Bioavailability and Bioactivity of Green Tea Catechins in Skin

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Abstract

Dietary flavonoids have been extensively researched in relation to health benefits in humans. The regular consumption of green tea catechins (GTC) has been associated with a reduction in the risk of developing diabetes, cardiovascular disease and cancer. Flavonoids are known to protect plants from the damage caused by exposure to UV radiation, and this effect has also been observed when flavonoids are applied topically to human skin cells. The effect of oral consumption of flavonoids on skin protection from UV exposure is not clear.

The work presented within this thesis aimed to investigate the effect of GTC on the response of skin cells to UV induced-stress. Keratinocyte cells from an immortalised human skin cell line (HaCaT) were assessed after exposure to various stress conditions *in vitro* (FBS starvation, hydrogen peroxide and UV), in combination with a pre-treatment of green tea extract or a purified mixture of GTC. GTC reduced cell death induced by stress (decrease in LDH release), and maintained viability (increase in MTT uptake) in HaCaT cells, relative to control treatments. The uptake of vitamin C, a photo-protective agent depleted after UV exposure, was enhanced by treatment with GTC during stress conditions, as monitored by uptake of ^{14}C-dehydroascorbic acid and evaluation of vitamin C transporters with qRT-PCR. In relation to *in vivo* conditions, GTC may provide protection and also enhance vitamin C uptake into skin cells undergoing stress.

Bioavailability of GTC and metabolites in human skin cells after daily consumption of green tea and vitamin C supplements for 3 months was also investigated. Catechin metabolites in a range of tissues (plasma, interstitial blister fluid, skin biopsies and urine) were identified with LC-MS-MS in unconjugated and conjugated (sulphate, methyl and glucuronide) forms. For the first time, conjugated catechin metabolites were identified in skin tissue samples and extracellular fluid surrounding skin cells; including M6/M6'-*O*-sulphate, *O*-methyl-EC-*O*-sulphate,

EC-*O*-sulphate and EGC-*O*-glucuronide, with metabolites identified in urine and plasma post-consumption similar to data reported in the literature.

The work presented in this thesis provides new knowledge on bioavailability of GTC and metabolites in human skin, which together with vitamin C, may exert UV protection and other health benefits. Further research is required *in vitro* using pure conjugated standards (methyl, glucuronide and sulphate moieties), and data corresponding to the inflammatory biomarkers post-UV exposure (analysis at the University of Manchester and University of Bradford) is also required before a conclusive relationship can be drawn between oral consumption of flavonoids and UV protection.

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Abbreviations

(6-4) PP	pyrimidine (6-4) pyrimidone photoproduct
8-OHdG	8-hydroxydeoxyguanosine
AA	ascorbic acid
amu	atomic mass unit
AO	ascorbate oxidase
AP-1	activator protein-1
BER	base excision repair
cDNA	complementary DNA
C	catechin
CD	cluster of differentiation
COMT	catechol-*O*-methyltransferase
COX	cyclooxygenase
CPD	cyclobutane pyrimidine dimer
CPM	counts per minute
C_T	threshold cycle
CV	coefficient of variation
DHAA	dehydroascorbic acid
DMSO	dimethyl sulfoxide
DPM	disintegrations per minute
EC	epicatechin
ECG	epicatechin gallate
ECM	extracellular matrix protein
EDTA	ethylenediaminetetraacetic acid
EG	ethyl gallate
EGC	epigallocatechin
EGCG	epigallocatechin gallate
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT	glucose transporter
GT	green tea
GTC	green tea catechin mixture
GTE	green tea extract
HaCaT	immortal human keratinocyte cell line
HBSS	Hanks Balanced Salt solution
HEPES	hydroxyethyl piperazineethanesulfonic acid

HPLC	high performance liquid chromatography
IL	interleukin
iNOS	inducible nitric oxide synthase
LCMS	liquid mass spectrometry
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LOD	limit of detection
LOQ	limit of quantification
LOX	lipoxygenase
M4	5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone
M6	5-(3',4'-dihydroxyphenyl)-γ-valerolactone
M6'	5-(3',5'-dihydroxyphenyl)- γ-valerolactone
MED	minimum erythema dose
MMP	matrix metalloproteinase
MRM	multiple reaction monitoring
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NER	nucleotide excision repair
NF-κB	nuclear factor-κB
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PPB	potassium phosphate buffer
qRT-PCR	quantitative real-time reverse transcription PCR
RCT	randomised controlled trial
ROS	reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute-1640 medium
SPF	sun protection factor
SULT	sulphotransferase
TNFα	tumour necrosis factor α
UDP	uridine diphosphate
UGT	uridine 5'-diphospho-glucuronosyltransferase
UVA	ultraviolet A
UVB	ultraviolet B
UVR	ultraviolet irradiation

Chapter 1. Literature Review

1.1. Green Tea: Composition, Metabolism and Protective Health Effects

1.1.1. Overview of Polyphenols

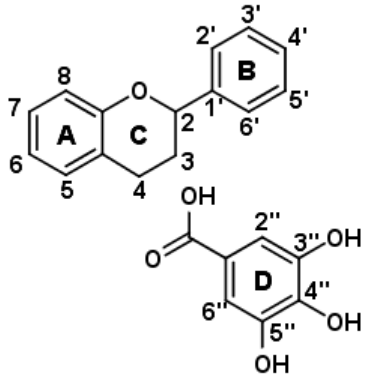
Polyphenols are secondary plant metabolites synthesised in plants for growth, reproduction, protection against predators, protection against ultraviolet radiation, and sensory and colour characteristics (Gould and Lister, 2005). Dietary polyphenols are researched widely *in vitro* and *in vivo* in connection to health benefits in humans. The proposed associated health benefits include, but are not limited to, reducing the incidence of cardiovascular disease, neurogenerative diseases, cancer and diabetes by modulation of gene expression, enzyme interactions and cell signalling pathways. The daily intake of polyphenols has been estimated to be 1 g per day (Scalbert and Williamson, 2000, Scalbert *et al*., 2005, Vauzour *et al*., 2010).

Each polyphenol contains a characteristic aromatic ring with at least one hydroxyl group (Figure 1-1). Polyphenols are separated into different classes dependent on structural features; including flavonoids, phenolic acids, lignans and stilbenes (Table 1-1). The largest class of polyphenols are the flavonoids which are identified by the diphenylpropane (C_6-C_3-C_6) structure; two aromatic rings joined through three carbon atoms (A, B and C ring, Figure 1-1; Ross and Kasum, 2002).

The flavonoids are separated into subclasses depending on modifications of the heterolytic C ring. The subclasses are flavones, isoflavones, flavonols, flavanones, catechins (or flavan-3-ols) and anthocyanidins (Ross and Kasum, 2002). The catechin flavanol structures are characterised by a dihydroxyl or trihydroxyl substitution on the B ring and also 5,7-dihydroxyl substitutions on the A ring, with no double bond in the C ring and a 3-OH on C ring (Figure 1-1; Yang *et al*., 2001).

Table 1-1. Structures and classes of polyphenols (Source: Balasundram *et al*., 2006).

Class	Structure
Simple phenolics	C_6
Hydroxybenzoic acids	C_6-C_1
Phenylacetic acids	C_6-C_2
Hydroxycinnamic acids	C_6-C_3
Stilbenes	C_6-C_2-C_6
Flavonoids	C_6-C_3-C_6
Lignans	$(C_6$-$C_3)_2$
Condensed tannins (proanthocyanidins)	$(C_6$-C_3-$C_6)_n$



Catechin	3	5	7	3'	4'	5'
(+)-Catechin	OH	OH	OH	OH	OH	
(-)-Epicatechin	OH	OH	OH	OH	OH	
(-)-Epicatechin Gallate	D	OH	OH	OH	OH	OH
(-)-Epigallocatechin	OH	OH	OH	OH	OH	OH
(-)-Epigallocatechin Gallate	D	OH	OH	OH	OH	OH

Figure 1-1. The flavonoid skeleton illustrating the position of the 3 rings (A, B and C, and the galloyl moiety, D) and the R groups of the catechins present in green tea.

1.1.2. Overview of Green Tea

After black tea, green tea is the second most popular tea beverage (USA Tea Council, 2013). Green tea is prepared from the *Camellia sinensis* plant species by roasting the tea leaves at high temperatures to inhibit polyphenol oxidation. In comparison to other teas, green tea has the highest flavanol content (35-50 %, black tea is 10 % flavanol; Cai and Chow, 2004), including catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin

gallate (EGCG). On analysis by dry weight, the most abundant flavanol in a cup of green tea is EGCG (Table 1-2).

Table 1-2. Polyphenols found in green tea (Source: Cabrera *et al*., 2003).

Class	Polyphenol
Flavan-3-ols	(-)-epicatechin
	(-)-epicatechin-3-gallate
	(-)-epigallocatechin
	(-)-epigallocatechin gallate
	(+)-catechin
	(+)-gallocatechin
	theaflavin
	theaflavin-3-3'-digallate
	theaflavin-3'-gallate
	theaflavin-3-gallate
	thearubigins
Flavones	Apigenin, luteolin
Flavonols	Kaempferol, myricetin, quercetin
Others	Gallic acid, chlorogenic acid, caffeic acid

1.1.3. Metabolism of Green Tea Catechins

After consumption, flavonoids enter the digestive system and undergo modification by phase II metabolism (Figure 1-2). Flavonoids bound naturally to sugar molecules (glucosides) undergo deglycosylation by lactase phlorizin hydrolase before absorption of the aglycone in the enterocyte cells of the small intestine (Day *et al*., 2000b). The hydroxyl groups of the green tea flavonoids are able to undergo phase II biotransformation by methylation, sulphation and glucuronidation, increasing the polarity of the flavonoid (recognised by the body as a xenobiotic) for excretion (Spencer *et al*., 1999, Lambert *et al*., 2007).

Green tea flavonoids can undergo glucuronidation by UDP-glucuronosyltransferases (UGT; EC 2.4.1.17), sulphation by sulphotransferases (SULT; EC 2.8.2.1) and methylation by catechol-*O*-methyl transferase (COMT; EC 2.1.1.6). In the small intestine, the enzymes involved in conjugation of green tea flavonoids are UGT1A1 and UGT1A8, and SULT1A1 and SULT1A3. Furthermore, modifications can also occur in the liver by UGT1A9 and SULT1A1 (Pai *et al*., 2001,

Williamson, 2004, Lambert *et al*., 2007). When high concentrations of flavonoids are present, metabolites are mainly conjugated with glucuronic acid as inorganic sulphate can be limiting and hence sulphation can be limited *in vivo* (Oddy *et al*., 1997).

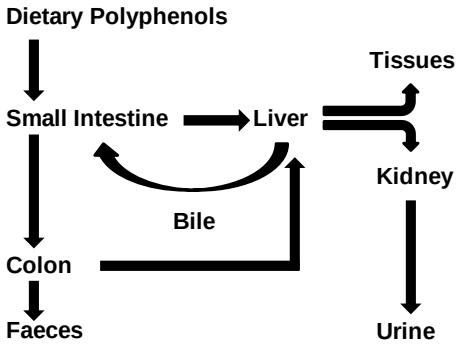


Figure 1-2. Simple overview of routes taken post-consumption of polyphenols (source: Scalbert and Williamson, 2000).

After metabolism in the liver, green tea flavonoids (both free and conjugated) are either excreted into the bile, or are circulated around the body to tissues, or to the kidneys for excretion in urine. After biliary excretion, the fate of the green tea flavonoids is the colon, where they can be metabolised by microbes to smaller compounds known as phenolic acids (Figure 1-3). Colonic catechin metabolites include hydroxyphenylpropionic acid, hydroxybenzoic acid, hydroxyhippuric acid, hippuric acid, p-coumaric acid, vanillic acid and hydroxyphenylacetic acid (Gonthier *et al*., 2003). Deconjugating enzymes are present in the colon and are able to remove glucuronide and sulphate moieties (Cardona *et al*., 2013). Both free and conjugated flavonoids in the colon can re-enter the enterohepatic cycle again to be further metabolised before eventual excretion from the body (Williamson, 2004).

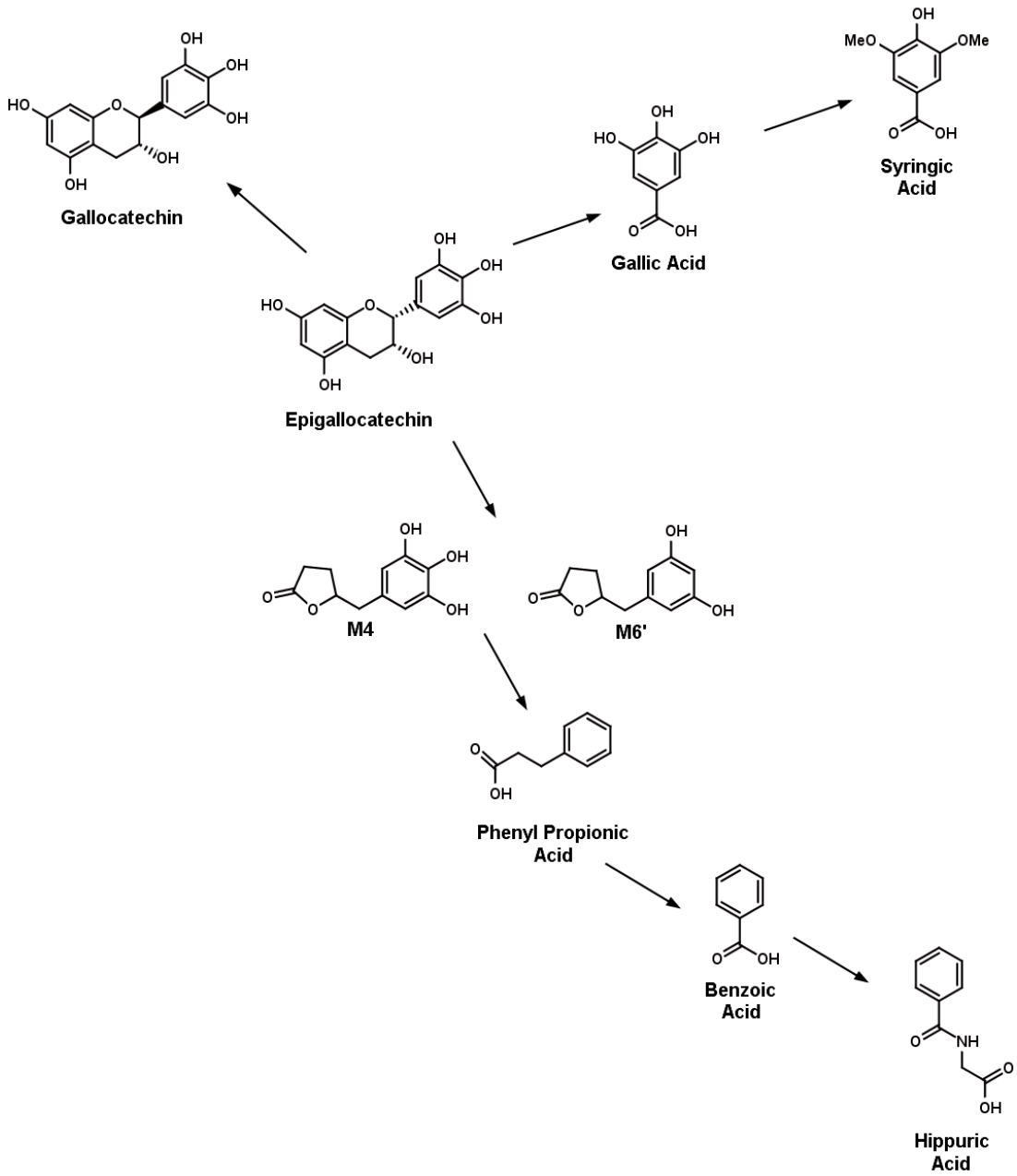


Figure 1-3. Possible degration and metabolite products of epigallocatechin (EGC) during digestion. Epimerisation is known to occur at a neutral pH (GC).

Green tea flavonoids have been identified in urine and plasma after human intervention studies in conjugated forms. Table 1-3 displays green tea catechin metabolites present in urine following a human intervention, with known conjugate positions.

Table 1-3. Green tea catechin metabolites identified in urine in two separate human intervention studies.

