the lumen and enterocytes of the digestive tract.
(Poly)phenols glycosides (PP-glycosides) reach small intestine with some being hydrolysed by lactase hydrolase phloridzin (LPH), or transported into the enterocytes by glucose transporters such as SGLT-1 and hydrolysed by cytosolic β-glucosidase (CBG) to release the aglycone (PP) which will be further metabolised by metabolising enzymes into conjugates. PP-glycosides that are not absorbed will reach the colon.

Some of these metabolites formed in the enterocyte efflux back into the small intestine with the help of adenosine triphosphate (ATP)-binding cassette (ABC) family transporters, multidrug resistance protein (MRP), and P-glycoprotein (P-gp) (Crozier et al., 2009). The metabolites are also absorbed into the portal vein the of blood stream, rapidly reach the liver for further phase II metabolism, and enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion (Donovan et al., 2006a). Some of (poly)phenol conjugates, such as rutinosides, have sugar moieties resistant to the action of LPH/CBG are not absorbed in the small intestine and so pass into the large intestine. In the large intestine, the colonic microflora will hydrolyse the conjugating sugars to release the aglycones which undergo ring fission yielding catabolites, including phenolic acids and hydroxycinnamates. These can be absorbed from the colon into the circulatory system and subsequently undergo phase II metabolism in the liver, and possibly the kidneys, before being eliminated in urine (Figure 1.16).

Recent findings on the bioavailability of dietary (poly)phenolic compounds have pointed out that (poly)phenol glucuronide, methyl and sulphate conjugates are treated as xenobiotics, as they are rapidly turned over and excreted via the kidneys (Tomás-Barbéran et al., 2012). As a result, AUC values of plasma pharmacokinetics do not necessarily provide accurate quantitative data on absorption. More reliable measurement of absorption can be obtained from urinary excretion, however, this is restricted by the inability to include metabolites sequestration in body tissues.

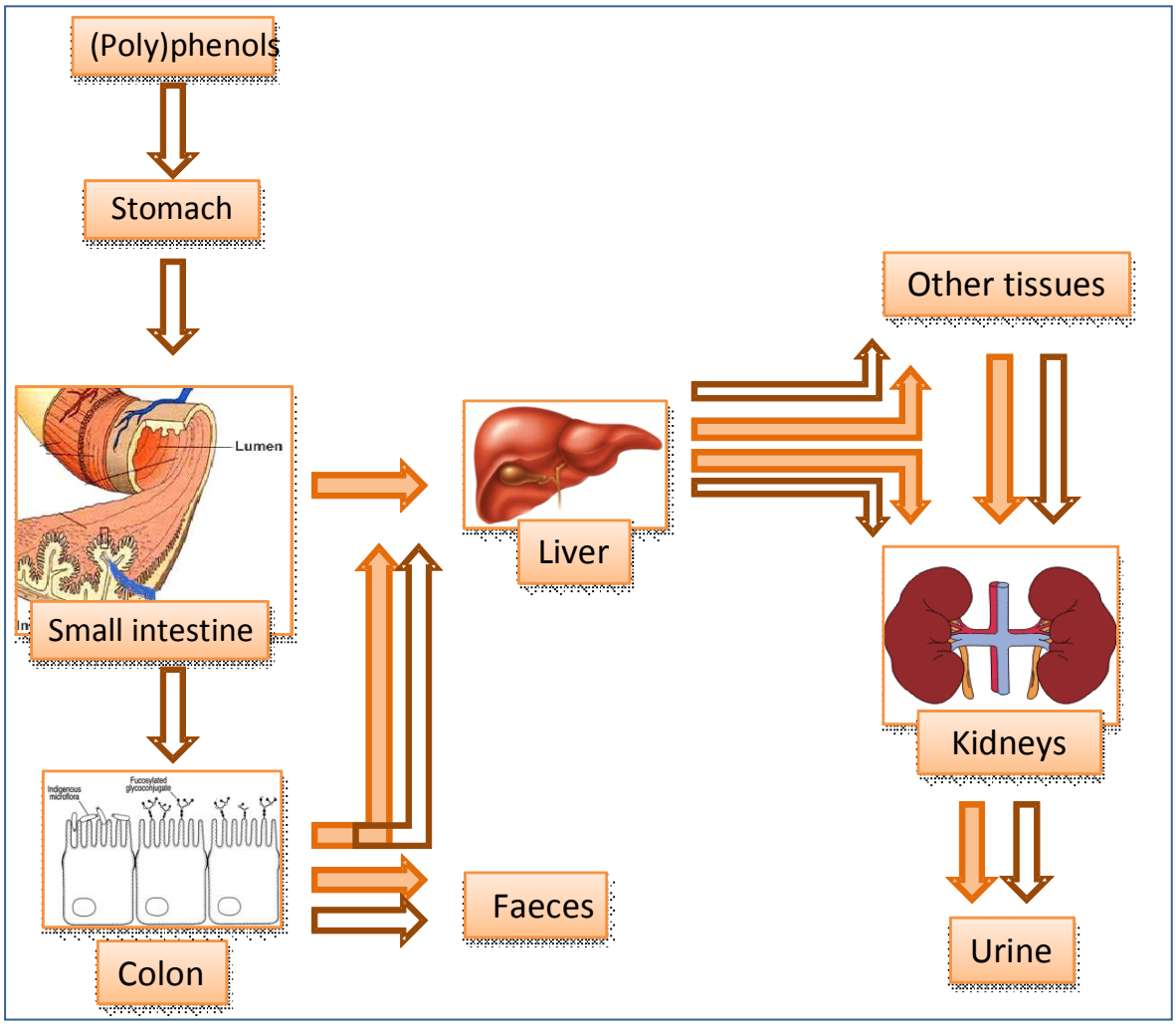


Figure 1. 16 Simplified routes of metabolism of dietary (poly)phenols. White arrows depict the flow of intact (poly)phenol compounds (i.e. flavonoid glucosides). Orange arrows depict the flow of colonic catabolites after the ring fission (i.e. phenolic acids and aglycones). Some metabolites are excreted into the faeces, while others will be reabsorbed and subjected to further metabolism in the liver.

1.6.1 Flavan-3-ols

The bioavailability of green tea in humans was investigated by Stalmach et al. (2009). Plasma and urine were collected 0-24 h after drinking 500 mL of green tea containing 648 μmol of flavan-3-ols. In plasma, 12 metabolites were detected in the form of *O*-methylated, sulphated and glucuronide conjugates of (epi)catechin and (epi)gallocatechin together with unmetabolised (-)-epigallocatechin-3-*O*-gallate and (-)-epicatechin-3-*O*-gallate with the T_{max} values ranging from 1.6 to 2.3 h. All metabolites are present in only trace amounts in plasma after 8 h and had cleared the circulatory system 24 h after intake. These T_{max} values and the pharmacokinetic profiles are indicative of the absorption in the small intestine.

No parent flavan-3-ols were found in urine, and the metabolite profile was similar to that detected in plasma except for two additional (epi)catechin-*O*-sulphates. Total urinary excretion was 8.1% of 648 μmol of flavan-3-ol intake. High levels of excretion relative to intake was observed for (epi)gallocatechin (11.4%) and (epi)catechin (24.5%) metabolites. In iloestomists, after they drank green tea (634 μmol of flavan-3-ols), 69% of intake was recovered in 0-24 h ileal fluid (Stalmach et al., 2010a), whereas in volunteers with a functioning colon, the majority of these ingested flavan-3-*O*-ols will subjected to further metabolism by the colonic microflora.

Cocoa is another important source of flavan-3-ols that has been well studied in humans. Humans consumed 40 g dark chocolate consisting of 282 μmol of (–)-epicatechin reached C_{max} of 355 nM with a T_{max} of 2 h, characteristic of absorption in the upper GI tract (Richelle et al., 1999). Further research by Baba et al. (2000) in which humans ingested 760 μmol of (–)-epicatechin and 210 μmol (+)-catechin, observed metabolites in plasma with a C_{max} of 4.8 μM and T_{max} of 2 h, with total urinary excretion equivalent to 29.9% of the ingested flavan-3-ols. This comparatively high bioavailability was also observed in another study in which human consume a cocoa beverage containing 22 μmol of catechin and 23 μmol of epicatechin. *O*-Methyl-(epi)catechin-*O*-sulphate and an (epi)catechin-*O*-sulphate were detected in the plasma, both with a C_{max} below 100 nM and a T_{max} of less than 1.5 h (Mullen et al., 2009) and a combined excretion equivalent to ~20% of intake. These high levels of excretion are similar with those obtained from green tea studies confirming that (-)-epicatechin and (+)-catechin are highly bioavailable in the human body.

Most flavan-3-ols are present in nature in different enantiomeric forms, such as (-)-epicatechin), (+)-epicatechin), (+)- catechin, and (-)-catechin (Figure 1.17). Transformation of enantiomers can take place in the gastrointestinal tract and/or during food processing (Hurst et al., 2011).

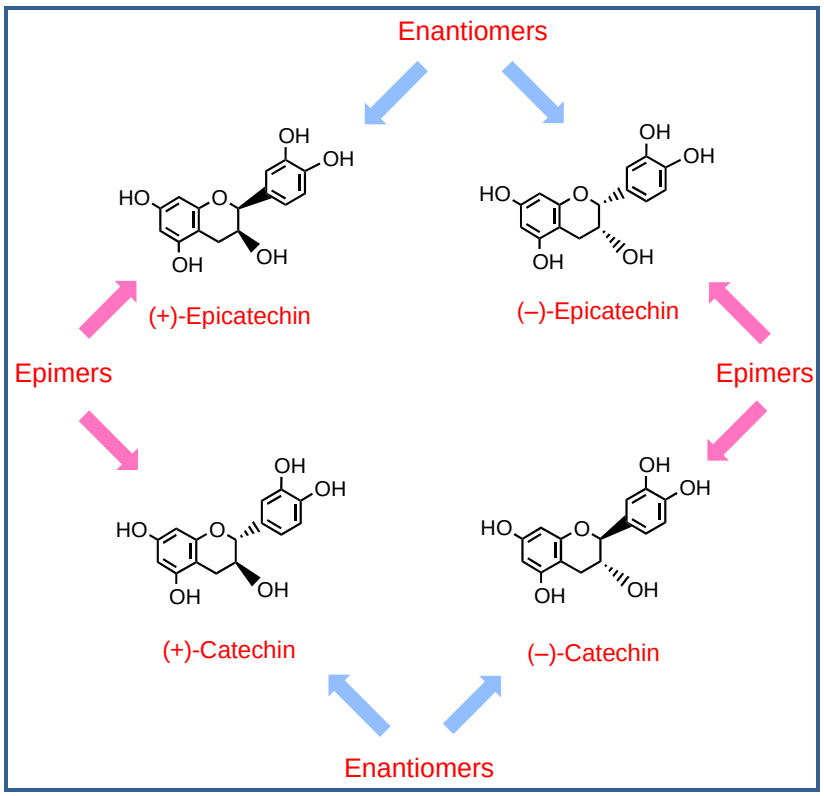


Figure 1. 17 Chemical structure of flavonol stereoisomers (-)-epicatechin, (+)-epicatechin, (-)-catechin, and (+)-catechin.

These pairs of enantiomers are not resolved by reverse phase chromatography, hence they are easily overlooked. They are called enantiomers which are one of the two steroisomers that are a mirror image of each other but non-superposable or not identical, whereas diastereoisomers are stereoisomers that are not enantiomers. Diastereoisomism are stereoisomers which have different configurations at a stereiocenter and are not mirror images of each other. Stereoisomers are absorbed differently, as reported in rats were (-)-catechin is absorbed more readily than (+)-catechin (Donovan et al., 2006b). A more detailed study on the bioavailability of different flavan-3-ol enantiomers was carried out in a cocoa feeding study by (Ottaviani et al., 2011). Based on plasma concentrations and urinary excretion, they reported that the bioavailability

of the stereoisomers was ranked as (-)-epicatechin > (-)-catechin > (+)-epicatechin = (+)-catechin. There were also differences in the flavonol metabolite profile obtained with catechin and epicatechin epimers. Both 3′- and 4′-*O*-methylated epicatechin derivatives are only present in urine and plasma after the intake of epicatechin enantiomers while the levels of nonmethylated metabolites of (-)-epicatechin were higher than those of its enantiomer (+)-epicatechin. In the study, the individual concentration of stereoisomers in the cocoa drink are not fully determined, which raises to the possibilities that the differences observed might also due to the varying stereochemical ratios presence in the cocoa drink (Crozier et al., 2012a).

1.6.2 Flavonols

Quercetin, particularly in onions, is the most studied flavonol. Among the early human bioavailability studies is an investigation by Aziz et al. (1998) in which volunteers ingested 300 g of lightly fried onions which contain conjugates of quercetin and isorhamnetin. Plasma peak concentration (C_{max}) was 1.3 h and 1.8 h for quercetin-4′-*O*-glucoside and isorhamnetin-4′-*O*-glucoside respectively and total urine recovery 24 h after intake was 17.4% for isorhamnetin-4′-glucoside and 0.2% as in quercetin-4′-glucoside. Later it became evident that these glucosides were misidentified and was probably flavonol glucuronides, standards of which were not available at the time. Subsequently, after pioneering work by Day and Williamson (2001), volunteers were fed lightly fried onions containing 275 μmol of flavonol glucosides principally quercetin-4′-glucoside and quercetin-3-4′-diglucoside. With the aid of HPLC-MS and reference compounds, a number of metabolites were identified in plasma including

quercetin-3′-*O*-sulphate, quercetin-3-*O*-glucuronide, isorhamnetin-3-*O*-glucuronide, quercetin-*O*-diglucuronide and quercetin-*O*-glucuronide-*O*-sulphate (Mullen et al., 2006). Rapid elimination of the metabolites was observed with C_{max} of some metabolites of <1 h being observed. Total metabolite excretion in urine 0-24 h after intake was 4.7% of the ingested parent flavonols.

In a human study with tomato juice containing 176 µmol of quercetin-3-*O*-rutinoside, two metabolites were detected in the plasma, quercetin-3-*O*-glucuronide and isorhamnetin-3-*O*-glucuronide (Jaganath et al., 2006). The C_{max} values obtained were much lower and T_{max} times were longer than those observed in the onion studies. The T_{max} values of ~4 h indicated absorption in the colon, which supported by evidence obtained in a parallel study with iloestomists where 86% of ingested quercetin-3-*O*-rutinoside was recovered in ileal effluent. This also indicates that the flavonol disaccharide was not cleaved by either LPH or CBG, confirming an earlier report by Day et al.(2000). In the healthy subjects with an intact functioning colon, the quercetin rutinoside reaching the colon would be subject to the action of the colonic microflora resulting in the release of quercetin which in the wall of the large intestine acts as a substrate for phase II enzymes resulting in low level absorption of quercetin metabolites. It is of interest to note that quercetin released in the small intestine was glucuronidated, sulphated and methylated (Mullen et al., 2006), while only glucuronide and methyl metabolites were formed in the colon. This suggests that the colon, unlike the small intestine, lacks SULTs capable of catalysing the formation of quercetin-3′-sulphate. It also implies that

further sulfation of quercetin metabolites does not occur after entry into the circulatory system (Jaganath et al., 2006).

1.6.3 Proanthocyanidins

Proanthocyanidins (condensed tannins), are dimers, oligomers and polymers of flavan-3-ols. (+)-Catechin and (–)-epicatechin are the main constituent sub-units but other monomers, namely (epi)gallocatechin and (epi)afzelechin are also found. They are widely distributed in cocoa, grapes, apples, strawberries, and red wine (Crozier et al., 2010). It has been estimated the intake of proanthocyanidin in the USA adults is 95 mg/d (Wang et al., 2011). Numerous studies indicated that polymeric proanthocyanidins are not absorbed in the small intestine. Most will reach colon where they are catabolised by the microflora forming phenolic acids such as 3-(3′-hydroxyphenyl)hydracrylic acid and 4-*O*-methyl-gallic acid which are absorbed into the circulatory system and excreted in urine (Déprez et al., 2000; Gonthier et al., 2003a).

Only small amounts of procyanidin dimers B1 and B2 were detected in human plasma after consumption of a grape seed extract (Sano et al., 2003) and cocoa (Holt et al., 2002).The absorption of B2 dimer was ~100 fold lower than that of flavan-3-ol monomers confirmed that polymerization impairs intestinal absorption. Feeding rats with ^{14}C-radiolabeled procyanidin B2 resulted in 80% of radioactive compound being absorbed and eliminated in urine, with only small amounts of radioactivity appearing in blood which reach maximum at 6 h after ingestion suggesting it was the result of catabolism by the colonic microflora (Stoupi et al.,

2010). The rate of procyanidin degradation by the microbiota depends on the degree of polymerization, with smaller dimers and trimers being broken down more rapidly than the large polymeric forms (Gonthier et al., 2003a).

1.6.4 Flavanones

Previous studies using enzyme hydrolysis reported that orange juice flavanone rutinoside are absorbed in the large intestine (Erlund et al., 2001; Manach et al., 2003). Mullen et al.(2008a) investigated the bioavailability of flavanones in humans who drank orange juice, containing 168 µmol of hesperetin-7-*O*-rutinoside and 12 µmol of naringenin-7-*O*-rutinoside. Circulatory and urinary flavanone metabolites were analysed by HPLC-MS^2. Hesperetin-7-*O*-glucuronide and a second hesperetin-*O*-glucuronide were detected in the plasma with a combined C_{max} of 922 nmol/L at T_{max} of 4.4 h. Additional metabolites were detected in urine, including a third hesperetin-*O*-glucuronide, a hesperetin-*O*-glucuronide-*O*-sulphate and hesperetin-*O*-diglucuronide. Differences in the metabolites found in plasma and urine indicate the post-absorption of phase II metabolism. Excretion 0-24 h after orange juice ingestion corresponded to 6.5% of hesperetin-7-*O*-rutinoside intake, whereas elimination of naringenin glucuronides in equivalent to 17.3% of naringenin-7-*O*-rutinoside intake. These differences of excretion of naringenin and hesperetin, relative to the amounts ingested, is a trend that had been reported previously with flavanones, but there are exceptions where such differences were not observed (Manach et al., 2005). There are two possibilities that influenced the apparent bioavailability of the orange juice flavanones. The lower excretion of hesperetin metabolites could be due to the higher intake of

hesperetin-7-*O*-rutinoside. Alternatively, it could be a consequence of hesperetin being less readily bioavailable than naringenin because of the positional impact of the hydroxy and methoxy substituents at the C3′ and C4′ positions on absorption (Crozier et al., 2010).

1.6.5 Isoflavones

Isoflavones occur principally in soy and soy products as aglycones or glycosides depend on the preparation process involved. In over age or cooked soybeans >95% are isoflavone glucosides, whereas on soybean-fermented products (i.e. tofu and tempeh) 20-40% isoflavones are aglycones (Wang and Murphy, 1994). Isoflavone glycosides are hydrolyzed into aglycones prior to absorption, with some of which is further metabolized by colonic microflora to metabolites such as equol.

Isoflavones are well absorbed with metabolites equivalent to 20-50% being excreted in urine (Manach et al., 2005).The bioavailability of daidzein and genistein have been reported to be are lower than that of their glucosides. When daidzein or daidzen-7-*O*-glucoside was ingested by humans, the plasma C_{max} at 8-9 h, was 3-6-fold higher in the glucoside feed. Several metabolites were detected after deconjugation including dihyrodaizein, *O*-desmethylangolensin, 6-hydroxy-daidzen, 8-hydroxy-daidzein, 3′-hydroxy-daidzein, and equol (Rüfer et al., 2008). In contrast, 5-fold higher plasma C_{max} concentrations, with a 4 h T_{max}, were observed with aglycones when a crude preparation of soya saponins was ingested with high amounts of daidzein and genistein or their mixed glucosides (Izumi et al., 2000). This similar to the findings of (Zubik and Meydani,

2003). A study in which 50 g of kinako (baked soya bean powder) was ingested by human volunteers resulted traces of glycosides being detected in plasma 1 h after intake, with most absorption occurs after deconjugation (Hosoda et al., 2008). The absorption of isoflavone glycosides appears to occur in the upper part of gastrointestinal tract, with the production of microbial metabolites equol, dihydrodaidzein and dihydrogenistein occurring in the colon (Crozier et al., 2010).

1.6.6 Anthocyanins

Anthocyanins are present in large amounts in some dietary components, especially berries, fruits and some red wines. The dietary intake of anthocyanins has been estimated to be 182 mg/mL (Manach et al., 2005) and 180–215 mg/d in the United States (Kühnau, 1976). In several studies showed that anthocyanins are very rapidly absorbed and excreted, but with only low efficiency. Typically ~0.1% of the ingested anthocyanins are detected in urine (Crozier et al., 2010). The absorption and excretion of anthocyanins are determined by the sugar moiety and structure of the aglycone (Wu et al., 2005). The complex array of information on anthocyanins bioavailability derived from human and animal models is due to the berry or fruit supplements containing complex anthocyanin profiles (Prior and Wu, 2006). Black raspberries, for instance, contain five cyanidin-3-*O*-glucosides, while blueberries consist 12 anthocyanins, mainly 3-*O*-glucosides, galactosides and arabinose of cyanidin, delphinidin, petunidin and malvidin where 3′-O-methlyation can transform these parent compounds into another anthocyanin, which makes assessing the content of

anthocyanins present in plasma and urine is difficult to interpret (Crozier et al., 2010).

Studies on bioavailability of anthocyanins are made more feasible by using strawberries which contain pelargonidin-3-*O*-glucoside and blackberries with cyanidin-3-*O*-glucoside as their predominant anthocyanins. When 200 g strawberries were fed to humans, anthocyanins are readily absorbed with T_{max} of 1 h with pelargonidin-3-*O*-glucoside as the main metabolite detected in plasma and 0.75% of total pelargonidin-3-*O*-glucoside intake excreted in urine (Mullen et al., 2008b). In other humans study using 200 g of blackberries, 12 anthocyanins were detected which is corresponds to 0.16% on anthocyanins intake (Felgines et al., 2005). It was suggested that the 4′-monohydroxy compound pelargonidin-3-*O*-glucoside is more readily absorbed than cyanidin-3-*O*-glucoside which has a 3′, 4′-dihydroxy structure.

1.6.7 Flavones

Compared to other flavonoid subclasses, the bioavailability of flavones has been investigated sparingly. The intestinal absorption of luteolin and luteolin-7-*O*-glycoside (Figure 1.18) in rats and humans was studied by Shimoi et al.(1998). Luteolin appears to be absorbed in the small intestine through passive diffusion, whereas its glycoside undergoes hydrolysis either by LPH or the colonic microflora before absorption. Luteolin was absorbed mainly in the jejunum (Zhou et al., 2008) and metabolized by phase II enzymes into glucuronides and sulphates during passage through the intestinal mucosa or the liver. Methylation of luteolin mediated by COMT

was reported in rats with chrysoeriol and diosmetin (Figure 1.18) being found in plasma and urine (Shimoi et al., 1998; Chen et al., 2011).

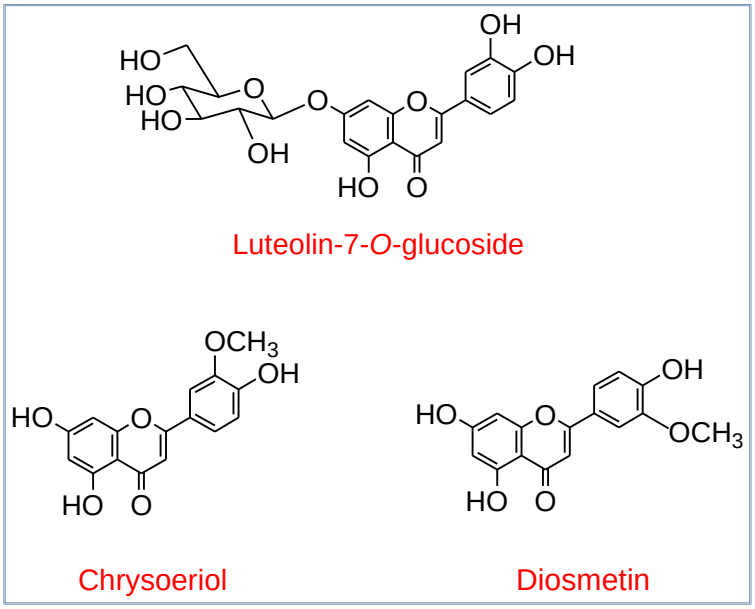


Figure 1. 18 Molecular structures of luteolin glucoside, chrysoeriol (3′-*O*-methylation) and diosmetin (4′-*O*-methylation).

Luteolin plasma C_{max} levels were lower in rats given pure luteolin as opposed to luteolin in a peanut hull extract (14.3 mg/kg of luteolin) (Zhou et al., 2008) and luteolin-7-*O*-glycoside in a *Chrysanthemum morifolium* extract (14.3 mg/kg) (Chen et al., 2007b). Luteolin and luteolin glucoside in extracts, were absorbed better than pure luteolin alone, suggesting the function of other components in the extract that could enhance the absorption efficiency.

Humans fed with 2 g of blanched parsley containing 65 µmol of apigenin, attained flavone C_{max} values after 7.2 h and 0-24 h urinary excretion was 0.22 % of intake (Meyer et al., 2006). In rats fed with 200

mg/kg of *Chrysanthemum morifolium,* the T_{max} of luteolin and apigenin was obtained at 1.1 and 3.9 h. Their total urinary excretion of apigenin and luteolin was 6.6% and 16.6%, respectively, up to 72 h after the intake (Chen et al., 2007b). These results are unusual as, in contrast to anthocyanins, it appears that the 3′,4′-dihydroxy luteolin is absorbed more readily than the 4′-monohydroxy apigenin. It has been reported that apigenin is oxidized to luteolin (Gradolatto et al., 2004), Chen et al.(2007b) failed to detect such a transformation in rat after oral administration of apigenin.

1.6.8 Hydroxycinnamic acids

Caffeic acid and ferulic acid are rapidly absorbed from the small intestine. These compounds are normally esterified in nature. Such conjugation has proved to hinder absorption. Conjugation of caffeic acid, as in 5-*O*-caffeoylquinic acid, markedly reduces absorption (Olthof et al., 2001). Although absorption of intact 5-*O*-caffeoylquinic acid was reported in some studies (Lafay et al., 2006a), hydrolysis of the parent compound by esterases prior to absorption remains the main process. Detailed studies on the bioavailability of caffeoylquinic acid has performed with healthy humans and volunteers with an ileostomy who ingested of 200 mL of instant coffee (Stalmach et al., 2010b). Caffeoylquinic acids and their metabolites were recovered in ileal effluent in amounts corresponding to 71 % of intake, with 78% as the parent chlorogenic acids and the remainder as free and sulphated caffeic acid. Excretion in 0-24 h urine was 8% of total intake, and the main metabolites were ferulic acid-4-*O*-sulphate, caffeic acid-3-*O*-sulphate, isoferulic acid-3-*O*-glucuronide and dihydrocaffeic acid-3-*O*-sulphate. In humans with an intact colon, urinary excretion was 29% of

caffeoylquinic acid intake. This is due to high accumulation of dihydroferulic acid and feruloglycine, sulphated and glucuronidated dihydrocaffeic and dihydroferulic acids in the urine produced by the colonic microflora metabolism that was absent in ileostomy patients.

