The metabolism and excretion of quercetin and (-)-epicatechin after co-consumption of onion soup and dark chocolate by healthy volunteers

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Dietary polyphenols have been associated with a decreased risk of diseases such as coronary vascular disease (CVD). However, the health benefits of these compounds depend on their bioavailability. In this thesis, the phase II enzymes; uridine diphosphate glucuronosyl transferase (UGT), sulfotransferase (SULT) and catechol-*O*-methyltransferase (COMT), which affect bioavailability of polyphenols were investigated.

Quercetin and (-)-epicatechin are representatives of the flavonol and flavanol subclasses of polyphenols. Thus, co-consumption of these compounds may provide interesting information on the role of phase II enzymes on their metabolism and excretion. Quercetin and (-)-epicatechin are found abundantly in onion and dark chocolate, respectively. Prior to providing food samples to healthy volunteers, the content of quercetin in onion soup and also (-)-epicatechin in dark chocolate were quantified by HPLC-DAD and HPLC-DAD/FLD, respectively. As expected red onion contained higher total quercetin content than yellow onion. In addition, homogenisation was an efficient method for extracting quercetin glycosides compared to vortex and sonication. Thus, red onion was selected to prepare a soup and found that there were no significant differences in total quercetin content in red onion soup after preparation (61.7±15.60 mg/ 130 ml soup) and before microwaving (60.4±9.6 mg/ 130 ml soup) or before microwaving and after microwaving (59.9±8.2 mg/130 ml soup), *p*-value = 0.818 and 0.666, respectively. For dark chocolate, the content of (-)-epicatechin was also analysed and quantified in different percentages of cocoa from different brands. However, 70% cocoa dark chocolate was selected for the human study. The content of (-)-epicatechin of 70% dark chocolate was 92.2±4.4 mg/100g FW, as detected with FLD.

Then, COMT activity was investigated *in vitro* followed by a pilot study *in vivo*. For the *in vitro* study, (-)-epicatechin was more efficiently methylated compared to quercetin. *In vivo*, in urine, the amounts of methylated and the parent compound of quercetin and (-)-epicatechin after single consumption of red onion soup, 70% cocoa dark chocolate and co-consumption of both types of food were underestimated when assessed after enzymatic deconjugation. In addition, this thesis revealed that the limitation was due to some compounds in urine which inhibited

sulfatase activity. Because of this, all main metabolites of quercetin and (-)-epicatechin found in urine without enzymatic hydrolysis were analysed.

Main conjugates of quercetin and (-)-epicatechin predicted to be found in urine were first synthesised using an enzymatic method prior to use as standards. Five quercetin conjugates and five (-)-epicatechin conjugates were successfully synthesised with a sufficient percentage yield. For the first time, 3′-*O*-methyl-quercetin-di-glucuronides were also produced and detected after glucuronidation of 3′-*O*-methyl-quercetin-glucuronide. In addition, methylated-(-)-epicatechin-glucuronides and methylated-(-)-epicatechin sulfates were originally synthesised by (-)-epicatechin-glucuronides and (-)-epicatechin sulfates which were the products of (-)-epicatechin glucuronidation and (-)-epicatechin sulfation reaction, using COMT from cytosol pig liver.

Then, the main metabolites of quercetin and (-)-epicatechin excreted in urine in 27 healthy volunteers were determined and compared between single consumption and co-consumption. The major metabolite of quercetin found most often in urine was quercetin glucuronide in single consumption of red onion soup and co-consumption of red onion and 70% dark chocolate. While, the methylated form of (-)-epicatechin was the most abundant form detected in urine after single consumption of 70% dark chocolate and co-consumption of both types of food. In addition, the % urinary excretion of quercetin was lower than quercetin in both single and co-consumption. Thus, different types of phase II enzymes (UGT, SULT and COMT) have a different affinity for substrates, quercetin and (-)-epicatechin, resulting in different types of the metabolic profiles in urine. However, at these low level of quercetin and (-)-epicatechin from red onion soup and 70% dark chocolate did not reveal a huge interact and had a large effect on their metabolism and excretion.

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List of Abbreviations

ACN	acetonitrile
AWAA	acetone water acetic acid
COMT	catechol-*O*-methyltransferase
D	daidzein
DAD	diode array detector
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EC	epicatechin
EG	ethyl gallate
EGCG	epigallocatechin gallate
HPLC	high performance liquid chromatography
HPLC-MS	high performance liquid chromatography coupled with mass spectrometry
HPLC-DAD	high performance liquid chromatography coupled with diode array detection/ fluorescence detection
HPLC-FLD	high performance liquid chromatography coupled with fluorescence detection
[M-H]-	negatively charge molecular ion
m/z	mass to charge ratio
mg	milligram
min	minute(s)
ml	millilitre
PAPS	3′-phosphoadenosine 5′-phosphosulfate
PMSF	phenyl methyl sulfonyl fluoride
PNS	*p*-nitrophenyl sulfate
PPB	potassium phosphate buffer

Q	quercetin
Q3,4′diG	quercetin 3, 4′-*O*-diglucoside
Q4′G	quercetin 4′-*O*-glucoside
SAM	S-adenosyl-L-methionine
SIM	single ion monitoring
SPE	solid phase extraction
SULT	sulfotransferase
T	taxifolin
TIC	total ion count
UDP	uridine diphosphate
UDPGA	uridine-5′-diphospho-glucuronic acid
UGT	uridine diphosphate glucuronosyl transferase
UPLC-MS	ultraperformance liquid chromatography coupled with mass spectrometry
µl	microlitre
µM	micromolar
µmol	micromole
3′MeQ	3′-methyl quercetin or isorhamnetin
4′MeQ	4′- methyl quercetin or tamarixetin

Chapter 1 Literature review

1.1 Introduction

Polyphenols are secondary metabolites found in many types of plant tissue. Polyphenols are non-nutritive compounds but they are the most abundant class of antioxidants in the human diet and they have been associated with a decreased risk of some diseases such as coronary vascular disease (CVD) and other chronic disease (Scalbert and Williamson, 2000). Quercetin and (-)-epicatechin are major flavonol and flavanol subclasses and found abundantly in onion and dark chocolate, respectively. Numerous studies have revealed that consuming onion or dark chocolate are associated with health benefits. According to the Netherlands Cohort Study on diet and cancer, Dorant et al. (1996) investigated the relationship between onion consumption and incidence of stomach carcinoma. They found that the highest onion consumption ($\geq$ 0.5 onions/day) reduced the risk of stomach carcinoma compared with the lowest consumption (0 onions/day) with the 0.50. Ebrahimi-Mamaghani et al. (2014) revealed that raw red onion consumption seems to be effective as a cholesterol-lowering food agent in women with polycystic ovary syndrome. In the case of red onion soup, Hubbard et al. (2006) provided evidence that a high dose of quercetin as onion soup inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway, contributing protective effects against cardiovascular disease. The first human clinical study with chocolate consumption was performed by Kondo et al. (1996). They found that 35 grams of delipidated cocoa decreased LDL oxidation between 2 and 4 hr after ingestion, indicating chocolate inhibits LDL oxidation *in vivo*. Subsequently, a number of studies reported that intake of dark chocolate improved lipoprotein profiles (Mursu et al., 2004; Allen et al., 2008; Nanetti et al., 2012). In recent years, West et al., (2014) stated that high-flavanol cocoa and dark chocolate consumption improves endothelial function by enhancing vasolidation and reducing stiffness in arteries in overweight women. In overweight men, dark chocolate consumption also improves vascular function and leukocyte adhesion factors in blood circulation (Esser et al., 2014). Additionally, consumption of dark chocolate also has beneficial effects on insulin, glucose, antioxidant status and oxidative stress responses to

prolonged exercise and carbohydrate metabolism performance during rest and exercise (Davison et al., 2012; Stellingwerff et al., 2014).

However, the health benefits of the main flavonoids, quercetin and (-)-epicatechin, present in onion and dark chocolate depend on their bioavailability. There are several factors that affect the bioavailability of polyphenols. The phase II enzymes in detoxification process are other important factors that affect the bioavailability and the biological effect of flavonoids. Quercetin and (-)-epicatechin are in different subclasses but these two compounds contain a similar structure called a catechol ring. In addition, quercetin is generally found in the form of glycosides form in foods, in contrast to (-)-epicatechin which is found as aglycone. Thus, prior to absorption and distribution into the human body, quercetin is de-glycosylated by luminal lactase phlorizin hydrolase and/or within the enterocyte by cytosolic β-glucosidase, resulting in the aglycone form. However, monomeric flavanols like (-)-epicatechin, they can be directly absorbed in the small intestine without deglycosylation. Quercetin and (-)-epicatechin are excreted out of the body in the same way as drugs or toxics using phase II enzymes; uridine diphosphate glucuronosyl transferase (UGT), sulfotransferase (SULT) and catechol-*O*-methyl transferase (COMT). Quercetin and (-)-epicatechin are generally conjugated with glucuronic acid, sulfate with or without methyl group by uridine diphosphate glucuronosyl transferase (UGT), sulfotransferase (SULT) and catechol-*O*-methyl transferase (COMT), respectively. These compounds are then changed to a variety of conjugated metabolites and made more water soluble and higher molecular weight. Conjugated with glucuronic acid have higher solubility in water than conjugated with sulfate and methyl group, leading to excretion out of the body by the kidneys and also losing their biological activity at the same time.

The metabolic profile of quercetin or (-)-epicatechin after a single consumption of flavonoid-rich foods, pure compounds or supplements have been studied by several research groups. There are two main publications which focus on the quercetin and (-)-epicatechin metabolites in the circulating system and revealed that quercetin or (-)-epicatechin has their own pattern of metabolites and inter-individual variation. First, Mullen et al. (2006) analysed plasma and urine samples from six human volunteers after the consumption of 270 g of fried red onions (containing 275 μmol quercetin 3, 4′di-*O*-glucoside and quercetin 4′-*O*-glucoside). Quercetin metabolites were detected in both plasma and urine collected

over a 24 hr period by using HPLC with a diode array and a tandem mass spectrometric detector. However, the metabolic profile of quercetin excreted in urine was different in the plasma. The quercetin-3′-*O*-glucuronide, two quercetin glucuronide sulfates, methylquercetin diglucuronide were found in urine but were absent or present only in small amounts in the blood. Other main metabolites such as quercetin diglucuronide, isorhamnetin-3-*O*-glucuronide, and quercetin-3-*O*-glucuronide were also found in the urine. The urinary excretion was 12.9 μmol (4.7% intake). In 2012, Actis-Goretta et al. analysed (-)-epicatechin metabolites in plasma and urine from 0 to 24 hr after the ingestion of 100 g dark chocolate (containing 79 mg or 272 μmol of (-)-epicatechin) in five volunteers. (-)-Epicatechin metabolites were detected in both the plasma and the urine (24 hr collection) by using the LC–MS/MS–MRM method. (-)-Epicatechin metabolites, including glucuronides, sulfates, methyl sulfates, and methyl glucuronides, excreted in urine were detected and were similar to those found in the plasma. The total urinary excretion of (-)-epicatechin was 20±2% of the intake. Although these two studies used high amount of quercetin and epicatechin for feeding the volunteers and also used more sensitive methods to detect all the metabolites in the biological samples, these studies were only performed using small numbers of volunteers and no publications studied all the metabolites of quercetin and (-)-epicatechin after co-consumption. As mentioned before, these two compounds contain similar structure but they are in different subclasses and have different step of metabolism. Co-consumption of these compounds may provide interesting information of their bioavailability in human body.

Thus, in this thesis, the metabolic profile of quercetin and (-)-epicatechin before and after 24 hr urine collection were analysed. The urinary metabolites are normally used as biomarkers of polyphenol intake in human. In addition, contrasting to genes and proteins, metabolites signify downstream biochemical end products that are closer to the phenotype. Therefore, it is easier to correlate metabolomic profiles with phenotype compared to genomic, transcriptomic and proteomic profiles.

The hypothesis of this study is that the interaction between quercetin and (-)-epicatechin with the phase II enzymes (UGT, SULT, COMT) would happen after co-consumption of these two compounds from red onion soup and 70% cocoa dark chocolate leading to change the urinary metabolic profiles in healthy volunteers.

To achieve the aim of this study, this thesis was divided into 7 chapters. The first chapter will begin with an introduction of main group of polyphenol named flavonoids; their structure and occurrence in foods followed by their metabolism and biological effects. The second chapter was materials and methods related to all experiments. Then, next four chapters are the research finding in this study. Before providing food samples, red onion soup and 70% cocoa dark chocolate, to healthy volunteers the exact amount of quercetin and (-)-epicatechin needed to be quantified (see in Chapter 3). After that the activity of COMT, one of the phase II enzymes, was investigated *in vitro* followed by the *in vivo* study. The percentage of urinary excretion of the methylated and the parent compound of quercetin and epicatechin after single consumption of red onion soup, 70% cocoa dark chocolate and co-consumption of both types of food were analysed as a pilot study. However, the amounts of these compounds were underestimated due to limitations in sulfatase activity. These results were explained in Chapter 4. Then, all main metabolites of quercetin and (-)-epicatechin predicted to be found in urine were decided to analyse. However, commercially relevant conjugated quercetin and (-)-epicatechin are not available. Therefore, main conjugates of these compounds, in different forms, were synthesised using an enzymatic method (See Chapter 5). Consequently, these compounds were used as standard compounds in Chapter 6. The main metabolites of quercetin and (-)-epicatechin excreted in urine were determined and compared between single consumption and co-consumption of red onion soup and 70% cocoa dark chocolate. Finally, summary and future perspectives relate to this research area will be explained in Chapter 7.

1.2 Flavonoids

1.2.1 General structure and occurrence in foods

Polyphenols are found widely in natural plants and have more than one phenol unit or building block per molecule in their structures. Polyphenols can be classified into two groups; non-flavonoids and flavonoids. Flavonoids are identified by having a common structure consisting of of 15 carbons, two aromatic rings (A ring and B ring) which are bound together by three carbon atoms (C ring). Thus, the chemical structures are a C6-C3-C6 skeleton (Figure 1.1). In addition, flavonoids can be divided into six subclasses according to the variation in the heterocyclic ring including; flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols. All of these subclasses share the same carbon skeleton but contain different substituents such as hydroxyl group and sugars presenting naturally as a glycosides which help to increase the water solubility of flavonoids. In contrast, others substituents such as methyl group decrease the water solubility of flavonoids.

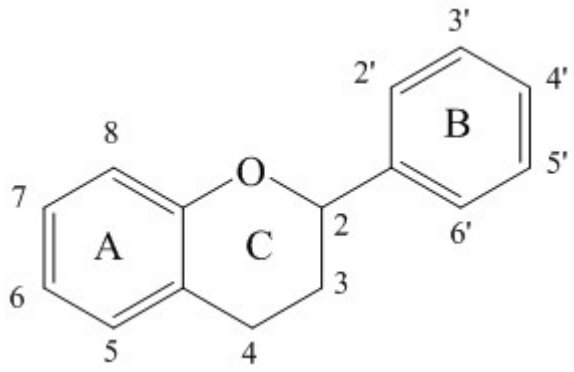
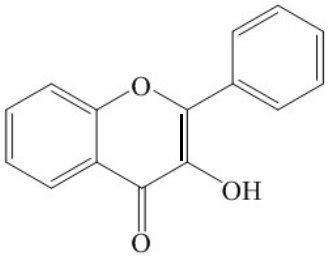
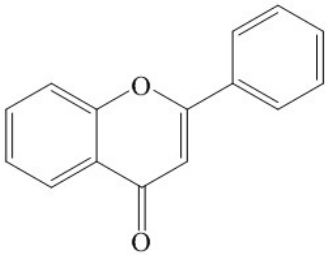
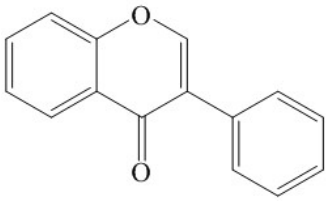
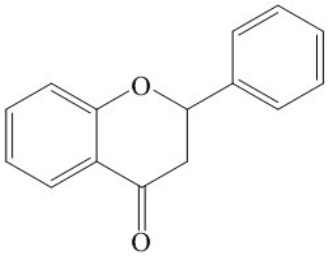
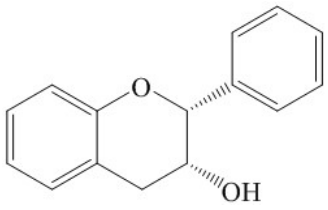
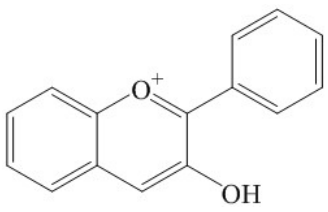


Figure 1.1: Carbon skeleton of flavonoids (C_6-C_3-C_6)

Flavonoids occur naturally as glycosides rather than aglycones. For example, flavonols are normally found as glycosides with conjugation occurring at the 5, 7, 3′, 4′, and 5′ positions. The main flavonol in our diet is quercetin. Other common flavonols include kaempferol, myricetin, isorhamnetin, tamarixetin, morin and fisetin. The richest sources of flavonols are onions, apples, and tea. Flavones have similar structural to flavonols but they lack oxygenation at C-3. For example, luteolin and apigenin which were identified in sweet red pepper and celery, respectively. Isoflavones have the B-ring attached at C-3 rather than at the C-2 position. They are found almost exclusively in leguminous plants for example. daidzein and genistein in soybean. Isoflavones also are classified as phytoestrogens

because of their structural similarity to estrogen. Flavanones are characterized by the absence of Δ2,3 double bonds and the presence of a chiral center at C-2 such as naringenin and hesperetin. Citrus fruits are the main food source of flavanones. Flavanols, unlike flavonols, do not exist as glycosides. They are the most complex subclass of flavonoids, ranging from the simple monomers to the oligomeric and polymeric proanthocyanidins. The two chiral centers at C2 and C3 of the monomeric flavanol produce four isomers for each level of B-ring hydroxylation. The main flavanols are catechins and very abundant in tea. Other sources are red wine and chocolate. Lastly, anthocyanins are red fruits pigments and found in berries fruits, grape, plums, red and black currants (Scalbert and Williamson, 2000; Crozier et al., 2009). Chemical structure and major food sources of each subclass was shown in Table 1.1.

Table 1.1: Occurrence of main subclasses of flavonoids in foods

Flavonoid subclass	**Chemical structure**	**Major food sources**
Flavonols		Onion, Tea, Apple
Flavones		Sweet red pepper, Celery, Parsley
Isoflavones		Soybean
Flavanones		Citrus fruit
Flavanols		Tea, Red wine, Chocolate
Anthocyanins		Berries, Grape, Plums

This thesis will focus only on 2 subgroups; the monomeric flavonol, quercetin and the monomeric flavanol (-)-epicatechin which are major flavonoids present in the human diet and are easily available as commercial standards. These two compounds behave as powerful antioxidants and free radical scarvengers and are able to interact with several key enzymes such as quercetin shows an effective inhibit lipoxygenase and xanthine oxidase (Duenas et al., 2010). Intestingly, these two compounds have a similar structure, containing a catechol ring with two hydroxyl groups that can be modified by uridine diphosphate glucuronosyl transferase (UGT), sulfotransferase (SULT) and catechol-*O*-methyl transferase (COMT) (see Figure 1.2).

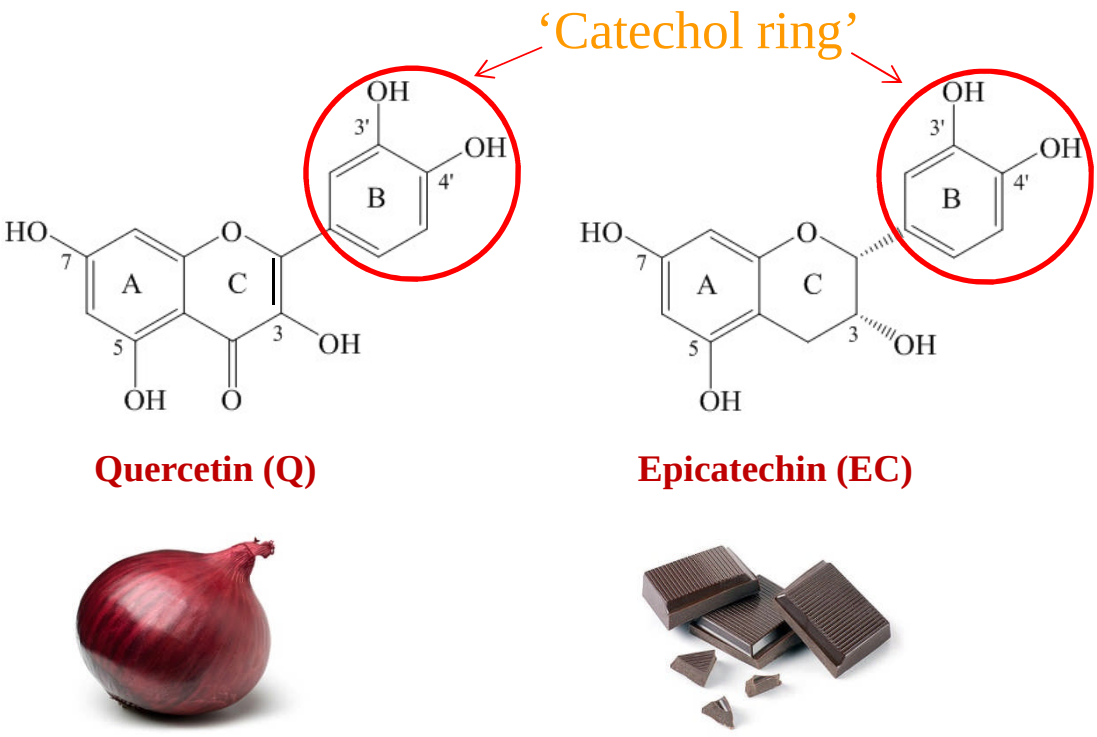


Figure 1.2: Structure of quercetin and (-)-epicatechin found mostly in onion and dark chocolate. They have a similar stucture characterised by a catechol ring.

1.2.2 Quercetin

Quercetin (3, 5, 7, 3′, 4′-pentahydroxyflavone) is a flavonol-type flavonoid most abundant in onion and apple, while present in other fruits and vegetables as well. It is mostly present in foodstuffs as *O*-glycosides. It means that at least one hydroxyl group is substituted by various types of sugar. In general, quercetin glycosides containing a sugar group at the 3-position, for example, quercitrin,

isoquercitrin, hyperoside and rutin, are commonly distributed in a wide variety of vegetables. However, in onion, the phenolic group at the 4′-position is necessarily bound by a sugar group and thus its major glycosides are quercetin-4′-*O*-β-D-glucoside (Q4′G) and quercetin-3, 4′-*O*-β-D-diglucoside (Q3, 4′diG) (Murota and Terao, 2003).

The chemical activities of quercetin can be ascribed to its electron-donating property (reducing activity) which is due to the presence of a phenolic hydroxyl group. This property is essential for exerting anti-oxidant activity by scavenging free radicals. Additionally, the *O*-dihydroxyl structure at the 3′- and 4′-positions in the B-ring, the so-called catechol group, is largely responsible for this activity. Murakami et al, (2008) reported that the hydroxyl groups at the 3′ and 4′-positions in the B-ring and the 3-position in the C-ring are responsible for the high reactivity of quercetin with free radicals. Furthermore, they suggested that quercetin and its analogues act as anti-oxidants by inhibiting oxidative enzymes such as xanthine oxidase (XOD), lipoxygenase (LOX) and NADPH oxidase which are also responsible for the attenuation of oxidative stress as they play key roles in the initial process of free radical-induced cellular damage. They also explained that quercetin is converted to *O*-quinone and the *O*-semiquinone radical. Then, oxygen molecules can react with this radical resulting in the production of O_2^- and H_2O_2. These reactive oxygen species (ROS) affect cellular redox signaling pathways and are capable of inducing cellular oxidative damage. Therefore, quercetin can possibly act as both a pro-oxidant and an anti-oxidant.

1.2.3 (-)-Epicatechin

(-)-Epicatechin is a flavanol that is commonly present in green teas, red wine, cocoa products, and many fruits, such as apples. However, cocoa bean has the highest levels of (-)-epicatechin (43,270 mg/kg fresh weight) and higher than green tea, which contains about 8,000 mg/kg fresh weight.

1.3 Bioavailability of quercetin and (-)-epicatechin

The bioavailability of dietary flavonoids is limited not only by the structural diversity of the molecule but it also depends on an enzyme in their metabolism, microbial-mediated biotransformation and active efflux (Yang, 2008). Though the bioavailabilities of flavonoids vary between different types of flavonoids, the

absorption and metabolism follow the same concept to all flavonoids. Flavonoid are metabolized both in tissues through the gut barrier and by the colonic microflora.

Most flavonoids, including quercetin in flavonol subclass, are glycosylated in food. Therefore, glycosylated flavonoids have to be deglycosylated before absorption. Duenas et.al (2010) suggested that the first step in quercetin metabolism is likely to be deglycosylation before absorption in the small intestine. Moreover, monoglucosides, in which only one glucose molecule is bound to quercetin, such as isoquercitrin and Q4′G, are absorbed in the small intestine as quercetin aglycone following hydrolysis with LPH. Alternatively, they are incorporated into intestinal epithelial cells via a SGLT-1 pathway. In the case of other glycosides which contain one sugar other than glucose (such as hyperoside) or disaccharides/oligosaccharides (such as rutin), absorption occurs in the large intestine as quercetin aglycone through deconjugation caused by the activity of enterobacteria (Murakami et al 2008).

The sugar is removed by two possible mechanisms. First, it involves the action of lactase phlorizin hydrolase (LPH) that is present in the brush border of the small intestine epithelial cells. For a specific substrate like flavonoid-*O*-β-D-glucosides, LPH will hydrolyse this substrate into an aglycone. Due to an increasing of lipophilicity and proximity to the cellular membrane of the aglycone, it then enters the epithelial cell by passive diffusion (Day et al., 2000). The second mechanism involves cytosolic β-glucosidase (CBG) that is present within the epithelial cells. In these epithelial cells, the polar glucosides are transported through the active sodium dependent glucose transporter SGLT1 (O'Leary et al., 2003). Then, the flavonoid glycosides will hydrolyse to the aglycone by CBG. In the case of (-)-epicatechin, it is acylated flavonoids which can pass through biological membranes and absorb without deconjugation. Prior to passage into blood circulation, simple aglycones are conjugated by glucuronidation, sulfation and methylation or a combination, using uridine-5′-diphosphate glucuronosyltransferase, sulfotransferases and catechol-*O*-methyltransferase, respectively. However, some conjugated flavonoids are probably efflux back to the gastrointestinal tract involving members of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters; multidrug resistance protein (MRP) and P-glycoprotein. In the case of quercetin and (-)-epicatechin, the efflux of these compounds involved with multi resistant protein 2 (MRP2) (O'Leary et al., 2003). After conjugation, the conjugated metabolites will reach the liver via the hepatic portal vein. In hepatocytes, these

metabolites will be subjected to further conjugation by phase II enzyme. In addition, the enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion**.** Some flavonoids and conjugated metabolites not absorbed in the small intestine will pass to the colon and can be absorbed at this site by the action of the colonic microflora (Crozier et al., 2009). The microflora in the colon promotes extensive modifications including hydrolysis, ring cleavage, and dehydroxylation, forming lower molecular weight phenolics (Scalbert and Williamson, 2000). The routes of dietary flavonoid and the mechanism for the absorption of flavonoids in the enterocyte are shown in Figure 1.3.

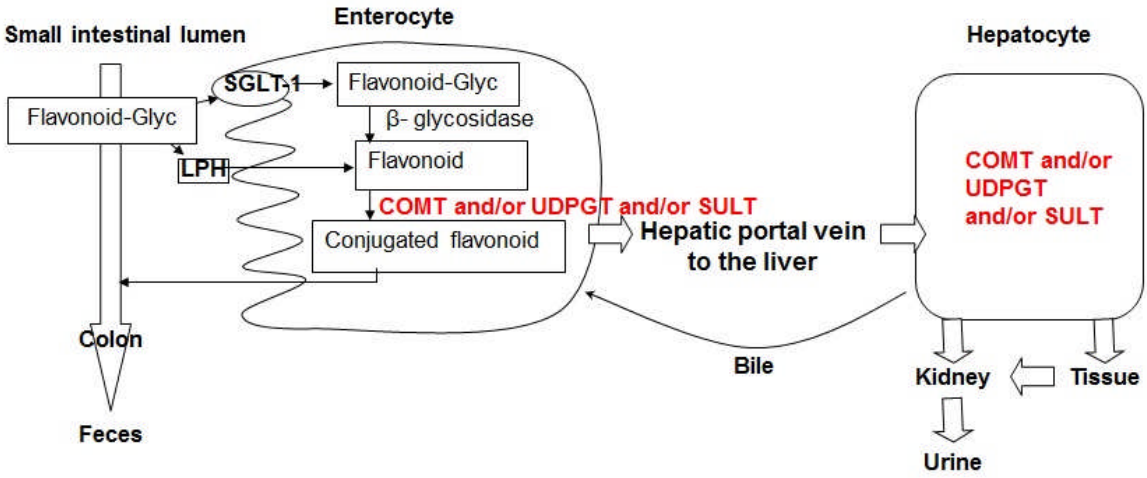


Figure 1.3: The metabolic pathway of a polyphenol. Glucuronidation, sulfation and methylation occurs at enterocyte and hepatocyte by uridine-5′-diphosphate glucuronosyltransferase (UGT), sulfotransferase (SULT) and catechol-*O*-methyltransferase (COMT), respectively.

The conjugation represents a metabolic detoxification process common to many xenobiotic compounds that restricts their potential toxic effects and facilitates their biliary and urinary elimination by increased solubility and a higher molecular weight. Thus, most dietary flavonoids are quickly eliminated in both urine and bile after ingestion. For quercetin, their metabolites were found in over 20 different forms in both plasma and urine. In addition, these metabolites were eliminated in urine at approximately 2–3% of the ingested dose (Mullen et al, 2006). For (-)-epicatechin, ten forms were found in plasma and fifteen forms were found in urine with 20±2% of the ingested dose (Actis-Goretta et al, 2012). Comparing between 4

enantiomers of flavanols, the bioavailability of these stereomers were ranked as (-)-epicatechin > (+)-epicatechin = (+)-catechin > (-)-catechin on the basis of plasma concentration and urinary excretion of the aglycones after enzymatic deconjugation (Ottaviani et al, 2011). Actis-Goretta et al, 2013 also studied the intestinal absorption and metabolism including the role of biliary and urinary excretion of 50 mg of purified (-)-epicatechin in healthy volunteers by an intestinal perfusion techinique. They found that approximately 44 % of dose of (-)-epicatechin did not absorbed but the predominant metabolite as sulfated form (around 80% of total metabolites analysed) was effluxed back to the intestinal lumen, reached to the large intestine and further metabolised by the gut microbiota. Additionally, they showed that (-)-epicatechin was a moderately excreted by bile. The conjugated enzymes involved in polyphenol metabolism are explained as follows.

1.3.1 Uridine-5′-diphosphate glucuronosyltransferase (UGT)

Glucuronidation mainly occurs as a result of the activity of glucuronosyl transferases with uridine-5′-diphospho-a-D-glucuronic acid (UDPGA) as glucuronide donor. The mechanism involves a nucleophilic attack of the oxygen nucleophiles (R-OH) or other atoms, such as nitrogen, sulfur and even carbon in the substrate molecule on the cofactor, leading to O-, N-, S- and C glucuronides, respectively (Ding et al, 2013). In this case, quercetin and (-)-epicatechin, containing hydroxyl group in their structures are easily conjugated by glucuronic acid to form *O*-glucuronided conjugates.

Human UGTs are classified into four families; UGT1, UGT2, UGT3 and UGT8, on the basis of amino sequence identify. UGT1A is the major subfamily responsible for xenobiotic glucuronidation and also show tissue-specific expression. UGTs variants are functionally different from the wide types. Most notably, UGT1A1 is a highly polymorphic isoform. Furthermore, the deficiency in its expression or activity may cause genetic diseases. Recently, UGTs polymorphisms are systematically identified and newly variant are updated at http://www.pharmacogenomics.pha.ulaval.ca /sgc/ugt_alleles (Wu et al., 2011).

The effects of UGT polymorphisms on flavonoid clearance have not been examined. However, several studies showed that UGT polymorphisms affect glucuronidation and clearance of drugs and other xenobiotic compounds. Then, it is possible that similar effects may be seen with dietary flavonoids (Lampe, 2009).

1.3.2 Sulfotransferases (SULT)

The sulfotransferases belong to an emerging superfamily of enzymes that catalyse the transfer of SO_3^- to hydroxyl or phenolic groups on susceptible substrates, or the nitrogen atom of N-substituted aryl or alicyclic compounds, or pyridine N-oxides, through the sulfating cofactor. SULT catalyses the transfer of sulfate from adenosine 3′-phosphate 5′-phosphosulfate (PAPS) to flavonoids, forming various flavonoid sulfates (Ding et al., 2013). The level of sulfation activity probably is limited by PAPS availability (Klaassen and Boles, 1997).

SULTs have been identified into two board classes; (1) membrane-bound SULTs that are responsible for the sulfonation of peptides, proteins, lipids, and glycosaminoglycans and (2) cytosolic SULTs that are responsible for the metabolism of xenobiotic and small endogenous substrates such as steroids, bile acids, and neurotransmitters (Gamage et al., 2005). Like other phase II enzymes, cytosolic SULTs compose a large superfamily of genes. To date, cytosolic SULTs have been classified into five families and share less than 40% similarity base on amino acid sequence. SULT1 and SULT2 families are the largest and responsible for sulfation of endo- and exogenous compounds (Kauffman, 2004).

Genetic variants in SULT genes with associated functional differences in the translated protein have been identified. Single-nucleotide polymorphisms in SULT1A1 and SULT2A1 are common and have been associated with altered response to therapeutic agents and sex steroid concentrations, respectively. *In vitro* study with recombinant SULT1A1 have been shown that the SULT1A1*2 variant is less effective than SULT1A1*1 at conjugating resveratrol, apigenin, and epicatechin. However, the influence of SULT1A1 on phytochemicals metabolism still remains to be evaluated *in vivo* (Lampe, 2009).

1.3.3 Catechol-*O*-methyltransferase (COMT)

COMT is an Mg^{2+} -dependent enzyme which catalyses the transfer of a methyl group to a hydroxyl group of catechol ring at 3′ or 4′ position, resulting in 3′ methylated or 4′ methylated product. In this catalytic reaction, S-adenosyl-L-methionine acts as a methyl donor, and then it will be changed to S-adenosyl homocysteine (Figure 1.4). COMT has a variety of substrates. These can be divided into two groups; endogenous substrate such as catecholamines neurotransmitters and catechol estrogens, and exogenous substrate catecholic drugs and dietary catechols.

The general function of COMT is to eliminate the biologically active or toxic catechols and some hydroxylated metabolites. For example, COMT introduces a methyl group to the catecholamine and thus inactivates catecholamine neurotransmitters (dopamine, epinephrine and norepinephrine) or converts the catechol estrogen to the inactive methoxy derivative (2-MeOE2 and 4-MeOE2).

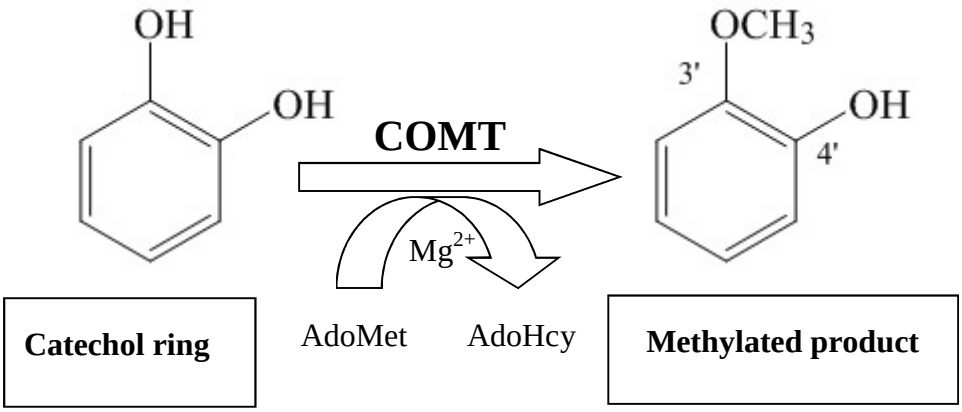


Figure 1.4: Catalytic reaction of COMT, Mg^{2+} dependent enzyme. COMT catalyses the transfer of a methyl group from AdoMet (S-adenosyl-L-methionine) to a 3′ or 4′ hydroxyl group on a substrate, containing catechol ring. (AdoHcy= S-adenosyl homocysteine)

COMT is encoded by a single gene with two different transcription start sites; at the P1 and P2 promoters. The COMT gene is localised on chromosome 22, band q11.22-23 (Figure 1.5). The shorter mRNA from the P2 promoter encodes a soluble COMT (S-COMT). The longer mRNA from the P1 promoter encodes mainly a membrane-bound COMT (MB-COMT) but to some extent S-COMT (Tunbridge et al., 2006). MB-COMT has higher substrate affinity but lower catalytic activity than S-COMT. Additionally, the MB-COMT is predominantly expressed in brain neurons whereas S-COMT is predominantly expressed in other tissues, such as liver, blood, and kidney (Chen et al., 2004).

Figure 1.5: COMT gene located on chromosome 22, band q11.22-23. It consists of six exons and has two promoters (sequences in grey are unique to the longer mRNA). P1 promoter and P2 promoter encodes MB-COMT and S-COMT, respectively. (Adapted from: Tunbridge et al., 2006; Tammimaki and Mannisto 2010)

Human COMT activity varies among individuals. Only three of the COMT mutants have been studied for their enzymatic activity and functional differences with the wild-type COMT (Bai et al, 2008) but the most well known mutant has two co-dominant alleles (G and A) in exon 4 of the COMT gene that influence the amino acid structure (Val or Met) at codon 108 in S-COMT or 158 in MB-COMT (rs165688 or rs4680, respectively). This variant causes the enzyme to be more thermolabile, structurally less stable, and has at least 40% lower enzyme activity than normal (Tammimaki and Mannisto 2010). Hence, the COMT enzyme activity is genetically polymorphic with a trimodal distribution; high activity in Val/Val genotype, intermediate activity in Val/Met genotype, and low activity in Met/Met genotype (Hosak, 2007). At present, a total of 14 polymorphisms are listed in the NCBI SNP database for the coding regions (exons) of the human COMT gene, and each of them are single point mutations (Table 1.2). In addition, the allele frequency of COMT polymorphism in different group of population is shown in Table 1.3.

Table 1.2: The COMT polymorphisms listed in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP) (Bai et al, 2008).

Amino acid positions (S/MB)	Amino acid residue		Effect on the catalytic activity of the enzyme
	Wild-type	Mutant	
-/9 (unpublished)	L	F	Unknown
-/34 (unpublished)	C	S	Unknown
12/62	H	H[a]	No change
22/72 and	A	S	Lower catalytic activity reduced thermostability
23/73	Q	Q[a]	No change
42/92 (unpublished)	V	M	Unknown
52/102 thermostability	A	T	Reduced
62/112	L	L[a]	No change
84/134	A	A[a]	No change
86/136	L	L[a]	No change
96/146	A	V	Unknown (unpublished)
108/158	V	M	Reduced thermostability
149/199	P	L	Unknown (unpublished)
153/203	L	L[a]	No change

[a] These nucleic acid mutations will not alter the amino acids that are encoded by the altered codons

Table 1.3: The allele frequency of COMT polymorphism in different group of population (adapted from Palmatier et al., 1999).

Population	N	Genotype distribution			Allele frequencies		Reference
		H/H	H/L	LL	H	L	
Japanese	153	74	70	9	0.71	0.29	Kunugi et al 1997a
	109	46	47	16	0.64	0.36	
Caucasian	173	40	88	45	0.48	0.52	Hoda et al 1996
	139	32	70	37	0.46	0.54	
Finnish	76	14	35	27	0.42	0.58	Syvanen et al 1997
	158	39	80	39	0.5	0.5	
Chinese	62	37	19	6	0.75	0.25	Xie et al 1997
	70	44	21	5	0.78	0.22	
North American Mixed European	148	49	73	26	0.58	0.42	Karayiorgo et al 1997
	73	14	36	23	0.44	0.56	
United Kingdom	78	20	33	25	0.47	0.53	Daniels et al 1996
	78	20	36	22	0.49	0.51	
North American Mixed European	87	30	44	13	0.6	0.4	Strous et al 1997
	54	12	28	14	0.48	0.52	
Chinese	99	52	40	7	0.73	0.27	Chen et al 1997
	177	92	72	13	0.72	0.28	
North American Mixed Europeana	129	31	70	28	0.51	0.49	Karayiorgo et al 1998
North American	157	45	72	40	0.52	0.48	
Japanese	150	58	77	15	0.64	0.36	Ohmori et al 1998

Population	N	Genotype distribution			Allele frequencies		Reference
		H/H	H/L	LL	H	L	
	150	81	56	13	0.73	0.27	
North American Mixed European	87	30	44	13	0.6	0.4	Lachman et al 1997
	63	20	35	8	0.6	0.4	
Spanish	113	35	57	21	0.57	0.43	Gutierrez et al 1997
	88	31	38	19	0.57	0.43	
Han Chinese	98	66	29	3	0.82	0.18	Li et al 1997
	93	44	41	8	0.69	0.31	
European	121	29	62	30	0.49	0.51	Kunugi et al 1997b
	169	42	85	42	0.5	0.5	
Japanese	135	58	59	18	0.65	0.35	Ohara et al 1998
	75	19	48	8	0.57	0.43	
	40	15	22	3	0.65	0.35	
North Americana	87	30	44	13	0.6	0.4	Lachman et al 1996a
North American, European, Hispanic	25	—	—	—	0.36	0.64	
	13	—	—	—	0.38	0.62	
	8	—	—	—	0	1	
North American	124	23	63	38	0.44	0.56	Vandenbergh et al 1997
	41	16	13	12	0.55	0.45	
	185	58	83	44	0.54	0.46	

North American White, Hispanic	28	12	11	5	0.63	0.38	Lachman et al 1998
	27	3	15	9	0.39	0.61	

Numerous epidemiological studies have examined an association between COMT polymorphism and health effect. Several studies showed that the val108/158met COMT polymorphism has been positively associated with neuropsychiatric conditions and a number of diseases such as breast cancer (Doyle, 2004). For example, differences in COMT activity relate to L-dopa level, resulting in Parkinson's disease susceptibility in difference ethnic groups (Tai and Wu, 2002). The low activity allele is also associated with progression and lymph node metastasis of breast cancer in Japanese women. In postmenopausal women, Wang et al., 2010 showed that carrying COMT-LL genotype contributes to estrogen-induced carcinogenesis of breast cancer. Additionally, other studies also showed that this polymorphism relates to coronary events. Happonen et al., (2006) examined the function of COMT gene on the risk of acute coronary events by modifying the effect of coffee intake. They found that heavy coffee consumption increases the incidence of acute coronary events in men with low COMT activity. In 2007 the same research group suggested that Val158Met polymorphism is associated with increased risk of acute coronary events and it may interact with high serum total homocysteine levels. Moreover, Annerbrink et al., (2010) suggested that the single nucleotide polymorphism is associated with various phenotypes, including different aspects of cognitive function, obesity and also blood pressure. Wang et al., (2007) supported that COMT polymorphisms play a role in large increases in BMI in obesity. However they showed that there was a significant association with Leu136Leu polymorphism but not for the Val158Met polymorphism. In the case of blood pressure, Annerbrink et al., (2008) found that Val158Met polymorphism associate with systolic and diastolic blood pressure, heart rate and body fat distribution in a sample of middle-aged Swedish men.

1.4 Biological activity of quercetin and (-)-epicatechin

During transfer across the enterocyte, and subsequently in the liver, the quercetin aglycone is immediately metabolized into its conjugated forms. The important step in quercetin metabolism seems to be methylation to 3′-*O*-methyl-

quercetin (isorhamnetin), the primary metabolite or 4′-*O*-methyl-quercetin (tamarixetin), a second metabolite. The methylation process may change the biological activity of quercetin because the hydroxyl group of quercetin is substituted, resulting in a decrease in the antioxidant activity of the parent compound. However, its metabolite (3′-*O*-methyl-quercetin or 4′-*O*-methyl-quercetin) still retain significant antioxidant/scavenging activity better than α-tocopherol. It is probably because of their stability (Duenas et al., 2011). This can be supported by Jaramillo et al., 2010. They suggested that the absorption and metabolic stability of methylated flavonoids are significantly increased when compared with unmethylated parent molecules, thus, 3′-*O*-methyl-quercetin is to be a higher bioavailability than quercetin. Furthermore, they showed that 3′-*O*-methyl-quercetin inhibited cell growth and induced both apoptosis and necrosis in human colon cancer cells hence it may have clinical significance with therapeutic and chemopreventive capabilities. However, another study showed that only quercetin, not its *O*-methylated metabolites, exerts protective effects against H_2O_2 cardiotoxicity and its action involves the modulation of PI3K/Akt and ERK1/2 signalling pathways (Angeloni et al., 2007).

The same as quercetin, *O*-methylation of the catechol B-ring resulted in a decrease of the antioxidant activity with regards to the parent compounds. However, the methylated metabolites still retain significant radical scavenging activity at pH 7.4. Some studied showed that (-)-epicatechin and its 3'-*O*-methyl (-)-epicatechin against UVA-induced damage and cell death in cultured human skin fibroblasts (Basu-Modak et al., 2003). Furthermore, Steffen et al. (2007) revealed that epicatechin scavenged O_2^- but its O-methylated metabolites prevented O_2^- generation through inhibition of endothelial NADPH oxidase activity, resulting in improvement of the bioactivity of NO.

There is consistent evidence from population studies that quercetin can reduce the risk of cardiovascular disease. In the case of (-)-epicatechin, the population data are less consistent. However, in intervention studies, flavan-3-ol–rich foods and beverages, such as tea and cocoa, consistently improve endothelial function. In the short-term effect, (-)-epicatechin diminished NO elimination through NADPH oxidase as caused by *O*-methylated-(-)-epicatechin metabolites (Figure 1.6). In the longer-term effect, (-)-epicatechin may additionally include

elevated generation of NO as a consequence of a higher cellular level of active eNOS protein (Steffen et al., 2007; 2008).

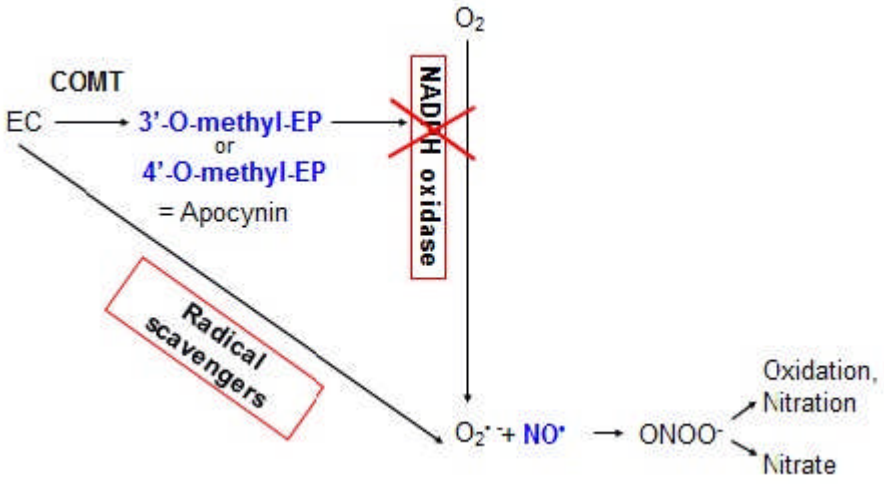


Figure 1.6: Inhibition of NADPH oxidase by *O*-methylated (-)-epicatechin metabolites (Adapted from: Steffen et al. 2007; 2008)

Additionally, several biomarkers related to (-)-epicachin or quercetin and its methylated metabolites have been studied (Table 1.4) and the summary is presented in Figure 1.7.

Table 1.4: Biomarkers related to (-)-epicachin or quercetin and its methylated metabolites

Compounds	Cell system	Concentration	Biomarkers	References
(-)-Epicatechin and/or its methylated metabolites	Human dermal fibroblasts	Up to 30 µM	- MMT reduction for mitochondrial function - Annexin-V binding staining for cell death - Caspase-3 activity for apoptosis	(Spencer et al., 2001)
	Human skin fibroblasts	1-50 µM	- MMT assay and confirmed by NR assay for mitochondrial function - AV and PI staining for cell death	(Basu-Modak et al., 2003)
	Mouse Primary cortical neurons	100- 300 nM	- Immunoblotting for detecting protein kinase signalling pathway (ERK and PI3K)	(Schroeter et al., 2007)
	Human umbilical vein endothelial cells	10 µM	- NO and also cGMP level	(Steffen et al., 2007)
	Human umbilical vein endothelial cells and cell lysates	10 µM	- O_2^- release - NADPH oxidation activity	(Steffen et al., 2008)
	Human and rat plasma	2.1- 42.8 µM	- Nitrotyrosine formation	(Natsume et al., 2008)

	Twelve healthy male subjects	200 mg (-)-epicatechin dissolved in 300 ml water	- Endogeneous NO production (S-nitrosothiols, Nitrite, Nitrate) - F2 isoprostanes	(Loke et al., 2008a)

Table 1.4: Biomarkers related to (-)-epicachin or quercetin and its methylated metabolites (Continued)

Compounds	**Cell system**	**Concentration**	**Biomarkers**	**References**
Quercetin and/or its methylated metabolites	Rat aorta	1- 10 µM	- Contractile response to phenylephrine or acetylcholine for endothelial function - Superoxide production and localize in vascular wall - $P47^{phox}$ expression by western blot and localization by immuno-histochemical	(Sanchez et al., 2007)
	Human peripheral monocytes and neutrophils	0-10 µM	Production of pro-inflammatory eicosanoids - Leucotrien B4 (LTB4) - Prostaglandin E2 (PGE2) Antioxidant activity - Formation of lipid hydroperoxides and F2 isoprostanes (stable biomarker for lipid peroxidation)	(Loke et al., 2008b)

Quercetin and/or its methylated metabolites	Rat aorta	10 µM	- Contractile response to phenylephrine or acetylcholine for endothelial function - Superoxide production and localize in vascular wall - $P47^{phox}$ and $P22^{phox}$ expression - Protein kinase C (PKC)	(Romero et al., 2009)
	Macrophage cell line, U937	30 µM	- Nitric oxide inhibitory activity - Catalase activity - Cytokine production: tumour necrosis factor-α (TNF- α), Interleukin 6 (IL-6), Interleukin 1 (IL-1)	(Okoko and Oruambo, 2009)
	Colon adenocarcinoma cell line, HCT-116	0- 100 µM	- MTT assay for cell viability - Annexin V and necrotic (PI) for apoptosis	(Jaramillo et al., 2010)
	Murine macrophage cell line, RAW264.7	0, 10-100 µM	The expression of proinflammatory marker - TNFα - Cytokines (iNOS, IL1β, IL6 and MIP1α	(Boesch-Saadatmandi et al., 2011)
	Human aortic endothelial cells	0- 15 µM	- TNFα induced cellular adhesion molecule (ICAM-1, E-selectin, VCAM-1) and release of monocyte chemotactic protein-1 (MCP-1)	(Lotito et al., 2011)

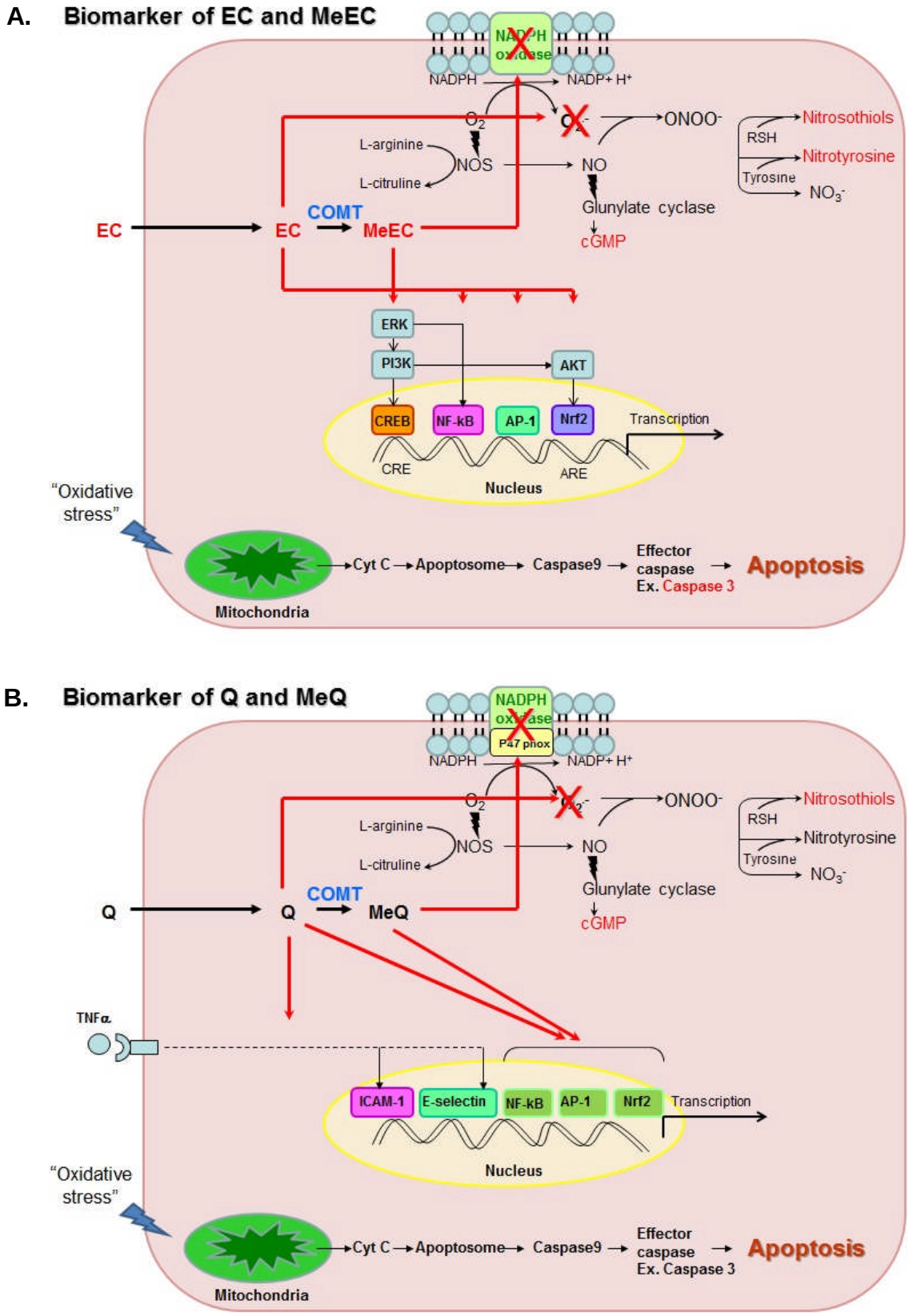


Figure 1.7: Biomarker of (-)-epicatechin (A), quercetin (B) and its methylated metabolites on a cellular level.