Metabolite	Reference
EC-3'-*O*-glucuronide, 4'-*O*-methyl-EC-3'-*O*-glucuronide, 4'-*O*-methyl-EC-5-*O*-glucuronide, 4'-*O*-methyl-EC-7-*O*-glucuronide	Natsume *et al*. (2003)
EGC-3'-*O*-glucuronide, EGC-7-*O*-glucuronide, 4'-*O*-methyl-EGC-3'-*O*-glucuronide, 4'-*O*-methyl-EGC-7-*O*-glucuronide, 4'-*O*-methyl-EGC-5-*O*-sulphate, 4'-*O*-methyl-EGC-3'-*O*-sulphate, EC-3'-*O*-glucuronide, EC-3'-*O*-sulphate, EC-4'-*O*-sulphate, 3'-*O*-methyl-EC-5-*O*-sulphate, 3'-*O*-methyl-EC-7-*O*-sulphate, 4'-*O*-methyl-EC-3'-*O*-sulphate, 4'-*O*-methyl-EC-5-*O*-sulphate, 4'-*O*-methyl-EC-7-*O*-sulphate, M4, M4-3'-*O*-glucuronide, M4-3'-*O*-sulphate, M4-4'-*O*-sulphate, 4'-*O*-methyl-M4-3'-*O*-sulphate, M6, M6-3'-*O*-glucuronide, M6-4'-*O*-glucuronide, M6-3'-*O*-sulphate or M6-4'-*O*-sulphate, M6', M6'-3'-*O*-glucuronide, M6'-3'-*O*-sulphate	Sang *et al*. (2008)

EC, epicatechin; EGC, epigallocatechin; M4, 5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone; M6, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone); M6', 5-(3',5'-dihydroxyphenyl)-γ-valerolactone

1.2. Bioavailability of Green Tea Catechins

Bioavailability is defined as “the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action” (FDA, 2003). The bioavailability of flavonoids can be influenced by various parameters, including binding to proteins present within the food matrix, efflux from intestinal cells and pH changes throughout the digestive tract. In the case of green tea catechins, pH is very influential on degradation. Catechins are stable at a low pH, but at a neutral pH and above, are rapidly degraded (Figure 1-3). Catechin stability is unaffected in the stomach (pH 1-3, gastric juice), but on entering the intestine (pH 7-8, pancreatic juice and secretions from epithelial cells of small intestine) the stability is greatly reduced (Chen *et al*., 1998, DeSesso and Jacobson, 2001). It has been estimated that there is an approximate loss of 80 % when the catechins reach the neutral pH of the intestine (Ferruzzi, 2010). Addition of ascorbic acid improved the stability of green tea catechins at neutral pH, as observed by a significantly reduced rate of degradation (e.g. 10 % catechins

remained with addition of ascorbic acid after incubation at 37 ºC and pH 7 for 10 hours, whereas no catechins were detected in control; Chen *et al*., 1998).

Uptake of xenobiotics into intestinal cells is an essential element of bioavailability. The intestinal epithelium has numerous uptake (oligopeptide transporters, organic anion transporters and organic anion transporting polypeptide transporters) and efflux transporters (breast cancer resistance proteins (BCRP; ABCG2), multidrug resistant associated proteins (MRP; ABCC2) and p-glycoproteins (ABCB1); all ATP-binding cassette transporters) that can transport flavonoids into and out of epithelial cells. Catechin, EC and ECG are substrates for MRP2, and flavonoid glucuronides and sulphates can be effluxed out of epithelial cells by MRP, BCRP and p-glycoprotein (Zhang *et al*., 2007, Gao and Hu, 2010), for example quercetin and resveratrol sulphates and glucuronides are effluxed out of Caco-2 cells by MRP2 (Wen and Walle, 2006).

As mentioned, ascorbic acid can improve the stability of green tea catechins which could potentially improve the bioavailability. With the addition of protein (casein, soy or skimmed milk, with no significance in bioavailability between protein source) to decaffeinated green tea, bioavailability of total catechins was reduced as shown by a reduction in peak areas (LC-MS analysis; by approximately 30 %) for catechins in plasma of 24 volunteers (Egert *et al*., 2013). The addition of caffeine to a beverage containing 95 mg EGCG enhanced bioavailability in plasma, but only at the low dose (addition of 40 mg caffeine and not 180 mg) in comparison to no caffeine (Nakagawa *et al*., 2009). In rats, co-administration of green tea with ascorbic acid and sucrose enhanced bioavailability (Peters *et al*., 2010), and consumption of cocoa with bread and sugar in a human intervention increased bioavailability of EC and C (Schramm *et al*., 2003). These results suggest that absorption and delivery of green tea catechins to target sites in the body can be influenced by the addition of other food constituents.

1.2.1. Green Tea Catechin Metabolites

Inter-individual variations in metabolites of green tea catechins identified in biological samples post-consumption have been noted (Lee *et al*., 2002). This is largely due to inter-individual differences in gut microbiota that break down catechins into smaller molecules (Gross *et al*., 2010), and explains variations within concentrations of metabolites present within biological samples (mainly urine and plasma).

Flavonoids have been associated with modifying the gut microbiota present which could lead to protective effects *in vivo*. The presence of polyphenols in the gut has been linked to proposed health benefits on the activity of gut bacteria (depending on increase or decrease of particular bacterial species), with research mainly focusing on reduction in cancer initiation by modulation of enzymes and enhanced anti-inflammatory activity by inhibition of cyclooxygenase (COX) 2, nuclear factor (NF)-κB, activator protein-1 (AP-1), tumour necrosis factor (TNF)α, interleukin (IL)-6 and vascular endothelial growth factor (VEGF) (Cardona *et al*., 2013).

A recent review of the effects of polyphenols on gut microbiota highlighted that approximately 90 % of ingested flavonoids pass through to the colon, and are not absorbed in the small intestine (Cardona *et al*., 2013). The bacterial species involved in the metabolism of polyphenols in the colon include *Escherichia coli*, *Clostridium sp.*, *Bifidobacterium sp*., *Lactobacillus sp*., *Bacteroides sp*. and *Eubacterium ep*.. Assessment of red wine polyphenols (28 % proanthocyanidin units, 4 % anthocyanins, 2 % phenolic acids, 1.4 % catechins, 1 % epicatechin and 0.8 % flavonols) on modification of microbiota in rats confirmed that polyphenols could modify gut microbiota by increasing lactobacilli (beneficial for intestinal function) and decreasing clostridia (considered detrimental to colonic mucosa), which could potentially protect against inflammation and oxidative stress *in vivo* (Dolara *et al*., 2005). Another study also assessed the changes in gut microbiota by

catechin alone. There were substantial increases observed in *Clostridium coccoides–Eubacterium rectal, Bifidobacterium spp*. and *Escherichia coli* microbiota, and inhibition of the *C.histolyticum* group when incubated with (+)-C (Tzounis *et al*., 2008). (-)-EC also increased the growth of *Clostridium coccoides–Eubacterium rectal*. This strain of bacteria has been associated with converting cholesterol into bile acid, and also inhibition of tumour initiation. The authors also reported that in colonic conditions, (+)-C can be converted to (+)-EC by bacteria present.

1.2.1.1. Green Tea Catechins and Metabolites in Urine

Many human and animal intervention studies have evaluated the metabolites excreted into urine after consumption of green tea, usually in a beverage form with studies lasting between 24 and 48 hours. The majority of studies investigating green tea catechin metabolites in biological samples, assess the free-form compounds (the term which will be used throughout this thesis for catechins or metabolites that are not bound to a sulphate ester or glucuronic acid) after enzyme deconjugation (using HPLC or LC-MS), or conjugates using changes in transitions from the removal of the conjugate moiety during fragmentation (LC-MS), as conjugate derivatives are not commercially available. Therefore, to examine conjugated derivatives the compound must either be hydrolysed by enzymes and the free-forms analysed, or samples assessed using LC-MS can be quantified relative to available standards.

Li *et al*. (2001) were the first to report an LC-MS method to analyse conjugated metabolites in urine. Five subjects consumed 1.2 g green tea extract in 300 mL of warm water and collected urine for up to 15 hours. Conjugates were monitored by the loss of the conjugated moiety from the parent transition. *O*-Methyl-EGC was synthesised with methyl iodine and EGC-*O*-glucuronides were synthesised by UDP glucuronic acid and mouse liver microsomes to confirm the

identification of *O*-methyl-EGC and EGC-*O*-glucuronide in urine samples. M4 (-5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone) and M6 (-5-(3',4'-dihydroxyphenyl)-γ-valerolactone) were also isolated from human urine and utilised for identification of retention times. It was noted that the main conjugates in the urine, or the conjugates with the largest peak area (not necessarily the highest in concentration as each compound has a different LC-MS response factor) were M4 and M6-*O*-glucuronides and *O*-sulphates, EGC-*O*-glucuronide, EC-*O*-glucuronide, EC-*O*-sulphate, *O*-methyl-EGC-*O*-glucuronide and *O*-methyl-EGC-*O*-sulphate. The authors reported the identification of the compounds, but did not attempt to quantify due to a lack of commercially available standards for catechin conjugates.

Sang *et al*. (2008) were the first to create a single LC-MS run for analysis of green tea catechin metabolites in urine (from 3 subjects who consumed 3 g green tea solids in 200 mL water). 3'-*O*-methyl-EC, 4'-*O*-methyl-EGC, M4 and M6 were synthesised and their structures confirmed by NMR. When the conjugated standard was not available, the identification of the compound was confirmed by loss of the conjugate moiety from the free-form transition, and also by relating the fragmentation pattern of the MS^3 spectra with that of the original free-form compound. The presence of M4, M6 and M6' (-5-(3',5'-dihydroxyphenyl)-γ-valerolactone) in the free-form, as well as sulphate and glucuronide forms, *O*-methyl-M4-*O*-sulphate, EGC-*O*-glucuronide, EGC-*O*-sulphate, *O*-methyl-EGC-*O*-glucuronide, *O*-methyl-EGC-*O*-sulphate, EC glucuronide, EC-*O*-sulphate and *O*-methyl-EC-*O*-sulphate was shown in the urine samples.

Del Rio *et al*. (2010a) reported that following the consumption of a 400 mL green tea drink after an overnight fast and 2 day polyphenol free diet in 20 volunteers, the main urinary conjugates excreted were valerolactones (no free-form present), *O*-methyl-EGC-*O*-glucuronide and EGC-*O*-glucuronide, and in total 37 metabolites were identified in the urine. All EC and valerolactone conjugates were quantified as EC equivalents, and EGC metabolites were quantified as

equivalents of EGC. As a response factor was not applied there was possibly a chance of over-quantification of metabolites. Response factors of hydroxycinnamic acids and their corresponding conjugated forms have been assessed, and it was clear that there was a wide range of values for response factors relative to free-form structures, from between 3 and 10 times, for most sulphate and glucuronide derivatives examined. This would result in over-estimation of conjugates present within samples (Farrell *et al*., 2011).

Double conjugation of metabolites has also been identified in the urine. These included EGC-*O*-sulphate-glucuronide, *O*-methyl-EGC-*O*-sulphate-glucuronide, M6/M6'-*O*-sulphate-glucuronide and M6/M6'-*O*-disulphate. No free-form (unconjugated) compounds were detected (Del Rio *et al*., 2010a). Conjugated metabolites were analysed by loss of the glucuronic acid or sulphate moiety from the parent transition to produce the daughter (free-form) transition. When the same group performed a similar intervention of 20 volunteers with 90 µmol of green tea in a ready to drink cold green tea beverage (500 mL), the main urinary metabolites identified were EGC-*O*-glucuronide and *O*-methyl-EC-*O*-sulphate (Del Rio *et al*., 2010b). An example of the potential conjugation positions (for both single or double) for EGCG is demonstrated in Figure 1-4.

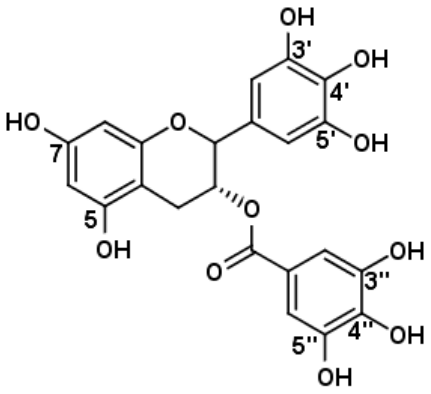


Figure 1-4. Possible conjugation positions for epigallocatechin gallate.

Wang *et al*. (2008) examined urine and plasma samples to determine biomarkers of green tea consumption. 124 volunteers were involved in a 3 month intervention, in which 41 were in the placebo group, 42 consumed 500 mg daily supplement of green tea and 41 consumed 1000 mg. In plasma there was a dose dependent increase of EGCG and ECG (assessed by maximum concentration, C_{max}), but not for EC or EGC, and therefore the investigators deemed EGCG and ECG as reliable biomarkers of green tea consumption in plasma samples. However, for 24 h urine, EC and EGC excretion dose dependently increased, whereas no change was seen for EGCG and ECG. EC and EGC were allocated as potential biomarkers of green tea consumption in urine. In the urine, EC was predominantly glucuronidated, whereas EGC was equally sulphated and glucuronidated.

The criteria for biomarkers of ingested flavonoids were evaluated by Spencer *et al*. (2008). The criteria included that the biomarker must be specific to the dietary flavonoid, that there must be a clear relationship between intake and concentration in biological samples and tissues and that this relationship is sensitive enough to changes in dose, and that the biomarker chosen is resistant to degradation. Most bioavailability studies assessing flavonoid intake have monitored dose changes in urine, but not dose changes in plasma. Urine is usually collected for 24 hours post ingestion which monitors initial metabolite excretion. Some studies have collected urine for up to 60 hours post ingestion of flavonoids, and metabolites were still present (polyphenol free diet pre and post-consumption of a flavonoid meal; Rechner *et al*., 2002, Spencer *et al*., 2008).

Many other studies have also identified phase II conjugated catechins and valerolactones as metabolites of green tea catechin (or cocoa for EC consumption) consumption. Roura *et al*. (2005) monitored EC-*O*-glucuronide and sulphate conjugates in urine after volunteers consumed a cocoa beverage, by the loss of the conjugate from the parent transition during LC-MS. Mullen *et al*. (2010) identified conjugated metabolites with LC-MS by use of synthetic conjugated standards and

by confirming that the fragmentation pattern of the compound after the loss of the conjugate moiety was identical to that of the initial free-form parent compound (MS^3). Stalmach *et al*. (2009) also identified the conjugated forms of the green tea catechin metabolites by analysing MS^3 fragmentation patterns, and confirming their resemblance to the fragmentation of the related free-form parent compound. As the conjugated forms of catechins and their metabolites were not available, quantification was performed by relating conjugates as EC or EGC equivalents, as has been mentioned previously.

1.2.1.2. Green Tea Catechins and Metabolites in Plasma

Green tea catechin metabolites circulating in plasma after consumption of green tea have been identified in numerous studies. Many studies have assessed the metabolites relative to the free-form by employing enzyme deconjugation. More recently, studies have monitored conjugated metabolites in the plasma, and quantified relative to free-form standards. As mentioned in section 1.2.1, a large inter-individual difference is noticeable in metabolites present *in vivo* post green tea catechin consumption. Williamson *et al.,* (2011) reviewed the analysis of plasma samples after green tea consumption and correlated data for different studies to 50 mg doses. This reviewed data displayed a visibly large difference between metabolism in individuals.

Consumption of 400 mL of a green tea beverage (400 µmol total catechins) by 20 volunteers resulted in a total of 12 metabolites identified in plasma along with ECG and EGCG in the free-form (Del Rio *et al*., 2010a). Green tea catechins and metabolites were monitored in plasma from 0 to 4 hours. The main metabolites were *O*-methyl-EGC-*O*-sulphate, *O*-methyl-EGC-*O*-glucuronide, EGC-*O*-glucuronide and EC-*O*-glucuronide which were all present below 100 nM, with T_{max} values between 1 and 3 hours.

Renouf *et al*. (2010) hydrolysed glucuronides and sulphates present in the plasma of 5 volunteers after consumption of 400 mL of a 1.25 % green tea infusion, by enzyme deconjugation, with T_{max} values between 1 and 2 hours for EC (C_{max} of 200 nM), EGCG (100 nM) and EGC (550 nM) when samples were analysed using HPLC Coularray. In 9 volunteers consuming the same infusion, but with analysis by LC-MS, T_{max} values were again between 1 and 2 h and C_{max} of 4'-*O*-methyl-EGC was 137 nM (Renouf *et al*., 2011).

The same group performed a dose response test on appearance of metabolites (deconjugated to free-forms) in plasma in a later study (Renouf *et al*., 2013). The three doses ranged from 180 to 415 mg total green tea catechins, and were consumed by 12 subjects in a cross-over study. There was saturation of EGC and 4'-*O*-methyl-EGC (no change in LC-MS peak area response in plasma samples after medium and high dose consumption, but an average fold increase of 2.5x between low and medium), but not for EGCG and EC which appeared to proportionally increase. The inter-individual variability was reported to be high (EGC after the high dose ranged from 78 to 830 nM). Ullmann *et al*. (2003) also reported a proportional increase in C_{max} of EGCG after a range of 50-1600 mg EGCG was consumed by 48 volunteers, with 8 volunteers consigned to one of the 6 dose groups (again with high inter-individual variability). A dose dependent increase of EGCG in plasma has also been reported, but not EC or EGC in 30 volunteers who consumed between 400 and 1200 mg green tea (polyphenol E, a decaffeinated green tea mixture) in a cross-over trial (Chow *et al*., 2005).

In a recent study by Henning *et al*. (2013), phenolic acids present in plasma were assessed after consumption of 6 cups of green tea daily for 3-6 weeks (randomised intervention of including control of 6 cups of water, 23 in green tea group and 24 in control group) by HPLC Coularray detection. Samples were enzymatically deconjugated. M4 and M6 were only detected in one sample, and

3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid were the only phenolic acids to change significantly in comparison to baseline plasma.

An earlier study by the same group compared the metabolites detected in plasma (analysed with HPLC coularray) after green tea, black tea and green tea supplement consumption (30 volunteers in a cross over study) (Henning *et al*., 2004). There was a notable delay in T_{max} after consumption of the supplement for all catechins (T_{max} for EGC was 1.3 h for green tea and 2.6 h for the green tea supplement).