Ferulic acid is the abundant hydroxycinnamate in tomatoes, beers and cereals. Rats fed with ferulic acid excreted metabolites including sulphated, glucuronidated and sulfo-glucuronidated ferulic acids (Azuma et al., 2000). Urinary recoveries of these metabolites varies from 10 to 72 % of the amount ingested which may resulted from the use of enzymes with different efficiencies to release the aglycones prior to quantitative analysis (Stalmach et al., 2012).

1.7 Catabolism of (poly)phenols and role of colonic microflora

(Poly)phenols largely present in food in the form of esters, glycosides, or polymers that typically undergo limited absorption in the proximal gastrointestinal tract. As noted above, prior to absorption into the small intestine, flavonoid glycosides are deglycosylated by LPH/CBG, and metabolised during the passage probably in the enterocytes of the gastrointestinal tract and the liver, with methylation, sulfation and glucuronidation steps. This process will increase their hydrophilicity and reduced any toxicity effects and facilitates their biliary and urinary elimination.

However, not all dietary (poly)phenol absorption starts in the upper GI tract. It has been reported that 90-95% of the dietary (poly)phenols are not absorbed in the small intestine (Clifford 2004). Among these (poly)phenols are

oligomeric and polymeric proanthocyanidins, (poly)phenols glycosylated with rutinose, chlorogenic acids and lignans which normally reach the colon and are catabolised by colonic microflora (Aura, 2008; Selma et al., 2009) which indicate the potential important of colon in the bioavailability equation. In colon, (poly)phenols and related compounds are catabolise by gut microflora enzymes (esterase, glucosidase, demethylation, and decarboxylation enzymes activities) forming low molecular weight phenolic compounds that can be absorbed in the large intestine (Rechner et al., 2004; Aura, 2008). These transformations include deglycosylation, the hydrolysis of esters and amides and deglucuronidation (Stalmach et al., 2010b), which may result in catabolites that might be more bioactive than the parent compounds. Released aglycones will undergo C-ring fission which produces more or less active compounds depending of the products formed. The type of catabolic products produce by ring fission where the heterocylic oxygen ring is split by the microflora depends on the type of flavonoids: flavonols mainly produce hydroxyphenylacetic acid, flavones and flavanones mainly transform to hydroxyphenylpropionic acids (Serra et al., 2012), where as flavan-3-ols are catabolized to phenylvalerolactones and hydroxyphenylpropionic acids, following this they will further converted to benzoic acid derivatives (Spencer, 2003). These catabolites are absorbed through the colonic barrier and further transformed by conjugation with glycine, glucuronic acid or sulphate groups (Rechner et al., 2002). These hepatic catabolites may undergo enterohepatic circulation, release in the blood and excreted from the body via urine, whereas non-absorbed phenolic catabolites are eliminated through the faeces.

Human gastrointestinal tract consists of the mouth, oral cavity, oesophagus, stomach, small intestine, and eventually the colon which is the

most heavily colonized region of the digestive tract comprising ~10^{12} bacteria/g colonic contents with many hundreds of different bacterial species (Gross et al., 2010). The identification and quantification of the colonic catabolites constitutes important research as a wide range of low molecular weight phenolic compounds are produced along with short chain fatty acids derived from carbohydrates that exert a prominent effect on human health.

The activity of colonic microflora on dietary (poly)phenols is often responsible for the modulation of its biological activity and beneficial health effects as in the case of the isoflavone daidzein which exist *in planta* principally as glycosides which require the action β-glucosidase for the uptake to the peripheral circulation. The aglycone is either absorbed or further metabolized by colonic microflora with daidzein being reduced to *O*-demethylangolensin and equol (Figure 1.19) (Kim et al., 2009). The production of equol varies greatly among individuals, with only 30-40% of Western populations being equol producers (Manach et al., 2005). The inability to produce equol was reported due to the lack of specific components of the intestinal microflora (Yuan et al., 2007). Equol producers may gain more health benefits from soy consumption than those non producer, since the (3*S*)-equol has been shown to be more estrogenic than its precursor (Setchell et al., 2002).

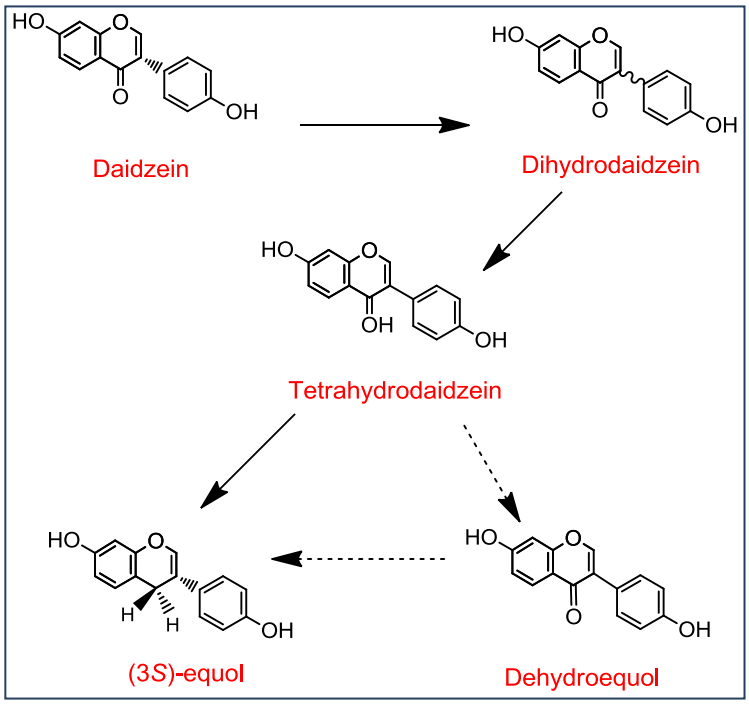


Figure 1. 19 Proposed pathway of equol formation from reduction of isoflavone daidzein by colonic microflora.

Certain large molecules require the action of microbes in order for it to be absorbed, as in the case of ellagitannins. Studies in human and animal microbiota, showed that ellagitannins are hydrolyzed to ellagic acid that is converted to a series of urolithins (González-Barrio et al., 2011). Urolithins occur as glucuronides and sulphates in human plasma, and the amounts produced vary markedly between individuals (González-Barrio et al., 2010). The potential benefit effect of urolithins have been reported, with the finding that urolithin A can inhibit colon cancer (Sharma et al., 2009).

2 PHYTOCHEMICAL ANALYSIS OF A MALAYSIAN MEDICINAL HERBAL PLANT: *FICUS DELTOIDEA*

2.1 Introduction

Ficus deltoidea (Figure 2.1A) is a member of the Moraceae and is one of about 40 genera of the mulberry family. *F. deltoidea,* also known as *F. diversifolia,* is referred to as mistletoe fig, which comes from its epiphytic habit of growing on larger trees. It is a shrub of upright cascading habit, which can reach 15 feet in height. Compared to other members of the fig family such as *F. microcarpa, F. carica* and *F. elastica*, it is small plant, and is widely cultivated as a houseplant because of its attractive spoon-shaped foliage and greenish-white fruits which turn purple when ripe. The plant has very small flowers massed on the inside walls called ostioles, it is invisible externally and in Chinese medicine is called "the no flower fruit" (Mat-Saleh and Latif, 2002; Lansky et al., 2008).

F. deltoidea is native to South East Asia, Borneo, and the Philippines. It is well known among the eastern practitioners of traditional medicine and is referred to by different names such as 'mas cotek' (gold droplet) or 'serapat angin' in Malaysia, 'tabat barito' in Indonesia and 'kangkalibang' in Africa. It has a unique feature with the underside of the leaves having dot-like mark (Figure 2.1B) referred to as "a drop of gold" from where the plant derives its common name.

Figure 2. 1 *A)* A young *Ficus deltoidea* plant: B) A dot-like mark on the underside of the leaves is a key characteristic of this species.

The fig family has been utilised for many different purposes, eaten as dried fruit, the leaves used to prepare a tea, while aerial roots and bark, even its wood ash, are used for making lye, an ingredient for preparing soba, a traditional Okinawa food (Ao et al., 2008). There are several studies with fig plants which have reported that their antioxidant activities are attributed by the presence of phenolic compounds such as flavonoids (Teixeira et al., 2006; Çalişkan and Aytekin Polat, 2011). These flavonoids include of flavones, isoflavones, flavan-3-ols and anthocyanins (Dueñas et al., 2008). However, the types of flavonoids present are seemingly related to differences between variant subspecies and/or geographical distribution (Abdel-Hameed, 2009; Shi et al., 2011).

F. deltoidea has been used as a traditional medicine for prevention and cure of various diseases such as diabetes, rheumatism, and breast cancer. The leaves are the main ingredient of herbal preparations, although other parts are also used including fruits, roots and even the whole plant. *F. deltoidea* is in high demand locally in south East Asian countries both as raw materials, such as dried leaves, or as processed herbal products (Figure 2.2).

A. *F. deltoidea* extract in a form of capsule

B. As ready to drink herbal tea

C. Prepared as herbal tea in a sachet

D. As an ointment for topical use

Figure 2. 2 *F. deltoidea* based herbal products.

However few, if any, of the products, such as those illustrated in Figure 2.2, have been tested scientifically and in reality are unproven in terms of their safety and efficacy. It is possible that such products may have toxicity effects due to the adulterations, and customers may well be misled by health claims and other information printed on the packaging.

Although fig plants such as *F. carica* has been investigated in some detail as a consequence of the economic value of their fruit (Oliveira et al., 2010; Gaaliche et al., 2011), there is little information on *F. deltoidea*. In the circumstances, using modern analytical techniques to analyse the phytochemical constituents of *F. deltoidea* is a key first step in determining the therapeutic value of this plant. This will also assist in providing information for the standardization of its diverse herbal products.

Aims of study

The aim of this study was to investigate the (poly)phenolic content of *F.deltoidea* to see if there were compounds present that could justify the reported efficacy and claimed medicinal properties of this Malaysia herbal spesies.

2.2 Materials and methods

2.2.1 Chemicals and reagents

HPLC grade methanol was purchased from Rathburn (Walkerburn, UK). The flavonoid standards, (+)-catechin, apigenin-8-*C*-glucoside (vitexin), apigenin-6-*C*-glucoside (isovitexin) and luteolin-6-*C*-glucoside (isoorientin) were obtained from Extrasynthese (Genay, France). Gallic acid and delphinidin-3-galactoside were supplied by AASC Ltd (Southampton, UK). Procyanidin B_2, (−)-epicatechin, formic acid, phosphoric acid, metaphosporic acid and Folin-Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (Dorset, UK). Other chemicals were supplied by Fisher Scientific (Leicestershire, UK).

2.2.2 Plant material

Dried leaves of *F. deltoidea,* purchased from a local market in Pahang, Malaysia, were dried further for 2 days at 45°C before being ground to a powder which was extracted with boiling water for 1 h after which the infusion was filtered and dried by Mini spray dryer B-290 (Buchi, Switzerland) to form a powder which was shipped to Glasgow by courier in an airtight plastic container.

2.2.3 Sample preparation

One hundred mg of a powdered *F. deltoidea* extract was dissolved in 10 mL of methanol-water (1:1, v/v), centrifuged at 10000 *g* for 5 min at 4 ºC prior to analysis of the supernatant.

2.2.4 Determination of total phenol content

The total phenolic of *F. deltoidea* extract was measured in gallic acid equivalents using the Folin-Ciocalteu assay of Singleton and Rossi (Singleton and Rossi, 1965). In brief, 200 µL of a 1:5 dilution sample was added to 10 mL of a 1:10 diluted Folin–Ciocalteau reagent and 1.8 mL of distilled water. After 5 min, 7 mL of a Na_2CO_3 solution (115 g/L) was added and the reaction mixture was left at room temperature for 2 h. The absorbance of the solution was read at 765 nm against water blank on Cecil 3000 series spectrophotometer (Cecil Instrument Ltd, Cambridge, UK). The absorbance was compared to a standard curve prepared with 50 to 500 mg/L gallic acid and results expressed as gallic acid equivalents (GAE).

2.2.5 Ferric reducing antioxidant potential (FRAP) assay

The antioxidant activity of *F. deltoidea* extracts were estimated using FRAP assay (Benzie and Strain, 1996). This method measures the ability of a solution to reduce a ferric-2,4,6-tri-2-pyridyl-s-triazine (TPTZ) complex (Fe^{3+}- TPTZ) to the ferrous form Fe^{2+}, producing an intense blue colour with absorption at 593 nm. The reaction is non-specific and any half time reaction, which has a less positive redox potential, under reaction conditions, than the Fe^{3+}/Fe^{2+}-TPTZ half-life reaction will drive the Fe^{3+}-TPTZ reduction. In the FRAP assay excess Fe^{3+} is used and the rate limiting factor of the Fe^{2+}-TPTZ, and hence colour

formation, is the reducing ability of the sample. 1.5 mL of FRAP reagent (10 mM TPTZ and 20 mM ferric chloride Fe^{3+} in acetate buffer pH 3.6) was added to 50 µL sample and 150 µL distilled water at 30 sec intervals. The mixture was incubated for 4 min prior absorbance reading at 593 nm. Optical density obtained was compared to a standard curve prepared with 0.1 to 1.0 mM ferrous sulphate ($FeSO_4$) and antioxidant activity expressed as the mean concentration of Fe^{2+} produced/mmol. A drawback of this assay is that some of polyphenol compounds such as tannic acid and quercetin react slowly to Fe^{3+} TPTZ, whereas compounds like thiols and proteins cannot be measured by this assay (Prior et al., 2005).

2.2.6 HPLC-PDA-MS^2 with on-line antioxidant detection

Use was made of a Surveyor HPLC system (Thermo Scientific, Waltham, MA, USA), comprising a pumping system, autosampler, and degasser coupled to a photodiode array absorbance (PDA) detector scanning from 200 to 700 nm controlled by Xcalibur software version 1.3. Separations were carried out using MAX-RP 4 µm, 250 mm x 4.6 mm C_{12} reverse phase column (Phenomenex, Torrance, CA) maintained at 40ºC and eluted at a flow rate of 1.0 mL/min with 60 min gradient from 15 to 50% methanol in water containing 0.1% formic acid. After passing through the flow cell of the PDA detector column eluate was directed to a fluorescence detector (Jasco FP-920 with excitation at 280 nm, and emission at 315 nm), then split and 20% directed to an LCQ Duo mass spectrometer (Thermo Scientific) with an electrospray interface operating with negative ionisation in full scan data dependent MS^2 mode from 150 to 1000 amu. For the detection of components with antioxidant activity, the remaining 800 µL/min of the HPLC eluate was mixed with an ABTS solution flowing at 0.5 mL/min and the resultant mixture passed through a holding coil before being

directed to a P2000 absorbance detector (Thermo Scientific) operating at 720 nm (Stewart et al., 2005) (Figure 2.3). Data were analysed using Xcalibur software and peaks were quantified in Trolox equivalents.

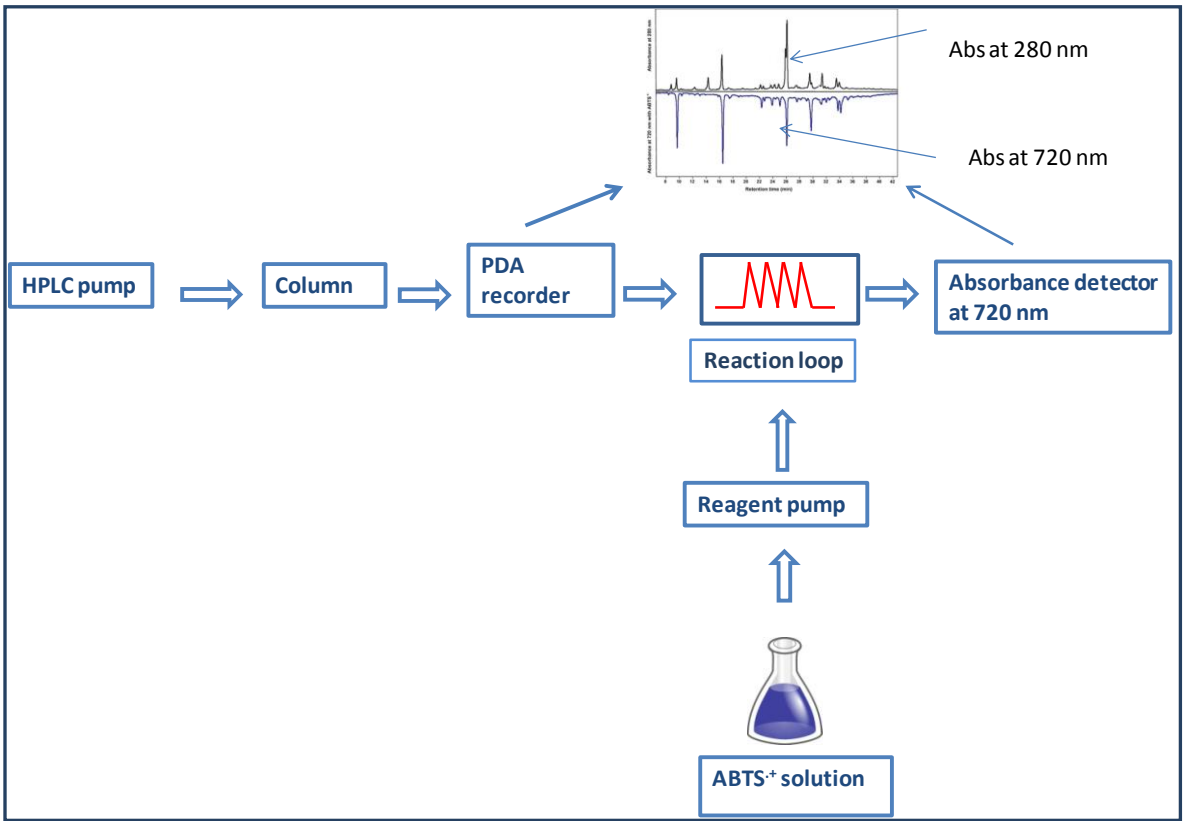


Figure 2. 3 ABTS-online HPLC system for the measurements of antioxidant activities of *F. deltoidea* extracts.

2.2.7 Analysis of procyanidins

Thiolytic degradation of *F. deltoidea* extract was carried out on a dried sample as described by Guyot et al.(2000) which were reacted with 400 µL of benzyl mercaptan (5% in methanol, v/v) in 200 µL of acidified methanol (3.3% HCl, v/v) at 40ºC for 30 min, with samples being vortexed every 10 min. The HPLC-PDA-FL-MS^2 analysis with conditions as described above except for the use of a 60 min gradient of 3–55% acetonitrile in 1% aqueous formic acid.

2.3 Results

2.3.1 Identification of phenolic compounds

Three aqueous extracts of *F. deltoidea* were analysed by gradient reverse phase HPLC with PDA, fluorescence, antioxidant and MS detection. HPLC fluorescence and absorbance profiles at 280 and 365 nm are presented in Figure 2.4. The mass spectral properties of the 26 numbered peaks along with their identification/partial identification are summarised in Table 2.1.

Identifications were based on the following criteria:

Peak 1 (t_R - 6.6 min, λ_{max} - 270 nm) was gallocatechin based on co-chromatography with a (+)-gallocatechin and a negatively charged molecular ion $[M-H]^-$ at *m/z* 305 which yielded MS^2 fragments at *m/z* 261, *m/z* 221 and *m/z* 179. Reversed phase HPLC does not separate the (+)- and (–)-gallocatechin so the enantiomer is not specified. This is also the case with the other flavan-3-ols that were detected.

Peak 2 (t_R - 11.4 min, λ_{max} - 275 nm) was identified as epigallocatechin as it co-eluted with a standard and both had a $[M-H]^-$ at *m/z* 305 and MS^2 ions at *m/z* 261, *m/z* 221 and *m/z* 179.

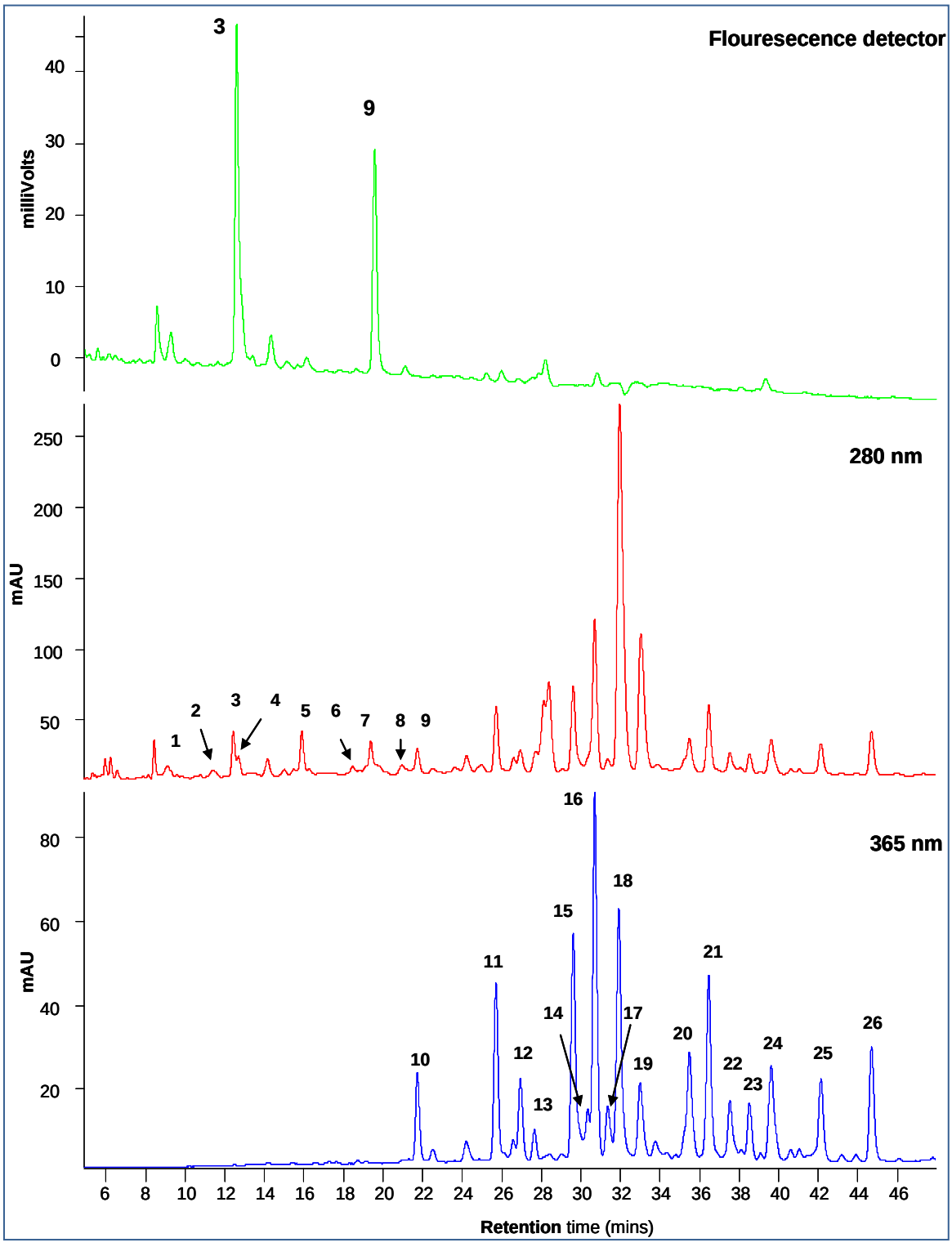


Figure 2. 4 HPLC profiles of *F. deltoidea.* The chromatograms were recorded at UV 365 nm and 280 nm. The numbering of their peaks indicates their subsequent identification by MS**.**

Table 2. 1 HPLC and mass spectral characteristic and identity of phenolics present in *F. deltoidea* analysed by HPLC-ESI/MS.

Peak	t_R (min)	λ_{max}	Compound	$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)
1	6.6	270	(+)-Gallocatechin	305	261, 221, 179
2	11.4	275	(-)-Epigallocatechin	305	261, 221, 179
3	12.5	280	(+)-Catechin	289	245, 205, 179
4	12.7	280	(Epi)catechin-(Epi)afzelechin	561	435, 289, 273
5	14.2	280	(Epi)catechin-(Epi)afzelechin	561	435, 289, 273
6	15.1	275	(Epi)catechin-(Epi)afzelechin-(Epi)afzelechin	833	561, 543, 289
7	15.9	280	(Epi)catechin-(Epi)afzelechin	561	435, 289, 271
8	18.5	275	(Epi)catechin-(Epi)afzelechin-(Epi)afzelechin	833	561, 289, 271
9	19.4	280	(-)-Epicatechin	289	245, 205, 179
10	21.8	350	Luteolin-6-8-*C*-diglucoside (Lucenin-2)	609	519, 489, 399
11	25.7	340	Apigenin-6, 8-*C*-diglucoside (Vicenin-2)	593	503, 473, 353
12	27.0	345	Luteolin-6-*C*-hexosyl-8-*C*-pentoside	579	489, 459, 399
13	27.8	345	Luteolin-6-*C*-glucosyl-8-*C*-arabinoside	579	489, 459, 399
14	29.6	335	Apigenin-6-*C*-arabinosyl-8-*C*-glucoside (Isoschaftoside)	563	503, 473, 443
15	30.4	345	Luteolin-6-*C*-arabinosyl-8-*C*-glucoside	579	489, 459, 399
16	30.7	335	Apigenin-6-*C*-glucoside-8-*C*-arabinoside (Schaftoside)	563	503, 473, 443
17	31.4	335	Luteolin-8-*C*-glucoside (Orientin)	447	369, 357, 327
18	31.9	320	Apigenin-6-*C*-pentosyl-8-*C*-glucoside	563	473, 443, 353
19	33.0	310	4-*p*-Coumaroylquinic acid	337	191, 173, 163
20	35.5	335	Apigenin-8-*C*-glucoside (Vitexin)	431	413, 341, 311
21	36.5	335	Apigenin-6-*C*-glucosyl-8-*C*-pentoside	563	473, 443, 353
22	37.5	335	Apigenin-6,8-*C*-dipentoside	533	515, 473, 443
23	38.5	335	Apigenin-6,8-*C*-dipentoside	533	515, 473, 443
24	39.6	335	Apigenin-6-*C*-glucoside (Isovitexin)	431	413, 341, 311
25	42.1	335	Apigenin-6,8-*C*-dipentoside	533	515, 473, 443
26	44.7	335	Apigenin-6,8-*C*-dipentoside	533	515, 473, 443

Peak numbers and retention times refer to **Figure 2.3** t_R-retention time, $[M-H]^-$ - negatively charged molecular ion.

Peak 3 (t_R - 12.5 min, λ_{max} - 280) was catechin based co-chromatography with a standard. Both compounds fluoresced and had a $[M-H]^-$ at *m/z* 289 which fragmented to produce daughter ions at *m/z* 245, *m/z* 205 and *m/z* 179.

Peaks 4, 5 and 7 (t_R - 12.7, 14.2 and 15.9 min, λ_{max} - 280 nm) were all identified as mixed type-B dimers of (epi)catechin and (epi)afzelechin. This identification was on the basis of each yielding a $[M-H]^-$ at *m/z* 561 which upon MS^2 produced ions at *m/z* 435, *m/z* 425 (Retro Diels-Alder reaction [RDA]), *m/z* of 407, *m/z* 289 (an (epi)catechin unit) and *m/z* 271 (an (epi)afzelechin unit) as reported by de Souza et al.(2008). The *m/z* of 407 results from water elimination of *m/z* 425. The heterocyclic ring fission (HRF) of the dimer produces *m/z* 435 (loss of 126 Da), indicated that ring A of the extension unit had a 1, 3, 5-trihydroxybenzene structure. The quinone methide (QM) cleavage of the interflavan bond gives rise to *m/z* 271 and *m/z* 289 which indicated the extension and terminal unit of this sequence as reported earlier by Verardo et al.(2010).