Generally, reactive species like ROS and RNS were produced in human body from several sources such as NADPH oxidase, the complex I and II in mitochondrial transport chain etc. These reactive species were controlled and balanced by antioxidants. When the level between reactive species and antioxidants are imbalance, it may cause oxidative stress. At the low level of ROS, it may has an effect on cell signalling or cellular messengers. While at the high level of ROS, it may leads to cytotoxicity, apoptosis and cell death, respectively. In addition, oxidative stress can induce by some stressors such as t-BOOH, oxidised LDL, H_2O_2, angiotensin-II, endothelin-1 etc. The effect of (-)-epicatechin, quercetin and/or its methylated metabolites on cytoprotectvie effects were studies by several research groups (Table 1.3). The oxidative stress was induced to different types of cells by different stressors, depending on the aim of each study. Then, the biological effects of (-)-epicatechin, quercetin and/or its metabolites were analysed using several types of biomarkers. In Figure 1.7A, a parent compound of (-)-epicatechin acts as a radical scavenger of superoxide while its methylated form acts like a aponin or NADPH oxidase inhibitor. Then, the production of superoxide was decreased, resulting in NO preservation and relating to the higher level of cGMP. The cGMP is 2^{nd} messenger response for the vasolidatory. When cGMP was increased, the vasolidation also was higher and can monitored by the flow mediated dilation or FMD. In addition, in the normal condition the oxidative stress can induces apoptosis and cell death by losing mitochondrial function, increasing caspase-3 and annexin-V binding protein. However, after pre-treatment human dermal fibroblast cells with 30 µM of (-)-epicatechin or 3´-methylated (-)-epicatechin prior to the addition of 50 µM of H_2O_2, this study showed that (-)-epicatechin and its methylated form significantly attenuated the loss of mitochondrial function and caspase-3 activity and these compounds also increased live cells (Spencer et al., 2001). In Figure 1.7B, quercetin and its methylated act as NADPH oxidase inhibitor similar to (-)-epicatechin and methylated (-)-epicatechin. In addition, after inducing cellular adhesion molecules (ICAM-1, E-selectin, VCAM-1) by TNF_α or tumour necrosis factor, quercetin, 3´- and 4´- methylated quercetin were equal effectively inhibited the expression of cellular adhesion molecules. Thus, this result indicated that the methylation of the catechol group of the B-ring of quercetin did not alter their anti-inflammatory activity (Lotito et al., 2011). Furthermore, in the normal condition angiotensin induced the phosphorylaion of cytoplasmic P47 subunit leading to

NADPH oxidase activation. However, quercetin and 3´-methylated quercetin can inhibit the overexpression of P47 subunit so the endothelial dysfunction was prevented (Sanchez et al., 2007). Another stressor like endothelin-1 can up-regulates of $P47^{phox}$ subunit and produced superoxide. However, quercetin and 3´-methylated quercetin prevented the increasing of vascular O_2^- production from uncoupled eNOS and NADPH oxidase through down-regulation of $P47^{phox}$ expression (Romero et al., 2009).

1.5 Aim and Objectives

The aim of this study was to investigate the metabolism and excretion of quercetin and (-)-epicatechin and their metabolites after co-consumption of onion and dark chocolate in healthy human. This will be achieved by the following objectives:

1) Quantification and comparison the amount of quercetin in yellow onion and red onion by HPLC-DAD.

2) Preparation a large batch of onion soup, re-quantification and comparison the amount of quercetin in this soup after preparation, before microwaving and after microwaving by HPLC-DAD.

3) Quantification the amount of (-)-epicatechin in different percentage of cocoa dark chocolate and also the selected 70% cocoa dark chocolate by HPLC DAD/FLD.

4) Evaluation the COMT activity in vitro using (-)-epicatechin or quercetin as substrates and extracted enzyme from pig liver cytosol.

5) Investigation the COMT variation in a small group of human volunteers by analysing urinary excretion of quercetin, (-)-epicatechin or its methylated forms after single consumption of red onion soup, 70% cocoa dark chocolate and co-consumption of both types of food by β-glucuronidase and sulfatase deconjugation and HPLC-MS analysis.

6) Investigation sulfatase activity using a commercial substrate and products, p-nitrocatechol sulfate (PNCS) and p-nitrocatechol (PNC).

7) Synthesis main metabolites of quercetin and (-)-epicatechin expected to be found in urine using enzymatic method and analysing by HPLC-MS.

8) Analysis main metabolites of quercetin and (-)-epicatechin excreted in urine in 27 healthy volunteers after single consumption of red onion soup, 70% cocoa dark chocolate and co-consumption of both types of food by UPLC-MS.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Standard flavonoids

Quercetin (Q) was purchased from Sigma Aldrich (Dorset, UK). Quercetin 3, 4′-*O*-diglucoside (Q3,4′diG), quercetin 4′-*O*-glucoside (Q4′G), isorhamnetin (3′MeQ), tamarixetin (4′MeQ), (-)-epicatechin (EC), daidzein (D) and taxifolin (T) were purchased from Extrasynthése (Genay, France). Ethyl gallate (EG) was purchased from Apin Chemicals Ltd (Oxfordshire, UK). All standards were of HPLC purity (above 98 %).

2.1.2 Food samples

Fresh yellow and red onions were purchased from a local supermarket (ASDA, Leeds, UK) prior to direct analysis or soup preparation. After red onion soup preparation, the soup samples were kept at -20ºC.

For dark chocolate, a preliminary sample (70% Cocoa Dark Chocolate, Lindt) was already available in the laboratory but others (Dark Chocolate French 70% Cocoa, Thorntons; the Best 85% Cocoa Dark Chocolate, Morrisons; excellence 90% Cocoa Dark Supreme Noir, Lindt) was obtained from a local supermarket (Morrisons, Leeds). Dark Chocolate French 70% Cocoa (Thorntons) was selected for use in human study. Then, the same batch of this 70% cocoa dark chocolate (batch number L120208) was purchased online through thorntons.co.uk from Thorntons PLC and kept at -20ºC.

2.1.3 General equipment

General equipment used in this study are as follows: First, two decimal balance (Percisa) and four decimal balance (Adventurer Analytical balance, Ohaus) were used for weighing some samples and chemicals or standards, respectively. Second, a Millipore Q water purifying system (Millipore, Hertfordshire, UK) was used to provide ultrapure, nuclease free water (≥18.2 MΩ cm at 25°C) both analytical purposes and as a mobile phase for HPLC and LC-MS. Third, for centrifugal evaporation, a Genevac (EZ-2 plus model; Fisher Scientific Ltd, Leicestershire, UK) was used for drying samples or standards. Fourth, pH meter (Hobiba compact pH meter, B-212 model) was used for buffer preparation. Fifth,

vortex (Vortex Genie 2, Scientific Inductries) was used for mixing samples. Additionally, microcentrifuge (MicroCL 17 Centrifuge, Thermo) and centrifuge (5810R, Eppendorf) were used for separating mixed solutions or samples. Finally, the water bath (NE2D Unstirred Digital Water Baths, Clifton Range®) and shaking water bath (GLS Aaqua 12 Plus, Grant) were used for sonicating HPLC solvent and incubating enzymatic synthesis reactions, respectively.

2.2 Methods for quantification of quercetin and (-)-epicatechin content in food

2.2.1 Determination of quercetin and its glycosides in onion

To compare the amount of quercetin in yellow and red onion, both types of these onions were used.

2.2.1.1 Freeze-drying preservation process

Freeze-drying (lyophilisation) is a dehydration process generally used to preserve an unstable compound or make samples more convenient for transportation. Freeze-drying was done by freezing the sample followed by reducing the surrounding pressure to allow the frozen water in the sample to sublimate, changing directly from the solid phase to the gas phase. To preserve some quercetin glycosides in fresh onion, in this study freeze-drying process used prior to quercetin glycoside extraction.

One bulb from yellow or red onion was skinned and chopped to increase the surface area prior to freezing. One hundred grams of each fresh sample was immediately frozen by putting in the freezer at -80°C for about 2 hr, and then freeze-dried using the “CoolSafe 55-9” at -55°C. The chopped onion samples were checked every 2 hr until dry. Each sample then was weighed again. Next, freeze-dried onions were finely ground into a powder with a coffee grinder. The grinder was primed with a small amount of onion from the freeze-dried sample, 2.5 g of yellow onion or 3.8 g of red onion, before grinding 10 g of each sample. To grind the sample, coarse level (No.12), the mid-level (No.6) and fine level (No. 1) were used in that order. The onion powder was weighed again (8.99 g for white onion and red 9.01 g for red onion). The percentage lost was calculated (10.1% for yellow onion and 9.9% for red onion). Finally, the onion powder was then kept in a 50 ml tube and stored at -

20°C. This method was adapted from published methods (Pérez-Gregorio et al., 2011, Mullen et al., 2004, Price and Rhodes, 1997).

2.2.1.2 Extraction of quercetin glycoside by homogenisation or sonication

To compare which extraction method is better to extract quercetin and its glycosides from fresh onion, homogenisation or vortex followed by sonication was tested in this step.

2.2.1.3 Homogenisation

To prepare 150 ml of extraction solution, 2.6 mg of ascorbic acid (MW= 175.12 g/mol) and 3.8 mg of diadzein (MW 254.25 g/mol) were added to 150 ml of 70% methanol. The solution was then mixed by stirring. The homogeniser was first cleaned with milliQ water before homogenising each sample. One g of each dry onion powder was homogenised in 10 ml of 70% methanol containing 100 µM ascorbic acid and 100 µM internal standard (diadzein) for 1 min using a Polytron PT1600E. During homogenisation, the onion sample was kept on ice. The mixture was then centrifuged (Eppendorf 5801R) at 3000xg at 4°C for 15 min. The supernatant was collected and the pellet further extracted and centrifuged two more times. The three supernatants (approximately 27.5 ml) were combined and reduced to dryness under vacuum using Genevac (setting a maximum temperature at 37°C and using HPLC fraction program for about 2-3 hr and followed by aqueous program for 2-3 hr). The dried extract was re-dissolved in 10 ml of 50% ethanol in water, vortexed and kept at -20°C before analysis by HPLC.

2.2.1.4 Vortex and sonication

To prepare 100 ml of extraction solution, 1.7 mg of ascorbic acid (MW= 175.12 g/mol) and 2.5 mg of diadzein (MW 254.25 g/mol) were added to 70 ml of 70% methanol and then the volume adjusted to 100 ml. The solution was then mixed. One g of each onion dry powder was mixed with 10 ml of 70% methanol containing 100 µM ascorbic acid and 100 µM internal standard (diadzein), vortexed for 2 min followed by sonicating at 25-27°C for 10 min. The samples were then mixed briefly again before centrifugation at 3000xg at 4°C for 15 min. The supernatant was collected and the pellet further extracted and centrifuged two more times. The three supernatants (approximately 27.5 ml) were combined and reduced to dryness in vacuum using a Genevac (Max. temperature = 37°C, program = HPLC

fraction, overnight). The dried extract was re-dissolved in 10 ml of 50% ethanol in water, vortexed and kept in -20°C before analysis.

2.2.1.5 Standard preparation of quercetin-3, 4′-*O*-diglucoside, quercetin-4′-*O*-glucoside and quercetin

To prepare 200 µM of stock mixture, 125 µl of 1.6 mM Q3,4′G (1 mg in 1 ml of 50% ethanol in MiiliQ water), 90.9 µl of 2.2 mM Q4′G (1 mg in 10 µl of DMSO and 990 µl of absolute ethanol) and 66.7 µl of 3 mM quercetin (1 mg in 10 µl of DMSO and 990 µl of absolute ethanol) were mixed and then adjusted to 1000 µl with 50% ethanol in MilliQ water. To prepare 1000 µM of diadzein (as internal standard), 256.4 µl of 3.9 mM of diadzein stock (1 mg in 1 ml of absolute ethanol) was mixed with 743.6 µl 50% ethanol in MilliQ water. Subsequently, nine dilutions of standard sample with internal standard were prepared as shown in Table 2.1.

Table 2.1: Preparation of standard mixtures of quercetin-3,-4′-*O*-di-glucoside, quercetin-4′-*O*-glucoside and quercetin dissolved in 50% ethanol. Each standard contained a final concentration of 100 µM daidzein (as an internal standard).

Standard No.	Final conc. (µM)	[Standard mixture] (200 µM) (µl)	50% Ethanol in milliQ water (µl)	[Daidzein] (1000 µM) (µl)	Total volume of each standard (µl)
1	100	200	160	40	400
2	75	150	210	40	400
3	50	100	260	40	400
4	25	50	310	40	400
5	10	20	340	40	400
6	7.5	15	345	40	400
7	5	10	350	40	400
8	2.5	5	355	40	400
9	1	2	358	40	400

2.2.1.6 Sample preparation

For homogenisation samples, 1 µl of re-constituted sample from 2.2.1.2.1 was diluted with 99 µl of 50% ethanol in MilliQ water (ratio 1:100) before preparing the sample as shown in Table 2.2.

For vortex and sonication samples, 10 µl of re-constituted sample from 2.2.1.2.2 were diluted with 90 µl of 50% ethanol in MilliQ water (ratio 1:10) before preparing the sample as shown in Table 2.2.

Table 2.2: Reaction mixture for non-spiked and spiked samples. Each sample contained a final concentration of 100 µM daidzein (as an internal standard). For spiked sample, a final concentration of 100 µM of mix standards, quercetin-3,4′-*O*-di-glucoside, quercetin-4′-*O*-glucoside and quercetin, were added into the reaction.

Reaction mixture	**Final conc. (µM)**	**Volume (µl)**	
		non-spike	**spike**
Diluted onion samples in 50% ethanol	-	100	100
1000 µM diadzein	100	40	40
200 µM of mix standards (Q3,4'diG, Q4'G, Q)	100	-	200
Solvent A (MilliQ water and 0.1% formic acid)	-	260	60
Total volume		**400**	**400**

2.2.1.7 Determination of quercetin and its glycoside in yellow and red fresh onion by high performance liquid chromatography-diode array detection (HPLC-DAD)

Reversed phase HPLC with diode array detection (Agilent technologies 1200 series, Binary SL) was used to analyse samples. For diode array detection (DAD), a variable wavelength (from 190 to 400 nm) of UV-visible spectra was scaned and recorded. It consisted of a solvent degassing unit, binary pump, autosampler, thermostatic column oven and diode array detector. The column, a ZORBAX Eclipse XDB-C18, 4.6x50 mm, 1.8 µm, 582 µl column volume (Agilent Technologies) was used in this study. Solvent A was composed of milli Q water and 0.1% formic acid. Solvent B was composed of acetonitrile and 0.1% formic acid. These two solvents were prepared using the following procedure. For solvent A, about 900 ml of water was added to a 1000 ml volumetric flask. Then, 1000 µl of

formic acid was added and the volume was adjusted to 1000 ml. The solvent A was mixed until it was homogeneous. This solvent A was transferred to a 1000 ml bottle. Solvent B was prepared as a similar method. About 900 ml of acetonitrile was added to 1000 ml volumetric flask. Then, 1 ml of formic acid was added and the volume was adjusted to 1000 ml with milli Q water. Solvent B was mixed until it was homogeneous and transferred to 1000 ml bottle. To protect it from UV light, the solvent B bottle was covered by aluminium foil. To remove air bubbles, both solvents were placed in the sonicator for approximately 30 minutes before performing HPLC. The optimal condition for quercetin analysis was modified from Morand et al (1998), initiated at 15% of solvent B, kept further for 2 min of 15% solvent B and increased linearly to reach 40% at 22 min, kept steady at this 40% for 2 min, the percentage of solvent B was finally increased to 90% at 25 min for 3 min before initial starting conditions at 29 min and were resumed for 5 min to re-equilibrate the column for the next injection. The total method length was 34 min. The flow rate and the injection volume were 0.5 ml/min and 10 µl, respectively.

2.2.1.8 Onion soup preparation

To prepare a meal rich in quercetin, red onion, which had a higher level of quercetin than yellow and white onion, was used to prepare a soup. Onion soups were prepared in the same manner on the same day in one batch and then frozen at -20°C. Six kilograms of peeled red onions were weighed. Each bulb was then chopped into a quarter and put into a blender (Kenwood Gourmet, PT800) to make small pieces. To make a stock, chicken flavour food seasoning powder (18g/1L of water) was pre-dissolved in 12 L of water before boiling it. The blended onion was then added to the chicken stock, stirred, covered and left to simmer for 7 min. To make the soup more homogenous, a large homogenizer (Mixer emulsifier, Silverson, Model AXL, UK) was used. The soup was left to cool, and then 130 ml portions were aliquoted into a clip and lock plastic container. Some onion soup was used to analyse quercetin content on the day of preparation, and others were kept frozen at -20°C until use (see Figure 1). On the day of use, the portions of soup were defrosted and then reheated in a microwave. Thus, the content of onion soup before and after reheating in a microwave also was analysed.

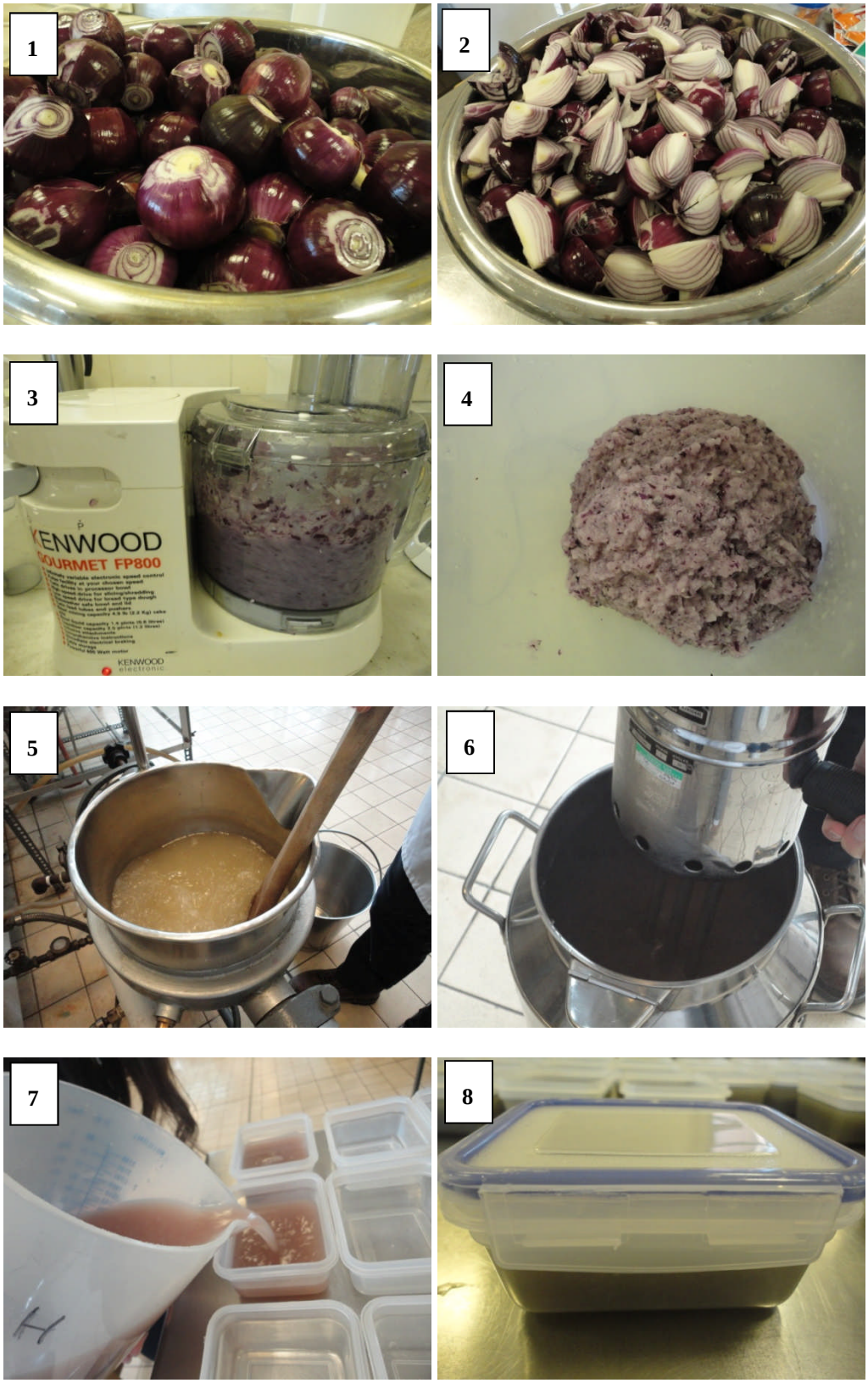


Figure 2.1: Onion soup preparation. 1 Red onions were peeled and weighed. 2. Each bulb was chopped into a quarter. 3 and 4 Put into a blender to make small pieces. 5 The stock was made followed by adding blended red onion into it and left to simmer for 7 min. 6 Soup was transferred to another container and homogenised after cooling down. 7 and 8 aliquoted and place in a clip and lock plastic container.

To test the quercetin content of the red onion soup from 2.2.1.6 before feeding to volunteers, three type of red onion soup (on the day of preparation, from freezer before or after reheating in microwave) were used to re-analyse the quercetin content. Five millilitres of each type of red onion soup was put into 50 ml tubes in duplicate. For the sample from the freezer (-20°C), this sample was defrosted by putting at room temperature followed by heating with or without microwaving for 1 min. Then, two volume of extraction solution (dissolved in absolute methanol or 70% methanol) was added to each sample. In total, there were 2 tubes of red onion soup on the day of preparation in absolute methanol solution or in 70% methanol, 2 tubes of defrosted sample before putting in microwave in absolute methanol solution or in 70% methanol and 2 tubes of defrosted sample after putting in microwave in absolute methanol solution or in 70% methanol. The samples were then mixed briefly using a vortex mixer for 2 min then centrifuged at 3000xg at 4°C for 15 min. The supernatant was collected and the pellet further extracted and centrifuged two more times. The three supernatants were combined and reduced to dryness under vacuum using Genevac (Max. temperature = 37°C, program = HPLC fraction, overnight). The dried extract was re-dissolved in 5 ml of 50% ethanol in water, vortexed and then analysed by HPLC-DAD as described in section 2.2.1.5.

2.2.1.10 Data analysis

All data are presented as mean ± standard deviation and results are expressed as mg/100 g of fresh weight and mg of quercetin equivalents. To compare the content of quercetin glucosides and total quercetin between the two different types of onion; yellow and red onion, independent t-test was used, while, dependent or paired t-test was used to compare the content of quercetin glucosides and total quercetin between different types of extraction method; homogenisation and vortex followed by sonication. For red onion soup, the content of quercetin glucosides and total quercetin between after preparation and before microwaving or before microwaving and after microwaving and between extracting with absolute methanol and 70% methanol were compared by dependent or paired t-test. The SPSS program was used with all comparisons at 95% confidence interval.

2.2.2 Determination of (-)-epicatechin in dark chocolate

The preliminary study consisted of testing the efficiency of the extraction method and comparing the content of (-)-epicatechin in different % cocoa in various commercial brands of dark chocolate. Four brands of dark chocolate; 70% cocoa dark chocolate (Thorntons and Lindt), 85% cocoa dark chocolate (Morrisons) 90% cocoa dark chocolate (Lindt), were extracted and analysed for their content of (-)-epicatechin. As a result of those analysed, 70% French dark chocolate (Thorntons, batch number L120208) was selected and the content of (-)-epicatechin of this type of chocolate was analysed once more prior to performing the human study.

2.2.2.1 Removal of the lipid from dark chocolate

Dark chocolate was milled with a grinder (Krups F203 Grinder) then 5 g of this chocolate sample was placed into a 50 ml centrifuge tube in duplicate. To remove the lipid fraction from dark chocolate, each tube was filled with hexane to the 45 ml mark and capped tightly. To completely disperse and dissolve, these samples were vortexed for at least 1 min before sonicating at 50°C for 5 min. They were then vortexed briefly followed by centrifuging for 5 min at 3000xg at room temperature. The hexane was decanted into a suitable waste container. The pellet was kept and the previous step repeated twice more. The residual hexane was then evaporated from the defatted solid fraction in a fume cupboard overnight.

2.2.2.2 (-)-Epicatechin extraction

To extract epicatechin from the defatted solid fraction, acetone water acetic acid (AWAA) extraction solution was prepared by combining 70 ml of HPLC grade acetone with 29.5 ml of milliQ water and 0.5 ml of glacial acetic acid (70%:29.5%:0.5%). Then, 2 g of defatted chocolate was weighed into a 15 ml centrifuge tube and 5 ml of AWAA added. It was mixed by shaking by hand then vortexing for at least 2 min to completely disperse the chocolate. The sample was sonicated at 50°C for 5 min, vortexed briefly and then centrifuged for 5 minutes at 3000xg. Finally, the supernatant was passed through a conditioned strong cation exchange SPE cartridge. To prepare the SPE, 5 ml of HPLC grade water was passed through the SPE column. To avoid samples diluted with water, 2 ml of supernatant was loaded onto the SPE cartridge and released to waste at a low flow rate. After the level of supernatant was above SPE bed, the valve of SPE was closed followed by taking the SPE cartridge out. To force some supernatant inside, the needle was taken

out followed by opening the SPE. To collect the sample, a 17 mm, 0.2 µm PTFE filter was placed at the end of the cartridge and then 1 ml of supernatant was loaded onto the SPE cartridge. The sample then was pushed through the SPE cartridge using the syringe plunger and syringe adaptor. Finally, the eluent was collected in a 1.5 ml Eppendorf tube and kept at 4°C in preparation for the next step.

2.2.2.3 Standard (-)-epicatechin (EC) preparation

To prepare 2000 µM of epicatechin stock solution for 800 µl, 470.8 µl of 3.4 mM epicatechin in AWAA (1 mg/ml) was mixed with 329.2 µl of AWAA. To prepare 1000 µM of daidzein stock solution for 1000 µl, 0.3 mg of diadzein was weighed and dissolved in 1 ml of absolute ethanol. Then, seven standards sample of epicatechin were prepared using Table 2.3.

Table 2.3: Preparation of standards (-)-epicatechin dissolved in extraction solution (AWAA). Each standard contained a final concentration of 100 µM daidzein (as an internal standard).

Standard No.	Final conc. (µM)	[Standard epicatechin] (2000 µM) (µl)	Extraction solution (AWAA) (µl)	[Daidzein] (1000 µM) (µl)	Total volume of each standard (µl)
1	1000	200	160	40	400
2	750	150	210	40	400
3	500	100	260	40	400
4	250	50	310	40	400
5	100	20	340	40	400
6	50	10	350	40	400
7	10	2	358	40	400

2.2.2.4 Sample preparation

Extracted dark chocolate was prepared with and without spiking using reaction mixtures as shown in Table 2.4 prior to inject on to the HPLC system.

Table 2.4: Reaction mixture for non-spiked and spiked samples. Each sample contained a final concentration of 100 µM daidzein (as an internal standard). For spiked sample, a final concentration of 500 µM of standard (-)-epicatechin was added into the reaction.

Reaction mixture	**Final conc. (µM)**	**Volume (µl)**	
		non-spike	**non-spike**
Extracted dark chocolate samples	-	100	100
1000 µM diadzein in absolute ethanol	100	40	40
2000 µM of standards epicatechin	500	-	100
AWAA extraction solution	-	260	160
Total volume		**400**	**400**

2.2.2.5 Determination of (-)-epicatechin in dark chocolate by high performance liquid chromatography-diode array detection/ fluorescence detection (HPLC-DAD/HPLC-FLD)

Reversed phase HPLC with diode array detection/fluorescence detection (Agilent technologies 1200 series, Quaternary) was used to analyse samples. The same type of column, solvent A and solvent B in section 2.2.1.5 were used in this study. The condition for epicatechin analysis was performed as follows; initiated at 5% of solvent B and increased to reach 10% within 5 min, 30% within 20 min, 50% for 7 more min, the percentage of solvent B was finally increased to 90% at 32 min and kept steady at this 90% for 4 min before back to initial starting conditions (5% of solvent B) at 37 min and were resumed for 3 min to re-equilibrate the column for the next injection The total method length was 40 min. The flow rate and the injection volume were 0.3 ml/min and 5 µl, respectively. In fluorescence detector mode, epicatechin was excited at 230 nm and detected by emission at 321 nm.

2.2.2.6 Data analysis

All data are presented as mean ± standard deviation and results are expressed as mg/100 g of fresh weight. The different between the content of (-)-epicatechin in 70% dark chocolate detected with DAD and FLD mode were compared using independent t-test at 95% confidence interval, SPSS program.

2.3 Methods for catechol-*O*-methyltransferase (COMT) *in vitro*

2.3.1 COMT extraction from pig liver

Pig liver COMT was extracted using the method adapted from Tilgmann et al, 1990.

All steps were performed at 4°C. 50 g of pig liver was weighed and homogenized in 150 ml of 0.05 M sodium phosphate, pH 7.2 and 0.2 mM phenyl methyl sulfonyl fluoride (PMSF) using a blender. The homogenate was separated into centrifuge tubes (approximately 40 ml for each tube). These homogenates were vortexed and then let stand in an ice bath for approximately 10 min. Then, the first centrifugation was performed at 10000xg for 25 min followed by the second centrifugation at 30000xg for 25 min. Supernatants were collected and the amount of protein were measured using Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). Then supernatants were aliquoted into 1.5 ml microcentrifuge tube (1 ml of each 1.5 ml microcentrifuge tube). Finally, all extracted enzyme were kept at -20°C for further COMT assay.

2.3.2 COMT assay

COMT assay was performed by the method adapted from Penning, 1979. COMT from pig liver was assayed with (-)-epicatechin (EC) or quercetin (Q) as the methyl acceptor. The standard incubation mixture consisted of: 0.05 M Tris-HCI buffer, pH 7.9, 1 mM S-adenosyl-L-methionine (SAM) dissolved in milliQ water (need to prepare fresh), 1 mM $MgC1_2$ dissolved in milliQ water, 1 mM EC dissolved in absolute ethanol or 1 mM Q dissolved in absolute ethanol containing 1% Dimethyl sulfoxide (DMSO), 1 mM dithiothreitol (DTT) dissolved in milliQ water (prepared fresh) and 0.25 ml of the partially purified COMT (200-400 µg of protein) (Table 2.5). Negative controls were prepared by omitting substrate or COMT. The reaction was started by adding the enzyme and terminated after carried out at 37°C

for 10 min in water bath by adding 2 volumes of absolute ethanol and 1 mM ascorbic acid followed by cooling on ice for 2 min. All samples were centrifuged at 10,000xg for 1 min. Supernatant was collected and filtered prior injected on the HPLC. In this study, 1 mM daidzein dissolved in milliQ water containing 1% DMSO also was added as the internal standard.

Table 2.5: The reaction mixtures of COMT assay using (-)-epicatechin or quercetin as a substrate. Negative control was prepared by omitting substrate or enzyme.

Reaction mixture	**Final Conc.**	**Volume (µl)**		
		No Substrate	**1 mM Substrate**	**No Enzyme**
MilliQ water	_	182.5	177.5	265
1 M Tris-HCl buffer, pH 7.9	0.05 M	25	25	25
10 mM Daidzein	1 mM	50	50	50
10 mM SAM (S-adenosyl-L-methionine)	1 mM	50	50	50
10 mM MgCl2	1 mM	50	50	50
50 mM Epicatechin or 50 mM Quercetin	1 mM	-	10	10
10 mM DTT (Dithiothreitol)	1mM	50	50	50
0.125 mg/ml Extracted COMT enzyme from pig liver	0.02 mg/ml	87.5	87.5	-
Absolute ethanol	6%	5	0	0
Total volume		**500**	**500**	**500**

2.3.3 Determination of (-)-epicatechin, quercetin and its methylated forms by HPLC-DAD

HPLC-DAD was used to analyse (-)-epicatechin, quercetin and its methylated forms in samples from section 2.3.2. The HPLC system, the type of column, the type of solvent and also solvent preparation were described in section 2.2.1.5. The HPLC condition for analysis of (-)-epicatechin and its methylated forms was as follows; initiated at 5% of solvent B and increased to reach 10% within 5 min, 30% within 20 min, the percentage of solvent B was finally increased to 90% at 25 min and kept steady for 4 min before returning back to initial starting conditions

at 30 min, which were resumed for 3 min to re-equilibrate the column for the next injection. The total method length was 33 min. The flow rate and the injection volume were 0.5 ml/min and 10 µl, respectively. The HPLC condition for analysis of quercetin and its methylated forms was the same as described in section 2.2.1.5.

2.4 Methods for enzymatic synthesis of the main quercetin and (-)-epicatechin conjugates found in urine

2.4.1 Glucuronidation of (-)-epicatechin or quercetin

Glucuronidation reagents were composed of (1) HEPES buffer solution containing 31.25 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) or HEPES (Fisher Scientific UK, Cas No. 7365-45-9), 100 mM $MgCl_2$ anhydrous (Cas No. 7786-30-3) and 2 mM ascorbic acid acid (Sigma UK, Cas No. 50-81-7) in the portion 8:1:1 and the pH was adjusted to 7.2 with 2N NaOH. (2) 5 mg alamethicin (Sigma UK, Cas No. 27061-78-5) dissolved in 1 ml of 25 mM HEPES. (3) 10 mM uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA, Sigma UK, Cas No. 63700-19-6) dissolved 25 mM HEPES. (4) 20 mM saccharolactone dissolved in 25 mM HEPES. (5) a substrate, 4 mM (-)-epicatechin (Sigma UK, Cas No. 490-46-0) dissolved in MilliQ water or 4 mM quercetin (Sigma UK, Cas No. 117-39-5) dissolved in dimethyl sulfoxide (DMSO). (6) the source of glucuronidase obtained from pig liver microsome available in the laboratory.

Briefly, pig liver homogenate was prepared according to Gibson et al., 1994 with slightly modification by Xu 2011. Fresh pig liver purchased from Morrison Supermarket (Leeds, UK) was immediately placed in ice-cold 0.25 M sucrose to rinse off excess blood. The tissue was then blotted dry, weighed and added to four times of its weight of 0.25 M sucrose to make 20% (w/v) homogenate. The tissue was finely chopped with scissors and homogenised with Potter-Elvehjem Tissue Grinder with Teflon Pestle (55 mL, Wheaton), which was dipped into ice bath. The homogenate was then centrifuged in a refrigerated centrifuge (Beckman J2-HS Centrifuge) to isolate subcellular fractions at 12,500 g for 15 min and the supernatant was carefully decanted. The microsomal tissue fraction was then prepared by calcium precipitation method. Aliquots of 50 ml of post-mitochondrial supernatant was mixed with 0.5 mL of 88 mM CaCl2 and left to stand on ice for 5 min, with occasional swirling. The mixture was centrifuged at 27, 000g for 15 min,

the supernatant was discarded and the pellet was resuspended in 2.5 mL of 0.05 M Tris-Cl, pH 7.5 containing 10 mM EDTA and 20% glycerol. The protein concentration was quantitated by Bradford protein assay (8.35g protein /mL). The final microsomal tissue fraction was then aliquoted in 250 µL per tube and stored at -80 °C for further assay.

To synthesise (-)-epicatechin or quercetin glucuronides, the pre-incubation mixture comprised of 40 µl of HEPES buffer solution (pH 7.2), 20 µl of 5 mg/ml alamethicin (UGT activator), 20 µl of 10 mM UDPGA (Glucuronic acid donor), 5 µl of 20 mM saccharolactone (a β-glucuronidase inhibitor) and 20 µl of substrate, 4 mM of (-)-epicatechin in milliQ water or 4 mM of quercetin in DMSO, were placed into 1.5 ml microcentrifuge tube followed by pre-incubated at 37°C for 5 min. To initiate the glucuronidation reaction, 5 µl of pig microsomal suspension (8.35 g protein/ml) was added to the reaction mixture. The total volume was 110 µl. In addition, negative control samples (without substrates or enzyme from pig microsomal suspension) also were prepared and treated the same way as samples. Then, all samples were incubated in a 37°C water bath for 4 hr. Next, the reaction mixture was stopped by the addition of 200 µl of ice cold methanol containing 1.6 mM ascorbic acid and cooled on ice for 15 min. The precipitated protein was removed by centrifugation at 17000xg at 4°C for 15 min. The supernatant was then taken and placed into a new 1.5 ml microcentrifuge tube. To dispose of precipitated protein from the sample, the previous centrifugation step was repeated once more. Finally, the supernatant was dried under centrifugal evaporation using HPLC fraction and lamp off program (approximately 45 min) and kept in -20°C before use.

In addition, to synthesise 3′-*O*-methylated quercetin glucuronide, a commercial compound, 3′-*O*-methylated quercetin (isorhamnetin) was used as a substrate.

2.4.2 Sulfation of (-)-epicatechin or quercetin

Sulfation reagents were composed of (1) 250 mM potassium phosphate buffer (PPB) containing 250 mM dipotassium hydrogen phosphate, K_2HPO_4 (Sigma UK, Cas No. 7758-11-4) and 250 mM potassium dihydrogen phosphate, KH_2PO_4 (Sigma UK, Cas 7778-77-0) in the portion 8:2 and the pH was adjusted to 7.4 with 5.6 mM ascorbic Acid (Sigma UK, Cas No. 50-81-7). (2) 1.97 mM adenosine 3′-phosphate 5′-phosphosulfate lithium, PAPS (Sigma UK, Cas No. 109434-21-1)

dissolved in 250 mM PPB. (3) 200 mM *p*-nitrophenyl sulfate potassium salt, PNS (Sigma UK, Cas No. 6217-68-1) dissolved in milliQ water (had to prepare fresh). (4) 5 mM DL-Dithiothreitol, DTT (Sigma UK, Cas No. 3483-12-3) dissolved in milliQ water (need to prepare fresh). (5) 100 mM sodium sulphite, Na_2SO_3 (Sigma UK, Cas No. 7757-83-7) dissolved in milliQ water. (6) a substrate, 4 mM (-)-epicatechin, EC (Sigma UK, Cas No. 490-46-0) dissolved in MilliQ water or 4 mM quercetin, Q (Sigma UK, Cas No. 117-39-5) dissolved in DMSO. (7) The source of sulfatase obtained from pig liver cytosol suspension available in the laboratory.

Briefly, pig liver cytosolic suspension was prepared using a modified version of a method by Campbell et al., 1987. Fresh pig liver purchased locally was placed in an ice-cold 250 mM sucrose solution to remove excess of blood. The liver was blotted dry and a sample of 13 g aliquot was finely chopped with scissors then homogenised with 50 mL of ice-cold Tris-HCl buffer (50 mM, pH 7.5) using a hand blender. The homogenate was separated in a refrigerated centrifuge (Beckman JA25.50; High Wycombe, UK) at 12,200 rpm for 10 min at 4°C. The resulting supernatant was decanted and further centrifuged for 90 min at 17,000 rpm at 4°C. The final supernatant was recovered, aliquoted and stored at -80°C for further use.

To synthesise (-)-epicatechin or quercetin sulfates, the pre-incubation mixture comprised of 15 µl 250 mM potassium phosphate buffer (pH7.4), 2.5 µl of 1.97 mM PAPS, 5 µl of 200 mM PNS, 10 µl of 5 mM DTT, 5 µl of 100 mM Na_2SO_3 and 1 µl of substrate, 4 mM of (-)-epicatechin in milliQ water or 4 mM quercetin in DMSO, were placed into a 1.5 ml microcentrifuge tube followed by pre-incubation at 37°C for 5 min. To initiate the sulfation reaction, 12.5 µl of pig liver cytosol suspension (85.6 mg protein/ml) was added to the reaction mixture. The total volume was 51 µl. In addition, negative control samples (without substrates or enzyme from pig microsomal suspension) were also prepared and treated the same way as test samples. Then, all samples were incubated in a 37°C water bath for 4 hr. Subsequently, the reaction mixture was stopped by adding 100 µl of ice-cold acetonitrile and cooling on ice for 15 min. The precipitated protein was removed by centrifugation at 17000xg at 4°C for 15 min. The supernatant was then taken and placed into a new 1.5 ml microcentrifuge tube. To dispose of all precipitated protein from the sample, the previous centrifugation step was repeated

once more. Finally, the supernatant was dried under centrifugal evaporation using HPLC fraction and lamp off program for 15 min and kept in -20°C before use.

2.4.3 Methylation of (-)-epicatechin, quercetin, (-)-epicatechin glucuronides or (-)-epicatechin sulfates

Methylation reagents were the same as described in Table 2.5 of section 2.3.2 with small modification. These reagents were composed of milliQ water, 500 mM Tris-HCl (pH 7.4), 10 mM SAM, 10 mM $MgCl_2$, 5 mM DTT, 4 mM substrate ((-)-epicatechin, quercetin, (-)-epicatechin glucuronides or (-)-epicatechin sulfates) and catechol-*O*-methyltransferase obtained from pig liver extraction in section 2.3.1.

To synthesise methylated-(-)-epicatechin or quercetin, all reaction mixtures; 7.5 µl of milliQ water, 25 µl of 500 mM Tris-HCl (pH 7.4), 25 µl of 10 mM SAM, 30 µl of 10 mM $MgCl_2$, 50 µl of 5 mM DTT, 62.5 µl of 4 mM substrate and 50 µl of pig liver cytosol suspension were placed into 1.5 ml microcentrifuge tube. The total volume was 250 µl. In addition, negative control samples (without substrates or enzyme from pig microsomal suspension) also were prepared and treated the same way as samples. Then, all samples were incubated in a 37°C water bath for 1 hr. The reaction mixture was then stopped by adding 2 volumes of absolute methanol contain 1% ascorbic acid followed by keeping on ice for 2 min. The precipitated protein was removed by centrifugation at 17000xg at 4°C for 15 min. The supernatant was then taken and placed into a new 1.5 ml microcentrifuge tube. Finally, the supernatant was dried under centrifugal evaporation using HPLC fraction and lamp off program and kept in -20°C before use.

Furthermore, methylated-(-)-epicatechin glucuronides and methylated-(-)-epicatechin sulfates were synthesised using dried (-)-epicatechin glucuronides and (-)-epicatechin sulfates from section 2.4.1 and 2.4.2 as a substrate.

2.4.4 Determination of total conjugated form yield

The % yields of conjugated forms were calculated according to the equation as shown below.

$$\text{The \% yield} = \frac{Peak\ area\ of\ conjugates}{Peak\ area\ of\ (conjugates+aglycone)} \text{ x } 100$$

2.5 Methods for urine analysis

2.5.1 Human subjects and study design

This study was approved by MEEC Faculty Research Ethics Committee, University of Leeds (Ethics reference: MEEC 11-041, Appendix B). All healthy volunteers (18 to 60 years of age, 18.5 and 24.99 kg/m^2 BMI) who were white Caucasians, non-smokers, took no dietary supplements or medication, had no GI tract, metabolic and chronic diseases and were not pregnant or lactating gave their written informed consent and participated in this study. Fifty participants were aimed to recruit at the beginning but only twenty seven subjects were positive response to participate and complete this human study. Subject's characteristics show in Table 2.6.

Table 2.6: Subject's characteristics

Parameters (units)	Male (n=6)		Female (n=21)	
	Mean±SD	Range	Mean±SD	Range
Age (years)	28±11	20-50	26±9	18-55
Weight (kg)	73±8	63-82	60±6	52-75
Height (cm)	181±9	170-194	166±7	153-176
BMI (kg/m^2)	22±3	19-25	22±1	19-25

The study consisted of 3 instances of a single-dose of food with 0-24 hr urine collection. In addition, Latin Square Design was used in this study. Some volunteer started to consume onion soup or 70% cocoa dark chocolate or both for the first day study followed by the rest of food samples in order for two more times. In the case of consuming onion soup for the first day study, for example, volunteers avoided quercetin and (-)-epicatechin rich food (see Appendix A) for 24 hr and observed over-night fasting for 12 hr before the study day. On day 1 study, volunteers were asked to void their bladder immediately after waking, and then to drink a glass of water (200-300 ml). Approximately an hour later, the volunteers again voided their bladder and this time urine was collected in the baseline-labelled urine container at the School of Food Science and Nutrition. Soon after the baseline urine collection, the volunteers were asked to consume onion soup (containing 50 g of onion) provided along with toast and butter for breakfast in the School of Food Science and

Nutrition. The time of consumption was recorded. Urine was then collected in a labelled urine container from 0-24 hr. All other foods consumed during the day were recorded using 24 hr dietary record (see Appendix A). Volunteers were asked to do the same procedure as day 1 study but 45 g of dark chocolate or onion soup and 45 g of dark chocolate together was provided to volunteers instead of onion soup for day 2 or day 3 study, respectively. Each study day was separated by at least 2 days, and each volunteer completed the study within 3 weeks

2.5.2 Urine collection and acellular urine

Approximately 3 g of ascorbic acid was placed into the clean urine collection bottle. Then, volunteers collected the urine samples at specified time in the designated urine containers. Urine samples were transported to the lab within 12 hr of the collection where they processed within 24 hr before being frozen at -20°C. Upon arrival, the volume of the specimen was measured by reading the scale on the urine container, and 2x50 ml aliquots taken. These aliquots were centrifuged at 2000xg, 4°C for 10 min to remove insoluble materials and cellular debris. After that the supernatant was carefully decanted to within approximately 1-2 ml of the cell pellet. Finally, the samples were labelled and stored at –20°C until analysis. The sediment of the centrifuged urine specimen was decontaminated before disposal by adding a disinfectant (1 % Virkon solution, 2x5 g tablet/l, in accordance with the manufacturer's guidelines) to the sediment and leaving overnight. The sediment was then discarded in the clinical waste bags.

2.5.3 Enzyme hydrolysis method

The urine was defrosted and vortexed. One ml of baseline or 24 hr urine sample was pipetted into 15 ml centrifuge tube (in triplicate for each biological sample). Then, 10 µl of internal standard (1 mg/ml of diadzein stock solution in absolute ethanol, final concentration 10 µg/ml) was added to each tube of urine followed by 150 µl of 0.2 M sodium phosphate buffer, pH 7.0 containing 5 U of β-glucuronidase (from *Escherichia coli* type IX-A) dissolved in 0.1 M sodium phosphate buffer pH 7 with 1 mg/ml BSA and 0.03 U of sulfatase (from *Aerobacter aerogenes* type IV) dissolved in 0.1 M sodium phosphate buffer pH 7 with 1 mg/ml BSA. Next, urine samples were incubated in a shaking water bath at 37ºC, 100 rpm for 2 hrs. One ml of ethyl acetate (1:1 v/v urine/ethyl acetate) was added to urine sample, vortexed for 2 min and centrifuged at 20ºC, 3000xg for 5 min. Supernatant

on the upper layer was collected and then the previous extraction step was repeated twice. Collected supernatant of each tube (approximately 3 ml) was dried in by centrifugal evaporation (lamp off, low BP mode) for 2 hr. Dried urine samples were reconstituted by adding 15 µl of solvent B (ACN and 0.1% formic acid), 83 µl of solvent A (MilliQ water and 0.1% formic acid) containing 100 µM ascorbic acid and 2 µl of 500 µM taxifolin in 50% ethanol. All samples were then vortexed for 2 min and centrifuge at 20ºC, 3000xg for 5 min. Supernatant of each sample was removed carefully and placed into amber HPLC vials prior to direct injection on LC-MS (see Section 2.5.8).

2.5.4 New enzyme hydrolysis conditions

A new enzyme hydrolysis condition, changing type of buffer, source and amount of enzyme and increasing the unit of enzyme, was used to analyse quercetin, (-)-epicatechin and its methylated metabolites in urine samples. The ABSS buffer at pH 5 was used instead of 0.2 M sodium phosphate buffer, pH 7.0. This buffer was composed of 20 g of ascorbic acid, 0.11 g of EDTA, 6.24 g of NaH_2PO_4 and 7.12 g of Na_2HPO_4 in total volume 100 ml and adjusted to pH 5 with 1 M NaOH. In addition, the unit of enzyme also was increased from 5 U to 200 U for β-glucuronidase and from 0.0 3U to 5 U and up to 20 U for sulfatase. The same source β-glucuronidase (*Escherichia coli* type IX-A) in section 2.5.3 was still used in this section. However, the source of sulfatase was changed from *Aerobacter aerogenes* type IV to *Abalone entrails* type VIII.

To perform an enzyme hydrolysis, all urine samples were defrosted and vortexed. Then, 200 µl of each urine sample was placed into 1.5 ml microcentrifuge tube followed by adding 200 µl of ABSS buffer, pH 5 containing 10 µl of 0.1 mg/ml daidzein dissolved in 0.2M sodium acetate buffer pH 5 (as an internal standard), 8 µl of 25KU/ml of β-glucuronidase dissolved in 75 mM sodium phosphate buffer pH 6.8 with 1 mg/ml BSA (final unit was 200U), 10 µl of 25 KU/ml sulfatase dissolved in 0.2 M Sodium acetate buffer, pH 5 (final unit was 5 U) and 172 µl of ABSS, pH 5. The ratio of urine sample to ABSS buffer, pH 5 was 1:1. All samples and reagents were handled on ice throughout extraction. Then, each sample was reverse mixed thoroughly, vortexed and incubated at 37ºC in a shaking water bath (100 rpm) for 1 hr. The reaction mixture was stopped by adding 200 µl of cold ethyl acetate. The sample was vortexed again for 30 sec before centrifuging at 17,000xg for 1 min at

room temperature. The upper layer was collected into a new 1.5 ml microcentrifuge tube then the previous extraction step was repeated twice. The combined extracts were dried under nitrogen and kept at -20ºC until analysis.

For analysis, the dried sample was re-dissolved with 10 µl of 0.1mg taxifolin in 1 ml of solvent A (5%ACN + 0.1% formic acid + milliQ water) containing 100 µM ascorbic acid and 90 µl of solvent A (5%ACN + 0.1% formic acid + milliQ water) containing 100 µM ascorbic acid. The samples were vortexed, centrifuged at 17,000xg for 1 min at RT before pipetting into amber HPLC vials and injected directly into LC-MS (see Section 2.5.8).

2.5.5 Urine concentration using freeze dryer

Due to the low percentage urinary excretion of quercetin and (-)-epicatechin detected in Chapter 4, accompanied by a low amount of quercetin and (-)-epicatechin spiked with synthetic conjugated standard in 24 hr urine samples in Chapter 5, the baseline and 24 hr urine samples were first concentrated by freeze-drying before analysis.

All urine samples from day 1, 2 and 3 of 27 volunteers (both baseline and 24 hr samples) were taken from -20ºC, defrosted by placing in the fridge overnight and vortexed before use. Five ml of each urine sample was placed into 15 ml centrifuge tube (duplicate for both baseline and 24 hr urine samples) followed by adding 62.5 µl of 0.1 mg/ml ethyl gallate in milliQ water (internal standard). After that these samples were kept in -80ºC prior to freeze drying. Urine samples from -80ºC were placed into flasks (around 15 tubes per/flask) then attached to a quick seal valve of freeze dryer (Virtis Benchtop Freeze dryer, Model Sentry 2.0, SP Scientific) and freeze dried for approximately 12 hr. Freeze dried urine samples were kept in -20ºC before analysis.

2.5.6 Concentrated urine preparation for LC-MS

Freeze dried urine samples from section 2.5.7 were re-constituted with 500 µl of 50% ethanol in milliQ water followed by vortexing and sonicating until completely dissolve. Then, samples were centrifuged at 3000xg, 4ºC for 10 min. Next, 400 µl of sample was transferred and placed into a pre-weighted 1.5 ml microcentrifuge tube. To eliminate ethanol from concentrated urine samples, these samples were then placed into a N_2 dryer for approximately 30 min (or until the

level of solution in samples left approximate 200 µl). These samples were then post-weighed prior to filtering with a 4 mm syringe filter 0.2 um ptfe (CRMA4-SF-02T, VWR International) and pipetted into amber HPLC vials. Subsequently, these samples were analysed by injecting two times per each sample onto UPLC-MS (see Section 2.5.9).

2.5.7 HPLC-MS

High performance liquid chromatography coupled with mass spectrometry (HPLC-MS) is a method for analysing and identifying flavonoid metabolites at the physiological levels because it is highly sensitive and applicable to complex mixtures like urine. Thus, this analytical method was used in this study. HPLC-MS analysis was performed on HPLC-2010C HT system (Shimadzu Scientific Instruments) coupled to a LCMS-2020 single quadrupole liquid chromatograph mass spectrometer equipped with electrospray ionization (ESI). Separation was achieved on a ZORBAX Eclipse XDB-C18, 4.6x50 mm, 1.8 µm, equipped with ZORBAX Eclipse XDB-C18 guard cartridge, 4.6 x 12.5 mm, 5 µm (Agilent Technologies). Mobile phases constituted water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid. The separation was achieved with the following gradient program: time 0.01 min, controller start; time 2 min, 15% solvent B; time 22 min, 40% solvent B; time 23 min, 90% solvent B; time 27 min, 90% solvent B; time 28 min, 15% solvent B; time 32 min, 15% solvent B; 32.01 min, controller stop. Mass spectrometric data were collected between 0.00 and 32.01 min. The flow rate was 0.5 ml/min and the injection volume, 10 µl. It has been reported that the negative mode was more sensitive than the positive mode. For example, quercetin and its derivatives were detected with the negative mode more sensitive than detected with positive mode, approximately ten times (Mullen et al., 2004). Thus, in this study MS analyses were performed in the negative mode, with nitrogen as nebulizer gas flow 1.5 l/min and drying gas (15 l/min), interface temperature 350°C, DL temperature 250°C, heat block 200°C and interface voltage set at −3.5 kV. The most intense product ion of each compound were recorded for total ion count (TIC) and single ion monitoring (SIM). Chromatographic data were recorded and integrated using Labsolutions LC-MS software.

To analyse quercetin, (-)-epicatechin and its methylated form from section 2.5.3, the separate $[M\text{-}H]^-$ ion at m/z 301 for quercetin, at 315 for 3′-*O*-methyl

quercetin and 4′-*O*-methyl quercetin, at 289 for (-)-epicatechin, at 303 for 3′-*O*-methyl (-)-epicatechin and taxifolin, at 175 for ascorbic acid and at 253 for daidzein were selected.

2.5.8 UPLC-MS

Ultraperformance liquid chromatography coupled with mass spectrometry (UPLC-MS) provides shorter chromatographic cycle and higher sensitivity than conventional analytical methods. Thus, this analytical method was used in this study. Analyses were carried out on a Nexera UPLC system (Shimadzu Scientific Instruments), coupled to a LCMS-2020 single quadrupole liquid chromatograph mass spectrometer equipped with electrospray ionization (ESI). Separation was achieved on a Kinetex^{TM} C18 column (2.6 µm, C18, 100A), size 150 x 2.1 mm (Serial No. 530411-87, Phenomenex, Cheshire, UK) equipped with a KrudKatcher Classic HPLC in-Line Filter, 0.5 µm Depth Filter (Part No. AF 0-5725, KrudKatcher), operating at 30°C. Mobile phases constituted 95%:5%, milliQ water: acetonitrile (solvent A) and 95%:5% acetonitrile: milliQ water (solvent B), both acidified with 0.1% formic acid. The separation was achieved with the following gradient program: 0.01, 0% B flow to waste; time 3 min, 0% B flow to PDA and MS; time 20 min, 15% B; time 50 min, 25% B; time 54 min, 100%; time 57 min flow to waste; time 57-61 min held at 100%, time 63 min back to 0% B; and a 7 min post run re-equilibration at 0% B. Mass spectrometric data were collected between 0 and 70 min. The flow rate was 0.26 ml/min and the injection volume, 10 µl. MS analyses were performed in the negative mode, with nitrogen as nebulizer gas flow 1.5 l/min and drying gas (15 l/min), interface temperature 350°C, DL temperature 250°C, heat block 200°C and interface voltage set at −3.5 kV. The most intense product ion of each compound were recorded for total ion count (TIC) and single ion monitoring (SIM). Chromatographic data were recorded and integrated using Labsolutions LC-MS software.

To analyse enzymatically synthesised quercetin and (-)-epicatechin conjugates (section 2.4) and also quercetin, (-)-epicatechin and the main metabolites excreted in urine samples from volunteers (section 2.5.7), the separate $[M-H]^-$ ion at 383 for methyl-(-)-epicatechin-sulfate, at 465 for (-)-epicatechin-glucuronide, at 369 for (-)-epicatechin-sulfate, at 479 for methyl-(-)-epicatechin-glucuronide, at 303 for 3′-*O*-methyl-(-)-epicatechin and taxifolin, at 381 for qquercetin-sulfate, at 477 for

quercetin-glucuronide, at 491 for isorhamnetin-glucuronide, at 289 for (-)-epicatechin, at 301 for quercetin, at 315 for 3′-*O*-methyl-quercetin and 4′-*O*-methyl quercetin, at 197 for ethyl gallate, at 175 for ascorbic acid at 653 for quercetin-diglucuronide, at 667 for methyl-quercetin-diglucuronide were selected.

2.6 Method for determining sulfatase activity using commercial substrate (PNCS) and product (PNC)

A model substrate and product of sulfatase called *p*-nitrocatechol sulfate (PNCS) and *p*–nitrocatechol (PNC) were used to estimate the sulfatase activity. 0.001 µmol of *p*-nitrocatechol sulfate were added to milliQ water, ABSS buffer, pH 5 and baseline urine, respectively. Then, this substrate was hydrolysed without or with 20 U of sulfatase (*Abalone entrails type* VIII) at 37°C for 1 hr. After that, this reaction was stopped by adding 2 volumes ice-cold acetonitrile and placed in on ice bath for 15 min. Next, the reaction mixture was centrifuged at 17,000 g for 15 min and the supernatant was then transferred into a new 1.5 ml microcentrifuge tube. The centrifugation step was performed one more time. Finally, the supernatant was transferred and placed into amber HPLC vials prior directly injected to LC-MS (see Section 2.5.7). One unit of this enzyme will hydrolyse 1.0 µmol of *p*-nitrocatechol sulfate per hr at pH 5.0 at 37°C (30 min assay). Thus, there was enough sulfatase to hydrolyse *p*-nitrocatechol sulfate in this reaction.