1.2.1.3. Green Tea Catechins and Metabolites in Tissues

The presence of flavonoids within tissue samples has not been explored widely. The degree of EGCG methylation in prostate tissue taken from 9 males (compared with 8 males on a water control) who had localised prostate cancer has been analysed (Wang *et al*., 2010a). The active group consumed 6 cups of green tea daily (one tea bag in 240 mL boiling water, brewed for 5 minutes) for 3 and 6 weeks. Prostate tissue was taken during a prostatectomy and was snap frozen in liquid nitrogen. 300 mg was used for analysis and tissue samples were subjected to enzyme deconjugation by sulphatase and β-glucuronidase, sulphatase alone, or β-glucuronidase only. After enzyme deconjugation it was observed that 48 % of the EGCG present in the tissue was methylated (10 % glucuronidated, 20 % sulphated and the rest free) and of the non-methylated form 25 % was glucuronidated and 5 % was sulphated.

The distribution of ^{3}H-EGCG after intragastric ingestion in rats has been explored. Twenty four hours after gavage there was a peak in radioactivity detected in brain, eye, thymus, lung, heart, spleen, liver and prostrate which related to 0.2 % total radioactivity distribution. The majority of radioactively labelled EGCG was excreted; 32 % identified in urine and 35 % total radioactivity in faeces (Kohri *et al*., 2001).

A similar study in mice also identified radioactivity in multiple tissues after 24 hours administration of ^{3}H-EGCG. Radioactivity was identified in brain, lung, heart, liver, spleen, pancreas, bone and skin, and also faeces and urine. The radioactivity in excreted products was reduced over 24 hours, whereas for tissues analysed the radioactivity accumulated over 24 hours (with percentages of less than 1 % for total radioactivity incorporated into tissues). These results demonstrate the ability of a green tea catechin (or degraded constituent or conjugate, as two ^{3}H labels were added to the B ring at positions 2' and 6', and two to the D ring at positions 2'' and 6'') to reach various tissues of the body (Suganuma *et al.,* 1998).

1.3. Protective Health Associations of Green Tea Catechins

The first connection between green tea consumption and beneficial health effects was published by a Japanese priest called Eisai in 1211 entitled “Maintaining Health by Drinking Green Tea” (Fujiki, 2005). Since 1211, and in particular the last few decades, extensive research has made a positive association between green tea consumption and protection against inflammation and disease. For instance, green tea has been inversely related to the incidence of lung cancer in Japanese smokers in comparison to American smokers. An epidemiological study in 1985 noted a lower incidence of lung cancer in Japanese smokers consuming more cigarettes a day than American smokers. The connection was made with the high consumption of green tea in Japan, in comparison to low consumption of black tea in America (Fujiki, 2005). However, in 2009 a systematic review stated that the evidence from 51 studies included in the review was insufficient and conflicting in supporting the proposal that green tea was positively related in reducing cancer prevalence, except for the inverse correlation between green tea consumption and prostate cancer (Boehm *et al*., 2009). Many reviews have been centred around the consumption of green tea and cancer reduction, with the most recent review identifying the positive body of evidence, but calling for mechanisms of action to be

identified and more human intervention studies questioning the bioavailability of green tea catechins with the putative effect of reducing the incidence of cancer onset (Lambert, 2013).

Reduction in the incidence of cardiovascular disease has also been associated with green tea consumption. Four separate studies have associated green tea catechins with a significant reduction in LDL cholesterol, and a meta-analysis of 14 randomised controlled trials related a positive effect of consumption green tea beverages and extracts on reducing total cholesterol and LDL cholesterol, which are risk factors for cardiovascular disease (Williamson *et al*., 2011a, Zheng *et al*., 2011). A recent systematic review of 11 randomised controlled trials again indicated a positive effect of green (and black) tea consumption in reducing the risk of cardiovascular disease, but the review suggested more long-term studies with longer-term follow up studies need to be performed to reinforce this connection (Hartley *et al*., 2013).

Green tea consumption has been linked to a reduction in fasting insulin and glucose concentrations in 17 trials (Liu *et al*., 2013). Green tea has also been linked to weight loss. Eleven studies suggested a slightly beneficial link in consumption of green tea with caffeine and weight loss or weight management (Hursel *et al*., 2009).

The associations quoted here are related to *in vivo* assessment of green tea consumption. There is a large body of *in vitro* research aimed towards uncovering mechanisms and providing evidence that the associations made have biological significance. The presence of phase II conjugates *in vivo* after ingestion of flavonoids has been emphasised in numerous studies and reviews. Many conjugates are not available commercially and therefore *in vitro* investigations rely largely on free-forms, or aglycones. For the studies that have used phase II conjugates, the association with beneficial protective effects is varied and can be dependent on position of the conjugate. Inhibition of xanthine oxidase and lipoxygenase activity by quercetin-*O*-glucuronides varied depending on the

conjugate position. The aglycone form was the most successful inhibitor of both enzymes, followed by 3'-*O*-methyl-quercetin and quercetin-4'-*O*-glucuronide for xanthine oxidase, and 3'-*O*-methyl-quercetin and quercetin-7-*O*-glucuronide for lipoxygenase inhibition. The conjugate with the highest K_i, and therefore least influence on inhibition of the two enzymes, was quercetin-3-glucuronide (Day *et al*., 2000a).

Assessment of cell damage when cortical neurons and dermal fibroblasts were treated with up to 30 µM of EC, 3'-*O*-methyl-EC or EC-*O*-glucuronides (mixture of 5- and 7-*O*-glucuronide) was monitored by measuring MTT uptake and caspase-3 activity. The EC-*O*-glucuronide mixture had no significant beneficial effect on either cell line, whereas the free-form and methyl conjugate appeared to significantly protect both cell lines against hydrogen peroxide-induced stress by limiting caspase-3 activity (Spencer *et al*., 2001).

The evaluation of the *in vivo* and *in vitro* data underlines the need for more research with regards to beneficial health claims associated with flavonoid consumption, and that when performing *in vitro* investigations, metabolically relevant compounds at biological concentrations should be examined.

1.4. Vitamin C: Structure, Metabolism and Protective Health Effects

1.4.1. Overview of Vitamin C

Vitamin C is an essential nutrient that is found in many plant sources of the human diet, including broccoli, oranges, kiwi and potatoes (Levine *et al*., 1999). Vitamin C has many valuable functions within the body, namely free radical scavenging and involvement as a cofactor for enzymatic reactions, and it is well known for protection against scurvy.

Vitamin C is present in foods either in the reduced form (ascorbic acid, AA) or in the oxidised form (dehydroascorbic acid, DHAA; Figure 1-5), and as humans do not have the gene encoding L-guluno-lactone oxidase which is required for the

synthesis of ascorbic acid from UDP-glucuronic acid, obtaining vitamin C from food sources is essential (Nishikimi and Yagi, 1991, Tsao, 1997). Vitamin C is one of the most popular supplements purchased, however studies have shown that the diet usually provides enough vitamin C to reach the recommended daily allowance, and therefore supplementation proves inefficient as excess ascorbic acid is excreted from the body, rather than absorbed.

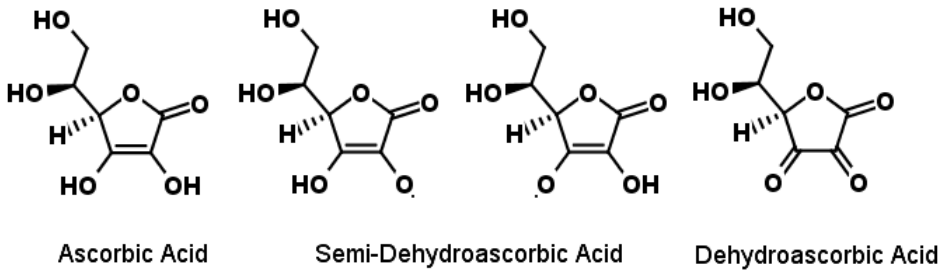


Figure 1-5. The various forms of vitamin C, including intermediates.

When a range of vitamin C doses were consumed daily for a 14 day period (30 to 2500 mg by 7 volunteers), vitamin C was not detected in the urine until after consumption of 100 mg. There was not a large inter-individual difference between the plasma concentration of AA for the 7 volunteers above 100 mg (~58+/-9 µM), however there was large a difference for plasma concentrations following daily consumption of 50 mg AA (~25+/-25 µM). Following consumption of >500 mg vitamin C daily for 14 days it was clear that the majority of AA consumed was excreted into the urine. A plateau of vitamin C in the plasma was reached after consumption of 250 mg vitamin C daily (250 mg daily, ~65+/-17 µM; 500 mg, ~72+/-17 µM; 1000 mg, ~75+/-12 µM; and 2500 mg, ~80+/-10 µM) (Levine *et al*., 1996). The current recommended daily allowance is 40 mg/day (NHS, 2012). Scurvy is developed after approximately 90 days of a diet deficient in ascorbic acid, and a study has shown the protection against scurvy from daily consumption of a minimum of 40 to 60 mg (Griffiths and Lunec, 2001).

Ascorbic acid is a six carbon acid ($C_6H_8O_6$) with a pK_a of 4.2. Ascorbic acid can be reversibly oxidised to DHAA. After the loss of one electron, a stable free radical, semi-dehydroascorbic acid, is formed (Figure 1-5). After the loss of the second electron DHAA is formed, and this can be reduced back to AA. However, under certain conditions the ring structure of DHAA can be irreversibly hydrolysed to diketogulonic acid, which is then transformed by metabolism into oxalate, threonate, xylose, xylonic acid and lynxonic acid (Rumsey *et al*., 1997).

As mentioned, vitamin C has many important functions. It is known that ascorbic acid is a cofactor for several enzymes, is involved in hormone synthesis, photo-protection and antioxidant protection, and is associated with immune responses. It is also essential for the maintenance of skin cells (Fuchs and Podda, 1997). Scurvy is a well documented disease that visualises the effects of a diet deficient in vitamin C. It highlights the importance of vitamin C in collagen synthesis due to complications with wound healing during deficiency (Fuchs and Podda, 1997). Ascorbic acid is a cofactor for hydroxylation of procollagen proline and lysine residues and it is also required for the secretion of procollagen out of cells. With reduced levels of ascorbic acid the secretion of procollagen is slower, and without the hydroxylation of proline and lysine the collagen formed is less stable (Peterkofsky, 1991).

Many *in vitro* analyses have illustrated the antioxidant potential of vitamin C (Figure 1-6). A recent human study of 33 volunteers identified that intake of 100 mg of calcium ascorbate per day for 4 weeks could result in a 22 % increase in radical scavenging activity in skin cells, assessed using electron paramagnetic resonance spectroscopy, in comparison to placebo volunteers. The results were evident after 2 weeks supplementation. Ingestion of 180 mg per day increased the activity by 37 % in comparison to the control (Lauer *et al*., 2013). Studies have also assessed the pro-oxidant activity of ascorbic acid (Buettner and Jurkiewicz, 1996, Mandl *et al*., 2009). Pro-oxidation can lead to formation of reactive oxygen species, reduction of

Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+}, and there is also some evidence of ascorbic acid selectively inducing apoptosis in cancerous cells only, which has been linked to pro-oxidation (Kojo, 2004).

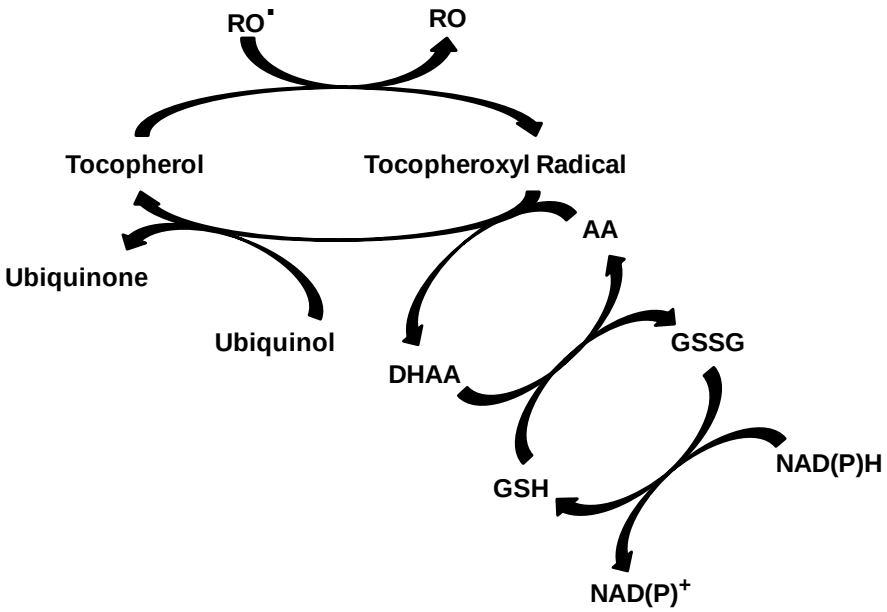


Figure 1-6. Conversion of AA to DHAA and DHAA to AA during radical scavenging (source: Pinnell, 2003).

1.4.2. Transporters of Vitamin C

There are five known transporters involved in vitamin C transport. The sodium dependent vitamin C transporters (SVCT1 and SVCT2) are specific for the transport of AA (Daruwala *et al*., 1999), whereas DHAA is transported by glucose transporters (GLUT1, GLUT3 and GLUT4; Mueckler *et al*., 1985, Kayano *et al*., 1988, Fukumoto *et al*., 1989). AA is the form of vitamin C found most predominantly (at 95%) in plasma, but under oxidative stress DHAA can be formed. Uptake of DHAA into cells through GLUT transporters can be inhibited by glucose, however this competition is cell specific. Glucose is present in the plasma at around 1000 fold higher concentrations than DHAA (Fuchs and Podda, 1997, Richelle *et al*., 2006). DHAA uptake by GLUT can also be modified by the presence of stomatin, a

membrane protein. In red blood cells, DHAA uptake is favoured over glucose whereas for immature erythrocytes without stomatin present, glucose uptake is favoured (Mandl *et al*., 2009).

Ascorbic acid is found to be circulating freely in the monovalent form in plasma, with a half life in the human body of between 10 – 20 days (Dhariwal *et al*., 1991). However, under periods of oxidative stress it is possible that cells will favour the uptake of DHAA over the uptake of AA (Dhariwal *et al.*, 1991, Fuchs & Podda, 1997, Richelle *et al*., 2006). Once inside the cells, DHAA reductases rapidly reduce DHAA to AA (reductases include glutathione, glutaredoxin, protein disulphide isomerase, GSH peroxidase, NADPH, thioredoxin reductase, 3α-hydroxysteroid dehydrogenase and NADH-lipoic acid-dependent lipoamide dehydrogenase; Catani *et al*., 2005). Many studies have highlighted the preference of DHAA uptake over AA, however this mechanism is cell specific and cannot always ensure an intracellular AA pool (Wilson, 2002, Levine *et al*., 2011). For example, SVCT2 knockout mice die at birth due to developmental problems caused by inefficient transport of ascorbic acid across the placenta (Mandl *et al.,* 2009). Savini *et al.* (2000) have illustrated the greater uptake of ^{14}C-DHAA in comparison to ^{14}C-AA in HaCaT (human keratinocyte) cells.

The SVCT transporters are unique transporter proteins as they cannot be linked by sequence homology to any other transporter family (Tsukaguchi *et al*., 1999). The function and sequence homology of SVCT1 and SVCT2 is closely related with a 65 % sequence identity at amino acid level. They both have a predicted twelve transmembrane domain with both N and C termini located on the cytoplasmic side of the membrane. The major difference between the SVCT transporters lies within the distribution of the two transporters in the body. SVCT1 is distributed in large epithelial systems such as the kidney and intestine, whereas SVCT2 is more specific to tissues, such as the brain and eyes (Tsukaguchi *et al*., 1999, Wang *et al*., 1999).

The SVCT proteins have a high specificity for ascorbic acid and there are no molecules that have been found to competitively inhibit the transport of ascorbic acid (Wang *et al*., 1999). However, a low affinity is exhibited for D-isoascorbic acid (Liang *et al*., 2001). The stoichiometry of the transporter is 2:1 (Na^+:ascorbate) and as ascorbic acid is a monovalent anion at pH 7.4, an electrogenic environment is created during transport due to the difference in charge (Wang *et al*., 1999, Mackenzie *et al*., 2008).

The GLUT transporters of dehydroascorbic acid belong to the Class I glucose transporters (Rogers *et al*., 2003). Similar to the SVCT transporters, the GLUT transporters have 12 transmembrane domains (Figure 1-7) and the amino and carboxyl termini are located on the cytoplasmic side of the transporter protein. As can be seen in Figure 1-7, the structure of the transmembrane domain appears to be separated into two halves by the large loop connecting TM6 and TM7 (Bell *et al*., 1993). Unlike the transport of ascorbic acid, the transport of dehydroascorbic acid can be inhibited by 2-deoxyglucose, D-glucose and 3-*O*-methylglucose (Rumsey *et al*., 1997).