Peaks 6 and 8 (t_R - 15.1 and 18.5 min, λ_{max} - 275 nm) exhibited a $[M-H]^-$ ion at *m/z* 833, and the resulting MS^2 spectrum comprised of ions at *m/z* 561 ($[M-H-272]^-$), *m/z* 543 ($[M-H-290]^-$), *m/z* 289 and *m/z* 271. The production of *m/z* 289 and 271 reveals that epi(afzelechin) is located in the upper unit, whereas (epi)catechin constitutes the lower one. The MS^2 of this ion also produced minor fragment ions at *m/z* 697 and *m/z* 679 which are keeping with RDA fission. MS^3 led to fragment ions at *m/z* of 425 and *m/z* of 407, RDA of a dimer. Both peaks therefore yield spectra indicative of the presence of a

type-B procyanidin trimer consisting of one (epi)catechin unit and two (epi)afzelechin units, in keeping with the findings de Souza et al.(2008).

Peak 9 (t_R - 19.4 min, λ_{max} - 280 nm), this also produced a strong fluorescent peak and was identified as epicatechin based on a $[M-H]^-$ at *m/z* 289 which gave rise to MS^2 ions at *m/z* 245, *m/z* 205 and *m/z* 179. The identification was confirmed by co-chromatography with an authentic standard.

Peak 10 (t_R - 21.8 min, λ_{max} - 350 nm) had a $[M-H]^-$ at *m/z* 609, and its MS^2 spectrum consisted of ions at *m/z* 489 ($[M-H-120]^-$), *m/z* 519 ($[M-H-90]^-$) and *m/z* 399 ($[M-H-120-90]^-$), corresponding to the fragmentation of a flavone *C*-diglycoside, which, based on the data of Ferreres et al.(2003) is tentatively identified as luteolin-6,8-*C*-diglucoside (aka lucenin-2).

Peak 11 (t_R - 25.7 min, λ_{max} - 340 nm) was characterised by a $[M-H]^-$ at *m/z* 593 which produced daughter ions at *m/z* 503 ($[M-H-90]^-$), *m/z* 473 ($[M-H-120]^-$), and indicative of the cleavage of a *C*-glycoside. These fragments, based on the findings of Han et al.(2008) are in keeping with the presence of apigenin-6, 8-*C*-diglucoside (aka vicenin-2).

Peak 12 (t_R - 27.0min, λ_{max} - 345 nm) exhibited a $[M-H]^-$ ion at *m/z* 579 and MS^2 fragments at *m/z* 489 ($[M-H-90]^-$), *m/z* 459 ($[M-H-120]^-$), in keeping with the presence of a *C*-linked hexose sugar and *m/z* 399 ($[M-H-120-60]^-$) corresponding to the further fragmentation of a pentose unit and *m/z* 285. These data indicate the presence of luteolin (*m/z* 285) + hexose (*m/z* 162) + pentose (*m/z* 132). Thus peak 12, in keeping with the data of Figueirinha et

al.(2008) is tentatively identified as either a luteolin-6-*C*-hexosyl-8-*C*-pentoside or luteolin-8-*C*-hexosyl-6-*C*-pentoside.

Peak 13 (t_R - 27.8min, λ_{max} - 345 nm) and peak 15 (t_R -30.4 min, λ_{max} - 345 nm) both had $[M-H]^-$ ion at *m/z* 579 and an MS^2 spectrum with ions at *m/z* 489 ($[M-H-90]^-$), *m/z* 459 ($[M-H-120]^-$), indicating the presence of a *C*-linked hexose moiety, and *m/z* 399 ($[M-H-180]^-$). This is a *C*-glycosylflavone fragmentation pattern similar to that reported by Ferreres et al.(2008) and on the basis of their elution sequence peak 13 is tentatively identified as luteolin-6-*C*-glucosyl-8-*C*-arabinoside and peak 15 as luteolin-6-*C*-arabinosyl-8-*C*-glucoside.

Peak 14 (t_R - 29.6 min, λ_{max} - 335 nm) and peak 16 (t_R - 30.7 min, λ_{max} - 335 nm), both had a $[M-H]^-$ at *m/z* 563 which produced daughter ions at *m/z* 503 ($[M-H-60]^-$), *m/z* 473 ($[M-H-90]^-$) and *m/z* 443 ($[M-H-120]^-$).The ($[M-H-60]^-$) indicates a pentose substitution, in addition to that of a hexose sugar. By reference to the mass spectrometric and chromatographic data of Han et al.(2008) peak 14 was tentatively identified as apigenin-6-*C*-arabinosyl-8-*C*-glucoside (aka isoschaftoside) and peak 16 as apigenin-6-*C*-glucosyl-8-*C*-arabinoside (aka schaftoside).

Peak 17 (t_R - 31.4 min, λ_{max} - 335 nm) had a $[M-H]^-$ at *m/z* 447 and an MS^2 spectra with ions at *m/z* 357 ($[M-H-90]^-$) and *m/z* 327 ($[M-H-120]^-$). In keeping with the findings of Kazuno et al.(2005) and co-chromatography with a reference compound this peak was identified as luteolin-8-*C*-glucoside (aka orientin).

Peak 18 (t_R - 31.9 min, λ_{max} - 320 nm) and peak 21(t_R -36.5 min, λ_{max} - 335 nm) gave the same $[M-H]^-$ ion at *m/z* 563 and its MS^2 spectrum consisted of ions at *m/z* 473 ($[M-H-90]^-$), the base peak, and *m/z* 443 ($[M-H-120]^-$). On examination of the fragment ions, it was observed that their relative abundances were similarly to those reported by Ferreres et al.(2003) suggesting that peak 18 was apigenin-6-*C*-arabinosyl-8-*C*-glucoside. The fragmentation pattern of peak 21 resembles data reported by Han et al.(2008) suggesting that the peak may be apigenin-6-*C*-glucosyl-8-*C*-pentoside.

Peak 19 (t_R - 33.0 min, λ_{max} - 310 nm) was a chlorogenic acid with a $[M-H]^-$ at *m/z* 337 and MS^2 fragments at *m/z* 191, *m/z* 173 and *m/z* 163. Based on the fragmentation patterns reported by Clifford et al.(2003) this peak is 4-*p*-coumaroylquinic acid.

Peak 20 (t_R - 35.5. min, λ_{max} - 335 nm) and peak 24 (t_R -9.6 min, λ_{max} - 335 nm) both had a $[M-H]^-$ ions at *m/z* 431 and MS^2 ions at *m/z* 341 ($[M-H-90]^-$) and *m/z* 311 ($[M-H-120]^-$). Co-chromatography and the mass spectrometric properties of reference compounds identified peak 20 as apigenin-8-*C*-glucoside (aka vitexin) and peak 24 as apigenin-6-*C*-glucoside (aka isovitexin).

Peak 22 (t_R - 37.5 min, λ_{max} - 335 nm), peak 23 (t_R - 38.5 min, λ_{max} - 335 nm), peak 25 (t_R - 42.1 min, λ_{max} - 335 nm) and peak 26 (t_R - 44.7 min, λ_{max} - 335 nm) all had a $[M-H]^-$ at *m/z* 533 which yielded MS^2 fragments at *m/z* 443 ($[M-H-90]^-$) and *m/z* 311 ($[M-H-60]^-$) which is likely to be due to the presence of a pentose substitution. There was no $[M-H-120]^-$ fragment indicating the absence of a *C*-hexose conjugate. The fragmentation suggests apigenin (271)

+ pentose (132) + pentose (132). Peaks 22, 23, 25 and 26 are therefore tentatively identified as apigenin-6, 8-*C*-dipentosides with likely pentose sugars being arabinose and xyloside.

2.3.2 Analysis of proanthocyanidins

No polymeric flavan-3-ols were detected by analysis after thiolytic degradation, as no adducts were formed during the reaction and the catechin/epicatechin peaks did not increase significantly.

2.3.3 Total phenols and antioxidant activity

The total phenolic content of the aqueous *F. deltoidea* extract was 6.3 ± 0.2 mmol/L of gallic acid equivalents while FRAP antioxidant activity was 7.2 ± 0.2 mmol/L of Fe^{2+}. These values are high, equivalent to those obtained with purple grape juice and cloudy apple juice by Mullen et al.(2007).

HPLC data obtained with the on-line ABTS antioxidant detection system are illustrated in Figure 2.5 along with absorbance traces at 280 and 365 nm. The chromatographic profiles are illustrated from 6-34 min as none of the later eluting peaks exhibited antioxidant activity. The peaks contributing the main antioxidant activity were the flavan-3-ol monomers (+)-gallocatechin (peak 1), catechin (peak 3) and epicatechin (peak 9) and the flavone apigenin-6, 8-*C*-diglucoside (peak 11) and the data on the contribution of individual compounds the overall ABTS antioxidant activity are summarized in Table 2.2. The flavan-3-ols were the main antioxidants contributing 85% compared to the 15.0% for the flavones that was due principally to apigenin-6, 8-*C*-diglucoside.

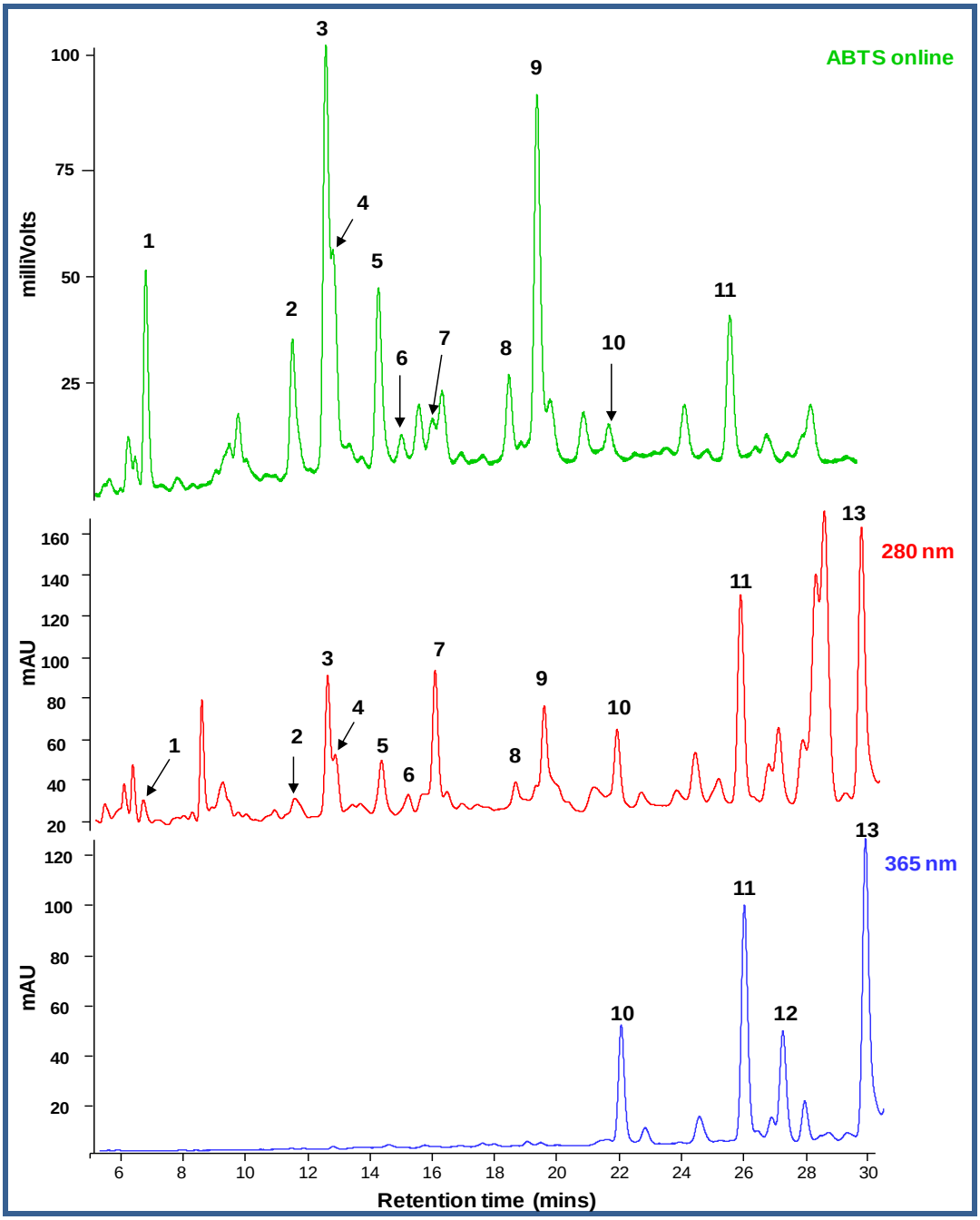


Figure 2. 5 HPLC-ESI/MS profile of *F. deltoidea* with on-line $ABTS^{+}$ antioxidant. Aqueous extract of *F. deltoidea* was analysed by gradient phase HPLC at 280 nm and 365 nm prior with $ABTS^{+}$ radical at 720 nm.

Table 2. 2 Content and antioxidant potential of individual compounds of water extract of *F. deltoidea.*

Peak	Compound	Concentration (µM)	Trolox equivalent (µM)	% antioxidant
	Flavan-3-ols			
1	Gallocatechin	44 ± 1	78 ± 3	9.4
2	Epigallocatechin	87 ± 2	68 ± 2	8.2
3	(+)-Catechin	98 ± 1	197 ± 4	24
4	(Epi)catechin-(Epi)afzelechin	18 ± 0	69 ± 1	8.3
5	(Epi)catechin-(Epi)afzelechin	21 ± 0	87 ± 1	11
6	(Epi)catechin-(Epi)afzelechin-(Epi)afzelechin	5.7 ± 0	11 ± 0	1.4
7	(Epi)catechin-(Epi)afzelechin	39 ± 0	7.2 ± 0	0.9
8	(Epi)catechin-(Epi)afzelechin-(Epi)afzelechin	5.1 ± 0	32 ± 1	3.9
9	(-)-Epicatechin	89 ± 3	148 ± 3	18
	Total	**407**	**697**	
	Flavones			
10	Luteolin-6-8-*C*-diglucoside (Lucenin-2)	13 ± 0	15 ± 1	1.8
11	Apigenin-6, 8-*C*-diglucoside (Vicenin-2)	15 ± 1	76 ± 1	9.1
12	Luteolin-6-*C*-hexosyl-8-*C*-pentoside	11 ± 0	0	0
13	Luteolin-6-*C*-glucosyl-8-*C*-arabinoside	3.9 ± 0	0	0
14	Apigenin-6-*C*-arabinosyl-8-*C*-glucoside (Isoschaftoside)	7.4 ± 0	0	0
15	Luteolin-6-*C*-arabinosyl-8-*C*-glucoside	2.0 ± 0	9.5 ± 0	1.1
16	Apigenin-6-*C*-glucoside-8-*C*-arabinoside (Schaftoside)	33 ± 0	13 ± 1	1.5
17	Luteolin-8-*C*-glucoside (Orientin)	10 ± 0	9.0 ± 0	1.1
18	Apigenin-6-*C*-pent-8-*C*-glu	117 ± 0	3.6 ± 0	0.4
20	Apigenin-8-*C*-glucoside (Vitexin)	7.8 ± 0	0	0
21	Apigenin-6-*C*-glucosyl-8-*C*-pentoside	7.0 ± 0	0	0
22	Apigenin-6,8-*C*-dipentoside	2.2 ± 0	0	0
23	Apigenin-6,8-*C*-dipentoside	1.1 ± 0	0	0
24	Apigenin-6-*C*-glucoside (Isovitexin)	15 ± 0	0	0
26	Apigenin-6,8-*C*-dipentoside	9.3 ± 0	0	0
	Apigenin-6,8-*C*-dipentoside	14 ± 0	0	0
	Total	**269**	**126**	
	Chlorogenic acids			
19	Coumaroylquinic acid	139 ± 3	3.3 ± 0	0.4
	Total	**813**	**826**	

*Results represent concentration of phenolics and antioxidant potential in aqueous extract of *F. deltoidea* (10 mg/mL).

2.4 Discussion

In the present study HPLC-ESI-MS was used to identify the phytochemical presence in the aqueous extract of *F. deltoidea*. The identification of related compounds was determined based on the fragmentation pattern and confirmed with the reference standard when available. Using this technique with negative ionisation, organic sample passes into the ionisation chamber of a mass spectrometer and bombarded by the electron forming a negative ion. The simplest case of the reaction as follows:

$$[M\text{-}H]^{\cdot} \longrightarrow X^{-} + Y.$$

The negatively charged molecular ion or parent ion is given the symbol $[M\text{-}H]^{-}$. Molecular ions are energetically unstable with some will breakdown into smaller fragments. In this case, a molecule ion will break into two parts, one is negative ion (X^{-}) whilst other is an uncharged free radical (Y). Only charged particles will be accelerated, deflected and detected by the mass spectrometer.The X^{-} or daughter ion reflects the fragmentation pattern of the parent ion and will be detected by the mass spectrometer. The fragmentation pathways of the parent ions depend on the substitution pattern, the class of flavanoids and also the collision energy applied during the collision-induced dissociation process which will show as host line in the mass spectrum. The mass spectrum *m*/*z* (mass to charge ratio) is reproducible, later used as an identifying 'fingerprint'. The identification will then be confirmed using a reference standard with the same fragmentation pattern.

In this study a total of 25 flavonoids (nine flavan-3-ols and 16 flavones) and one chlorogenic acid, 4-*p*-coumaroylquinic acid, were identified or tentatively identified in *F.deltoidea* with proanthocyanidins and flavones as major compounds detected (Figure 2.5). No other isomers were detected that could correspond to a *p*-coumaroylquinic acid when the total ion current chromatogram was scanned for the molecular ion at *m/z* 337. Such a compound, if present would be in very small amounts which is unusual since plant producing them generally contains several subgroups of similar compound or isomers (Clifford et al., 2003). It is notable that the dominance of 4-*p*-coumaroylquinic acid relative to other compounds in *Ficus* spp. according to our knowledge this has never been reported before. Thus, it makes the herbal extract *F. deltoidea* a convenient source for 4-*p*-coumaroylquinic acid.

Four flavan-3-ol monomers were detected, namely catechin, epicatechin, gallocatechin and epigallocatechin which also occur in green tea prepared from young leaves of *Camellia sinensis* (Del Rio et al., 2004; Chan et al., 2007). Five oligomers of proanthocyanidins were also detected consisting, unusually, of (epi)catechin and (epi)afzelechin sub-units. The main fragmentation pathways for these proanthocyanidins are RDA fission and subsequent elimination of water, HRF and interflavanic bond cleavage through the QM mechanism (Lansky et al., 2008). Further analysis of the *F. deltoidea* extract after thiolytic degradation did not detect the presence of additional proanthocyanidin with a higher degree of polymerisation than three.

Sixteen flavone *C*-glycosides were detected. Luteolin and apigenin derivatives were the main flavones present in *F. deltoidea*, and among them were isomers of schaftoside and vitexin (Figure 2.6) which have not been previously reported to occur in other *Ficus* species (Lansky et al., 2008; Leong et al., 2008). The differences in the flavonoid content of *F. deltoidea* and other fig species may be related to species differences, geographical distribution and environmental factors (Pistelli et al., 2000). The high level of phenolics contents in *F. deltoidea* is similar to that reported in other *Ficus* spp. (Ao et al., 2008) although the main phenolics may vary from one species to another (Lansky et al., 2008). Since *F. deltoidea* is typically consumed as herbal tea, it is worth noting that the high level of total phenolics are comparable with those occurring in black and green tea (Fan et al., 2007) as well as fruit juices (Mullen et al., 2007). This is similar to the findings by Chan et al.(2010) which indicates that herbal tea prepared from *F. deltoidea* contains a level of antioxidants comparable to black tea.

The *F. deltoidea* tea exhibited both a high total phenol content and antioxidant activity. The HPLC with on-line detection of antioxidant activity demonstrated that the flavan-3-ol monomers and the (epi)catechin-(epi)afzelechin dimers and trimers accounted for 85% of the antioxidant activity of the aqueous infusion of *F. deltoidea* leaves. Proanthocyanidins, which are widely distributed in pine bark, berries, grapes and cocoa products (Crozier et al., 2006) have become a focus of interest due to their radical-scavenging properties and anti-inflammatory activity and anti-carcinogenic effects (Duthie and Crozier, 2000; Gonçalves et al., 2005; Mantena and Katiyar, 2006; Nandakumar et al., 2008). Procyanidins in Madiran red wine

produced from Tannat grapes have been linked with increased male longevity in the Gers region of south west France (Corder et al., 2006).

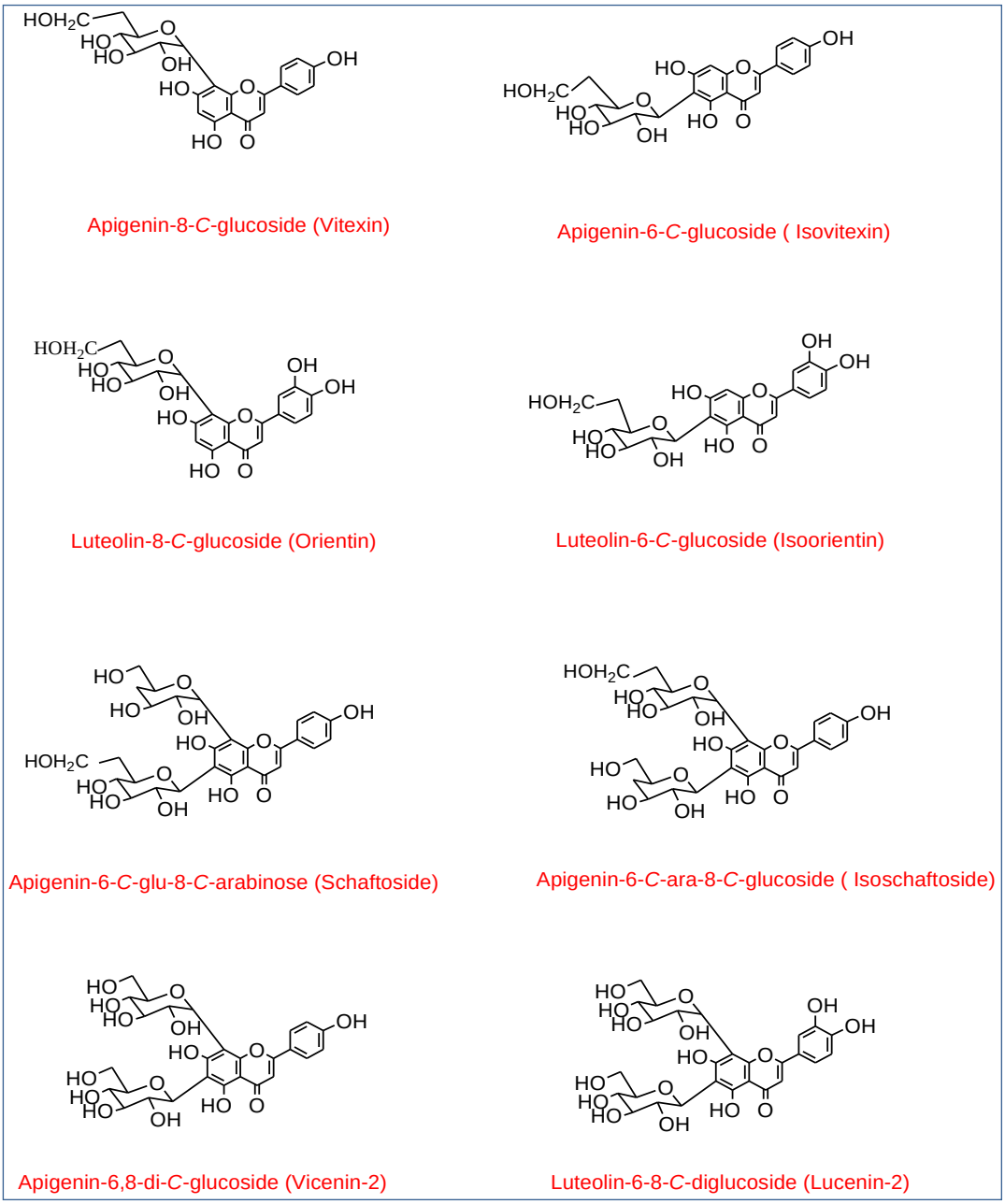


Figure 2. 6 Flavones identified from *F. deltoidea* aqueous extract.

Flavones are active components in many herbs that have been found to have impact on human health. Apigenin possess pharmacological effects with its antioxidant, antiviral, anti-inflammatory and anti-cancer properties (Duthie and Crozier, 2000; Shui and Leong, 2004) while luteolin has anti-

inflammatory effects (Chen et al., 2007a). Both apigenin and luteolin are reported to have protective effects against vascular diseases (Ma et al., 2008) and they are also potential inhibitors of cellular autoimmunity (Verbeek et al., 2004). As mentioned earlier, *F. deltoidea* has anti-diabetic properties (Abdel-Hameed, 2009), and arguably this could at least in part be due to the presence of *C*-glycosylflavonoids, such as vitexin, isovitexin and orientin. Owing to *C*-3′hydroxylation of the B ring of flavones, these *C*-glycosylflavones possess strong α-glucosidase inhibitory activity, higher than that of acarbose, a drug used for treating type 2 diabetes mellitus (Li et al., 2009). Considering the benefits of these herbal formulations, generating practical data on their safety is a prerequisite to their increase use in herbal medicine.

It was revealed the high antioxidant properties of an aqueous extract of *F. deltoidea* are due to the presence of large amounts of proanthocyanidins together with flavones. Arguably the reported therapeutic actions of *F. deltoidea* could be due to the synergistic actions of these components. Synergism derives from Greek word *syn-ergo*, meaning working together, refers to phenomenon where two or more herbal ingredients enhance each other's effects more than any individual contributions (Williamson, 2008). This synergism may also arise as one herbal preparation enhancing the therapeutic effect of other active ingredients by modulating its ADME (absorption, distribution, metabolism and excretion) via coalistic combinations (Ma et al., 2009), where the ingredient in combination is more active than the compound alone. One reported example of a synergistic effect was in randomized trial of 10 healthy volunteers given caffeine, green tea and placebo, which later showed that green tea has thermogenic properties and promotes fat oxidation greater than its caffeine content per se

(Ma et al., 2009). These synergistic effects has also been reported in some herbal preparation, such as St. John`s wort (*Hypericum perforatum*) which is more active as antidepressant in a combination of ingredients (Spinella, 2002).

In recent years, synergy research in Phytomedicine has been established where its main activity is to find the scientific rationale of many herbal remedies derived from traditional medicines which exhibit superior therapeutic activities then their individual constituents. These synergistic effects are claimed to be due to the mixture of bioactive constituents and their bi-products contained in plant extracts which improve the effectiveness of many extracts (Wagner and Ulrich-Merzenich, 2009).

To fully understand the mode of actions of *F. deltoidea*, more research is needed, particularly in terms of safety and efficacy before human intervention studies can be carried out. With its sweet taste, unlike green tea or black tea, together with its pleasant aroma, *F. deltoidea* herbal tea has promising potential to be utilized and commercialized as a healthy beverage.