Chapter 3 Quantification of Quercetin and (-)-Epicatechin Content in Food

3.1 Abstract

Onion and dark chocolate are rich dietary sources of quercetin and (-)-epicatechin, respectively. The main aim of this chapter was to determine the content of quercetin in onion soup using HPLC-DAD and also (-)-epicatechin in dark chocolate using HPLC-DAD/FLD in preparation for a human bioavailability study. Prior to selecting the type of onion to prepare an onion soup, two main quercetin glucosides, quercetin-3,4′-di-*O*-glucoside (Q3,4′diG) and quercetin-4′-*O*-glucoside (Q4′G), from freeze dried yellow and red onion were extracted and compared. As expected red onion contained higher total quercetin content than yellow onion: 414±83 mg/ 100 g FW for red onion and 325±63 mg/100 g FW for yellow onion (*p*-value = 0.353). However, the content was very dependent on the method of extraction. For re-suspended freeze-dried ground onion powder, homogenisation was an efficient method for extracting quercetin glycosides compared to vortex and sonication. Thus, red onion was selected to prepare a soup containing 50 g of red onion per portion (130 ml). To determine the heat stability of quercetin in red onion soup, three different cooking methods were examined, and it was found that there were no significant differences in total quercetin content in red onion soup after preparation (61.7±15.60 mg/ 130 ml soup) and before microwaving (60.4±9.6 mg/ 130 ml soup) or before microwaving and after microwaving (59.9±8.2 mg/130 ml soup), *p*-value = 0.818 and 0.666, respectively. The total levels of quercetin glucosides, expressed as quercetin equivalents, ranged from 25.1±3.6 to 33.1±1.8 mg per portion of soup. In dark chocolate, the content of (-)-epicatechin was also analysed and quantified in different percentages of cocoa from different brands: 114.7 mg/ 100 g FW for 90% cocoa, 123.3 mg/ 100 g FW for 85% cocoa and 109.1 mg/ 100 g FW for 70% cocoa. Although 90% and 85% cocoa dark chocolate contained a higher content of (-)-epicatechin than 70% cocoa, 70% cocoa dark chocolate was less bitter and so was selected for the human study. The content of (-)-epicatechin of 70% dark chocolate of selected brands from the same batch was then analysed prior to feeding to volunteers. It was 92.2±4.4 mg/100g FW, as detected with FLD. To sum up, based on the content of quercetin and (-)-

epicatechin, one portion of red onion soup (containing 109 µmol of quercetin in 130 ml) and a half bar of 70% dark chocolate (containing 167 µmol of (-)-epicatechin in 45 g) were analysed for subsequent feeding to healthy volunteers as described in Chapter 4.

3.2 Introduction

Onion is one of the richest sources of quercetin and is commonly consumed in Western countries. For example, in The Netherlands, 16 mg of quercetin was mostly consumed per day from approximately 25 mg/day of total flavonol (quercetin, myricetin and kaempferol) and flavone (luteolin and apigenin) (Hertog et al., 1993). In addition, onions and apples were the most common sources of flavonoid intake in the United States, Finland, former Yugoslavia and Greece and highly negatively correlated to heart disease mortality (Hertog et al., 1995). Hertog et al. (1992) reported that the highest content of quercetin was found in the edible part of onions (28.4-48.6 mg/100 g FW) followed by kale (11 mg/100 g FW), apples (2.1-7.2 mg/100 g FW), tea infusion (1-2.5 mg/100 g FW) and red wine (4-16 mg/L). In onions, the amount of quercetin varies depending on the colour and type. According to the USDA Database for flavonoid content, yellow onions contain 21.4 mg/100g FW which is in the same range as found by Tsushida and Svzuki (1996) and Makris and Rossiter (2001); 22.7 mg/ 100 g FW and 30 mg/100 g FW, respectively. The mean content of quercetin in raw yellow onion is also reported in the Phenol Explorer Database; 36.0 mg/100 g FW for quercetin-3,4′-di-*O*-glucoside, 23.5 mg/100 g FW for quercetin-4′-*O*-glucoside and 0.3 mg/100 g FW for quercetin,. In red onions, the same USDA Database reported that quercetin was present at 33.4 mg/100 g FW, whereas the Phenol Explorer Database stated that the mean quercetin -3,4′di-*O*-glucoside, quercetin-4′-*O*-glucoside and quercetin contents were 101.3, 43.7 and 1.3 mg/100 g FW, respectively. This information shows that red onion contains a higher amount of quercetin then yellow onion. Furthermore, Crozier et al. (1997) found 20.1 mg/100 g of quercetin in red onion which was higher than in white onion (18.5-63.4 mg/ 100 g). Additionally, the outer layers have a higher level of quercetin than the inner layers, i.e. it decreases from the outside to the inside. Therefore, a whole bulb of the edible part of the onion was used in this study. During cooking or processing, quercetin aglycone may increase or decrease. For example, quercetin glycosides in cooked onion were not degraded but leached

into the cooking water. Frying an onion with oil and butter for 40 min did not change the total flavonoid content, but the quercetin derivatives were not specifically mentioned. Microwave cooking without water for 1 min increased 50% of total quercetin content probably due to better extractability (Ioku et al., 2001). Hence, onion soup, which still contains quercetin glycosides in the boiling water, seems to be a better source of quercetin than fried onion and so was used in this study.

Chocolate is an abundant source of (-)-epicatechin, representing more than 35% of the total polyphenol content in cocoa bean. In addition, it is a well-liked and highly consumed product, with around 58% and 43% of European people eating milk chocolate and dark chocolate, respectively (Cooper et al., 2008). In the US, people prefer milk chocolate and approximately 87% prefer to consume as a form containing nuts, wafer or fruit than consuming as pure chocolate (Vinson et al., 2006). Chocolate is made from cocoa liquor, prepared from fermented and roasted cocoa bean. There are various types of chocolate such as dark chocolate, milk chocolate and white chocolate. Dark chocolate commonly contains more cocoa liquor than milk chocolate. However, there is no cocoa liquor in white chocolate. The higher cocoa liquor in dark chocolate, containing more flavonoids, may also have a better biological effect than in milk chocolate because the intestinal absorption of flavonoids is supposedly inhibited by the milk contained in it (Serafini et al., 2003). The polyphenol content reflected the percentage of cocoa content in chocolate. Therefore, a high percentage of cocoa in dark chocolate appears to have the highest content of polyphenols (Cooper et al., 2008). However, there are exceptions depending on the method of processing. This argument is supported by Langer et al. (2011) that the labelled cocoa content of the chocolate did not consistently match analysed levels of polyphenols. Several research groups reported the content of (-)-epicatechin in dark chocolate. Arts et al. (2000) found (-)-epicatechin level at 32.7-50.3 mg/100 g in dark chocolate (54% cocoa) and lower but still substantial levels in milk chocolate (34% cocoa). Other researchers such as Cooper et al. (2007) reported that (-)-epicatechin was in the range of 7.1-194.2 mg/100 g, while Andres-Lacueva et al. (2008) found a range from 11.6-73.0 mg/100 g. In the Phenol Explorer database, the mean (-)-epicatechin content was 70.4 mg/100 g FW. Recently, Langer et al, 2011 tested different brands of chocolate and found that all dark chocolates tested contained higher levels of total flavanols than

milk or white chocolate; 93.5-651.1 mg of (-)-epicatechin equivalent/100 g for dark chocolate, 40.6 mg of (-)-epicatechin equivalent/100 g for milk chocolate and 0.0 mg of (-)-epicatechin equivalent/100 g for white chocolate.

The main aim of this chapter was to determine the content of quercetin in onion soup using HPLC-DAD and also (-)-epicatechin in dark chocolate using HPLC-DAD/FLD. First, due to the different content of quercetin in different types of onion, the amount of quercetin in two different types of freeze dried yellow and red onion powder were analysed and compared. Then, red onion, which contains a higher amount of quercetin than yellow onion, was selected to prepare onion soup. As mentioned previously, cooking methods may affect the content of quercetin, the bioavailability and finally the biological effects. Thus, the content of quercetin in red onion soup was quantified again after preparation and before and after heating in a microwave. In dark chocolate, the content of (-)-epicatechin was also analysed and quantified in different percentages of cocoa from different brands. Finally, since the amount of (-)-epicatechin can vary depending on the manufacturer of the chocolate, the amount of (-)-epicatechin from the same batch of selected 70% dark chocolate was analysed again prior to feeding to volunteers. The content of these flavonoids was then used for calculating a percentage of intake of the human bioavailability study in the next chapter.

3.3 Materials and Methods

See Section 2.2

3.4 Results

3.4.1 Determination of quercetin content in freeze-dried yellow and red onion

As stated above, different types of onions contain different amounts of quercetin. Thus, the quercetin contents of yellow and red onions were analyzed and compared. Quercetin-3,4′-di-′*O*-glucoside and quercetin-4′-*O*-glucoside are major quercetin glucosides and identified in freeze-dried yellow and red onion powder by HPLC-DAD. They were eluted at 3.6 min for quercetin-3,4′-di-*O*-glucoside and 10.0 min for quercetin-4′-*O*-glucoside at the maximum wavelength of 370 nm in comparison with retention times (Figure 3.1) and diode array spectra of their

commercial standards (Figure 3.2). In this case, daidzein was used as an internal standard and eluted at a retention time of 13.3 min with a maximum wavelength of 280 nm (chromatogram not shown). In Figure 3.2, the maximum wavelength of quercetin-3,4′-di-*O*-glucoside was shifted followed by quercetin-4′-*O*-glucoside because quercetin-3,4′-di-*O*-glucoside has a greater degree of conjugation than quercetin-4′-*O*-glucoside, resulting in a larger shift of the maximum wavelength. These shifts also related to the retention time of each compound in Figure 3.1. The amount of quercetin was analysed on a molar basis using a standard curve of authentic compounds (Figure 3.3). Standard curves were constructed for each standard sample at nine concentrations. The regression analysis showed that the on-column amount, ranging from 10 to 1000 pmol was linearly correlated with a peak area of R^2 = 0.9970 for quercetin-3,4′-di-*O*-glucoside, R^2 = 0.9999 for quercetin-4′-*O*-glucoside and R^2 = 1 for quercetin. In addition, some extracted onion samples were spiked with compounds to confirm identity. The percentage recovery of quercetin-3,4′-di-*O*-glucoside and quercetin-4′-*O*-glucoside is shown in Table 3.1. The results indicated that the recovery for red onion was higher than yellow onion in the case of quercetin-3,4′-di-*O*-glucoside but not quercetin-4′-*O*-glucoside. In addition, homogenization had a better percentage recovery than vortex and sonication.

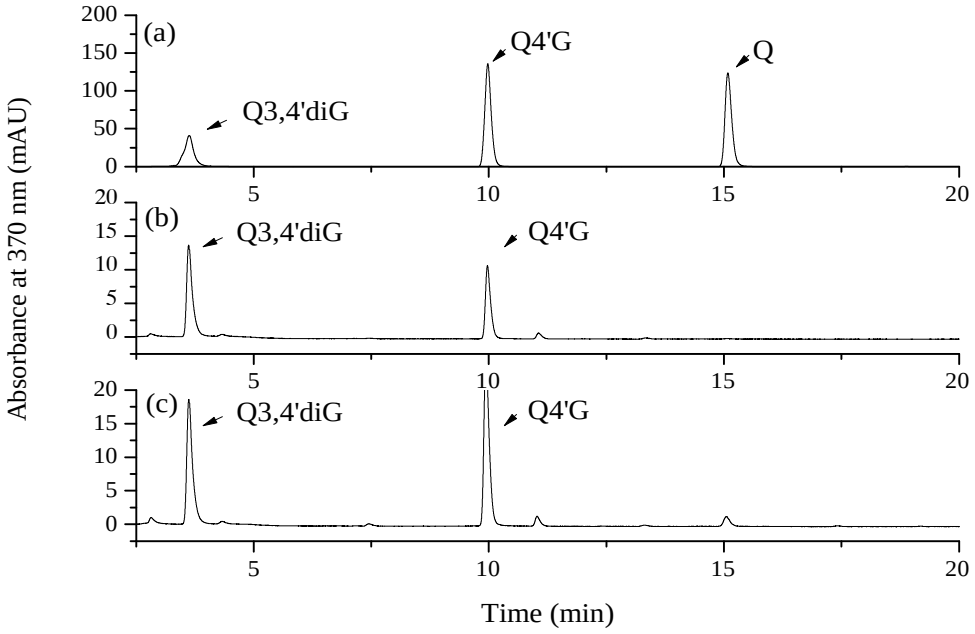


Figure 3.1: HPLC chromatograms showing analysis of the main quercetin glycosides of freeze dried onion: (a) standard compounds composed of quercetin-3,4′-di-glucoside (Q3,4′diG), quercetin-4′-*O*-glucoside (Q4′G) and quercetin (Q), each 100 µM with an injection volume of 10 µl (1 nmol per injection) (b) yellow onion (c) red onion.

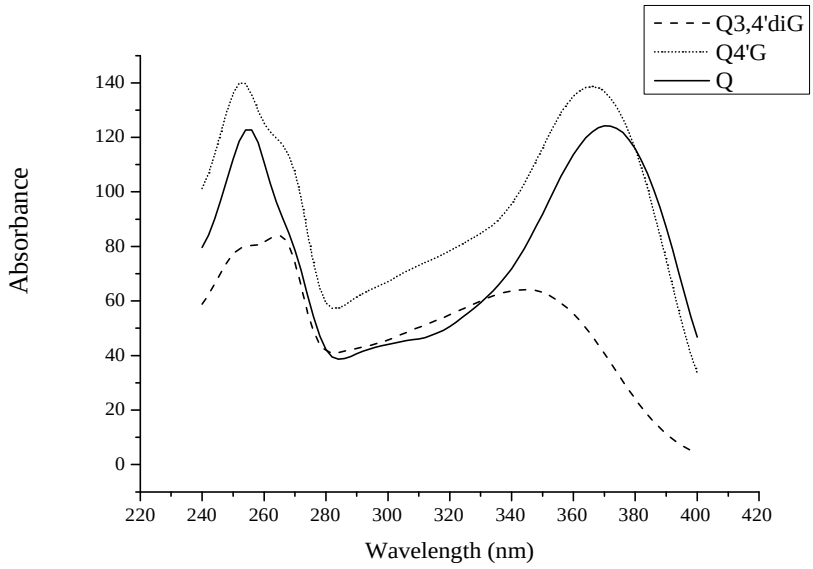


Figure 3.2: UV absorption spectra of quercetin standards; quercetin 3,4′-di-glucoside, quercetin 4′-*O*-glucoside and quercetin. Spectra were produced by scanning the standard in 50% ethanol between 240 and 400 nm using diode array detection.

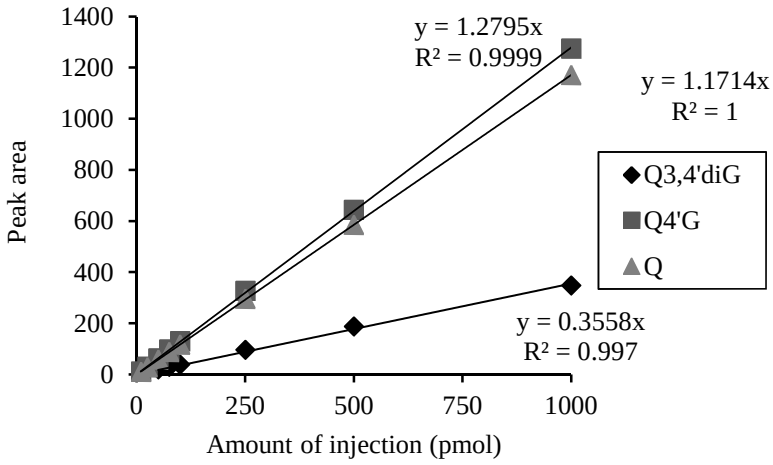


Figure 3.3: Standard curves of quercetin-3,4′-di-glucoside, quercetin-4′-*O*-glucoside and quercetin for quantification in freeze dried yellow and red onion.

Table 3.1: Recovery of quercetin-3,4′-di-glucoside and quercetin-4′-*O*-glucoside in extracted yellow and red onion, spiking with 100 µM of each standard prior to injection into HPLC.

Extracted samples	**% Recovery**			
	Homogenization		**Vortex and sonication**	
	Quercetin-3,4′-di-*O*-glucoside	**Quercetin-4′-*O*-glucoside**	**Quercetin-3,4′-di-*O*-glucoside**	**Quercetin-4′-*O*-glucoside**
Yellow onion	114.5±3.5	100±0.0	110.5±0.7	95.0±1.4
Red onion	119.5±7.8	99.5±0.7	113.0±0.0	95.5±0.7

The summary of quercetin content in freeze-dried yellow and red onion is shown in Table 3.2 and Figure 3.4. These data illustrate the difference in quercetin content of freeze-dried yellow and red onion using two different extraction methods, homogenisation and vortex followed by sonication. It can be seen that red onion contains a higher total amount of quercetin than yellow onion in both extraction methods (in homogenization, *p*-value between yellow and red onion is 0.353 while, in vortex and sonication *p*-value is 0.092). Only the amount of quercetin-4′-*O*-glucoside in red onion, extracting with vortex and sonication, was significantly different in yellow onion (*p*-value = 0.044). The total quercetin content was

413.8±82.9 mg quercetin glucosides/100 g FW or 217.3±44.4 mg quercetin equivalent/100 g FW for red onion and 325.4±63.1 mg quercetin glucosides/100 g FW or 165.2±32.4 mg quercetin equivalent/100 g FW for yellow onion. In addition, extracting quercetin with homogenisation apparently produced a higher total amount of quercetin than vortexing followed by sonication by approximately 4-5 times. However, there were no significant differences between quercetin-3,4′-di-*O*-glucosides, quercetin-4′-*O*-glucoside and total quercetin content in yellow onion or in red onion extracted with homogenisation and vortex and sonication. The *p*-value of quercetin-3,4′-di-*O*-glucoside, quercetin-4′-*O*-glucoside and total quercetin content compared between homogenisation and vortex and sonication in yellow onion were 0.102, 0.143, 0.108 respectively. For red onion, the *p*-value was 0.125 for quercetin-3,4′-di-*O*-glucoside content, 0.202 for quercetin-4′-*O*-glucoside and 0.144 for total quercetin content, comparing between homogenisation and vortex and sonication.

Table 3.2: Different content of quercetin-3,4′-di-*O*-glucoside, quercetin-4′-*O*-glucoside and total quercetin content in yellow and red onion, using two different extraction methods, homogenisation and vortex followed by sonication.

Method	Q content (mg/100 g FW)			Q equivalent (mg/100 g FW)		
	Quercetin-3,4′di-*O*-glucoside	**Quercetin-4′-*O*-glucoside**	**Total**	**Quercetin-3,4′di-*O*-glucoside**	**Quercetin-4′-O-glucoside**	**Total**
Homogenisation						
Yellow	272.2±51.0	53.1±12.2	325.4±63.1	130.7±24.5	34.5±7.9	165.2±32.4
Red onion	304.1±55.6	109.7±27.3	413.8±82.9	145.9±26.7	71.3±17.7	217.3±44.4
Vortex and Sonication						
Yellow	54.3±1.4[a]	16.0±0.2[a]	70.3±1.6[a]	26.0±0.7[a]	10.4±0.1[a]	36.5±0.8[a]
Red onion	70.1±10.5[a]	37.0±6.5[b]	107.1±16.9[a]	33.6±5.0[a]	24.1±4.2[a]	57.7±9.2[a]

(Results are expressed as mean ± standard deviation. Different superscripts indicate significant different of quercetin 4′-*O*-glucoside content between yellow and red onion extracted with vortex and sonication (p-value <0.05), *p*-value of a,b is 0.044)

3.4.2 Determination of quercetin content in red onion soup

Red onion, which contains a higher amount of quercetin than yellow onion, was selected to prepare onion soup and test the effect of microwave cooking on quercetin content in the soup. The quercetin content in red onion soup was quantified again after preparation, before and after heating in a microwave for 1 min. Additionally, extraction efficiencies of two different types of extraction solution, absolute methanol and 70% methanol, were tested. After soup preparation and before and after microwave cooking, quercetin was still present as glycosides, quercetin-3,4′-*O*-di glucosides and quercetin-4′-*O*-glucoside, were similar to freeze-dried yellow and red onion (Figure 3.5). Again, quercetin content in red onion soup was quantified using a standard curve (Figure 3.6). Seven standards of mix quercetin glycosides and quercetin were prepared in the same way as Table 1 in Section 2.2.1.1.3 after that the standard curves were constructed. The regression analysis showed that the on-column amount, ranging from 10 to 1000 pmol, was linearly correlated with the peak area with R^2 = 0.9993 for quercetin-3,4′-di-*O*-glucoside, R^2 = 1 for quercetin-4′-*O*-glucoside and R^2 = 1 for quercetin. The total quercetin content of red onion soup (from 50 g fresh weight) varied from 25.1±3.6 to 33.1±1.8 mg quercetin equivalents/130 ml (Table 3.3). Compared to the quercetin content of 50 g freeze-dried red onion in Table 2, this amount was still in the same range. In addition, there were no significant differences in total quercetin content in red onion soup after preparation (61.7±15.60 mg/ 130 ml soup) and before microwaving (60.4±9.6 mg/ 130 ml soup) or before microwaving and after microwaving (59.9±8.2 mg/130 ml soup), *p*-value = 0.818 and 0.666, respectively (Figure 3.7) (using paired t-test at 95% confidence interval, SPSS program). However, the amount of quercetin-4′-*O*-glucoside and total quercetin in red onion soup after microwaving were significantly different between extracted with absolute methanol or 70% methanol solution (*p*-value = 0.021 and 0.0478). The ratio of quercetin-3,4′-di-*O*-glucoside to quercetin-4′-*O*-glucoside after preparation, before and after microwave cooking were 2.6±0.0:1, 2.4±0.1:1 and 2.3±0.1:1. It was slightly different, indicating heat stability of quercetin in onion after heating in a microwave oven for 1 min. Finally, the extraction method using absolute methanol and 70% methanol seemed to provide the same level of recovery yields.

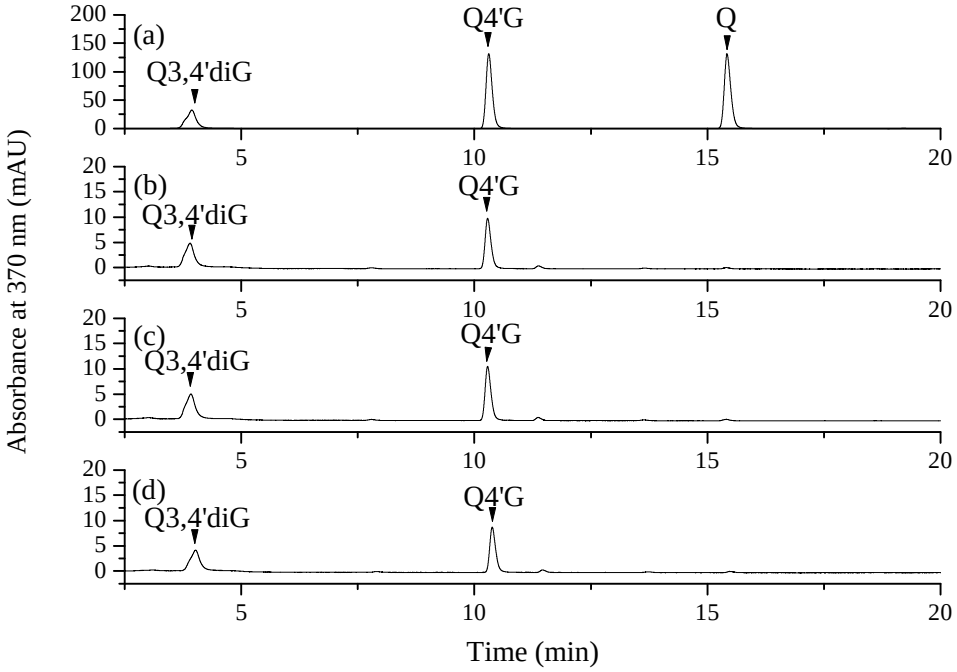


Figure 3.4: HPLC chromatograms showing analysis of the main quercetin glycosides of red onion soup at 370 nm: (a) standard compounds composed of quercetin-3,4′ di-*O*-glucoside, quercetin 4′-*O*-glucoside and quercetin (b) after preparation (c) before microwaving (d) after microwaving.

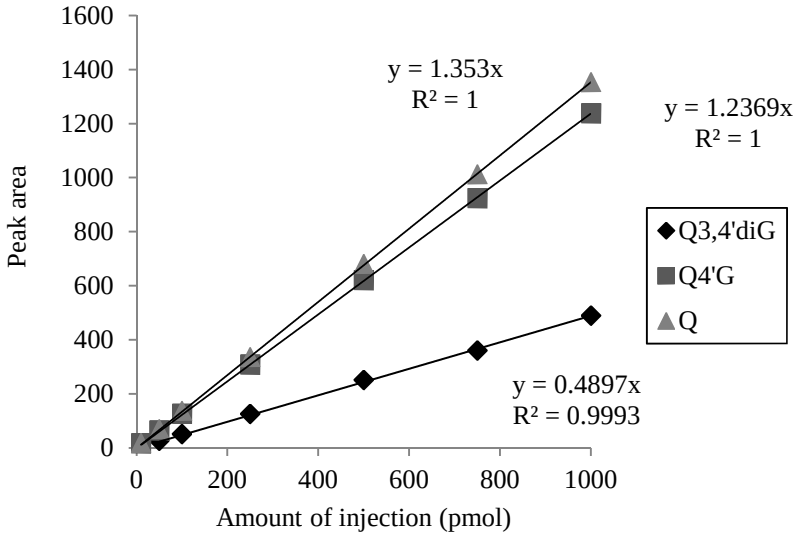


Figure 3.5: Standard curves of quercetin-3,4′-di-*O*-glucoside, quercetin-4′-*O*-glucoside and quercetin for red onion soup quantification.

Table 3.3: Different content of quercetin-3,4′-di-*O*-glucoside, quercetin-4′-*O*-glucoside and total quercetin content in red onion soup (130 ml) after 3 different cooking methods, using two different extraction solutions, absolute or 70% methanol.

Treatment	**Q content, mg/130 ml**			**Q equivalent, mg/130 ml**		
	Quercetin-3,4′-di-*O*-glucoside	**Quercetin-4′-*O*-glucoside**	**Total**	**Quercetin-3,4′-di- *O*-glucoside**	**Quercetin - 4′-*O*-glucoside**	**Total**
Absolute methanol						
After preparation	40.2±12.4	15.6±4.0	55.8±16.4	19.3±5.9	10.1±2.6	29.4±8.5
Before microwaving	44.8±2.4	17.8±0.9	62.6±3.4	21.5±1.2	11.6±0.6	33.1±1.8
After microwaving	32.3±5.0[a]	14.8±1.9[a]	47.1±6.9[a]	15.5±2.4[a]	9.6±1.2[a]	25.1±3.6[a]
70% methanol						
After preparation	44.5±10.8	17.2±4.8	61.7±15.6	21.3±5.2	11.2±3.1	32.5±8.3
Before microwaving	42.3±5.1	18.2±4.5	60.4±9.6	20.3±2.4	11.8±2.9	32.1±5.4
After microwaving	42.1±6.4[a]	17.8±1.7[b]	59.9±8.2[b]	20.2±3.1[a]	11.6±1.1[a]	31.8±4.2[a]

(Results are expressed as mean ± standard deviation. Different superscripts indicate significant different of quercetin 4′-*O*-glucoside and total quercetin content after microwaving between extracted with absolute or 70% methanol (p-value < 0.05), p-value of a,b for quercetin 4′-*O*-glucoside is 0.021 and for total quercetin is 0.047)

3.4.3 Determination of (-)-epicatechin content in dark chocolate

The amount of (-)-epicatechin in chocolate with different percentages of cocoa (90%, 85% and 70% cocoa) from different brands were quantified and compared using HPLC-DAD/FLD. (-)-Epicatechin was separated at 15.4 min at a maximum wavelength of 280 nm using a diode array detector and at 15.6 min at Ex = 230, Em = 321 using a fluorescence detector. There were 114.7 mg/ 100 g FW for 90% cocoa, 123.3 mg/ 100 g FW for 85% cocoa and 109.1 mg/ 100 g FW for 70% cocoa. Although 90% and 85% cocoa dark chocolate contained a higher content of (-)-epicatechin than 70% cocoa, 70% cocoa dark chocolate was less bitter and so 70% cocoa chocolate was selected and quantified for the human study in the next step. Typical chromatograms of (-)-epicatechin in 70% dark chocolate detected by DAD and FLD are shown in Figure 3.8. Seven standards of (-)-epicatechin were prepared as in Table 2.3 in Section 2.2.2.3 then the standard curves were constructed. The regression analysis showed that the on-column amount, ranged from 50 to 5000 pmol for the DAD mode and 50 to 3750 pmol for FLD were linearly correlated with the peak area with R^2 = 0.9912 for (-)-epicatechin detected with DAD and R^2 = 0.9992 for (-)-epicatechin detected with FLD, respectively. For the DAD mode, the limit of detection and quantification were calculated from the regression linear of Figure 3.9A and there were 635 and 2117 pmol while, the limit of detection and quantification of the FLD mode were 9 and 31 pmol (Figure 3.9B). From these results, FLD has a lower limit of detection and quantification than DAD or is more sensitive to detect a low amount of (-)-epicatechin in the same sample than DAD, resulting a higher (-)-epicatechin content determined by use of the FLD mode. The content of (-)-epicatechin was calculated based on the standard curve in Figure 3.9. (-)-Epicatechin was determined as 81.2±8.5 mg/100g FW detected using DAD and 92.2±4.4 mg of (-)-epicatechin/100g FW detected using FLD and shown in Table 3.4. The content of (-)-epicatechin in 70% dark chocolate detected using the DAD mode showed no difference compared with the FLD mode (using an independent t-test, *p*-value = 0.247).

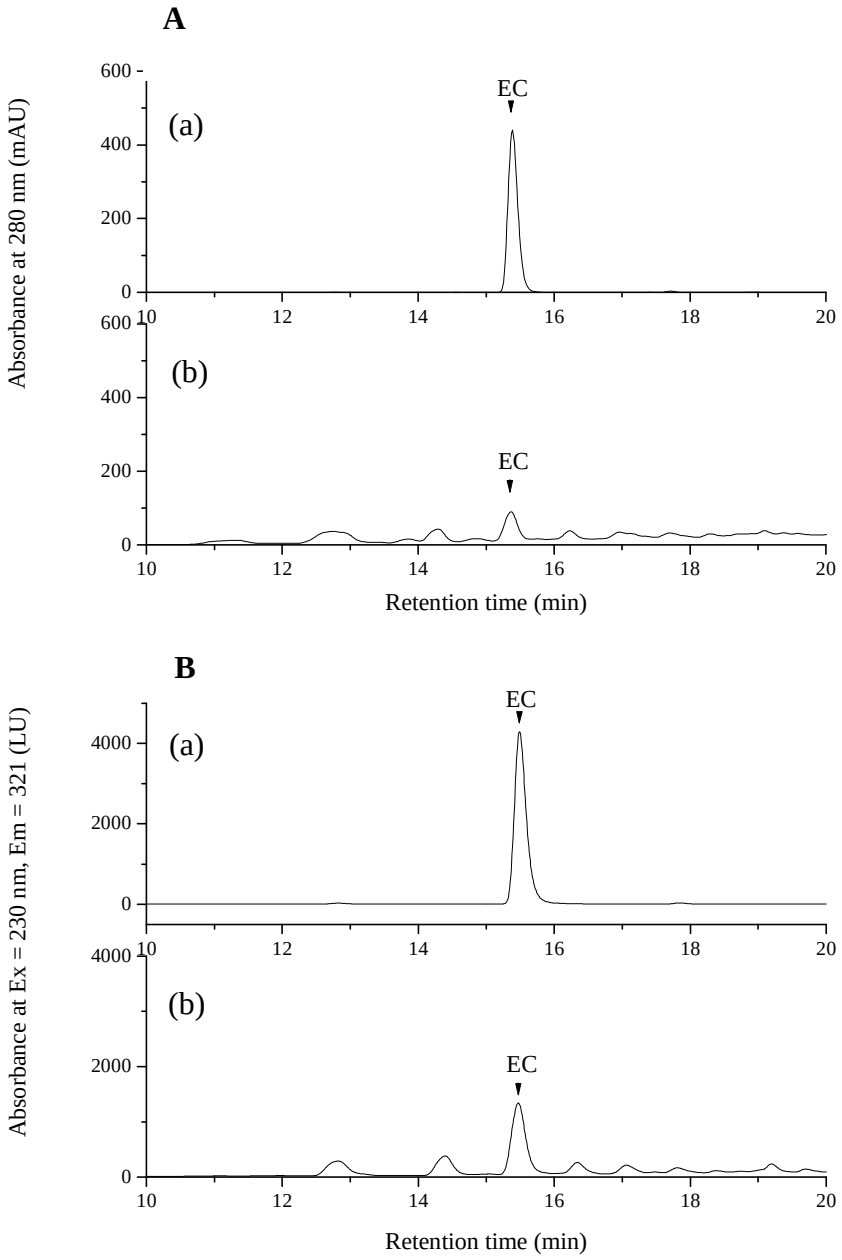


Figure 3.6: HPLC chromatograms showing analysis of (-)-epicatechin with 70% dark chocolate with DAD at 280 nm (A) and with FLD at Ex = 230, Em = 321 (B); (a) standard epicatechin (EC), (b) in 70% dark chocolate.

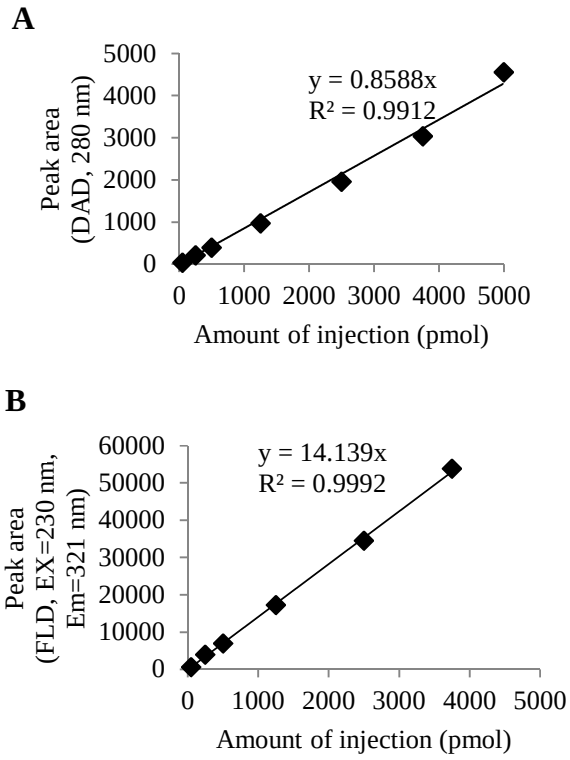


Figure 3.7: Standard curve of (-)-epicatechin for 70% dark chocolate quantification.

Table 3.4: (-)-Epicatechin content in 70% dark chocolate using HPLC-DAD or FLD

Detector	**(-)-Epicatechin content in 70% dark chocolate (mg/100g FW)**
DAD	81.2±8.5
FLD	92.2±4.4

3.5 Discussion

The amount of quercetin in onion varies depending on bulb colour and type. The findings in this study shows that yellow onion contains lower amounts of quercetin than red onion. For yellow onion, the amount of quercetin was 272.2±51.0 mg/100 g FW for quercetin-3,4′-di-*O*-glucoside, 53.1±12.2 mg/100 g FW for quercetin -4′-*O*-glucoside or 325.4±63.1 mg/ 100g FW in total. Price and Rhodes (1997) identified the main flavonoids in four varieties of onion bulb; white, brown, hybrid pink and red skinned varieties by freezing in liquid nitrogen and freeze-

drying prior to analysis. They confirmed that quercetin-3,4′-di-*O*-glucoside is the main component, ranging from 5-130 mg/100 g FW, followed by quercetin-4′-*O*-glucoside, ranging from 3.6-39.4 mg/100 g FW and quercetin but at low levels in all four types. For yellow onion, they found 111.7 mg/100g of quercetin-3,4′ di-*O*-glucoside, 36 mg/100g of quercetin-4′-*O*-glucoside, 3.9 mg/100g of quercetin or 151.6 mg/100g in total. This data revealed that the total amount of yellow onion in our study is higher than those reported by Price and Rhodes (1997) by around 2 times. The maximum quercetin content in yellow onion in our study also showed that it is higher than the quercetin content reported in both USDA (21.4 mg quercetin/100 g FW) and the maximum Phenol Explorer Database (135.8 mg/100 g FW for quercetin-3,4′-di-*O*-glucoside, 83.0 mg/100 g FW for quercetin-4′-*O*-glucoside) by approximately 2 times. However, the minimum value of quercetin-3,4′di-*O*-glucoside and quercetin-4′-*O*-glucoside are still in the range of the Phenol Explorer Database (Table 3.5).

Prior to determining quercetin and its glycosides in onion in our study, a freeze-drying preservation process was performed (Price and Rhodes, 1997). Some different steps were used and compared to Price and Rhodes (1997). First, a whole bulb of onion was skinned and chopped into small pieces then immediately frozen by putting in the freezer at -80ºC so samples were easier to grind to a powder for the next step, while, Price and Rhodes (1997) cut five bulbs of onion into quarters and then they were frozen by immersion in liquid nitrogen. Another difference is the extraction. Although this study used 70% methanol as an extraction solution in Price and Rhodes (1997), here 100 µM ascorbic acid also was added into the extraction solution to preserve all quercetin glycosides. In addition, all extraction processes were performed on ice to prevent degradation of quercetin glycosides. These are possible reasons why the amount of quercetin in yellow onion found in this study was higher than that found by Price and Rhodes (1997). Thus, it is important to consider this in every single step prior to conducting an estimation of content.

For red onion, the level of quercetin (304.1±55.6 mg/100 g FW for quercetin-3,4′-di-*O*-glucoside, 109.7±27.3 mg/100 g FW for quercetin-4′-*O*-glucoside and 413.8±82.9 mg/100 g FW in total) is clearly higher than the total quercetin levels stated in the USDA database (33.4 mg/100 g FW) by approximately 12.5 times. For Phenol Explorer Database comparison, the minimum content of both

quercetin-3,4′-di-*O*-glucoside and quercetin-4′-*O*-glucoside are in the range of the Phenol Explorer Database but the maximum value of quercetin-3,4′-di-*O*-glucoside is out of the range (higher around 1.5 times) except for quercetin-4′-*O*-glucoside (Table 3.5).

Table 3.5: Comparison of quercetin glycosides found in yellow and red onion between this study and the Phenol Explorer Database

Onion types	This study (mg/100 g FW)		Phenol Explorer Database (mg/100 g FW)			
	Votex and sonication	Homogenisation	Average	Min	Max	Standard deviation
Yellow						
Q3,4′diG	54.3±1.4	272.2±51.0	36.0	11.4	135.8	49.5
Q4′G	16.0±0.2	53.1±12.2	23.5	13.8	83.0	16.2
Red onion						
Q3,4′diG	70.1±10.5	304±55.6	101.3	20.2	207.5	58.9
Q4′G	37.0±6.5	109.7±27.3	43.7	30.0	114.3	21.2

Again, there were several possible reasons affecting quercetin content in onions, for example plant distribution and variety, seasonal variation, light and climate, food preparation and processing. The data from the Phenol Explorer database was obtained by collecting data from different publications. Each study may have used different types of onion from different areas and also different techniques when preparing and analysing samples, resulting in different quercetin content. A freeze-drying step prior to determining quercetin glycoside content was used in this study and may be another important reason why found the quercetin content was higher compared to other publications. The freeze-drying step may assist in altering the structure of tissue making it easier for quercetin extraction in the next step. This reason is supported by Perez-Gregorio et al. (2011). They elucidated that the freeze-drying process increases the extraction of flavonoids up to 32% for flavonols and 25% for anthocyanins in red onion.

Our study also revealed that red onion is a good source of quercetin and it contains a higher amount of quercetin glucosides than yellow onion similar to most publications except Crozier et al. (1997). They found that red onion (20.1 mg/ 100g)

contains less quercetin than white onion (18.5-63.4 mg/100 g). In this case Crozier analysed only one type of red onion and determined the content of quercetin by acid hydrolysis of quercetin conjugates. This acid condition may not be suitable or completely hydrolyse all conjugates in red onion, leading to underestimation of the quercetin content. Variation of quercetin content in onion may probably be genetic in origin and storage factors. Patil et al. (1995) analysed quercetin content of 55 yellow, 3 pink, 6 red and 11 white onions and found different levels, ranging from 0.021 to 28.6 mg/ 100 fresh weight. It was apparent that the total quercetin content varied about 1360-fold. They also studied the effect of storage conditions on the variation of total quercetin content and found that total quercetin content of bulbs changes significantly at 24ºC compared to other treatments (5ºC, 30ºC and controlled atmosphere for 0, 1, 2, 3, 4 and 5 months), rising in mid-storage and dropping later. Thus, from this evidence, it is clear that colour may not be the only criterion affecting total quercetin content, but also genetic origin and storage factors. Cooking processes are another factor which should be considered and may affect the content of quercetin, the bioavailability and finally the biological effects. Price et al. (1997) studied the effect of boiling for 15 min of a composition of quercetin glucosides in red and brown-skinned onions and found 12% of quercetin-3,4′-di-*O*-glucoside and 13% of quercetin-4′-*O*-glucoside leaching into the cooking water but no free quercetin. Rodrigues et al (2009) also reported that quercetin glycosides of onions are lost during boiling for 30 min and leached into the boiling water without being degraded at 37% for quercetin-3,4′-di-*O*-glucoside and 29% for quercetin-4′-*O*-glucoside. Additionally, they found that boiling for 60 min had more serious effects because of the degradation of quercetin derivatives (53% and 44% for quercetin-3,4′-di-*O*-glucoside and quercetin-4′-*O*-glucoside, respectively). However, in this study, the boiling step was used for only 7 min in preparing red onion soup. Then, onion glucosides in onion soup probably will not be lost or degraded in large amounts and it will remain in the water. Therefore, onion soup seems to be a better source of quercetin. The content of quercetin in red onion soup was quantified again after preparation and before and after heating in a microwave oven (data shown in Table 3.3). There were no significant differences in total quercetin content in red onion soup after preparation (61.7±15.6 mg/ 130 ml soup), or before (60.4±9.6 mg/ 130 ml soup) and after microwaving (59.9±8.2 mg/130 ml soup) as a result of a short microwave cooking time (only 1 min). Rodrigues et al. (2009) showed that

moderate microwave cooking (450 watt for 4 min) did not affect the quercetin content, but intense microwaving (750 watt for 4 min) treatment caused flavonol losses of 16% and 18% for quercetin-3,4′-di-*O*-diglucoside and quercetin-4′-*O*-glucoside, respectively. Thus, to warm onion soup for only 1 min before giving to volunteers in the next experiment did not cause losses of quercetin in large amounts.

In dark chocolate, the content of (-)-epicatechin determined in this study was 81.2±8.5 mg/100g FW for DAD mode and 92.2±4.4 mg/100g FW for FLD mode. The content of (-)-epicatechin in 70% dark chocolate detected using the DAD mode showed no difference compared with the FLD mode, *p*-value = 0.247. It also was in the same range as (-)-epicatechin levels reported in the Phenol Explorer Database (average content is 70.4 mg/100 g FW and ranging from 32.7-125 mg/100 g FW) and the research of Langer et al, 2011 which ranged from 93.5-651.1 mg of (-)-epicatechin equivalent/100 g for dark chocolate. Thus, the amount of (-)-epicatechin in our study is consistent with other publications.

3.6 Conclusion

In summary, the findings of this study showed that red onions contain a higher amount of quercetin than yellow onions in agreement with most publications. Although cooking processes such as microwaving have an effect of the quercetin content of the onions, this study revealed no significant differences between red onion soup after preparation and before and after microwaving for 1 min. For (-)-epicatechin content in dark chocolate, different percentages of cocoa dark chocolates were studied but 70% cocoa dark chocolate was then used in this study. Using higher than 70% of cocoa dark chocolate may negatively affect the taste perception of volunteers. Although, FLD is more sensitive to detecting a low amount of (-)-epicatechin than DAD, resulting in a higher (-)-epicatechin content determined using the FLD mode, but the amount of (-)-epicatechin measuring in the same 70% cocoa dark chocolate sample is the same for both detection method. The dose of quercetin and (-)-epicatechin supplied by red onion soup (containing 109 µmol of quercetin in 130 ml) and 70% dark chocolate (containing 167 µmol of (-)-epicatechin in 45 g) was then applied for the human bioavailability study in the next chapter.

Chapter 4 Determination of Quercetin, (-)-Epicatechin and Their Methylated Forms in Urine Samples After Enzyme Hydrolysis

4.1 Abstract

Catechol-*O*-methyl transferase is an important phase II enzyme that affects the bioavailability of quercetin or (-)-epicatechin. The activity of this enzyme also varies between people because of genetic variation. Thus, it is interesting to study the COMT variation in individuals and relate this to the bioavailability of quercetin or (-)-epicatechin in healthy volunteers. The main aim of this chapter was to study the COMT activity and the metabolism of two dietary flavonoids, quercetin or (-)-epicatechin. A preliminary study of COMT activity was first tested *in vitro* prior to performing a pilot study *in vivo*. In humans, COMT activity was measured by the ratio of total urinary methylated quercetin or (-)-epicatechin to its aglycone excreted in urine after consumption of onion soup (containing 109 µmol of quercetin in 130 ml) or dark chocolate (containing 167 µmol of (-)-epicatechin in 45 g), or both of them together. Baseline and 24 hr urine samples from some subjects were hydrolysed by β-glucuronidase and sulfatase to release aglycone and methylated forms followed by HPLC-MS analysis. The result from the *in vitro* study showed that the conversion rate and COMT activity of (-)-epicatechin (9.1 pmol/min, 827 pmol/mg protein/min) was higher than quercetin (1.3 pmol/min and 126 pmol/mg protein/min). In the human study, the urinary excretion of quercetin, 3′-*O*-methylated quercetin and 4′-*O*-methylated quercetin were 0.70, 0.18 and 0.07% after onion soup intake. In comparison, there was 0.38% urinary excretion of (-)-epicatechin after 70% cocoa dark chocolate intake and no 3′-*O*-methylated (-)-epicatehcin and 4′-*O*-methylated (-)-epicatechin were detected in urine. These results were unchanged after co-consumption of both type of food and low compared to publications (Manach et al., 2005). A limiting factor may be enzyme hydrolysis using β-glucuronidase and sulfatase. Although new hydrolysis conditions were tested, the urinary excretion of quercetin and (-)-epicatechin was still low, and so variation of COMT activity in individuals cannot be compared. After *in vitro* assays, it was revealed that the amount of quercetin, (-)-epicatechin and their methylated forms were underestimated due to some compounds in urine which inhibited sulfatase activity. Thus, glucuronidase or sulfatase cannot be used for

quantitative urine analysis and so the approach was modified and the intact conjugated metabolites of quercetin and (-)-epicatechin excreted in the urine will be analysed in (Chapter 6) instead.

4.2 Introduction

As explained in Chapter 1, phase II enzymes (UGT, SULT and COMT) are an important factor influencing bioavailability of flavonoids. In the case of COMT, during passage of flavonoids across the intestinal tract and to the liver through blood circulation, COMT will add a methyl group from the co-factor SAM to a hydroxyl group of the catechol ring of flavonoids at the 3′ or 4′ position, resulting in a 3′-*O*-methylated or 4′-*O*-methylated product. The extent of this chemical change can affect the bioavailability and the biological effects of flavonoids. Some studies suggested that this modification probably reduced the biological effect of the parent compound (Duenas et al., 2011); whereas other studies suggested that the metabolites may have superior benefits for health (Jaramillo et al., 2010; Basu-Modak et al., 2003; Steffen et al., 2007). For example, Duenas et al. (2011) showed that quercetin metabolites, quercetin 3′-*O*-sulfate, quercetin 4′-*O*-sulfate, quercetin 3-*O*-glucuronide and isorhamnetin 3-*O*-sulfate decreased the antioxidant activity, investigated by ABTS and FRAP assays, comparing to the parent compound. However, an *O*-methylated metabolite (-)-epicatechin, 3′-*O*-methylated epicatechin, was shown to inhibit NADPH oxidase activity in human umbilical vein endothelial cells, resulting in improved function of vascular endothelium (Steffen et al., 2008).

COMT activity was originally analysed *in vitro* by Axelrod and Tomchick (1958). For a COMT substrate like dietary catechol flavonoids, some research groups have shown that these exogenous substrates are methylated by COMT faster than endogenous substrates *in vitro*. For example, quercetin, a subclass of dietary flavonoid, and fisetin, a structural analogue of quercetin, are rapidly *O*-methylated by cytosolic COMT, with rates two to three times higher than for both catechol estrogens and catecholamines, because quercetin and fisetin have a higher affinity for COMT than the endogenous substrates. (Zhu et. al. 1994; 2000). Zhu et al. (2000) also studied several common catechol-containing tea polyphenols which contain a catechol structure and share certain degrees of structure similarity to quercetin and fisetin. They found that common tea polyphenols such as (-)-

epicatechin, (+)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, and (-)-epigallocatechin gallate are rapidly *O*-methylated by human placental cytosolic COMT. In addition, in 2004 they showed that tea polyphenols, quercetin and fisetin were strong inhibitors of *O*-methylation of 2- and 4-hydroxyestradiol by cytosolic COMT from human liver. The same research group showed that major polyphenolic components present in coffee, chlorogenic acid and caffeic acid, act as inhibitors in the human liver and placental COMT-mediated *O*-methylation of catechol estrogens with mix mechanism, competitive and non-competitive inhibition (Zhu et al, 2009). Additionally, Zhu et al, (2010) further determined cytosolic COMT activity for the *O*-methylation of catechol estrogen. They found that there are positive correlations between the sensitivity of human placenta COMT to heat inactivation and to inhibition by EGCG but an inverse correlation between heat inactivation and inhibition by quercetin. They suggested that the differences in inhibition probably were due to different mechanisms of COMT inhibition involved. In addition, COMT inhibition may enhance the biological effect of the green tea polyphenol EGCG in human breast cancer cells (Landis-Piwowar et al., 2010).

For COMT activity *in vivo*, the relationship between tea catechins consumption, COMT genotype and risk of breast cancer in Asian-American women had been reported by Wu et al. (2003). They found that people who carried the low activity COMT allele, yielded less *O*-methylation resulting in a less efficient in elimination of tea catechins, and a reduced breast cancer risk after tea catechin consumption. However, Shrubsole et al. (2009) revealed that the same COMT genotypes did not affect the relationship between green tea consumption and breast cancer risk among pre- and postmenopausal women in a Chinese population. Miller et al. (2012) showed that COMT genotype did not intensely affect EGCG absorption and metabolism. Recently, Lorenz et al. (2014) also revealed that a high dose of EGCG from supplement does not impair COMT activity *in vivo*. However, in the case of (-)-epicatechin, no any publication has reported the effect of COMT activity on the absorption and metabolism after cocoa or cocoa product consumption. In the case of quercetin, Wang et al. (2012) reported that quercetin is not only increased the cellular absorption and decreased methylation of EGCG *in vitro* but this flavonoid also enhanced of total and non-methylated EGCG in lung, kidney and liver of severe combined immunodeficient mice after administration of brewed green tea and quercetin supplement. In addition, this study suggested that the

combination of quercetin and green tea may enhance the tumour inhibitory effect of green tea polyphenols.

Therefore, the different COMT activity levels in individuals possibly may affect the different amounts of polyphenol circulating throughout the human body. In addition, in the case of quercetin and (-)-epicatechin combination, quercetin which acts as a COMT inhibitor may increase or decrease methylation of (-)-epicatechin during the absorption and hepatic steps, influencing biological activity. Thus, it is necessary to study the COMT variation in individuals and relate this to the bioavailability of quercetin or (-)-epicatechin in healthy volunteers. The main aim of this chapter was to study the correlation between COMT activity and the metabolism of two dietary flavonoids, quercetin or epicatechin. The preliminary study of COMT activity was first tested *in vitro* prior to performing a pilot study *in vivo*. In humans, COMT activity was estimated by measuring the ratio of total urinary methylated quercetin or (-)-epicatechin to aglycone excreted in urine after consumption of onion soup (containing 109 μmol of quercetin in 130 ml) or dark chocolate (containing 167 μmol of (-)-epicatechin in 45 g), or both of them together. However, the free form of quercetin and (-)-epicatechin could not be found in urine but were transformed into glucuronidated, sulfated and methylated conjugates. Then, β-glucuronidase and sulfatase were used to hydrolyse the glucuronidated and sulfatated forms into methylated forms and its aglycone prior to determination by LC-MS. Furthermore, several factors that affected the enzyme hydrolysis condition were studied and the optimal condition was used to analyse urine sample in a small group of volunteers.

4.3 Materials and Methods

See Section 2.3 for catechol-*O*-methyltransferase (COMT) *in vitro*, section 2.5.1-2.5.4 for urine analysis and section 2.6 for determining sulphatase activity using commercial substrate (PNCS) and product (PNC).

4.4 Results

4.4.1 COMT activity *in vitro*

4.4.1.1 (-)-Epicatechin as a substrate of COMT

COMT activity was first tested *in vitro* prior to estimation *in vivo*. COMT can be found in several tissues, but is highest in liver and kidney. In this study, pig liver was used as a source of COMT, since it has high activity, and is feasible, cheap and easier to handle than human liver. There was approximately 125 ug/ml or 0.125 mg/ml protein content in supernatant, extracting from pig liver.

For COMT assay using (-)-epicatechin as a substrate, eight reaction mixtures of COMT assay were prepared according to Table 2.5. The first two samples were used as control samples and the other assays were varied by incubation time. After that, samples were injected onto the HPLC using the optimal gradient for epicatechin separation (see Section 2.3.3). In this assay, (-)-epicatechin was *O*-methylated by COMT and two metabolites of (-)-epicatechin, 3′-*O*-methylated-(-)-epicatechin and 4′-*O*-methylated-(-)-epicatechin, were produced. The retention time were approximately 13.6 and 14.9 for 3′-*O*-methylated-(-)-epicatechin and 4′-*O*-methylated-(-)-epicatechin, respectively (Figure 4.1). Furthermore, as expected, the amount of 3′-*O*-methylated-(-)-epicatechin and 4′-*O*-methylated-(-)-epicatechin was higher with increasing the incubation time. The chromatogram of samples and positive control are shown in Figure 4.1.