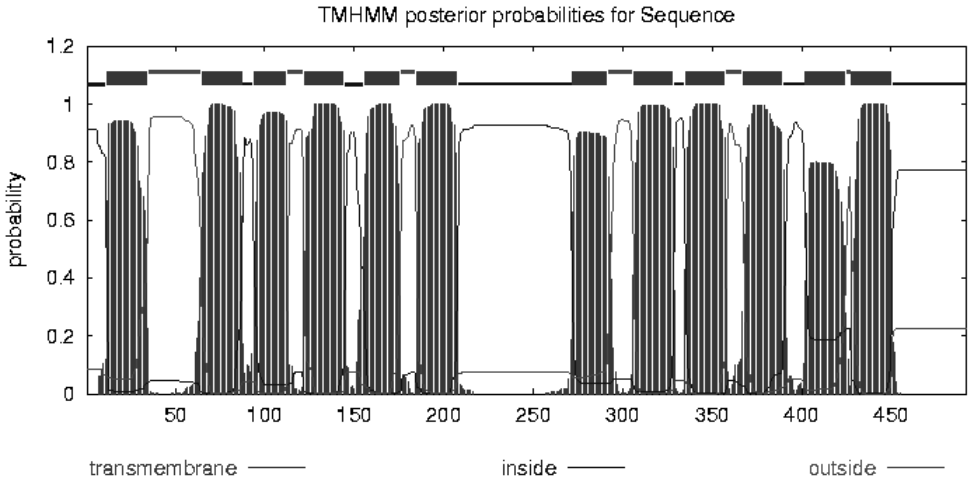


Figure 1-7. Predicted 12 transmembrane domain of GLUT1 (T-Coffee, 2000).

GLUT transporters can be separated by their location. GLUT1 has a wide tissue distribution and is thought to be the main transporter of DHAA, whereas GLUT3 is found in the brain, placenta, testis and platelets, and GLUT4 is located in muscle and also in adipose tissue (Mueckler *et al*., 1985; Kayano *et al*., 1988; Fukumoto *et al*., 1989).

The five transporters are found in both the epidermal and the dermal layers of the skin. The transporters that are located in the epidermis are SVCT1, SVCT2 and GLUT1. Those found in the dermis are SVCT2, GLUT3 and GLUT4 (Gherzi *et al*., 1992, Steiling *et al*., 2007).

Modification of the ascorbic acid transporters has been assessed previously by Nestle Research Centre (unpublished data). Regulation of SVCT1 and SVCT2 was monitored after incubation of HaCaT cells for 24 hours with flavonoids (EGCG, genistein, curcumin, quercetin and hesperetin), however there was no change in comparison to control cells.

Regulation of the GLUT transporters (in relation to glucose uptake) has been explored in diabetes research. A green tea extract consumed by rats up-regulated mRNA levels of GLUT1 and GLUT4 in liver samples (Cao *et al.,* 2007), and a polyphenolic extract of cinnamon up-regulated GLUT1 and GLUT3 expression in mouse macrophages (Cao *et al.,* 2008).

1.5. UV Irradiation and Skin

1.5.1. Background to UV Irradiation of Skin

The skin is the largest organ in the human body and it is made up of 3 layers; the epidermis, dermis and subcutaneous layer (Figure 1-8). The skin acts as a barrier, and one of the main functions of the skin is to protect the internal organs from the external environment, including ultraviolet radiation. The epidermis is the top layer of skin and is the first layer to come into contact with the external environment. It consists of many different cell types (keratinocytes, melanocytes,

Langerhans cells and Merkel cells) with keratinocytes as the majority (90-95 %) (Haake *et al*., 2001).

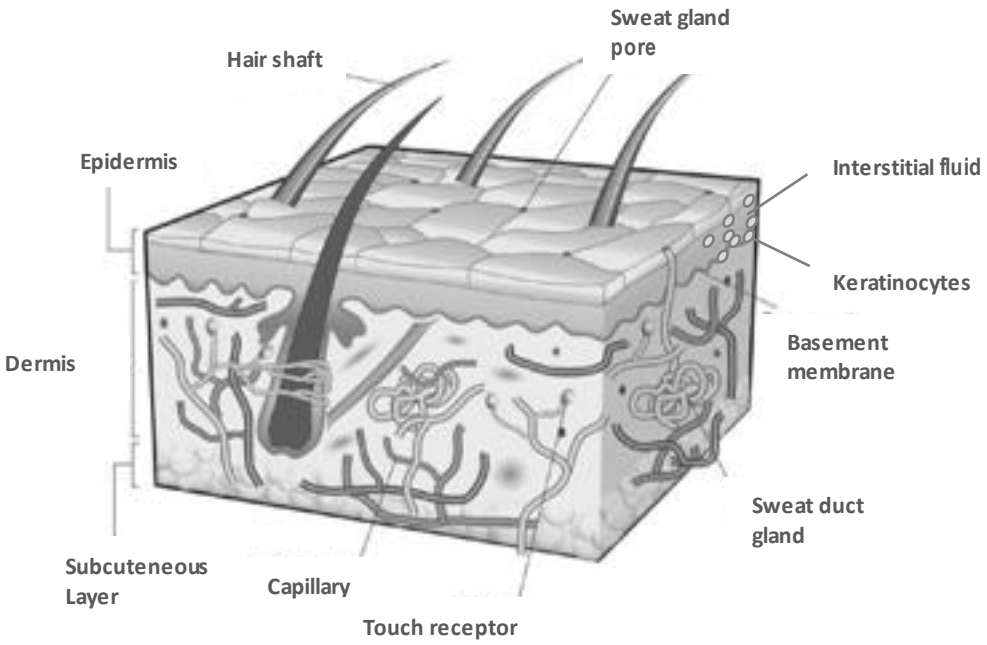


Figure 1-8. Structure of the skin (Source: MacNeil, 2007).

As for any cell, an increase in age is associated with a reduction in cellular function. Overexposure to the sun can prematurely result in skin cell aging, or photo-aging. It is well known that exposure to ultraviolet radiation (UVR) can result in sunburn, and prolonged and repeated exposure of skin to the sun can cause damaging consequences, specifically epidermal tumours. These include basal and squamous cell carcinomas, and malignant melanoma (Peterson, 2001, Sies, 2007, Pfeifer *et al*., 2005). Melanoma is the most fatal form of skin cancer, and in the UK the incidence of malignant melanoma has significantly increased from between 1975 and 2010 (4 and 7 times higher for females and males, respectively; with an incidence rate of 4 and 2.5 to 17.5 per 100,000 population for females and males, respectively) (Cancer Research, 2012). The incidence has increased over the years due to a change in behaviour towards sun protection. Sunscreens can be applied

poorly which reduces protection and sunscreens are not re-applied during the day (Swindells and Rhodes, 2004).

1.5.2. UV Radiation

There are three wavelength bands of UVR that span between 200-400 nm. UVR is highly associated with photo-aging of skin and skin cancer. The two wavelength bands that can penetrate the ozone layer and come into contact with skin are UVA and UVB. It has been estimated that UVA causes around 10-20 % of all skin cancers, with the majority being the result of UVB exposure (Pfeifer *et al.*, 2005). UVB is associated with tumour initiation, whereas UVA is linked to tumour promotion (Pinnell *et al.,* 2003).

To cause detrimental damage, UVR must first interact with a chromophore inside the cell (Sies and Stahl, 2004). Chromophores in skin cells include nucleic acid, protein, NADH and 7-dehydrocholesterol. Depending on the type of chromophore, the damage caused by UVR can be directly damaging or indirectly damaging to DNA (Trautinger, 2001; Figure 1-9). The damage caused by these reactions varies from changes in gene expression, activation of inflammatory events and also stimulation of kinase-dependent signalling pathways (Trautinger, 2001; Sies & Stahl, 2004).

UVB ranges from 290-320 nm and is the main form of radiation that both causes sunburn and induces skin cancer. UVB penetrates into the epidermis and can directly damage cellular DNA that is present in skin cells. DNA bases can absorb incident photons within the UVB band range and this directly affects DNA as cyclobutane pyrimidine dimers can be formed. UVB is also able to up-regulate genes that contribute to the progression of tumours at the promotion stage (Ichihashi *et al*., 2003, Wang *et al*., 2010c).

UVA is between 320-400 nm and this type of radiation is able to penetrate further into the skin, reaching the dermal layer. There is 20 times more UVA emitted

in sunlight than UVB (Pinnell *et al.,* 2003). UVA can indirectly damage DNA by producing reactive oxygen species (ROS) within the cell via a cascade of events. Indirect damage occurs as UVA is only weakly absorbed by DNA (Wang *et al*., 2010c).

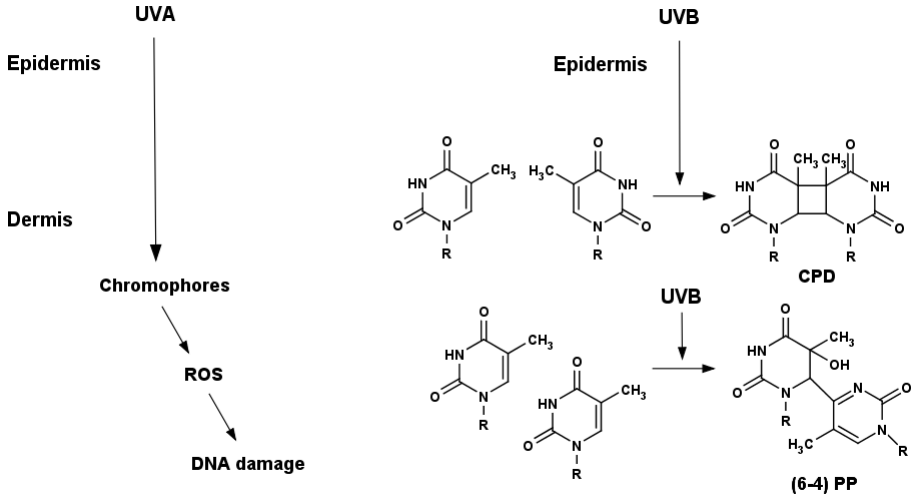


Figure 1-9. An overview of UV damage in skin cells. ROS, reactive oxygen species; CPD, cyclobutane pyrimidine dimers; (6-4) PP, pyrimidine (6-4) pyrimidone photoproducts.

Some examples of the indirect damage caused by UVA include hydrogen peroxide synthesis from activation of oxidases (containing flavins, for example riboflavin and nicotinamide coenzymes) and from activation of free iron from ferritin. The ROS can then oxidise lipids, proteins and DNA within the cell. The double stranded DNA can be damaged by oxidation of the DNA bases by ROS (Lehmann *et al*., 1998; Pfeifer *et al.*, 2005), which can also produce O_2^- (superoxide anion radical) and $^1O_2$ (singlet oxygen). Neutrophils are another generator of O_2^- and $^1O_2$, and the number of neutrophils present in cells is increased after UVR exposure (Wlaschek *et al*., 2001).

1.5.3. Photoproducts and Mutations

The products generated in cells after exposure to UVR are called photoproducts (or lesions). Approximately one photon in 500 produces a lesion after exposure. These lesions can be repaired by DNA repair mechanisms; however those that are not can lead to the development of skin cancer (Trautinger, 2001).

Two examples of photoproducts produced in skin after exposure to UVB are cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts ((6-4) PP) in DNA; an example of direct DNA damage. They are produced when covalent bonds on the same strand of DNA are formed between adjacent pyrimidines. This in turn reduces transcription and replication of DNA (Lehmann *et al*., 1998; Sies & Stahl, 2004; Pfeifer *et al.*, 2005).

CPD are the most frequently formed lesion (in mammalian cell models they account for approximately 80 % of lesions) and they occur between the 5,6 bonds of two adjacent pyrimidine bases. They can also be formed indirectly by UVA. The (6-4) PP lesions are formed when a bond is created between position 6 and position 4 of two pyrimidines. Purine dimers and pyrimidine mono-adducts can also be created following exposure (Pfeifer *et al.*, 2005).

The CPD formed from UVB with the largest effect on mutagenicity are thymine-cytosine (Hong *et al*., 2004) and cytosine-cytosine (CC), as 5-methylcytosine is a major site for UVB mutagenesis. These mutations are TC to TT and CC to TT and can be related to the mutations of the *p53* gene. Mutations to the *p53* gene are a well known component of skin cancer as they have been found in approximately 50 % of basal cell carcinoma cells and 90 % of squamous cell carcinoma cells. Other genes involved are *ras* and *PTCH* (patched gene) (Ichihashi *et al.*, 2003; Pfeifer *et al.*, 2005).

Mutations of C to T can occur at the 5’-PyCG trinucleotide sequence within the gene, especially in human keratinocytes where this sequence includes 5-methylcytosine. There are 5 frequent codons that contain the 5’-PyCG sequence

and these can be mutated as described by UVB (codons 196, 213, 245, 248 and 282; Pfeifer *et al.*, 2005).

After CPD have formed, they can be replicated by DNA polymerase η (POLH) or other error-prone DNA polymerases. These polymerases do not identify the error (or lesion) formed. Error-prone DNA polymerases are able to integrate an adenine base opposite the cytosine involved in the CPD. This eventually converts the cytosine bases into thymine bases; a transition mutation. Another outcome of CPD formation is deamination of the cytosine opposite a guanine to a thymine (with adenosine eventually being integrated opposite thymine). This error can be bypassed by POLH during replication, and again a transition mutation occurs (Pfeifer *et al.*, 2005). By converting the mutagenic dimer to AT, the mutagenicity of the CPD lesions formed by UVB can be decreased (Ichihashi *et al.*, 2003; Pfeifer *et al.*, 2005).

As mentioned (6-4) PPs are less frequently formed in comparison to CPD, by 5-10 fold. Up to 90 % of (6-4) PPs can be repaired 3 hours after exposure to UVB. After formation, (6-4) PPs can be converted to Dewar isomers (bicyclic structures) by further UVB exposure. Once CPD dimers are formed they are stable to further exposure to UVB. If the (6-4) PP has a cytosine at the 5’ end then it is possible that deamination can occur (Trautinger, 2001, Ichihashi *et al.*, 2003).

Reactive oxygen species have a major role in induction of skin cancer. A marker of ROS formation is the presence of 8-hydroxydeoxyguanosine (8-OHdG) which is formed after oxidative attack by either singlet molecular oxygen or molecular oxygen formed by electron transfer on guanine. The other bases can also be modified (to form 8-OHdA, 5-OHdC and thymine glycol), however it is 8-OHdG that is present under all conditions of oxidative stress (Ichihashi *et al.*, 2003).

1.5.4. Repair Mechanisms of DNA

DNA excision repair systems are present within cells to reduce mutations created by UVR (Pfeifer *et al.*, 2005). Nucleotide excision repair (NER) modifies lesions formed by UVR (i.e. CPDs and (6-4) PP) before DNA replication can occur. NER can be divided into two repair mechanisms; global genome repair and transcription coupled repair. Another repair mechanism is base excision repair (BER) (D'Errico *et al*., 2007). BER is used within cells to repair changes that have occurred to DNA bases such as the formation of 8-OHdG and other products of ROS. Another mechanism is the synthesis of DNA by post replication repair specific DNA polymerase (Ichihashi *et al.*, 2003). The lesions that are not repaired can lead to cancer initiation in cells.

1.5.5. Evidence of Cellular Damage by UVR

There is both *in vivo* and *in vitro* evidence to support the cellular changes that occur as a result of UVR exposure. A study of UVR on hairless mice showed the progression of tumorigenic and metastatic states of melanomas by the cytokine IL-8. It was apparent that IL-8 was induced by UVR as analysed at both an mRNA and protein level. The data also showed the association of an increase in IL-8 concentration with an increase in type IV collagenase matrix metalloproteinase (MMP)-2 activity, and consequently a degradation of type IV collagen (Singh *et al*., 1995).

As reviewed by Wlaschek *et al*. (2001), collagen can be lost from skin cells after exposure to UVR because of modifications in the expression of genes by the ROS produced, resulting in a decrease in collagen metabolism and an increase in MMP activity. The ROS that are indirectly generated from UVA can increase the expression of MMP1, 2 and 3, whereas UVB can increase MMP1 and 3. UVR also reduces tissue inhibitors of MMP. MMP can also be increased by MAPK kinase pathways, including the ERK-kinase signalling pathway, that are activated by singlet

oxygen. This also results in the secretion of cytokines (IL-1α, IL-1β and IL-6) (Trautinger, 2001; Wlaschek *et al*., 2001; Sies & Stahl, 2004).

Both H_2O_2 and $^1O_2$ are involved in up-regulating MMP after UVA exposure. The intermediates produced from lipid peroxidation (including malondialdehyde, as well as •OH) are able to up-regulate MMP following UVB by stimulating c-Jun amino-terminal kinase (JNK) 2 (Wlaschek *et al*., 2001).

Photo-damage in skin also causes changes to the dermal extracellular matrix (ECM) by decreasing the synthesis of ECM proteins, reducing procollagen I and III, and up-regulating MMPs. UV-induced inflammation involves the production of cytokines, including TNFα, IL-1β, IL-6, which in turn can lead to synthesise of other cytokines, chemokines and cellular adhesion molecules. Cytokines and activation of kinase pathways (e.g. p38 MAPK) can lead to the activation of NF-κB, a transcription factor involved in regulation of cell survival. Under certain conditions, such as activation in cancerous cells, NF-κB activity needs to be inhibited to prevent the growth of cancer cells. The inflammatory responses involve up-regulation of genes synthesising phospholipase A2, lipoxygenase (LOX), cyclooxygenase (COX) and inducible nitric oxide synthase (Godoy *et al*., 2007, Huang *et al*., 2004, Swindells and Rhodes, 2004). The regulation of these genes is usually investigated when applying topical lotions *in vivo*, and when analysing cells *in vitro*.