2.5 Conclusion

In conclusion, these finding suggests that the RP-HPLC-ESI-MS^2 analytical method has successfully identified a total of twenty six phenolics, with newly reported proanthocyanidins and the exclusive occurrence of a single chlorogenic acid in *F. deltoidea e*xtract. The chromatographic fingerprinting obtained is also a useful reference for *F. deltoidea* herbal products. All the above findings lend credence to the potential beneficial use of *F. deltoidea* extract as a healthy beverage. Nevertheless, the detailed

mechanism of action and the interactions among the different compounds responsible for this potential activity needs further investigation.

In addition, the Information gained from this study offers a stepping stone towards the documentation of herbal medicinal tea and inputs to policy makers on the way towards regulating and standardization of *F. deltoidea* herbal products.

3 ABSORPTION, DISPOSITION, METABOLISM AND EXCRETION OF [3-^{14}C]CAFFEIC ACID IN RATS

3.1 Introduction

Hydroxycinnamates, such as caffeic acid and ferulic acid, are of dietary significance being widely distributed in fruits, vegetables, grains, and coffee. They occur predominantly as esterified forms linked with sugars or organic acids such as quinic acid and tartaric acid (Figure 3.1) (Clifford, 1999; Crozier et al., 2006). Esterification with quinic acid produces a family of compounds collectively known as chlorogenic acids. The most widely distributed chlorogenic acid is 5-*O*-caffeoylquinic acid, which can occur along with its 3-*O* and 4-*O* isomers (Figure 3.1), and is especially abundant in coffee, apple and mate (Clifford et al., 2003; Marks et al., 2007; Clifford and Crozier, 2011). These compounds can be major constituents in the human diet and are of interesting as they have antibacterial and anti-inflammatory properties, (Almeida et al., 2006; Larrosa et al., 2009) and have been linked to potential protective effects against cardiovascular disease, diabetes type 2, Alzheimer's disease and ischemia reperfusion injury (Lindsay et al., 2002; Federico et al., 2004; Gordon and Wishart, 2010).

The potential biopharmacological properties of hydroxycinnamates are dependent on their absorption in the gastrointestinal tract and the associated metabolism. The bioavailability of caffeoylquinic acids and their primary metabolite, caffeic acid, have been investigated using a diversity of ex vivo test systems, human and animal models (Olthof et al., 2001; Gonthier et al.,

2003b; Crozier et al., 2010; Stalmach et al., 2012). It has been reported that both caffeic acid and its ester, chlorogenic acid have shown a protective effects against ischemia-reperfusion injury, with the main contribution coming from caffeic acid which exhibit stronger antioxidant activity than its ester (Sato et al., 2011). Recent detailed studies with chlorogenic acid after the ingestion of coffee by healthy subjects, and volunteers with an ileostomy has been reported by (Stalmach et al., 2009; Stalmach et al., 2010b).

Nevertheless, there is still a gap on the extent to which hydroxycinnamates are sequestered in body tissues and organs, a topic on which detailed information can only be obtained with animal-based investigations. Hydroxycinnamate disposition and metabolism are most readily monitored using an animal model and feeding radiolabeled substrates as demonstrated in flavonol bioavailability study in which [2-^{14}C]quercetin-4′-*O*-glucoside was fed to rats (Mullen et al., 2008c).

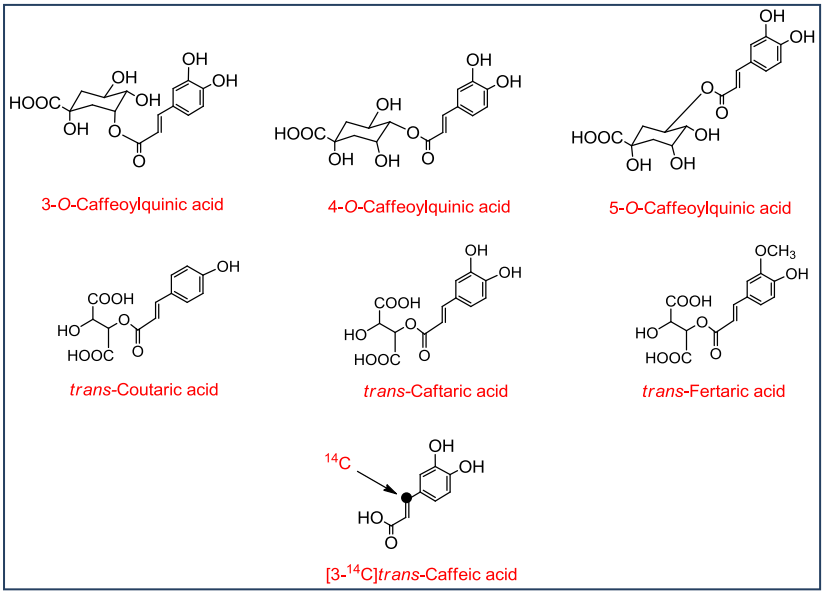


Figure 3. 1 Structures of common dietary hydroxycinnamates.

Aims of study

The aims of the current study focused on the absorption, disposition, metabolism and excretion of [3-^{14}C]*trans*-caffeic acid (Figure 3.1) over a 72 h period following ingestion of the labeled hydroxycinnamate by male Sprague-Dawley rats.

3.2 Material and methods

3.2.1 Synthesis of [3-^{14}C]*trans*-caffeic acid

[3-^{14}C]*trans*-Caffeic acid (Figure 3.1) (specific activity 2.2 mCi/mmol) was synthesized by Dr. S.T. Caldwell and DR. R.C. Hartley (Department of Chemistry, University of Glasgow) as described by Omar et al.(2012).

3.2.2 Chemicals and reagents

trans-Caffeic acid was obtained from AASC Ltd. (Southampton, UK) and *cis*-caffeic acid was prepared by exposing a methanolic solution of *trans*-caffeic acid to UV light (Hartley and Buchan, 1979). HPLC grade methanol and acetonitrile were purchased from Rathburn Chemicals (Walkerburn, UK). Formic acid and scintillation cocktail (Optiflow Safe One) were supplied by Fisher Scientific (Leicestershire, UK). Tissue solubilizer was obtained from National Diagnostic (Hull, UK).

3.2.3 Rats and feeding procedures

The rat feeding and samples collection (urine, faeces, blood, plasma, cage washing, tissues, organs, and GI tract) were carried out at the University of Montpellier by Dr. Cyril Auger and Dr. Jean-Max Rouanet (Omar et al., 2012). In brief, the description of animal handling are as follows.Sprague-

Dawley rats (Iffa Credo, L'Arbresle, France) weighing ~250 g, handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and NIH guidelines, were housed individually in stainless steel metabolic cages with free access to deionised water.They were kept under a 0700 to 1900 h light regime, at a temperature of 23 ± 1°C, with constant humidity.

The rats were deprived of food for 16 h before being fed by gavage with 0.5 mL of water/ethanol (92:8, v/v) containing of 1.52 mg (140 x 10^6 dpm) [3-^{14}C]*trans*-caffeic acid, a dose equivalent to 6.08 mg/kg body weight. At the following time points: 1, 3, 6, 12, 24, 48, and 72 h, three rats were anaesthetized with pentobarbital (60 g/L pentobarbital, 60 mg/kg body weight). Blood was drawn by cardiac puncture with a heparin-moistened syringe and plasma and erythrocytes were separated by centrifugation at 2300 *g* for 10 min at room temperature, before being frozen in liquid nitrogen and stored at –80°C. The whole body tissues were perfused *in situ* with chilled 0.15 M NaCl to remove residual blood, after which brain, heart, lungs, kidneys, liver, testes, muscle and spleen were removed, rinsed in saline, blotted dry, frozen and stored at –80 °C. The gastrointestinal (GI) tract was removed intact along with its contents, and then separated into stomach, duodenum, ileum/jejunum, caecum and colon. All tissues were weighed before being frozen. Faeces and urine were collected from each rat during the course of the study and they too were frozen and stored at –80°C. Samples were shipped to the University of Glasgow on dry ice. Upon arrival tissues were freeze-dried, weighed, ground to a powder and stored at –80°C, along with plasma and urine prior to analysis. The weight of all freeze-dried

organs and total volumes of liquid samples are summarized in the Appendix 3.1 and 3.2.

3.2.4 Measurement of total radioactivity in tissues and body fluids

Ten milligram aliquots of powdered freeze-dried samples and 100 μL volumes of plasma and urine were treated overnight with 1 mL of tissue solubilizer at 50°C in shaking water bath. This produced clear solutions to which was added 10 mL of scintillation cocktail before the determination of radioactivity using a LS 6500 multi-purpose liquid scintillation counter (Beckman, USA).

3.2.5 Sample preparation

One hundred milligram aliquots of powdered tissues and 100 μL of plasma were extracted with 1.5 mL methanol:water (1:1, v/v) containing 0.1% formic acid. If only very low amounts of total radioactivity had been detected in a sample, it was not extracted and further processed. After mixing 30 min the extract was centrifuged at 1000 *g* for 10 min, the methanolic supernatant decanted and the pellet re-extracted. The two supernatants were combined, and concentrated to one third of volume in vacuo after which 10 μL aliquots were taken to measure radioactivity and ascertain the amount of sample required for analysis by HPLC with radioactivity counting (RC) and tandem mass spectrometry (MS^2) detection. Urine was analyzed directly without further processing.

3.2.6 HPLC analysis with PDA, radioactivity and MS^2 detection

HPLC was used with RC and MS^2 detection for identification and quantification of radiolabeled compounds as described previously (Mullen et al., 2002). Samples were analyzed on a Surveyor HPLC system comprising an autosampler cooled to 4 °C, an HPLC pump, and a PDA detector, scanning from 250 nm to 700 nm (Thermo Finnigan, CA). HPLC was carried out using a 250 mm x 4.6 mm i.d. 4 µm Synergi C_{18} RP-Max column (Phenomenex, Macclesfield, UK) maintained at 40 °C and eluted at flow rate 1 mL/min with a 60 min gradient of 5-40% acetonitrile in 0.1 % formic acid. After passing through the flow cell of the absorbance monitor, the column eluate was split and 0.3 mL directed to a Finnigan LCQ Duo tandem mass spectrometer with an electrospray interface in negative-ionisation mode operating in full scan mode from *m/z* 150 to 600. The remaining 0.7 mL of eluate was mixed at a "T" with scintillation cocktail pumped at 3.0 mL/min and directed to a radioactivity monitor (Reeve Analytical Model 9701, LabLogic, Sheffield, UK) fitted with a 1.0 mL homogeneous flow cell.

The mass spectrometer was operated in negative ionisation mode. Tuning was optimized by infusing a standard of ferulic acid dissolved in the initial HPLC mobile phase into the source at flow rate of 0.3 mL/min. Analyses were carried out using full scan, data-dependant MS^2 scanning from *m/z* 100 to 600. Capillary temperature was 300 °C; sheath and auxiliary gases were 80 and 60 units/min, respectively, and collision energy was set at 35%. Data was processed by Xcalibur software programme version 2.1. Each samples was analysed in triplicate and data were presented as mean values ± standard error (n=3).

3.3 Results

3.3.1 Distribution of radioactivity in rat GI tract

Three rats were sacrificed 1, 3, 6, 12, 24, 48, and 72 h after each ingested 140 x 10^6 dpm of [3-^{14}C]caffeic acid and the distribution of radioactivity in tissues, plasma, urine and faeces, as well as the cage washings, which consisted of a mixture of urine and faeces was determined. The data obtained are presented in Tables 3.1 and 3.2. In Table 3.1 data are presented as the amounts of radioactivity recovered in the various tissues and fluids while in Table 3.2 it is presented as radioactivity recovered as a percentage of the ingested 140 x 10^6 dpm of [3-^{14}C]caffeic acid. The radioactivity in the GI tract, tissues, organs, and plasma are the amounts present at each time point, while that in urine, faeces, and cage washings are the cumulative amounts that have left the body after ingestion.

One hour after ingestion, 62% of the ingested radioactivity remained in the stomach with 7.9% having moved to the duodenum and 9.1% reaching the jejunum/ileum. Overall 80% of the radioactivity was still present in the GI tract but after 3 h, this dropped to 45% of intake. After 3 h, the level in the stomach declined to 7.1% as radioactivity appeared in increased amounts in the jejunum/ileum, caecum and colon. After 6 h, radioactivity in the GI tract had fallen to 28% of the quantity ingested and was present mainly in the caecum and colon. At later time points the amount of radioactivity declined further and what remained was present in the distal rather than the proximal GI tract (Tables 3.1 and 3.2).

3.3.2 Distribution of radioactivity in rat plasma, urine, faeces and cage washings

After 1 h 2.4% of the ingested radioactivity was detected in plasma. However, there was rapid decline and at subsequent time points, radioactivity in the circulatory system (plasma and red blood cells) had fallen to <0.1% of intake (Tables 3.1 and 3.2). Over the 3-72 h period radioactivity in the GI tract fell from 45% to 0.3% of intake while there was a 68% excretion in urine. This indicates that the [^{14}C]caffeic acid-derived compounds, despite passing through the circulatory system in quantity do not accumulate to any extent as they are removed extremely rapidly by urinary excretion from the kidneys. The majority of the radioactivity in urine was excreted within 24 h of the ingestion of [3-^{14}C]caffeic acid.

The total radioactivity passing out of the body in faeces over the 72 h period after ingestion was 4.6% of intake, and even if the cage washings which comprised mainly of faecal material were added to this figure it is still much lower than the 68% excreted in urine.

Table 3. 1 Distribution of radioactivity (dpm x 10^3) in the tissues, GI tract, plasma, red blood cells, cage washing, urine and faeces 1-72 h after ingestion of 140 x 10^6 dpm of [^{14}C]*trans*-caffeic acid by rats [a]

Tissue	1 h	3 h	6 h	12 h	24 h	48 h	72 h
Stomach	87,349 ± 14,248	9,940 ± 8,426	260 ± 89	519 ± 275	105 ± 25	313 ± 263	108 ± 31
Duodenum	11,134 ± 2,672	11,325 ± 9,052	262 ± 70	408 ± 243	84 ± 6	78 ± 5	15 ± 3
Jejunum/Ileum	12,701 ± 1,460	22,343 ± 4,141	1,508 ± 308	1,891 ± 766	1,472 ± 851	960 ± 119	60 ± 6
Caecum	18 ± 4	8,446 ± 5,215	27,484 ± 19,727	5,441 ± 1,493	1,896 ± 320	440 ± 107	205 ± 96
Colon	34 ± 19	10,303 ± 7,683	9,273 ± 2,124	5,503 ± 2,839	2,593 ± 545	411 ± 194	57 ± 31
Subtotal GI tract	***111,236 ± 16,507***	***62,356 ± 12,761***	***38,787 ± 18,749***	***13,769 ± 2,760***	***6,150 ± 1,234***	***2,202 ± 521***	***444 ± 114***
Kidney	4,496 ± 1,257	1,678 ± 384	4,493 ± 2,956	195 ± 25	96 ± 24	49 ± 15	13 ± 1
Brain	9 ± 3	3 ± 0	4 ± 0	3 ± 2	1 ± 0	1 ± 0	1 ± 0
Testes	86 ± 36	20 ± 2	18 ± 3	8 ± 4	7 ± 4	1 ± 0	1 ± 0
Lungs	119 ± 18	54 ± 10	28 ± 5	6 ± 2	12 ± 6	3 ± 1	1 ± 0
Heart	81 ± 11	20 ± 5	18 ± 2	3 ± 9	5 ± 3	2 ± 1	60 ±1
Muscle	129 ± 42	69 ± 42	48 ± 19	9 ± 3	10 ± 3	4 ± 1	3 ±1
Liver	474 ± 40	142 ± 35	190 ± 44	50 ± 6	46 ± 17	13 ± 2	6 ± 0
Spleen	10 ± 2	2 ± 0	3 ± 1	0.4 ± 0	0.6 ± 0	0.8 ± 0	0.2 ± 0
Red blood cell	4 ± 2	34 ± 9	12 ± 4	17 ± 12	6 ± 6	0	0
Subtotal tissues (B)	***5,407 ± 1,340***	***2,021 ± 390***	***4,815 ± 2,953***	***293 ± 17***	***184 ± 41***	***75 ± 14***	***26 ± 2***
Total (A+B)	**116,643 ± 17,596**	**64,377 ± 13,128**	**43,601 ± 21,692**	**14,055 ± 2,762**	**6,333 ± 1,275**	**2,276 ± 531**	**470 ± 113**

Sample	0-1 h	0-3 h	0-6 h	0-12 h	0-24 h	0-48 h	0-72 h
Plasma	3,388 ± 3,299	41 ± 11	37 ± 5	5 ± 1	9 ± 8	2 ± 1	nd[b]
Urine	5,435 ± 1,868	11,157 ± 2,759	23,089 ± 1,651	24,178 ± 3,719[c]	87,363 ± 11,381	93,708 ± 33,689	95,597 ± 34,018
Faeces	nd	nd	nd	1,519 ± 1,463	1,928 ± 739	5,701 ± 1,265	6,419 ± 936
Cage washings	3,950 ± 3,667	2,841 ± 759	4,818 ± 1,693	12,531 ± 2,194	5,940 ± 1,883	7,153 ± 1,941	8,928 ± 2,143
Subtotal (C)	***12,773 ± 5,001***	***14,039 ± 3,298***	***27,944 ± 1,269***	***38,233 ± 2,539***	***95,240 ± 34,986***	***106,564 ± 35,498***	***110,944 ± 35,240***
Total (A+ B+C)	**129,417 ± 17,268**	**78,416 ± 10,685**	**71,546 ± 22,939**	**52,308 ± 4,866**	**101,573 ± 35,408**	**108,840 ± 34,968**	**111,414 ± 35,231**

[a]Radioactivity in the cage washings, urine and faeces are the cumulative amounts that have left the body since ingestion at 0 h. [b]nd = not detected. [c]This value is low because of spillage of an undetermined volume of urine during collection. 0-24, 0-48 and 0-72 h estimates based on a combination of data obtained with 0-12, 12-24, 24-48 and 48-72 h samples.

Table 3. 2 Percentage distribution of radioactivity expressed as a percentage intake in tissues, GI tract, plasma, red blood cells, cage washing, urine and faeces 1-72 h after ingestion of 140 x 10^6 dpm of [^{14}C]trans-caffeic acids by rats [a].

Tissue	1 h	3 h	6 h	12 h	24 h	48 h	72 h
Stomach	62 %	7.1%	0.2%	0.4%	0.1%	0.2%	0.1%
Duodenum	7.9%	8.1%	0.2%	0.3%	0.1%	0.1%	<0.1%
Jejunum/Ileum	9.1%	16%	1.1%	1.4%	1.1%	0.7%	<0.1%
Caecum	<0.1%	6.0%	19.6%	3.9%	1.4%	0.3%	0.1%
Colon	<0.1%	7.4%	6.6%	3.9%	1.9%	0.3%	<0.1%
Subtotal GI tract (A)	***80%***	***45 %***	***28 %***	***9.8%***	***4.4%***	***1.6 %***	***0.3%***
Kidney	3.2%	1.2%	3.2%	0.1%	0.1%	<0.1%	<0.1%
Brain	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
Testes	0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
Lungs	0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
Heart	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
Muscle	0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
Liver	0.3%	0.1%	0.1%	<0.1%	<0.1%	<0.1%	<0.1%
Spleen	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
Red blood cell	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	Nd
Subtotal tissues (B)	***3.9 %***	***1.4%***	***3.4%***	***0.2%***	***0.1%***	***0.1%***	***0%***
Total (A + B)	**83%**	**46%**	**31%**	**10%**	**4.5%**	**1.6%**	**0.3%**

Sample	0-1 h	0-3 h	0-6 h	0-12 h	0-24 h	0- 48 h	0-72 h
Plasma	2.4%	<0.1%	<0.1%	<0.1%	0.1%	<0.1%	nd[b]
Urine	3.9%	8.0%	16%	17%[c]	62%	67%	68%
Faeces	nd	nd	nd	1.1%	1.4%	4.1%	4.6%
Cage washings	2.8%	2.0%	3.4%	9%	4.2%	5.1%	6.4%
Total (C)	***9.1%***	***10%***	***20%***	***27%***	***68 %***	***76%***	***79%***
Total (A + B + C)	**92%**	**56%**	**51%**	**37%**	**73%**	**78 %**	**80%**

[a]Radioactivity in the cage washings, urine and faeces are the cumulative amounts that have left the body since ingestion at 0 h. [b]nd = not detected. [c]This value is low because of spillage of an undetermined volume of urine during collection. 0-24, 0-48 and 0-72 h estimates based on a combination of data obtained with 0-12, 12-24, 24-48 and 48-72 h samples.

3.3.3 Distribution of radioactivity in rat tissues

The amount of radioactivity detected in the various body tissues outside the GI tract are also presented in Tables 3.1 and 3.2. The quantities detected in brain, testes, lungs, heart, muscle, and spleen were minimal, <0.1% of intake. However 1.2–3.2% of intake was present in the kidneys with ~10-fold lower levels in the liver after 1 h and 3 h. Despite the 140 x 10^6 dpm intake of [3-^{14}C]caffeic acid the maximum amount of radioactivity transferred to the brain of the rats was a mere 9 x 10^3 dpm 1 h after ingestion (Table 3.1).

3.3.4 Identification of radiolabeled compounds

Aliquots of methanolic extracts of urine, faeces and sections of the GI tract containing >20 x 10^3 dpm of radioactivity were analyzed with HPLC-PDA-RC-MS^2 and MS^3 the consecutive reaction monitoring (CRM). The data obtained from MS^2 and MS^3 daughter ions were then compared with the available standards for *trans*- and *cis*-caffeic acid and in the case of other metabolites were referred to published MS^n fragmentation patterns (Poquet et al., 2008; Stalmach et al., 2009; Farrell et al., 2011). A total of 13 radiolabeled compounds were detected and nine were identified on the basis of the properties summarized in Table 3.3. These are described below.

Peaks 3 (t_R- 16.8 min) and 6 (t_R- 18.3 min) had a negatively charged molecular ion ($[M-H]^-$) at *m*/*z* 355 which yielded an M–176 daughter ion at *m*/*z* 179 which equates with the loss of a glucuronide moiety, and a further MS^2 fragment at *m*/*z* 135 corresponding to caffeic acid. These metabolites were detected in urine, and in keeping with data of Farrell et al.(2011), were

tentatively identified as a caffeic acid-4′-*O*-glucuronide (peak 3) and caffeic acid-3′-*O*-glucuronide (peak 6).

Peaks 4 (t_R- 18.1 min) and 5 (t_R- 19.2min) both having a $[M\text{-}H]^-$ at *m/z* 179, which upon MS^2 produced an ion at *m/z* 135, were caffeic acid constituents. Based on cochromatography with reference compounds, the earlier eluting peak 4 was identified as *trans*-caffeic acid, the radiolabeled substrate ingested by the rats, while the later eluting minor component, peak 5 was *cis*-caffeic acid. Both compounds were detected in the GI tract at the 1 h and 3 h time points.

Peak 7 (t_R- 20.2 min), which was detected in urine had a $[M\text{-}H]^-$ at *m/z* 369, that produced an MS^2 ion *m/z* 193, a 176-*amu* loss indicative of cleavage of a glucuronide moiety.The fragmentation pattern and chromatographic properties suggest this peak is ferulic acid-4′-*O*-glucuronide and this was confirmed by cochromatography with a chlorogenic acid metabolite previously identified as ferulic acid-4′-*O*-glucuronide by Stalmach et al.(2009).

Peaks 8 (t_R- 22.8 min) and 9 (t_R- 25.4 min) both had a $[M\text{-}H]^-$ at *m/z* 259, which upon MS^2 produced an ion at *m/z* 179, with an 80 amu loss of a sulphate unit. Peak 8 was identified as caffeic acid-4′-*O*-sulphate and peak 9 as caffeic acid-3′-*O*-sulphate on the basis of cochromatography with previously identified chlorogenic (Stalmach et al., 2009).

Peak 10 (t_R- 26.1 min) and peak 11 (t_R- 29.7 min) both had a $[M\text{-}H]^-$ at *m/z* 273, which produced a daughter ions at *m/z* 193, an 80 *amu* loss of sulphate. The identity of these metabolites were confirmed as ferulic acid-4′-*O*-sulphate (peak 10) and isoferulic acid-3′-*O*-sulphate (peak 11) on the basis

of cochromatography with a previously identified metabolite of chlorogenic acids (Stalmach et al., 2009).

It was not possible to identify peak 1, 2, 12 and 13 (see Figure 3.2, 3.3 and 3.4), named respectively as unknowns A, B, C and D, since they did not ionise and produce recognizable mass spectra. Unknown A and D were detected in caecum and colon, unknown B in the faeces, and unknown C in urine (Table 3.3).

3.3.5 Radiolabeled compounds in the GI tract

HPLC-RC profiles of radioactivity in the various segments of the GI tract 1, 3, and 6 h after [^{14}C]*trans*-caffeic intake are illustrated in Figure 3.2. The amounts of the various radiolabeled compounds detected are expressed both as dpm x 10^3 and as a percentage of the total radioactivity of each segment of the GI tract in Table 3.4.

One hour after ingestion of [^{14}C]*trans*-caffeic acid most radioactivity remained in the GI tract. The main components in the stomach, duodenum, and ileum were the unmetabolized trans isomer (76-87%) along with smaller amounts of *cis*-caffeic acid (13-24%). Three hours after intake the radioactivity had progressed down to the distal GI tract with *trans*-caffeic acid remaining the main radiolabeled component in the ileum (72%) and caecum (30%). The ileum also contained *cis*-caffeic acid (16%). The caecum, contained unknown A (41%), unknown D (11%), and caffeic acid-3′-*O*-sulphate (18%). No radiolabeled compound was present in the colon in detectable amounts at this stage.

Six hours after [^{14}C]*trans*-caffeic acid intake most of the radioactivity remaining in the GI tract was in the caecum and colon in the form of the unknown metabolite D, peak 13 (Figure 3.2, Table 3.4). At the later 12, 24, 48, and 72 h time points only traces of radioactivity remained in the GI tract with no metabolites present in sufficient quantities to facilitate identification (Figure 3.3).

3.3.6 Radiolabeled compounds in urine and faeces

Radioactivity in urine and faeces (Tables 3.1 and 3.2) are the cumulative amounts that had been excreted from the body of the rats after ingestion of [^{14}C]*trans*-caffeic acid. HPLC-RC profiles of radiolabeled metabolites detected in urine are illustrated in Figure 3.4, with mean quantitative data presented in Table 3.5.

In the first hour after intake, seven radiolabeled metabolites were excreted in urine. These were caffeic acid-4′-*O*-glucuronide (38%), caffeic acid-3′-*O*-glucuronide (2.1%), ferulic acid-4′-*O*-glucuronide (3.2%), caffeic acid-4′-*O*-sulphate (16%), caffeic acid-3′-*O*-sulphate (11%), ferulic acid-4′-*O*-sulphate (28%) and isoferulic acid-3′-*O*-sulphate (1.4%).

Analysis of the urine samples, detected the presence of unmetabolized *trans*-caffeic acid in the 0-3 h, 0-6 h, 0-12 h and 12-24 h samples. Caffeic acid-3′-*O*-glucuronide and ferulic acid-4′-*O*-glucuronide were detected only in 0-1 h urine. Caffeic acid-4′-*O*-sulphate, caffeic acid-3′-*O*-sulphate, ferulic acid-4′-*O*-sulphate, and the smaller quantities of isoferulic acid-3′-*O*-sulphate were all excreted in the 0-1 h urine and the amounts remained relatively constant in the 0-3 h, 0-6 h and 0-12 h samples but declined in the 0-24

urine. Caffeic acid-4′-*O*-glucuronide followed a similar profile, but it excretion was more prolonged with relatively high amounts in the 12-24 h urine. None of these metabolites were excreted after 24 h and the 24-48 h and 48-72 h samples contained only low levels of the unknown metabolite C in quantifiable amounts (Figure 3.4, Table 3.5).