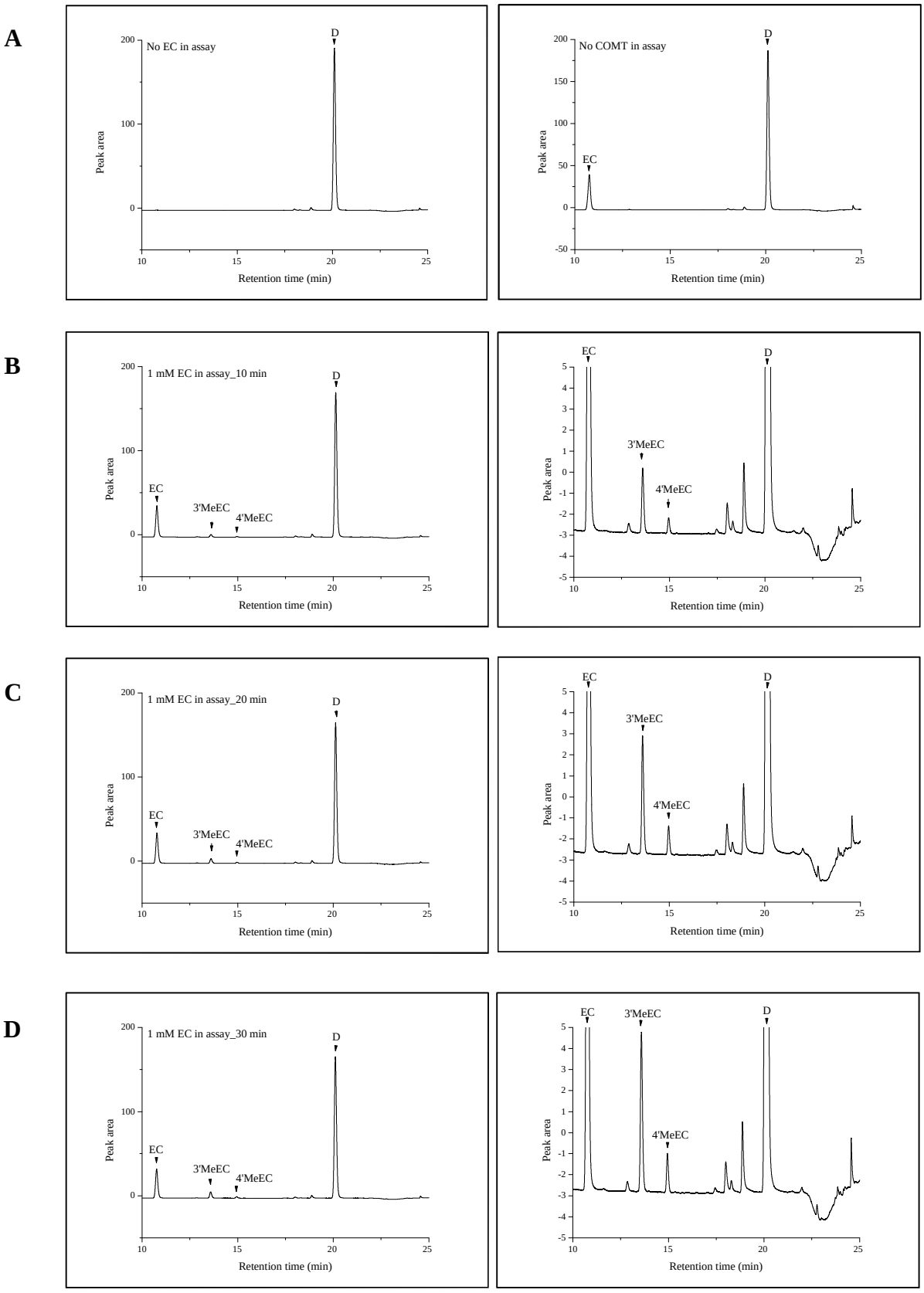


Figure 4.1: Chromatogram of (-)-epicatechin and its metabolite (3′-*O*-methylated (-)-epicatechin and 4′-*O*-methylated (-)-epicatechin) after different incubation time (A: negative control, B-G: COMT assay, incubated at 10 min, 20 min, 30 min, 40 min, 50 min and 60 min, respectively). For B to G, the left panels are an original chromatogram and the right panels are magnified.

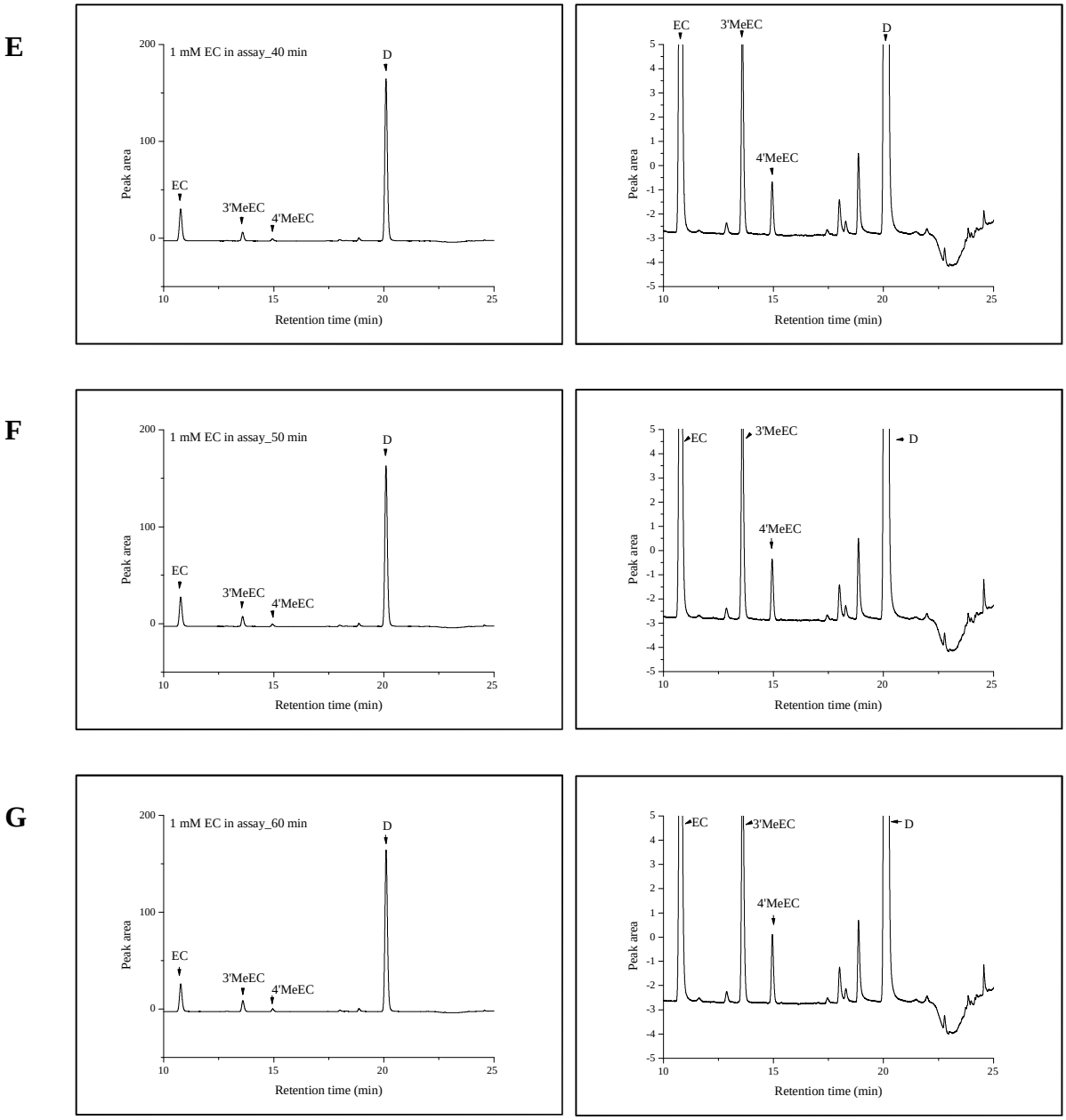


Figure 4.2: Chromatogram of (-)-epicatechin and its metabolite (3′-*O*-methylated (-)-epicatechin and 4′-*O*-methylated (-)-epicatechin) after different incubation time (A: negative control, B-G: COMT assay, incubated at 10 min, 20 min, 30 min, 40 min, 50 min and 60 min, respectively). For B to G, the left panels are an original chromatogram and the right panels are magnified (Continued).

For the rate of COMT, the amount of 3′-*O*-methylated-(-)-epicatechin and 4′-*O*-methylated-(-)-epicatechin were calculated using the equation from the (-)-epicatechin standard curve. Then, the conversion rate of COMT was constructed as shown in Figure 4.2.

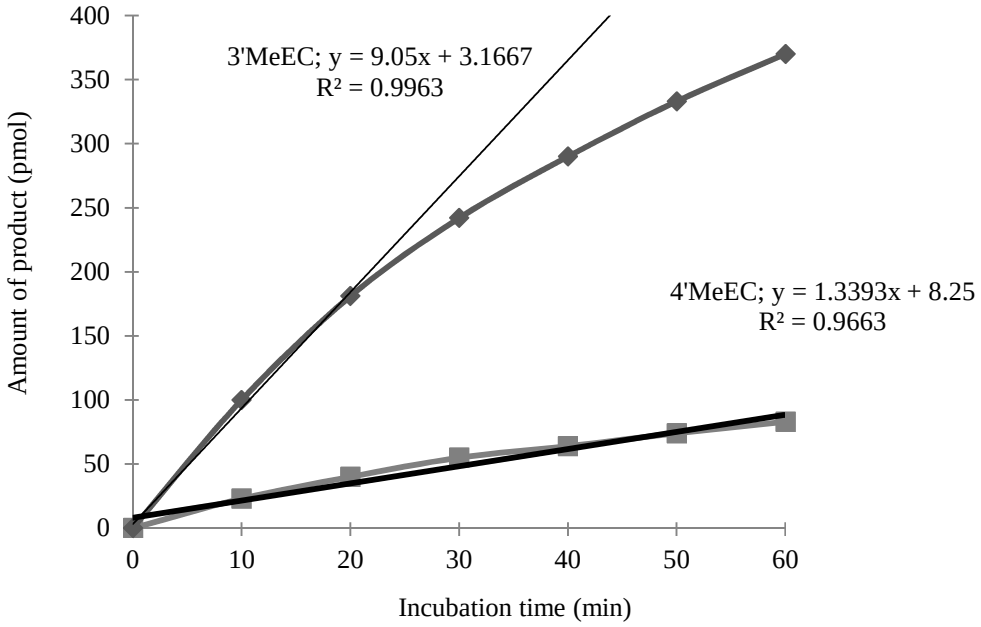


Figure 4.3: Conversion rates of COMT. The slope of the upper and the lower curve represented conversion rate for 3′-*O*-methylated-(-)-epicatechin and 4′-*O*-methylated-(-)-epicatechin, respectively.

For 3′-*O*-methylated form, there was 9.1 pmol/min, whereas, the conversion rate of 4′-*O*-methylated-(-)-epicatechin was relatively low (1.3 pmol/min). This data showed that 3′-*O*-methylated-(-)-epicatechin had an overall conversion rate higher than 4′-*O*-methylated-(-)-epicatechin.

4.4.1.2 Quercetin as a substrate of COMT

Using quercetin as a substrate, COMT *O*-methylated quercetin to produce 3′-*O* methylated quercetin (retention time = 19.892 min and a small peak of 4′-*O* methylated quercetin, eluting later). The chromatogram of samples and positive control are shown in Figure 4.3 and Figure 4.4, respectively.

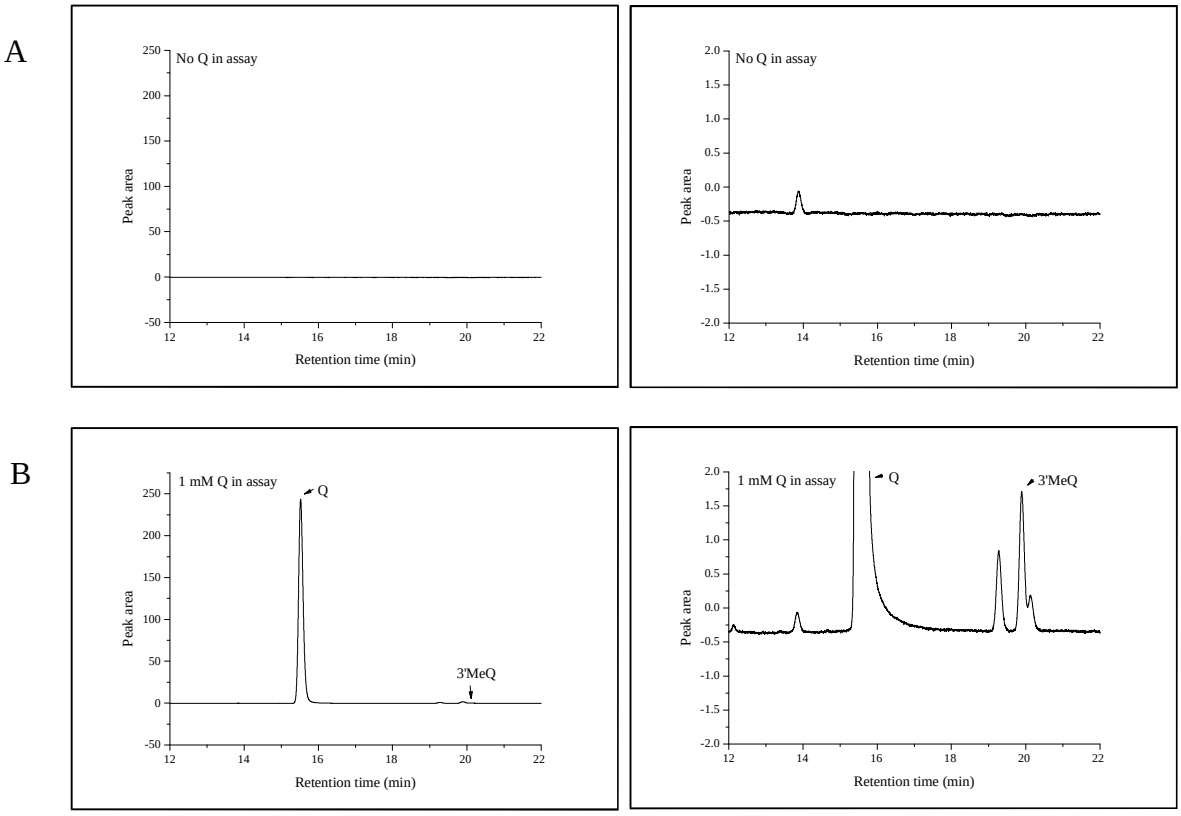


Figure 4.4: Chromatogram of quercetin and its metabolite (3′MeQ) in assay (B). A is negative control. The left panels are an original chromatogram and the right panels are magnified.

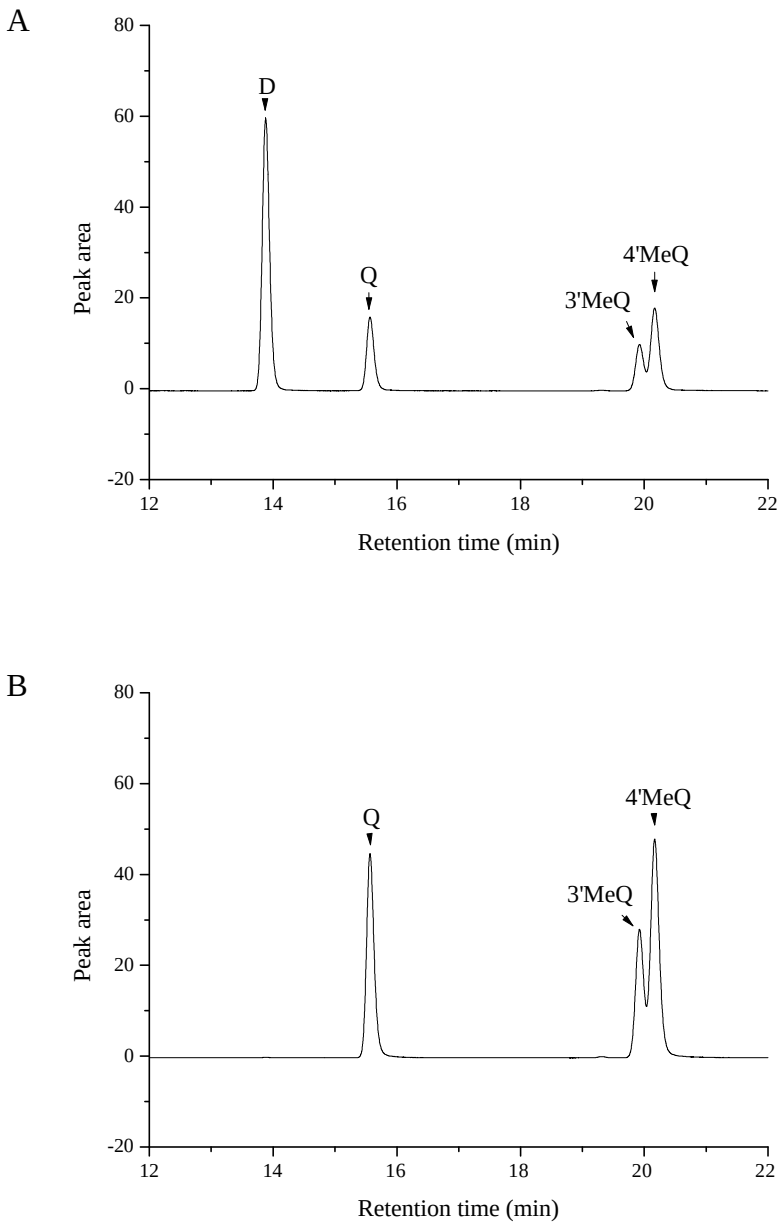


Figure 4.5: Chromatogram of 100 µM each standard; daidzein (D), quercetin (Q), 3′-*O*-methyl quercetin (3′MeQ) and 4′-*O*-methyl quercetin (4′MeQ) detected at 302 nm (A) and 370 nm (B).

The formation rate of 3′-*O*-methylated quercetin and COMT activity were 126 pmol/mg protein/min and 1.4 pmol/min, respectively. Next, the study focused on the methylated products of COMT in human body.

4.4.2 COMT activity *in vivo*

After assessing the COMT activity *in vitro*, it also was then estimated *in vivo*. The COMT activity was analysed by measuring the ratio of product to substrate or the ratio of total methylated of quercetin or (-)-epicatechin to its aglycone in this case. After single consumption of onion soup, 70% cocoa dark chocolate and co-consumption onion soup and 70% cocoa dark chocolate in different days, the baseline and 24 hr urine were then collected from healthy volunteers. 5 U of β-glucuronidase (*E. coli*, type IX-A) and 0.03 U of sulfatase (*A. aerogenes*, type IV) in 0.2 M sodium phosphate buffer, pH 7.0 were used to release the parent aglycone and methylated forms before quantifying by LC-MS (see Section 2.5.7). The amount of quercetin, (-)-epicatechin and their methylated products were identified and quantified based on HPLC retention time and MS analysis using standard curves of quercetin, (-)-epicatechin and their 3′-*O*-methylated or 4′-*O*-methylated forms dissolved in baseline urine (Figure 4.5). The regression analysis of each standard curve showed that the on-column amount, ranging from 20 to 100 pmol, was linearly correlated with a peak area ratio of each standard to taxifolin (act as internal standard II) of R^2 = 0.9685 for quercetin, R^2 = 0.9204 for 3′-*O*-methyl quercetin, R^2 = 0.9776 for 4′-*O*-methyl quercetin, R^2 = 0.9998 for (-)-epicatechin, R^2 = 0.9850 for 3′-*O*-methyl (-)-epicatechin and R^2 = 0.9802 for 4′-*O*-methyl (-)-epicatechin.

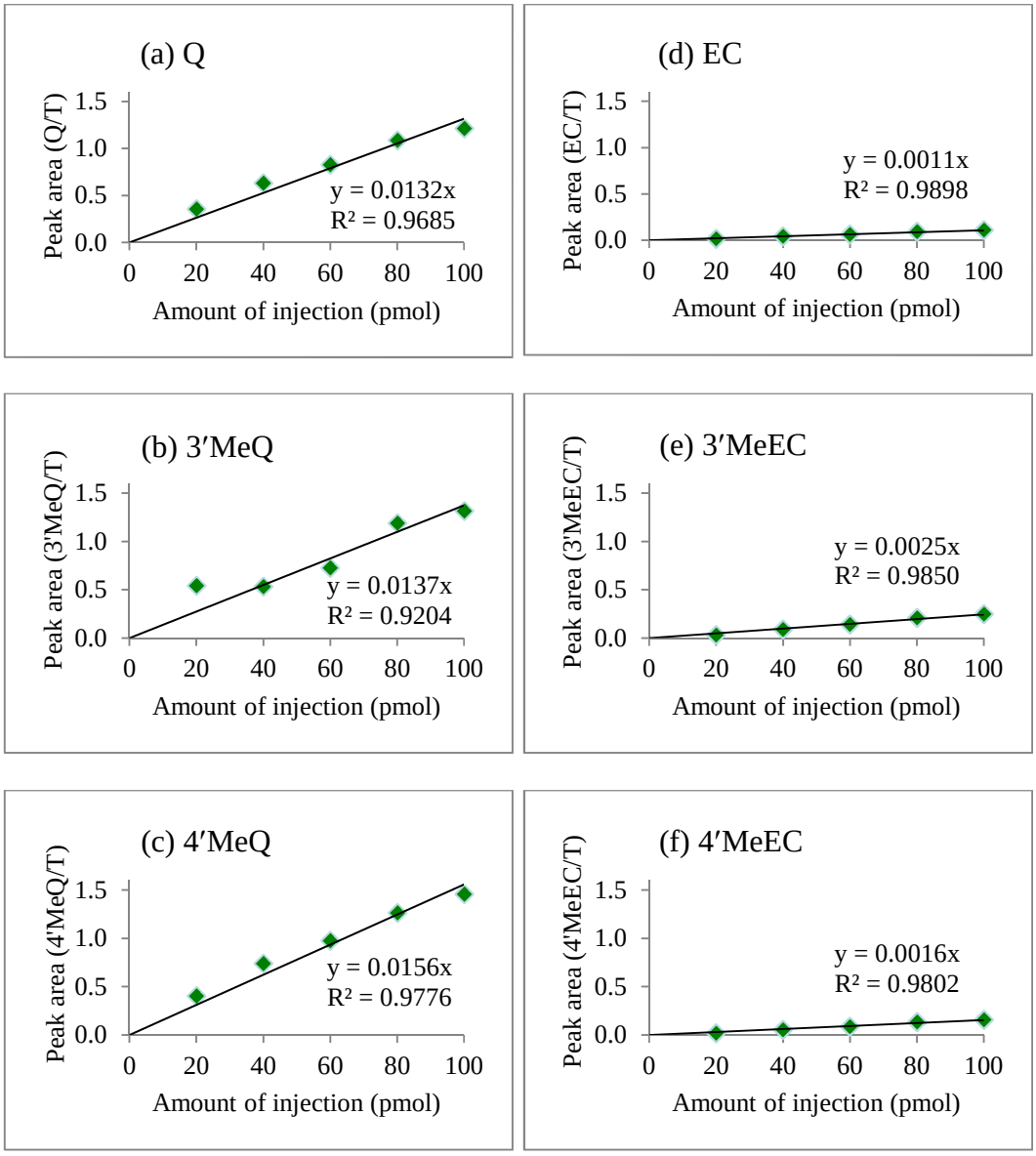


Figure 4.6: Standard curves of (a) quercetin (b) 3′-*O*-methyl quercetin (c) 4′-*O*-methyl quercetin (d) (-)-epicatechin (e) 3′-*O*-methyl (-)-epicatechin and (f) 4′-*O*-methyl (-)-epicatechin dissolved in baseline urine.

The percentage urinary excretion of each compound was calculated by the amount excreted divided by the amount ingested and multiplied by 100. From this equation, the percentage urinary excretion of quercetin, (-)-epicatechin and its methylated forms was calculated and is shown in Table 4.1. In day 1, quercetin and its methylated forms; 3′-*O*-methylated and 4′-*O*-methylated quercetin, were excreted in urine after red onion soup intake. The percentages of excretion in urine were 0.70%, 0.18% and 0.07%, respectively. In day 2, only (-)-epicatechin was detected

in urine (0.38% of dose) after single consumption of 70% cocoa dark chocolate. After co-consumption in day 3, the percentage urinary excretion of quercetin, (-)-epicatechin and their methylated forms were similar to results in day 1 and day 2 but the percentage of excretion was higher by approximately 2 fold.

The total percentage of urinary excretion of quercetin (0.95%) and (-)-epicatechin (0.38%) found in this subject was then compared with other publications (Table 4.2). The percentage of total quercetin in urine was in the reported range but the urinary excretion of total (-)-epicatechin was lower by around 7 times.

Table 4.1: The percentage of urinary excretion of quercetin, (-)-epicatechin and its methylated forms

Compounds	**% Urinary excretion**		
	Day 1 study red onion soup consumption	**Day 2 study 70% cocoa dark chocolate consumption**	**Day 3 study both consumption**
Quercetin	0.70	-	1.10
3′-*O*-Methyl quercetin	0.18	-	0.28
4′-*O*-Methyl quercetin	0.07	-	0.16
(-)-Epicatechin	-	0.38	0.72
3′-*O*-Methyl (-)-epicatechin	-	nd	nd
4′-*O*-Methyl (-)-epicatechin	-	nd	nd

Table 4.2: The percentage urinary excretion adapted from Monach et al., 2005. These were converted to correspond to a supply of 50 mg aglycone equivalent.

Compound	**%Urinary excretion (Manach et al., 2005)**	
	Mean	**Range**
Quercetin glucosides	2.5±1.2	0.31-6.4
(Epi)catechin	18.5±5.7	2.1-55.0

Some factors which may possibly affect enzyme hydrolysis or stability of analysed compounds such as pH, efficiency of extracted solvent and the different solvents for reconstitution more considered and tested. Firstly, the pH of 0.2M sodium phosphate buffer or solvent B in different urine preparation step was prepared and measured (Table 4.3). The pH in Table 4.3 shows that the pH of tube No 2, a mixture between 150 µl of 0.2 M Sodium phosphate, pH 7.0 and 1 ml of 50% Ethanol in milliQ water was 8.2. This condition may degrade quercetin in the reaction. For other tubes (No 3, 4, 5), the pH are in acid condition which be able to preserve compounds from degradation.

Table 4.3: The pH of 0.2 M sodium phosphate buffer or solvent B in different urine preparation step

Tube No.	**Reaction mixture**	**pH**
1	150 µl of 0.2 M Sodium phosphate, pH 7	7
2	150 µl of 0.2 M Sodium phosphate, pH 7.0 + 1 ml of 50% Ethanol in milliQ water	8.2
3	Solvent B (Acetronitrile + 0.1% Formic acid)	4.4
4	Solvent B (Acetronitrile + 0.1% Formic acid) + Solvent A (MilliQ water + 0.1 Formic acid) containing 100 µM ascorbic acid	2.6
5	Solvent B (Acetronitrile + 0.1% Formic acid) + Solvent A (MilliQ water + 0.1% Formic acid) containing 100 µM ascorbic acid + 500 µM Taxifolin in 50% Ethanol in milliQ water	2.6

Secondly, 1 µM quercetin, 1 µM (-)-epicatechin and 1 µM daidzein (internal standard) were prepared in 50% ethanol and extracted with and without ethyl acetate prior to analysis by LC-MS. Then, the peak area of each sample was compared (Figure 4.6). Extracting quercetin, (-)-epicatechin and daidzein with ethyl acetate produced a higher yield than without ethyl acetate extraction. Lastly, the different

solvents, 50% ethanol in milliQ water and 15% solvent B, were used to prepared 1 µM EC then directly injected to LC-MS and the peak area of these two samples were compared. The peak area of (-)-epicatechin dissolved in 50% ethanol in milliQ water and in 15% solvent B were 376,049 and 372,922 which was not different.

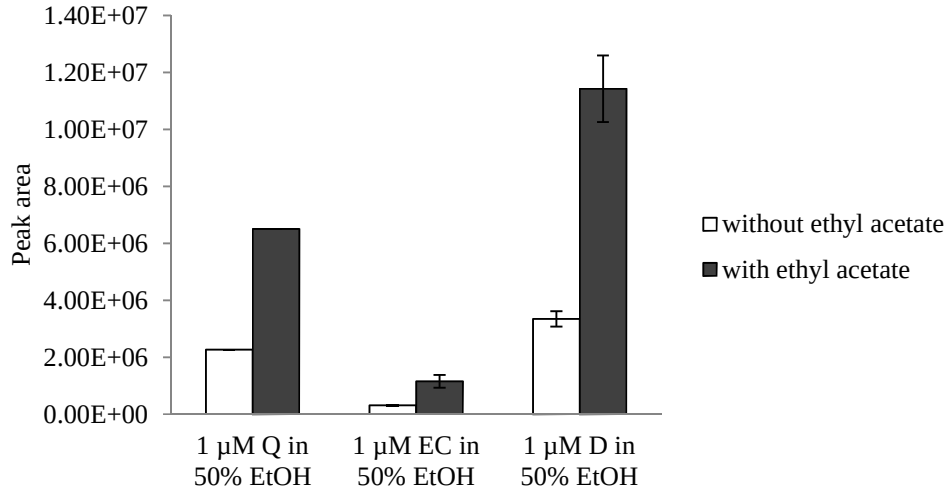


Figure 4.7: Efficiency of extracted solvent, ethyl acetate, on quercetin (Q), (-)-epictechin (EC) and diadzein (D) extraction

From these results, ethyl acetate for quercetin, (-)-epicatechin and daidzein extraction and 50% ethanol in water or 15% solvent B for reconstitution seems not to have any effect on the stability of analysed compounds in different urine preparations, except pH in hydrolysis condition may cause the activity of enzyme and the stability of some compounds. Therefore, the type of buffer used for enzyme hydrolysis needs to re-considered and changed. A new enzyme deconjugation method optimised by Dr Kayleigh Clarke was then applied in this section. The ABSS buffer at pH 5 was used instead of 0.2 M sodium phosphate buffer, pH 7.0. However, this pH condition is optimal for a different type of β-glucuronidase and sulfatase activity. Then, sulfatase, not glucuronidase, was changed from *Aerobacter aerogenes* type IV to *Abalone entrails* type VIII. In addition, the low amount of deconjugated enzyme in the hydrolysis reaction may possibly be another reason causing a low amount of (-)-epicatechin and no methylated (-)-epicatchin to be detected in urine. Thus, the unit of enzyme also was increased from 5 U to 200 U for β-glucuronidase and from 0.03 U to 5 U and up to 20 U for sulfatase, according to Saha et al, 2012. The units of enzyme were varied and tested in urine sample of

volunteer No 004 after single consumption of 70% dark chocolate. The result in Table 4.4 is shown that 200 U of β-glucuronidase (*Escherichia coli* type IX-A) and 20 U of sulfatase (*Abalone entrails* type VIII) are better to hydrolyse (-)-epicatechin metabolites in urine than other conditions. However, only (-)-epicatechin and 4'-*O*-methylated-(-)-epicatechin were detected after enzyme deconjugation and no 3'-*O*-methylated-(-)-epicatechin was detected.

Table 4.4: The amount of (-)-epicatechin, 3'-*O*- methylated-(-)-epicatechin and 4'-*O*- methylated-(-)-epicatechin (nmol) after hydrolyse with vary unit of β-glucuronidase (*Escherichia coli* type IX-A) and sulfate (*Abalone entrails* type VIII).

Volunteer 004					
Units of enzyme (U)		**The amount of compounds (nmol)**			
β-glucuronidase	**Sulfatase**	**EC**	**3'-*O*-MeEC**	**4'-*O*-MeEC**	**Total EC**
0	0	nd	nd	nd	nd
5	0.03	nd	nd	nd	nd
200	5	1019	nd	464	1483
200	20	1786	nd	1289	3074

After that, this condition, 200U of β-glucuronidase (*Escherichia coli* type IX-A) and 20U of sulfatase (*Abalone entrails* type VIII), was used to analysed (-)-epicatechin and its methylated metabolites in more volunteers to check whether the % urinary excretion in these volunteers were in the same range of volunteer No 004 or not. Again, only (-)-epicatechin was detected in urine after enzyme deconjugation and no any methylated forms of (-)-epicatechin found in urine. The % urinary excretions of these volunteers were shown in Table 4.5.

Table 4.5: The urinary excretion of (-)-epicatechin after 70% dark chocolate intake in selected volunteers

Volunteer Number	% of dose (-)-epicatechin
003	1.8
004	1.1
117	2.1
118	1.0
120	2.3

Although the amount of enzyme was increased, the % urinary excretion of (-)-epicatechin was unaffected. As mentioned previously, the % urinary excretion reported by (-)-epicatechin of Manach et al. (2005) was hugely wide-ranging from 2.1% to 55.0% because the data originated from various types of food and not only from cocoa. Baba et al., 2000 demonstrated that urinary excretion of total (-)-epicatechin was 29.8±5.3% for chocolate intake and 25.3±8.1% for cocoa intake. In addition, Actis-Goretta et al. (2012) showed that the total urinary excretion of (−)-epicatechin is 20±2% after consuming 100 g of Nestlé Noir 70% chocolate containing 79 mg of (−)-epicatechin (272 μmol), 26 mg of (±)-catechin, and 49 mg of procyanidin B2. The present study provided a half bar of 70% dark chocolate (containing 167 μmol of (-)-epicatechin in 45 g) then the expected % of urinary excretion in urine should be around 12%, calculated based on the Actis-Goretta et al. study. However, the % urinary excretion analysed from some volunteers in this study showed that it is lower than the expected amount around 6 times. It is proposed that β-glucuronidase and sulfate did not hydrolyse completely. Something in urine may possibly inhibit enzyme activity, and this was assessed in the next section.

4.4.3 The limitation of sulfatase activity

In 2012, Saha demonstrated that epicatechin sulfate and methylepicatechin sulfates, both in urine and as the authentic compounds, are poor substrates for six commercial sulfatases (*Helix pomatia* Type H-1 or H-2, *Patella vulgate* Types IV or V, *Aerobacter arerogenes* Type VI or *Abalone entrails* Type III) after incubation with 20 U of these sulfatase and 200 U of β-glucuronidase for 2 hr at pH 5.0.

Therefore, the efficiency of sulfatase used in this study was investigated. Sulfated quercetin and (-)-epicatechin were synthesised from commercial quercetin or (-)-epicatechin and then these synthesised compounds were used as substrates for sulfatase hydrolysis. For quercetin sulfation, quercetin was converted to quercetin sulfate by SULT from pig liver cytosol. The sulfuryl group of the universal sulfuryl group donor, adenosine-3′-phosphate-5′-phosphosulfate (PAPS) was transformed to the hydroxyl group of quercetin by SULT, resulting in quercetin sulfate. For (-)-epicatechin sulfation, similar enzymatic reaction was prepared but (-)-epicatechin was used as a substrate instead of quercetin. In addition, control samples, sulfation reaction mixtures without cytosol pig liver, were also prepared. After enzymatic synthesis, sulfated quercetin and (-)-epicatechin were analysed by LC-MS using the total ion current (TIC) and selected-ion monitoring (SIM) mode (at specific m/z- = 381 for quercetin sulfate and at m/z- = 369 for (-)-epicatechin sulfate). The chromatogram of quercetin sulfate and (-)-epicatechin sulfate are shown in Figure 4.7 and Figure 4.8, respectively. In Figure 4.7A, quercetin and quercetin sulfate were found in (a) no enzyme and quercetin sulfation sample when detecting with TIC mode. However, in SIM mode (m/z- = 381) in Figure 4.7B, only quercetin sulfate was detected (b). In Figure 4.8, two forms of (-)-epicatechin were generated and detected in both TIC (a) and SIM (b) mode. Again, no (-)-epicatechin was found in SIM mode (m/z- = 369). Next, the % yields of these products were calculated according to the equation in section 2.4.4.

There were 82% yield for sulfated quercetin and 96% yield for sulfated epicatechin. Subsequently, some of these sulfated samples were dried and added to ABSS buffer, pH 5 with or without 20 U of sulfatase (*Abalone entrails* type VIII) and varied the incubation time (for 1, 2, 3 and 4 hr). The sufatase hydrolysis result is presented in Figure 4.9.

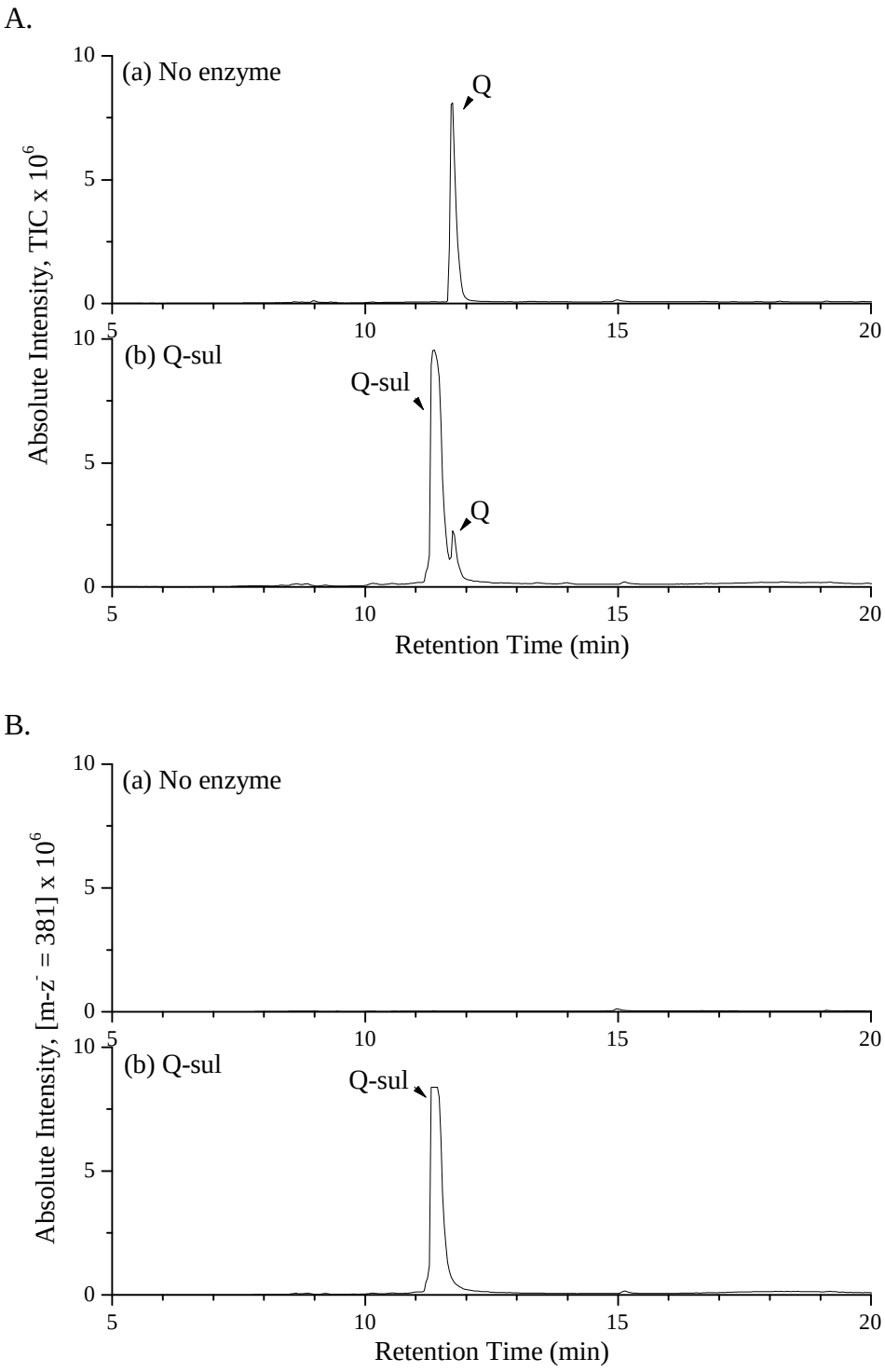


Figure 4.8: Chromatogram of quercetin and quercetin sulfate in TIC mode (A) and specific m/z- at 381 (B). (a) No cytosol pig liver added to the sulfation reaction (control) (b) sulfated quercetin synthesised from sulfation reaction.

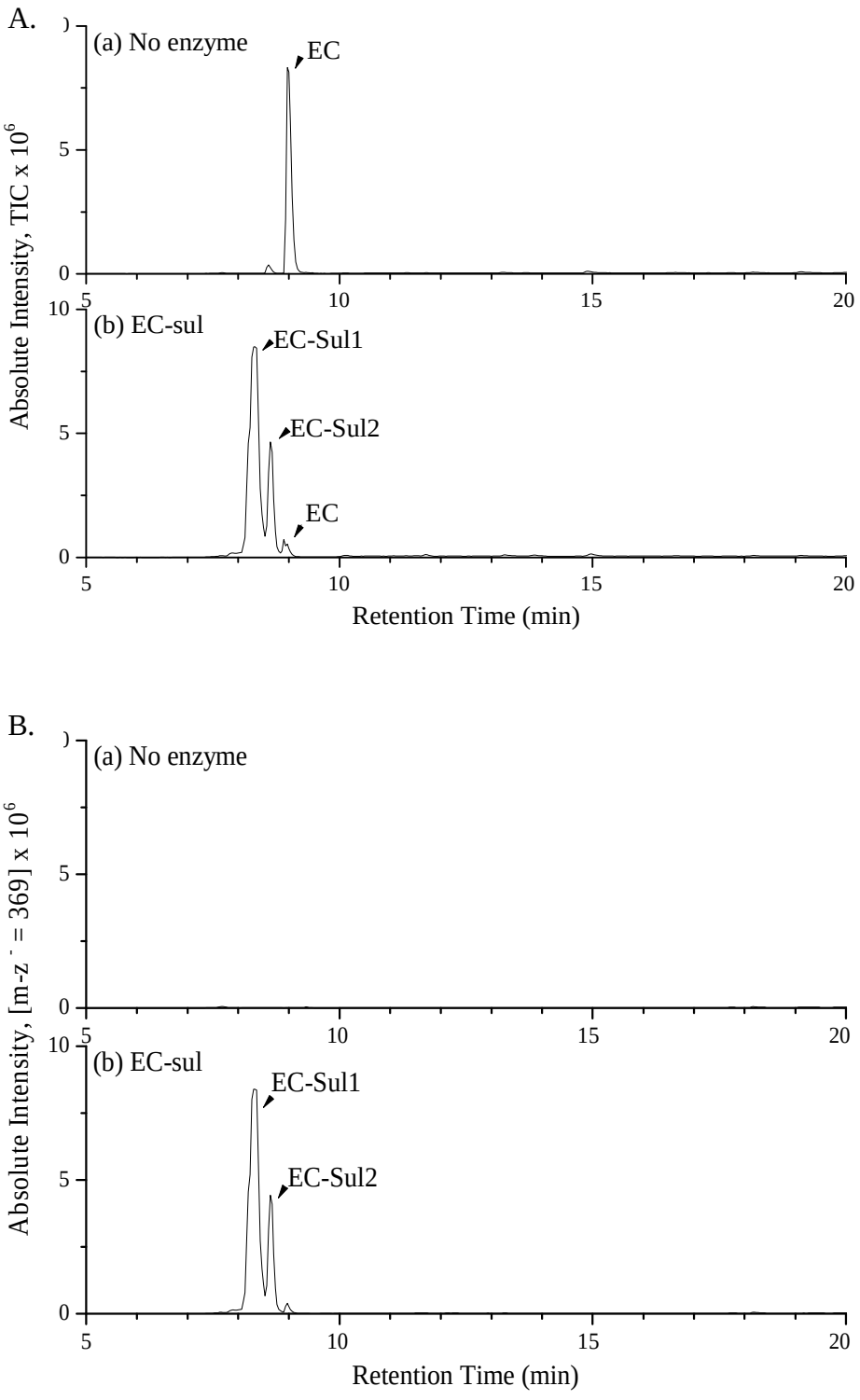


Figure 4.9: Chromatogram of (-)-epicatechin and (-)-epicatehin sulfate in TIC mode (A) and specific m/z- at 369 (B). (a) No cytosol pig liver added to the sulfation reaction (control) (b) sulfated (-)-epicatechin synthesised from sulfation reaction.

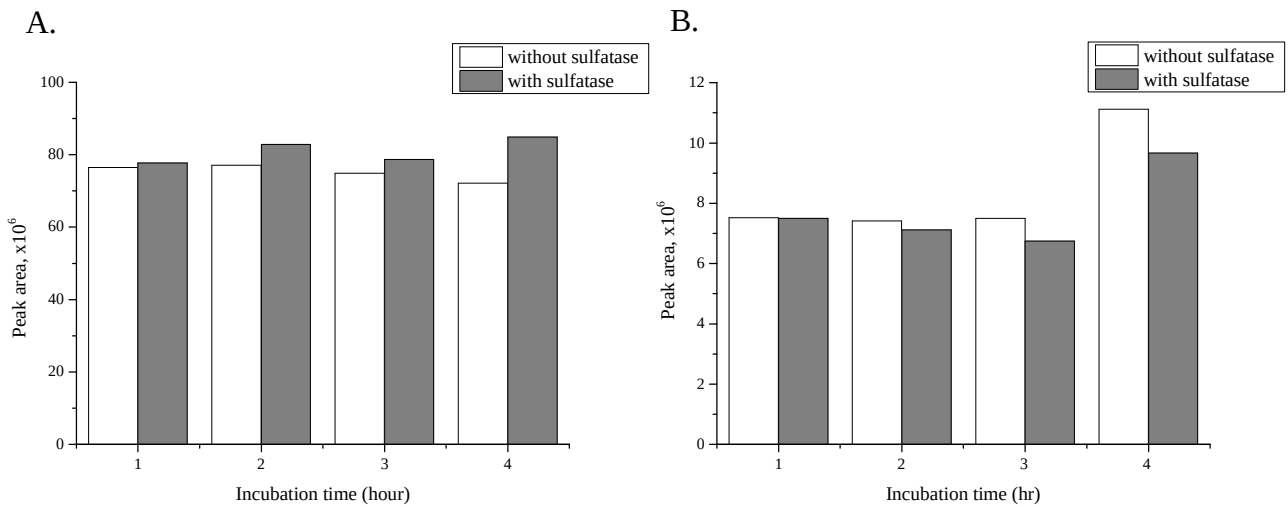


Figure 4.10: Peak area of sulfated quercetin (A.) and sulfated (-)-epicatechin (B.) after hydrolyse without or with 20 U of sulfatase (*Abalone entrails type VIII*) at different incubation times (1, 2, 3 and 4 hr)

The result in Figure 4.9 shows that there are no differences between the amount of sulfated forms of quercetin and (-)-epicatechin without or with sulfatase. It seems sulfatase does not hydrolyse, sulfated quercetin and (-)-epicatechin, and so these substrates remain in the reaction.

Finally, a model substrate, *p*-nitrocatechol sulfate (PNCS) was used to test sulfatase activity. 0.001 µmol of *p*-nitrocatechol sulfate were added to milliQ water, ABSS buffer and baseline urine, respectively. Then, this substrate was hydrolysed with or without 20 U of sulfatase (*Abalone entrails type* VIII) at 37°C for 1 hr and processed by the same procedure as above. One unit of this enzyme will hydrolyse 1.0 µmol of *p*-nitrocatechol sulfate per hr at pH 5.0 at 37°C. Thus, there was enough sulfatase to hydrolyse *p*-nitrocatechol sulfate in this reaction. In general, *p*-nitrocatechol sulfate was hydrolysed to *p*–nitrocatechol and sulfate after treatment with sulfatase (Figure 4.10). In Figure 4.10A and 4.10D, (a) is a substrate, *p*-nitrocatechol sulfate, added to milliQ water and (b) is a product, *p*-nitrocatechol, added to milliQ water. These two samples act as positive controls, eluting at 9.7 min for *p*-nitrocatechol sulfate and 10.7 min for *p*–nitrocatechol. In Figure 4.10B and 4.10E, *p*-nitrocatechol sulfate was dissolved in ABSS buffer and hydrolysed to *p*-nitrocatechol after treatment with sulfatase (d). Thus, a high amount of product was observed. However, there are some substrates still left in the reaction. Without sulfatase treatment (c), a tiny peak of *p*-nitrocatechol also was observed. It is

possible due to the degradation of *p*-nitrocatechol sulfate during sample preparation. In baseline urine (Figure 4.10C and 4.10F), sulfatase can hydrolyse *p*-nitrocatechol sulfate to *p*-nitrocatechol but not completely (f). A little peak of product was observed and substrate still found in the reaction. In addition, unidentified peaks were also observed in this chromatogram. This peak may be a product of other biological compounds, still remaining in urine and acting as a sulfatase inhibitor. Thus, the evidence from this study strongly indicates that some compounds in urine inhibit sulfatase activity.

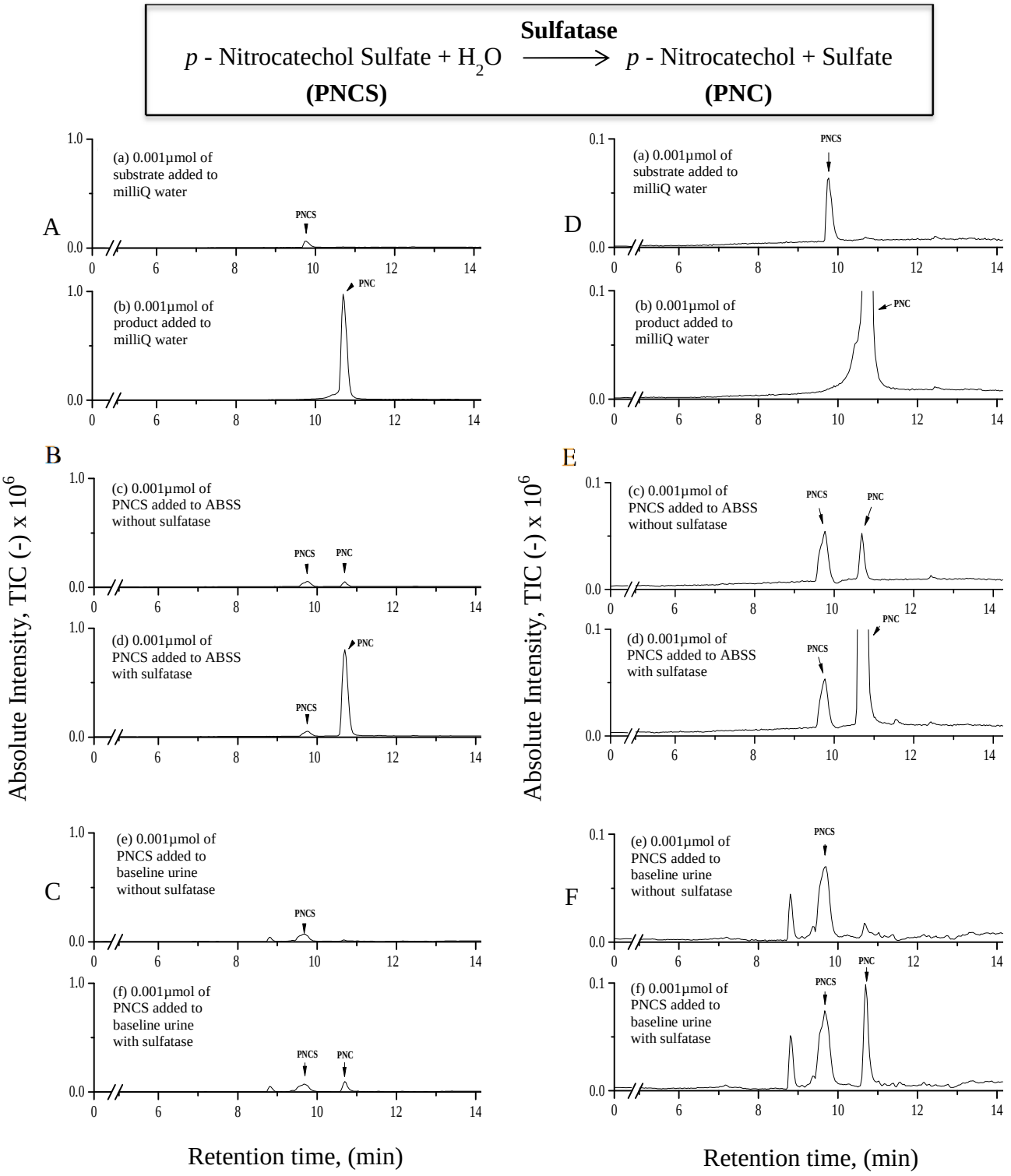


Figure 4.11: Sulfatase reaction. *p*-Nitrocatechol sulfate was hydrolysed to *p*-nitrocatechol and sulfate by sulfatase (above). Chromatogram of model substrate (PNCS) and product (PNC) of sulfatase (A), (D) added in milliQ water (B), (E) added in ABSS buffer and (C), (F) added in baseline without and with sulfatase treatment. Chromatogram A, B and C are 1X magnification (in the left panel) and D, E and F are 10X magnification (in the right panel).

4.5 Discussion

The main aim of this chapter was to study the correlation between COMT activity and the metabolism of two dietary flavonoids, quercetin or (-)-epicatechin. The preliminary study of COMT activity was first tested *in vitro* prior to performing a pilot study *in vivo*.

4.5.1 COMT activity *in vitro*

The main finding in this section is that (-)-epicatechin was *O*-methylated by COMT from pig liver into two forms; 3′-*O*-methylated-(-)-epicatechin and 4′-*O*-methylated-(-)-epicatechin with a conversion rate 9.1 pmol/min and 1.3 pmol/min, respectively. Thus, this data showed COMT preferred to add a methyl group onto the 3′ than 4′ position. These data agree with the previous result studied by Zhu et al, 2000. They found that the major monomethylated metabolite of (-)-epicatechin is the 3′-*O*-methyl isomer whereas the minor metabolite is 4′-*O*-methyl isomer. COMT activities in this present study were 827 pmol/mg protein/min for 3′methylated epicatechin and 122 pmol/mg protein/min for 4′methylated epicatechin. The rate for the *O*-methylation of epicatechin by pig liver COMT is different to the reported for *O*-methylation of tea polyphenol by human placental cytosolic COMT (150-500 pmol/mg of protein/min) (Zhu et al, 2000). This is probably because other factors such as different substrates, incubation conditions and COMT enzyme source influence enzyme activity (Pihlavisto and Reenila, 2002).

For COMT with quercetin as a substrate, the COMT activity was 126 pmol/mg protein/min. According to Zhu et al (1994), who also studied the *O*-methylation of quercetin, fisetin, catechol estrogen and catecholamines by either pig liver or hamster kidney, quercetin was rapidly *O*-methylated, and the *O*-methylation rate was higher than catechol estrogen and catecholamine in both tissues. COMT activity was 14,870 pmol/mg protein/min for pig liver and 200 pmol/mg protein/min for hamster kidney. The COMT activity in this study was lower than the previous studied of Zhu et al. more 118 times. The possible explanation is that the sources of both COMT studies were different. This study used a crude COMT extracted from pig liver whereas the study of Zhu et al. used commercial COMT which is more purified. Additionally, Zhu et al (1994) also showed that the main methylated product of COMT assay using quercetin as a substrate was 3′-*O*-methylated

quercetin. They found about 97% of 3′-*O*-methylated quercetin recover in urine of hamster. Thus, these data is in good agreement with our study. The reason for favouring 3′-*O*-methylation over 4′-*O*-methylation is that 4′-*O*-hydroxyl group approaches the adenosyl-L-methionine and unfavorable position with the hydrophobic protein residues of the catalytic site (Mannisto et al, 1999).

In this study, the conversion rate and enzyme activity of (-)-epicactechin were higher than quercetin. (-)-Epicatechin is probably a better substrate for *O*-methylation by COMT from pig liver extracted in this study than quercetin. Thus the result from this section showed that an extract of pig liver displayed a COMT enzyme activity towards (-)-epicatechin and quercetin, producing their methylated products; 3′-*O*-methylated (-)-epicatechin and 4′-*O*-methylated (-)-epicatechin, and 3′-*O*-methylated quercetin *in vitro*. Then, COMT from pig liver probably have the same action of COMT in human liver. Thus, COMT activity in human body needs to be analysed.

4.5.2 COMT activity *in vivo*

In human, COMT activity was estimated by determining quercetin, (-)-epicatechin and its methylated metabolites in urine samples after β-glucuronidase and sulfatase enzyme hydrolysis followed by calculating the ratio of total urinary methylated quercetin or (-)-epicatechin to corresponding aglycone in urine after single consumption of onion soup or dark chocolate and co-consumption both. In this study, quercetin and its methylated forms, 3′-*O*-methylated and 4′-*O*-methylated quercetin, were detected in urine after enzyme hydrolysis in both single and co-consumption studies. The percentage of total quercetin excreted in urine in this study (0.95%) was still in the range when compared to Manach et al. (2005), ranging from 0.31%-6.4%. Compare to Mullen et al. (2006), they provided 270 g fried red onion (containing 275 μmoles of quercetin) to volunteers, resulting in 12.9 μmole of total quercetin excreted in urine or 4.7% of intake but this study provided red onion soup, containing 109 μmol of quercetin in 130 ml, was to volunteers. The amount of intake in this study was lower that Mullen's study approximately 2.5 times. Thus, it is reasonable that the total quercetin excretion in this study was lower than Mullen's study. In addition, the total % urinary excretion of 3′-*O*-methylated and 4′-*O*-methylated quercetin after onion soup intake was 0.25%, while, Mullen et al (2006) revealed that there was 0.91% urinary excretion of isorhamnetin-3-glucuronide and

isorhamnetin-4′-glucuronide. Thus, providing a higher dose to volunteers seems produce a higher % in urine.