In the skins' defence against oxidative stress induced by UV irradiation, antioxidants are present. These include ascorbic acid, tocopherol and glutathione, and also antioxidant enzymes, including superoxide dismutase, catalase, glutathione reductase and thioredoxin reductase (Swindells and Rhodes, 2004).

1.5.6. Bioactives and UV Inflammation

Current sun protection practise involves application of sunscreen which blocks absorption of UV. However there are many problems associated with sunscreen application. Most sun protection factors (SPF) are allocated using an

application of 2 mg/cm^2 skin, but in practise on average only 0.5 mg/cm^2 is applied, which vastly diminishes protection (SPF 50 applied at 2 mg/cm^2 is SPF 50, but applied at 0.5 mg/cm^2 is reduced to the protection provided by SPF 3; Pinnell *et al.,* 2003). Another is that vitamin D synthesis is inhibited by sunscreen. Vitamin D is required for maintenance of bone health, and can be synthesised in the body by conversion of 7-dehydrocholesterol after exposure to UVB. Vitamin D can be consumed in the diet (oily fish, eggs, fortified breakfast cereals or supplements), but adequate amounts can be produced *in vivo*. In theory, blocking UVB absorption by sunscreen should reduce the synthesis of vitamin D within skin, but the evidence for this is conflicting with some studies showing an increase in vitamin D synthesis with sunscreen application, and others showing a reduction in synthesis but not in bone metabolic markers (Burnett and Wang, 2011).

Many associations have correlated treatment of food bioactives with prevention against UV-induced inflammation, studied in animal models and humans, and *in vitro*. The treatments have involved topical application or oral consumption of the food bioactive. Vitamin E, grape seed proanthocyanins, green tea and selenium have been associated in reduction of melanoma (Jensen *et al*., 2010), and folate, vitamins A, C, D and E, selenium, green tea and black tea have been affiliated with reducing incidence of non-melanoma skin cancers (Payette *et al*., 2010).

Many studies have assessed the effect of oxidative stress induced by UV *in vitro* using the HaCaT cell line which is a non-tumourgenic cell line that was initially from adult human skin. HaCaT cells maintain the normal keratinocyte morphology and also epidermal differentiation capacity as normal human skin and are easier to handle than a primary cell line (Boukamp *et al.,* 1988).

1.5.7. Vitamin C and UV Inflammation

As an essential component for the maintenance of skin cells, levels of vitamin C in skin layers have been assessed in mice. There was approximately

1.3 µmol AA/g of epidermis and 1.3 µmol DHAA/g. In the dermis there was slightly less vitamin C; 1.0 µmol AA/g and 0.9 µmol DHAA/g (Fuchs and Podda, 1997). After exposure to a UVR simulator, the levels of AA and DHAA were depleted in the epidermis and dermis of mice (Shindo *et al.,* 1993). As ascorbic acid is a strong reducing agent and a free radical scavenger, it has an important function in reducing inflammation induced by UV radiation and could be diminished in this process (Shindo *et al*., 1994; Savini *et al*., 1999). Ascorbic acid content in human skin has also been assessed. Skin biopsies were obtained from the forearm of 15 young volunteers and 15 older volunteers, also from the inner arm to compare photo-aged skin against skin exposed to limited irradiation. In comparison to the young skin, AA was significantly lower in the epidermis (69 and 61 % of the concentration in young skin) and dermis (63 and 70 %) of photo-aged and aged skin, respectively (Rhie *et al*., 2001).

After exposure to UVR, a vitamin C concentration gradient can be formed from the outer layer of the epidermis (stratum corneum) to the deeper layers in the skin of mice. The ascorbic acid concentration within skin cells is lowest in the stratum corneum and increases with the deeper layers, implying that UVR exposure is more destructive to vitamin C upon first contact with the skin. It is unclear whether AA depletion in the skin after exposure to UVR is solely due to involvement in reducing inflammation, whether AA has been oxidised to DHAA during recycling of the tocopheryl radical (vitamin E) or whether there is difficulty with recycling of DHAA back to AA by DHAA reductases (Shindo *et al*., 1994, Packer, 1997, Weber *et al*., 1999).

Numerous *in vitro* studies have demonstrated prevention of UV-induced damage after incubation of skin cells with vitamin C, by monitoring reduction in biomarkers of inflammation or reduction in apoptosis. These have included NFκB (a transcription factor activated under inflammation) and DNA repair mechanisms (Table 1-4).

Table 1-4. Incubation of skin cell lines with vitamin C pre- or post- exposure of cells to UVR, and modifications in inflammation markers observed.

Vitamin C and Conditions	Cell Type	Outcome	Reference
100 µM AA, 12 hours post UVB exposure	Colo 16 (human squamous cell carcinoma)	↓ ROS ↓ apoptosis	(Jin *et al*., 2007)
1 µM AA incubation every 2 days, for 7 days total, pre-UVA exposure	Primary keratinocytes (neonatal foreskin)	↓ lipid peroxidation ↓ lactate dehydrogenase release ↓ IL-1α and IL-6	(Tebbe *et al*., 1997)
1 mM AA for 20 minutes pre-UVA	Mouse embryonic fibroblasts	↓ riboflavin	(Besaratinia *et al*., 2007)
5 mM AA for 1 hour pre-TNFα stimulation	ECV304 (endothelial cell line) and Huvecs	↓ NF-κB activation by TNF and IL-1	(Bowie and O'Neill, 2000)
30 and 100 AA µM, 30 minutes pre-UVB	Primary keratinocytes (neonatal foreskin)	↓ ERK1/2 and p38,	(Peus *et al*., 1999)
2.8 mM AA-Mg for 24 hours pre-UVA	HaCaT human keratinocytes	↓ DNA damage	(Lehmann *et al*., 1998)
200 µM AA-2P and DHAA, 24 hours pre-UVB	Mouse epidermal keratinocytes (Pam212)	↓ CPD, (6-4) PP and p53	(Sugimoto *et al*., 2009)
1 mM AA-2P, 5 hours pre-UVB (assessed 10 µM to 1 mM)	HaCaT human keratinocytes	↓ cell death and radical formation ↓ AP-1 activation ↑ fra-1 expression ↓ phosphorylation of JNK	(Catani *et al*., 2005)
100 µM VC-IP, 24 hours pre-UVB	HaCaT human keratinocytes	↓ PGE_2 and IL-1α	(Ochiai *et al*., 2006)
5 µM AA, 2 days pre-UVB	Mouse keratinocytes (BALB/c MK-2)	↓ 8-OHdG	(Stewart *et al*., 1996)

AA, ascorbic acid; AA-2P, AA 2-phosphate; VC-IP, vitamin C tetra-isopalmitate; UVA, ultraviolet A; UVB, ultraviolet B; TNFα, tumour necrosis factor-α; ROS, reactive oxygen species; IL, interleukin; NFκB, nuclear factor κB; ERK, extracellular signal related kinase; CPD, cyclobutane dimer; (6-4) PP, pyrimidine (6-4) pyrimidone photoproduct; AP-1, activator protein 1; JNK, c-Jun N-terminal kinase; PGE2, prostaglandin E2; 8-OHdG, 8-hydroxydeoxyguanosine.

There have been a number of human trials that have demonstrated the effects of vitamin C supplementation in association with UVR. In an intervention study with 18 participants, volunteers were given 1 g of ascorbic acid and 335 mg of α-tocopherol twice a day for 3 months. The protection against UVB was analysed by evaluating changes in minimal erythemal dose (MED). After one week, the median MED significantly increased by 21 %. After 3 months the increase in MED was 41 % suggesting that pro-longed intake increased the capacity of the skin to protect against exposure to UV. The plasma serum levels of the two vitamins also increased over the 3 month period and the levels of the vitamins in the plasma became stable after one month (Placzek *et al*., 2005). An earlier study by Fuchs and Kern (1998) emphasised the synergistic effect of ascorbic acid (500 mg) and α-tocopherol (500 mg) on increasing MED, as when consumed individually no effect was seen in comparison to placebo consumption.

Another study on the effects of vitamin C supplementation (500 mg ascorbic acid per day) on UVR exposure was conducted *in vivo* on 12 humans for 8 weeks. The results again indicated that the MED did not change after supplementation. However the concentration of ascorbic acid increased in the plasma but stayed at a consistent concentration in the skin after exposure to UVR. The group concluded that the UVR source used only generated an environment of mild oxidative stress as levels of vitamin E and fatty acids also remained consistent after exposure to UVR (McArdle *et al*., 2002).

A human trial involving daily consumption of 500 mg of ascorbic acid for 6 weeks indicated a reduction in the oxidative stress marker 8-OHdG, alongside an increase in blood plasma levels of ascorbic acid, implying that vitamin C is an important component in the regulation of DNA repair enzymes (Cooke *et al*., 1998). In a human trial involving 46 volunteers, 23 consuming a diet rich in AA (600 mg/day) compared with the other 23 who normally had a poor intake of fruit and vegetables, the DNA repair was increased in lymphocytes after the volunteers

were exposed to hyperbaric oxygen therapy after 1 week on the diet (Alleva *et al*., 2012).

Topical application of 10 % vitamin C solution to the skin of pigs reduced the number of erythema developed from exposure to UVB by approximately half. Again, the levels of ascorbic acid after exposure to UV depleted drastically to almost a complete loss in the skin in some cases (Darr *et al*., 1992). For optimal absorption of topical solutions containing ascorbic acid into skin, pH must be lower than 3.5 to remove the ionic charge and the topical solution should contain 20 % AA (above or below reduces uptake into skin; Pinnell *et al.,* 2001).

1.5.8. Green Tea Catechins and UV Inflammation

Flavonoids have also been linked to protection of skin against UV inflammation. As mentioned, flavonoids are secondary metabolites utilised by plants for defence against pests and also protection against ultraviolet radiation, whilst also promoting favourable colour and sensory characteristics. Most association between flavonoids and protection against UV has been performed using cells *in vitro* or topical treatment to humans. Animal studies have also been employed, and treatment with flavonoids has been monitored largely by topical application or by addition of flavonoids to drinking water.

Pharmacokinetic studies after consumption of green tea have shown the presence of the conjugated forms of the catechins in plasma, with minimal presence of the free-forms (usually EGCG and ECG) (Del Rio *et al*., 2010a, Fung *et al*., 2013) so the catechins could be expected to reach the skin in the conjugated form. As green tea catechin conjugates (glucuronide and sulphate) are not commercially available, *in vitro* studies have been performed using treatment of skin cells with unconjugated (free-form) catechins, which indicate the putative protection that green tea catechins could have against UV-induced inflammation. Information from analysis of plasma has also indicated that the maximum concentration reached is

<10 µM, and due to stability of flavonoids during digestion (in particular, instability of catechins at or below neutral pH), the concentrations used for *in vitro* experiments should be less than this (Kroon *et al*., 2004). Similar to the vitamin C studies, skin cells were pre-treated with flavonoids before UV exposure and different markers of UV-induced inflammation were assessed.

Protection of flavonoid treatment against UV-induced inflammation in skin cells *in vitro* is displayed in Table 1-5. Research has highlighted a reduction in many markers of UV inflammation, including regulation of genes including NFκB and MMPs, formation of 8-OHdG and phosphorylation of MAPK by flavonoids *in vitro*.

In animal models, protection against UV-induced inflammation has also been shown in mice treated either orally or topically with green tea catechins. Oral consumption of 6 mg green tea solids/mL water for 23 weeks (Lu *et al*., 2001) reduced the incidence of tumours, and topical application of 6.5 µM EGCG over 18 weeks reduced tumour formation (Lu *et al*., 2002). Wang *et al*. (1991) compared the oral and topical treatment of green tea catechins in reducing tumour formation, over a 25 week period. Oral consumption of the green tea extract reduced the incidence of tumours greater than topical application.

Oral consumption of green tea catechins in mice has also been shown to protect against carcinogenesis by repair and removal of damaged DNA. This corresponded with an increase in IL-12, and also TNFα, IL-1β and IL-6 (Meeran *et al*., 2009). Positive mutant *p53* patches can be indicators of carcinoma development. Oral consumption of green tea catechins before UVB exposure in mice reduced the amount of patches present. This was also true post-treatment of UVB (Conney *et al*., 2008).

Heinrich *et al*. (2011) performed a three month human intervention of daily consumption of 1 L green tea and the effects on protection against UV inflammation in skin. The erythema produced by 1.25 x MED was reduced after consumption of green tea, skin elasticity was improved, skin hydration was increased and dermal

blood flow was also increased. Topical application of EGCG (1mg/cm^2 skin in 50 µL acetone) to the skin of 6 human volunteers 20 minutes prior to UV exposure reduced the production of H_2O_2 and NO in both the epidermis and dermis, and also inhibited the infiltration of inflammatory leukocytes ($CD11b^+$ cells) into the skin (Katiyar *et al.,* 2001).

Topical treatment using green tea catechins in a hydrophilic cream on hairless mice (SKH-1 mice) was shown to inhibit protein oxidation and lipid peroxidation, as well as inhibit the phosphorylation of MAPK proteins (*p38*, JNK and ERK1/2). The depletion of antioxidant enzymes after UV exposure, such as glutathione peroxidase, was also inhibited (Vayalil *et al*., 2003).

In summary, both vitamin C and green tea catechins have a strong positive connection with reduction of UV-induced inflammation in skin, with evidence from both *in vitro* and *in vivo* research.

Table 1-5. Incubation of skin cell lines with flavonoids pre or post exposure of cells to UVR, and modifications in markers observed.

Flavonoids and Conditions	Cell Type	Outcome	Reference
1.4-140 mg/L GTC, 1 hour pre-UVB	HaCaT kerationcyte cells	↓ degeneration of nucleus and mitochondria	(Wu *et al*., 2009)
10 µM EGCG, 24 hours pre-UVB	NHEK (primary human keratinocytes)	↓ NF-κB	(Afaq *et al*., 2003)
44 µM EGCG, 3 hours pre-UVB	NHEK (primary human keratinocytes)	↓ H_2O_2 production ↓ phosphorylation of MAPK signalling pathways (p38, JNK, ERK 1/2)	(Katiyar *et al*., 2001)
5 µM EGCG, 10 hours pre-UVB	HCL 14 (human keratinocyte cells)	↓ AP-1 activation	(Barthelman *et al*., 1998)
2 µM EGCG, 30 minutes pre-UVB	HaCaT human keratinocytes	↓ NO and iNOS ↓ NF-κB	(Song *et al*., 2006)
10-100 µM ECG, 2 hours pre-UVB	HaCaT human keratinocytes	↓ activation of p38, JNK, ERK 1/2; incubation with 100 µM ECG ↓ H_2O_2 production; 10 µM incubation	(Huang *et al*., 2007)
100 µM dihydrocaffeic acid, TNFα and UVB (glucuronide conjugates also tested but no effect)	HaCaT keratinocyte cells	↓ IL-6 and IL-8 (after TNFα) ↓ IL-8 after UVB	(Poquet *et al*., 2008)
10 µM ellagic acid, 24 hours pre-UVB	Human dermal fibroblasts and HaCaT keratinocyte cells	↓ MMP formation	(Bae *et al*., 2010)
1-20 µM delphinidin for 24 hours pre-UVB	HaCaT keratinocyte cells	↓ lipid peroxidation ↓ 8-OHdG formation ↓ PARP cleavage	(Afaq *et al*., 2007)
1-5 µM apigenin and luteolin, 24 hours pre-UVA	HaCaT keratinocyte cells	↓ MMP1 collagenase, cJun and cFos expression ↓ phosphorylation of 3 separate MAPK upstream of AP-1	(Hwang *et al*., 2011)

GTC, green tea catechins; EGCG, epigallocatechin gallate; ECG, epicatechin gallate; UVA, ultraviolet A; UVB, ultraviolet B; TNFα, tumour necrosis factor-α; NFκB, nuclear factor κB; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal related kinase; AP-1, activator protein 1; NO, nitric oxide; iNOS, inducible NO synthase; IL, interleukin; MMP, matrix metalloproteinase; 8-OHdG, 8-hydroxydeoxyguanosine; PARP, poly adenosine diphosphate ribose polymerase.

1.6. Human Intervention Studies Investigating the Effect of Green Tea and Ascorbic Acid Supplementation on Skin Protection

The human intervention studies evaluated within this thesis involved daily consumption of supplements containing green tea catechins and ascorbic acid, to improve stability during digestion in the intestine. Therefore it is possible that green tea catechins within this human model could support the uptake of vitamin C after exposure of skin to UV. ^{3}H-EGCG has been previously identified in the skin of mice following consumption (Suganuma *et al*., 1998). We hypothesised that conjugated forms of catechins and metabolites would be the predominant forms present in human skin after consumption of green tea and ascorbic acid supplements. For the first time, a method that efficiently identifies both free-form and conjugated forms of catechins in biological samples (plasma, urine, skin biopsies, skin interstitial blister fluid) was employed. Any protective effects of skin against UV-induced inflammation identified post supplementation (samples currently processed by the University of Manchester and University of Bradford) could be accredited to the daily consumption of green tea catechins and vitamin C.

1.7. Thesis Objectives

The aims of this thesis were to investigate the effects of green tea catechin treatments on skin cell metabolism during stress *in vitro*, and to study the bioavailability of green tea catechin, metabolites and conjugates in skin *in vivo* (Figure 1-10).