The 0-12 h and 0-24 h faeces contained the unknown metabolite B (Figure 3.3, Table 3.5), and although increased amounts of radioactivity were present in the 24-48 and 48-72 h faecal material, no metabolites were present in sufficient amounts to facilitate identification and quantification.

Table 3. 3 HPLC-MS^2 identification of *trans*-caffeic acid metabolites in extracts of tissues and body fluids after ingestion of 140 x 10^6 dpm [^{14}C]trans-caffeic acid by rats [a].

Peak No	HPLC t_R (min)	Compound	[M-H]$^-$ (m/z)	MS^2 (m/z)	MS^3 (m/z)	Location
1	15.6	unknown A	-			Caecum
2	16.4	unknown B	-			Faeces
3	16.8	caffeic acid-4′-*O*-glucuronide	355	179	135	urine
4	18.1	*trans*-caffeic acid[a]	179	135		GI tract, urine
5	19.2	*cis*-caffeic acid[a]	179	135		GI tract
6	18.3	caffeic acid-3′-*O*-glucuronide[a]	355	179	135	urine
7	20.2	ferulic acid-4′-*O*-glucuronide[a]	369	193, 113, 175		urine
8	22.8	caffeic acid-4′-*O*-sulphate[a]	259	179	135	urine
9	25.4	caffeic acid-3′-*O*-sulphate[a]	259	179	135	jejunum/ileum,
10	26.1	ferulic acid-4′-*O*-sulphate[a]	273	193	178, 149, 134	urine
11	29.7	isoferulic acid-3′-*O*-sulphate[a]	273	193	178	caecum, urine
12	30.4	unknown C	-			urine
13	43.0	unknown D	-			caecum, colon

[a]Indicates cochromatography with a reference compound.

Table 3. 4 Levels of ^{14}C-labeled compounds in the GI tract 1, 3, 6, and 12 h after ingestion of 140 x 10^6 dpm of [^{14}C]*trans*-caffeic acid by rats [a].

Compound (Peak)	1 h Stomach	1 h Duodenum	1h Ileum	3 h Ileum	3 h Caecum	6 h Caecum	6 h Colon	12 h Caecum	12 h Colon
unknown–A (1)	nd[b]	Nd	nd	nd	3,460 *(41%)*	nd	nd	Nd	nd
trans-caffeic acid (4)	73,681 *(87%)*	9,060 *(81%)*	9,712 *(76%)*	16,128 *(72%)*	2,538 *(30%)*	nd	nd	Nd	nd
cis-caffeic acid (5)	11,739 *(13%)*	2,075 *(19%)*	2,988 *(24%)*	3,567 (*16%)*	n.d.	nd	nd	Nd	nd
caffeic acid-4′-*O*-sulphate (8)	nd	Nd	nd	nd	n.d.	nd	nd	Nd	nd
caffeic acid-3′-*O*-sulphate (9)	nd	Nd	nd	2,647 *(12%)*	1,536 *(18%)*	nd	nd	Nd	nd
unknown –D(13)	nd	Nd	nd	n.d.	912 *(11%)*	27,484 (*100%)*	9,272 *(100%)*	5,441 *(100%)*	5,502 (100%)

[a]Data expressed as dpm x 10^3 and in parentheses as a percentage of the radioactivity in each sample at individual time point. [b]nd= not detected.

Table 3. 5 Levels of ^{14}C-labeled metabolites in urine 0-72 h and faeces 12 and 24 h after ingestion of 140 x 10^{6} dpm of [^{14}C]*trans*-caffeic acid by rats[a].

	Urine							Faeces	
Compound (Peak)	**0-1 h**	**0-3 h**	**0-6 h**	**0-12 h**	**0-24 h**	**24-48 h**	**48-72 h**	**0-12 h**	**0-24 h**
caffeic acid-4'-*O*-glucuronide (3)	2,084 *(38%)*	1,536 *(14%)*	3,308 *(14%)*	5,671 *(24%)*	35,489 *(42%)*	nd[b]	nd	Nd	nd
unknown–B (2)	nd	nd	nd	nd	nd	nd	nd	1,519 *(100%)*	1,928 *(100%)*
trans caffeic acid (4)	nd	2,437 *(22%)*	7,466 *(32%)*	6,185 *(26%)*	8,358(*9.9%)*	nd	nd	nd nd	nd
caffeic acid-3′-*O*-glucuronide (6)	112 *(2.1%)*	nd	nd	nd	nd	nd	nd	Nd	nd
ferulic acid-4′-*O*-glucuronide (7)	171 *(3.2%)*	nd	nd	nd	nd	nd	nd	Nd	nd
caffeic acid-4′-*O*-sulphate (8)	875 *(16%)*	2,242 *(20%)*	2,995 *(13%)*	3,073 *(13%)*	6,813 *(8.1%)*	nd	nd	nd	nd
caffeic acid -3′-*O*- sulphate(9)	569 *(11%)*	1,364 *(12%)*	2,563 *(11%)*	3,506 *(15%)*	7,383 *(8.8%)*	nd	nd	nd	nd
ferulic acid -4′-*O*- sulphate (10)	1,545 *(28%)*	3,169 *(28%*)	5,642 *(24%)*	4,691 *(19%)*	9,693 *(12%)*	nd	nd	nd	nd
isoferulic acid-3′-*O*-sulphate (11)	684 *(1.4%)*	409 *(4.8%)*	1,114 *(5%)*	1,052 *(4.4%)*	nd	nd	nd	nd	nd
unknown–C (12)	nd	nd	nd	nd	16,627*(20%)*	6,345 *(100%)*	1,889 *(100%)*	nd	nd

[a]Data expressed as dpm x 10^{3} and in parentheses as a percentage of the radioactivity in each sample at individual time point. nd-not detected.

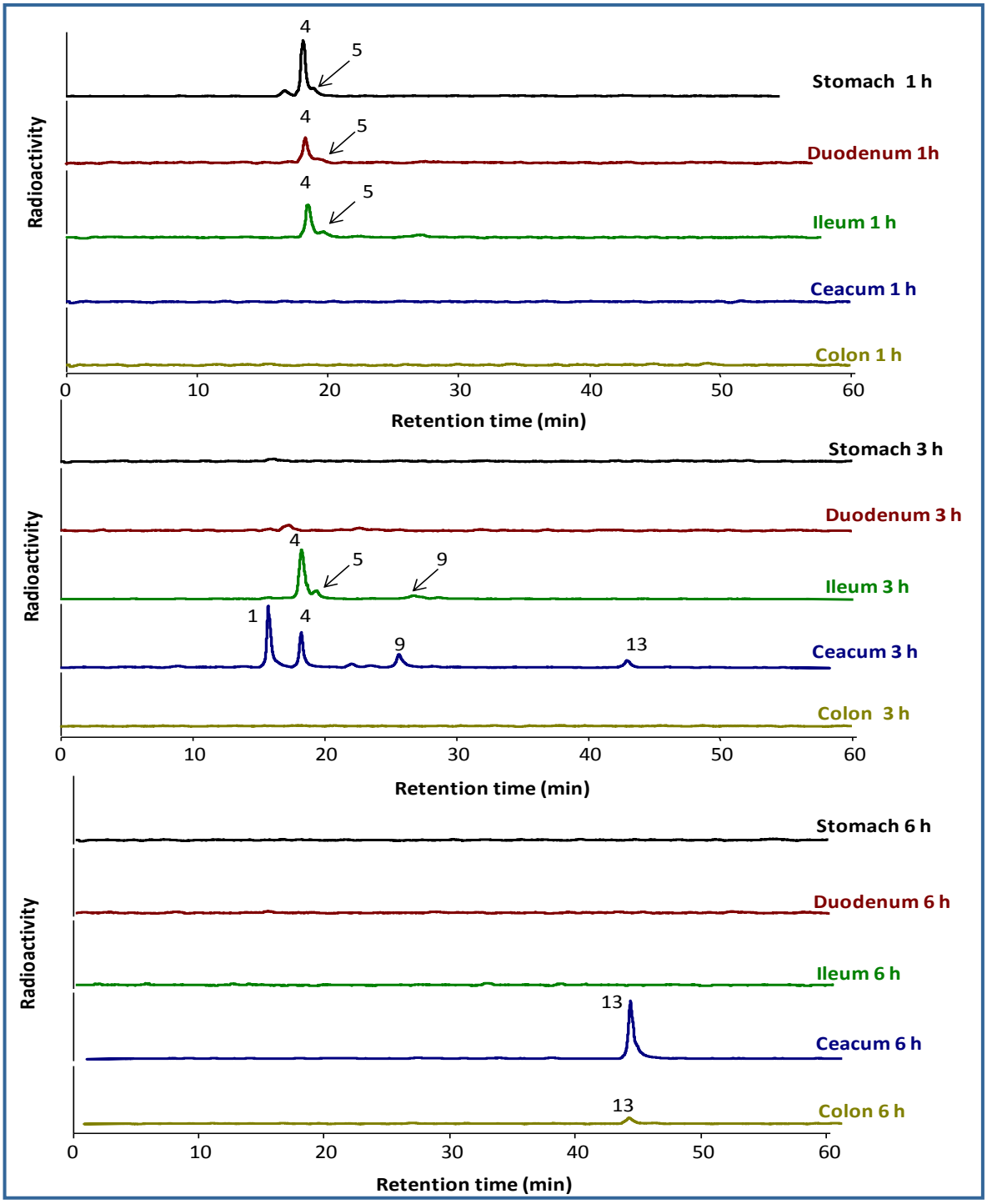


Figure 3. 2 HPLC-RC analysis of radiolabeled compounds in the individual sections of the GI tract rats, 1, 3, and 6 h after the ingestion of [^{14}C]caffeic acid. For the identification of numbered peaks see **Table 3.3.**

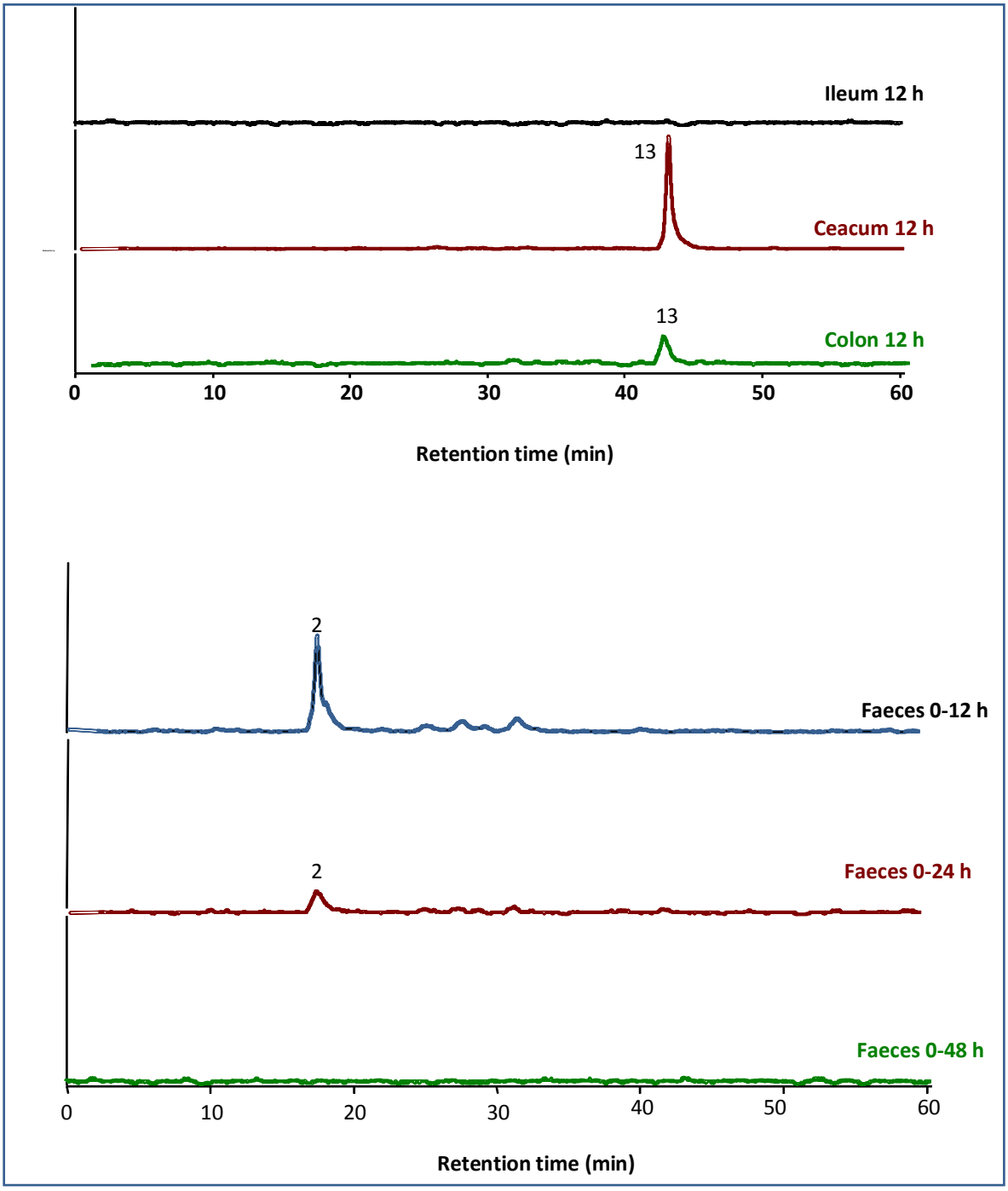


Figure 3. 3 Radiolabeled compounds in the GI tract and faeces at 12, 24, and 48 h after the ingestion of [^{14}C]caffeic acid. For the identification of numbered peak see **Table 3.3**

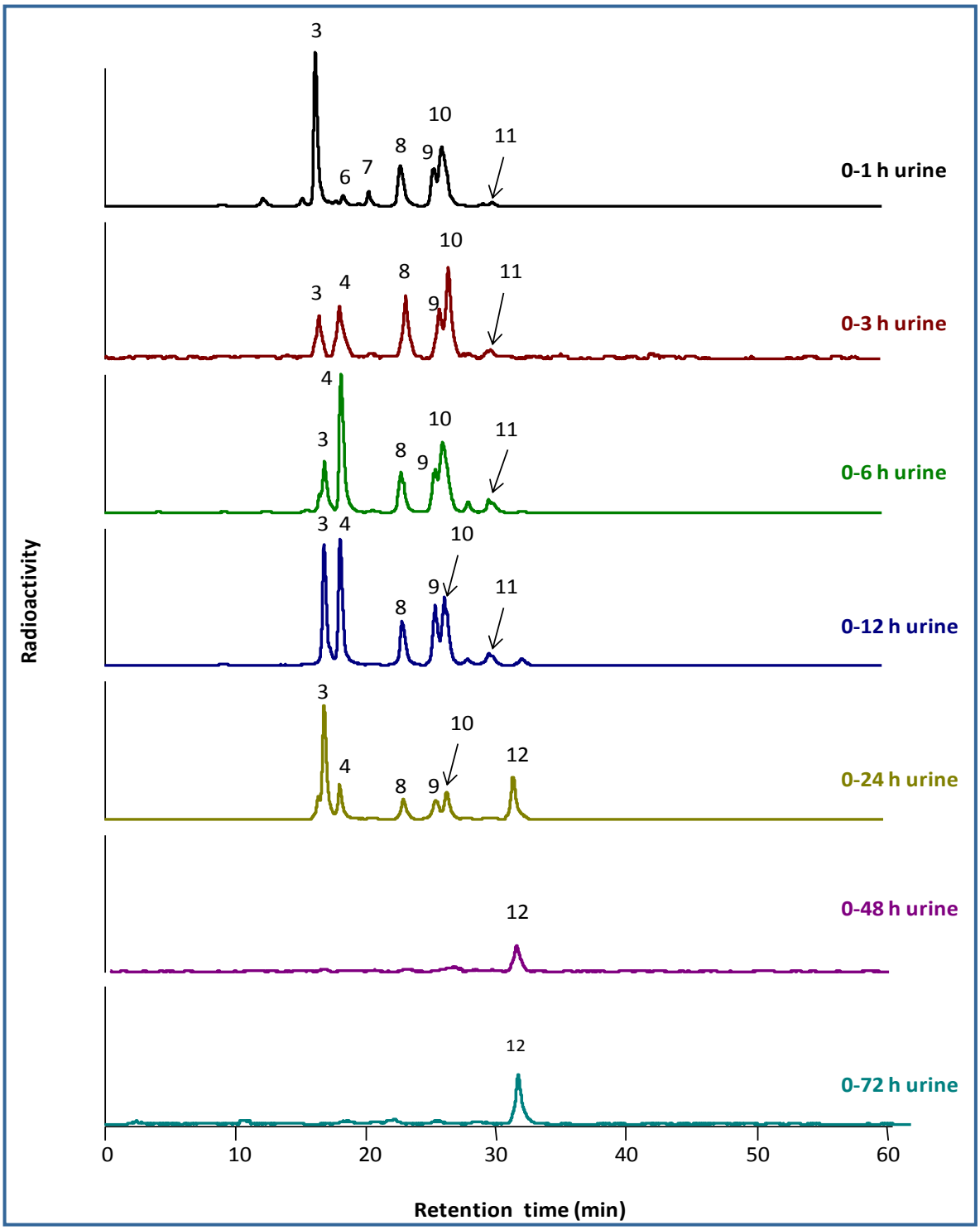


Figure 3. 4 HPLC-RC analysis of radiolabeled compounds in the urine 0-72 h after ingestion [^{14}C]caffeic acid. For the identification of numbered peak see **Table 3.3.**

3.3.7 Radiolabeled compounds in kidney, liver and other tissues

The amount of the radioactivity accumulated in tissues outside GI tract was minimal throughout the study with most being detected in the kidneys 1, 3 and 6 h after the intake (1.2–3.2%). Despite the high amount of the radioactivity excreted in the urine, only trace amounts of radioactivity were detected in plasma. Due to low level of radioactivity in all these samples, no metabolites were identified by HPLC-RC-PDA-MS^2.

3.4 Discussion

The study was designed to obtain the information on the absorption, metabolism, and overall bioavailability of [^{14}C]*trans*-caffeic acid following its ingestion by rats. Based on the data obtained from HPLC-PDA-RC-MS^2 analyses, information on the metabolism and catabolism of the radiolabeled substrate as it passed through the GI tract and was excreted in urine and, to a lesser degree, faeces are presented in Tables 3.4 and 3.5 and summarised in Figure 3.5.

After 1 h, most of the radioactivity remained in the stomach in the form of *trans*-caffeic acid and its *cis* isomer. Smaller amounts of these compounds were also present in the duodenum and the ileum/jejunum. At this point, the plasma contained 2.4% of the ingested radioactivity indicating some absorption in the small intestine (Renouf et al., 2010), however no metabolite was present in identifiable quantities. Urine collected in the first hour contained caffeic acid metabolites, indicative of absorption in the proximal GI tract. The metabolites were caffeic acid-4′-*O*-glucuronide, caffeic acid-3′-*O*-glucuronide, ferulic acid-4′-*O*-glucuronide, caffeic acid-3′-*O*-

sulphate, caffeic acid-4′-*O*-sulphate, ferulic acid-4′-*O*-sulphate, and smaller amounts of isoferulic acid-3′-*O*-sulphate which reflect phase II metabolism of caffeic acid upon its removal from the lumen of the GI tract, possibly in the intestinal epithelium or the liver. Previous published results have demonstrated that caffeic acid incubation with human hepatoma HepG2 cells produced methylated, glucuronidated and sulphated conjugates, which suggested the phase II metabolism occurs in the liver (Mateos et al., 2006). The same pattern of caffeic acid metabolism was also observed in rat hepatocytes and isolated microsomal fractions (Moridani et al., 2002).

Absorbed caffeic acid are subjected to conjugation by various enzymes before entering the blood stream: methylation by COMT catalyses the transfer of a methyl group S-adenosyl-L-methionine to (poly)phenols, which activity is highest in the liver and kidneys, sulfation by SULT and β-glucuronidation by UDP-glucoronosyltransferases, a membrane bound enzymes that catalyze the transfer of glucuronic acid (Manach et al., 2004; Williamson and Clifford, 2010). In humans, 15 isoforms of UDP-glucuronosyltranferases have been identified, one known as UGT1A located in the intestine, may play an important role in the first-pass metabolism of (poly)phenols (Fisher et al., 2001).

In the jejunum/ileum *trans*- and *cis*-caffeic acid were the main compounds detected after 1 and 3 h of ingestion of ^{14}C-caffeic acid. At this juncture, the main radioactive compound in the caecum was *trans*-caffeic acid along with smaller amounts of caffeic acid-3′-*O*-sulphate and the unidentified compounds, metabolites A and D. Radioactivity in the caecum and colon at 6 and 12 h was exclusively in the form of the unknown

metabolite D while the unknown B was the sole radiolabeled metabolite to be detected in faeces.

Urine collected 0-6 h after [^{14}C]*trans*-caffeic acid intake contained radiolabeled caffeic acid-4′-*O*-glucuronide, caffeic acid-3′-*O*-sulphate and caffeic acid-4′-*O*-sulphate, ferulic acid-4′-*O*-sulphate, isoferulic acid-4'-*O*-sulphate and caffeic acid. The increased amounts of radiolabeled caffeic acid-4′-*O*-glucuronide in the 0-12 and 0-24 h, and isoferulic acid-4′-*O*-sulphate in the 0-12 h, urine samples suggest that the formation of these derivatives may occur in the lower GI tract. Sulphates were the main hydroxycinnamate metabolites but it is not possible to determine the extent sulfation occurs in the wall of the small intestine and the colon or is hepatic in origin. Similarly in humans, sulfation is more dominant than glucuronidation for caffeic acid as shown with human intestinal S9 homogenates (Wong et al., 2010). SULT catalyze the transfer of sulphate moiety from 3′-phosphoadenosine-5′-phosphosulphate to a hydroxyl group of (poly)phenols and occur mainly in the liver (Falany, 1997; Piskula and Terao, 1998). However, later a human study has indicated that SULT 1A1 which is the most active isoform for sulfation of caffeic, isoferulic and dihydrocaffeic acid (Wong et al., 2010) is highly expressed in the liver and intestine (Teubner et al., 2007).

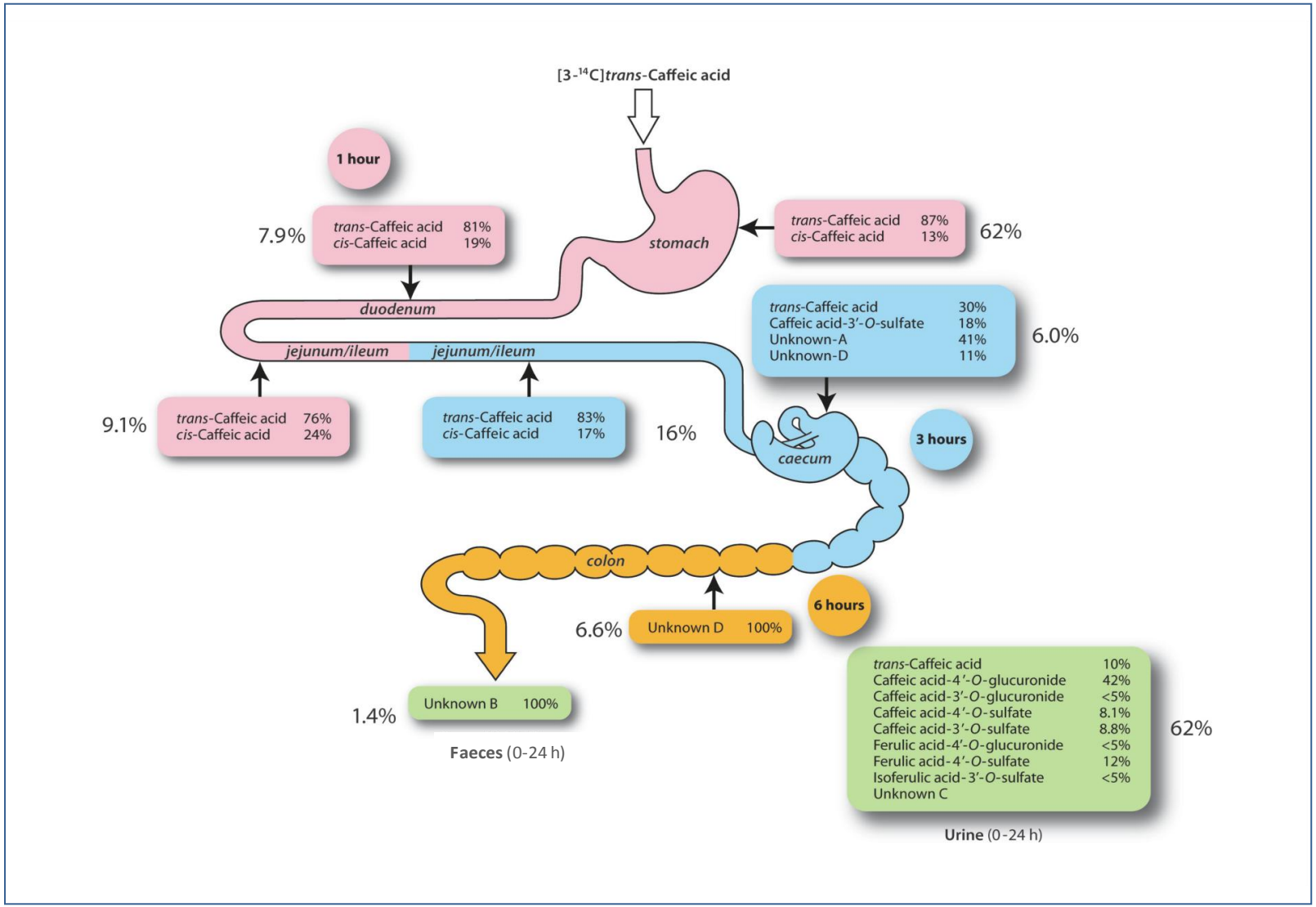


Figure 3. 5 Schematic of the metabolism of [^{14}C]caffeic acid in rats following ingestion, movement down the GI tract, and subsequent appearance of radiolabeled metabolites in faeces and excretion in urine. Values for caffeic acid and metabolites inside the boxes represent their percentage of total radioactivity in the individual sections of the GI tract and/or faeces and urine. Values outside the boxes indicate radioactivity as a percentage of the amount ingested.

Over the 72 h post-ingestion period, a total of 68% of the radioactivity was excreted in urine indicating that, despite the low levels in plasma, substantial amounts of radioactivity were passing through the circulatory system en route to the kidneys and excretion in urine. Urine and to the lesser extent faeces were reported to be the main route of radioactivity disposal from the body, where 48% and 68% radioactivity were recovered in the urine with 25% and 19% in the faeces of rats 24 h following the injection of [3-^{14}C]cinnamic and [2-^{14}C]ferulic acid (Teuchy and Van Sumere, 1971). High recovery of radioactivity (82-90%) was also observed in the urine of rats and mice after administration [3-^{14}C]-cinnamic acid (Nutley et al., 1994).