For urinary (-)-epicatechin, only (-)-epicatechin was detected in urine and not 3′-*O*-methylated and 4′-*O*-methylated (-)-epicatechin. The possible reasons why the percentages of quercetin, (-)-epicatechin and its methylated urinary metabolites in this study were lower than other publications may due to; the low dose of food samples were provided to volunteers, the low amount of deconjugated enzyme using in the hydrolysis reaction or these flavonoids were poor substrates for sulfatase (Saha et al, 2012), the matrix effect from urine sample, and these flavonoids maybe less excreted into urine but still retain in the body and derive greater biological effects (Inoue-Choi M, 2010). There were possible solutions to solve these problems. In case the low dose of food sample, the larger amount of urine sample probably need to use for urine analysis. In the case of the low amount of deconjugated enzyme in the hydrolysis reaction, the unit of enzyme needs to be varied or increased. In addition, other urine analysis method such as non-enzymatic hydrolysis can be used. However, this non-enzymatic hydrolysis method is suitable for analysing all conjugated metabolites excreted in urine. In the case of matrix effect in urine, sample preparation techniques such solid phase extraction or protein precipitation probably need pior inject the sample to LC-MS. In addition, chromatographic or mass spectrometric condition needs to be optimized to reduce the matrix effect. In this study, the standard curves were performed in baseline urine and extracted as the same condition of 24 hr urine sample so this procedure also useful for evaluating the matrix effect urine sample.

In this study, some factors that may affect enzyme hydrolysis condition or stability of analysed compounds were tested and a new deconjugation method was used to analyse urine samples. Only (-)-epicatechin was detected in volunteers with a low percentage, ranging from 1.0-2.3% urinary excretion. As mentioned previously, though the unit or amount of enzyme in deconjugation reaction was increased but it was not alter or increase the % urinary excretion of (-)-epicatechin. In addition, β-glucuronidase and sulfate seems hydrolysed (-)-epicatechin metabolites in urine but incomplete. This result is in agreement with previously result reported by Saha et al 2012. Although, they used 20U of sulfatase and 200U of β-glucuronidase from variety source of enzyme and also varied the incubation time. They found that approximately 90% of various form of (-)-epicatechin

glucuronide was hydrolysed. In addition, no commercial sulfatases were effective to hydrolysed (-)-epicatechin sulfates or methyl-(-)-epicatechin-sulfates in urine. Thus, something in urine may possibly inhibit enzyme activity. This assumption was supported by Taylor, 2005. They suggested that the presence of interfering compounds and also the type of conjugated forms in urine have influence on the ability of enzymatic hydrolysis. Then, the activity of enzyme needs to investigate in the next step.

4.5.3 The limitation of sulfatase activity

In this study, synthetic quercetin sulfate and (-)-epicatechin sulfates were used to test a sulfatase activity. However, sulfatase seems not hydrolyse these substrates adding in the ABSS buffer into their products because there were indifferent between the peak area of substrates hydrolysed without and with sulfatase. In addition, different amount of synthetic (-)-epicatechin sulfates also was spiked into baseline urine and extracted the same way as 24 hr urine sample. Again, there were not different between the peak area of substrates hydrolysed without and with sulfatase (data not shown). Saha et al, 2012 also used authentic (-)-epicatechin, 3′-methylated-(-)-epicatechin and 4′-methylated-(-)-epicatechin spike into urine and test sulfatase activity. They claimed that none of sulfates test was effectively hydrolyse but no data was shown. Moreover than this, the present study used *p*-nitrocatechol sulfate (PNCS) and *p*–nitrocatechol (PNC), a model substrate and product of sulfatase to investigate its activity. The result in Figure 4.10 was originally confirmed that some compounds in urine inhibit the sulfatase activity for flavonoids determination in urine. This observation agrees with the results obtained by Graef, (1975). They found that incubation of urine with β-glucuronidase and arylsuphatase from *Helix pomatia* results in the incomplete hydrolysis of urinary steroid conjugates due to inhibitors of these enzymes, presenting in urine. They also revealed that these inhibitors can be separated from the steroid conjugates by chromatography of the urine onto a specific column prior to perform enzymatic hydrolysis using optimal condition.

Sulfatases play an important role in regulating the sulfation states in biological systems (Hanson, 2004). Sulfatase or arylsulfatase enzymes (aryl-sulfate sulphohydrolase, EC 3.1.6.1) catalyse hydrolysis of phenolic sulfate esters to a phenolic compound and sulfate ion. There were two major types of arylsulfatase

enzymes occur in man; Type I enzyme (arylsulfatase C) and Type II enzymes (arylsulfatase A and B). For type II isoenzymes, it has been reported that their sensitivity was inhibited by various anionic species, including sulfite, sulfate, and phosphate (Bostick, 1978). Chruszcz, 2003 also stated that arylsulfatase A is inhibited by phosphate by forming a covalent bond with the active site 3-oxoalanine of this enzyme. For arylsulfatase B, Rao, (1984) purified this enzyme from rabbit liver and found that it is also competitively inhibited at modest concentrations by a variety of phosphate esters derived from amino acids, amines and simple sugars.

In the case of urine, it consists of inorganic salts (such as Na^+, K^+, PO_4^{2-} and SO_4^{2-}), urea, creatinine and uric acid. Although urine is a biological fluid that more clean compare to plasma but the inorganic ion, phosphate and sulfate, in these samples have been reported that are potential substances to inhibit the ability of enzyme hydrolysis of steroid sulfate conjugates of (Shibasaki, 2001). However, Shibasaki (2001) suggested that removal this ion from the urine before enzyme hydrolysis may increase the rate of hydrolysis but some steroids also lost during the removal step. One more important factor may cause the sulfatase activity need to be considered. It is the type of buffers, 0.2 M Sodium phosphate buffer, pH 7 and ABSS, pH 5, using in enzyme hydrolysis condition in this study. These two buffers contain NaH_2PO_4 and Na_2HPO_4. Although there is equilibrium between the acid and its conjugate base in the buffer solution but some factors probably influent the stability of this equilibrium, resulting in an access ion of PO_4^{2-} and possibly inhibiting sulfatase activity.

4.6 Conclusion

An extract of pig liver displayed COMT activity towards (-)-epicatechin and quercetin, producing their methylated products; 3′-O-methylated-(-)-epicatechin, 4′-O-methylated-(-)-epicatechin and 3′-O-methylated-quercetin. In addition, the conversion rate and enzyme activity of (-)-epicatechin in this study were higher than quercetin.

The low percentage urinary excretion of quercetin, (-)-epicatechin and their methylated forms in urine samples is underestimated due to limitations in sulfatase activity. The result from sulfatase activity showed that interfering substances in urine probably inhibit its activity.

To avoid this underestimation and lost the important information of natural metabolites circulating in human body, deconjugation of quercetin and (-)-epicatechin metabolites in urine sample by co-treatment with β-glucuronidase and sulfatase cannot be used but all conjugated metabolites of quercetin and (-)-epicatechin found in the urine must be analysed directly. However, to identify and quantify these metabolites, all conjugated quercetin and (-)-epicatechin found in urine must firstly be synthesised.

Chapter 5 Enzymatic Synthesis of the Main Quercetin and (-)-Epicatechin Conjugates Found in Urine

5.1 Abstract

On the basis of the results in Chapter 4, sulfatase does not sufficiently hydrolyse urinary metabolites of quercetin and (-)-epicatechin to the aglycone and methylated forms. Consequently, this will lead to lower amounts of compounds in urine, resulting in underestimation. Thus, enzyme treatment was not used in the next study but instead all conjugated metabolites of quercetin and (-)-epicatechin found in the urine were analysed. To identify and quantify all metabolites excreted in urine samples, standard compounds must first be synthesised. Thus, the main aim of this chapter was to synthesise major conjugates of quercetin and (-)-epicatechin found in urine and used as a reference for urine analysis in Chapter 6. For synthesis of quercetin conjugates, quercetin was used as a substrate to produce quercetin glucuronides and quercetin sulfates by uridine diphosphate glucuronosyl transferase (UGT) from microsomal pig liver and sulfotransferase (SULT) from cytosol pig liver. In the case of 3′-*O*-methyl-quercetin-glucuronides, they were produced by using a commercial substrate, 3′-*O*-methyl-quercetin (or isorhamnetin). In the glucuronidation reaction of quercetin-glucuronides and 3′-*O*-methyl-quercetin-glucuronides, quercetin-di-*O*-glucuronides and 3′-*O*-methyl-quercetin-di-glucuronides were also produced and detected, respectively. For synthesis of (-)-epicatechin conjugates, (-)-epicatechin was used as a substrate to produce (-)-epicatechin-glucuronides, (-)-epicatechin-sulfates and methylated-(-)-epicatechin, respectively. The same source of enzymes for quercetin glucuronidation and sulfation were used for (-)-epicatechin glucuronidation and sulfation. For methylated-(-)-epicatechin-glucuronides and methylated-(-)-epicatechin sulfates synthesis, they were produced by (-)-epicatechin-glucuronides and (-)-epicatechin sulfates which were the products of (-)-epicatechin glucuronidation and (-)-epicatechin sulfation reaction, using COMT from cytosol pig liver. In summary, five quercetin conjugates and five (-)-epicatechin conjugates, the major metabolites found in urine, were successfully synthesised using the enzymatic method in this present study with a sufficient percentage yield of synthesised conjugates. Based on the retention time and the specific mass to ion ratio of each conjugate (e.g.

98.9±0.2% for methylated-(-)-epicatechin-sulfates, 79.3±0.7% for quercetin glucuronides, 62.1±0.4 for (-)-epicatechin glucuronides), it was reasonable to conclude that all enzymatic syntheses of quercetin and (-)-epicatechin were correct and so able to be used as standard compounds for urine analysis in Chapter 6.

5.2 Introduction

To estimate the bioavailability of polyphenols in a biological system, β-glucuronidase and sulfatase enzymes are generally used to release the parent aglycone. Then, the aglycone is quantified by HPLC or LC-MS. However, on the basis of the results in Chapter 4, sulfatase does not hydrolyse sufficiently and this will lead to lower amounts of compounds, resulting in underestimation. Additionally, glucuronidase and sulfatase cause loss of vital information on natural conjugates. Numerous supportive evidences also showed that the polyphenol metabolites in the circulation system (concentration from nM to μM) have other biological effects compared to their parent compounds (Crozier et al. 2009). Thus, enzyme treatment using glucuronidase or sulfatase was not used to hydrolyse conjugated forms of quercetin or epicatechin to aglycone in urine samples in this study but instead all conjugated metabolites found in the urine were analysed. To identify and quantify all metabolites excreted in urine samples, standard compounds need to be used as a reference. Due to a lack of commercially available quercetin and (-)-epicatechin conjugates for identification and quantification, synthetic sulfates, glucuronides and methyl derivatives of quercetin or epicatechin need to be synthesised as standards to identify and quantify quercetin or epicatechin metabolites present in urine samples after the consumption of onion soup and 70% cocoa dark chocolate.

As mentioned in Chapter 1, two research groups have studied previously the quercetin and (-)-epicatechin metabolites in the circulating system. First, Mullen et al. (2006) analysed plasma and urine samples from six human volunteers after the consumption of 270 g of fried onions (containing 275 μmol quercetin 3,4′di-*O*-glucoside and quercetin 4′-*O*-glucoside). Quercetin metabolites were detected in both plasma and urine collected over a 24 hr period by using HPLC with a diode array and a tandem mass spectrometric detector. However, the metabolic profile of quercetin excreted in urine was different to the profile found in the plasma.

Quercetin-3′-*O*-glucuronide, two quercetin-glucuronide-sulfate, methyl-quercetin-diglucuronide were found in urine but were absent or present only in small amounts in the blood. Other main metabolites such as quercetin-diglucuronide, isorhamnetin-3-*O*-glucuronide, and quercetin-3-*O*-glucuronide were also found in the urine. In 2012, Actis-Goretta et al. analysed (-)-epicatechin metabolites in plasma and urine from 0 to 24 hr after the ingestion of 100 g dark chocolate (containing 79 mg or 272 µmol of (-)-epicatechin) in five volunteers. (-)-Epicatechin metabolites were detected in both the plasma and the urine (24 hr collection) using LC–MS/MS–MRM. (-)-Epicatechin metabolites, including glucuronides, sulfates, methyl sulfates, and methyl glucuronides, excreted in urine were detected and were similar to those found in the plasma. The total urinary excretion of (-)-epicatechin was 20±2% of the intake. The metabolic profiles of quercetin and (-)-epicatechin excreted in urine, arranging from the maximum to minimum amount, from Mullen et al.'s study (2006) and Actis-Goretta et al.'s study (2012) study are shown in Table 5.1 and Table 5.2, respectively.

Table 5.1: The amount of quercetin metabolites (µmol) excreted in urine from 0 to 24 hr after the ingestion of of 270 g fried onion by six volunteers.
(Adapted from Mullen et al., 2006)

Quercetin metabolites	**Total 0-24 hr (µmol)**	
	Mean	**SE**
Quercetin-diglucuronide	2.22	0.42
Quercetin-3'-*O*-glucuronide	1.85	0.19
Isorhamnetin-3-*O*-glucuronide	1.79	0.24
Quercetin-glucuronide-sulfate	1.38	0.16
Quercetin-glucuronide-sulfate	1.23	0.19
Methylquercetin-diglucuronide	1.00	0.16
Quercetin-3-*O*-glucuronide	0.91	0.15
Quercetin-glucoside-sulfate	0.82	0.16
Isorhamnetin-4'-*O*-glucuronide	0.70	0.11
Methylquercetin-diglucuronide	0.43	0.10
Quercetin-glucoside-sulfate	0.39	0.06
Quercetin-glucuronide-glucoside	0.16	0.02
Total	12.89	1.04

Table 5.2: The amount of (-)-epicatechin metabolites (μmol) excreted in urine from 0 to 24 hr after the ingestion of 100 g dark chocolate by five volunteers (Adapted from Actis-Goretta et al., 2012)

(-)-Epicatechin metabolites	**Total 0-24 hr (μmol)**	
	Mean	**SD**
3'-*O*-methyl-(-)-epicatechin-5-sulfate	14.10	3.88
(-)-Epicatechin-3'-β-D-glucuronide	13.30	3.85
(-)-Epicatechin-3'-sulfate	8.53	2.71
(-)-Epicatechin-7-β-D-glucuronide	7.27	1.35
3'-*O*-methyl-(-)-epicatechin-7-sulfate	2.33	0.68
3'-*O*-methyl-(-)-epicatechin-4'-sulfate	1.67	0.62
4'-*O*-methyl-(-)-epicatechin-5-sulfate	1.37	0.34
3'-*O*-methyl-(-)-epicatechin-β-D-glucuronide	1.20	0.29
(-)-Epicatechin-5'-sulfate	1.15	0.20
3'-*O*-methyl-(-)-epicatechin-β-D-glucuronide	1.08	0.43
(-)-Epicatechin-4'-β-D-glucuronide	1.03	0.06
4'-*O*-methyl-(-)-epicatechin-7-sulfate	0.73	0.23
3'-*O*-methyl-(-)-epicatechin-β-D-glucuronide	0.73	0.10
(-)-Epicatechin-4'-sulfate	0.56	0.13
4'-*O*-methyl-(-)-epicatechin-β-D-glucuronide	0.56	0.08
Total	55.70	4.60

Based on the information of metabolic profiles of quercetin and (-)-epicatechin from the two publications mentioned above, only the main conjugated forms of these compounds were then synthesised in this study (Table 5.3).

Table 5.3: The target quercetin and (-)-epicatechin metabolites to be synthesised in this chapter.

Metabolite	[m/z-]
Quercetin metabolites	
Quercetin-diglucuronide	653
Quercetin-glucuronide	477
Isorhamnetin-glucuronide	491
Methylquercetin-diglucuronide	667
Quercetin sulfate	381
(-)-Epicatechin metabolites	
Methyl-(-)-epicatechin-sulfate	383
(-)-Epicatechin-glucuronide	465
(-)-Epicatechin-sulfate	369
Methyl-(-)-epicatechin-glucuronide	479

Several methods have been used to synthesise flavonoid conjugates such as extracting from plants, isolating from blood or urine, synthesising using microorganisms, and chemical or enzymatic syntheses. Numerous studies have reported synthesis of quercetin or (-)-epicatechin conjugated metabolites using chemical methods but other studies have been conducted using enzymatic synthesis (Table 5.4). Donovan et al. (1999) synthesised 3′ and 4′ methylated catechin and epicatechin by chemical synthesis followed by separating the synthesised compounds using several methods. They found that HPLC with fluorescence detection and GC with mass spectrometric detection are sensitive and selective for analysing a small amount of these compounds in biological samples. Aside from this, Day et al. (2001) synthesized quercetin 3′-*O*-sulfate specifically using chemical reactions between quercetin and sulfamic acid to determine the exact nature of the plasma metabolites. Quercetin 3′-*O*-sulfate was synthesised again by Jones et al. (2005) but using a different chemical, sulfur trioxide-N-triethylamine instead of sulphamic acid. In 2006, Needs and Kroon also synthesised the human plasma metabolites of quercetin using 4′,7-di-*O*-benzylquercetin as a precursor and claimed that this method was more convenient than the previous enzymatic method (Boersma et al., 2002; O'Leary et al., 2003). In 2012, quercetin-3′-*O*-sulfate,

quercetin-4′-*O*-sulfate and (-)-epicatechin-4′-*O*-sulfate were scalable (hemi) synthesised and characterised by Duenas et al. (2012). Four biological (-)-epicatechin conjugates; 3′-*O*-glucuronide, 4′-*O*-glucuronide, 3′-*O*-sulfonate and 3′-*O*-methyl-4′-*O*-sulfonate, relating to the B-ring of (-)-epicatechin were chemically synthesised by Romanov-Michailidis et al. (2012) and then used as analytical-grade standards for (-)-epicatechin analysis in a human study. A year later, monoglucuronide and monosulfates of (-)-epicatechin, 3′-*O*-methyl and 4′-*O*-methyl-(-)-epicatechin were also chemically synthesised by Zhang et al., (2013) but using an orthogonally protected (-)-epicatechin intermediate which is a crucial compound in the synthetic step. At present, most studies tend to focus on the chemical synthetic method probably due to the potential for producing a higher yield of conjugated compounds. For the enzymatic synthesis method, the first report was produced by Shali et al. , 1991. They synthesised quercetin and catechin sulfate using sulfotransferases from rat liver. In addition, mixed quercetin glucuronides (quercetin-di-glucuronides, quercetin-3-*O*-glucuronide, quercetin-7-*O*-glucuronide, quercetin-4′-*O*-glucuronide and quercetin-3′-*O*-glucuronide) were produced enzymatically from pig liver by O'Leary et al. (2001). Then, Boersma et al. (2002) investigated the regioselectivity of UGT by incubating luteolin and quercetin with different sources of microsomal samples (liver or intestine from a rat or a human), resulting in different forms of luteolin and quercetin glucuronides. In 2008, Duenas et al., using a modified method described by Plumb et al. (2003), produced quercetin and isorhamnetin glucuronides enzymatically using microsomal enzyme from pig liver and also isolated quercetin-3-*O*-glucuronide (Q3GA) from green beans. Four peaks of quercetin glucuronides (quercetin-di-glucuronides, quercetin-3-*O*-glucuronide, quercetin-4′-*O*-glucuronide and quercetin-3′-*O*-glucuronide) were identified after a 2 hr incubation period using microsomal pig liver. In 2012, Blount et al. investigated the potential of recombinant human glucuronosyl transferases of the UGT1A and UGT2B families to glucuronidate epicatechin or 3′-*O*-methyl epicatechin *in vitro* and found that UGT1A9 was the most effective to produce epicatechin 3'-*O*-glucuronide. 3'-*O*-methyl-epicatechin 5-*O*-glucuronide was also produced when incubating UGT1A9 with 3'-*O*-methyl-epicatechin. Although the enzymes and cofactor concentrations in the enzymatic synthesis reaction were probably a limiting factor for generating metabolites using the enzymatic method, however, using animal tissues as sources of enzymes produced a structure of

metabolites that were closer to the biological system than synthesis using chemical reactions.

The main aim of this chapter, therefore, was to synthesise conjugated metabolites of quercetin and epicatechin, specifically quercetin sulfate, quercetin glucuronide, quercetin-di-glucuronide, 3′ methyl quercetin glucuronide, methyl quercetin di-glucuronide, epicatechin glucuronide, methyl epicatechin glucuronide, methyl epicatechin, epicatechin sulfate and methyl epicatechin sulfate by enzymatic synthesis. To achieve the aim of this study, glucuronides, sulfates, sulfates and glucuronides were conjugated with methyls of quercetin or epicatechin which were synthesised using enzyme from pig liver. Then, dried conjugated compounds were re-dissolved in milliQ water, baseline and 24 hr urine before analysing with UPLC-MS. The percentage yield of each enzymatic synthesised compound was finally calculated. The relative relation time and m/z- of all synthesised compounds will be used as a reference for quantification of quercetin and epicatechin metabolites present in urine samples in Chapter 6.

Table 5.4: Chemical and enzymatic synthesis of conjugated quercetin and (-)-epicatechin found in several publications

Method	Conjugated compounds	Publications
Chemical synthesis	3′ and 4′-*O*- methyl catechin 3′ and 4′-*O*- methyl-(-)-epicatechin	Donovan et al. (1999)
	Quercetin 3′-*O*-sulfate	Day et al. (2001)
	Quercetin 3′-*O*-sulfate	Jones et al. (2005)
	Quercetin 3′-*O*-sulfate Isorhamnetin 3-glucuronide Quercetin 3-*O*-glucuronide Quercetin 3′-*O*-glucuronide	Needs and Kroon (2006)
	Quercetin-3′-*O*-sulfate Quercetin-4′-*O*-sulfate Epicatechin-4′-*O*-sulfate	Duenas et al. (2012)
	3′-*O*-glucuronide-(-)-epicatechin 4′-*O*-glucuronide-(-)-epicatechin 3′-*O*-sulfonate-(-)-epicatechin 3′-*O*-methyl-4′-*O*-sulfonate-(-)-epicatechin	Romanov-Michailidis et al. (2012)
	Glucuronide- (-)-epicatechin Sulfate-(-)-epicatechin 3′and 4′-*O*-methyl-(-)-epicatechin	Zhang et al. (2013)

Table 5.4 continued: Chemical and enzymatic synthesis of conjugated quercetin and (-)-epicatechin found in several publications.

Enzymatic synthesis	Quercetin sulfate Catechin sulfate	Shali et al. (1991)
	Quercetin-di-*O*-glucuronide Quercetin-3-*O*-glucuronide Quercetin-7-*O*-glucuronide Quercetin-3′-*O*-glucuronideQuercetin-4′-*O*-glucuronide	O'Leary et al. (2001)
	Quercetin-3-*O*-glucuronide Quercetin-7-*O*-glucuronide Quercetin-3′-*O*-glucuronideQuercetin-4′-*O*-glucuronide	Boersma et al. (2002)
	Quercetin di-*O*-glucuronide Quercetin-3-*O*-glucuronide Quercetin-4′-*O*-glucuronide Quercetin-3′-*O*-glucuronide	Duenas et al. (2008)
	(-)-Epicatechin 3′-*O*-glucuronide 3′-*O*-methyl-(-)-epicatechin 5-*O*-glucuronide	Blount et al. (2012)

5.3 Materials and Methods

See section 2.4 for enzymatic enzymatic synthesis of the main quercetin and (-)-epicatechin conjugates found in urine.

5.4 Results

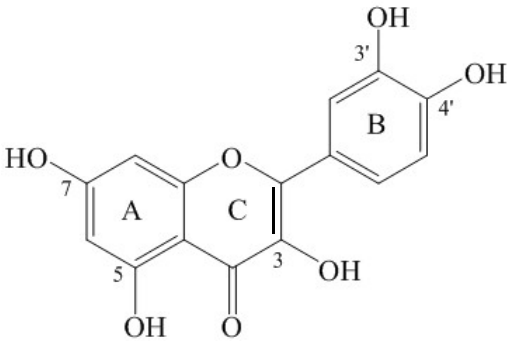
To identify and quantify all metabolites excreted in urine samples, standard conjugated quercetins or (-)-epicatechins need to be synthesised and used as a reference.

5.4.1 Quercetin conjugates synthesis

Glucuronidation of flavonoids often occurs at the -OH group attacted to the aromatic ring. In the case of quercetin, there are five hydroxyl groups in its structure at position 3, 5, 7, 3′ and 4′ (Figure 5.1: A.), and then glucoronic acid, sulfate or

methyl can be added to these positions, resulting in five possible different forms of quercetin conjugates.

A.



B.

UGT **UGT**
Q → Q-G → Q-di-G

SULT
Q → Q-S

UGT **UGT**
3′MeQ → 3′MeQ-G → 3’MeQ-di-G

Figure 5.1: A. Five possible positions for uridine diphosphate glucuronosyl transferase (UGT), sulfotransferase (SULT) and two possible positions for catechol-*O*-methyl transferase (COMT) to conjugate glucuronic acid, sulfate and methyl group to the structure of quercetin. B. Summary of the synthesised step of each conjugated quercetin.

To synthesise quercetin-glucuronides, quercetin was used as a substrate and converted to quercetin-glucuronides by UGT from microsomal pig liver to work alongside alamethicin and UDPGA to act as a UGT activator and glucuronic donor, respectively. Saccharolactone, a β-glucuronidase inhibitor, was also added into the reaction mixture and then the glucuronidated products were still maintained. The glucuronidation reaction of quercetin was prepared and performed under the conditions described in section 2.4.1. In this reaction, glucuronic acid can be further conjugated by UGT using quercetin-glucuronide as a substrate. So, quercetin-di-*O*-glucuronide also can be produced and was detected in this sample.

For quercetin-sulfates, quercetin also was used as a substrate but SULT from cytosol pig liver played an important role by adding a sulfate group to quercetin instead of UGT. SULT will transform the sulfuryl group from the universal sulfuryl group donor, adenosine-3′-phosphate-5′-phosphosulfate (PAPS) to the hydroxyl group of quercetin, resulting in quercetin sulfates. Sulfation of quercetin was described in section 2.4.2 Quercetin glucuronide sulfate, which is another main metabolite excreted in urine (Table 5.1), was not able to be synthesised from already

synthesised quercetin glucuronide and SULT. This was possibly due to conjugation of quercetin glucuronide compound with the sulfate group; one more step may cause instability of the quercetin glucuronide at some point during the sulfation synthesis process.

For 3′-*O*-methylated quercetin glucuronide, a commercial compound, 3′-*O*-methylated-quercetin (isorhamnetin) was used as a substrate. Again, UGT will add glucoronic acid to the substrate to produce 3′-*O*- methylated-quercetin-glucuronide and also further produce 3′-*O*-methylated-quercetin-diglucuronide in the same reaction. The summary of synthesised steps of each conjugated quercetin is shown in Figure 5.1B.

The products of each enzymatic synthesis were subsequently dried under centrifugal evaporation and reconstituted with 100 µl of MilliQ water, baseline urine and 24 hr urine and further analysed on a UPLC-MS based on the relative retention time and the ratio of mass to ion. In addition, control samples, glucuronidation or sulfation of quercetin without substrate or enzyme from pig liver, was also prepared by reconstituting in MilliQ water and analysing on UPLC-MS at the same time. The scheme of quercetin glucuronidation and sulfation are shown in Figures 5.2 and 5.4 respectively. Figure 5.2 shows a chromatogram of quercetin glucuronide after enzymatic synthesis in TIC mode (A) and specific m/z- at 477 (B). For TIC or the total ion scan mode (Figure 5.2A), ethyl gallate (EG, Retention time = 14.1) and taxifolin (T, Retention time = 18.7) were internal standards and added to every sample. Ethyl gallate and taxifolin can be detected in all samples in this total ion scan mode. However, as expected no peak of ethyl gallate and taxifolin were detected in the ionization mode at m/z- at 477 which was specific for detecting quercetin glucuronides. In Figures 5.2A and 5.2B, (a) and (b) are chromatograms of control samples, glucuronidated quercetin without substrate and enzymes from pig liver microsomes, respectively. Quercetin was not added to the reaction mixture (a), thus no glucuronidated quercetin was detected in this sample. For (b), microsomal pig liver suspension was absent and so quercetin as substrate was still found in this sample. In Figure 5.2A (c), (d) and (e) some quercetin glucuronides were produced, and after re-dissolving in MilliQ water, baseline or 24 hr urine, were observed in the total ion scan mode. These data were confirmed in the specific ion mode in Figure 5.2B (c), (d), (e) and it was found that five different forms of quercetin glucuronides

were produced and eluted at different retention times. These were 19.0 min for quercetin glucuronide 1, 20.1 min for quercetin glucuronide 2, 21.1 for quercetin glucuronide 3, 25.1 min for glucuronide 4 and 27.3 min for quercetin glucuronide 5. To check whether there were any other matrixes in the baseline and 24 hr urine affecting the properties of the synthesized compounds, dried samples were re-dissolved in baseline and 24 hr urine samples. The results showed that the peak height and the peak area of quercetin glucuronides re-dissolved in baseline (d) and 24 hr urine (e) were not different to those dissolved in milliQ water (c). Thus, the matrix of baseline urine did not affect the chromatographic properties of enzymatically synthesized quercetin glucuronides. In addition, to investigate how much quercetin glucuronide was found in 24 hr urine after a single consumption of onion soup, synthesised quercetin glucuronides were spiked in 100 µl of the 24 hr urine sample of some volunteers. The result from chromatogram in Figure 5.2B (e), however, revealed that the peak height and the peak area of this sample seemed the same as synthesized quercetin glucuronides dissolved in baseline urine (Figure 5.2B (d)). Furthermore, 100 µl of non-spiked baseline and 24 hr urine sample after onion soup intake also were analysed at the same time as spiked samples. However, no peaks of any synthesized standards were detected in this non-spiked urine sample (chromatogram not shown). It is possible, due to a low volume of 24 hr urine samples being used in this experiment, which this resulted in a low amount of quercetin metabolites in the urine sample and therefore undetectable.

In the quercetin glucuronidation reaction, quercetin-di-*O*-glucuronides were also produced and detected at m/z- at 653. The major two products, quercetin di-glucuronide 1 and quercetin di-glucuronide 2, were observed and separated at 15.7 min and 19.8 min, respectively (Figure 5.3; c, d and e).

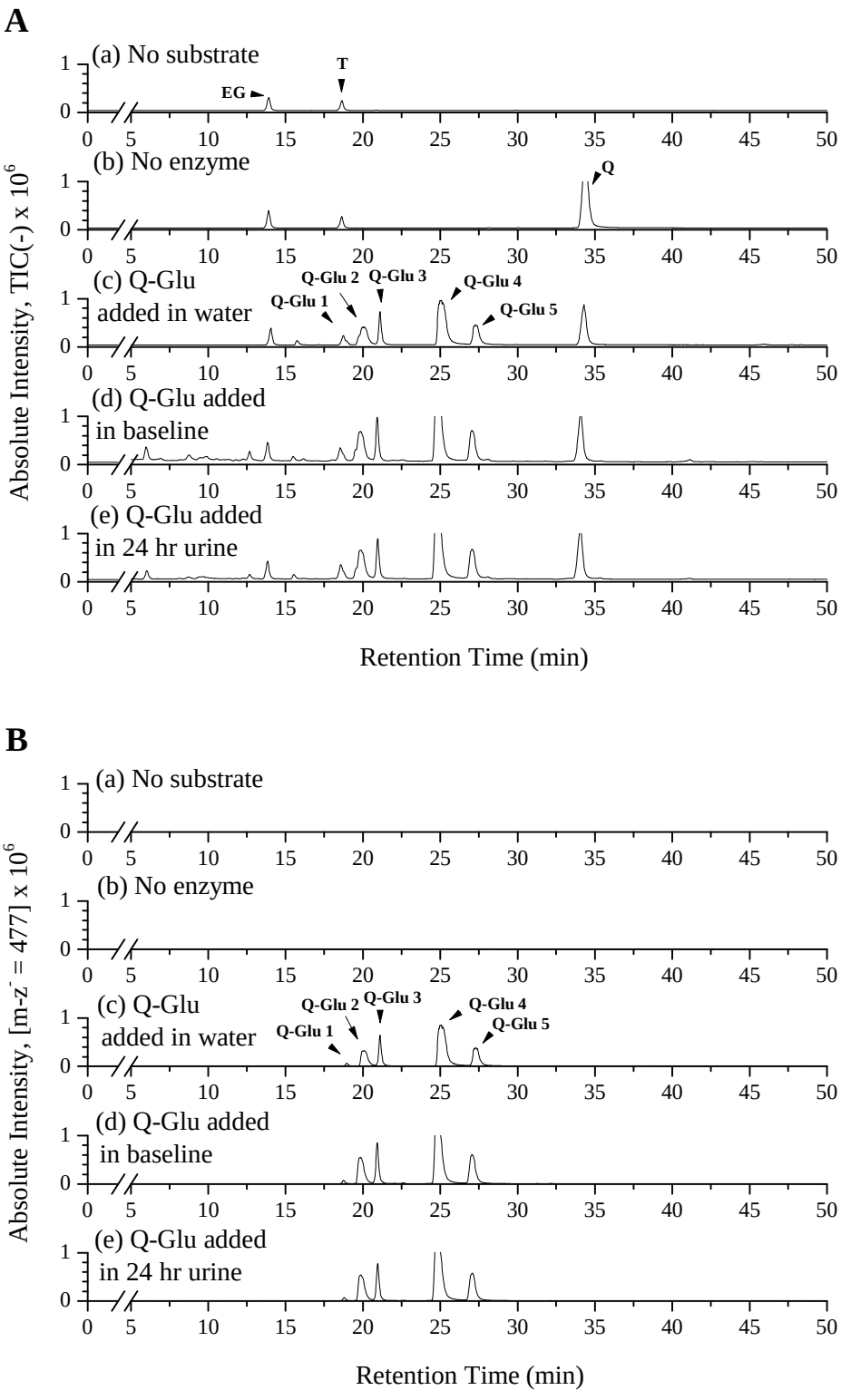


Figure 5.2: Chromatogram of quercetin-glucuronides after enzymatic synthesis in TIC mode (A) and specific m/z- at 477 (B). (a) and (b) are control. (c), (d) and (e) are quercetin-glucuronides, re-dissolved in water, baseline urine and 24 hr urine, respectively.

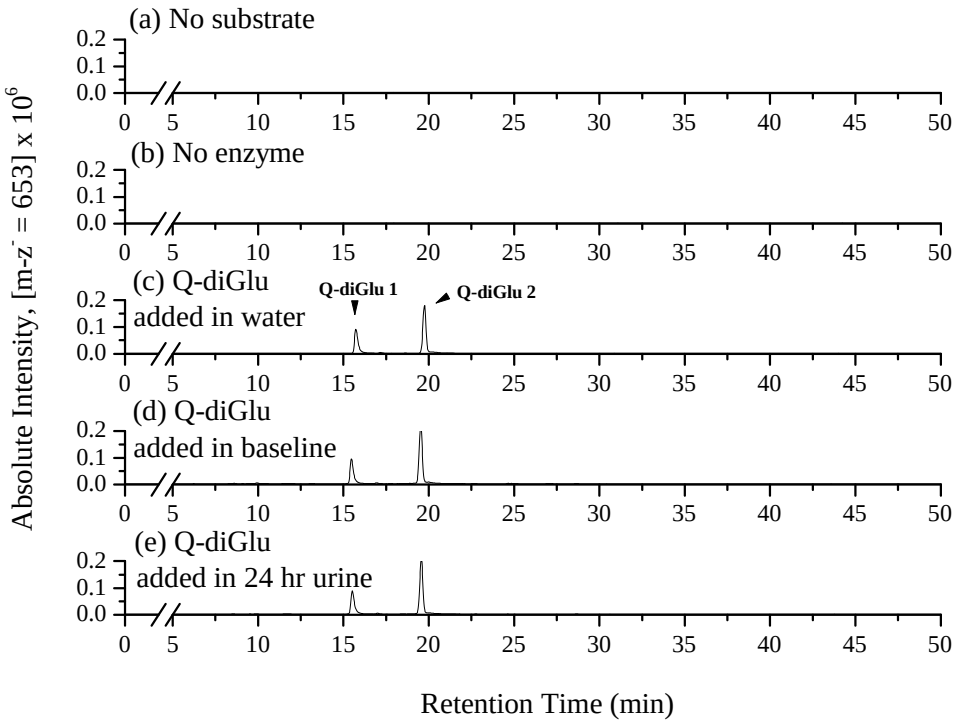


Figure 5.3: Chromatogram of quercetin-di-glucuronides after enzymatic synthesis in specific m/z- at 653. (a) and (b) are control. (c), (d) and (e) are quercetin-di-glucuronides, re-dissolved in water, baseline urine and 24 hr urine, respectively.

The same process was applied to other enzymatically conjugated quercetin derivatives. For quercetin sulfates, only two sulfated quercetin derivatives were produced and observed in the sulfation reaction. They were eluted at 27.5 min and 34 min, representing sulfated quercetin 1 and sulfated quercetin 2 (Figure 5.4B; c, d and e). For 3′-*O*-methyl quercetin glucuronide, a commercial substrate, 3′-*O*-methyl-quercetin (or isorhamnetin) was converted into three different 3′-*O*-methyl quercetin glucuronides. These were separated and eluted at 24.6, 25.7 and 28 min, respectively (Figure 5.5B; c, d and e). In addition, some *O*-methylated-quercetin-di-*O*-glucuronides were also produced and detected at m/z- at 667. They eluted at 17.7 min for *O*-methylated-quercetin-di-*O*-glucuronide 1 and 18.9 for *O*-methylated-quercetin-di-*O*-glucuronide 2 (Figure 5.6; c, d and e).

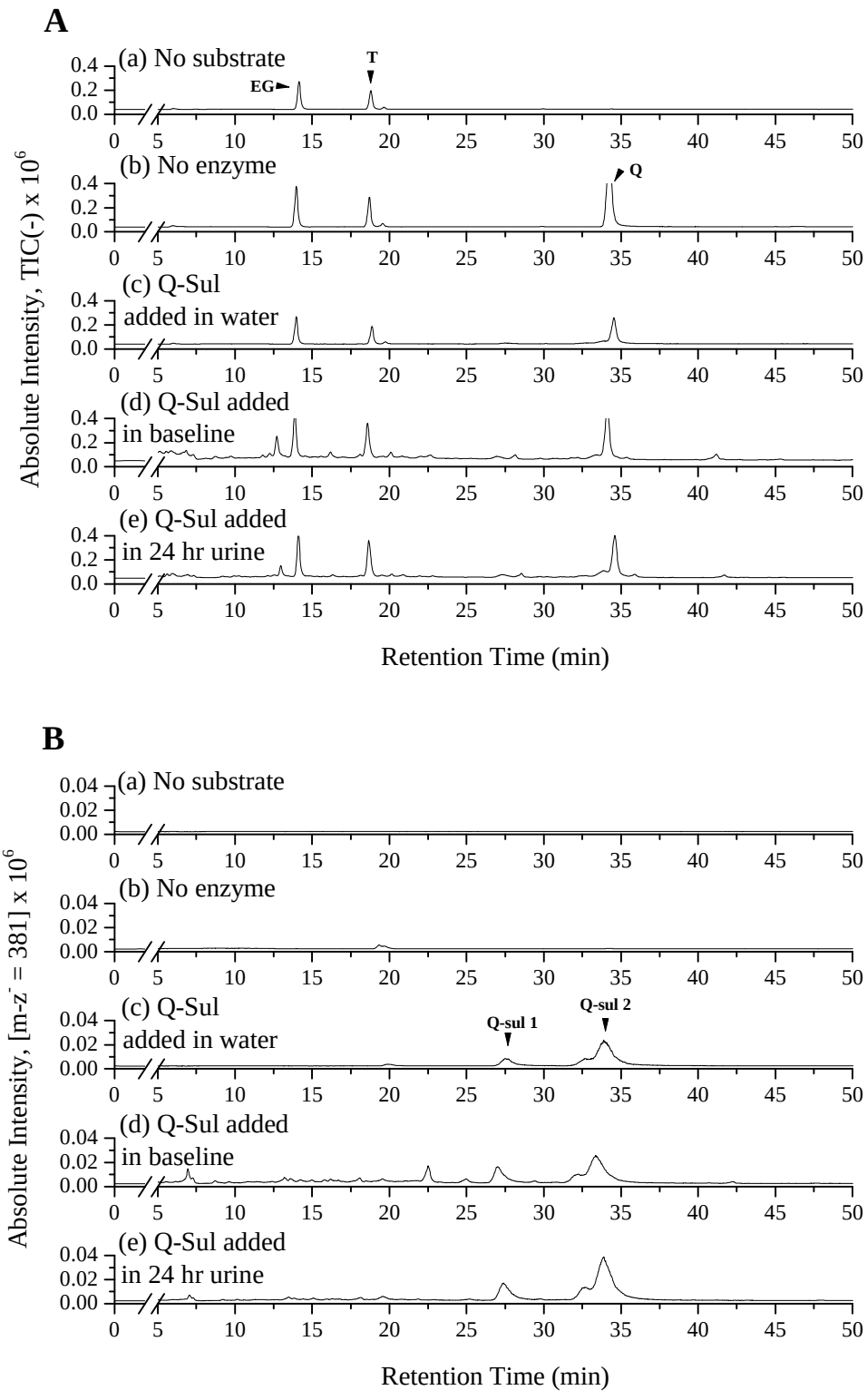


Figure 5.4: Chromatogram of quercetin-sulfates after enzymatic synthesis in TIC mode (A) and specific m/z- at 381 (B). (a) and (b) are control. (c), (d) and (e) are quercetin-sulfates, re-dissolved in water, baseline urine and 24 hr urine, respectively.

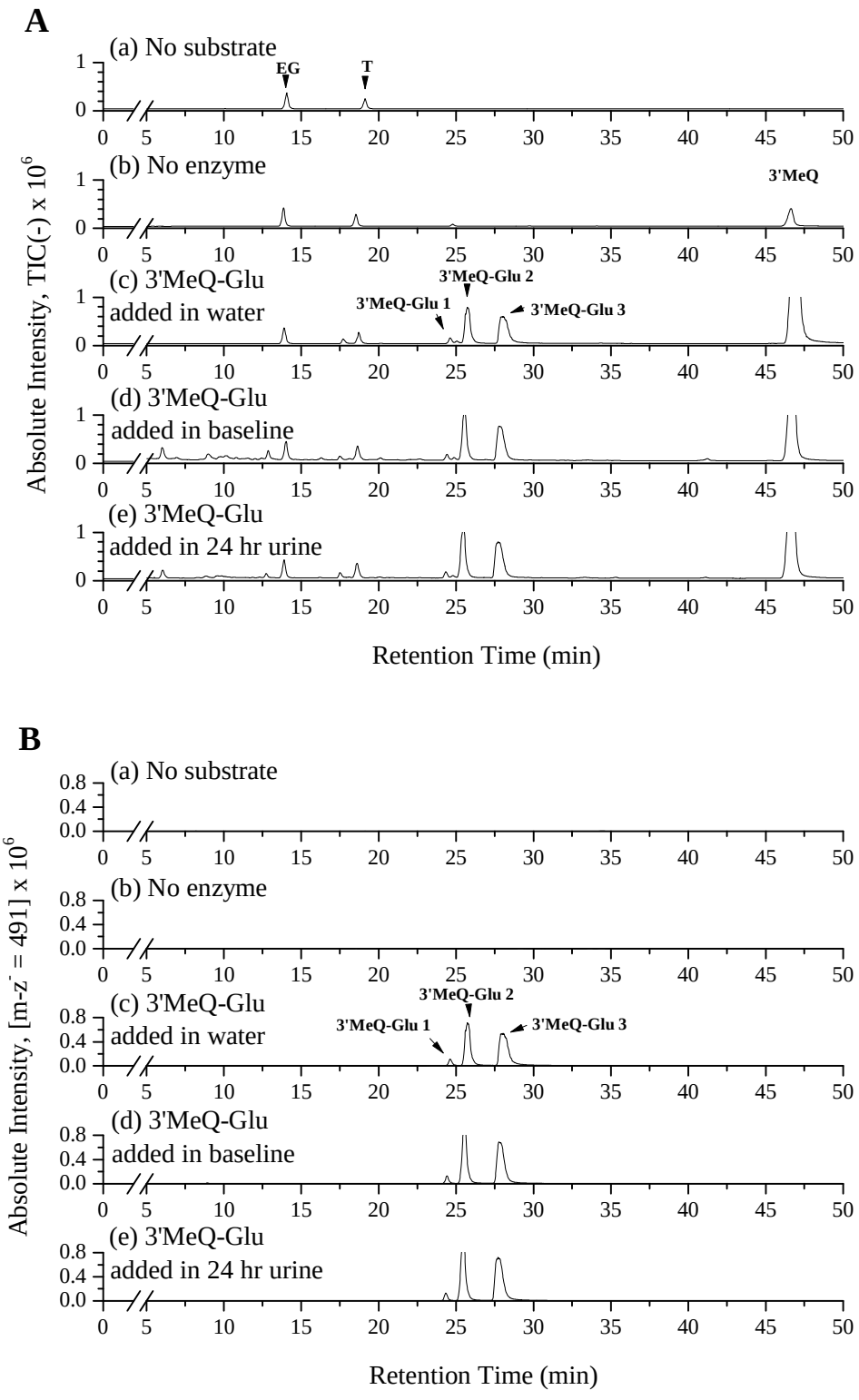


Figure 5.5: Chromatogram of 3′-methyl-quercetin-glucuronides after enzymatic synthesis in TIC mode (A) and specific m/z- at 491 (B). (a) and (b) are control. (c), (d) and (e) are 3′-methyl-quercetin-glucuronides, re-dissolved in water, baseline urine and 24 hr urine, respectively.

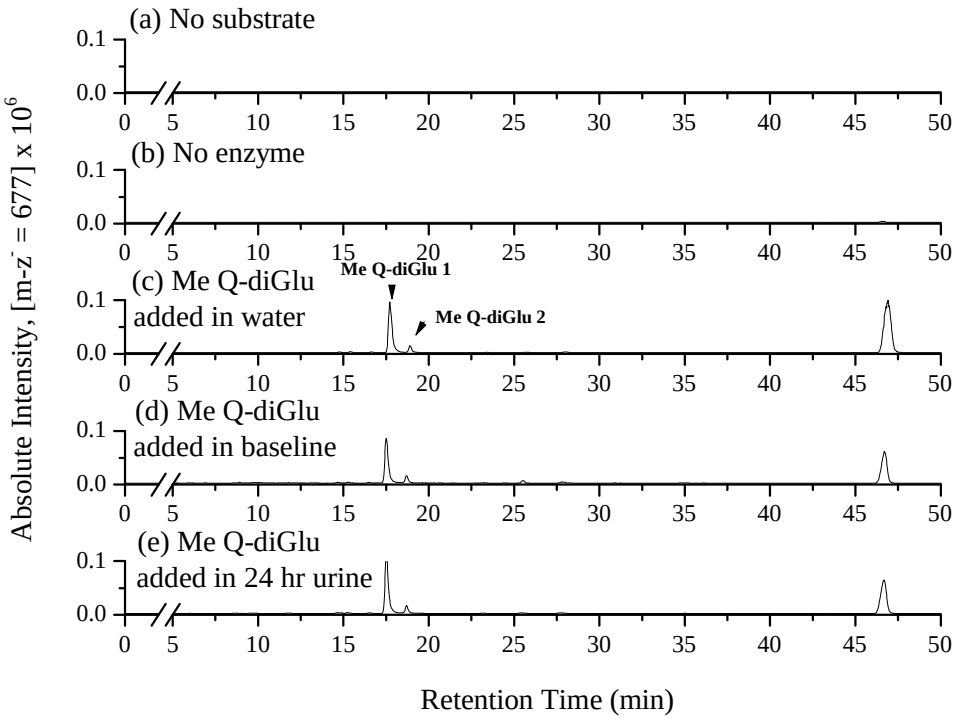


Figure 5.6: Chromatogram of methyl-quercetin-di-glucuronides after enzymatic synthesis in specific m/z- at 667. (a) and (b) are control. (c), (d) and (e) are methyl-quercetin-di-glucuronides, re-dissolved in water, baseline urine and 24 hr urine, respectively.

The chromatogram of all enzymatic quercetin metabolites dissolved in milliQ water and detected in specific m/z- of each conjugate are shown in Figure 5.7. This data accompanied by the retention time of each conjugate form of quercetin in Table 5.5 confirmed that all synthetic conjugates of quercetin were correct, glucuronided forms were separated first followed by sulfated and methylated forms.

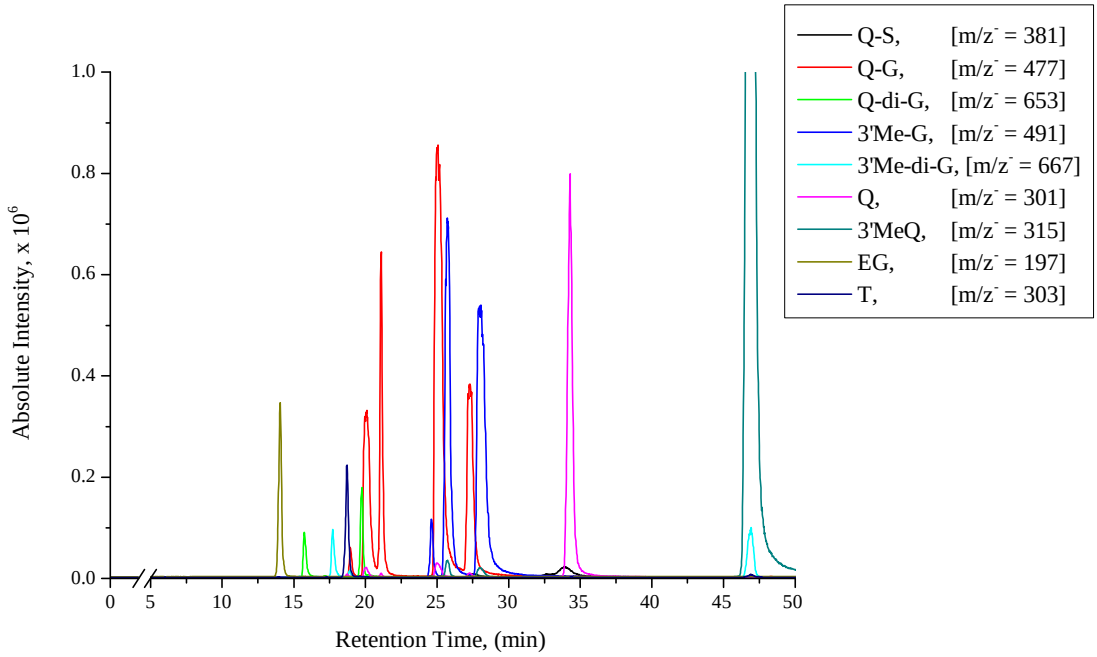


Figure 5.7: Chromatogram of all enzymatic quercetin metabolites dissolved in milliQ water and detected in specific m/z- of each conjugate. (EG and T act as internal standard). Glucuronidated forms were eluted first followed by sulfated and methylated forms.

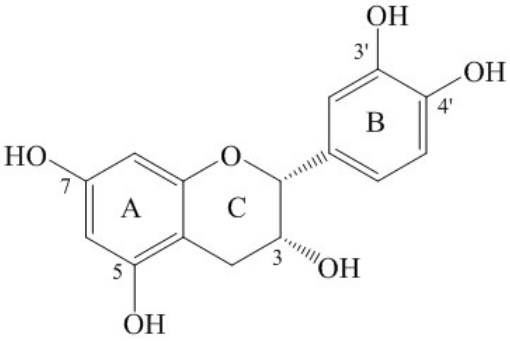
Table 5.5: Retention time of each conjugated form of enzymatic synthesised quercetin and internal standards (EG: Ethyl gallate and T: Taxifolin), detected at specific [m/z-].

Compound	[m/z-]	Peak No	Retention time, (min)
EG	197	1	14.1
Q-di-G	653	1	15.7
		2	19.8
3'-*O*-Me-di-G	667	1	17.7
		2	18.9
T	303	1	18.7
Q-G	477	1	19.0
		2	20.1
		3	21.1
		4	25.1
		5	27.3
Q-S	381	1	27.5
		2	34.0
3'-*O*-Me-G	491	1	24.6
		2	25.7
		3	28.0
Q	301	1	34.3
3'-*O*-MeQ	315	1	46.9

5.4.2 (-)-Epicatechin conjugate synthesis

In the case of (-)-epicatechin, there are again five hydroxyl groups in the structure (Figure 5.8A) similar to quercetin so that five possible forms of glucuronided, sulfated or methylated quercetin or (-)-epicatechin could theoretically be generated.

A.



B.

UGT **COMT**
EC → EC-G → Me-EC-G

SULT **COMT**
EC → EC-S → Me-EC-S

COMT
EC → Me-EC

Figure 5.8: A. Five possible positions for uridine diphosphate glucuronosyl transferase (UGT), sulfotransferase (SULT) and two possible positions for catechol-*O*-methyl transferase (COMT) to conjugate glucuronic acid, sulfate and methyl group to the structure of (-)-epicatechin. B. Summary of synthesised step of each conjugated (-)-epicatechin.

Glucuronidation and sulfation of (-)-epicatechin were prepared under the same conditions as described for quercetin glucuronides and quercetin sulfates which were in sections 2.4.1 and 2.4.2, respectively. In the glucuronidation reaction, (-)-epicatechin was converted to (-)-epicatechin glucuronides by UGT enzymes in microsomal pig liver while in the sulfation reaction, (-)-epicatechin was converted to (-)-epicatechin sulfates by SULT from cytosol pig liver. After drying, (-)-epicatechin glucuronides and (-)-epicatechin sulfates were then used as substrate for COMT in methylation reactions catalysed by cytosol from pig liver. Methylated (-)-epicatechin also was synthesised using the same procedure and used as control. The summary of synthesised steps of each conjugated (-)-epicatechin is shown in Figure 5.8B.

Again, the products of all enzymatic synthesis conjugates were subsequently dried under centrifugal evaporation and reconstituted with 100 µl of MilliQ water,

baseline urine or 24 hr urine and further analysed on a UPLC-MS based on the relative retention time and the ratio of mass to ion. In addition, control samples, glucuronidation or sulfation of quercetin without substrate or enzyme from pig liver, were also prepared by reconstituting in MilliQ water and analysing by UPLC-MS.

Then, glucuronidated (-)-epicatechins were analysed by UPLC-MS using a two detector mode; total ion count (TIC) and specific ionization mode (SIM) at m/z^- = 465 for epicatechin glucuronide detection. The chromatogram of (-)-epicatechin glucuronides after enzymatic synthesis is displayed in Figure 5.9. For the total ion mode (Figure 5.9A), ethyl gallate (EG, retention time = 14.5) and taxifolin (T, retention time = 19.2) were internal standards and added to every sample. Ethyl gallate and taxifolin was detected in all samples in this total ion scan mode but, as expected, no peak of ethyl gallate and taxifolin in specific ionization mode at m/z- at 465 was observed since this was specific for detecting (-)-epicatechin glucuronides.

In Figure 5.9A and 5.9B, (a) and (b) are chromatograms of control samples, glucuronidation (-)-epicatechin without (-)-epicatechin and UGT from microsomal pig liver, respectively. Substrate was not added to the glucuronidation reaction (a) then no glucuronidated (-)-epicatechins were produced and detected in both TIC and specific mode. In addition, no enzyme added to the glucuronidation reaction of (-)-epicatechin (b) thus no (-)-epicatechin glucuronides were produced.

Figure 5.9A, (c) and Figure 5.9B, (c) synthesized epicatechin glucuronides, re-dissolved in milliQ water, and are shown using different detector modes; TIC and specific m/z- at 465. The specific ionization mode confirms that (-)-epicatechin glucuronides were produced in four different forms eluting at retention time 10.2 min for (-)-epicatechin glucuronide 1, 10.5 min for (-)-epicatechin glucuronide 2, 11.0 min for (-)-epicatechin glucuronide 3 and 12.7 min for (-)-epicatechin glucuronide 4, respectively. Again, dried conjugated compounds were also dissolved in baseline and 24 hr urine. Similar results as obtained for the quercetin glucuronidation were observed. The peak height and the peak area of (-)-epicatechin glucuronides re-dissolved in baseline (d) and 24 hr urine (e) were not different to those dissolved in milliQ water (c). Therefore, the matrixes in baseline urine did not affect enzymatically synthesized quercetin glucuronides. Moreover, the results from the chromatogram in Figure 5.9B (e) showed that the peak height and peak area of this sample was no different to the synthesized peak height and the peak area of (-)-epicatechin glucuronides dissolved in baseline urine (Figure 5.9B (d)). In addition,

100 µl of non-spiked baseline and 24 hr urine samples after a single consumption of 70% dark chocolate were also prepared and analysed as the same time as the spiked baseline and 24 hr urine samples. However, no peaks of synthesized conjugates were detected in the 24 hr urine sample. This is likely to be due to the same reason as explained previously. A low volume of 24 hr urine samples was used in this experiment, resulting in a small amount of (-)-epicatechin metabolites contained in urine making it undetectable.