The aims were achieved by:

1) Synthesis of ^{14}C-DHAA for uptake studies
2) Evaluation of the effects of treatments with a purified mixture of green tea catechins (GTC) or green tea extract (GTE) on the viability of HaCaT cells in culture

3) Evaluation of the effects of various stresses (FBS depletion, hydrogen peroxide and UVB) on the viability of HaCaT cells in culture
4) Evaluation of the effects of GTC and GTE treatment on the viability of HaCaT cells in culture that had been subjected to various stresses
5) Evaluation of the effects of stress, GTC and GTE treatments on ^{14}C-vitamin C uptake in HaCaT cells in culture
6) Evaluation of the effects of stress, GTC and GTE treatments on the expression of vitamin C transporters and COX2
7) The development of an analytical LC-MS method that efficiently identifies both free-form and conjugated forms of catechins in biological samples (plasma, urine, skin biopsies and skin interstitial blister fluid)

Experimental Hypotheses:

1) Stress causes a decrease in viability of cells
2) GTC and GTE treatments enhance vitamin C uptake in stressed cells with positive effects on viability
3) The enhanced vitamin C uptake is mediated through the up regulation of vitamin C transporters in cells
4) Conjugated catechins are present in biological tissues following consumption of GT and ascorbic acid in human volunteers

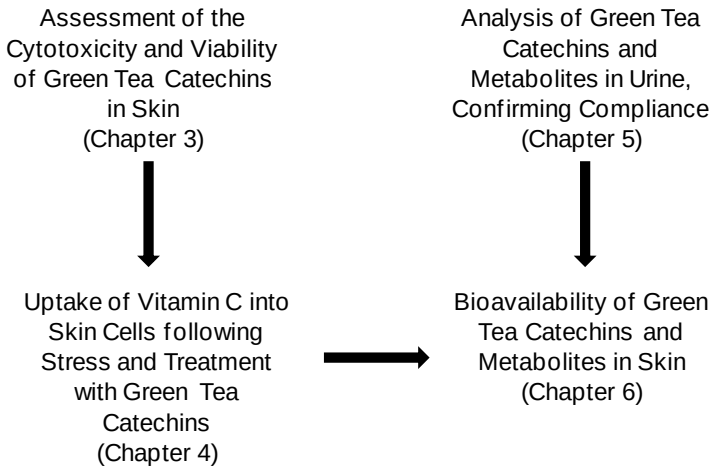


Figure 1-10. Thesis objectives.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Equipment

All high performance liquid chromatography (HPLC) analysis was performed on an Agilent 1200 SL system (Agilent Technologies, Dorset, UK) which comprised of a binary pump, degasser, well plate autosampler (5 ºC), and column oven (35 ºC). A Zorbax Eclipse Plus C18 microbore column (1.8 µm, 100 x 2.1 mm; Agilent Technologies, Dorset, UK) was used for HPLC analysis. For analysis using liquid chromatography mass spectrometry (LC-MS), a Kinetex C18 microbore column (2.6 µm, 150 x 2.1 mm; Phenomenex, Cheshire, UK) was used and the HPLC system was connected to a 6410 triple quadrupole LC-MS/MS (Agilent Technologies, Santa Clara, CA, USA). The flow was passed into an electrospray source with a gas temperature of 350 ºC, a 30 psi nebulizer pressure and nitrogen flowing at 11L/min. Analytes were detected in negative mode using multiple reaction monitoring (MRM) acquisition. A Millipore Q water purifying system (Millipore, Hertfordshire, UK) was used to provide ultrapure, nuclease free water (≥18.2 MΩ cm at 25 °C) for HPLC, LC-MS and polymerase chain reaction (PCR) experiments.

For centrifugal evaporation a Genevac (EZ-2 plus model; Fisher Scientific Ltd, Leicestershire, UK) was used with the setting HPLC fraction. Absorbance measurements for the ascorbate oxidase assay were performed using a Cecil CE 201 spectrophotometer (Cecil Instruments Ltd., Cambridgeshire, UK) and two matched quartz cuvettes (Hellma UK Ltd, Essex, UK). A Multiskan FC Microplate Photometer was used for absorbance measurements for cell culture assays and the Bradford assay, (Thermo Fisher Scientific, Loughborough, UK) with 96 well plates (Greiner Bio-One Ltd, Gloucestershire, UK).

For quantification of ^{14}C-ascorbic acid (AA) and ^{14}C-dehydroascorbic acid (DHAA), a Tri-Carb 1600TR (Packard Instruments, Canberra Ltd, Oxfordshire, UK) scintillation counter was used with 10 mL Ecoscint XR scintillation fluid and 20 mL scintillation vials (National Diagnostics, Fisher Scientific Ltd, Leicestershire, UK). Quantification of messenger RNA (mRNA) was performed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Loughborough, UK) and conversion of mRNA to complementary DNA (cDNA) and also quantitative real-time reverse transcription PCR (qRT-PCR) reactions were performed using a StepOne Real time PCR System (Applied Biosystems, Life Technologies Ltd, Cheshire, UK).

Ultraviolet irradiation of an immortal human keratinocyte (HaCaT) cell line was performed using an ultraviolet B (UVB) lamp at 312 nm (LF 206MS using a 6W bulb, Uvitec, Cambridgeshire, UK) and a UVB sensor (UVX-31) and radiometer (UVX radiometer using the 20 mW/cm^2 setting, UVP, Cambridgeshire, UK). The UVB lamp was placed at the top of a matt black box, 9 cm directly above the 6 well plates.

2.1.2. Standards

The green tea extract used in cell culture treatments was supplied by Nestlé (Nestlé Research Centre, Switzerland). Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), catechin (C) and taxifolin were purchased from Extrasynthése (Genay, France). Ethyl gallate (EG) and 3-methyl gallic acid were obtained from Apin Chemicals Ltd (Oxfordshire, UK), hippuric acid, benzoic acid, 3-(2,4-dihydroxyphenyl) propionic acid, vanillic acid and 3-hydroxybenzoic acid were purchased from Fluka (Dorset, UK), 4-hydroxybenzoic acid was from Sigma Aldrich (Dorset, UK), 2H hippuric acid, 2,4-dihydroxybenzoic acid, 3-x-dihydroxybenzoic acid (possibly 3-4 or 3-5) and 2,4,6-trihydroxybenzoic acid were from Acros Organics (Leicestershire, UK), and 2-hydroxypropionic acid, 3,4-dihydroxyphenylacetic acid, 3-(3-hydroxyphenyl) propionic acid,

3-(4-hydroxyphenyl) propionic acid, 3-hydroxyphenyl acetic acid, 4-hydroxyphenyl acetic acid, homovanillic acid, syringic acid, mandelic acid and gallic acid were purchased from Alfa Aesar (Lancashire, UK). All standards were of HPLC purity (above 98 %).

2.2. Methods for *In Vitro* Studies

2.2.1. Cell Culture

HaCaT cells (a monolayer of keratinocytes, a gift from Dr Laure Poquet, Nestlé Research Centre, Switzerland) were used between passages P48 and P66. They were cultured in Roswell Park Memorial Institute (RPMI-1640) medium containing 11 mM glucose and supplemented with 10 % fetal bovine serum (FBS), 584 mg/L L-glutamine, 100 U/mL penicillin-streptomycin and 0.25 µg/mL amphotericin B (Sigma Aldrich, Dorset, UK) for 7 days in T75s (60,000 cells/cm^2, Corning, Sigma Aldrich, Dorset, UK) at 37 ºC under a humidified atmosphere of 95 % air: 5 % CO_2. This medium will be referred to throughout as serum medium. Serum medium was changed every 2 or 3 days.

When the cells were 80-90 % confluent, they were washed with Hanks Balanced Salt Solution (HBSS) and then treated with a 0.25 % trypsin/ethylenediaminetetraacetic acid (EDTA) solution for 15 minutes. The trypsin was inactivated with equal amounts of serum medium (Sigma Aldrich, Dorset, UK). The cells removed via trypsin were centrifuged at 1200 rpm for 5 minutes and the cell pellet was resuspended in serum medium. To assess for viability, the cells were mixed with trypan blue (Sigma Aldrich, Dorset, UK) and counted using a haemocytometer under a microscope. Cells that were frozen down for long term storage and future use, were centrifuged again and the cell pellet was resuspended in freezing medium (10 % dimethyl sulfoxide (DMSO, Sigma Aldrich, Dorset, UK), 70 % FBS and 20 % RPMI-1640) and placed in a cryo-vial at 5,000,000 cells/mL (Nunc, Thermo Fisher Scientific, Loughborough, UK). The cryo-vial was placed for

24 hours at -80 °C in a freezer container filled with isopropanol (Mr Frosty, VWR, Leicestershire, UK) to ensure a cooling rate of 1 ºC/min and was then removed from the container and left at -80 °C. Ideally after 24 hours at -80 °C, the cryo-vial would be moved to liquid nitrogen for long term storage.

Prior to experiments, cells were seeded in 6 well plates (density of 60,000 cells/cm^2, or 190,000 cells/well; Corning, Sigma Aldrich, Dorset, UK) in serum medium and differentiated over 4 days. Experiments were performed in serum free medium and before treatments began serum medium was aspirated and HaCaTs were washed with HBSS to remove any remaining FBS.

2.2.2. Treatment of HaCaT Cells with Green Tea, Hydrogen Peroxide and Ultraviolet B

HaCaT cells were treated with an amount of green tea extract (GTE) or a purified mixture of flavanols (green tea catechins, GTC) that contained 10 µM EGCG (other flavanols present were 0.4 µM C, 1.8 µM ECG, 1.9 µM EC and 10 µM EGC). A solution of the GTC or GTE was dissolved in HBSS and then diluted in serum free medium to the required concentrations. Due to degradation of the catechins at neutral pH (epimerisation; Figure 1-3), the treatment medium was prepared immediately before the experiment and was added rapidly to the cells.

The cells were then incubated with green tea (GT, corresponding to either GTE or GTC; 2 mL per well) for up to 24 hours, before assessment with the lactate dehydrogenase assay (LDH) for cytotoxicity of the GT, with the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for viability, change in expression of vitamin C transporters by qRT-PCR, and uptake of ^{14}C-vitamin C (Figure 2-1A).

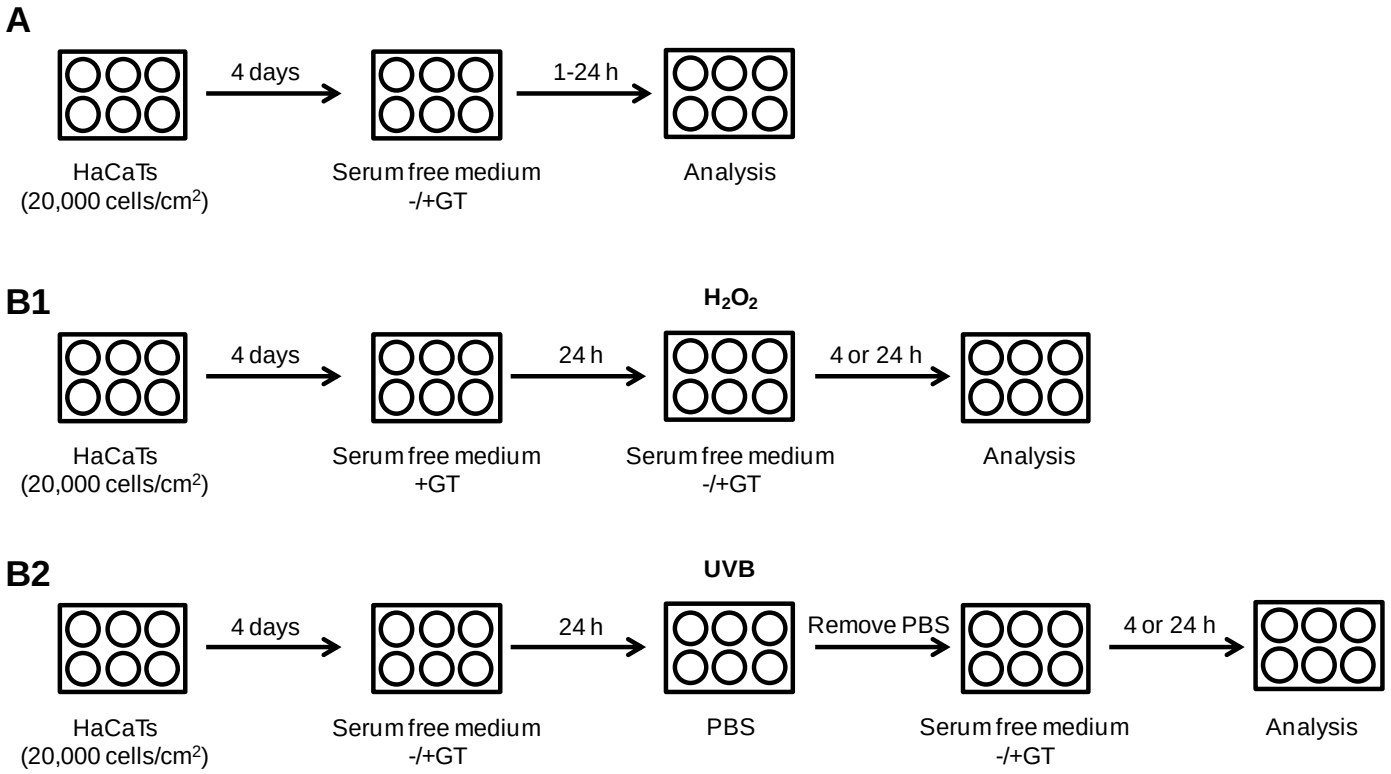


Figure 2-1. Protocols for treatment of HaCaT cells with green tea (GT, either GTE or GTC). A. Treatment with GT for 1 to 24 hours. B. Pre-treatment without GT for 24 hours followed by treatment with (1) hydrogen peroxide simultaneously with or without GT for 24 hours, or (2) UVB and subsequently treatment with or without GT. Analysis was by qRT-PCR (regulation of vitamin C transporters), assessment of cytotoxicity (LDH) and viability (MTT), and by uptake of radio-labelled vitamin C (^{14}C-AA and ^{14}C-DHAA).

Oxidative stress experiments were performed by pre-treating HaCaT cells with 2 mL GT for 24 hours and then subjecting the cells to either UVB irradiation or hydrogen peroxide stress (Figure 2-1B). For hydrogen peroxide stress, cells were treated initially with GT for 24 hours, and then were treated with 0, 0.1 or 1 mM hydrogen peroxide simultaneously with or without GT (2 mL/well) for a further 24 hours before analysis as mentioned previously.

For UVB stress, HaCaTs were treated with 2 mL GT for 24 hours. Cells were washed with phosphate buffer solution (PBS, Sigma Aldrich, Dorset, UK) and then 1 mL PBS was added to each well for UVB irradiation. The UVB lamp was switched on 10 minutes prior to the experiment and then the intensity of the UVB emitted was measured by placing the sensor directly below the lamp (in place of the 6 well plate). The amount of time that the cells experience irradiation depends on the energy required for the experiment (10 or 50 mJ/cm^2) and the intensity of the lamp, and so the following calculation was used: time (sec) = energy (mJ/cm^2) / intensity (mW/cm^2), where 1 J = 1 W sec. PBS was removed after irradiation and the cells were treated with or without 2 mL GT for a further 24 hours before analysis.

HaCaT cells were also treated for 24 hours with or without EC-*O*-sulphate and EGC-*O*-glucuronide for 24 hours prior to UVB exposure and then again for 24 hours post-exposure, followed by analysis of ^{14}C-vitamin C uptake. For synthesis of EC-*O*-sulphate and EGC-*O*-glucuronide, see section 2.3.1.2.1.

2.2.3 Analysis of HaCaT Cells

2.2.3.1 Lactate Dehydrogenase Cytotoxicity Assay

The release of LDH from the cytoplasm (indicating membrane damage) was analysed with the cytotoxicity detection kit plus (LDH, Roche Diagnostics Ltd, West Sussex, UK). LDH present in the removed medium converts lactate to pyruvate which in turn reduces NAD^+ to $NADH/H^+$. The kit provides a catalyst (NAPDH-diaphorase) that transfers two hydrogens from $NADH/H^+$ to a yellow

tetrazolium salt (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) to form a red formazan salt that can be monitored at 492 nm.

After experiments, medium was collected from the 6 well plates and stored at 4 °C (for 3 days maximum). Excess treatment medium (not incubated with the cells) was also stored at 4 °C, and was used as a blank. 100 µL sample was added to a 96 well plate in technical triplicates. Then 100 µL reaction mixture (catalyst and dye solution from the kit) was added before shaking the plate for 10 seconds and incubating for 10 minutes at room temperature. Then 50 µL stop solution was added before shaking the plate for another 10 seconds and then reading the absorbance at 492 nm.