The urinary excretion and the low levels of radioactivity in faecal samples also demonstrate efficient absorption and high bioavailability of caffeic acid. Studies in humans which utilized coffee as main source for the hydroxycinnamic acids have provided the supporting evidence of the high bioavailability of these compounds where plasma concentration peaks were observed 0.5 h after the administration of green coffee and decaffeinated coffee, with more than 30-50% of the ingested moieties were recovered in plasma (Olthof et al., 2003; Monteiro et al., 2007; Farah et al., 2008) This is in keeping with the high urinary excretion of hydroxycinnamate metabolites after the consumption of caffeoylquinic acid-rich coffee by human volunteers (Stalmach et al., 2009).

The high absorption of caffeic acid observed in the study is in line with the fact that free form of hydroxycinnamic is more efficiently absorbed than its esterified form. Ingested caffeic acid in rats showed the maximum concentration of metabolites 100-fold greater than that of chlorogenic acid metabolites (Azuma et al., 2000; Olthof et al., 2001). Ferulic acid is reported to be

efficiently absorbed (up to 25%) from tomatoes in human, with 50% of its excreted by rats and the absorption markedly reduced to 3% when ingested with wheat bran (Bourne and Rice-Evans, 1998; Adam et al., 2002; Kern et al., 2003a; Mateo et al., 2009).

The proposed pathway for the metabolism of caffeic acid as it passes through the body of the rats is illustrated in Figure 3.6. *trans*-Caffeic acid underwent some degree of conversion to its *cis* isomer, as well as methylation, sulfation, and glucuronidation, events that were readily monitored through the use of a ^{14}C-labeled substrate. The routes are similar, but not identical, to those operating in humans after the ingestion of 5-*O*-caffeoylquinic acid (Stalmach et al., 2009; Stalmach et al., 2010b). In humans, methylation via COMT appears more extensive as evidenced by the occurrence of a more diverse spectrum of ferulic acid, isoferulic acid, dihydroferulic acid metabolites appearing in plasma and urine as major components (Rechner et al., 2001; Monteiro et al., 2007). Also in humans in the small intestine and colon, in contrast to rats, dehydrogenation of the hydroxycinnamate side chain occurs as indicated by formation of dihydrocaffeic acid and dihydroferulic acid metabolites (Peppercorn and Goldman, 1972). Arguably the absence of these metabolites in rats reflects differences in the composition of their colonic microflora. Humans, unlike rats, also produce feruloglycine as a caffeic and ferulic acid metabolite, and this is probably a reflection of differences in hepatic enzymes (Kern et al., 2003b).

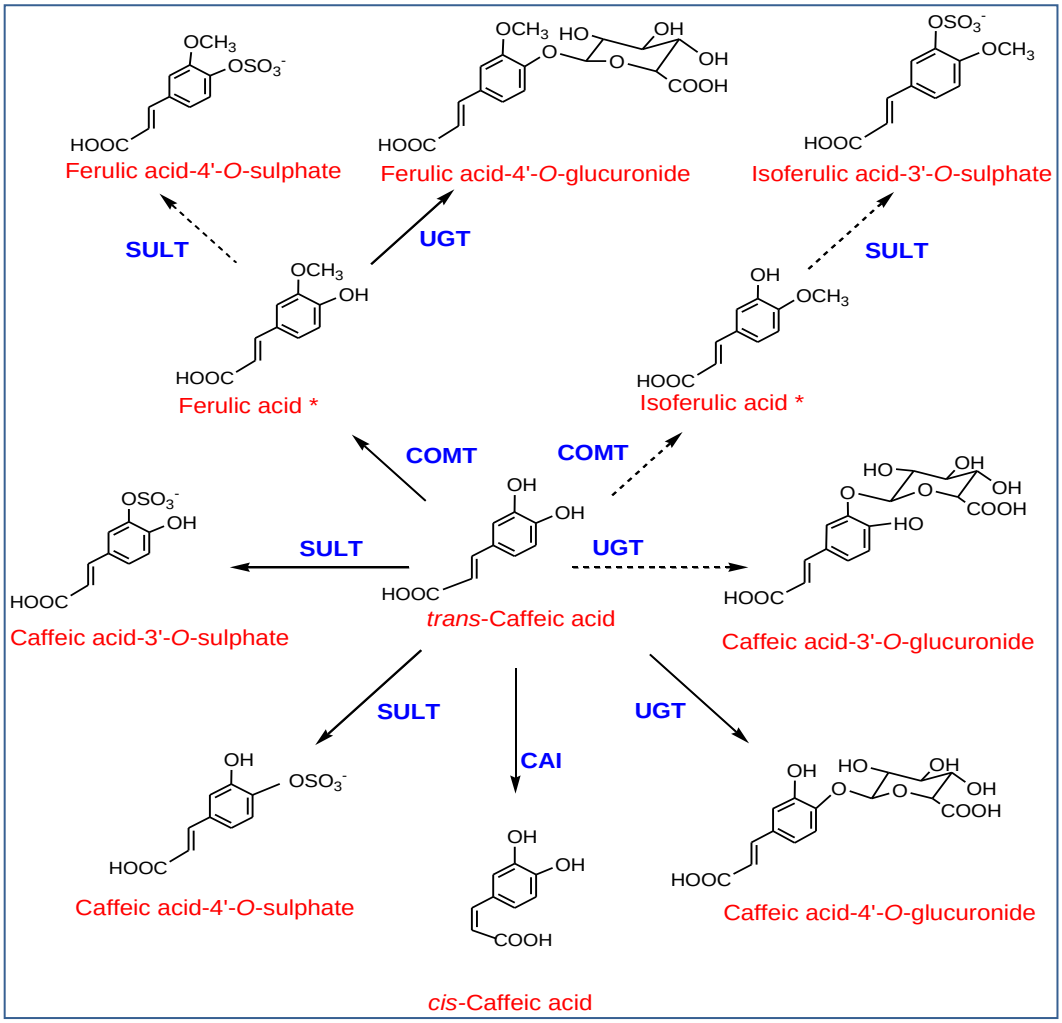


Figure 3. 6 Proposed routes of metabolism of *trans*-caffeic acid following ingestion by rats. CAI-caffeic acid isomerise, COMT-catechol-*O*-methyltransferase, SULT-sulfotransferases, UGT-uridine-5′-diphosphate-glucuronosyltransferase.

The formation of *cis*-caffeic acid in the stomach within 1 h is of interest, as it was not a contaminant of the [^{14}C]*trans*-isomer that was ingested by the rats. Possibly there is a mammalian or gut bacterial enzyme able to produce this transformation, but reduction to dihydrocaffeic acid and non-specific oxidation to yield both *cis*- and *trans*-caffeic acid is another possibility. However, the absence of dihydrocaffeic acid does not support this option.

The relative proportions change little after the bolus has left the stomach (Table 3.4), suggesting that the gut microflora are not involved. Although Poquet et al.(2008) have observed a putative *cis*-ferulic acid glucuronide, no radiolabeled metabolites of *cis*-caffeic acid were detected in the present study.

3.5 Conclusion

In conclusion this study has proved the advantages of using radiolabeled compound to monitor the absorption, disposition, metabolism, and excretion compound of interest in this case, caffeic acid. This is support by the evidence that ~80% of [3-^{14}C]caffeic acid intake has been recovered in rats. Caffeic acid is highly bioavailable and based on the pathway proposed, its metabolism in rats although is not identical, is following the same routes as in human.

4 CAFFEIC ACID METABOLISM BY COLONIC MICROFLORA

4.1 Introduction

Caffeic acid is the prominent hydroxycinnamic acid representing between 75-100% of the total hydroxycinnamic acid content of fruits either in the free form or esterified as in chlorogenic acids such as 5-*O*-caffeoylquinic acid (5-CQA) (Manach et al., 2004; Crozier et al., 2006). There is evidence indicating that 5-CQA is absorbed less readily in the small intestine than caffeic acid. Some esterase-catalysed hydrolysis of 5-CQA, releasing caffeic acid, occurs in the small intestinal mucosa but most progresses along the GI tract and enters the large intestine where it is subject to the action of the colonic microbiota (Booth et al., 1957; Scalbert et al., 2002). Caffeic acid released by hydrolysis of 5-CQA in the colon can be absorbed into the circulatory system but can also be subject the further metabolism prior to absorption (Chesson et al., 1999). There was evidence of absorption of intact 5-CQA in the stomach of rats (Lafay et al., 2006b) but in humans most evidence points to only limited absorption of 5-CQA per se (Stalmach et al., 2012).

In rats, ingested 5-CQA appears to be hydrolyzed in the upper part of the GI tract with caffeic acid and aromatic metabolites being rapidly detected in plasma (Azuma et al., 2000; Nardini et al., 2002). 5-CQA which is not hydrolysed passes to the colon where it will be hydrolyzed by the microflora releasing caffeic acid and quinic acid (Figure 4.1).

The colonic microflora will transform caffeic acid to dihydrocaffeic acid [aka 3-(3′,4′-dihydroxyphenyl)propionic acid], 3-(3′-hydroxyphenyl)propionic acid, 3-hydroxybenzoic acid, *m*-coumaric acid, hippuric and 3′-hydroxyhippuric acid. Dihydrocaffeic acid generated by gut microflora also associated with the coffee intake (Clifford, 2000). Many of these compounds have been considered to exert physiological effects with evidence for the prevention of different cancer and cardiovascular diseases being obtained in several studies (Tanaka et al., 1993; Jin et al., 2005).

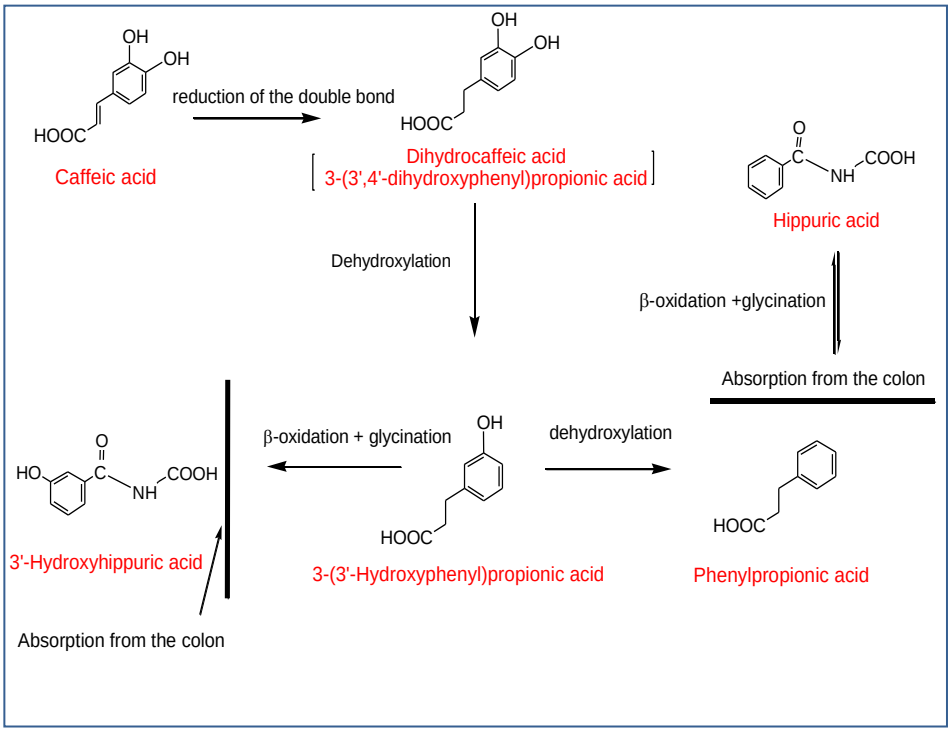


Figure 4. 1 Degradation of caffeic acid by colonic microflora.

Aims of the study

The aim of this study was to investigate the metabolism of caffeic acid during in vitro incubation with human colonic microflora. Analysis of catabolites was performed by GC-MS.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Caffeic acid was purchased from AASC Ltd (Southampton, UK). Analytical grade chemicals were used in preparing the buffer, macromineral, micromineral and reducing solutions. Manganese chloride, cysteine hydrochloride, sodium sulphate nonahydrate, resazurin were purchased from Sigma-Aldrich (Dorset, UK). Ammonium bicarbonate and calcium chloride were obtained from Fisher Scientfic (UK), while cobalt chloride and magnesium sulphate were supplied by VWR International (UK). HPLC grade methanol and formic acid were purchased from Fisher Scientfic Ltd (Leichestershire, UK).

4.2.2 Fermentation medium

The fermentation medium was prepared by mixing 2.5 g of tryptone in 450 mL of distilled water and 112.5 μL of micromineral solution (consisting of 13.2 g of $CaCl_2.2H_2O$, 10 g of $MnCl_2.4H_2O$, 1 g of $CoCl_2.6H_2O$, 8 g $FeCl_3.6H_2O$ and distilled water up to 100 mL). This mixture was agitated to dissolve the chemicals and then 225 mL of buffer solution (consisting of 2 g of NH_4HCO_3, 17.5 g $NaHCO_3$ and distilled water added up to 500 mL), 225 mL of macromineral solution (consisting of 2.85 g of Na_2HPO4, 3.1 g KH_2PO4, 0.3 g $MgSO_4.7H_2O$ and 500 mL distilled water was added) and 1125 μL of 0.1% (w/v) resazurin solution (a redox indicator) were added. This fermentation medium was adjusted to pH 7 using 6 M HLC and subsequently sterilised by boiling for a few minutes and allowed to cool under oxygen-free nitrogen (OFN) around 30 minutes to remove oxygen (medium colour changed from blue to pink). For each 10 mL of the

medium, 0.5 mL reducing solution (consisting of 312.5 mg of cysteine hydrochloride, 2 mL of 1 M NaOH, 312.5 mg $Na_2S.9H_2O$ and make up to 50 mL with distilled water) was added.

4.2.3 Subjects

Five subjects, (3 female and 2 male) all Asian, were recruited for the study, designated as volunteer 1 (V-1), volunteer 2 (V-2), volunteer 3 (V-3), volunteer 4 (V-4) and volunteer 5 (V-5). They were all non-smokers, aged between 25-40 years, had no history of gastrointestinal problems and had not taken antibiotics for at least three months prior to the study. The volunteers were asked to follow a low (poly)phenol diet for 48 h prior to faecal collection (See Appendix 4.1). All collected faecal samples were processed within 30 min of passage.

4.2.4 In vitro fermentation

The in vitro fermentation was performed according to the method previously described by (Jaganath et al., 2009) For each donor, 6.4 g of freshly voided faecal sample was homogenized with 20 mL of sodium phosphate buffer to obtain 32 % faecal slurry. Five mL of the slurry was added to 44 mL pre-reduced fermentation medium and 1 mL of substrate (with or without glucose) in 100 mL fermentation bottles (total volume of 50 mL). The substrates used were as follows:

Control- distilled water:

1. 55 µmoles of caffeic acid with 0.5 g of glucose.

2. 55 µmoles of caffeic acid without glucose

After the addition of the substrate, the fermentation bottles were purged with oxygen OFN and sealed airtight. Samples were placed horizontally in a shaking water bath at 60 strokes/min and incubated at 37°C for 48 h, aiming to stimulate the conditions in the colonic lumen. Aliquots of the fermented faecal samples (3-5 mL) were collected after 0, 1, 2, 4, 6, and 8 h and stored immediately at -80°C.

4.2.5 Extraction of phenolic acids for GC-MS analysis

Phenolic acid extraction, derivatization, and analysis of faecal slurries for GC-MS analysis were adapted after a method of Roowi et al.(2010). A 60 μg aliquot of 2,4,5-trimethoxycinnamic acid was added to 1 mL of faecal slurry. Samples were acidified by adding 300 μL of 1M HCL and partitioned twice against 1.5 mL volumes of ethyl acetate. The upper organic phases were combined, transferred to an amber glass vial and dried at 40°C under a stream of nitrogen. Samples were further dried by the subsequent addition and evaporation of 200 μL of dichloromethane. Three hundred microliter of *N,*methyl-*N*-(trimethylsily)trifluoroacetamide and pyridine mixture (1:4, v:v) was added to each vial, and the head space flushed with a gentle flow of nitrogen before sealing. Samples were derivatized at 80°C for 20 min for complete silylation. A set of standard calibrations solutions of phenolic acids ranging from 54-216 μg/mL was prepared and treated in a similar manner to the extracted samples.

4.2.6 GC-MS analysis of faecal slurries

Derivatized samples were analyzed with GC-MS (Agilent 6890 Series) equipped with a split/splitless injector and a Agilent 7683 autosampler. Separations were carried out on a ZB-5MS (30 m x 0.25 id x 0.25 μm) capillary

column (Phenomenex, UK) with helium carrier gas (1.2 mL/min). Derivatized samples (1 μL) were injected with 1 μL syringe into the GC injection port in split mode with a 1:25 ratio. The GC-MS conditions were as follows: The inlet temperature was maintained at 220°C. The oven was programmed from 40°C (held 0.1 min) to 160°C at 20°C/min, to 200°C at 1.5°C/min, to 250°C at 10°C to a final temperature of 300°C at 40°C/min held for 5 min. The transfer line was maintained at 310°C. Data analysis and acquisition were performed using MSD Chemstation software. Phenolic compounds were identified based on their retention time, mass spectra of authentic standards and NIST library screening.

4.2.7 Statistical analysis

All samples were analyzed in triplicate for GC-MS. Data are presented as mean values ± standard error (n=3). When appropriate, data were subjected to statistical analysis using (ANOVA) to determine the significance difference of glucose supplement between samples. Statistical analyses were performed using PSPP (free software).

4.3 Results

4.3.1 Degradation of caffeic acid incubated with faecal slurries

Analysis of the faecal slurries incubated with caffeic acid revealed the degradation of caffeic acid by the colonic microflora. The rate of caffeic acid degradation at 6 time points over 8 h periods during incubation with 55 μmoles of caffeic acid in 5 volunteers are presented in Figure 4.2.

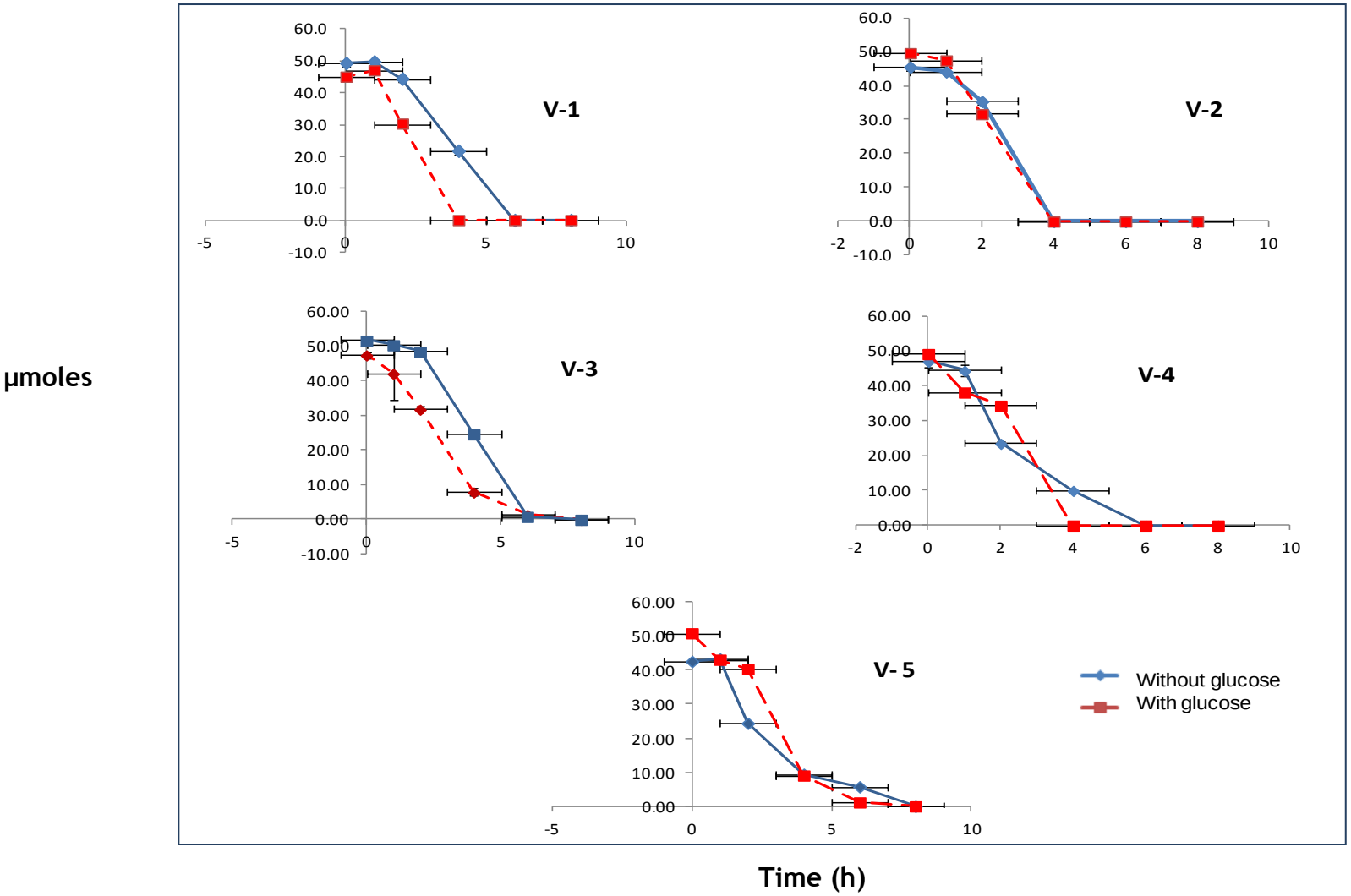


Figure 4. 2 Degradation of caffeic acid (55 µmoles).

Caffeic acid was rapidly degraded by human microbiota, from all the volunteers. The initial substrate dose was 55 µmoles, from which 77-89% (43-49 µmoles, without glucose) and 82-92% (46-52 µmoles, with glucose) of caffeic acid, was recovered at the initial 0 h time point. In the absence of glucose caffeic acid was completely metabolized at 6 h in samples from three volunteers, V-1, V-2 and V-5, and the clearance was enhanced with the addition of glucose as all the caffeic acid had disappeared. However, in V-3 and V-5 the disappearance of caffeic was not affected by the addition glucose where caffeic acid clearance was 8 h under both conditions.

Overall the results demonstrate the effect of glucose supplement was different among the five volunteers ($p< 0.5$). Based on the graph presented in Figure 4.3, only V-1 and V-3 showed clear effect of glucose supplement with the rate of degradation of caffeic acid with slower degradation of caffeic acid being enhanced. This is keeping with glucose acting as a carbon source for the bacteria (Wang and Gibson, 1993; Edwards et al., 1996) which enhanced colonic catabolism of caffeic acid, although the profiles varied substantially between individual volunteers.

4.3.2 Identification of phenolic acid catabolites

A summary of the four identified catabolites identified by GC-MS is presented in Table 4.1 and illustrated in Figure 4.3. 3-Phenylpropionic acid was detected in trace amounts in the faecal incubates but was also present in the control (distilled water) indicating that it was not a product of caffeic acid catabolism. It was therefore, not quantified.

Incubation with active human microbiota resulted in rapid degradation of caffeic acid. Disappearance of caffeic acid was associated with the appearance of three catabolites which were identified as phenylacetic acid, 3-(3′-hydroxyphenyl)propionic acid and dihydrocaffeic acid (aka 3′,4′,-dehyroxyphenyl)propionic acid). Accumulation of these metabolites are presented in Table 4.2, and illustrated in Figure 4.4 respectively.

Table 4. 1 GC-MS identification of phenolic acids detected in human faecal slurries after incubation with caffeic acid.

Compound	t_R (min)	Identified ion (*m/z*)	Qualifier ion (*m/z*)	Identification
Phenylacetic acid	6.59	73	164,193,91	Standard
3-Phenylpriopionic acid	7.75	104	73,207,91,222	Standard
3-(3'-Hydroxyphenyl)propionic acid	13.3	205	192,310,177,73	Standard
Dihydrocaffeic acid	20.8	179	398,267,73,280	Standard
Caffeic acid	29.4	396	73,219,307,384	Standard

Phenylacetic acid was detected in incubates from all volunteers with and without the addition of glucose. Low levels of this catabolite were detected as earlier at 0 h with the addition of glucose in all the volunteers. Its amount increased slightly over the time. In all volunteers the highest accumulation of this catabolite was between 6-8 h, with the amount varying depending upon the presence or absence of glucose. The highest amount of phenylacetic acid was detected in V-2 (21.2 µmoles) in the absence of glucose.

3-(3′-Hydroxyphenyl)propionic acid (3HPPA) was detected in incubates from all volunteers with and without the addition of glucose. This catabolite was estimated as the second product of caffeic catabolized during the incubation. It

was initially detected at 2 h. Its accumulation was enhanced by the presence of glucose, in samples from all volunteers. The highest amount of this catabolite was detected at 8 h, with the main producers being V-1 and V-2 (32 μmoles).

Dihydrocaffeic acid was the main catabolite detected during incubation of human faecal slurries with caffeic acid. In all volunteers the disappearance of caffeic acid was marked with a gradually increase of dihydrocaffeic acid, which was first detected at 1 h. The production of dihydrocaffeic acid was enhanced with the addition of glucose in all volunteers. The formation of dihydrocaffeic acid reached a peak between 4-6 h in faecal samples from all volunteers with the highest amount recorded being 45 μmoles in V-2 in the presence of glucose.

Overall the results demonstrate that caffeic acid degradation varied in the faecal samples from different individuals. The impact of the presence of glucose in the incubation medium also varied ($p< 0.5$). Based on the results presented in Table 4.2, the effect of glucose supplement varied between individuals and also in terms of formation of the catabolites. In the absence of glucose, the formation of phenylacetic acid was higher in V-1, V-2 and V-3, whereas in V-4 and V-5 the amount of phenylacetic acid was higher in the presence of glucose. In all volunteers except V-5, glucose addition increased the formation of 3-(3′-hydroxyphenyl)propionic acid. Interestingly, the formation of dihydrocaffeic acid was enhanced in all volunteers in the presence of glucose. Therefore, although the effect of glucose supplement varied between these Asian volunteers, it appears that the formation of dihydrocaffeic is enhanced by the addition of glucose to the faecal incubates.