Next, synthesized (-)-epicatechin-glucuronides were used as a substrate for synthesizing methyl-(-)-epicatechin-glucuronides using an enzyme preparation described in section 2.3.1. In this case, S-adenosyl-L-methionine was used as a methyl donor. Dried conjugated compounds were prepared and analysed in the same way as mentioned previously. In Figure 5.10, the chromatogram (c), (d), (e) indicates that the methyl group from S-adenosyl-L-methionine added to 4 forms of synthesized (-)-epicatechin-glucuronides, resulted in four methyl-(-)-epicatechin-glucuronide products, separating at different retention times (12.8 min, 13.9 min, 14.8 min and 15.7 min, respectively). In addition, methyl-(-)-epicatechin also synthesized using the same reaction as the methyl-(-)-epicatechin-glucuronide synthesis. From the chromatogram in Figure 5.11 (c), (d), (e), only one form of methylated (-)-epicatechin was produced at a retention time of 17.4 min. Taxifolin which has m/z^- at 303, is the same as methylated epicatechin, was also detected.

(-)-Epicatechin-sulfate was synthesised using (-)-epicatechin as a substrate. From the chromatogram in Figure 5.12 (c), (d), (e), three forms of (-)-epicatechin-sulfate were produced at retention times of 10.8 min, 13.9 min and 14.8 min, respectively. Again, methyl-(-)-epicatechin-sulfate was synthesised using (-)-epicatechin-sulfate as a substrate. Three methyl-(-)-epicatechin-sulfates were produced, eluting at 13.6 min for methyl-(-)-epicatechin-sulfate 1, 15.5 min for methyl-(-)-epicatechin-sulfate 2 and 17.5 min for methyl-(-)-epicatechin-sulfate 3 (Figure 5.13; c, d and e).

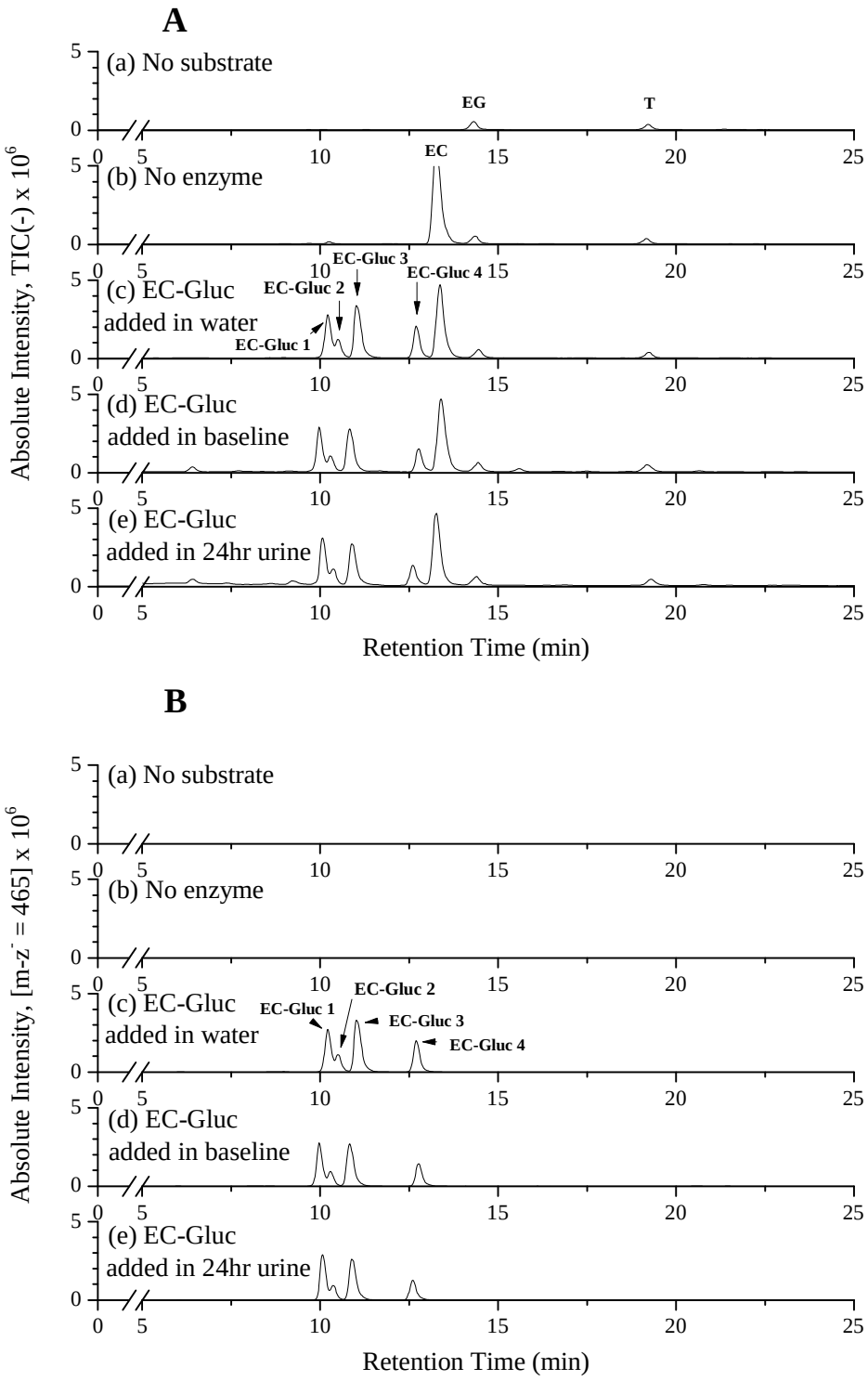


Figure 5.9: Chromatogram of (-)-epicatechin-glucuronides after enzymatic synthesis in TIC mode (A) and specific m/z- at 465 (B). (a) and (b) are control. (c), (d) and (e) are (-)-epicatechin-glucuronides, re-dissolved in water, baseline urine and 24 hr urine, respectively.

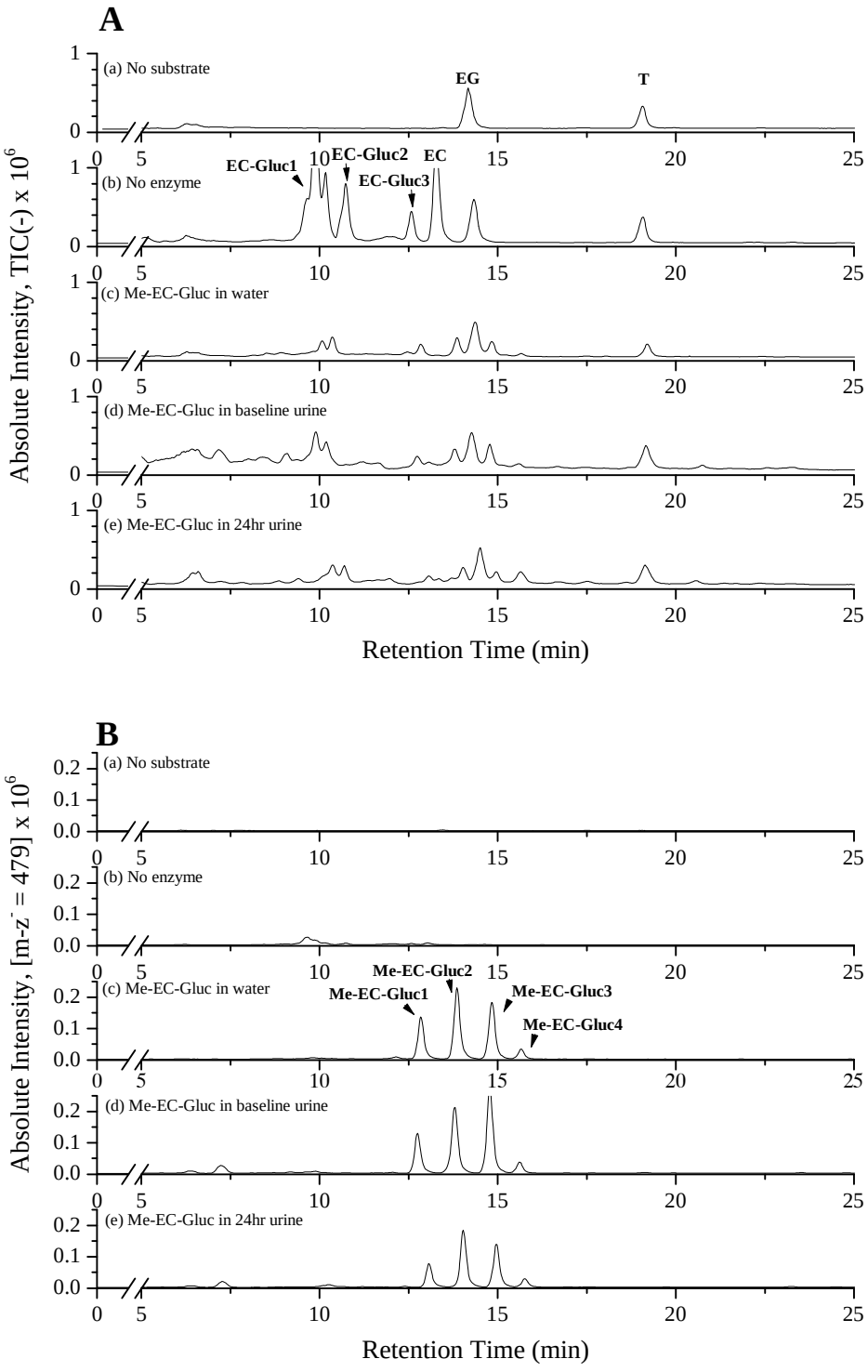


Figure 5.10: Chromatogram of methyl-(-)-epicatechin-glucuronides after enzymatic synthesis in TIC mode (A) and specific m/z- at 479 (B). (a) and (b) are control. (c), (d) and (e) are methyl-(-)-epicatechin-glucuronides, re-dissolved in water, baseline urine and 24 hr urine, respectively.

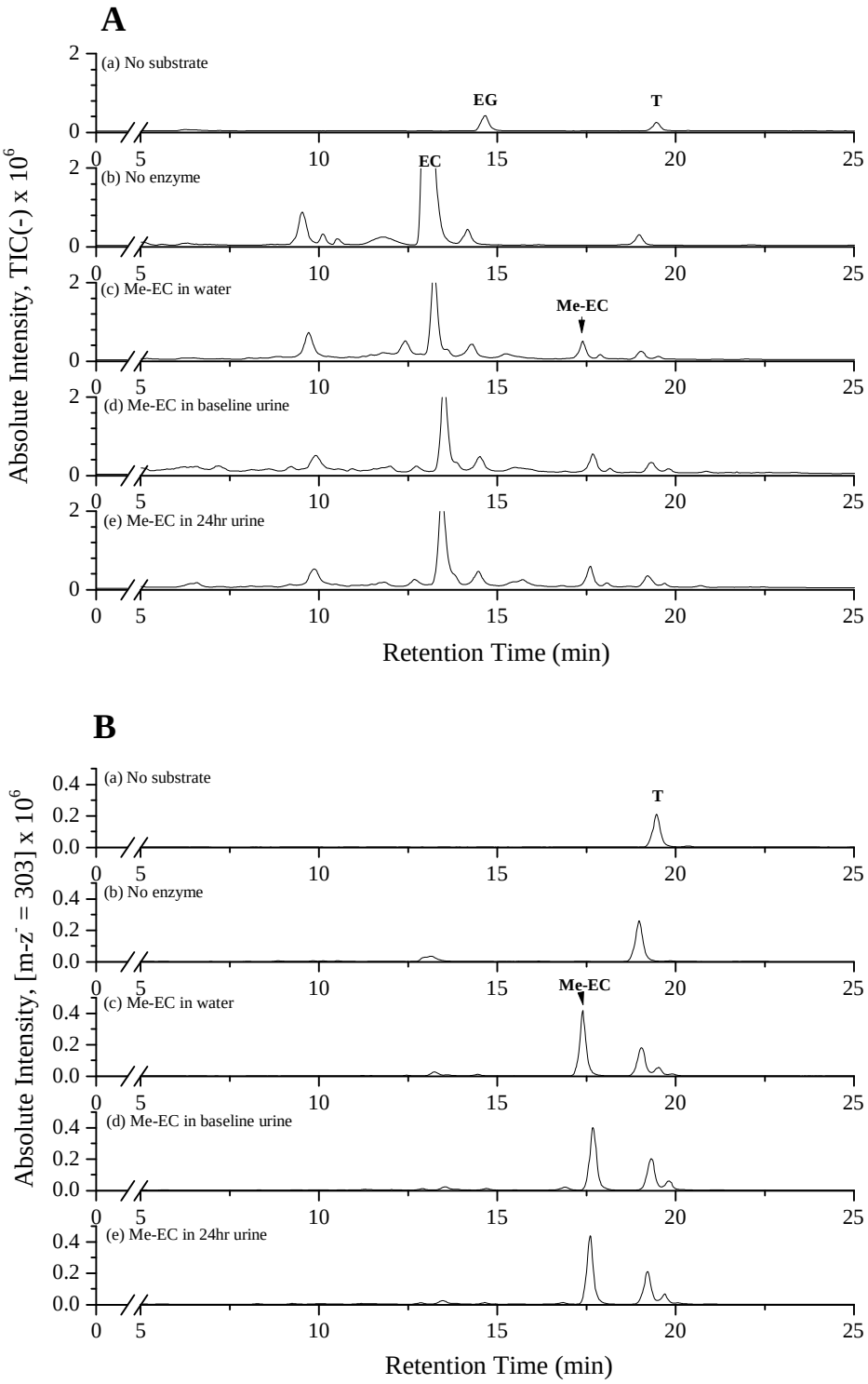


Figure 5.11: Chromatogram of methyl-(-)-epicatechin after enzymatic synthesis in TIC mode (A) and specific m/z- at 303 (B). (a) and (b) are control. (c), (d) and (e) are methyl-(-)-epicatechin, re-dissolved in water, baseline urine and 24 hr urine, respectively.

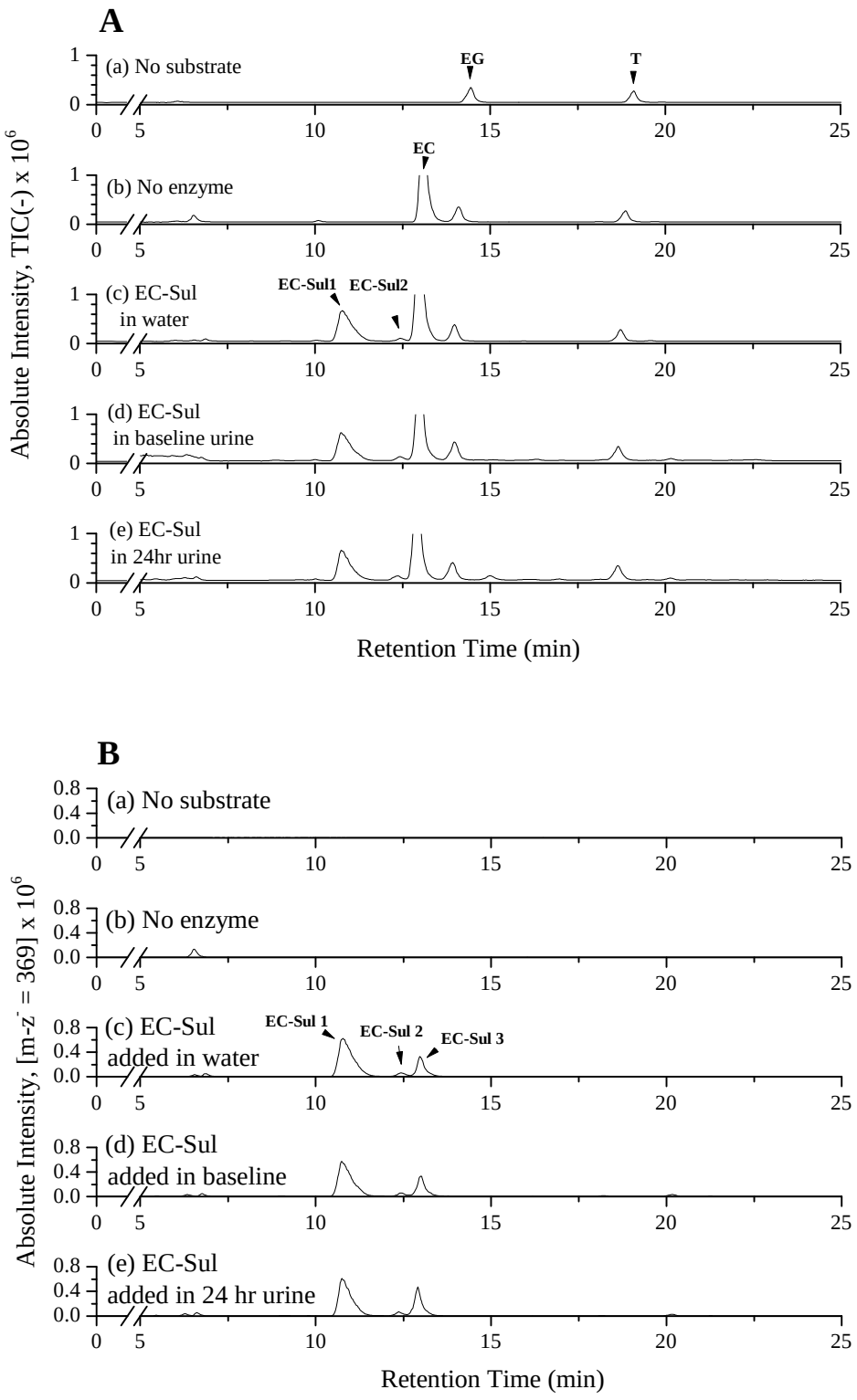


Figure 5.12: Chromatogram of (-)-epicatechin-sulfates after enzymatic synthesis in TIC mode (A) and specific m/z- at 369 (B). (a) and (b) are control. (c), (d) and (e) are (-)-epicatechin-sulfates, re-dissolved in water, baseline urine and 24 hr urine, respectively.

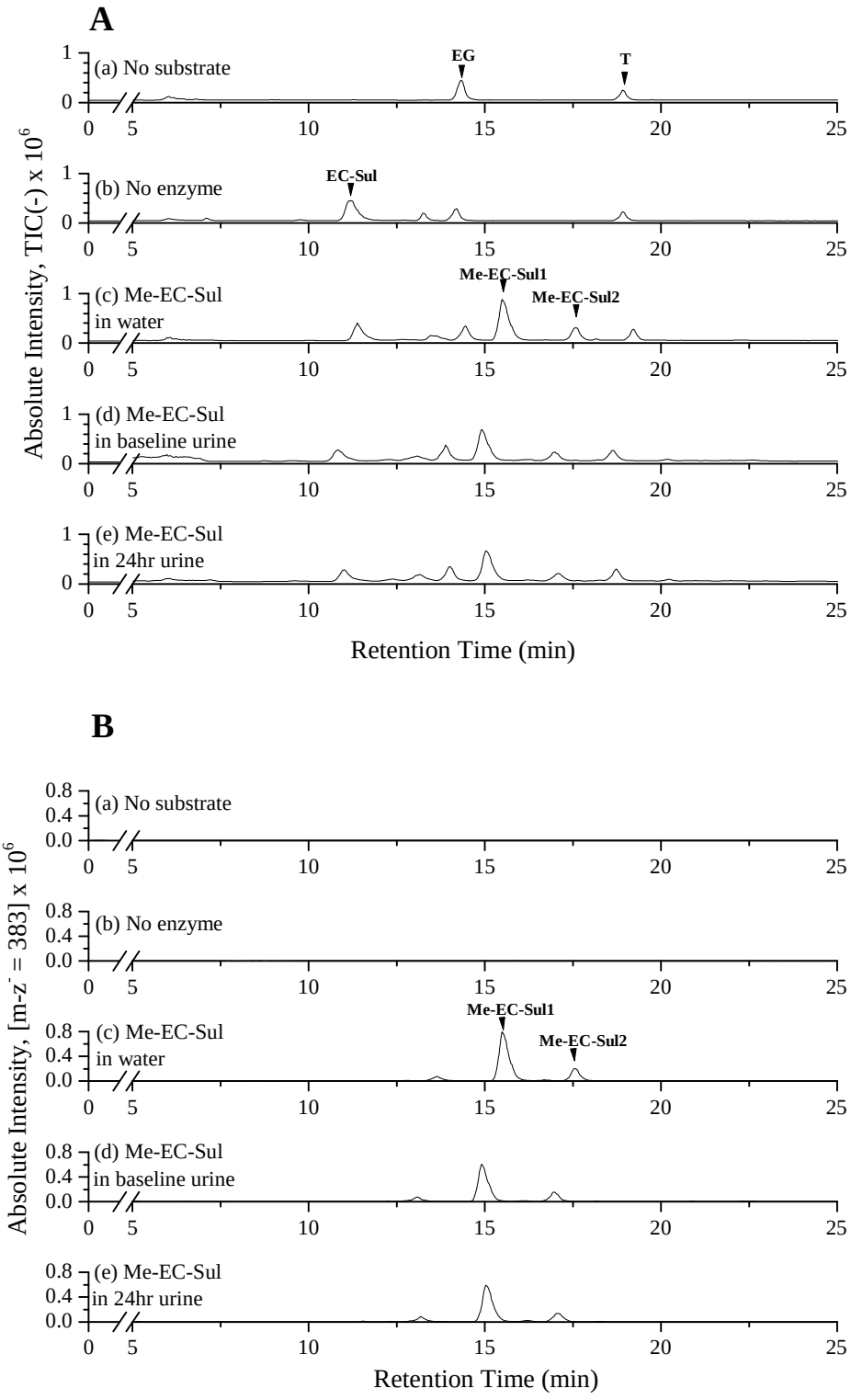


Figure 5.13: Chromatogram of methyl-(-)-epicatechin-sulfates after enzymatic synthesis in TIC mode (A) and specific m/z- at 383 (B). (a) and (b) are control. (c), (d) and (e) are methyl-(-)-epicatechin-sulfates, re-dissolved in water, baseline urine and 24 hr urine, respectively.

The chromatogram of all enzymatic (-)-epicatechin metabolites dissolved in milliQ water and detected in specific m/z- of each conjugate is shown in Figure 5.14. The different retention times of all synthesised (-)-epicatechins are illustrated in Table 5.6. Again, these data confirm that all synthetic conjugates of (-)-epicatechin were correct. Glucuronidated forms, more water soluble than sulfated and methylated, were separated first followed by sulfated and methylated forms.

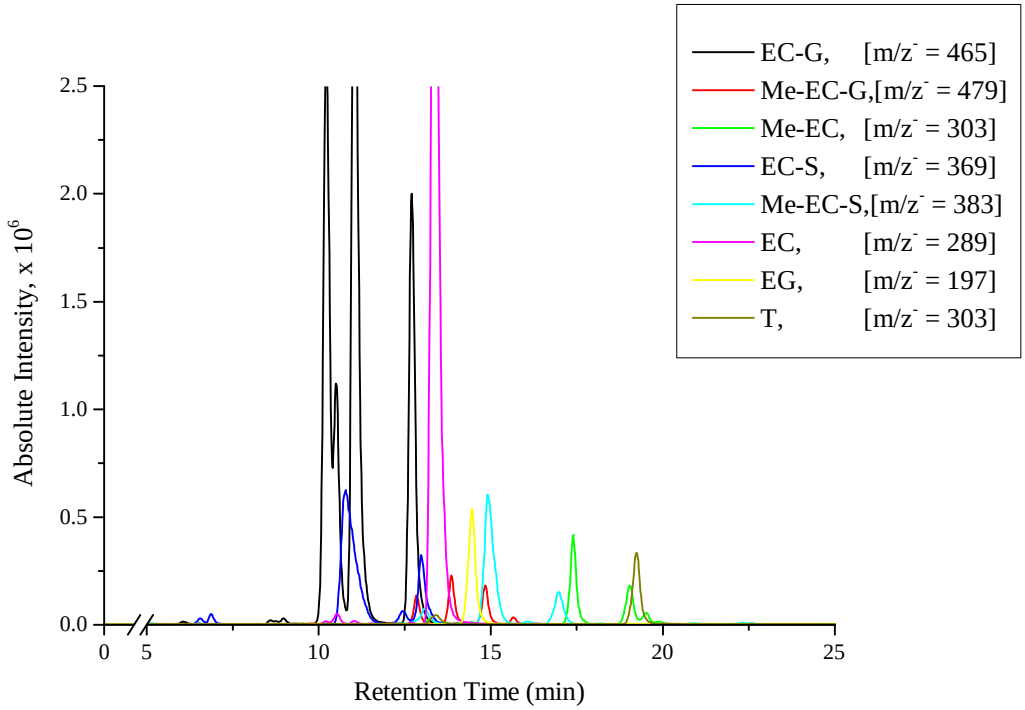


Figure 5.14: Chromatogram of all enzymatic epicatechin metabolites dissolved in milliQ water and detected in specific m/z- of each conjugate. (EG and T act as internal standard). Glucuronidated forms were separated first followed by sulfated and methylated forms.

Table 5.6: Retention time of each conjugated form of enzymatically synthesised (-)-epicatechin and internal standards (EG: Ethyl gallate and T: Taxifolin), detected at specific [m/z-].

Compound	[m/z-]	**Peak No.**	**Retention time, (min)**
EC-G	465	1	10.2
		2	10.5
		3	11.0
		4	12.7
EC-S	369	1	10.8
		2	12.4
		3	13.0
Me-EC-G	479	1	12.8
		2	13.9
		3	14.8
		4	15.7
Me-EC-S	383	1	13.6
		2	15.5
		3	17.5
Me-EC	303	1	17.4
EC	289	1	13.4
EG	197	1	14.5
T	303	1	19.2

5.4.3 The percentage yields of synthesised conjugates

The conversion rates of quercetin and (-)-epicatechin into their conjugated products is shown in Table 5.7. For quercetin conjugate synthesis, quercetin glucuronide showed the highest percentage yield (79.3±0.7%) in baseline followed by 3′-*O*-methylated-quercetin-glucuronides (39.1±0.5%) and quercetin sulfates (23.3±0.7%). For (-)-epicatechin conjugates synthesis, methylated-(-)-epicatechin-sulfate added in 24 hr urine has a greater percentage yield (98.9±0.2%) than (-)-epicatechin glucuronide (58.0±6.0%) and methylated-(-)-epicatechin-glucuronides (56.9±0.9%) followed by quercetin sulfate (47.6±2.5%). The lowest percentage yield was methylated-(-)-epicatechin which was 14.8±0.0%.

Table 5.7: Summary of the percentage yields of synthesised quercetin and (-)-epicatechin conjugates in this study (each sample was performed biological replicates).

Synthesised conjugates	Peak No.	% Yields	Total (%)
Quercetin conjugates			
Q-G added to MQ	1	0.9±0.0	75.8±0.0
	2	12.2±0.0	
	3	9.9±0.0	
	4	3.9±0.0	
	5	13.9±0.0	
Q-G added to baseline	1	0.8±0.0	79.3±0.7
	2	13.7±0.2	
	3	10.3±0.2	
	4	39.3±0.2	
	5	15.2±0.2	
Q-G added to 24 hr urine	1	0.7±0.1	77.8±2.2
	2	14.0±0.5	
	3	9.5±0.1	
	4	39.5±0.9	
	5	14.2±0.7	
Q-S added to MQ	1	5.5±0.0	33.1±0.0
	2	27.6±0.0	
Q-S added to baseline	1	5.5±0.4	23.3±0.7
	2	17.8±0.2	
Q-S added to 24 hr urine	1	6.4±0.2	34.1±0.7
	2	27.7±0.5	
3′Me-Q-G added to MQ	1	1.0±0.0	24.6±0.0
	2	10.9±0.0	
	3	12.7±0.0	
3′Me-Q-G added to baseline	1	1.5±0.0	39.1±0.5
	2	17.7±0.0	
	3	19.9±0.5	
3′Me-Q-G added to 24 hr urine	1	1.4±0.1	39.3±1.5
	2	17.5±0.4	
	3	20.4±1.0	

Continued…

Table 5 7 Summary of the percentage yields synthesised quercetin and (-)-epicatechin conjugates in this study (Continued)

Synthesised conjugates	Peak No.	% Yields	Total (%)
Epicatechin conjugates			
EC-G added to MQ	1	17.8±0.1	62.1±0.4
	2	6.5±0.1	
	3	24.8±0.1	
	4	13.1±0.1	
EC-G added to baseline	1	19.3±0.2	58.4±0.8
	2	5.9±0.1	
	3	22.3±0.2	
	4	10.9±0.3	
EC-G added to 24 hr urine	1	18.9±2.1	58.0±6.0
	2	5.2±0.9	
	3	22.9±1.2	
	4	11.0±1.8	
Me-EC-G added to MQ	1	14.0±0.4	62.2±1.1
	2	24.7±0.2	
	3	20.3±0.4	
	4	3.1±0.1	
Me-EC-G added to baseline	1	12.5±2.4	65.8±12.0
	2	22.4±3.8	
	3	27.8±5.2	
	4	3.1±0.6	
Me-EC-G added to 24 hr urine	1	9.8±0.1	56.9±0.9
	2	25.0±0.0	
	3	19.5±0.8	
	4	2.5±0.0	
Me-EC added to MQ	1	14.2±0.7	14.2±0.7
Me-EC added to baseline	1	15.5±0.5	15.5±0.5
Me-EC added to 24 hr urine	1	14.8±0.0	14.8±0.0
EC-S added to MQ	1	30.9±0.5	40.8±0.6
	2	1.4±0.0	
	3	8.5±0.0	
EC-S added to baseline	1	34.1±0.1	48.1±1.0
	2	1.7±0.3	
	3	12.2±0.6	
EC-S added to 24 hr urine	1	31.7±0.5	47.6±2.5
	2	1.2±0.5	
	3	14.8±1.5	
Me-EC-S added to MQ	1	6.0±0.3	97.6±1.1
	2	75.4±0.4	
	3	16.3±0.4	
Me-EC-S added to baseline	1	7.5±0.4	98.7±1.2
	2	74.1±0.6	
	3	17.1±0.3	
Me-EC-S added to 24 hr urine	1	7.9±0.1	98.9±0.2
	2	75.1±0.1	
	3	15.9±0.0	

5.5 Discussion

The important finding in the present study was that almost all conjugated quercetin and (-)-epicatechin derivatives, except for quercetin-glucuronide-sulfates, which were expected to be in urine were successfully synthesised using enzymatic synthesis using microsomal and cytosolic preparations from pig liver. These are sources of phase II enzymes; UGT, SULT and COMT. From the basic structure of quercetin and (-)-epicatechin, glucuronic acid, sulfate or methyl groups were added to the hydroxyl group, leading to five possible forms of conjugated products, dependent on the activity and sources of phase II enzymes . Several sources of phase II enzymes from different species such as rat, pig and human were used. However, in this study pig liver was the best choice because it contains a high content of phase II enzyme and is more similar to human liver than rat liver. Moreover, it is more convenient than human liver. Shali et al. (1991) also stated that guinea pig liver can produce quercetin sulfate and secrete into bile with a higher rate than values for the rat. The specific position of conjugated quercetin or (-)-epicatechin in this study, however, cannot be defined by UPLC-MS. A higher performance technique like LC-MS/MS or NMR could be another way to identify their structure.

The results in this present study have shown that glucuronic acid from UDPGA was conjugated to all five hydroxyl groups of quercetin, consequently generating into five forms of quercetin glucuronides. Although peak No 1 in Figure 5.2 is a tiny peak, the specific m/z- at 477 corresponded to quercetin glucuronide. Thus, this data confirmed that the first small peak is also quercetin conjugated with glucuronide. In this study, the main conjugated quercetin and (-)-epicatechin were synthesised which is greater than that described in other publications (Table 5.4). O'Leary et al., (2001) focused on monoglucuronide of quercetin and revealed that only four quercetin glucuronides; quercetin-3-*O*-glucuronide, quercetin-7-*O*-glucuronide, quercetin-3′-*O*-glucuronide and quercetin-4′-*O*-glucuronide, were generated by a cell-free extract of pig liver. There were 6.5 molar % of 3-*O*-glucuronide-7-*O*-glucuronide, 85 molar % of 4′-O-glucuronide, 6.5 molar % of 3′-*O*-glucuronide and 2 molar % of free quercetin. The apparent affinity of UGT from a cell-free extract of pig liver to quercetin followed the order 4′-> 3′-> 7-> 3 with no conjugation at the 5-position, and the biological activity of these conjugates to inhibit xanthine oxidase and lipoxygenase was still maintained (Day et al., 2000).

The main form of quercetin glucuronide found by Day et al. 2000 was 4′-*O*-glucuronide, while Boersma et al., 2002 found 7-*O*-glucuronide. Boersma et al., (2002) assumed that it may due to the different concentrations of quercetin in the reaction. In addition, the preferential position of glucuronidation is different in different types of flavonoids and also varies according to the isoform of UGT. All five forms of quercetin glucuronides produced and detected in this study may be due to alamethicin, saccharolactone and ascorbic acid being added into the glucuronidation reaction owing to enhanced activity of UGT and also preservation of glucuronidated quercetin products. Alamethicin is a UGT activator leading to higher yields. After quercetin glucuronides were produced, saccharolactone and ascorbic acid, β-glucuronidase inhibitor and reducing agent in the glucuronidation reaction respectively, may assist to preserve the synthesised conjugates. These assumptions are supported by the high percentage yield of each synthesised conjugate. This study demonstrated that quercetin glucuronide was produced with a maximum percentage yield of 79.3±0.7%. In addition, (-)-epicatechin glucuronide and methylated-(-)-epicatechin-glucuronides were the second conjugates that generated four forms with yields of 58.0±6.0% and 56.9±0.9%, respectively. The rest of the conjugates were produced in only a few forms. Duenas et al. (2008) demonstrated that quercetin metabolites are generated at different pH values using a 2 hr incubation time, however, pH at 8.3 in water improved quercetin glucuronide yield up to 19% for quercetin 4′-glucoronide and 7% for quercetin 3′-glucuronide. Although the conjugated position of synthesised quercetin glucuronides cannot be distinguished in this study but their profile (see chromatogram of quercetin glucuronides in Figure 5.2) can compare with the quercetin glucuronide profile synthesised by Duenas et al. (2008). Based on the chromatogram of quercetin glucuronides from Duenas et al., (2008), quercetin glucuronide 4 and quercetin glucuronide 5 in this study should be quercetin 4′-glucuronide and quercetin 3′-glucuronide, respectively. Then, the percentage yield of quercetin glucuronide 4 and quercetin glucuronide 5 are 39.3±0.2 and 15.2±0.2, respectively (Table 5.7). This data supported that glucuronidation of quercetin using the enzymatic method in this study provide a better yield than the previous method optimised by Duenas et al, 2008.

As mentioned above, quercetin-glucuronide-sulfate synthesis was attempted but was unsuccessful. Since sulfate is more chemically unstable than glucuronide or

methyl groups, it can be assumed that sulfate moieties of quercetin-glucuronide-sulfate may hydrolyse during synthesised step.

Similar research was carried out on the enzymatic synthesis of (-)-epicatchin conjugates and showed that 3'-*O*-methyl-epicatechin 5-*O*-glucuronide was produced when incubating recombinant human UGT1A9 with 3'-*O*-methyl-epicatechin to 50% yield after 24 hr (Blount et al., 2012). However, the findings of this present study showed that the percentage yield of methylated-(-)-epicatechin-glucuronides is 62.2±1.1% after 1 hr incubation. Although, Blount et al. attempted to optimise the condition of 3'-*O*-methyl-epicatechin 5-*O*-glucuronide synthesis by using room temperature incubation and adding an extra glucuronic acid donor every 4 hr and extra UGT1A9 of both initial reaction and every 4 hr but the percentage yield of (-)-epicatechin conjugates synthesised based on the condition in this study still seems higher than Blount et al. (2012). In addition, Blount et al. (2012) synthesised 3'-*O*-methyl-epicatechin-5-*O*-glucuronide by methylating (-)-epicatechin or (-)-epicatechin glucuronide first then subsequently by glucuronidation. The opposite line was also performed but no glucuronidated methyl epicatechin was produced. However, this study uses the later procedure and successful synthesis.

Conjugation with glucuronic acid to quercetin and (-)-epicatechin structure leads to increase a water solubility higher than conjugation with sulfate and methyl. Thus, glucuronided forms were separated first followed by sulfated and methylated forms (Figure 5.7 and Figure 5.14). Again, these data confirmed that all synthetic conjugates of quercetin and (-)-epicatechin were correct and could be used for urine analysis in next study.

5.6 Conclusion

Due to the absence of commercially available relevant conjugated quercetin and (-)-epicatechin derivatives, the main conjugates of these compounds, in different forms, were synthesised using an improved enzymatic method. Five quercetin and five (-)-epicatechin conjugates were successfully synthesised with a higher percentage yield compared to previous reports. These synthesised compounds will be used as standards in Chapter 6.

Chapter 6 Determination of Quercetin, (-)-Epicatechin and Main Metabolites in Urine Samples from Healthy Volunteers

6.1 Abstract

Previously, the metabolic profile of flavonoids after a single consumption of flavonoid-rich foods, pure compunds or supplements have been studied by several research groups. Each flavonoid has their own pattern of metabolites and inter-individual variation. However, in daily life, humans do not consume only a single type of flavonoid per meal. When two flavonoids like quercetin and (-)-epicatechin are co-administered, these two flavonoids may interact with each other during absorption and distribution, especially with regard to phase II enzyme conjugation. Then, the phase II metabolites of quercetin and (-)-epicatechin may change and affect their bioactivities. The main aim of this chapter was to assess an absorption and metabolism of quercetin and (-)-epicatechin using urine as a biomarker. Baseline and 24 hr urine samples were taken from twenty seven volunteers and were collected after consumption of red onion soup, 70% cocoa dark chocolate and co-consumption at three different times. Due to the low percentage urinary excretion of quercetin and (-)-epicatechin detected in Chapter 4, accompanied by a low amount of quercetin and (-)-epicatechin spiked with synthetic conjugated standard in 24 hr urine samples in Chapter 5, the baseline and 24 hr urine samples were first concentrated by freeze-drying before protein precipitation, filtration and injection into UPLC-MS. In the case of onion intake, eight conjugated quercetin metabolites were identified. Glucuronic acid conjugated with quercetin was the major urinary metabolite and there were three different forms. In addition, the total metabolites of quercetin excreted in the urine of each volunteer was very different. For the single consumption of 70% cocoa dark chocolate, six urinary (-)-epicatechin metabolites were detected and the main form was methylated (-)-epicatechin. In contrast to the metabolic profiles of quercetin, the total metabolites of (-)-epicatechin did not show a vast variation in each volunteer. After co-consumption, all urinary quercetin and (-)-epicatechin metabolites were found to be the same as those metabolites after single consumption. Interestingly, the total quercetin metabolites were also apparently increased in four of the volunteers. For total (-)-epicatechin metabolites, there were no large differences. The total (-)-epicatechin metabolites in some volunteers increased, while others were decreased

and did not change between single and after co-consumption. These differences were possibly due to the variation of phase II enzymes in each volunteer.

6.2 Introduction

A number of studies investigated the metabolic profile of flavonoids after consumption of a supplement or a single food. Each flavonoid has their own pattern of metabolites and inter-individual variation. Eighteen urinary quercetin metabolites after the ingestion of fried red onions by six volunteers were quercetin-diglucuronides, quercetin-glucoside-sulfates, quercetin-4′-*O*-glucuronide and quercetin-3′-*O*-sulfate, quercetin-3-*O*-glucuronide, quercetin-3′-*O*-glucuronide, quercetin diglucuronide, quercetin glucuronide glucoside, quercetin glucuronide sulfates, quercetin glucoside sulfates, isorhamnetin-3-*O*-glucuronide, isorhamnetin-4′-*O*-glucuronide and methylquercetin diglucuronides (Mullen et al., 2006). In addition, (-)-epicatechin metabolites in urine after the ingestion of 100 g dark chocolate by five volunteers were found as fifteen forms; three (-)-epicatechin-glucuronides, three (-)-epicatechin-sulfates, five *O*-methyl-(-)-epicatechin-sulfates and four *O*-methyl-(-)-epicatechin-glucuronides (Actis-Goretta et al, 2012).

It has been suggested that glucuronidation, sulfation and methylation in the gut and/or liver result in poor oral bioavailability of flavonoids. The metabolic profile of flavonoids may modify the protective effects and other biological activites in humans. For example, Khan et al. (2012) found that regular cocoa consumption alters lipid profile by significantly increasing plasma HDL cholesterol levels and decreasing oxidised LDL concentrations in high-risk coronary heart disease subjects, associated with increases in the phase II metabolites level in urine and the microbial-derived metabolites of cocoa flavonoids.

However, foods are consumed by humans not as a single serving but normally as mixtures. Only a few research groups have studied the effect of the type of consumed foodstuffs on the metabolite patterns in human. Borges et al. (2010) studied the bioavailability of combinations of polyphenolic compounds in a polyphenol-rich juice drink. This juice contained green tea flavan-3-ols, grape seed and pomace procyanidins, apple dihydrochalcones, procyanidins and chlorogenic acid, citrus flavanones and grape anthocyanins. They found that the metabolic profile of flavanones, dihydrochalcones and chlorogenic acids in plasma and urine

after acute consumption of polyphenol rich beverage were not significantly different when compared to other single component studies. However, they noticed that there were quantitative differences between (epi)catechin and (epi)gallocatechins metabolites. In addition, they suggested that although the absorption of these flavan-3-ols were not influenced by other phenolic compounds, this difference is possibly due to some competition between phase II enzymes; uridine-5′-diphosphate glucuronyltranferases, sulfotransferases and catechol-*O*-methyltransferases, resulting in differences in the plasma and urinary flavan-3-ol metabolic profiles. Lee and Mitchell (2012) studied the glycosidic moiety which is a key factor influencing the absorption and elimination of flavonoids. Two dietary sources of quercetin, apple peel and onion powder, containing quercetin but linked with different types of sugar moiety, and also a mixture of these two powders, were fed to healthy volunteers (eight female and eight male). They found that consuming onion powder alone led to faster absorption, higher plasma concentration and better bioavailability than single consumption of apple peel powder. For a mixture of apple peel and onion powder, the absorption rate of this mixture was intermediate between the absorption rate of apple peel and onion powder. Thus, the result from this research group showed that feeding two different sources of quercetin glycosides to healthy volunteers at the same time can modify pharmacokinetic parameters and also metabolic profiles in the circulation. The effect of other key factors, food matrix and dose ingestion, on the bioavailability and also biological effects of (-)-epicatechin was performed by Hollands et al. (2013). Although, they did not provide a co-consumption of food samples to volunteers, they demonstrated that a whole apple puree, containing not only (-)-epicatechin but other flavonoids (quercetin glycosides, phloretin glycosides, and chlorogenic acid) reduced the bioavailability of (-)-epicatechin compared to (-)-epicatechin-rich extracted drink from apple. In addition, consuming a double dose of epicatechin-rich apple extract increased more than two fold the (-)-epicatechin bioavailability. Recently, the co-consumption of two types of dietary flavonoids was reported (Nakamura et al., 2014). A combination meal of sautéed onion and tofu, containing quercetin and isoflavones, was fed to five healthy volunteers and then the plasma metabolic profile of quercetin and isoflavones were analysed. Glucuronidated and methylated quercetin were most abundant a after single serving of quercetin while glucuronide and sulfate conjugates of isoflavone, were major metabolites after tofu ingestion. In the case of combined ingestion, only

quercetin sulfate was detected along with a reduction of sulfated isoflavones. In both HepG2 cells and Caco-2 cells, they also found that quercetin is a better substrate for UGT and SULT than genistein isoflavone.

From these data, different chemical structures of flavonoids in foods have important consequences on bioavailability. In addition the affinity of phase II enzyme for various flavonoid substrates affects their absorption and bioavailability. One of the current trends in nutrition is to focus on metabolomics, the study of metabolites or chemicals in the biological system which can provide an overview of the metabolic status (Llorach et al., 2012). Thus, it would be interesting to investigate the interaction between the metabolic profiles of the exogenous metabolome or all metabolites derived from the ingestion of foods like quercetin and (-)-epicatechin. To obtain the metabolomic data of quercetin and (-)-epicatechin, Ultra Performance Liquid Chromatography coupled with Mass Spectrometry (UPLC-MS) was used in this study.

The main aim of this study was to investigate the metabolism and excretion of quercetin and epicatechin after co-consumption of onion soup and dark chocolate by healthy volunteers (n=27). The hypothesis of this study is that inhibitory interaction between quercetin and (-)-epicatechin will occur after co-administration of these two flavonoids. This interaction may change an individual compound's absorption and urinary metabolites of other flavonoids. In this case the flavonol quercetin may affect absorption and metabolism of flavanol (-)-epicatechin by modifying (inducing or inhibiting) the phase II enzyme activity, resulting in higher or lower bioavailability of (-)-epicatechin.

Due to the low level of analytes, the baseline and 24 hr urine required concentrating by freeze-drying and protein precipitation, filtration prior to further analysis by the Ultra Performance Liquid Chromatography coupled with Mass Spectrometry. Then, to understand the effect of phase II enzymes on bioavailability of quercetin and (-)-epicatechin, their main metabolites of quercetin (quercetin-sulfate, quercetin-glucuronide, isorhamnetin-glucuronide) and (-)-epicatechin (methyl-(-)-epicatechin-sulfate, (-)-epicatechin-glucuronide, (-)-epicatechin-sulfate, methyl-epicatechin-glucuronide) were identified in human urine without enzyme hydrolysis. Thus, a more complete picture of these two compounds relating to bioavailability and possible correlation to bioefficiency can be obtained.

6.3 Materials and Methods

See Section 2.5.5, 2.5.6 and 2.5.8 for determination main metabolites of quercetin and (-)-epicatechin in urine samples from 27 healthy volunteers.

6.4 Results

6.4.1 Urine metabolites of quercetin after a single consumption of red onion soup in healthy volunteers

To identify all metabolites found in urine, the relative retention time (relation time of compounds/relation time of internal standard) and the mass to ion ratio of synthesised standards from previous chapters were used as a reference. In this study, eight conjugates of quercetin metabolites identified as three forms of quercetin glucuronides, two forms of quercetin sulfates, quercetin-di-*O*-glucuronides, 3′-*O*-methyl-quercetin-*O*-glucuronide and 3′-*O*-methyl-quercetin-di-*O*-glucuronide were investigated in urine from human volunteers who consumed a single dose of red onion soup. The Ultra Performance Liquid Chromatography coupled with Mass Spectrophotometer results confirmed the occurrence of metabolites eluting at different retention times. First, three peaks of quercetin glucuronides were observed in urine samples. The first quercetin glucuronide was eluted at a retention time of 21 min (Figure 6.1A). The second quercetin glucuronide was eluted at retention time of 22 min but in some volunteers this peak was co-eluted at retention times of 22 and 23 min. Therefore, peak areas of these forms were combined and calculated together (Figure 6.1B). The last quercetin glucuronide form was found most in all volunteers and eluted at a retention time of 29 min (see Figure 6.1C).

To distinguish whether which glucuronidated form was produced and excreted in urine, known standard compounds which we already have in the laboratory were used to spike the urine sample of some volunteers. In volunteers 4, 5, 10 and 13, a high peak of quercetin glucuronide at a retention time of 22 and 23 min were found. Then, the 24 hr urine sample of volunteer 4, which had the highest peak, was spiked with quercetin-3-*O*-glucuronide and the peak eluted at the retention time of 22 and 23 min was not quercetin-3-*O*-glucuronide but actually was peak No 1, eluting at 21 min (see Figure 6.2). These peaks were also found in a few volunteers in low amounts (see Figure 6.1A). In volunteer 9, the highest peak of

quercetin glucuronide retention time at 29 min was found in this volunteer. Then, a standard compound of quercetin-7-*O*-glucuronide was spiked in this 24 hr urine and found that this peak eluted at 29 min was not quercetin-7-*O*-glucuroide. A small peak of spiked quercetin-7-glucuconide was appeared at 20.4 min.

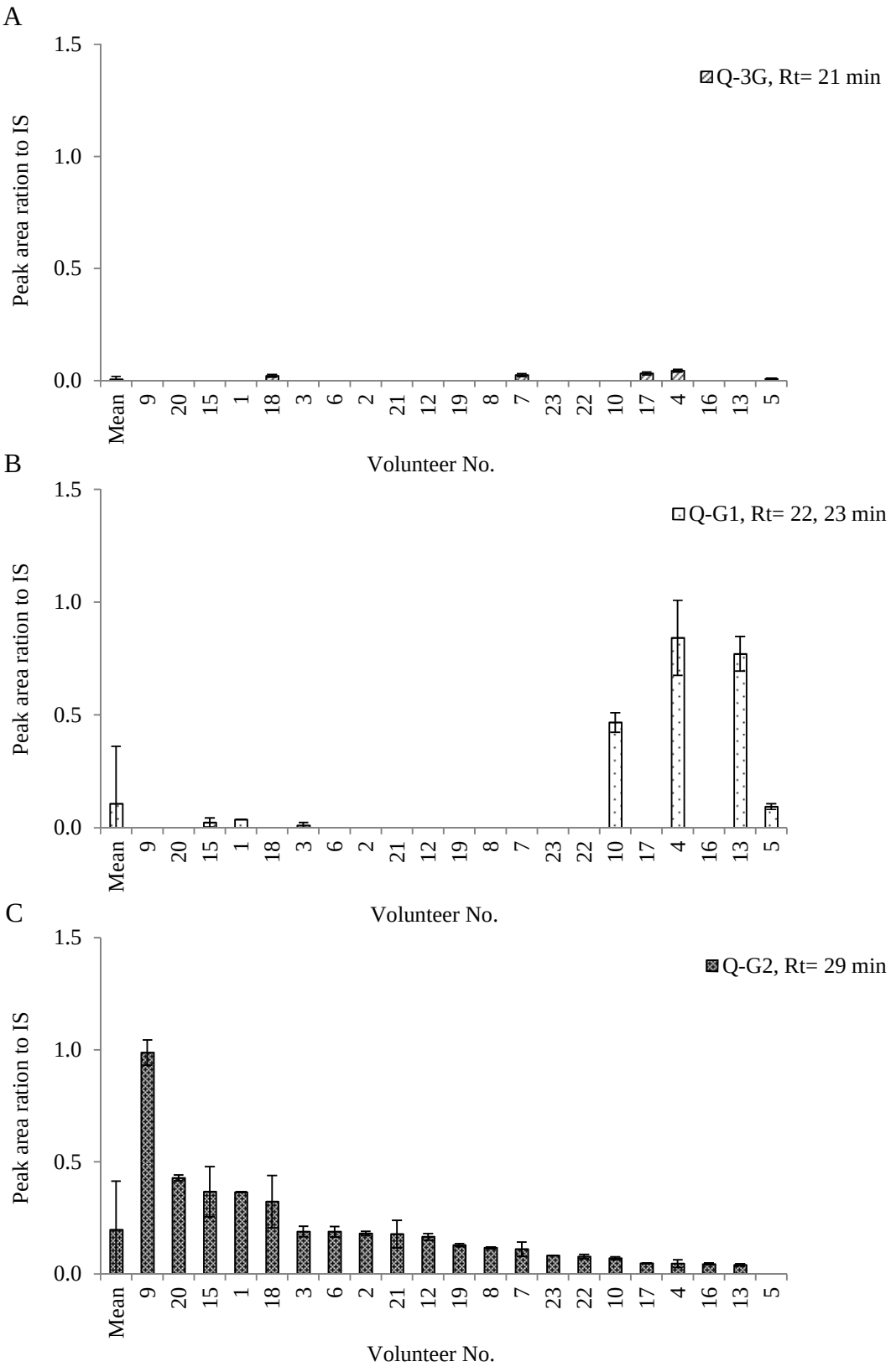


Figure 6.1: Three main quercetin glucuronides were found in the urine samples of volunteers. A. quercetin-3-*O*-glucuronide, B. quercetin-*O*-glucuronide 1 (retention time at 22, 23 min) and C. quercetin-*O*-glucuronide 2 (retention time at 29 min).

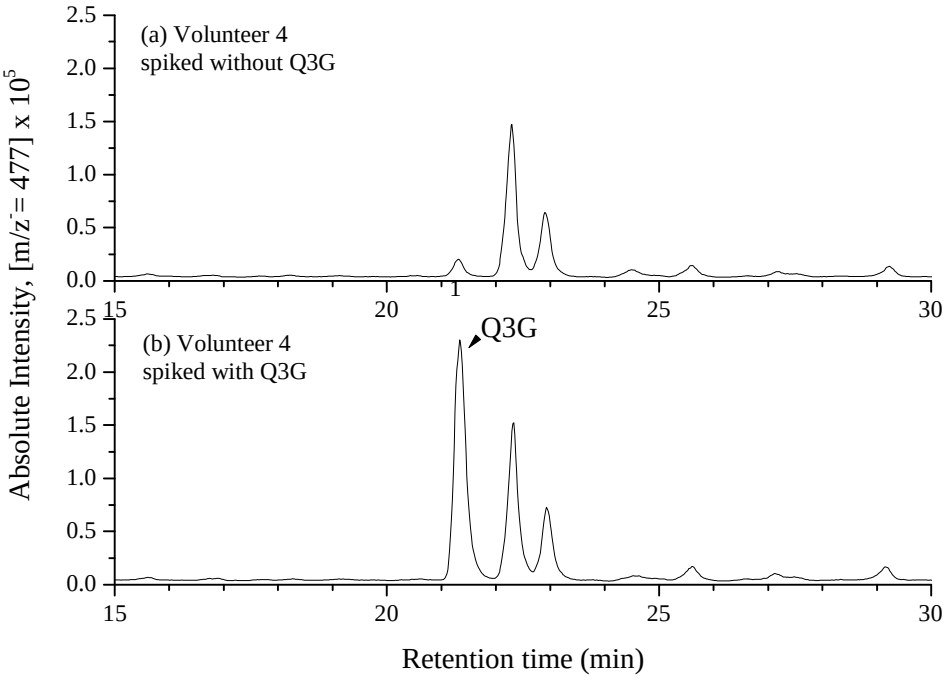


Figure 6.2: Chromatogram of quercetin-glucuronides found in volunteer 4 spiked (a) without and (b) with quercetin-3-*O*-glucuronide standard and detected at a specific mass to ion ratio (m/z^- = 477).

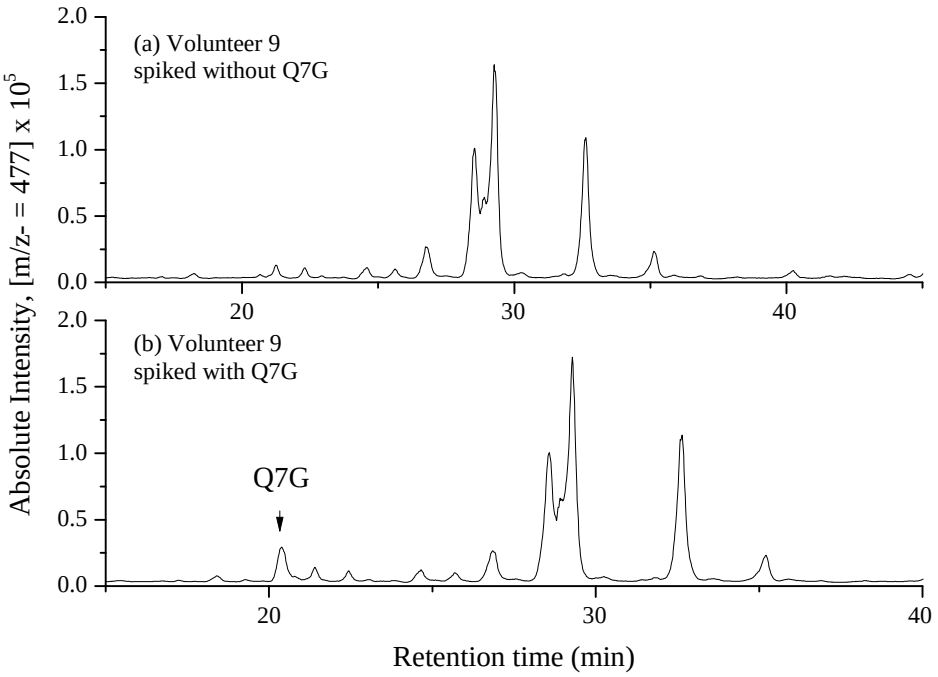


Figure 6.3 Chromatogram of quercetin-glucuronides found in volunteer 9 spiked (a) without and (b) with quercetin-7-*O*-glucuronide standard and detected at a specific mass to ion ratio (m/z^- = 477).