2.2.3.2. MTT Viability Assay

MTT is a yellow tetrazolium salt which is taken up by viable cells and becomes purple as it is reduced by dehydrogenases present within the cells. For the MTT assay, medium was aspirated from the cells and then 2 mL of MTT solution was added to each well (0.5 mg MTT/mL serum free medium). Cells were incubated with the MTT solution for 2 hours. The MTT solution was aspirated and 2 mL of destaining solution was added (100 mM HCl and 10 % Triton-X-100 (Fisher Scientific Ltd, Leicestershire, UK) in isopropanol (Sigma Aldrich, Dorset, UK)), solubilises the purple tetrazolium which is insoluble in aqueous solutions) and then the well plates were covered in aluminium foil and left on a plate shaker for 15 minutes. 200 µL sample was added in technical triplicates into 96 well plates and measured at 595 nm. For hydrogen peroxide and UVB treated cells, 100 µL sample was added to a 96 well plate and diluted with 100 µL destaining solution (also used as the blank for all MTT reactions).

2.2.3.3. RT-PCR for Changes in Expression of Vitamin C Transporters

mRNA from HaCaT cells was isolated using the Qiagen RNeasy Kit (Valencia, CA, USA) according to the instructions. In brief, the cell monolayer was lysed using the RLT buffer (containing guanidine-thiocyanate) supplied in the kit and subsequently scraped using a cell scraper (Greiner Bio-One Ltd, Gloucestershire, UK), followed by disruption of plasma membranes and cell walls by sheering through a 20-gauge needle and syringe which simultaneously homogenised the sample. The lysed sample was then mixed with ethanol (Sigma Aldrich, Dorset, UK) to improve binding of RNA to the silica membrane present within the inner column of the supplied eppendorf tube. This allowed for the other components present within the sample to be eluted during centrifugation. The samples were then washed three times (once with RW1 buffer which contains a guanidine salt, and twice with RPE buffer which contains ethanol) before the RNA sample was finally eluted with water.

Quantification and purity of mRNA was assessed using a Nanodrop (absorbance at A_{260} and purity ratio between 1.9-2.1; A_{260}/A_{280}) before conversion to cDNA (using 1 µg mRNA and the High Capacity RNA to cDNA Kit, Applied Biosystems, Cheshire, UK). Again the manufacturer's instructions were followed, but in brief, 1 µg mRNA was added to a 0.1 mL tube (8 tube strip, Applied Biosystems, Cheshire, UK) on ice and then nuclease free water was added to take the total sample volume to 9 µL. 10 µL 2x RT buffer and 1 µL 20x RT enzyme mix (provided in the kit) were added and the tube was vortexed and spun down before loading the 8 tube strip onto a thermal cycler (37 °C for 60 minutes, 95 °C for 5 minutes and then 4 °C until sample collection). The sample was placed at -20 °C until it was required for qRT-PCR.

Standard curves were performed for all gene probes and all were normalised to the endogeneous control (housekeeping) gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All genes assessed (apart from laminin, 50 ng cDNA)

required 100 ng cDNA per reaction. Quantitative changes in gene expression were assessed using real time reverse transcription (TaqMan Gene Expression Master Mix, Applied Biosystems, Cheshire, UK). Reverse transcription was performed by mixing 2 µL of the cDNA sample with 1 µL of the gene probe, 1 µL of GAPDH, 6 µL RNase free water and 10 µL master mix (which contains AmpliTaq Gold DNA polymerase, uracil-DNA glycosylase, dNTPs with dUTP, ROX passive reference and other buffer components) in a 48 well plate (Applied Biosystems, Cheshire, UK) before spinning down the plate to remove any air bubbles. A blank was added to each plate in triplicate (cDNA was replaced with water) to confirm that there was no contamination.

The plate was transferred to the StepOne Real-Time PCR system and then entered into the following thermal cycling conditions: 2 minutes at 50 °C, 10 minutes at 95 °C and then 40 cycles of 15 seconds at 95 °C followed by 1 minute at 60 °C. The 15 second step allows for denaturing of the double strand and the 1 minute step provides the time for the annealing and extension of the primer onto the cDNA and also the binding of the TaqMan MGB probe (containing a reporter at the 5' end and a non-fluorescent quencher and minor groove binder at the 3' end) to the strand. If the probe binds to the strand then the reporter is cleaved by the DNA polymerase present within the mastermix and a fluorescent signal is detected.

The results were expressed as C_T values (or threshold cycle) which is the cycle number at which the fluorescent signal is significantly detected for the first time. Changes in expression are normalised to the endogenous control using the following calculation:

$$\text{Change in gene expression} = 2^{-\Delta\Delta CT}$$

where ΔC_T = Gene of interest - GAPDH and $\Delta\Delta C_T = \Delta C_T$ - average ΔC_T for control (untreated) cells only.

The primers were obtained from Applied Biosystems (Gene Expression Assays, Cheshire, UK); GLUT1 (SLC2A1, Hs00892681_m1), GLUT3 (SLC2A3, Hs00359840_m1), GLUT4 (SLC2A4, Hs00168966_m1), SVCT1 (SLC23A1, Hs00195277), SVCT2 (SLC23A2, Hs00192765), COX2 (PTGS2, Hs00153133), 12LOX (ALOX12, Hs00167524) and Laminin (Hs00165042_m1) which were all labelled with the FAM dye and GAPDH (NM_002046.3) which was labelled with the VIC dye. The two different dyes allowed for duplex reactions. Each experiment was performed in three biological replicates and for the PCR quantitative reactions technical triplicates of each were used.

2.2.3.4 Uptake of ^{14}C-Vitamin C

50 µCi (1.85 MBq, 1.02 mg) ^{14}C-AA was obtained from Perkin Elmer (Cambridgeshire, UK) with a specific activity of 2-10 mCi (74-370 MBq)/mmol (and the radiolabel on carbon 1). For 50,000 dpm/500 µL of a 250 µM solution, 42.98 mg "cold" AA was added to 1.02 mg "hot" ^{14}C-AA before dilution in 25 mL ethanol. The solution was vortexed until the ascorbic acid dissolved creating a 10 mM (1.76 mg/mL) solution. This was portioned into 500 µL aliquots (0.88 mg). The ethanol was evaporated using centrifugal evaporation for 15 minutes on the low boiling point fraction with the lamp off. Dried down aliquots were stored at -80 °C before reconstitution in 20 mL glucose-free and serum-free medium (a 250 µM solution; Sigma Aldrich, Dorset, UK).

After completion of cell treatment with GT or stress (Figure 2-1), cells were washed with PBS and then they were incubated for 10 minutes with 100 or 250 µM ^{14}C-AA or ^{14}C-DHAA. As ^{14}C-DHAA is not commercially available, ^{14}C-AA was incubated with ascorbate oxidase (AO, 0.8 U/10 µL in 50 % glycerol; Sigma Aldrich, Dorset, UK) for 5 minutes.

To stop the uptake of ^{14}C-vitamin C, medium was removed and cells were washed twice with ice cold HBSS. The cell monolayer was then lysed with 500 µL

1 M NaOH for 5 minutes before scraping with a cell scraper. 500 µL 0.1 M HCl was added to begin neutralising the samples (otherwise too many bubbles form when mixing the lysed cells in sodium hydroxide only, and adding 1 M HCl causes the cells to aggregate making it difficult to form a homogenous solution (Figure 2-2). 800 µL of the sample was added to a 20 mL scintillation vial containing 405 µL 1 M HCl which neutralises the sample and 10 mL scintillation fluid (alkaline solutions interfere with the scintillation fluid causing a high background noise). The vials were counted for 10 minutes and results were expressed as counts per minute (cpm). Cells were normalised to mg protein present in 800 µL of sample using a Coomassie (Bradford) protein assay kit (Fisher Scientific Ltd, Leicestershire, UK).

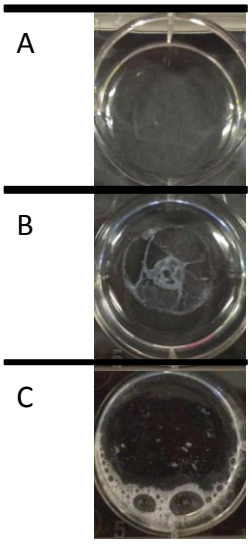


Figure 2-2. Cell lysis of ^{14}C-vitamin C samples using 500 µL 1 M NaOH and 500 µL 0.1 M HCl (A), the aggregation caused by 1 M NaOH neutralised with 1 M HCl (B) and the bubbles formed when using 1 M NaOH alone (C).

2.2.3.4.1. Protein Quantification using the Bradford Assay

The remaining radiochemistry sample was processed with the Bradford Assay kit (Pierce, Thermo Fisher Scientific, Loughborough, UK) following the instructions. In brief, 5 µL sample was added to a 96 well plate and 250 µL Coomassie reagent was added. The plate was mixed with the shaker inside the

plate reader for 30 seconds before 10 minute incubation at room temperature. The plate was then read at 595 nm and samples were converted into µg/mL using a standard curve created with the bovine serum albumin standard provided.

2.2.3.4.2. Conversion of ^{14}C-Ascorbic Acid to ^{14}C-Dehydroascorbic Acid

To confirm the conversion of ^{14}C-AA to ^{14}C-DHAA with AO (50 % glycerol), the methanol method created by Badrakhan *et al*. (2004) was employed. Concentrations of DHAA in water or glucose free and serum free medium (400 µL) were mixed with 10 µL 50 % glycerol and left at room temperature for 5 minutes (mimicking AO with AA reaction). Then 30 µL deferrioxamine mesylate (15.4 mM, 10 mg/mL, discarded after 6 hours of dissolving), 30 µL water treated with Chelax 100 (a chelating agent that removes transition metals from samples, 0.4 mg/10 mL treated for 24 hours prior to the experiment and adjusted to pH 5.5), 200 µL methanol and 400 µL phosphate/citrate buffer (400 mM NaH_2PO_4 and 125 mM citric acid L-hydrate) and then incubated at 37 °C for 30 minutes before reading at 346 nm using a spectrophotometer (all Sigma Aldrich, Dorset, UK). To confirm conversion, identical concentrations of AA (to the DHAA standards, again 400 µL) were incubated with 10 µL AO (0.8 U) at room temperature for 5 minutes and then mixed with the same components as for the DHAA reaction before incubating for 30 minutes at 37 °C and analysis at 346 nm.

2.3. Methods for the Green Tea Human Studies

The human studies formed part of a collaborative project undertaken by three Universities (University of Leeds, University of Manchester and the University of Bradford), each with different roles. In total, there were three separate green tea intervention studies performed, each occurring over a three month period.

The three human intervention studies included a pilot study, a double-blind randomised controlled trial (RCT) and a bioavailability study. The pilot study was an

open study in which 16 subjects consumed a low dose supplement (equivalent of 2 cups; three 450 mg supplements each containing 2.1 mg C, 12.5 mg EC, 49.3 mg EGC, 26 mg ECG and 72.6 mg EGCG) of green tea daily for three months. This allowed for an initial investigation to be performed into the protection of green tea supplementation against UV-induced skin damage, and also provided samples for method development at the three Universities.

The three month double-blind RCT involved daily consumption of supplements containing either a placebo or green tea (high dose; equivalent to 5 cups of green tea) by 50 volunteers. The purpose of the RCT was to assess any beneficial changes to biomarkers of skin damage by green tea after UV irradiation by comparing both irradiated and non-irradiated samples (blisters and biopsies) for placebo and green tea volunteers at the beginning of the intervention (pre-supplementation) and at the end (week 12).

The bioavailability study involved the consumption of the high dose supplementation by 11 volunteers daily for 3 months. Four different biological samples (interstitial blister fluid, skin biopsies, plasma and urine) were collected on day 1 of the study and after 3 months supplementation, to identify if any association was apparent between green tea catechin metabolites present in each of the samples after daily consumption for 3 months.

The University of Manchester conducted the oral intervention studies and distributed samples to all three sites. During the process the mimimal erthyma dose (MED) was assessed using solar simulated UVR on the upper buttock skin to verify if green tea could reduce sunburn over the three month time course for the pilot and RCT. Blister fluid and skin tissue from the upper buttock were collected for eicosanoid analysis (prostaglandin E2 and 12-hydroxyeicosatetraenoic acid) at the University of Bradford (pilot and RCT), green tea catechin metabolite analysis at the University of Leeds (pilot and bioavailability) and immunohistochemical analysis (quantification of neutrophils, cluster of differentiation (CD)3^+ cells and $CD8^+$ cells)

at the University of Manchester (RCT). Plasma samples were also collected for the bioavailability study and these were analysed by the University of Leeds only, for green tea catechin metabolite profiling.

To test for compliance to the studies, urine excreted over a 24 h period was collected from volunteers at various time points during the intervention (baseline sample at day 0, first post-supplementation sample on day 1 and then again at week 6 and finally week 12) and green tea catechin metabolites present within the urine were tested for. During the RCT, the urine was also used to identify whether the volunteers had been consuming the placebo or the green tea supplement, and for those consuming the green tea, the compliance was confirmed. Ethical approval was received from the North Manchester Research Ethics Committee (reference 08/H1006/79) and the study conformed to the Declaration of Helsinki principles.

2.3.1. Testing Urine for Compliance

Compliance was assessed by counting the residual capsules in the dispensed containers returned by the volunteers, and through analysis of 24 h urine samples collected from all volunteers pre-supplementation (day 0) and then after 1 day, 6 weeks and 12 weeks supplementation. Urine was collected in HCl-washed flasks containing AA (approx 1 g/L), and stored in aliquots at –20°C.

2.3.1.1. Enzyme Deconjugation of Pilot Study Urine

Urine was defrosted and the pH was adjusted to pH 5 with 0.1 M NaOH. The procedure was performed on ice. 40 µL of urine (two technical replicates for each biological sample) was combined with 4 µL NaH_2PO_4 solution (0.4 M, pH 5, 200 g/L AA, 1 g/L EDTA) and 20 µL sodium acetate buffer (0.2 M, pH 5, 0.012 µg of the internal standard taxifolin, and 5 U sulphatase, type VIII, Abalone entrails; Sigma Aldrich, Dorset, UK). 200 U β–glucuronidase (type X, E. coli; Sigma Aldrich, Dorset, UK) in NaH_2PO_4 (75 mM, pH 6.8) was also added and the sample was

gently vortexed before incubation at 37 ºC for 1 hour. The sample was then extracted with 250 µL ice cold ethyl acetate three times (Fisher Scientific Ltd, Leicestershire, UK), with a 30 second vortex and 2 minute centrifugation at 17,000 g step in between each extraction. Supernatants were combined and then dried down under nitrogen flow on ice. Samples were placed at -80 ºC until required.

Reconstitution of the dried down sample involved the addition of 12 µL of 20 % acetonitrile (with 1 g/L AA; VWR, Leicestershire, UK). Samples were sealed in a microwell plate (Eppendorf, Leicestershire, UK) and then 5 µL of the sample was injected onto a Kinetex C18 microbore column running a binary gradient of LC-MS grade water vs. acetonitrile both with 0.2 % formic acid, at 0.3 ml/min. The gradient started at 5 % acetonitrile for 5.8 minutes and then increased to 30 % over 29.2 minutes, before rising to 95 % over 2.4 minutes. The column was washed at 95 % acetonitrile for 3.6 minutes before returned back to 5 % over 3.6 minutes with a re-equilibration over 10.9 minutes. The total method length was 55.5 minutes, and a list of free-form catechins and metabolites and retention time, is displayed in Table 2-1.

Table 2-1. The green tea catechin metabolite transitions present in urine for the pilot study.

Compound	M-H (m/z)	MS^2 (m/z)	Fragmentor	Collision Energy	Retention Time (min)
EGCG	457.1	168.9	120	15	19.6
GCG	457.1	168.9	120	2	19.9
ECG	441	169	140	20	24.4
CG	441	169	140	20	25.5
3'-Me-EGC	319.2	260	130	15	19.2
4'-Me-EGC	319.2	137	110	10	19.5
GC	305	124.9	100	15	5.4
EGC	305	124.9	100	15	13.8
Taxifolin	303	284.9	110	2	23.6
3'-Me-EC	303	136.8	110	12	22.5
4'-Me-EC	303	136.8	110	12	24.3
C	289	244.8	120	8	14.6
EC	289	245	120	8	18.5
M4	223	179	130	12	9.8
M6'	207	163	120	12	15.5
M6	207	163	120	15	17.2
Syringic Acid	197	181.9	90	8	16.5
2H Hippuric Acid	194	149.9	80	8	17.8
3-Me-Gallic Acid	183	167.9	90	8	8.4
4-Me-Gallic Acid	183	167.9	80	5	9.8
3-(2,4-Dihydroxphenyl) Propionic Acid	181	136.9	90	8	12.9
Homovanillic Acid	181	136.9	90	8	15.4
Hippuric Acid	178.1	134	80	5	11.6
Gallic Acid	169	124.9	90	10	2.7
2,4,6-Trihydroxybenzoic Acid	169	150.9	80	8	5.7
3,4-Dihydroxyphenylaceti c Acid	167	123	60	0	17.8
Vanillic Acid	167	151.9	80	8	14.1
3-(4-Dihydroxyphenyl) Propionic Acid	165	121.1	110	5	6.9
3-(3-Dihydroxyphenyl) Propionic Acid	165	121	80	5	19.7
2,4-Dihydroxybenzoic Acid	153.1	109	80	8	12.6
3,x-Dihydroxybenzoic Acid	153	108.8	80	8	5.3
Mandelic Acid	151	106.9	70	2	4.4
4-Hydroxyphenyl Acetic Acid	151	106.9	70	2	7.9
3-Hydroxyphenyl Acetic Acid	151	107	60	0	13.9
2-Hydroxypropionic Acid	151	107	70	8	15.3
4-Hydroxybenzoic Acid	137	92.9	80	10	9.6
3-Hydroxybenzoic Acid	137	92	80	8	13.3
Benzoic Acid	121	77	70	8	21.5

Me, methylated; epigallocatechin, EGC; epigallocatechin gallate, EGCG; epicatechin gallate, ECG; epicatechin, EC; hippuric acid, HA; gallic acid, GA; syringic acid, SA; benzoic acid, BA; 3-hydroxybenzoic acid, 3-HBA; -5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone, M4; -5-(3',4'-dihydroxyphenyl)-γ-valerolactone, M6; -5-(3',5'-dihydroxyphenyl)-γ-valerolactone, M6'.