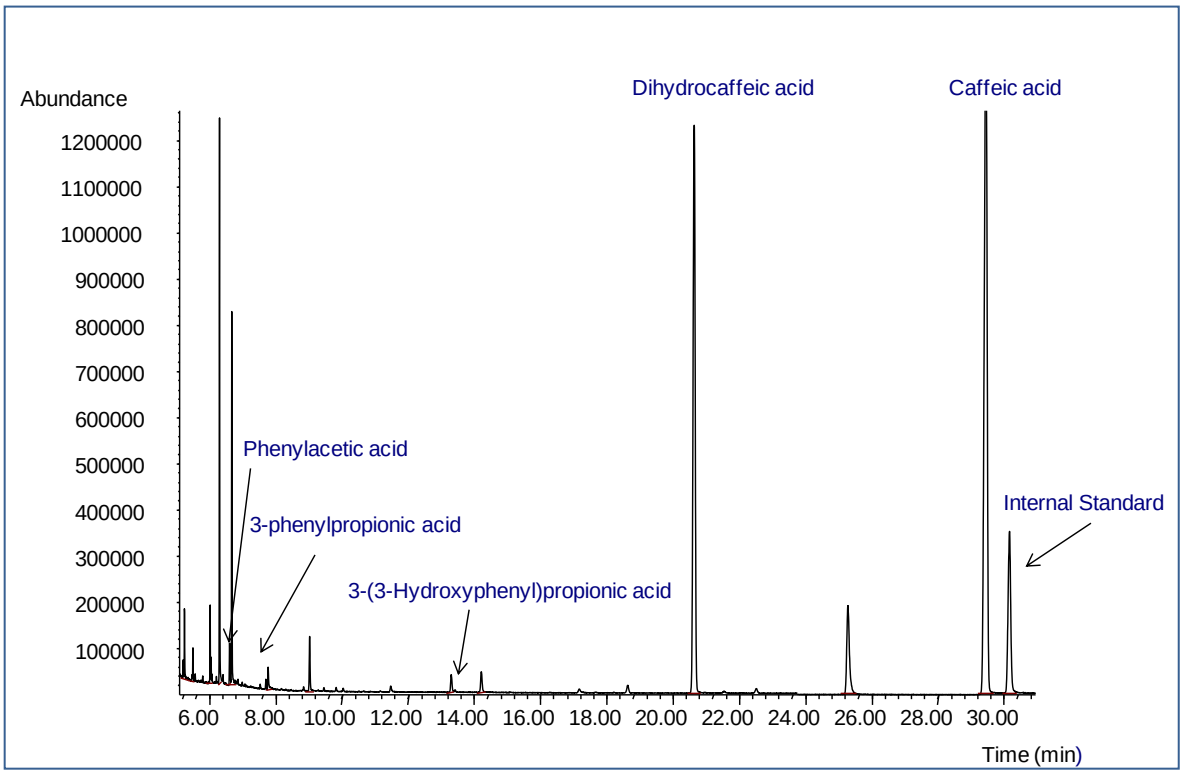


Figure 4. 3 Chromatogram of faecal material from V-1 (at 4 h) during incubation with caffeic acid analysed by GC-MS.

Volunteer	Metabolite	Glucose	0 h	1 h	2 h	4 h	6 h	8h
V-1	Caffeic acid	-	49 ± 5.9	50 ± 0.8	44 ± 1.7	22 ± 0.7	0	0
		+	*45 ± 1.2*	*47 ± 1.3*	*30 ± 2.5*	*0*	*0*	*0*
	Phenylacetic acid	-	0	0	1.0 ± 0.2	2.1 ± 0.1	4.9 ± 0.2	7.1 ± 0.1
		+	*0.7 ± 0.1*	*1.4 ± 0.1*	*2.1 ± 0.3*	*2.4 ± 0.2*	*2.9 ± 0.1*	*2.9 ± 0.1*
	3-(3′-hydroxyphenyl)propionic acid	-	0	0	0	2.8 ± 0.1	13 ± 0.7	32 ± 1.0
		+	*0*	*0*	*0.5 ± 0.0*	*4.0 ± 0.2*	*6.1 ± 0.1*	*8.8 ± 0.3*
	Dihydrocaffeic acid	-	0	0	4.4 ± 0.1	20 ± 0.7	30 ± 1.1	4.7 ± 0.1
		+	*0*	*0*	*10 ± 5.1*	*35 ± 1.5*	*37 ± 1.6*	*42 ± 1.7*
	Total	**-**	**49 ± 2.6**	**50 ± 0.4**	**50 ± 0.7**	**47 ± 0.2**	**48 ± 0.4**	**44 ± 0.4**
		+	***46 ± 0.5***	***48 ± 0.5***	***43 ± 2.0***	***42 ± 0.6***	***46 ± 0.7***	***53 ± 0.7***
V-2	Caffeic acid	-	46 ± 3.0	44± 2.5	35 ± 1.8	*0*	*0*	*0*
		+	*48 ± 3.3*	*48 ± 0.5*	*32 ± 2.5*	*0*	*0*	*0*
	Phenylacetic acid	-	0.4 ± 0.0	0.8 ± 0.0	1.5 ± 0.1	14 ± 5.6	18 ± 0.9	21 ± 0.5
		+	*0.5 ± 0.0*	*1.0± 0.0*	*1.5 ± 0.1*	*3.9 ± 0.3*	*5.4 ± 0.2*	*6.8 ± 0.4*
	3-(3′-hydroxyphenyl)propionic acid	-	0	0	0	*3.0 ± 0.1*	*20 ± 3.6*	*32 ± 6.7*
		+	*0.2± 0.2*	*0.3 ± 0.3*	*0.6 ± 0.5*	*1.6 ± 1.2*	*2.0 ± 1.7*	*2.6 ± 2.1*
	Dihydrocaffeic acid	-	0	0.3 ± 0.0	3.9 ± 0.2	25± 8.2	8.6 ± 0.1	0
		+	*0*	*2.7 ± 0.0*	*13 ± 0.3*	*45± 7.5*	*37 ± 2.9*	*32 ± 0.7*
	Total	**-**	**46 ± 1.3**	**45 ± 1.1**	**41 ± 0.7**	**41 ± 3.6**	**46 ± 0.5**	**53 ± 1.2**
		+	***50± 1.4***	***52 ± 2.1***	***47 ± 0.2***	***51 ± 3.1***	***44± 1.2***	***41 ± 0.8***
V-3	Caffeic acid	-	52 ± 5.5	50 ± 8.9	47 ± 5.7	25 ± 4.8	0.8 ± 0.1	0
		+	*48 ± 0.9*	*42 ± 7.8*	*32 ± 0.7*	*7.9 ± 1.1*	*1.4 ± 0.1*	*0*
	Phenylacetic acid	-	0	1.0 ± 0.2	1.6 ± 0.1	4.1 ± 0.6	6.7 ± 1.7	13 ± 0.9
		+	*0.4 ± 0.0*	*0.8 ± 0.0*	*1.5 ± 0.1*	*1.1 ± 0.9*	*4.1 ± 0.2*	*5.4 ± 0.5*
	3-(3′-hydroxyphenyl)propionic acid	-	0	0	0	0.6 ± 0.0	13 ± 5.0	15 ± 5.0
		+	*0*	*0*	2.4 ± 0.8	5.2 ± 0.9	9.8 ± 1.6	7.0 ± 0.5
	Dihydrocaffeic acid	-	*0*	*0*	*0*	*21 ± 5.8*	*26 ± 3.4*	*24 ± 1.8*
		+	0	0.3 ± 0.0	6.9 ± 0.3	29 ± 1.1	31 ± 0.6	30 ± 1.4
	Total	**-**	**52 ± 2.4**	**51 ± 3.8**	**50 ± 2.5**	**51 ± 2.5**	**46 ± 1.8**	**52 ± 1.9**
		+	***47 ± 0.4***	***43 ± 3.4***	***43 ± 0.3***	***43 ± 0.1***	***46 ± 0.6***	***42 ± 0.5***
V-4	Caffeic acid	-	47 ± 1.8	44 ± 1.5	23 ± 0.7	9.9 ± 0	0	0
		+	*49 ± 2.1*	*38 ± 0.3*	*34 ± 1.5*	*0*	*0*	*0*
	Phenylacetic acid	-	0	5.9 ± 0	2.5 ± 0.3	1.5 ± 0.0	5.1 ± 0.3	*11 ± 0.9*
		+	*0.4 ± 0.0*	*0.8 ± 0*	*2.8 ± 1.8*	*6.7 ± 5.5*	*14 ± 1.0*	*14 ± 0.3*
	3-(3′-hydroxyphenyl)propionic acid	-	*0*	*0*	*0.1 ± 0.0*	*0.1 ± 0.0*	*2.6 ± 0.2*	*14 ± 0.8*
		+	*0*	*0*	*1.2 ± 1.5*	*2.1 ± 1.5*	*1.3 ± 1.0*	*1.8 ± 2.0*
	Dihydrocaffeic acid	-	*0*	*0*	*17 ± 1.2*	*30 ± 0.7*	*33 ± 2.0*	*24 ± 2.6*
		+	*0*	*0.4 ± 0.0*	*13 ± 1.0*	*44 ± 2.7*	*35 ± 1.6*	*27 ± 4.3*
	Total	**-**	***47 ± 0.8***	***50 ± 0.6***	***43 ± 0.4***	***41 ± 0.3***	***40 ± 0.8***	***49 ± 0.9***
		+	***50 ± 0.9***	***39 ± 0.1***	***51 ± 0.2***	***52 ± 1.7***	***49 ± 0.6***	***43 ± 1.7***
V-5	Caffeic acid	-	*43 ± 2.1*	*43 ± 1.8*	*24 ± 5.9*	*9.4 ± 0.5*	*5.7 ± 1.1*	*0*
		+	51 ± 0.7	43 ± 3.2	40 ± 0.4	9.0 ± 0.5	1.2 ± 0.4	0
	Phenylacetic acid	-	*0*	*0.2 ± 0.1*	*0.4 ± 0.2*	*1.2 ± 0.1*	*1.0 ± 0.1*	*1.3 ± 0.4*
		+	*1.1 ± 0.3*	*1.8 ± 0.1*	*0.9 ± 0.1*	*4.6 ± 1.1*	*4.2 ± 2.9*	*3.1 ± 2.1*
	3-(3′-hydroxyphenyl)propionic acid	-	*0*	*0*	*0*	*1.4 ± 0.5*	*2.8 ± 0.8*	*9.0 ± 0.8*
		+	*0*	*0*	*0*	*0*	*2.8 ± 0.3*	*6.6 ± 1.1*
	Dihydrocaffeic acid	-	*0*	*0*	*23 ± 11*	*32 ± 6.0*	*33 ± 12*	*37 ± 11*
		+	*0*	*0*	*6.5 ± 0.7*	*27 ± 1.3*	*37 ± 4.7*	*31 ± 4.2*
	Total	**-**	***43 ± 0.9***	***43 ± 0.8***	***48 ± 4.6***	***44± 2.6***	***42 ± 5.0***	***47 ± 4.6***
		+	***52 ± 0.3***	***45 ± 1.4***	***48 ± 0.3***	***41± 0.5***	***46 ± 1.8***	***42 ± 1.5***

Table 4. 2 Degradation of caffeic acid (55 µmoles) and formation of catabolites in human faecal slurries. Data are expressed as µmoles ± standard error ; (+) incubated with 0.5 g glucose, (-) incubated without glucose.

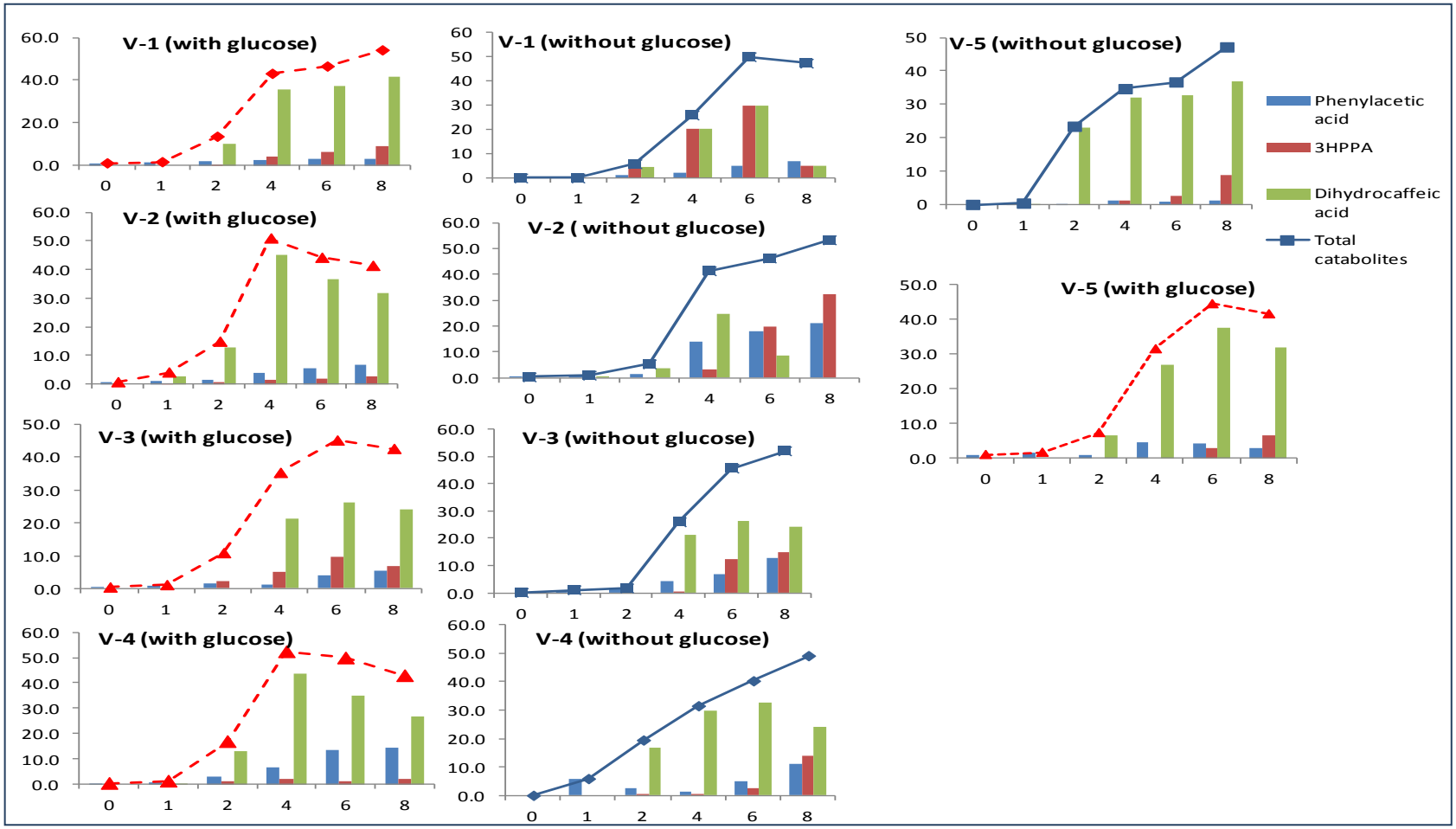


Figure 4. 4 Formation of 3HPPA (3-(3′-hydroxyphenyl)propionic acid), phenylacetic acid, and dihydrocaffeic acid during incubation of caffeic acid (55 µmoles) with human faecal slurries.

4.4 Discussion

Caffeic acid is a common dietary component found in a large range of plant-derived food products. The C_6-C_3 hydroxycinnamate is reported to have potential health effects with in vitro chemoprotective, antioxidant, and anti-inflammatory properties (Natella et al., 1999; Karlsson et al., 2005). For these events to occur in vivo, caffeic acid must be readily bioavailable. Studies on the bioavailability of hydroxycinnamates suggest that they are absorbed in both the small and large intestine with the colonic microflora playing a key role. In the present study, the microbial metabolism of caffeic acid (3′,4′-dihydroxycinnamic acid) was investigated using an in vitro fermentation model of human faecal samples obtained from Asian volunteers.

In vitro fermentation to study the colonic metabolism has been used by many researchers with the number of samples collected varying, as seen in the study of colonic degradation of rutin and green flavan-3-ols (Jaganath et al., 2009; Roowi et al., 2010) with only three donors, while in another study used four donors (Aura et al., 2005). Though the sample size were small, statistical analyses showed that the subsequent degradation of substract was dependent upon the interindividual composition of the colonic microflora and also the presence of glucose (Jaganath et al., 2009).

Earlier work on caffeic acid degradation by colonic microflora was studied by Gonthier (2006) using human faecal sample. 3-(3-Hydroxyphenyl)propionic and benzoic acid were identified as the major catabolites produced by the colonic microflora. In the present study, the effect of of glucose supplement and bacteria composition upon caffeic acid

degradation was investigated using 55 µmoles of caffeic acid incubated with human faecal slurries obtained from 5 different individuals. A higher concentration of caffeic acid compared to 1 µmoles utilised by Gonthier (2006) was used to see if the concentration of the substrate could effect the production of the catabolites.

Caffeic acid was shown to be rapidly degraded in vitro by the colonic microflora. This is shown by the reduced amount of caffeic acid recovered at 0 h in all faecal slurries samples although the amount detected was significantly different between individuals ($p < 0.5$). The effect of glucose supplemention was also studied and thus demonstrated that the caffeic acid degradation was enhanced with the addition of sugar to the faecal incubates with time point clearance 4 h while it was 6 h in that absence glucose.

Incubation of caffeic acid (55 µmoles) into human faecal slurries resulted in the appearance of a number of catabolites namely phenylacetic acid, 3-(3′-hydroxyphenyl) propionic acid and dihydrocaffeic acid. Colonic bacteria potentially involved in such catabolism include *Escherichia coli, Bifidobacterium lactis*, and *Lactobacillus gasseri* (Couteau et al., 2001).

In human faecal samples caffeic acid was rapidly reduced to form dihydrocaffeic acid and 3-(3′-hydroxyphenyl)propionic acid, which in vivo, are absorbed via large intestine and converted to 3-hydroxyhippuric acid, presumably in the liver, prior to excretion in urine (Rechner et al., 2004). Dihydrocaffeic has been reported to be the major microbial transformation product of caffeic acid (Renouf et al., 2010). This supported by the current findings.

Free dihydrocaffeic acid has been detected in plasma and urine after coffee consumption (Goldstein et al., 1984), while the presence of conjugated dihydrocaffeic acid has been detected in human plasma after intake of artichoke leaf extracts (Wittemer et al., 2005). More recently Stalmach et al. (2009) detected dihydrocaffeic acid, and dihydrocaffeic acid-3-*O*-sulphate in plasma and dihydrocaffeic acid-3-*O*-sulphate and dihydrocaffeic acid-3-*O*-glucuronide in urine after the consumption of a 5-CQA-rich coffee. These conjugates were not detected in the faecal incubations indicating that while the colonic bacteria produce dihydrocaffeic acid from caffeic acid, further phase II metabolism producing glucuronide and sulphate conjugates probably occurs in the wall of the colon and/or is hepatic in origin. Dihydrocaffeic acid which resulted from the microbial hydrogenation of the side chain of caffeic acid exerts antioxidant activity are similar to caffeic acid when tested using FRAP assay (Lekse et al., 2001). Dihydrocaffeic acid unlike 5-CQA can enter erythrocytes that help to reduce the trans-membrane oxidant stress generated by extracellular ferricyanide (Poquet et al., 2008). The biological effects after the intake of green tea and chocolate may also due to dihydrocaffeic acid as it is also a microbial metabolite of catechin and procyanidins (Cos et al., 2004; Cabrera et al., 2006).

In a previous study (Chapter 3), ingestion of [3-^{14}C]caffeic acid by rats resulted the excretion of radiolabeled caffeic acid-3′-*O*-glucuronide, ferulic acid-4′-glucuronide, caffeic acid-4′-*O*-sulphate, caffeic acid-3′-sulphate, ferulic acid-4′-sulphate and isoferulic acid-3′-*O*-sulphate in urine, with faeces containing of two unknown metabolites. In the in vitro study none of the above conjugated metabolites were found in human faecal samples indicating the role of the liver and/or wall of the colon as sites of sulfation and glucuronidation. Interestingly

the absence of ferulic acid and isoferulic acid catabolites in the in vitro study also indication that the colon and/or the liver, rather than that microbiota, are the potential site of methylation reactions catalysed by COMT.

Reduction of the side chain of caffeic acid and dehydroxylation at the C4 position of dihydrocaffeic acid results in the formation of 3-(3′-hydroxyphenyl) propionic acid (Aura, 2008). 3-(3′-Hydroxyphenyl)propionic acid is a known metabolite of colonic degradation of flavonols, flavan-3-ols, flavanones, and hydroxycinnamates (Gonthier et al., 2006; Serra et al., 2012), and has been proposed as the likely precursor of 3′-hydroxyhippuric found in urine (Rechner et al., 2004).

Dehydroxylation, β-oxidation and glycination in the liver will convert 3-(3′-hydroxyphenyl)propionic acid to the urinary metabolite hippuric acid (Rechner et al., 2004). Hippuric acid (*N*-benzoyl-glycine) a glycine conjugate of benzoic acid is primarily derived from plant phenolics and aromatic amino acids via bacterial transformation, however it is also synthesized from compounds such as benzoic acid, quinic acid, tryptophan, tyrosine and phenylalanine (Spencer, 2003). The liver is the active site for the formation of hippuric acid, which is why it was not produced by the faecal incubations in the present study.

The dehydroxylation of 3-(3′-hydroxyphenyl)propionic acid will produce 3-phenylpropionic acid which is subject to β-oxidization with the resultant 3-phenylpropionic acid being converted to benzoic acid (Peppercorn and Goldman, 1971; Scheline, 1991). In vivo, benzoic acid is conjugated with glycine either in the liver and excreted as hippuric acid in urine (Olthof et al., 2003). In the present study there was only traces amount of 3-phenylpropionic acid which was

also presence in the control, indicating that this is not solely a product of caffeic acid catabolism, whereas only traces of benzoic acid were detected in the faecal samples of some volunteers. The results obtained thus support the operation of the proposed catabolic pathway illustrated in the Figure 4.5.

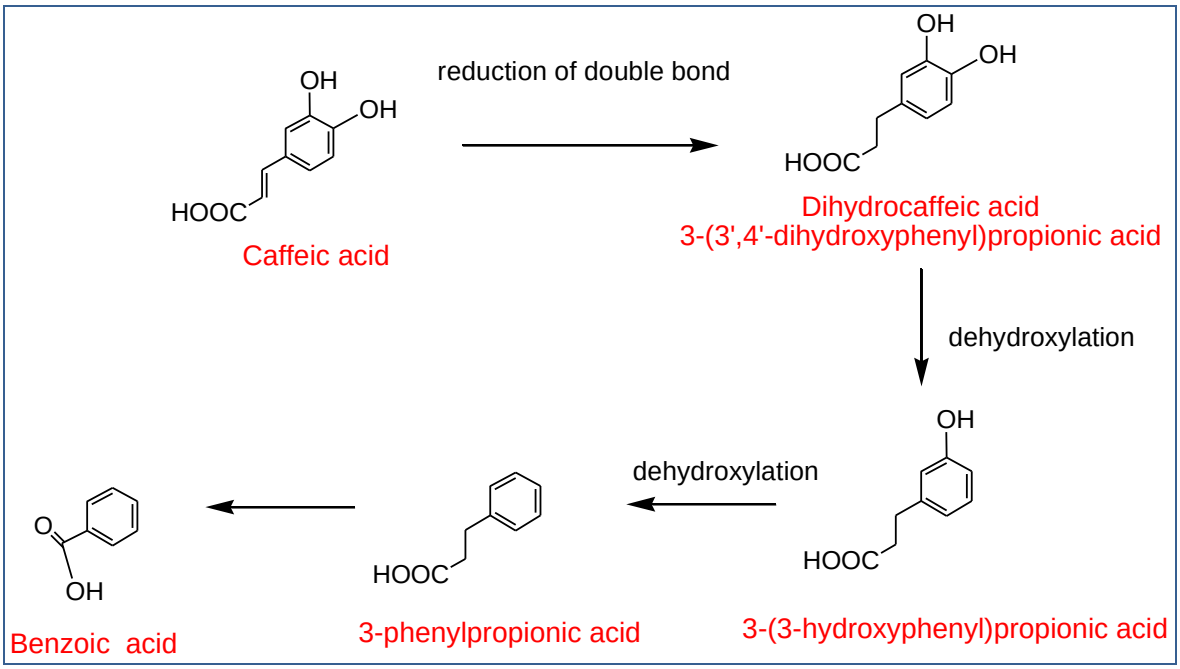


Figure 4. 5 Proposed scheme of colonic degradation of caffeic acid in humans.

However in a previous study, benzoic acid and 3(3′-hydroxyphenyl)propionic were the main catabolites detected when 1 µmol of caffeic acid was incubated with human faecal samples (Gonthier et al., 2006). In this study caffeic acid was cleared after 2 h of incubation. The differences observed could be due to the high amount of 55 µmoles caffeic acid used in the present study where the earliest clearance of caffeic acid was observed at 4 h of incubation (V-1, V-2 and V-4). The different amount of faecal material used between these studies in which resulted in a different density of colonic microflora, could also be the reason for these variations.

The rate of caffeic acid degradation and catabolites produced also varied among individuals, and thus may reflect the individual variations in intestinal bacterial colonization (Meijer-Severs and van Santen, 1986). It has been proposed there is a high degree of complexity of bacteria species among human individuals that is determined by the diet, which supplies nutrients not only to the host but also to the intestinal bacteria (Blaut and Clavel, 2007). In the present study all volunteers were of Asian origin, which should have reduced the variations. Their diet is different from the typical western diet. Thus raises other questions as to whether diet and ethnicity could have some affect on the colonic microbial population.

In vitro fermentation was used to assess the colonic metabolism of caffeic acid, which aimed to mimic the events occurring in the human colon. It has been used by other researchers to investigate the fate of flavonoids catabolised by the colonic microbial (Peppercorn and Goldman, 1971; Rechner et al., 2004; Aura, 2008). However in many cases, the experimental approach used were varied in terms of its inoculums size, type of media, anaerobic conditions, choice of buffer and mixing rate in which could affect the bacterial ecotypes that resulting the catabolism of flavonoids (Edwards et al., 1996). The advantage of using this in vitro model to elucidate the pathway of the degradation of caffeic acid, is that it can be determined in a reliable and affordable manner (Edwards et al., 1996). It must be noted that this in vitro model may not fully depict the in vivo conditions, where the concentration of catabolites produced is based on the combination of catabolism and absorption which cannot be induced in vitro.

4.5 Conclusions

In summary, the rate and extent of in vitro caffeic acid degradation varied somewhat in the faecal samples provided by the five volunteers. It was clearly observed that dihydrocaffeic is the main catabolite of caffeic acid with its formation being enhanced by the addition glucose. The glucose supplement also affected the formation of phenylacetic acid and 3-(3′-hydroxyphenyl) propionic acid but the extent to which this occurred varied between individuals.

5 SUMMARY

Dietary (poly)phenols are naturally occurring compounds widely distributed in higher plants, such as in fruits and vegetables, and a number of derived products such as fruit juices, tea, coffee and chocolate. (Poly)phenols are the most abundant antioxidants in the human diet and are of interest due to their potential beneficial effects on human health.

In the first series of analyses presented in the thesis, a study was carried out with herbal medicinal plant, *Ficus deltoidea* from Malaysia. This plant is locally known as 'Mas cotek' and its phytochemical contents antioxidant activity were discussed in Chapter 2. Flavones, flavan-3-ols and a single of *p*-coumaroylquinic acid, a hydroxycinnamic acid, are groups of flavonoids and non-flavonoid compound detected in the aqueous extract of *F. deltoidea*. The herbal product derived from this plant has been claimed to possess therauptic effects.

With the exception of a protective impact on the gastrointestinal tract, dietary (poly)phenols must be absorbed before any reported beneficial effects can take place. The absorption, distribution, metabolism and excretion (ADME) is an important area in determining the effect of consumed (poly)phenols in human health. In an attempt to obtain more information about ADME, a study has been conducted with rats into the bioavailability of caffeic acid which is a hydroxycinnamate related to *p*-coumaroylquinic acid which was found to occur in *Ficus deltoidea*. The study was designed toinvestigate fate of the hydroxycinnamate by feeding a radiolabeled substrate, [3-^{14}C]caffeic acid, after

which at a number of time points various tissues, organs, urine, faeces and cages washing were collected and analysed as described in Chapter 3.