In this study, two forms of quercetin sulfates were also found in urine samples, eluting at retention times of 27 min and 31 min, respectively (Figure 6.4). Additionally, the spiked urine sample in volunteer 13 confirmed that the peak eluted at a retention time of 31 min is quercetin 3′-*O*-sulfate (Figure 6.5).

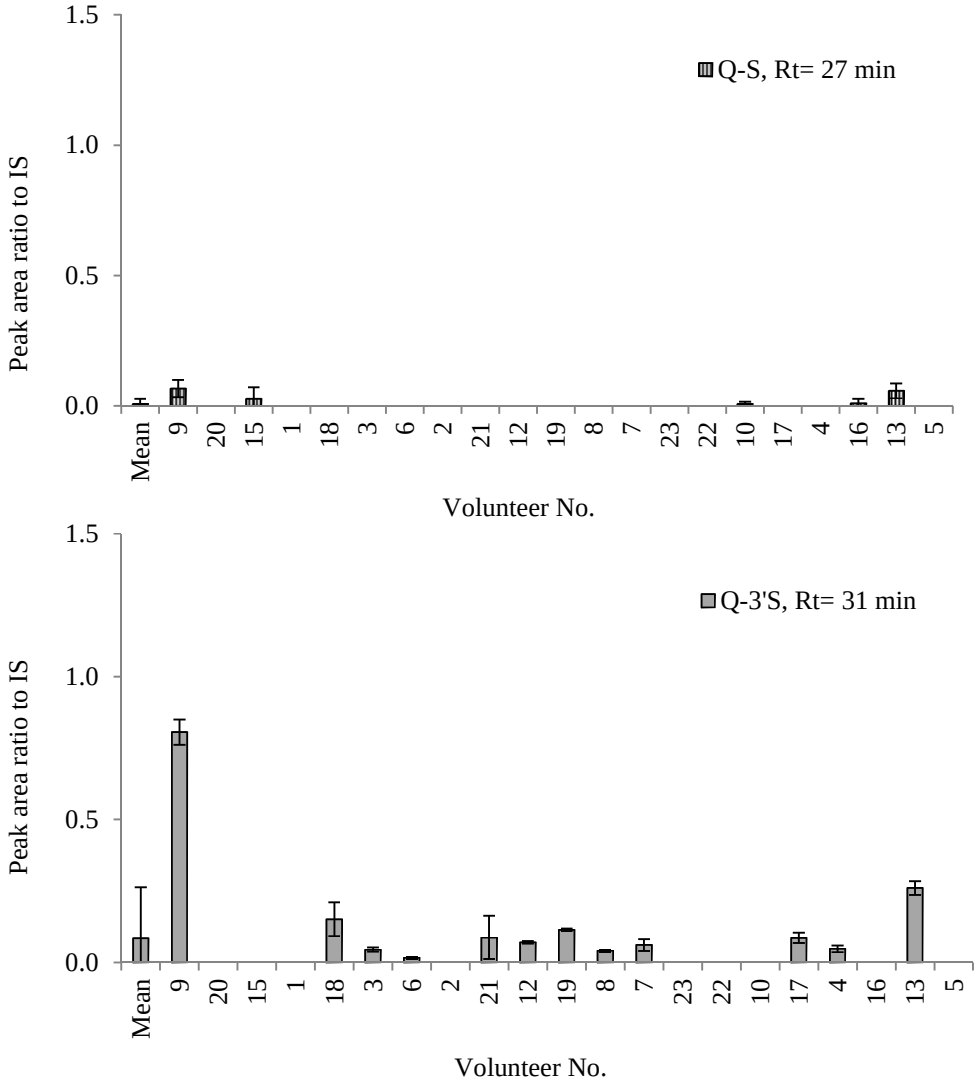


Figure 6.4: Two main quercetin-sulfates found in urine samples of volunteers. A. quercetin-*O*-sulfate (retention time at 27 min) and B. quercetin-3′-*O*-sulfate (retention time at 31 min).

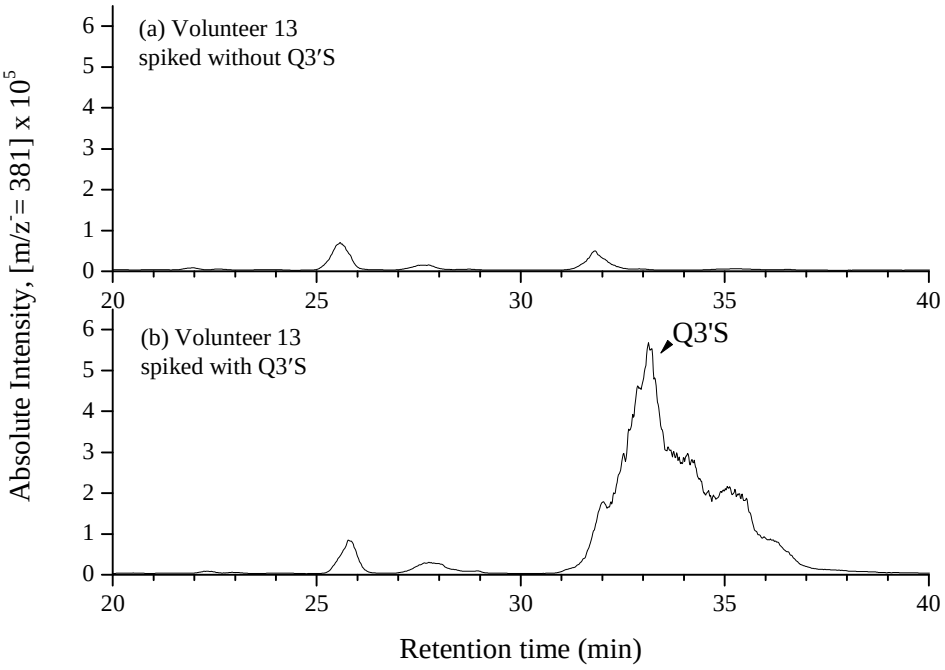
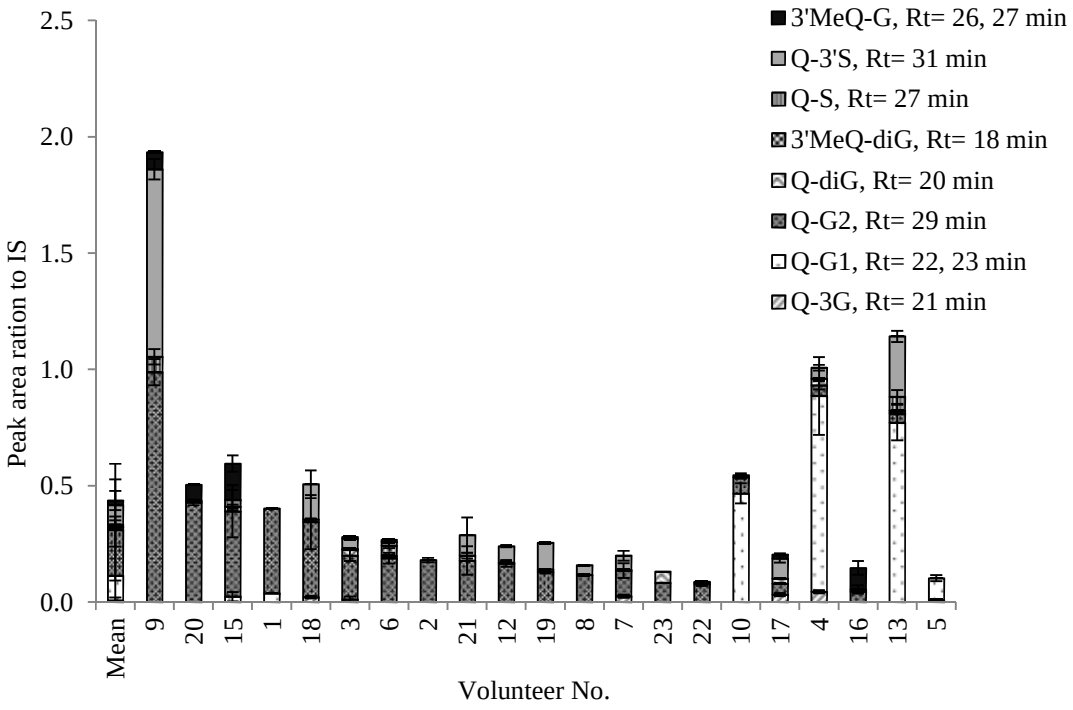


Figure 6.5: Chromatogram of quercetin-glucuronide found in volunteer 13 spiked (a) without and (b) with quercetin-3′-*O*-sulfate standard and detected at a specific mass to ion ratio (m/z^- = 381).

Furthermore, other quercetin conjugates (quercetin-di-*O*-glucuronide, Rt = 20 min; 3′-*O*-methyl quercetin-*O*-glucuronide, Rt = 26 and 27 min; 3′-*O*-methyl quercetin-di-*O*-glucuronide, Rt = 18 min) were also excreted and found in urine in only small amounts. All combined urinary quercetin metabolites, arranged in order of the main quercetin glucuronide (Rt = 29 min), in each volunteer is shown in Figure 6.6A. From this bar chart, a huge difference of quercetin metabolites excreted in urine was observed in each volunteer who consumed onion soup only. Additionally, the glucuronidated form of quercetin was most abundant in urine followed by sulfated quercetin and methylated quercetin (74%, 21% and 5%, respectively (Figure 6.6B)).

A.



B.

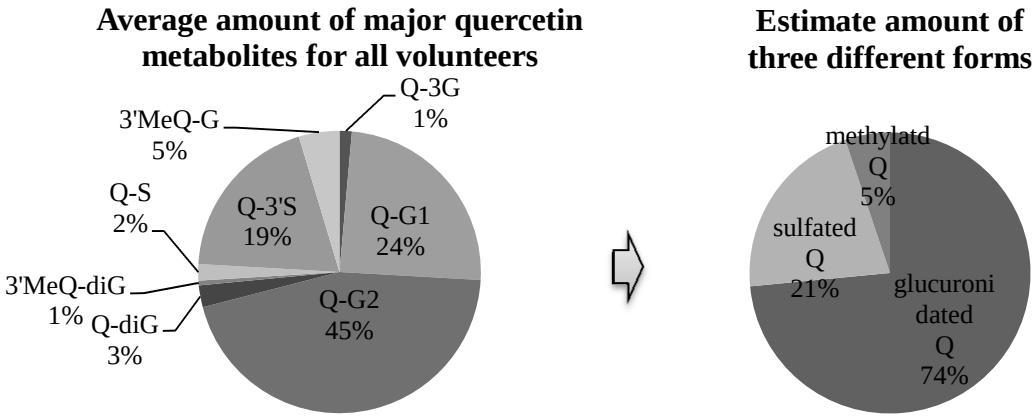


Figure 6.6: A. All eight urinary quercetin metabolites were found in 24 hr urine samples of each volunteer. B. The percentage of average amount of major quercetin metabolites for all volunteers (on the left). These amounts can be converted into three main forms (on the right).

6.4.2 Urine metabolites of (-)-epicatechin after a single consumption of 70% cocoa dark chocolate in healthy volunteers

For a single consumption of 70% cocoa dark chocolate, six (-)-epicatechin metabolites were found in urine; methylated-(-)-epicatechin-sulfate, methylated-(-)-epicatechin-glucuronide, methylated-(-)-epicatechin, (-)-epicatechin-sulfates (two forms) and (-)-epicatechin-glucuronide. In this case, methylated-(-)-epicatechin-sulfate was found most (Figure 6.7A, Rt = 15 min) followed by (-)-epicatechin-sulfate (Figure 6.8B, Rt = 12-13 min). (-)-Epicatechin-glucuronide, eluting at 12 min, also found in the urine samples of almost all volunteers (Figure 6.9) but lower than the amount of methylated (-)-epicatechin-sulfate and epicatechin-sulfate.

A tiny amount of other (-)-epicatechin metabolites; methylated-(-)-epicatechin-glucuronide (Rt = 13 min), methylated-(-)-epicatechin (Rt = 18 and 20 min) and also (-)-epicatechin-sulfate (Rt = 11 min) were detected in a few volunteers (Figure 6.7B and 6.7C).

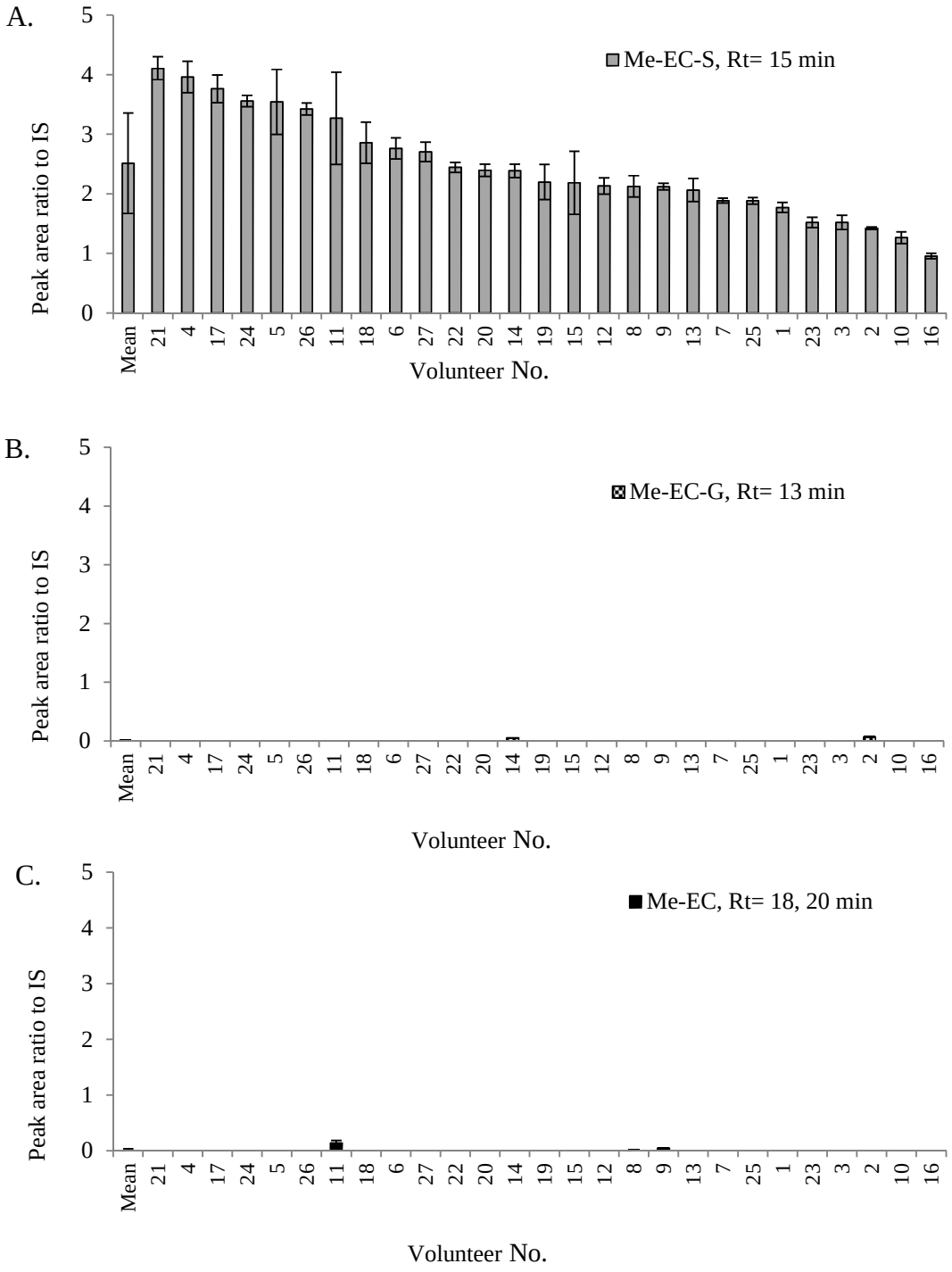


Figure 6.7: Three different methylated forms of (-)-epicatechin were found in urine samples, after a single consumption of 70% cocoa dark chocolate, in each volunteer. A. methyl-(-)-epicatechin-sulfate, eluting at 15 min, B. methyl-(-)-epicatechin-glucuronide, eluting at 13 min and C. methyl-(-)-epicatechin, eluting at 18 min and 20 min.

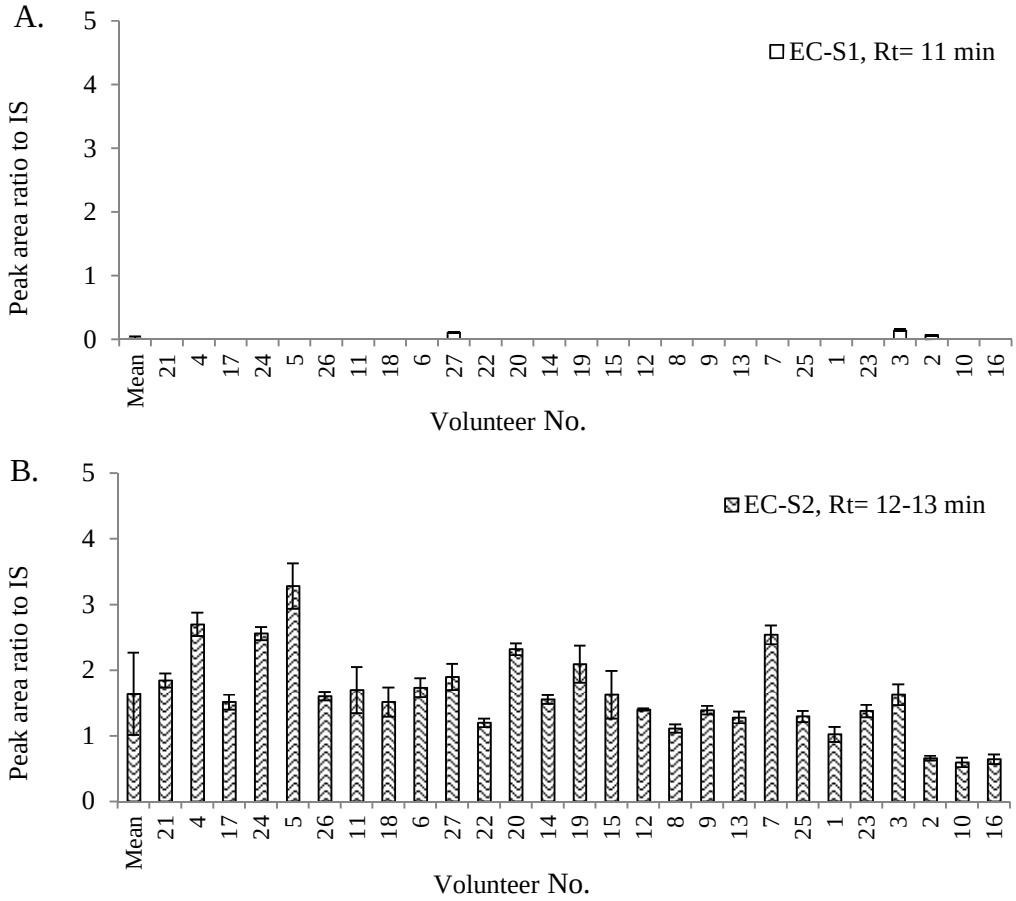


Figure 6.8: Two sulfated forms of (-)-epicatechin were found in urine samples, after a single consumption of 70% cocoa dark chocolate, in each volunteer. A. (-)-epicatechin-sulfate 1, eluting at 11 min, B. (-)-epicatechin-sulfate 2, eluting at 12 and 13 min.

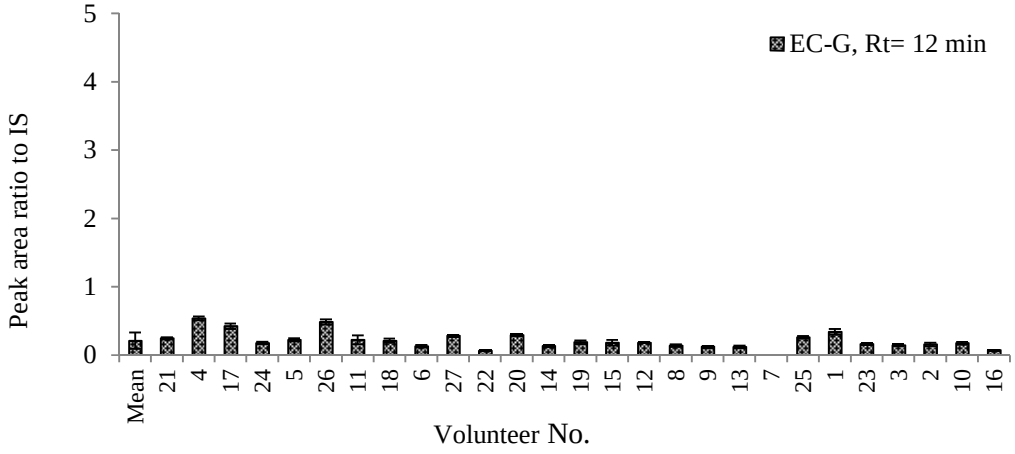


Figure 6.9: Glucuronidated forms of (-)-epicatechin were found in urine samples, after a single consumption of 70% cocoa dark chocolate, in each volunteer. The retention time was 12 min.

Again, all combined urinary (-)-epicatechin metabolites, arranged in order of the main methylated (-)-epicatechin sulfate (Rt = 15 min), in each volunteer is shown in Figure 6.10A. From this bar chart, urinary (-)-epicatechin metabolites after a single consumption of 70 % cocoa dark chocolate were not vastly different in each volunteer. However, unlike quercetin metabolites, after a single consumption of 70% dark chocolate, methylated forms of (-)-epicatechin were found in most urine samples followed by sulfated (-)-epicatechin and glucuronidated (-)-epicatechin (60%, 37% and 3%, respectively (Figure 6.10B)).

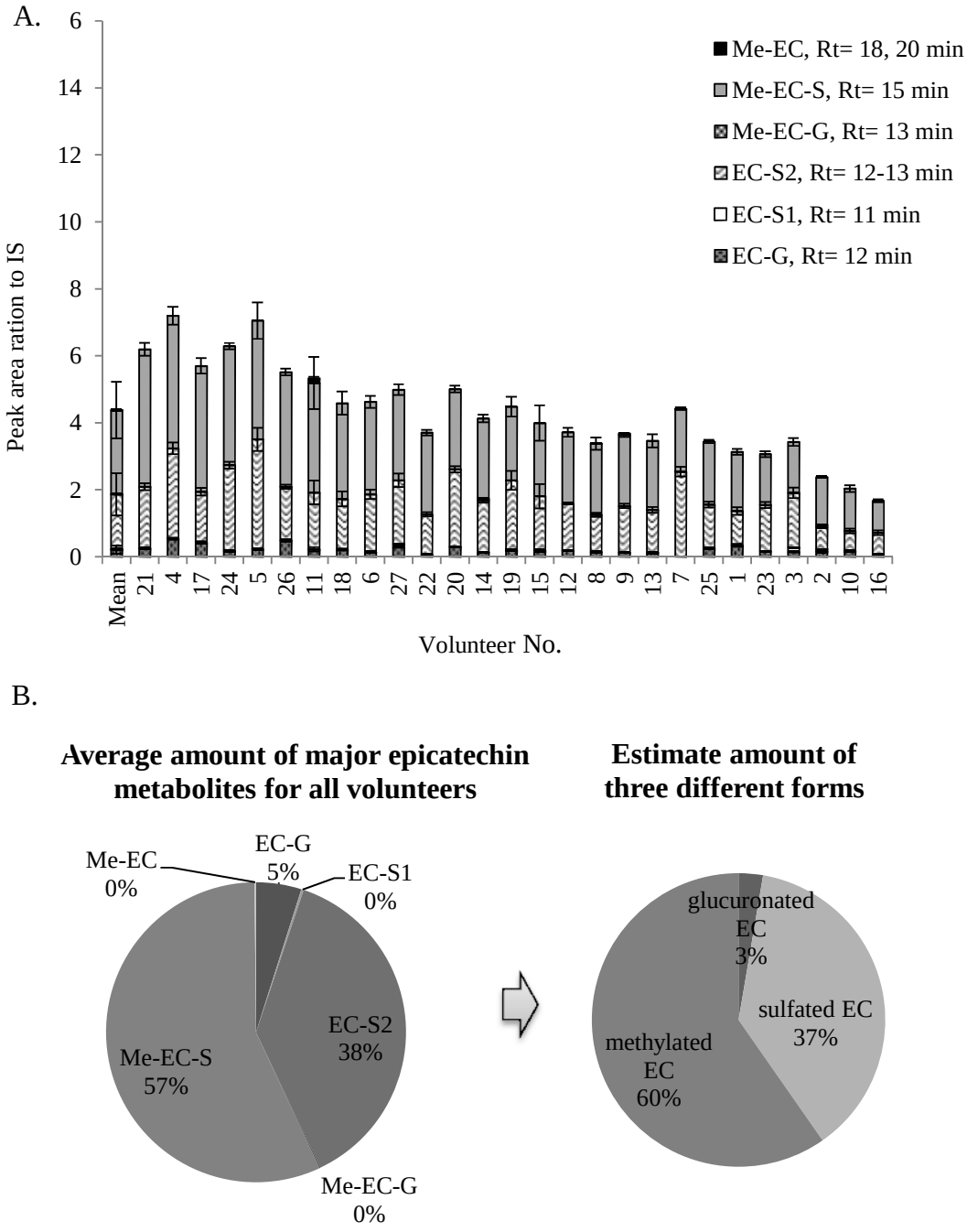


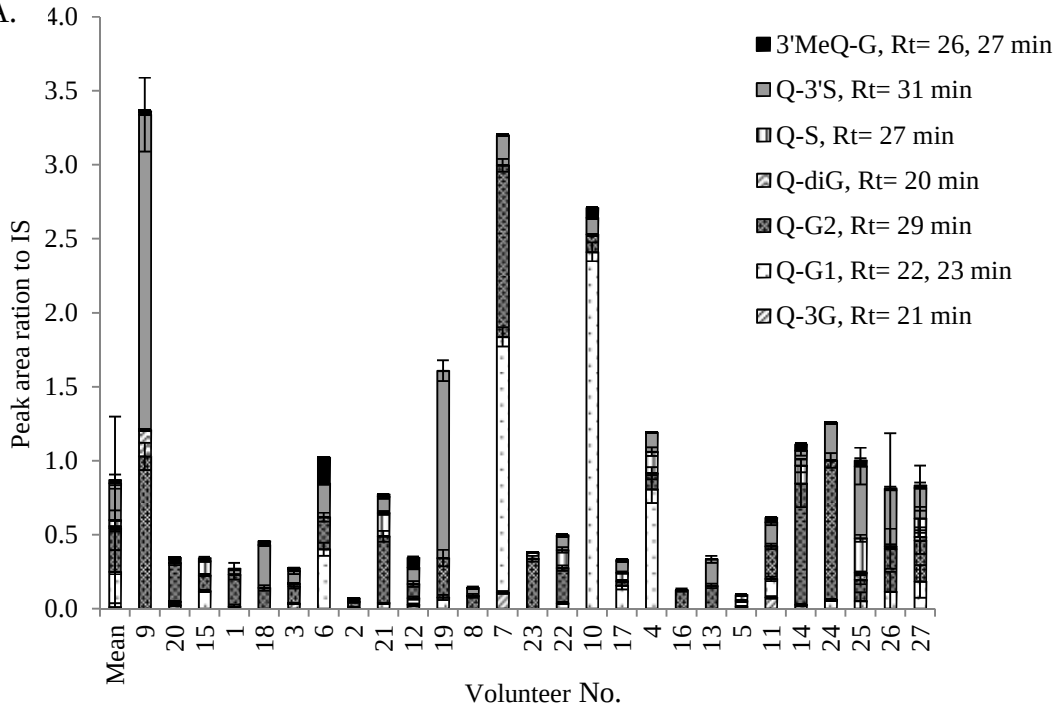
Figure 6.10: A. All six urinary (-)-epicatechin metabolites found in 24 hr urine samples from each volunteer. B. The percentage of the average amount of major (-)-epicatechin metabolites for all volunteers (on the left). These amounts can be converted into three main forms (on the right).

6.4.3 Urine metabolites of quercetin after co-consumption of onion soup and 70% cocoa dark chocolate in healthy volunteers

After co-consumption of onion soup and 70% cocoa dark chocolate, all urinary quercetin metabolites were found to be the same as those metabolites after consuming a single dose of red onion soup except 3′-*O*-methyl quercetin-di-*O*-glucuronide (Rt = 18 min). Again, all quercetin metabolites excreted in urine varied in different volunteers (Figure 6.11A). In addition, the glucuronidated form of quercetin was still the major form found in urine. There were 65% of glucuronidated quercetin, 32% of sulfated quercetin and 3% of methylated quercetin (Figure 6.11B).

The total urinary quercetin metabolites between single and co-consumption were compared. The bar chart from Figure 6.12B shows that the mean of total quercetin metabolites after a single consumption was different from the mean of total quercetin metabolites after co-consumption but not significantly (*p*-value = 0.057). The line graph of the total urinary quercetin metabolites between single and co-consumption also constructed and revealed that the total urinary quercetin metabolites were increased in volunteers 7, 9, 10, 19 after co-consumption of red onion soup and 70% cocoa dark chocolate (Figure 6.13A). However, it is quite difficult to interpret the results of other volunteers using this line graph. Thus, another type of line graph, the difference of total quercetin urinary metabolites between co- and single consumption, was prepared (Figure 6.13B). From this data, the total quercetin urinary metabolites were increased after co-consumption of two types of food. Furthermore, it can be divided into 3 levels; high level for volunteers 7 and 10, intermediate level for volunteers 9 and 19 and low level for the rest, except volunteer 13. In volunteer 13, the total quercetin metabolites decreased after co-consumption of red onion soup and 70% cocoa dark chocolate.

A.



B.

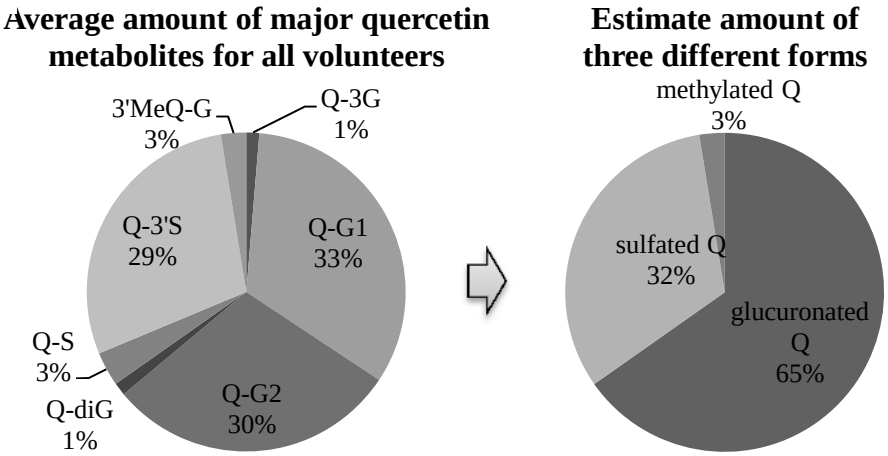


Figure 6.11: A. Seven urinary quercetin metabolites found in 24 hr urine samples from each volunteer. B. The percentage of the average amount of major quercetin metabolites for all volunteers (on the left). These amounts can be converted into three main forms (on the right).

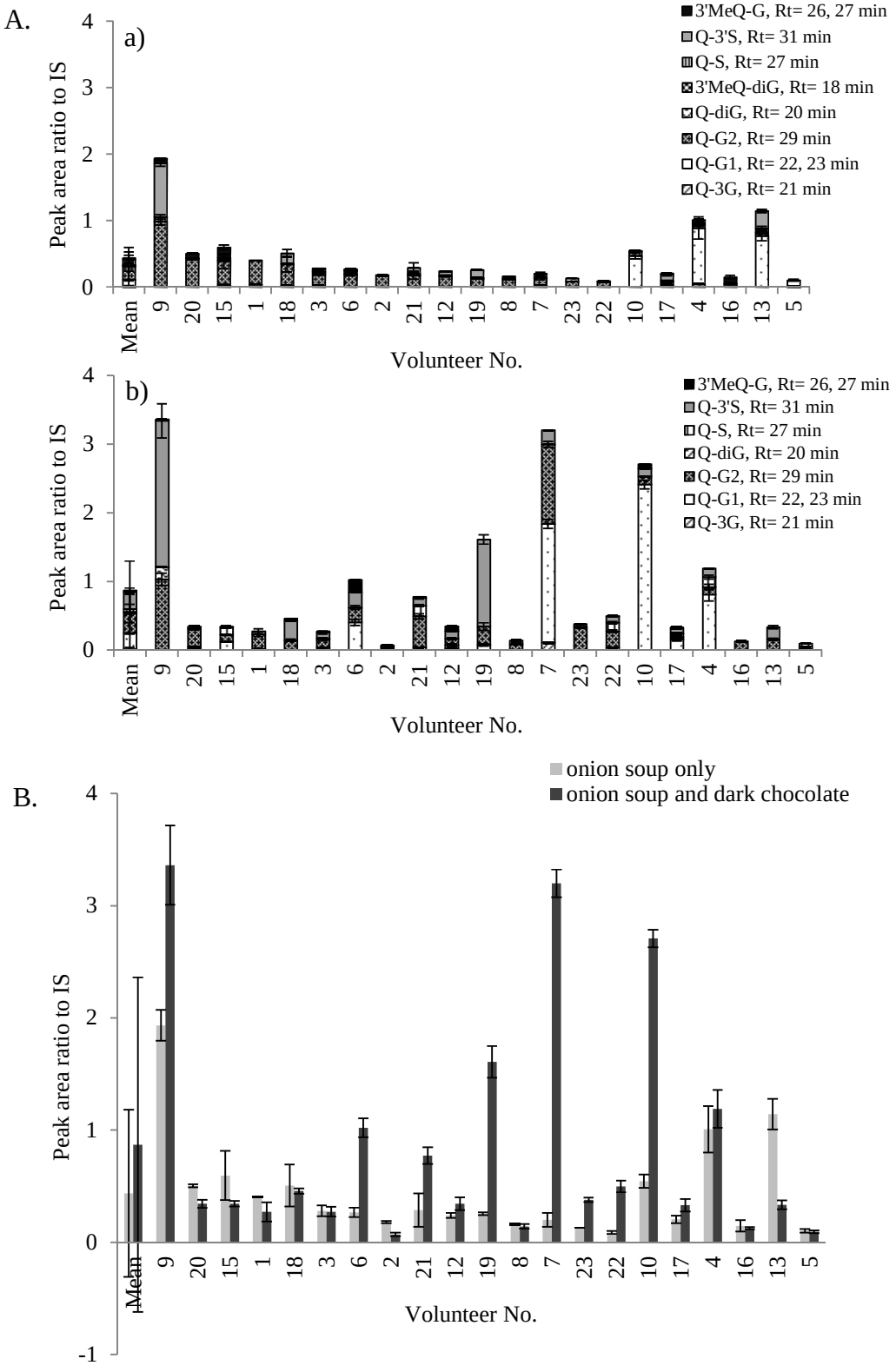


Figure 6.12: A. All urinary quercetin metabolites after (a) single and (b) co-consumption. B. Comparison of total urinary quercetin metabolites between single and co-consumption. There was no difference between the mean of single and co-consumption, *p*-value = 0.057

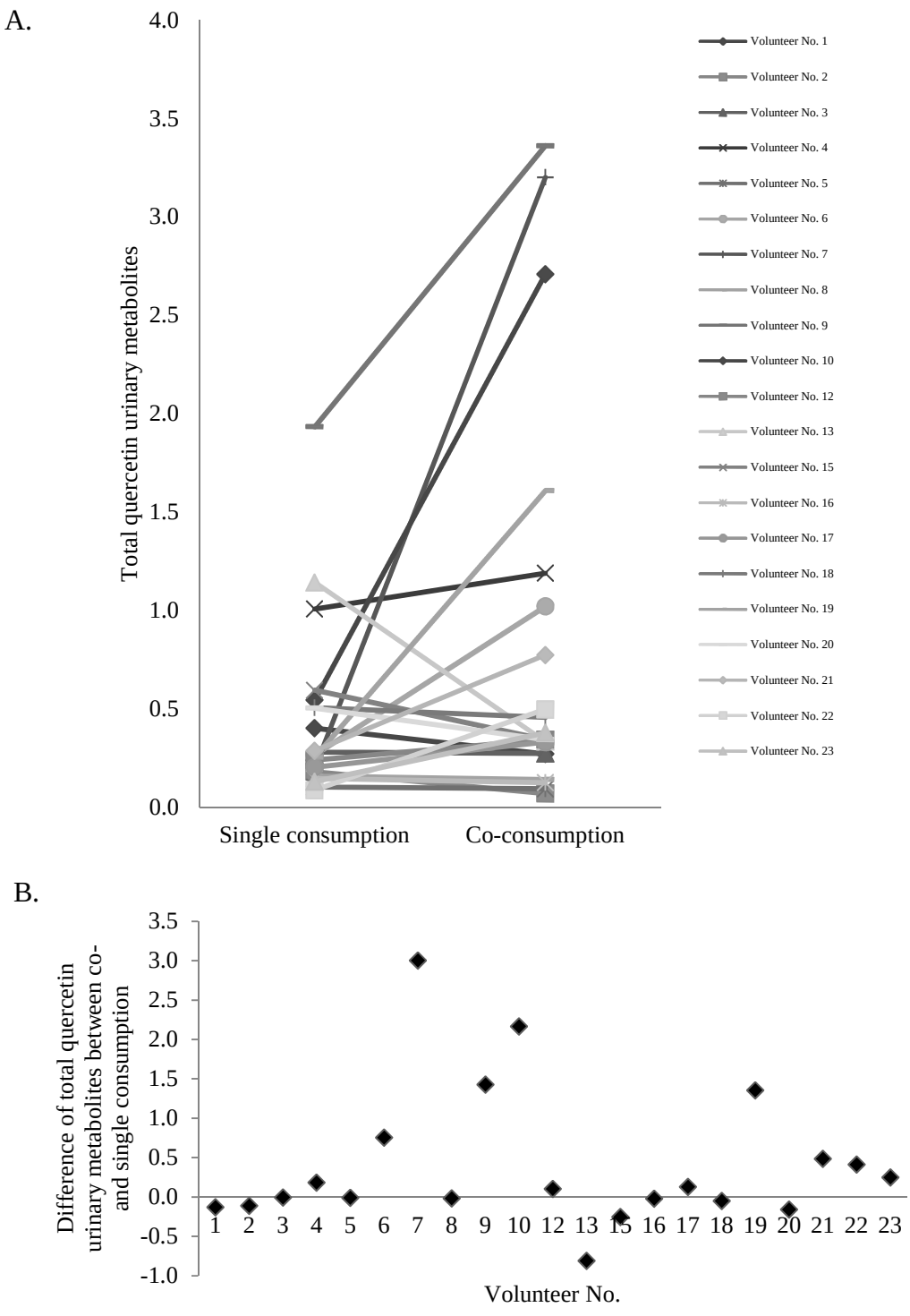


Figure 6.13: A. Trend of total quercetin urinary metabolites from single to co-consumption in each volunteer. B. The difference in total quercetin urinary metabolites between co-and single consumption.

6.4.4 Urine metabolites of (-)-epicatechin after co-consumption of onion soup and dark chocolate in healthy volunteers

After co-consumption of onion soup and 70% cocoa dark chocolate, all urinary metabolites were found to be the same as those metabolites after consuming a single dose of 70% cocoa dark chocolate. All urinary (-)-epicatechin metabolites did not show a massive variation from one volunteer to another (Figure 6.14A). In addition, methylated-(-)-epicatechin was still the main form detected in urine. There were 53%, 40% and 7% for methylated-(-)-epicatechin, sulfated-(-)-epicatechin and glucuronidated-(-)-epicatechin, respectively (Figure 6.14B).

The total urinary (-)-epicatechin metabolites between single and co-consumption were compared (Figure 6.15B). This bar chart illustrates that the mean of total (-)-epicatechin metabolites after a single consumption was no different to the mean of total (-)-epicatechin metabolites after co-consumption, *p*-value is 0.095. The line graph of the total urinary (-)-epicatechin metabolites between single and co-consumption (Figure 6.16A) and the difference of total (-)-epicatechin urinary metabolites between co- and single consumption (Figure 6.16B) are represented and show that the total urinary (-)-epicatechin metabolites vary. The highest increase in the total (-)-epicatechin urinary metabolites after co-consumption of red onion soup and 70% cocoa dark chocolate was observed in volunteer 24. The total (-)-epicatechin urinary metabolites also increased in volunteer 4, 6, 7, 18 and 19. In other volunteers, the total (-)-epicatechin metabolites had changed, increasing or decreasing, but in moderation.

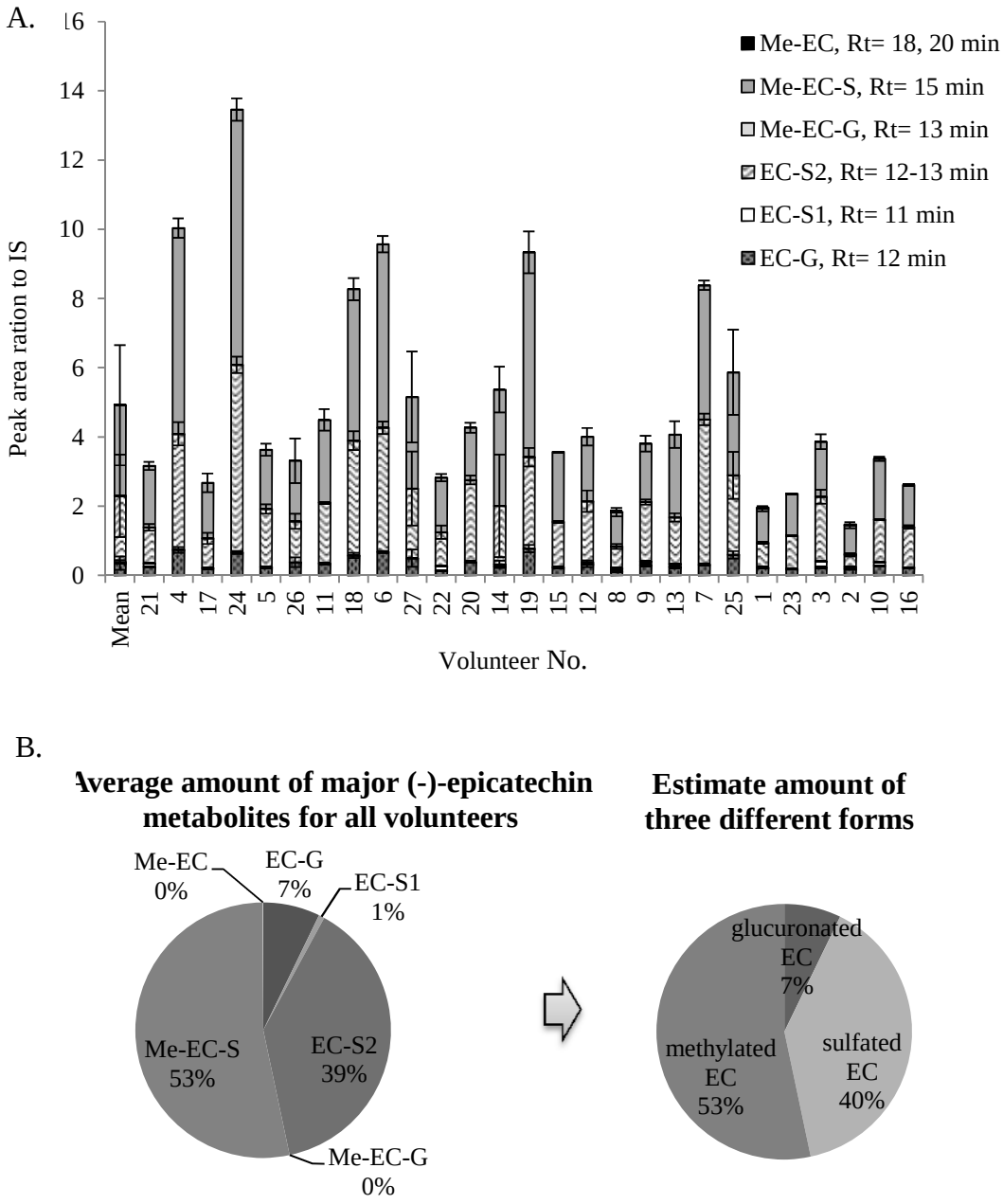


Figure 6.14: Six urinary (-)-epicatechin metabolites found in 24 hr urine in each volunteer. B. The percentage of the average amount of major quercetin metabolites for all volunteers (on the left). These amounts can be converted into three main forms (on the right).

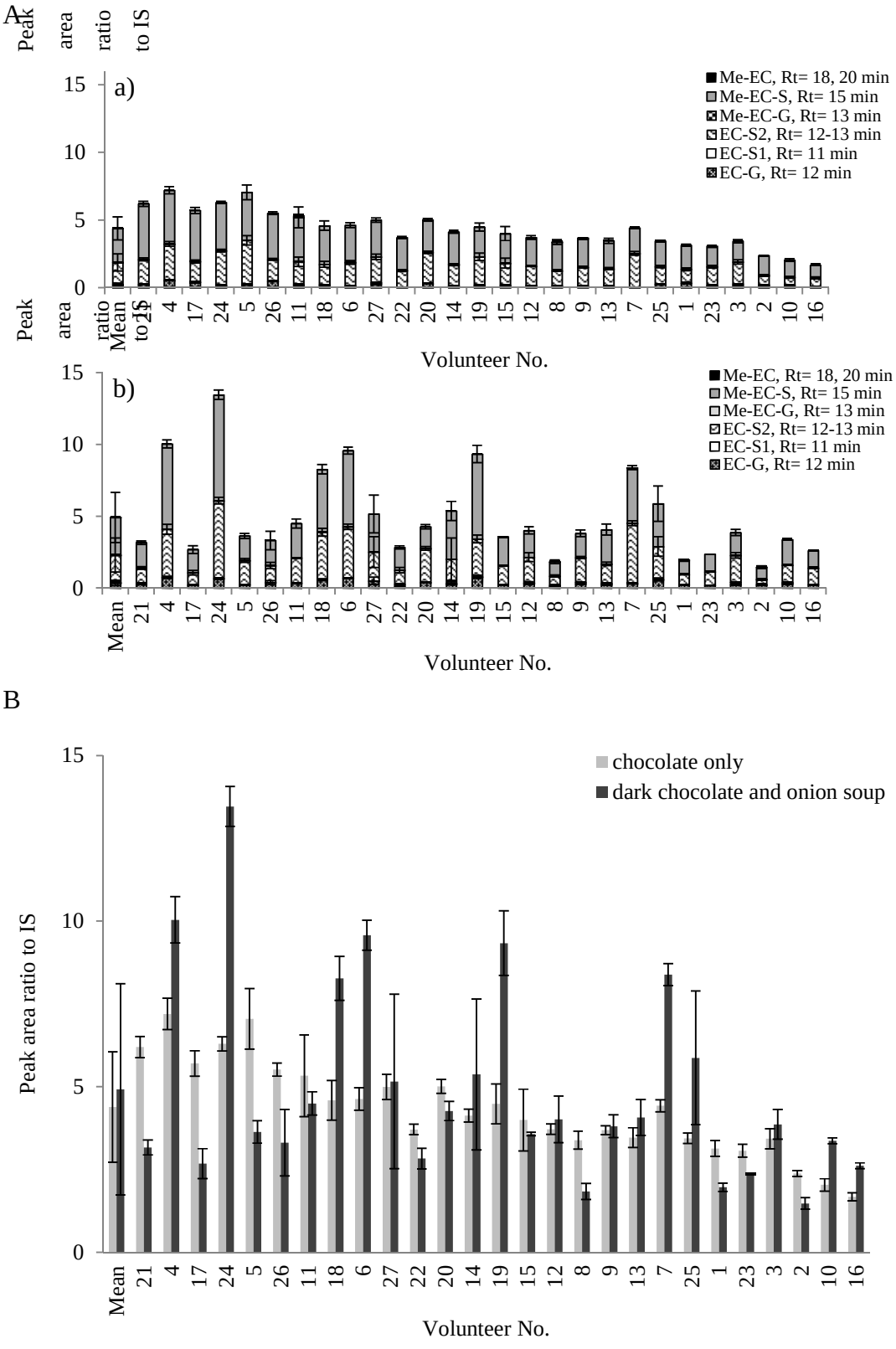


Figure 6.15: A. All urinary (-)-epicatechin metabolites after (a) single and (b) co-consumption B. Comparison of total urinary quercetin metabolites between single and co-consumption. There was no difference between the mean of the single or co-consumption, *p*-value = 0.095

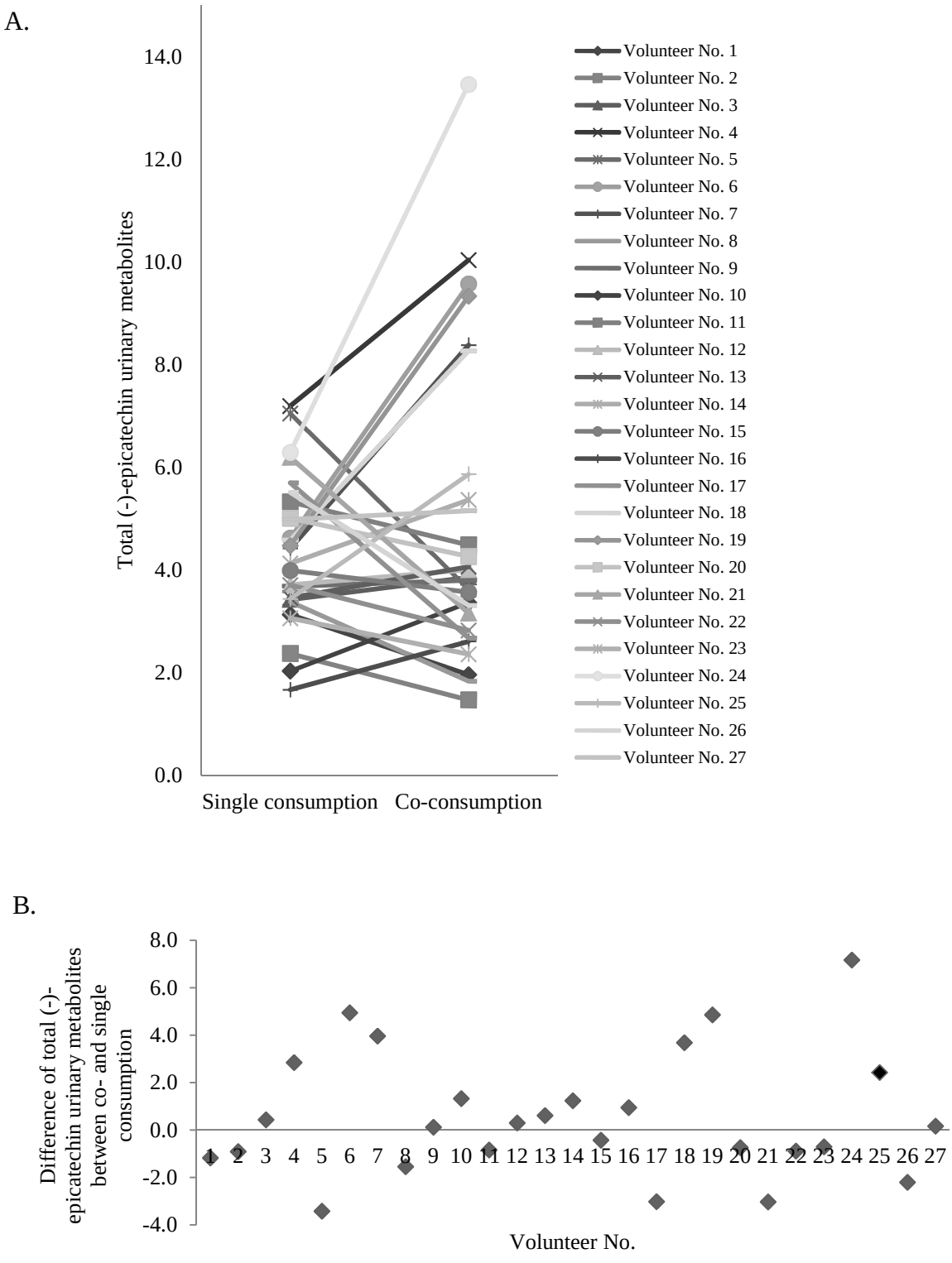


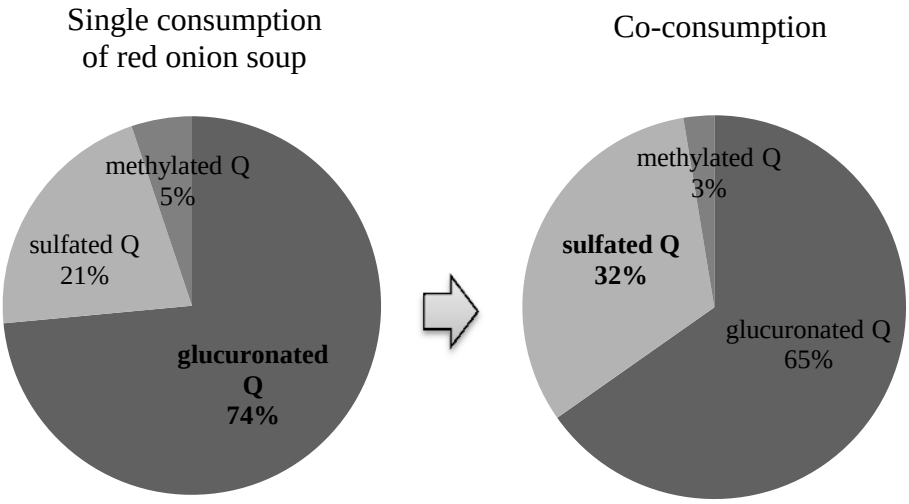
Figure 6.16: A. The total urinary (-)-epicatechin metabolites of 24 hr urine collection after a single consumption of red onion soup and co-consumption red onion and 70% cocoa dark chocolate in each volunteer. B. The difference of total (-)-epicatechin urinary metabolites between co-and single consumption.

6.4.5 Comparison of estimated amount of three different forms between single consumption and co-consumption of onion soup and dark chocolate in healthy volunteers

The estimated amount of three different forms after consumption of onion soup and co-consumption is compared (Figure 6.16A). This pie chart shows that glucuronidated quercetin and methylated quercetin excreted in urine decreased, while sulfated quercetin increased after consuming red onion soup and 70% cocoa dark chocolate.

For 70% cocoa dark chocolate and co-consumption, this pie chart illustrates that methylated (-)-epicatechin decreased, whereas, glucuronidated and sulfated (-)-epicatechin improved after consuming red onion soup and 70% cocoa dark chocolate (Figure 6.16B).

A.



B.

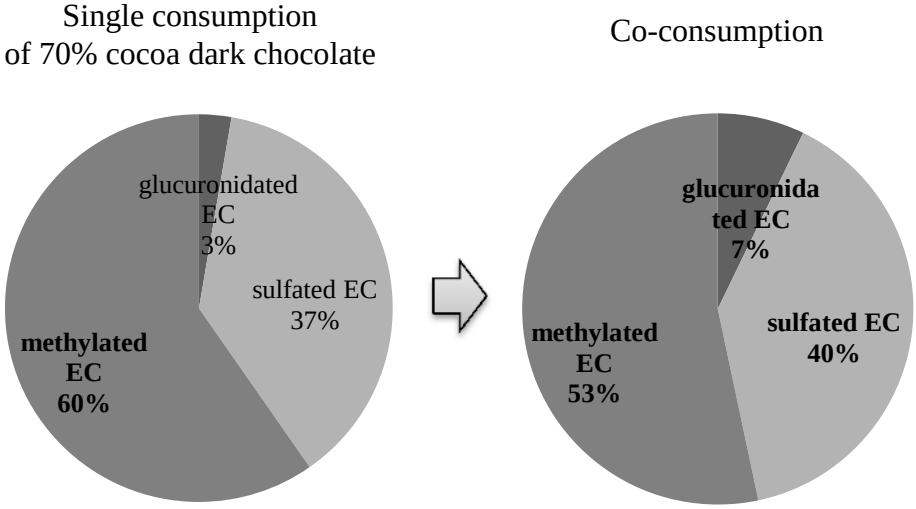


Figure 6.17: Comparison of three different conjugated forms; glucuronidated, sulfated and methylated forms, between single and co-consumption. A. Single consumption of red onion soup and co-consumption. B. Single consumption of 70% cocoa dark chocolate and co-consumption.