Previous studies have demonstrated that not all consumed (poly)phenol compounds are absorbed directly into the circulation from the small intestine and are readily bioavailable. In such circumstances, substantial quantities of the compounds ingested will reach the colon. In the colon they will undergo transformation by microbiota with some of the resultant catabolites being absorbed and released into the circulatory system. The important role of the colonic microflora was investigated in a study described in the last chapter of this thesis, Chapter 4. In this study, the catabolism of caffeic acid by the colonic microbiota was studied using human faecal slurries obtained from five Asian donors.

5.1 Phytochemical analysis of *F. deltoidea* extract and its antioxidant properties

The use of medicinal plants for prevention and treatment of various diseases has been documented since ancient times. In modern medicine many drugs developed are of natural origin, which can be categorized as original natural products, products derived semi-synthetically from natural products, or synthetic products based on natural product models (Cragg et al., 1997). It has been reported that at least 119 compounds derived from 90 plant species are considered important drugs have been used in one or more countries, with 77% of these plant-derived products being used in traditional medicine (Farnsworth et al., 1985).

In Malaysia the use of herbal medicinal plants is linked with traditional medicines that are still being used by the major ethnic communities (Chen, 1981). However, the information about traditional medicines and their uses are not fully documented thus leaving a huge gap in gathering the information on their potential beneficial effects. Chapter 2 has described the analysis of the phytochemical content of *F. deltoidea* a Malaysian medicinal plant. This plant was chosen because its popularity in Malaysia where it is used by the traditional practitioner for healing and prevention of several diseases with some of its products already being marketed commercially. In the present study, aqueous extract of *F. deltoidea* was analysed for their flavonoid contents and antioxidant activity. The identification and quantification of its phytochemicals was made feasible with the use of HPLC-ESI-MS^2 and HPLC coupled with ABTS online detection which enable the quantification of individual compound that contribute to antioxidant activity. In Malaysia, *F. deltoidea* is usually prepared from leaves and consumed as herbal tea. Other parts of the plant such as fruit, stem and roots are however also used as the basis for herbal preparations. These extracts are among many herbal medicinal plants that have been used by the traditional practitioners for the treatment of diseases such as diabetes, rheumatism and breast cancer. Although it has been claimed by traditional practitioner that this *F. deltoidea* extracts have a potential beneficial effects on several aspects of human health, there is a lack of scientific evidence to support the claims.

Therefore the underlying objectives in Chapter 2 were to investigate, identify and quantify the phenolic compounds presence in *F. deltoidea* aqueous extract as a baseline for a standardized *Ficus* extract. The phytochemical profile is of importance for herbal drug standardization since it has a direct bearing on

the activity and the quality of the herbal potions (Nikam et al., 2012). Total phenolic contents and their antioxidant activities were also evaluated. Based on the results obtained it was demonstrated that *F. deltoidea* tea contains high levels of flavonoids mainly flavones and flavan-3-ols. The principal flavan-3-ols identified were gallocatechin, epigallocatechin, catechin and epicatechin, which were presence in high amount (44 to 98 μM) in the aqueous infusion (10 mg/mL). The high concentration of flavan-3-ols found in the extract is comparable to those reported in the fruit juices (Mullen et al., 2007) and tea (Chan et al., 2007; Fan et al., 2007). In addition, the *Ficus* extract also contained five oligomers of proanthocyanidins consisting of (epi)catechin and (epi)afzelechin sub-units which are considered unusual and have not been reported in other *Ficus* species. Overall, these flavan-3-ols accumulated in the tea in concentrations of ~400 μM. Flavones were also found in the aqueous infusion in the forms of luteolin and apigenin glucosides. In addition to this, 4-*p*-coumarylquinic acid at a concentration of 139 μM was identified. There was an absence of other chlorogenic acids which is unusual (Clifford et al., 2003). In terms of the antioxidant activity of the *Ficus* tea, flavan-3-ols were the main contributor.

The findings of the study have established that flavan-3-ols and flavones as the main flavonoids found in the tea that can be used as reference for the standardization extract of various *Ficus* products. These flavonoids which exhibited high antioxidant activities may be the basis of the potential health effects of *Ficus* products. Further study should be carried out, particularly in terms of their safety and efficacy before human intervention study can take place.

5.2 The bioavailability of [3-^{14}C]caffeic acid in rats

Caffeic acid is hydroxycinnamic compound, normally esterified with quinic acid to form chlorogenic acid with high concentration found in coffee (Clifford 2004). Apart from being antioxidant agents, caffeic acid has exhibited anticancer and antimutagenic activities, antidiabetic effect in streptozocin-induced in diabetic rats and antihyperglycemia properties in mice (Hsu et al., 2000; Jung et al., 2006; Weng et al., 2012). It has been shown that the absorption of hydroxycinnamic acid occurs throughout the gastrointestinal tract, with findings indicated that caffeic acid is more readily available than its conjugated that chlorogenic derivative caffeic acid ((Olthof et al., 2001; Konishi et al., 2006; Lafay et al., 2006b).

This second study, discussed in Chapter 3, investigated the bioavailability of caffeic acid using the rat as an animal model. This study was carried out using [3-^{14}C]caffeic acid. After supplementation, samples were collected from various organs and body fluids were analysed using HPLC-ESI-MS^2 coupled to a radioactive detector. Monitoring the metabolism of caffeic acid was made feasible with the use of a radiolabeled substrate in which all derivatives and by-product produced derived from caffeic acid metabolism can easily be discriminated.

It was found that feeding rats with radiolabeled caffeic acid resulted in some transformation of the parent *trans*-caffeic acid into *cis*-caffeic acid in the stomach within 1 h, with movement to duodenum and ileum/jejunum also taking place. At this time point, small amounts of radioactivity were detected in the plasma indicating early absorption of caffeic acid into the circulatory system.

However the identification of metabolites in the plasma was not possible because low levels of radioactivity accumulated. Urine collected 0-1 h, after feeding contained metabolites thereby providing evidence of caffeic acid absorption in the proximal GI tract, despite the low level accumulation in plasma. These metabolites were caffeic acid-3′ and -4′-*O*-glucuronides, caffeic acid-3′ and -4-sulphates, ferulic acid-4′-*O*-glucuronide and-4′-sulphate, and smaller amounts of isoferulic acid-3′-*O*-sulphate. These metabolites reflect phase II metabolism of caffeic acid possibly in the intestinal epithelium or the liver. Urine collected from 0-6 h revealed sulphates as the main hydroxycinnamate metabolites. In this context caffeic acid is similarly metabolised in humans. It is not possible to determine the extent to which sulfation occurs in the wall of the small intestine and the colon, or in the liver. The study also demonstrated urine as the main route of caffeic acid metabolite excretion, with 48-68% of radioactivity recovered in urine, compared with 19-25% found in rat faeces collected over a 24 h period. The high urinary excretion and the comparatively low level radioactivity found in faeces are evidence of the rapid absorption and high bioavailability of caffeic acid. Results observed in this study are in keeping with the previous study with unlabeled caffeic acid by Gonthier et al.(2006) who concluded that free caffeic acid was more efficiently absorbed that its esterified form. The present study also established that caffeic acid metabolism comprised methylation, sulphation, and glucuronidation steps via routes that are similar, although not identical with those found in humans (Stalmach et al., 2009; Stalmach et al., 2010a).

5.3 Caffeic acid metabolism by colonic microflora

(Polyp)henols have attracted considerable interest due to their numerous beneficial effects toward human healths. These (poly)phenols are abundant in numerous plants but many of them are not readily bioaccesible in the small intestine and therefore pass to the colon. This is supported by the feeding studies iloestomists, which showed that only one third of the ingested chlorogenic acids can be absorbed in the upper gastrointestinal tract, with rest remainder moving to the colon to be hydrolyzed by bacteria releasing free caffeic and quinic acids (Chesson et al., 1999; Couteau et al., 2001; Gonthier et al., 2003b).

The colon is the active site of microbiota activities which result in dehydroxylation, demethylation, dehydrogenation, glycination, and hydrogenation to form several catabolites such as 3′,4′-dihydroxyphenylpropionic acid, 3′-methoxy-4′-hydroxyphenylpropionic acid, 3′- and 4′-hydroxyphenylpropionic acid, 3′-and 4′-hydroxyphenylacetic acid, 3′,4′-dihydroxyphenylacetic acid, 3′-methoxy-4′-hydroxyphenylacetic acid, 3′- and 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid, 3′-hydroxycinnamic acid (*m*-coumaric acid), hippuric acid and 3′-hydroxyhippuric acid (Choudhury et al., 1999; Gonthier et al., 2003b; Olthof et al., 2003; Gonthier et al., 2006).

The colon is rich in nutrients providing a non-hostile anaerobic environment that is suitable for microbial colonization. Thanks to recent advances in microbial ecology, immunology and metabolomics the role of these colonic microbes in the overall well being of the host have been highlighted. The colonic

bacteria develop mutual relationship with the host, and in return for the food supply and protected environment provided by the host, colonic microbiota help in maintaining host nutrition, immune modulation, pathogen colonization resistance, intestinal epithelial development and activity, and energy metabolism (Hooper et al., 2002; Bäckhed et al., 2004). The vital role of colonic bacteria to the host system has been revealed by results obtained from the studies in germ-free animals which shown interference in cells functionality; reduction in cytokine production and mucosal cell turnover, underdeveloped mucosal associated lymphoid tissue and malfunctioning of the cell mediated immunity (Falk et al., 1998; Mazmanian and Kasper, 2006). The intestinal ecosystem is build by the interaction between its microbes, epithelium, mucosal immune system, microvasculature, and enteric nervous system. Between them there is cell signalling to ensure the system is kept in harmony, any disturbance could result a chronic and physiological inflammation (Clavel and Haller, 2007; Peterson et al., 2007; Liu et al., 2008).

Microflora or microbiota refers to the collection of microbes colonizing a particular host niche, whereas microbiome refers to all the genes embedded in the genomes of species represented in microbiota (Hooper and Gordon, 2001). The microbial densities are low in the proximal and middle small intestine but become very dense in the colon (10^{11}-10^{12}/g) (Savage, 1986). In humans, these microbial are mainly anaerobic such as bifidobacteria, lactobacilli and bacteroides (Meijer-Severs and van Santen, 1986), with those that appear to participate in phenolic metabolism being Bacteroides, Clostridium, Eubacterium, Ruminococcus, and Eggertheilla (Blaut and Clavel, 2007). Therefore, the colon is an active site for (poly)phenol catabolism with biotransformation into simple phenols, phenolic acids, aromatics, and lactones (Aura, 2008; Selma et al.,

2009). Catabolites produced in the colon can be excreted in faeces but can also be absorbed into the enterocytes via passive diffusion and further metabolized in the liver by phase II enzymes, prior to re-entering to the circulation system and elimination in urine.

Dietary (poly)phenols are known to be an important factor in modulating immunocompetence of an individual, therefore understanding the role of the diet in relation to colonic microflora is essential when promoting functional foods or nutritional foods with supposed beneficial effects on human health. To understand the effect of that colonic microflora on the catabolism of (poly)phenols, studies were conducted using caffeic acid, a prominent compound of hydroxycinnamic acids. Caffeic acid is widely found in vegetables, fruits, tea, coffee, and wine. Despite the high intake of this compound in human diet the information regarding its metabolism is still scarce. In Chapter 4, the breakdown of the hydroxycinnamic acid by human microbiota was investigated in vitro by incubating 55 μmoles of caffeic acid with human faecal slurries obtained from five Asian donors. No radiolabeled caffeic acid remained so the study was carried out using unlabeled substrate with catabolites being analysed by gas chromatography–mass spectrometry (GC-MS). This GC-methodology, despite requiring TMSi derivatization, is better suited to the analysis of phenolic and aromatic catabolites that our HPLC-MS instrumentation as a consequences of poor ionisation of the free acids (Sánchez-Patán et al., 2011).

From results obtained, it can be concluded that caffeic acid catabolism by colonic microflora is very much dependent on two main factors. First is the composition and density of the colonic microflora. Inter-individual differences in terms of bacteria composition have resulted in different amounts of catabolites

produced and variations in the time of their formation. Caffeic acid catabolism by colonic microflora resulted the production of three main catabolites, identified as dihydrocaffeic acid, 3-(3′-hydroxyphenyl)propionic acid and phenylacetic acid. Although the main catabolites produced were the same in all faecal samples, there were quantative variations in the amounts produced. For example, dihydrocaffeic acid which is the main caffeic acid catabolite and formed in all individuals. The earliest formation of dihydrocaffeic acid was detected at 1 h in faecal samples from V-2, V-3 and V-4, but it only appeared at 2 h from samples of V-1 and V-5. Beside the time differences in the formation of dihydrocaffeic acid, the quantity produced also varied with faecal samples from different individuals. These inter-individual variations were observed in terms of the catabolites produced, presumably due to differences in microflora composition. Variable densities of colonic microflora have be reported with *Lactobacillus* and *Bifidobacterium* sub-populations between and within adult individuals (McCartney et al., 1996). It has also been suggested that the difference in the microbial ecology may be prompted by an Asian diet. This is suggested due to the fact that intestinal tract is sterile at birth and it is only during postnatal development that the human microbial ecology will undergo dramatic changes (Isolauri, 2012). The microbial ecology may be influenced, by the human diets, as it has been proposed that Western diets may contribute to environmental changes in colonic microbiota that affect energy storage and obesity (Bäckhed et al., 2004).

Glucose supplement was a second factor that influenced the formation of caffeic acid catabolites. The addition of glucose to the medium in this study was important to mimic in vivo conditions which reflect the dietary fiber intake. Glucose is required as an energy source, to stimulate the growth and the

production of bacteria (Edwards et al., 1996). In the presence of glucose, the colonic microflora catabolism of caffeic acid catabolism moved towards the production of dihydrocaffeic acid and 3-(3′-hydroxyphenyl)propionic acid, whereas without glucose more dihydrocaffeic acid was produced along with phenylacetic acid. After 4 h incubation in the presence of glucose no caffeic acid remained in two faecal samples from two volunteers (V-1 and V-4). The effect of glucose addition was also reported by Jaganath et al.(2009) in which glucose enhanced deglycosylation of rutin to quercetin.

5.4 Future research

Phytochemical analysis of *F. deltoidea* lead to the identification of potentially bioactive flavonoid compounds in this herbal medicinal plant. As a consequence, further study in terms of its bioavailability should be conducted. This study is essential, as it can provide more information of absorption and metabolism of different groups of flavonoids that occur in the herbal tea. Prior to that, toxicological studies are required in terms of its safety and efficacy before human intervention study can be conducted. With more information at hand, it is possible that one day *Ficus* products can be further targeted for drug development or at least could be recognize as a beverage for health as seen in tea and coffee.

The bioavailability of caffeic acid has been investigated using rats fed with ^{14}C-radiolabeled material. Although preparations of radiolabeled compound are laborious, difficult to achieved, the data obtained in the present study demonstrates the value of feeding radiolabeled substrate. The ready discrimination of the ^{14}C-labeled metabolites produced allowed the ready

identification of caffeic acid-derived components. In rats, caffeic acid was not retained in the body and it was shown that after 72 h, ~80 % of ingested radioactivity had been excreted in urine. Perhaps in the future ethical permission can be obtained to feed radiolabeled caffeic acid and other compounds, with seemingly low bioavailability such flavones to humans.

Human or animal intervention studies are important in the study of bioavailability of (poly)phenols. However, the additional use of in vitro gastrointestinal models is important as they can provide mechanistic knowledge regarding microbial (poly)phenol bioconversions. Metabolomic datasets from in vitro studies when linked with in-depth microbiome analyses offers the possibility of the discovery of new (poly)phenol metabolites metabolites with potential health-promoting effects. In addition they will also identify the particular microbial groups and species that are responsible for the biotransformation of (poly)phenols, which can be used to evaluate strategies that target the enrichment of (poly)phenol-converting species such as *Clostridium orbiscidens* and *Eubacterium ramulus* (Schoefer et al., 2003; Clavel et al., 2006). With more emerging technologies to come such as molecular approaches using ribosomal RNA (rRNA), high-throughput diversity approaches such as phylogenetic microarrays, quantitative technologies such as fluorescence in situ hybridization (FISH), and real-time quantitative PCR (RT-qPCR) will help in assessing more information regarding the of the microbiota species responsible for the (poly)phenol biotransformation in the colon (van Duynhoven et al., 2010).

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APPENDICES

Appendix 3.1 Dry weight (in gram) of tissues/ organ samples of rats after feeding with ^{14}C-radiolabeled compound. n.a- samples were not available. Rats were divided into three groups A, B and C. Each group consisted of animal that were sacrificed at 1, 3,6,12, 24, 48 and 72 h.

Rat (group)	Time (h)	Liver	Kidney	Brain	Heart	Lungs	stomach	Muscle	Ceacum	Colon	Spleen	Duodenum	Ileum/jej
A	1	2.65	1.90	1.51	0.83	0.99	2.20	2.67	0.62	0.66	0.15	0.62	0.95
	3	2.37	1.42	1.48	0.74	1.01	1.91	3.32	0.59	1.66	0.14	0.81	0.87
	6	2.61	1.70	1.06	0.83	0.93	1.37	3.51	0.85	1.06	0.15	0.82	0.93
	12	2.25	1.69	0.98	0.82	1.03	1.56	2.82	1.51	0.97	0.12	0.65	0.61
	24	2.43	1.51	1.39	0.82	1.04	2.05	3.52	0.51	0.60	0.12	0.71	0.69
	48	2.31	1.66	1.29	0.73	2.13	1.49	2.60	0.55	0.92	0.14	0.75	0.90
	72	2.13	1.28	1.45	0.74	1.10	1.74	2.86	0.34	0.35	0.13	0.59	0.43
B	1	2.28	1.80	1.46	0.86	1.09	1.60	3.49	0.66	0.98	0.14	0.77	1.08
	3	2.67	1.64	1.15	0.75	1.04	2.89	3.01	0.80	1.41	0.15	0.84	0.93
	6	2.40	1.88	1.31	0.79	1.33	2.41	3.33	0.63	1.04	0.17	0.76	0.89
	12	2.56	1.91	1.32	0.79	1.30	2.01	3.57	0.90	1.28	0.14	0.69	0.67
	24	2.98	1.60	1.41	0.87	2.10	2.17	2.94	0.51	0.68	0.12	0.54	0.96
	48	2.31	1.62	1.48	0.70	1.02	1.56	3.32	0.83	1.55	0.14	0.70	0.71
	72	2.14	1.75	1.20	0.75	1.24	1.67	3.78	0.62	0.64	0.15	0.65	0.57
C	1	2.05	1.46	1.44	0.71	0.80	3.17	4.26	0.89	1.56	0.17	0.83	1.06
	3	2.15	1.64	1.41	0.76	0.99	1.88	1.23	1.24	1.75	0.13	0.74	0.89
	6	2.92	1.68	1.49	0.89	0.97	1.91	1.96	0.74	1.24	0.19	0.81	0.84
	12	2.44	1.77	1.47	0.73	2.59	1.87	1.31	0.37	0.77	0.12	0.66	0.71
	24	2.57	1.71	1.47	0.78	1.10	1.50	2.51	0.43	n.a	0.15	0.62	0.64
	48	2.29	1.70	1.33	2.05	0.91	2.47	2.51	1.13	n.a	0.19	0.59	0.68
	72	2.22	1.56	1.36	0.76	0.00	2.53	3.08	0.48	0.31	0.14	0.72	0.71

Appendix 3.2 Total volume of liquid samples (mL) and faeces (g) collected from rats after feeding with ^{14}C-radiolabeled. n.a- samples were not available. Rats were divided into three groups A, B and C. Each group consisted of animal that were sacrificed at 1, 3,6,12, 24, 48 and 72 h.

Rat Group	Time (h)	Urine mL	Time (h)	Cages washing	RBC mL	Plasma mL	Time (h)	Faeces (g)
A	1	n.a	1	31.6	0.5	2	1	n.a
	3	1.8	3	37.5	4.0	3	3	n.a
	6	1.5	6	36.5	3.6	3	6	n.a
	12	3.1	12	42.5	4.0	3.8	12	n.a
	24	8.9	24	26.0	4.2	4.2	24	1.56
	48(0-24)	6.3	48	31.5	5.0	3	48(0-24)	2.59
	48 (24-48)	14.4	72	26.0	4.2	3.5	48(24-48)	1.83
	72(0-24)	10.6					72(0-24)	3.12
	72 (24-48)	4.5					72 (24-48)	0.24
	72 (48-72)	1.4					72(48-72)	0.10
B	1	-	1	32.5	5.7	5.3	1	n.a
	3	1.4	3	40.0	4.7	4.2	3	n.a
	6	2.6	6	41.8	3.6	3.3	6	n.a
	12	2.7	12	33.0	4.8	3.8	12	n.a
	24	12.3	24	27.0	3.0	3	24	0.58
	48(0-24)	5.9	48	30.0	3.2	4.4	48(0-24)	1.86
	48 (24-48)	10.4	72	31.5	2.2	2.2	48(24-48)	1.45
	72(0-24)	-					72(0-24)	2.84
	72 (24-48)	-					72 (24-48)	1.56
	72 (48-72)	5.4					72(48-72)	1.92
C	1	-	1	19.0	2.3	2.3	1	n.a
	3	0.7	3	32.5	4.0	3	3	n.a
	6	2.2	6	26.5	2.9	3.3	6	n.a
	12	1.4	12	42.5	4.2	3.8	12	n.a
	24	4.3	24	30.0	2.0	2	24	1.98
	48/0-24	3.9	48	48.0	3.6	4	48(0-24)	1.12
	24-48	5.1	72	29.5	4.0	3.2	48(24-48)	1.30
	72/0-24	3.5					72(0-24)	2.92
	24-48	5.0					72 (24-48)	3.73
	48-72	4.9					72(48-72)	1.23

Appendix 4.1 -PARTICIPANT INFORMATION SHEET

Title of study

Invitation to take part
Thank you for reading this.
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part

What is the purpose of the study?
In previous study, the bioavailability of caffeic acid in rats was investigated using Caffeic acid which helps to determine the possible pathway for the caffeic acid using animal model. Therefore this study will help to monitor the degradation products of dietary flavonoids and caffeic acid and to delineate the possible catabolic pathways involved in the degradation of caffeic acid.

Why have I been chosen?
You are:
i) a healthy individual, aged 18-60, not obese, non-smoker, in good general health, not taking any supplement or medication
ii) you do not suffer from any allergy or condition affecting bowel health

Do I have to take part?
If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?
You will meet a member of the Research Team from the University of Glasgow who will explain the study at a convenient time. All transport costs as well as costs associated to the study will be reimbursed, but we cannot offer any fees.

What do I have to do?
You will be invited to follow low polyphenol diet for two days (48 h). Diet sheets will be provided, and you will also be given a telephone number, to be able to call a member of the Research Team with any queries. You will be asked to tell us if you think you may have slipped up with any of the dietary advice.

Example of a typical intervention

Day 1	8 am		Start of the low phenolic diet (see examples)
Day 2	8 am	(24 hr)	Low phenolic diet
Day 3	8 am	(48h)	Return in the morning with feces sample

The diet involves foods with *low polyphenol* content, such as meats, fish, pasta, bread (but avoiding tea, coffee, colourful fruits and vegetables). During this period, you will be asked to keep a dietary intake record.
You will be asked to record any possible slip-ups (e.g. eating high polyphenol foods by mistake during the low-polyphenol diet). It is very easy to make mistakes, but vital that we know in order to get reliable results.
At the end of the diet (after 48 h of low polyphenol diet) a faecal sample will be collected. The faecal sample should be collected in a special pot provided together with anaerobic pouch to keep the sample in anaerobic condition and send on the morning of day 3.

What are the possible disadvantages and risks of taking part?
There are no risks or disadvantages associated with this study other than time loss and inconvenience of following dietary restrictions.

What are the possible benefits of taking part?
There are no direct benefits to the volunteers associated with taking part. This study will provide us with a better understanding of the association between polyphenol-rich food consumption and colonic health. All participants who completed the study will be compensated (£30).

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/university will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?
Results will be presented at meetings of learned societies and published in scientific journals. Results will also be included in student project reports, when applicable. We will arrange a meeting to discuss the results with participant volunteers if they would like that.

Who is organising and funding the research?
This project is being organised by the Plant Product and Human Nutrition Group, at the University of Glasgow.

Who has reviewed the study?
This project has been reviewed by the University of Glasgow, Faculty of Medicine, ethics committee.

Contact for further information
If you require further information please contact
Thank you for reading this information sheet

CONSENT FORM

Title of Project:

Name of Researcher:

1. I confirm that I have read and understand the information sheet dated. ..... (version.....) for the above study and have had the opportunity to ask questions. ☐

1. I confirm that I do not suffer from allergies to foods involved in this study. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time , without giving any reason, without my legal rights being affected. ☐

3. I agree to my samples (urine and faecal samples) being stored and used for further analysis to look at biochemical compounds and bacteria, as new techniques are being available ☐

5. I agree to take part in the above study. ☐

_____	_____	_____
Name of subject	***Date***	***Signature***

_____	_____	_____
Researcher	***Date***	***Signature***

1 for subject; 1 for researcher
Centre Number:

Study Number:
Subject Identification Number for this trial

Dietary guidelines for lowpolyphenol diet, Baseline diet:

Avoid ***all fruits, vegetables, onions, coffee, tea, chocolate, fruit juices, soft drink (i.e. irn brun, coca cola), alcohol, vanilla and similar flavourings***, ***wholemeal products, spices (i.e. curry), tomato sauce, and beans (i.e. lentils, green beans, peas)***. Avoid all dietary supplements (***vitamins, mineral, and herbal products***).

Examples of suitable breakfast include:
Eggs
Cheeses
Sausage
Beacon
Fish
Toast
Bread
Croissant (NO chocolate)
Waffles, pancakes with butter and sugar (NO jam)
Butter
Milk
Biscuits (NOT wholemeal)
Rice based cereals (NOT Coco pops)

Examples of suitable lunch & dinners include:
Tuna, chicken and egg sandwiches (mayonnaise ok)
Burger and chips (NO ketchup, relish, gherkins)
Sausage rolls
White pasta and cheese / cream
Chicken / sausages and mashed potatoes (NO gravy)
Omelette (with cheese, ham)
Potatoes without skin
Meats (NO ketchup, brown sauce)
Cheese and cream cheese (NO garlic or onion-based cheeses)
Fish and chips with salt and vinegar (NO ketchup)
Sardines on white toast (no tomato sauce)
Chicken fried rice with eggs and oyster sauce (NO soya products or vegetables
Roast Chicken/ prawns with white rice
Noodle
Chicken nuggets and chips
Roast Beef Sandwich with crisps
Salmon with white rice
Steak and mashed potatoes

Examples of suitable snacks include:
Biscuits (NOT wholemeal, chocolate, or fruit contains)
Shortbreads
Custard rice puddings
Crisps (ready salted)
Plain Frozen Yoghurt
Cheese and crackers
Plain donut without chocolate or fruit fillings
Salted rice crackers, Rice cakes , **Examples of suitable drinks include:** Water and milk

DIETARY INTAKE RECORD

During the day which you have to follow the diet described previously, we are going to ask you to record what you have been eaten at every meals.
By asking you to record your daily diet (quantities do not have to be written down), this may be easier for you to choose and plan what you can eat, and this may also help you remembering that you are on a `special diet'.
Please write down and food/drink consumed

DAY 1- Date:_____

Breakfast:

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Lunch:

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Dinner:

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All-day snacks:

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DAY 2- Date:_____
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Lunch:
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Dinner:
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All-day snacks:
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