6.5 Discussion

6.5.1 Single consumption of red onion soup

For a single consumption of red onion soup, eight conjugated quercetin metabolites; three forms of quercetin glucuronides, two forms of quercetin sulfates, quercetin-di-*O*-glucuronides, 3′-*O*-methyl-quercetin-*O*-glucuronide and 3′-*O*-methyl quercetin-di-*O*-glucuronide was identified in this study. These metabolites are also the predominant ones out of eighteen urinary quercetin metabolites found by Mullen et al, (2006). They found quercetin-diglucuronide was the major metabolite in urine followed by quercetin-glucoside-sulfates, quercetin-4′-glucuronide and quercetin-3′-sulfate. In our study, quercetin glucuronides were the major urinary metabolites and found in three different forms, while, quercetin-diglucuronide was the major metabolite in Mullen et al.'s (2006) study. These major metabolites were different, possibly due to the differences in the amount of quercetin intake. In our study we provided a lower amount of quercetin (50 g of red onion soup containing 109 µmol of quercetin in 130 ml) to volunteers than Mullen et al. (2006). They provided 270 g of fried onions, containing 275 µmol quercetin 3, 4′di-*O*-glucoside and quercetin 4′-*O*-glucoside. Thus, the initial amount of substrate for uridine diphosphate glucuronosyl transferase in glucuronidation of Mullen et al. (2006) study is high enough to produce quercetin conjugates and further conjugated with glucuronic acid, resulting di-*O*-glucoside form. The second most common urinary metabolite in our study was quercetin-3′-sulfate, while, quercetin glucoside sulfates was the second most metabolite excreted in urine found in Mullen et al.'s (2006) study. In this study quercetin-3′-sulfate was confirmed by spiking into the 24 hr urine sample with synthesised standard quercetin-3′-sulfate which was available in the laboratory. This data agree with Mullen et. al, (2006) that although it did exist in urine, it was a low amount and insufficient for quantification. In the case of quercetin-glucoside-sulfates, it may be excreted and found in urine in our volunteers but due to being unsuccessful in synthesising quercetin-glucoside-sulfates in Chapter 5 and the lack of commercial standard then these quercetin glucoside sulfates were not investigated in all volunteers. For red onion soup consumption, quercetin seems to have a better affinity to uridine diphosphate glucuronosyl transferase (UGT) than sulfotransferase (SULT) and catechol-O-methyl transferase (COMT). That is the reason why the glucuronides form found in most urine samples followed by sulfate form and a tiny

amount in methylated form. The result from this study showed 74% for glucoronided forms, 21% for sulfated forms and 5% for methylated forms. In addition, the different activity of each uridine diphosphate glucuronosyl transferase isoform and also the position of *O*-glucuronidation of uridine diphosphate glucuronosyl transferase resulted in different products and different conjugated forms, respectively. In 2002, Boersma et al. revealed that isoenzyme of uridine diphosphate glucuronosyl transferase were involved in the glucuronidation of quercetin. Moreover, individual uridine diphosphate glucuronosyl transferase can convert quercetin into different percentages of metabolites. For example, Boersma et al. (2002) found that UGT1A1 and UGT1A8 found most in the human intestine can convert quercetin to its product around 30% (6% for 7-*O*-Glucuronide, 0% for 3-*O*-Glucuronide, 9% for 4′-*O*-Glucuronide and 85% for 3′-*O*-Glucuronide) and 20% (13% for 7-*O*-Glucuronide, 8% for 3-*O*-Glucuronide, 9% for 4′-*O*-Glucuronide and 70% for 3′-*O*-Glucuronide), while, UGT1A9 (50% for 7-*O*-Glucuronide, 26% for 3-*O*-Glucuronide, 5% for 4′-*O*-Glucuronide and 19% for 3′-*O*-Glucuronide) found most in humans and its conversion rate was 36%. In addition, the results from our study showed that the total metabolites of quercetin excreted in urine by each volunteer was largely different. This difference could be due to the polymorphism of uridine diphosphate glucuronosyl transferase in each volunteer. For example, UGT1A1 is a highly polymorphic isoform, deficiency in its expression or activity may lead to genetic and developed diseases.

6.5.2 Single consumption of 70% cocoa dark chocolate

For the single consumption of 70% cocoa dark chocolate, methylated-(-)-epicatechin-sulfate was mainly found in urine samples similar to the results obtained by Actis-Goretta et al. (2012). They found 3′-*O*-methyl-(-)-epicatechin-5-sulfate metabolites most in 24 hr urine after the single consumption of 100 g of chocolate. Thus, (-)-epicatechin seems to be a suitable substrate for catechol-*O*-methyl transferase (COMT) and sulfotransferase (SULT) than uridine diphosphate glucuronosyl transferase (UGT). Then, methylated forms of (-)-epicatechin were found most in urine samples followed by sulfated (-)-epicatechin and glucuronidated (-)-epicatechin. There were 60%, 37% and 3%, respectively. However, data from Actis-Goretta et al. (2012) showed 43% of methylated forms followed by 39% of glucuronidated and 18% sulfated form of epicatechin excreted in 24 hr. The

methylated and sulfated form in our study seems to be higher than Actis-Goretta et al. (2012) but glucuronidated forms are much lower. This difference may possibly be due to the amount of (-)-epicatechin fed to volunteers and also the number of volunteers attending in this study. A greater amount of substrate such as (-)-epicatechin in the circulation may enhance more the activity of phase II enzymes and the large number of volunteers will give more precise metabolic results. Furthermore, this study found that the total metabolites of (-)-epicatechin after a single consumption of 70% cocoa dark chocolate did not show a vast variation in each volunteer.

6.5.3 Co-consumption of red onion soup and 70% cocoa dark chocolate

After co-consumption of red onion soup and 70% cocoa dark chocolate, interestingly, the total quercetin metabolites was apparently increased in volunteer 7, 9, 10, 19. For (-)-epicatechin metabolites, there were no obvious differences. The total (-) epicatechin metabolites of some volunteers were increased, while, others were decreased and did not change between single and after co-consumption. From the review papers, the percentage of urinary excretion of quercetin is 4.7%, while it was 21% for (-)-epicatechin (Mullen et. al, 2006; Actis-Goretta et al., 2012) In this study, the amount of (-)-epicatechin found in urine was higher than quercetin. Thus, the results of this study support the previous publication. The urinary quercetin metabolites in all volunteers were vastly different when compared to urinary (-)-epicatechin metabolites. From the huge difference in their urinary excretion, quercetin seems not to be eliminated by the kidney with the same efficacy as (-)-epicatechin. It is possibly due to the polymorphism of phase II enzyme in some volunteers. In addition, different groups of the population may have a different pattern of metabolites, in this case a group of Caucasian people were used to study, possibly leading to the unique metabolic profile. The relationship between the polymorphic of phase II enzyme like COMT and the urinary excretion of polyphenol has been investigated by Inoue-Choi et al. (2010). They found that subjects who carry the homozygous low-activity (LL) associated COMT genotype significanly reduce the urinary metabolites of tea polyphenols (EGC and EC) and its metabolites (4′-MeEGC, M4, and M6) after daily drinking of green tea approximately 35-45% compare to HH genotype. In addition, they suggested that the remaining metabolites may stay longer in the circulation and derive greater benefits for health. However, Miller et al. (2012) also investigated the influence of COMT genotype on plasma

metabolites of green tea catechins in humans and found that they were indifferent to the metabolic profiles of the plasma catechin between COMT groups. Thus, COMT polymorphic has no effect on the absorption and elimination of green tea catechin, epigallocatechin gallate (EGCG).

6.5.4 Problems and solutions of concentrated urine samples to Electrospray Ionisation (ESI)

Due to the low percentage of urinary excretion of quercetin and (-)-epicatechin detected in Chapter 4, accompanied by a low amount of quercetin and (-)-epicatechin spiked with synthetic conjugated standard in 24 hr urine in Chapter 5, thus the baseline and 24 hr urine samples were then concentrated by freeze-drying first. Concentrated urine samples may cause ion suppression or reduce a detector response of Electrospray Ionisation (ESI), the most common atmospheric pressure ionisation technique, used in LC-MS system in this study. The presence of other species (endogenous or exogenous species) in the sample matrix (in this case is urine) can reduce the ionisation efficiency of a sample by competing for ionisation or inhibiting the efficiency of ionisation then has an effect on detector response. First, a high concentration of concentrated urine in this study led to saturating the droplet surface, reduced charge excess then loss of detector response. Second, although concentrated urine contains a high concentration of quercetin or (-)-epicatechin and is easy to detect with MS, this concentrated urine also contains a high concentration of interference, leading to an increase in surface tension and viscosity, reducing in desolvation or solvent evaporation, and consequentely affecting ionisation efficiency. Finally, the non-volatile species ex. protein or salt, found in urine can cause co-precipitation of analyte in the droplet thus preventing ionisation. To evaluate the effect of other matrix in urine sample on ion suppression, baseline urine was used as a control in every single sample. In addition, to avoid an ion suppression, protein precipication was used to remove all protein species from concentrated urine followed by filtrating this urine again with 0.2 µM to make the sample clearer. In addition, to avoid or prevent co-elution of suppressing species, the chromatograpic separation was modified in this study. Finally, LC and MS sources need to be cleaned every 24 hr throught the running time in LC-MS to make sure the machine work effectively.

6.6 Conclusion

In this study, eight urinary metabolites of quercetin and six urinary metabolite of (-)-epicatechin from human urine samples collected at 24 hr were identified by UPLC-MS. Major metabolite of quercetin found most in urine is quercetin glucuronide in both single and co-consumption of onion soup. While, methylated form of (-)-epicatechin most abundant form in urine after single and co-consumption of 70% dark chocolate. To our knowledge, this is the first study on the metabolites profile of quercetin and (-)-epicatechin in human. Then, the precise biological activities or mechanism of these metabolites should be study further.

Chapter 7 Summary and Future Perspectives

7.1 Summary of thesis findings

In summary, the findings of this study showed that red onions contain a higher amount of quercetin than yellow onions in agreement with most publications. As mentioned previously, homogenisation was an efficient method for extracting quercetin glycosides than vortex and sonication. Thus, using this extraction method may produce a higher yield and can be applied for the quantification of the content of other flavonoids in food samples.

The result from the *in vitro* study showed that an extract of pig liver displayed COMT activity towards (-)-epicatechin and quercetin, producing their methylated products; 3′-*O*-methylated-(-)-epicatechin, 4′-*O*-methylated-(-)-epicatechin and 3′-*O*-methylated-quercetin. In addition, (-)-epicatechin seems to generate methylated forms better than quercetin. The results in chapter 6 also revealed that methylated conjugates occur in higher amounts in urine after consumption of (-)-epicatechin. These data are in agreement with *in vitro* data which indicated that a preference of COMT towards (-)-epicatechin.

In addition, sulfatase activity study showed that interfering substances in urine probably inhibit its activity. Then, co-treatment urine samples with β-glucuronidase and sulfatase cannot be used but all conjugated metabolites of quercetin and (-)-epicatechin found in the urine must be analysed directly. Thus, the full picture of all quercetin and (-)-epicatechin metabolites will be obtained.

To identify and quantify these metabolites, all conjugated quercetin and (-)-epicatechin found in urine must firstly be synthesised. Five quercetin conjugates and five (-)-epicatechin conjugates were successfully synthesised with a sufficient percentage yield of the synthesised conjugates. For the first time, 3′-*O*-methyl-quercetin-di-glucuronides were also produced and detected in the glucuronidation reaction of 3′-*O*-methyl-quercetin-glucuronides. In addition, methylated-(-)-epicatechin-glucuronides and methylated-(-)-epicatechin sulfates were originally synthesised by (-)-epicatechin-glucuronides and (-)-epicatechin sulfates which were the products of (-)-epicatechin glucuronidation and (-)-epicatechin sulfation reaction, using COMT from cytosol pig liver. These enzymatic conjugates are not

only used for identifying quercetin and (-)-epicatechin (in chapter 6). Furthermore, these enzymatic conjugates can be use to study the biological effect of quercetin or (-)-epicatechin metabolites in cells or other tissue in the future.

In this study, eight urinary metabolites of quercetin and six urinary metabolite of (-)-epicatechin from human urine samples collected at 24 hr were identified by UPLC-MS. The major metabolite of quercetin found most in urine is quercetin glucuronide in both single and co-consumption of onion soup. While, the methylated form of (-)-epicatechin is the most abundant form in urine after single and co-consumption of 70% dark chocolate. To our knowledge, this is the first study on the metabolites profile of quercetin and (-)-epicatechin in human. Then, the precise biological activities or mechanism of these metabolites should be study further.

7.2 Future Perspectives

To fulfil this research work, the biological effects of quercetin or (-)-epicatechin and its metabolic conjugated products should be investigated in cell culture with or without phase II enzyme expression. As reviewed, COMT will generate 3′ or 4′ methylated (-)-epicatechin from (-)-epicatechin. The overexpression of COMT may lead to the higher product of 3□ or 4□□methylated (-)-epicatechin, resulting in the NADPH inhibition and have a health benefit in the endothelial function.

In addition, COMT also link with DNA methylation. Some polyphenol like EGCG can inhibit COMT and subsequent to inhibit DNA methyltransferase indirect way. The Figure 7.1 shows SAH can inhibit DNMT and then can interrupt or reverse the carcinogen process of cancer. Thus, this is another aspect which needs to be considered.

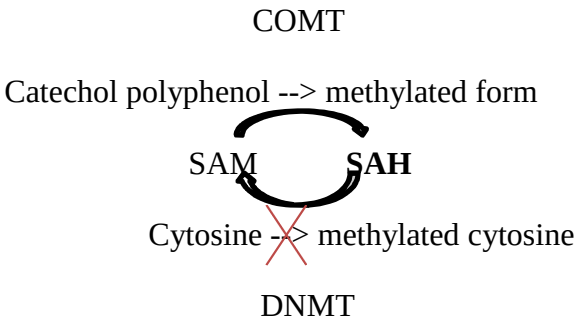


Figure 7.1: The relationship between catechol-*O*-methyltransferase (COMT) and DNA methyltransferase (DNMT)

In the case of quercetin, it has exhibited a wide range of beneficial biological activities including act as drug bioavailability enhancers. The bioavailability, blood levels and efficacy of various drugs such as diltiazem and digoxin were increased by quercetin. In addition, the absorption of flavonoid like epigallocatechin gallate was enhanced after consumption red onion supplement, containing as well a rich source of quercetin (Kesarwani, Gupta and Mukerjee 2013). Thus, co-consumption of quercetin with other types of flavonoids may enhance its bioavailability and biological activity.

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Appendices

A. Ethical Application Form

UNIVERSITY OF LEEDS RESEARCH ETHICS COMMITTEE APPLICATION FORM [1]

UNIVERSITY OF LEEDS

Ethics reference number:	
Grant reference and/ or student number:	

PART A: Summary

A.1 Which Faculty Research Ethics Committee would you like to consider this application? [2]

- ○ Arts and PVAC (PVAR)
- ○ Biological Sciences (BIOSCI)
- ○ ESSL/ Environment/ LUBS (AREA)
- ◉ MaPS and Engineering (MEEC)
- ○ Medicine and Health (Please specify a subcommittee):
  - ○ Leeds Dental Institute (DREC)
  - ○ Health Sciences/ LIGHT/ LIMM
  - ○ School of Healthcare (SHREC)
  - ○ Medical and Dental Educational Research (EdREC)
  - ○ Institute of Psychological Sciences (IPSREC)

A.2 Title of the research [3]

Effect of combination of foods on flavonoid absorption into the body.

A.3 Principal investigator's contact details [4]	
Name *(Title, first name, surname)*	Miss Patthamawadee Charoensuk
Position	PhD student
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	Faculty of Mathematics and Physical Sciences
Work address *(including postcode)*	School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK
Telephone number	07740291870
University of Leeds email address	fspc@leeds.ac.uk

A.4 Purpose of the research: [5] (Tick as appropriate)

- [x] Research
- [x] Educational qualification: ***Please specify:*** _____*PhD*_____
- [ ] Educational Research & Evaluation [6]
- [ ] Medical Audit or Health Service Evaluation [7]
- [ ] Other

A.5 Select from the list below to describe your research: (You may select more than one)

- [x] Research on or with human participants
- [ ] Research with has potential significant environmental impact.[8] If yes, please give details:

  _____
- [ ] Research working with data of human participants
  - [ ] New data collected by questionnaires/interviews
  - [ ] New data collected by qualitative methods
  - [ ] New data collected from observing individuals or populations
  - [ ] Research working with aggregated or population data
  - [ ] Research using already published data or data in the public domain
- [ ] Research working with human tissue samples [9]

A.6 Will the research involve any of the following: [10] (You may select more than one)

If your research involves any of the following an application must be made to the National Research Ethics Service (NRES) via IRAS www.myresearchproject.org.uk as NHS ethical approval will be required. There is no need to complete any more of this form. Contact governance-ethics@leeds.ac.uk for advice.

- ☐ Patients and users of the NHS (including NHS patients treated in the private sector) [11]
- ☐ Individuals identified as potential participants because of their status as relatives or carers of patients and users of the NHS
- ☐ Research involving adults in Scotland, Wales or England who lack the capacity to consent for themselves [12]
- ☐ A prison or a young offender institution in England and Wales (and is health related) [14]
- ☐ Clinical trial of a medicinal product or medical device [15]
- ☐ Access to data, organs or other bodily material of past and present NHS patients [9]
- ☐ Use of human tissue (including non-NHS sources) where the collection is not covered by a Human Tissue Authority licence [9]
- ☐ Foetal material and IVF involving NHS patients
- ☐ The recently deceased under NHS care
- ☑ None of the above

You must inform the Research Ethics Administrator of your NRES number and approval date once approval has been obtained.

If the University of Leeds is not the Lead Institution, or approval has been granted elsewhere (e.g. NHS) then you should contact the local Research Ethics Committee for guidance. The UoL Ethics Committee need to be assured that any relevant local ethical issues have been addressed.

A.7 Will the research involve NHS staff recruited as potential research participants (by virtue of their professional role) or NHS premises/ facilities?

☐ Yes ☑ No

If yes, ethical approval must be sought from the University of Leeds. Please note that NHS R&D approval is needed in addition, and can be applied for concurrently: www.myresearchproject.org.uk. Contact governance-ethics@leeds.ac.uk for advice.

A.8 Will the participants be from any of the following groups? (Tick as appropriate)

- ☐ Children under 16 [16]
- ☐ Adults with learning disabilities [12]
- ☐ Adults with other forms of mental incapacity or mental illness
- ☐ Adults in emergency situations
- ☐ Prisoners or young offenders [14]
- ☑ Those who could be considered to have a particularly dependent relationship with the investigator, e.g. members of staff, students [17]
- ☐ Other vulnerable groups
- ☐ No participants from any of the above groups

Fifty participants, both male and female, will be recruited after a positive voluntary response to a local advertisement in the School of Food Science and Nutrition (by using the email distribution list for the School) and by poster on the campus, targeting primarily students and staff of the university (see **Appendix 1**).

Positive respondents may include members of the same department (Food Science and Nutrition) or members of the university from unrelated departments. No student or staff member will be approached directly or coerced. The participants will be free to withdraw from the study at any point without giving a reason. Students and staff will not have to travel purposely to participate in the experiments, but would be already present on site.

A Criminal Record Bureau (CRB) check will be needed for researchers working with children or vulnerable adults (see www.crb.gov.uk)

A.9 Give a short summary of the research [18]

Flavonoids are a class of water-soluble plant pigments which are found in a wide range of foods and may reduce the risk of chronic diseases such as cardiovascular diseases[1]. These biological effects depend on the amount of flavonoid taken up by the intestine and distributed to the target tissues.

One important factor that affects absorption, distribution and excretion from the body of flavonoids is an enzyme in our body called "Catechol-O-methyltransferase or COMT". When a flavonoid moves across the intestinal tract and to the liver through blood circulation, COMT will add a methyl group to the flavonoid, resulting in a chemical change. The extent of this change can affect the bioavailability (absorption, distribution and excretion) and the biological effect of flavonoids and varies between people.

This research will study the correlation between COMT activity and the metabolism of two dietary flavonoids, quercetin or epicatechin. In this research, the urinary content (over 24 hr) of quercetin, epicatechin and their total methylated products will be measured after consumption of 50 g of quercetin from onion soup or 50 g of epicatechin from dark chocolate or co-consumption of both of them in 50 volunteers. (For more details see C2.)

The outcome of this human study should provide bioavailability data on quercetin and epicatechin which could be important for individual dietary advice.

References:

*LOKE W.M., HODGSON J.M., et al. 2008. Pure dietary flavonoids, quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy human volunteers. Am. J. Clin. Nutr. 88: 1018-1025.

A.10 What are the main ethical issues with the research and how will these be addressed? [19]

The major ethical issue is using human participants in the proposed research. This issue will be addressed by:

1) Obtaining informed consent from the participant

The study coordinator will provide a clear, concise Participant Information Sheet (see **Appendix 2**) and Informed Consent Form (see **Appendix 3**) for the volunteers to read, fill in, and sign prior to commencement of the study. When the study coordinator has determined that the candidate is an appropriate subject for the study, the background of the study (including purpose, duration, procedures, potential discomforts and their minimization, lack of risk associated with participation in the study, potential benefits, confidentiality, disclosure of results, participation and withdrawal, questions and concerns of the participant) will be described and explained orally to the subject. The study coordinator will fully answer all questions to the satisfaction of the individual. Written informed consent will be obtained from each subject by the study coordinator prior to enrolment in the study (7 days before). The consent form will be signed and dated by the subject and the study coordinator. The consent form will be copied twice; one copy for the records of the study (held by the study coordinator), and one copy for the personal records of the subject. No subject will participate in the study before completion of the written informed consent. More importantly, subjects will be free to withdraw at any time, without giving a reason.

2) Pre-study questionnaire

In order to assess whether the volunteers are suitable for the study or not, they will be asked to complete questions related to their life style and relevant medication history (see **Appendix 4**). All volunteers will be assured that their participation is completely voluntary. Asking about personal information for example; weight, height, age, race, sex and medical history may be considered as a sensitive issue.

3) Slight discomfort/inconvenience caused by the restricted diet 24h and over-night fasting 12h prior to a study day.

The discomfort suffered by the participant due to the restricted diet (avoidance of quercetin and epicatechin rich food, **Appendix 5**) 24 hours before, and the over-night fasting 12 hours before, a study day can be considered as mild. However, there are many foods which are allowed for this study (see **Appendix 6**).

4) Storage and confidentiality of data

Confidentiality of all study participants will be maintained; codes (ID numbers) for participant identification will be used, and they will be assigned on the day of the study. All data collected will be considered as confidential, and stored securely in a locked filing cabinet according to current University regulations. It will not be possible to identify the individuals from the codes in any way. The linkage between the participant's identity and ID number will be kept in written form only, and stored in a locked filing cabinet in a restricted access area. Data evaluation will only be performed using ID numbers. Anonymised data will be stored for 5 years in accordance with the University of Leeds guidelines on the password protected M-drive of the University server.

5) Urine collection and acellular urine samples

Urine specimens collected from the participants will be rendered acellular within 6 hours of collection before storage. (For the standard protocol being used for removing cellular debris see **Appendix 10**) Urine specimens thus treated are not classified as human tissue. The analysis of the urine specimens will be limited to flavonoids and their metabolites only, and therefore no specific data on the health status of volunteers will be obtained from them.

After the end of this research study (31st September 2014), urine specimens will be disposed using "waste disposal" method the same as Appendix10.

6) Ethics Committee Approval:

This human study will be submitted to the Ethics Committee of the Faculty of Mathematical and Physical Sciences (MAPS) at the University of Leeds.

PART B: About the research team

B.1 To be completed by students only [20]	
Qualification working towards (eg Masters, PhD)	PhD
Supervisor's name *(Title, first name, surname)*	Professor Gary Williamson
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	Faculty of Mathematics and Physical Sciences
Work address *(including postcode)*	School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK
Supervisor's telephone number	01133438380
Supervisor's email address	g.williamson@leeds.ac.uk
Module name and number *(if applicable)*	-

B.2 Other members of the research team (eg co-investigators, co-supervisors) [21]	
Name *(Title, first name, surname)*	Doctor Andrea Day
Position	Co-supervisor
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	Faculty of Mathematics and Physical Sciences
Work address *(including postcode)*	School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK
Telephone number	01133432974
Email address	a.j.day@leeds.ac.uk

Name (Title, first name, surname)	Doctor Caroline Orfila
Position	Co-supervisor
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	Faculty of Mathematics and Physical Sciences

Work address (including postcode)	School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK
Telephone number	01133432966
Email address	c.orfila@leeds.ac.uk

Part C: The Research

C.1 What are the aims of the study? [22]

The aim of this research is to study the correlation between COMT activity and the metabolism of two dietary flavonoids, quercetin or epicatechin. This will be achieved by measuring the urinary content of quercetin, epicatechin and their total methylated products after consumption of onion soup or dark chocolate, or both of them together, in healthy human subjects.

C.2 Describe the design of the research. Qualitative methods as well as quantitative methods should be included.

1) Study design and programme:

This study is three instances of a single-dose with 24 hours urine collection. All volunteers will be requested to complete the consent form and pre-study questionnaire prior to participation in this study. It is anticipated that 50 volunteers will be recruited in this study. The study can be divided into three separate days, each will have a minimum of 24 hrs between then:

Day 1:

Volunteers will be asked to void their bladder immediately after waking, and then have a glass of water (200-300 ml). Approximately an hour later, the volunteers should again void their bladder and this time collect the urine sample in the baseline-labelled bottle at the School of Food Science and Nutrition.

Soon after the baseline urine collection, the volunteers will be asked to consume **onion soup** (containing 50 g of onion) provided along with bread or toast and butter for breakfast in the School of Food Science and Nutrition. The time of consumption should be recorded.

Urine should be collected in labelled bottles for time point 0-24 hr. Lunch will be provided. All other foods consumed during the day should be recorded using 24 hour dietary record (Appendix 8). Crucially, quercetin and epicatechin rich foods should be avoided (see Appendix 5).

Day 2:

Volunteers will be asked to do the same procedure as Day 1 study but **50 g of dark chocolate** will be provided to volunteers instead of onion soup.

Day 3:

Volunteers will be asked to void their bladder immediately on waking, and then have a glass of water (200-300 ml). Again, approximately an hour later, the volunteers should again void their bladder and this time collect the urine sample in the baseline-labelled bottle at the School of Food Science and Nutrition.

Soon after the baseline urine collection, the volunteers will be asked to consume **onion soup (containing 50 of onion) and 50 g of dark chocolate together** provided along with bread or toast and butter for breakfast in the

School of Food Science and Nutrition. The time of consumption should be recorded.

Urine should be collected in labelled bottles for time point 0-24 hr. Lunch will be provided. All other foods consumed during the day should be recorded using 24 hour dietary record (Appendix 8). Crucially, quercetin and epicatechin rich foods should be avoided (see Appendix 5).

Remark: The collection bottles which have a secure lid will be labelled and provided by the study coordinator. The participants will be transporting only one urine sample (time point 0-24 hr) to the university on the next study day.

2) Statistical Analysis

COMT activity and methylated product of quercetin or epicatechin in each subject will be analysed using "Pearson Correlation Analysis".

C.3 What will participants be asked to do in the study? [23]

At recruitment, each participant is asked to fill in a questionnaire asking for information on gender, race, age, weight, height, histories of tobacco, usual dietary habit and medical history. This information is used for the assessment of the subjects. Answer to the questionnaire will determine the subjects suitability based on the following parameters:

- Healthy male and female
- Caucasian (For more details see C8.)
- 18 to 60 years of age
- Normal body mass index (BMI) between 18.5 and 24.99 kg/m^2 (principal cut-off points according to World Health Organization)
- Non-smoker
- No dietary supplements or medication
- No GI tract or metabolic and chronic diseases (eg. Hepatic or Kidney disease, Cardiovascular disease, Cancer)
- Not pregnant (For more details see C8.)

Written informed consent is then obtained from all subjects (7 days before the beginning of the study) after being advised of the study programme regime in full prior to the start of the study.

All 50 volunteers will undergo Day 1, Day 2 and Day 3 study, respectively. During the study day subjects are required to follow the instruction and avoiding quercetin and epicatechin rich foods in their diet for the previous 24h and for the duration of the study day.

Urine sample collection will occur with the needs of each subject between to 0 to 24 hours with a control sample collection immediately before the consumption of onion soup or dark chocolate or both of them. Therefore, 2 urine samples will provide to the researcher for each study day (24 hours duration). The number of visits will be 3 times in total.

C.4 Does the research involve an international collaborator or research conducted overseas: [24]
(Tick as appropriate)

☐ Yes ☑ No

If yes, describe any ethical review procedures that you will need to comply with in that country:

Describe the measures you have taken to comply with these:

Include copies of any ethical approval letters/ certificates with your application.

C.5 Proposed study dates and duration

Research start date (DD/MM/YY): As soon as ethical approval is obtained

Research end date (DD/MM/YY): 31st September 2014

Fieldwork start date (DD/MM/YY): _____-_____ Fieldwork end date (DD/MM/YY): _____-_____

C.6. Where will the research be undertaken? (i.e. in the street, on UoL premises, in schools) [25]

The study will be conducted in the School of Food Science and Nutrition, University of Leeds.

RECRUITMENT & CONSENT PROCESSES

C.7 How will potential participants in the study be:

The identification of the subjects who will participate in the study will be done by codes, as reported in question A10, and we will not need or store any personal details of the volunteers for our aim of the study prior the beginning of the research. If they participate, they will fill in a pre-study questionnaire which will be signed by each volunteer and by the coordinator of the study.

We will recruit volunteers by showing an invitation sheet in the School of Food Science and Nutrition and in other locations at University of Leeds. Subjects will be voluntarily recruited from the student and staff population of the University of Leeds.

C.8 Will you be excluding any groups of people, and if so what is the rationale for that? [27]

The selection criteria are chosen to minimize the interference with flavonoid metabolism. Thus, subjects with any GI tract or metabolic diseases (eg. Hepatic or Kidney disease, Cardiovascular disease, Cancer) and subjects with regular dietary supplements or medication will be excluded because the criteria mentioned above could modify the absorption, distribution and the excretion of flavonoids in human body.

Moreover, subjects in other races or pregnant will be excluded because these people have a different COMT levels compare to Caucasians or non-pregnant, respectively.

C.9 How many participants will be recruited and how was the number decided upon? [28]

Based on previous studies, 50 people would be sufficient to account for inter-individual variation in flavonoid metabolism.

C.10 Will the research involve any element of deception? [29]

No.

C.11 Will informed consent be obtained from the research participants? [30]

☑ Yes ☐ No

If yes, give details of how it will be done. Give details of any particular steps to provide information (in addition to a written information sheet) e.g. videos, interactive material. If you are not going to be obtaining informed consent you will need to justify this.

Written informed consent will be sought from the participant prior to study begin. The potential participant will receive a Participant Information Sheet and the Informed Consent Form (see **Appendix 2 and 3**) after a positive reply to the recruitment advert. The main investigator will verbally explain the Participant Information Sheet, and the Informed Consent Form, and will be available for questions there and then, and at any time after. The prospective participant has approximately until 7 days prior to study begin to finally decide, sign and hand in/pass back the signed Informed Consent Form (in duplicate: a copy for the participant and one copy for the main investigator; and co-signed by the main investigator).

If participants are to be recruited from any of potentially vulnerable groups, give details of extra steps taken to assure their protection. Describe any arrangements to be made for obtaining consent from a legal representative.

Copies of any written consent form, written information and all other explanatory material should accompany this application*. The information sheet should make explicit that participants can withdrawn from the research at any time, if the research design permits.*
Sample information sheets and consent forms are available from the University ethical review webpage at http://researchsupport.leeds.ac.uk/index.php/academic_staff/good_practice/ethical_review_process/university_ethical_review-1.

C.12 Describe whether participants will be able to withdraw from the study, and up to what point (eg if data is to be anonymised). If withdrawal is not possible, explain why not.

Yes, it will be made clear to the potential participant that withdrawal is possible at any time, without giving a reason, and without questions being asked. No negative consequences or change of treatment of the participant will ensue.

C.13 How long will the participant have to decide whether to take part in the research? [31]

The participant should decide if to participate or not in the study in about 7 days prior to study starting.

C.14 What arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? [32]*(e.g. translation, use of interpreters etc. It is important that groups of people are not excluded due to language barriers or disabilities, where assistance can be given.)*

Participants who don't understand the English language will be not recruited in the study. Information about the researcher such as e-mail, and mobile phone number will be available on the information sheet.

C.15 Will individual or group interviews/ questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews/group discussions, or use of screening tests for drugs)? [33]

☐ Yes ☑ No

If Yes, give details of procedures in place to deal with these issues

The information sheet should explain under what circumstances action may be taken

C.16 Will individual research participants receive any payments, fees, reimbursement of expenses or any other incentives or benefits for taking part in this research? [34]

☑ Yes ☐ No

If Yes, please describe the amount, number and size of incentives and on what basis this was decided.

The participants will receive a Love2shop gift voucher of £15 as a compensation of their time at the termination. It can be counted as only £5 per each time which is small compared to their participation. Therefore, there should not be any potential for conflict of interest. The participants can spend this gift voucher for general merchandise from Wilkinson or Boots, school stationary and book from WH smith etc. This is also not the potential for conflict of interest.

For more details of the stores available see http://www.highstreetvouchers.com/gift-vouchers/redeemers/high-street-gift-voucher-redeemer-list.jsp

RISKS OF THE STUDY

C.17 What are the potential benefits and/ or risks for research participants? [35]

There is very low potential risk involved in the present study. The potential benefits associated to the present study are related with the consumption of fruits and vegetables, naturally rich in flavonoids, antioxidants which can prevent or minimise oxidative stress in the human body.

C.18 Does the research involve any risks to the researchers themselves, or people not directly involved in the research? *Eg lone working* [36]

☐ Yes ☑ No

If yes, please describe: _____

Is a risk assessment necessary for this research?

☐ Yes ☑ No If yes, please include a copy of your risk assessment form with your application.

Further information on fieldwork risk assessments is available at http://www.leeds.ac.uk/safety/fieldwork/index.htm.

DATA ISSUES

C.19 Will the research involve any of the following activities at any stage (including identification of potential research participants)? (Tick as appropriate)

- ☐ Examination of personal records by those who would not normally have access
- ☐ Access to research data on individuals by people from outside the research team
- ☐ Electronic transfer of data
- ☐ Sharing data with other organisations
- ☐ Exporting data outside the European Union
- ☐ Use of personal addresses, postcodes, faxes, e-mails or telephone numbers
- ☐ Publication of direct quotations from respondents
- ☐ Publication of data that might allow identification of individuals to be identified
- ☐ Use of audio/visual recording devices
- ☐ FLASH memory or other portable storage devices

Storage of personal data on or including any of the following:

- ☐ Manual files
- ☐ Home or other personal computers
- ☐ Private company computers
- ☐ Laptop computers

Only coded data will be stored in the university research drive and this cannot be linked in any way to individual participant.

C.20. How will the research team ensure confidentiality and security of personal data? E.g. anonymisation procedures, secure storage and coding of data.[37] You may wish to refer to the data protection and research webpage.

See the answer explained in Question A10.

C.21 For how long will data from the study be stored? Please explain why this length of time has been chosen.[38]

____5____ years, _____ months

NB: RCUK guidance states that data should normally be preserved and accessible for ten years, but for some projects it may be 20 years or longer.

Students: It would be reasonable to retain data for at least 2 years after publication or three years after the end of data collection, whichever is longer

CONFLICTS OF INTEREST

C.22 Will any of the researchers or their institutions receive any other benefits or incentives for taking part in this research over and above normal salary or the costs of undertaking the research? [39]

☐ Yes ☑ No

If yes, indicate how much and on what basis this has been decided

_____

C.23 Is there scope for any other conflict of interest? [40] *For example will the research funder have control of publication of research findings?*

☐ Yes ☑ No ***If yes, please explain*** _____

C.24 Does the research involve external funding? (Tick as appropriate)

☐ Yes ☑ No ***If yes, what is the source of this funding?*** _____

PART D: Declarations

Declaration by Chief Investigators

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
2. I undertake to abide by the University's ethical and health & safety guidelines, and the ethical principles underlying good practice guidelines appropriate to my discipline.
3. If the research is approved I undertake to adhere to the study protocol, the terms of this application and any conditions set out by the Research Ethics Committee.
4. I undertake to seek an ethical opinion from the REC before implementing substantial amendments to the protocol.
5. I undertake to submit progress reports if required.
6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer.
7. I understand that research records/ data may be subject to inspection for audit purposes if required in future.
8. I understand that personal data about me as a researcher in this application will be held by the relevant RECs and that this will be managed according to the principles established in the Data Protection Act.
9. I understand that the Ethics Committee may choose to audit this project at any point after approval.

Sharing information for training purposes

Optional – please tick as appropriate:

☐ I would be content for members of other Research Ethics Committees to have access to the information in the application in confidence for training purposes. All personal identifiers and references to researchers, funders and research units would be removed.

Principal Investigator

Signature of Principal Investigator: .....
(This needs to be an actual signature rather than just typed. Electronic signatures are acceptable)

Print name: Patthamawadee Charoensuk Date:(dd/mm/yyyy): 16/04/12

Supervisor of student research

I have read, edited and agree with the form above.

Supervisor's signature: .....
(This needs to be an actual signature rather than just typed. Electronic signatures are acceptable)

Print name: Professor Gary Williamson Date:(dd/mm/yyyy): 17/04/12

Co-supervisor's signature: .....
(This needs to be an actual signature rather than just typed. Electronic signatures are acceptable)

Print name: Doctor Andrea J. Day Date:(dd/mm/yyyy): 18/4/12

Co-supervisor's signature: .....
(This needs to be an actual signature rather than just typed. Electronic signatures are acceptable)

Print name: Doctor Caroline Orfila Date:(dd/mm/yyyy): 18/04/12

Please submit your form **by email** to J.M.Blaikie@leeds.ac.uk or if you are in the Faculty of Medicine and Health FMHUniEthics@leeds.ac.uk. **Remember to include any supporting material** such as your participant information sheet, consent form, interview questions and recruitment material with your application.

Checklist:

- ☑ I have used layman's terms to describe my research (applications are reviewed by lay members of the committee as well).
- ☑ I have answered all the questions on the form, including those with several parts (refer to the guidance if you're not sure how to answer a question or how much detail is required)
- ☑ I have included any relevant supplementary materials such as
  - ☑ Recruitment material (posters, emails etc)
  - ☑ Sample participant information sheet
  - ☑ Sample consent form.

  Include different versions for different groups of participants eg for children and adults.
- ☑ If I am not going to be using participant information sheets or consent forms I have explained why not and how informed consent will be otherwise obtained.
- ☑ If you are a student have you discussed your application with your supervisor and are they satisfied that you have completed the form correctly? (This will speed up your application).
- ☑ I have submitted a signed copy of my application. (If you are a student your supervisor also needs to sign the form).

Appendices:

Contents

Appendix 1: Invitation sheet, advertisement

1.1 The wording of the e-mail message

Subject of message

Healthy male and female **volunteers required** to eat onions and chocolate!

Body of message

You are invited to participate in a **PhD research study**

What you have to do:

- Certain foods (naturally containing quercetin and epicatechin) will have to be avoided 24 hours prior to a study day.
- Eat **onion soup, dark chocolate and both of them** on 3 different occasions.
- You will collect your urine before and after eating within 24 hour each time.

If you are interested, please contact:
Patthamawadee Charoensuk,
PhD student at the School of Food Science and Nutrition,
University of Leeds
fspc@leeds.ac.uk

If you decide to take part you can still withdraw at any time without any consequences. You do not have to give a reason. All the information that we collect about you during the course of the research will be kept strictly confidential, and you will not be able to be identified.

Thank you very much
Patthamawadee Charoensuk

PS. If this e-mail is not relevant to you, I apologize for any inconvenience.

1.2 Poster

Healthy male and female VOLUNTEERS REQUIRED to eat ONIONS and CHOCOLATE !

You are invited to participate in a PhD research study

WHAT YOU HAVE TO DO:

- **Certain foods (containing quercetin and epicatechin) will have to be avoided 24 hours prior to a study day.**
- **Eat onion soup, dark chocolate and both of them in 3 different occasions.**
- **You will collect your urine before and after eating within 24 hour each time.**

If you are interested, please contact:
Patthamawadee Charoensuk, PhD student
School of Food Science and Nutrition,
University of Leeds

fspc@leeds.ac.uk

A reward voucher of £15 will be offered.

THANK YOU VERY MUCH !

This study is supervised by Professor Gary Williamson, Food Biochemistry group, School of Food Science and Nutrition,University of Leeds, UK.

UREC Ethics form version 11 (updated 17/01/12)

Appendix 2: Participant information sheet

1. Research Project Title:

Effect of combination of foods on flavonoid absorption into the body.

2. Invitation paragraph

You are being invited to take part in a research project. 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. 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. Thank you for reading this.

3. What is the project's purpose?

Flavonoids are a class of water-soluble plant pigments which are found in a wide range of foods and may reduce the risk of chronic diseases such as cardiovascular diseases[1]. These biological effects depend on the amount of flavonoid absorbed in the intestine and distributed to the body.

One important factor that affects absorption, distribution and excretion from the body of flavonoids is an enzyme in our body called "Catechol-O-methyltransferase or COMT". When a flavonoid moves across the intestinal tract and to the liver through blood circulation, COMT will add a methyl group to the flavonoid, resulting in a chemical change. The extent of this change can affect the biological effect and varies between people.

This research will study the correlation between COMT activity and the metabolism of two dietary flavonoids, quercetin or epicatechin. In this research, the urinary content (over 24 hr) of quercetin, epicatechin and their total methylated products will be measured after consumption of 50 g of onion soup or 50 g of dark chocolate or co-consumption both of them.

4. Why have I been chosen?

The participant's selection criterion includes:

- Healthy male and female
- Caucasian
- 18 to 60 years of age
- Normal body mass index (BMI) between 18.5 and 25 kg/m^2
  (principal cut-off points according to World Health Organization)
- Non-smoker
- No dietary supplements or medication
- No GI tract or metabolic and chronic diseases
  (eg. Hepatic or Kidney disease, Cardiovascular disease, Cancer)
- Not pregnant

5. Do I have to take part?

It is up to you to decide whether to take part or not. If you do decide to take part you will be given this information sheet to keep (and be asked to sign a consent form) and you can still withdraw at any time without affecting any benefits that you are entitled to in any way. Your participation in the research is entirely voluntary. You do not have to give a reason for declining or withdrawing.

6. What will happen to me if I take part?

Your participation in the study will last for 24 hours which involves urine collection prior the study and after food consumption on 3 different days. Before the study starts, you have to fill in the subject informed consent form and pre-study questionnaire in the room provided during the study. You are expected to attend to the School of Food Science and Nutrition at the time scheduled for you. A flow chart of the study will provide on page 25 of this information sheet.

7. What do I have to do?

You have to avoid foods listed in the "Food list to avoid in this study" list 2 days prior to commencement of the study. You also have to fast for overnight (12 hr) before each experiment day. After one week interval, you need to follow the same protocol for the second and third visits.

8. What are the possible disadvantages and risks of taking part?

There are no risks and dangers associated with your participation in the study. Consumption of onion soup and dark chocolate is safe. However you may feel slight discomfort due to the restricted diet (avoidance of quercetin and epicatechin rich food, Appendix 6). The opportunity to stop, or withdraw from the study, exists at any time, without giving any reasons. All data will be used even from subjects who withdraw (provided that the volunteer consents to this).

9. What are the possible benefits of taking part?

You may not personally benefit from participating in the study, but results may be used for the advancement of knowledge and the future benefit of other individuals.

10. Will my taking part in this project be kept confidential?

The result of the data obtained will be reported in a collected manner with no reference to a specific individual. Hence, the data from each individual will remain confidential. As a subject only you have the right to know the results of the total analysis.

11. What type of information will be sought from me and why is the collection of this information relevant for achieving the research project's objectives?

Data on your general health and samples of your urine will be collected in the study. The content of quercetin, epicatechin and their total methylated products in urine sample will be measured. These content will be used a marker for absorption, distribution and excretion study and linked to COMT activity in each participant.

12. What will happen to the results of the research project?

Once all participants have completed the study, the information obtained will need to be collected and analysed before any results are published. This is likely to take at least one year to be finalised. If you would also like to know the results of the study, the research team will be able to give this information to you when it becomes available. You will not be identified in any report or publication.

13. Who is organising and funding the research?

This study is not funded by any organisation or company.

14. Contact for further information

Patthamawadee Charoensuk
Study coordinator
School of Food Science & Nutrition
Faculty of Mathematics & Physical Sciences
University of Leeds
Email address: fspc@leeds.ac.uk
Telephone number: 07740291870 (For emergencies)

Code No.....
22

Appendix 3: Informed Consent form

Title of Research Project: Effect of combination of foods on flavonoid absorption into the body.
Name of Researcher: Patthamawadee Charoensuk

Initial the box if you agree with the statement to the left

1 I confirm that I have read and understand the information sheet explaining the above research project and I have had the opportunity to ask questions about the project. ☐

2 I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without there being any negative consequences. In addition, should I not wish to answer any particular question or questions, I am free to decline. You can contact the study coordinator via this e-mail address: fspc@leeds.ac.uk ☐

3 I understand that my responses will be kept strictly confidential. I give permission for members of the research team to have access to my anonymised responses. I understand that my name will not be linked with the research materials, and I will not be identified or identifiable in the report or reports that result from the research. ☐

4 I agree for the data collected from me to be used in future research. ☐

5 I agree to take part in the above research project and will inform the principal investigator should my contact details change. ☐

6 I agree to be re-contacted for future research projects related to this study. ☐

(The participants do not have to agree to this statement)

Name of participant (*or legal representative*)	Date	Signature
Name of person taking consent (*if different from lead researcher*) *To be signed and dated in presence of the participant*	Date	Signature
Lead researcher *To be signed and dated in presence of the participant*	Date	Signature

Appendix 4: Pre-study questionnaire

Part 1: Identifying information

Full name of participant: .....

Date of birth:.....

Weight: .....kg., Height: .....m., BMI: .....kg/m^2

Contact phone No. : .....

E-mail address: .....

Code number: .....

"This identifying information will be kept strictly confidential."

Code No.....
24

Part 2: Questionnaire

Please give me a few minutes to complete all 7 questions.

Q1. How **OLD** are you?

A. 18 to 60 years old B. Lower or higher than A.

Q2. What is your ETHNIC group?

Choose one option that best describes your ethnic group or background

A. White (ex. British, Scottish, or any white background, *please describe*.....)

B. Asian (ex. Indian, Chinese, or any Asian background, *please describe*.....)

C. Black (ex. African, Caribbean, or any Black background, *please describe*.....)

D. Mixed or multiple or any other ethnic group. Please describe.....

Q3. Are you **MALE** or **FEMALE**?

A. MALE B. FEMALE

Q4. Are you **NON-SMOKER** or **SMOKER**?

A. NON-SMOKER B. SMOKER

Q5. Do you take any **DIETARY SUPPLEMENTS** or take any **MEDICATIONS** in the last 2 weeks before participating this study?

A. NO B. YES

Q6. Do you have any **GASTROINTESTINAL (GI) TRACT, METABOLIC AND CHRONIC DISEASES (eg. Hepatic or Kidney disease, Cardiovascular disease) or SERIOUS DISEASES?**

A. NO B. YES

Q7. Are you **PREGNANT**?

A. NO B. YES

---Thank you for considering joining this study---

Appendix 5: Food list to avoid in this study

Subjects are required to **avoid** certain foods for one day prior to the study.

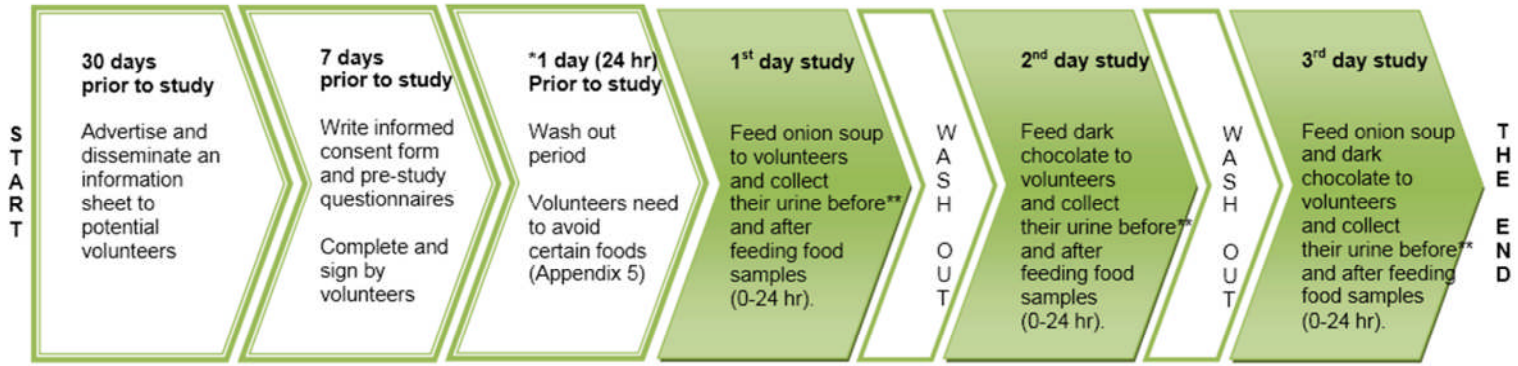
Type of Foods	Food containing	
	Quercetin	Epicatechin
Alcoholic Beverages	Beer Wine Champagne	Beer Wine Champagne Cider Sherry
Cereals and cereal products	Buckwheat, refined flour Buckwheat, whole grain flour	
Cocoa	Chocolate, dark	Chocolate, dark/milk Cocoa, powder
Fruits and fruit products	Fruits - Berries eg. Biberry, blackberry, raspberry Fruits – Pomes eg. Apple	Fruits - Berries eg. Blackberry, blackcurrant, cloudberry, grape, blueberry, raspberry, redcurrant, strawberry Fruits and jams - Drupes eg. Apricot, nectarine, peach, plum, cherry Fruits - Pomes eg. Apple, pear, quince Fruits - Stalk vegetables eg. Rhubarb Fruits - Other fruits eg. Fig, banana, kiwi, pomegranate
Non-alcoholic Beverages	Berry juices eg. Raspberry, grapefruit juice Citrus juices eg. Orange juice Pome juices eg. Apple juice Tropical fruit juices eg. Pomegranate Tea	Cocoa beverage - Chocolate Berry juices eg. Blackberry juice Drupe juices eg. Apricot juice Pome juices eg. Apple juice Tropical fruit juices Tea
Seasonings	Oregano, vinegar, capers, cloves	Vinegar
Seeds	Nut eg. Almond, pistachio	Nut eg. Almond, cashew nut, hazel nut peacan nut, pistachio Bean, Lentils
Vegetables	Onion and its family (eg. Shallot), Tomato	Avocado. Pod vegatables eg. Broad bean pod, green bean Pulse vegatables eg. Fresh pea

Reference: INRA. Database on polyphenol contents in foods [online]. 2011. [Accessed 17 March 2011]. Available from: http://www.phenol-explorer.eu

Appendix 6: Food list to allow in this study

- Meat (which is not processed), e.g. "fresh" chicken, beef, burgers, kebab
- Milk and dairy products
- Pasta, rice, bread, cereals (except buckwheat cereal products), chips
- *In moderation*: all fruit and vegetables which are **NOT** mentioned in **Appendix 5**
- Sweets (except cocoa-containing), cakes, pastry, crisps, nuts

Appendix 7: Study flow chart



Remark:* **Wash out period** should be performed 1 day (24 hr) before study day in each time.
** Participants have to fast 12 hr then collect their baseline urine sample (urine before eating food sample).

Appendix 8: 24 hour dietary record

Date: ..... **Code No: .....**

This form used to record a detailed description of your food intake. You need to state the types and amounts of food/beverage you consume for a day. You need to include the information of the ingredients, preparation of the meal, brand name and also amount that you consumed.

Time	Type of food/beverage	Details	Amount*

* **Amount**: description of portion size of item eaten ex. tea spoon, glass of water, bowl of soup etc.

Appendix 9: Urine collection form

Time of urine collection: Baseline / 0-24 hours

i) To be completed by the subject for each individual urine sample

Subject Code:	
Collection Date: **Collection Time:**	
Sample Collection Irregularities **(eg. Loss of sample):**	
Other Comments:	

ii) To be completed by Study Coordinator:

Subject Code:	
Collection Date: **Collection Time:**	
Total Sample Volume (ml):	
Comments:	

Appendix 10: Protocol for urine collection and acellular urine

Approximately 3 g of ascorbic acid (vitamin C) will be placed into the clean urine collection bottle. Then, subjects will collect the urine samples at specified time in the designated bottle.

Preparation of acellular urine:

Urine samples will be transported to the lab within 12 hours of the collection where they will be processed within 24hr before being frozen at -20 ° C.

1 Upon arrival, the volume of the specimen will be measured, and 2 x 50-ml aliquots taken.
2 These aliquots will be centrifuged at 2000 x g, 4° C for 10 min *to remove insoluble materials and cellular debris*.
3 The supernatant will be carefully decanted to within approximately 1-2 ml of the cell pellet.
4 The samples will be stored at –20°C until analysis.

Waste disposal:

1 The sediment of the centrifuged urine specimen will be decontaminated before disposal by adding a disinfectant (1 % *Virkon* solution, 2 x 5-g tablet /1 L, in accordance with the manufacturer's guidelines) to the sediment and leaving over night.
2 The sediment can then be discarded in the clinical waste bags.

General good laboratory practice and safe operation:

1 The urine samples will only be processed on a designated bench area highlighted by biohazard labels.
2 The centrifuge is disinfected before and after centrifugation by spraying 70% ethanol inside the chamber and wiping.
3 Personal biosafety guidelines and precautions will be adhered to at all times.

Reference:

Magistroni R. *et al.*; 2009; " Proteomic analysis of urine from proteinuric patients shows a proteolitic activity directed against albumin"; *Nephrol Dial Transplant*; **24**; pp. 1672-1681.

B. Ethics Committee Letter of Approval

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UNIVERSITY OF LEEDS

Patthamawadee Charoensuk
Food Science and Nutrition
University of Leeds
Leeds, LS2 9JT

MEEC Faculty Research Ethics Committee
University of Leeds

16 May 2012

Dear Patthamawadee

Research title **Effect of combination of foods on flavonoid absorption into the body.**
Ethics reference **MEEC 11-041**

I am pleased to inform you that the application listed above has been reviewed by the MaPS and Engineering joint Faculty Research Ethics Committee (MEEC FREC) and following receipt of your response to the Committee's initial comments, I can confirm a favourable ethical opinion as of the date of this letter. The following documentation was considered:

Document	*Version*	*Date*
MEEC 11-041 Ethical application_Patthamawadee_Submit190412.pdf	1	19/04/12
Summary form for MEEC 11-041.pdf	1	11/05/12
MEEC 11-041 Ethical application_Patthamawadee_Response to committee.pdf	1	11/05/12

Please notify the committee if you intend to make any amendments to the original research as submitted at date of this approval, including changes to recruitment methodology. All changes must receive ethical approval prior to implementation. The amendment form is available at www.leeds.ac.uk.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. There is a checklist listing examples of documents to be kept which is available at http://researchsupport.leeds.ac.uk/index.php/academic_staff/good_practice/other_information_nhs_sites in the '*Other useful documentation*' section.

Yours sincerely

Jennifer Blaikie
Senior Research Ethics Administrator, Research & Innovation Service
On behalf of Professor Gary Williamson, Chair, MEEC FREC

CC: Student's supervisor(s)