2.3.1.2. Presence of EC-*O*-Sulphate and EGC-*O*-Glucuronide in Urine

The protocol for urine preparation was based on a method developed by Sang *et al*. (2008). The urine was defrosted and vortexed for 30 seconds before sonication for 5 minutes. The urine was vortexed again for 30 seconds and 200 µL was removed for analysis (technical duplicates from one biological sample). This was mixed with 100 µL internal standard 1 (ethyl gallate in acetonitrile, 2 µg/mL), 100 µL ethanol and 800 µL acetonitrile (for protein precipitation; Fisher Scientific Ltd, Leicestershire, UK) for 30 seconds before sonication for 5 minutes. The sample was vortexed again for 30 seconds before centrifuging at 17,000 g for 10 minutes. The supernatant was placed in a new eppendorf and dried down using centrifugal evaporation (46 ºC, HPLC fraction). Samples were stored at -20 ºC until analysis.

For analysis, 50 µL ethanol and 50 µL internal standard 2 (taxifolin, 0.5 µg/mL in 0.1 % ascorbic acid in 5 % acetonitrile) were added to each dried down sample before vortexing for 30 seconds, sonicating for 5 minutes and vortexing again for 30 seconds. The sample was centrifuged at 17,000 g for 10 minutes and the supernatant placed into amber HPLC vials (Fisher Scientific Ltd, Leicestershire, UK).

A 5 µL sample aliquot was injected onto a Kinetex C18 microbore column running a binary gradient of 95 % LC-MS grade water (5 % acetonitrile) vs. 95 % acetonitrile (5 % water) both with 0.1 % formic acid, at 0.3 ml/min. The gradient started at 0 % acetonitrile and increased to 100 % over the first 8.4 min (7 column volumes), then it was held at 100 % for 3.6 min (3 column volumes) to wash the column before returning to 0 % acetonitrile over 2.4 min (2 column volumes), re-equilibrating over 4.8 min (4 column volumes). The total method length was 19.2 min.

Analyte and MS^2 transition parameters were individually optimized using standards that were commercially available; EC (289>245), EGC (305>125) ethyl

gallate (internal standard 1, 197>124) and taxifolin (internal standard 2, 303>285). Conjugates analysed were EC-*O*-sulphate (369>289) and EGC-*O*-glucuronide (481>305).

2.3.1.2.1. Synthesis of EC-*O*-Sulphate and EGC-*O*-Glucuronide

For synthesis of EC-*O*-sulphate and EGC-*O*-glucuronide, a method reported by Wong *et al*., (2010) was employed. EC-*O*-sulphate was synthesised by the following method, adjusting volumes accordingly. First, 15 µL potassium phosphate buffer (PPB; pH 7.4), 1 µL EC substrate (4 mM in water; final concentration 80 µM), 10 µL dithiothreitol (5.1 mM in PPB; final concentration 1 mM) and 5 µL p-nitrophenyl sulphate (200 mM in water; final concentration 20 mM) were mixed with 5 µL of an inhibitor (100 mM Na_2SO_3 in water; final concentration 10 mM) and 2.5 µL 3'-phosphoadenosine-5'-phosphosulphate (2 mM in PPB; final concentration 100 µM). 12.5 µL cytosolic pig liver enzyme was added and the mixture was incubated for 4 hours at 37 ºC. Sulphation was terminated by the addition of 100 µL ice cold acetonitrile (500 mM HCl) and placed on ice for 15 minutes before centrifugation at 17,000 g (4 ºC) for 15 minutes. The supernatant was placed at -80 ºC until analysis.

For synthesis of EGC-*O*-glucuronide, firstly 40 µL hydroxyethyl piperazineethanesulfonic acid (HEPES, pH 7.2) was combined with 20 µL EGC substrate (4.4 mM in 25 mM HEPES; final concentration 800 µM) and 20 µL alamethicin (22 mM in 25 mM HEPES; final concentration 1 µM). This was mixed with 20 µL uridine-5'-diphospho-glucuronic acid (11 mM in 25 mM HEPES; final concentration 2 mM) and 5 µL saccharolactone (22 mM in 25 mM HEPES; final concentration 1 mM). 5 µL microsomal pig liver enzyme was added and the mixture was incubated for 4 hours at 37 ºC before termination by the addition of 200 µL ice cold methanol (1.6 mM AA), and centrifuged and stored as mentioned for sulphation.

2.3.2. Analysis of Bioavailability Samples

A 3 month intervention study was completed in which 11 volunteers consumed a green tea supplement and then biological samples (interstitial blister fluid, biopsies, plasma and urine) were collected to assess the bioavailability of green tea in skin by comparison of green tea catechin metabolites present in plasma 3 hours post green tea supplementation with metabolites present in skin samples (blister and biopsy) at the same time. This allows for an understanding of the target sites reached by green tea catechin metabolites, and will also support any changes seen in the immunohistochemistry and inflammatory marker results performed at the University of Bradford and the University of Manchester.

Skin samples were collected before and after irradiation of buttock skin with a solar simulator (Newport Spectra-Physics Ltd) which mimics sunlight by irradiating with both UVA and UVB (290-400 nm). The light source was distanced 10 cm away from the skin and a radiometer was used to calibrate the source and confirm consistency of application. The skin was irradiated with 10 doses of UV, and after 24 hours the lowest dose which produced an erythema was noted and this minimal erythema dose (MED) was monitored between the first irradiation on day 0 and the week 12 irradiation. A dose that was 3 times higher than the MED was applied to induce any inflammatory responses, and then blister and biopsy samples were collected.

2.3.2.1. Urine Analysis

Urine was processed similarly to the description in 2.3.1.2. To improve the recovery of spiked conjugates and free-form (unconjugated) derivatives of green tea catechin metabolites, the protocol was adjusted. A combination method was employed that allowed for isolation of conjugates in acetonitrile (used to precipitate protein from the urine) and extraction of free-form catechins and metabolites into ethyl acetate.

After the urine had been defrosted, 50 µL AA (6 mM solution in water, to give a final concentration of 1 mM) and 50 µL EG (4 µg/mL in water) were added to 200 µL urine (technical duplicates from one biological sample which allowed for handling errors, but showed little variation between samples) on ice. Samples were vortexed for 10 seconds and 500 µL ice cold ethyl acetate was added. Samples were vortexed for 30 seconds and left on ice for 2 minutes before centrifuging at 17,000 g (4 ºC) for 2 minutes. The supernatant was removed to a new eppendorf and the process was repeated again (resulting in a total of 1 mL ethyl acetate present in the supernatant eppendorf). The supernatant was dried down under nitrogen flow on ice and when eventually dry it was placed at -80 ºC.

The urine sample (still on ice) remaining after ethyl acetate extraction was then mixed with 800 µL ice cold acetonitrile and vortexed for 2 minutes before centrifuging at 17,000 g (4 ºC) for 10 minutes. The supernatant was placed into a pre-weighed eppendorf tube and the organic solvent was removed by centrifugal evaporation (HPLC fraction, lamp off). The remaining aqueous portion of the sample remaining in the eppendorf was calculated (using 1 g = 1 mL) after the eppendorf was re-weighed. Samples were placed at -80 ºC until required. For analysis on the LC-MS, 90 µL of the aqueous sample was added to the dried down sample. 10 µL taxifolin (20 µg/mL in 50 % acetonitrile and 1 % AA, to give a final concentration of 5 % and 0.1 %, respectively) was also added before 1 minute of vortexing followed by 5 minutes sonicating. The sample was then centrifuged for 5 minutes at 17,000 g (4 ºC) and placed into an amber HPLC vial.

The LC-MS method was also extended to allow for analysis of conjugates, as well as free-form (unconjugated), derivatives of green tea catechin metabolites, providing a more extensive profile (Table 2-2). The structures and fragmentation patterns are displayed in Chapter 5 (Figure 5-5). Again, a 5 µL sample aliquot was injected onto a Kinetex C18 microbore column running a binary gradient of 95 % LC-MS grade water (5 % acetonitrile) vs. 95 % acetonitrile (5 % water) both with

0.1 % formic acid, at 0.3 ml/min. The gradient started at 0 % acetonitrile and was held at 0 % for 6 minutes (5 column volumes), then it was increased to 100 % over 15 column volumes (by 24 minutes). 100 % was held for 3.6 min (3 column volumes) to wash the column before returning to 0 % acetonitrile over 1.2 min (1 column volumes), re-equilibrating over 4.8 min (4 column volumes). The total method length was 33.6 min (Figure 2-3).

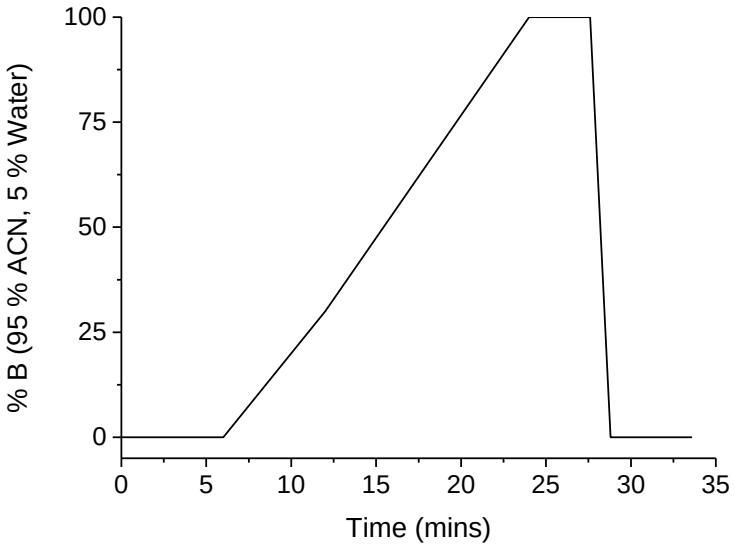


Figure 2-3. Gradient of acetonitrile during LC-MS analysis of biological samples (urine, plasma, skin interstitial fluid and skin biopsies) from the human intervention studies.

Table 2-2. The transitions for the green tea related compounds assessed for in plasma, blisters, biopsies and urine for the three month bioavailability study. The quantitative transitions are highlighted in bold. The purpose of the other transitions was to qualify that the compounds had been identified correctly (peak in the same position for both quantifier and qualifier transitions).

Compound	M-H (m/z)	MS^2 (m/z)	Compound	M-H (m/z)	MS^2 (m/z)
EGCG-*O*-Glucuronide	**633**	**457**	*O*-Me-M4-*O*-Sulphate	**317.1**	**222**
ECG-*O*-Glucuronide	**617**	**441**		317.1	237.1
EGCG-*O*-Sulphate	**537**	**457**	3-HBA-*O*-Glucuronide	**313**	**137**
	537	376.9		313	233.2
ECG-*O*-Sulphate	**521**	**441**	EGC	**305**	**124.9**
O-Me EGC-*O*-Glucuronide	**495**	**319**		305	174.9
	495	174.9	3'-*O*-Me-EC	**303.1**	**136.8**
EGC-*O*-Glucuronide	**481**	**305**		303.1	165
	481	343.3	Taxifolin	**303.1**	**284.9**
O-Me-EC-*O*-Glucuronide	**479**	**303**		303.1	124.9
	479	74.8	M4-*O*-Sulphate	**303.1**	**223**
Quercetin-*O*-Glucuronide	**477.1**	**300.8**		303.1	179.2
	477.1	150.8	4'-*O*-Me EC	**303.1**	**136.8**
EC-*O*-Glucuronide	**465**	**289**		303.1	165
	465	214.1	Quercetin	**301.1**	**150.8**
Kaempferol-*O*-Glucuronide	**461.1**	**284.9**		301.1	178.8
EGCG	**457**	**168.9**	EC	**289**	**245**
	457	304.9		289	203
ECG	**441**	**169**	C	**289**	**244.8**
	441	289		289	202.9
O-Me-M4-*O*-Glucuronide	**413**	**217**	M6/M6'-*O*-Sulphate	**287.1**	**207.1**
	413	195		287.1	162.9
M4-*O*-Glucuronide	**399.1**	**222.9**	Kaempferol	**285**	**186.9**
	399.1	179.1		285	92.9
O-Me-EGC-*O*-Sulphate	**399.1**	**319**	SA-*O*-Sulphate	**277**	**197.2**
	399.1	216.7		277	181.9
EGC-*O*-Sulphate	**385**	**305**	*O*-Me-GA-*O*-Sulphate	**263.1**	**127**
	385	217		263.1	145.1
O-Me-EC-*O*-Sulphate	**383.1**	**303**	HA-*O*-Sulphate	**258**	**177.8**
	383.1	137.1	GA-*O*-Sulphate	**249**	**169**
M6/M6'-*O*-Glucuronide	**383.1**	**206.9**	M4	**223**	**179**
	383.1	113.2		223	137.8
Quercetin-*O*-Sulphate	**381.1**	**300.8**	3-HBA-*O*-Sulphate	**217**	**93.1**
	381.1	150.8		217	136.9
SA-*O*-Glucuronide	**373**	**153**	M6	**207**	**163**
	373	197		207	122
EC-*O*-Sulphate	**369**	**289**	M6'	**207**	**163**
	369	245.1		207	122
Kaempferol-*O*-Sulphate	**365**	**285.1**	BA-*O*-Sulphate	**201**	**121**
	365	284.9	SA	**197.1**	**181.9**
O-Me-GA-*O*-Glucuronide	**359**	**183.3**		197.1	166.9
	359	112.8	Ethyl Gallate	**197**	**124**
HA-*O*-Glucuronide	**354**	**178**		197	169.1
GA-*O*-Glucuronide	**345.2**	**113.1**	3-Me GA	**183**	**167.9**
	345.2	75		183	123.8
3'-*O*-Me-EGC	**319.2**	**260**	HA	**178.1**	**134**
	319.2	275	GA	**169**	**124.9**
4'-*O*-Me-EGC	**319.2**	**137**		169	78.8
	319.2	166	3-HBA	**137**	**92.9**
			BA	**121**	**77**

Epigallocatechin, EGC; epigallocatechin gallate, EGCG; epicatechin gallate, ECG; epicatechin, EC; hippuric acid, HA; gallic acid, GA; syringic acid, SA; benzoic acid, BA; 3-hydroxybenzoic acid, 3-HBA; -5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone, M4; -5-(3',4'-dihydroxyphenyl)-γ-valerolactone, M6; -5-(3',5'-dihydroxyphenyl)-γ-valerolactone, M6'.

2.3.2.2 Plasma Analysis

Plasma samples were defrosted and the whole procedure was carried out on ice. The samples were gently vortexed before 380 µL (technical duplicate for each biological sample was performed) was removed and placed in a 2 mL eppendorf with 20 µL AA (22.5 mM in water, final concentration of 1 mM) and 50 µL EG (4 µg/mL in water). Samples were vortexed for 10 seconds and then 1 mL of hexane (Sigma Aldrich, Dorset, UK) was added dropwise to the eppendorf tube. Samples were then vortexed for 1 minute and centrifuged at 17,000 g (4 ºC) for 10 minutes. The top fat layer was removed to waste and the sample was placed back on ice. If the sample still contained a notable amount of fat, then another 1 mL hexane was added. 500 µL of ice cold ethyl acetate was added and the procedure continued as for urine, with the only change being the addition of 1.5 mL acetonitrile to the sample that remained after ethyl acetate extraction (therefore the pre-weighed tube was a 2 mL eppendorf) and 10 µL was injected onto the column.

2.3.2.3. Blister Analysis

Interstitial blister fluid was produced through use of suction cups under a vacuum on two areas of the buttock; non-irradiated skin and skin irradiated with 3x MED. On average, a blister was formed after 3 hours of vacuum, and it was removed with a needle and combined with 25 µL NaH_2PO_4 (0.4 M, pH 3.6 with 200 g/L AA and 1 g/L EDTA) before being snap frozen in liquid nitrogen and eventually stored at -80 ºC.

Blister samples were defrosted on ice and initial volumes were recorded. The whole procedure was performed on ice. If there was more than 80 µL initially then two technical replicates were performed for each biological sample. The samples were gently vortexed and then briefly spun down. 40 µL of blister fluid was added to an eppendorf, along with 10 µL 0.4 M NaH_2PO_4 solution (containing 200 g/L AA, 1 g/L EDTA and 0.04 µg/10 µL EG). 300 µL ice cold ethyl acetate was

added and the sample followed the same procedure as for urine, however 160 µL acetonitrile was used, the sample was only centrifuged at 17,000 g (4 ºC) for 5 minutes and after re-weighing the eppendorf tubes samples were reconstituted back to 20 µL using water before vortexing for 1 minute. 18 µL of the sample was collected and added to the corresponding dried down ethyl acetate tube with 2 µL taxifolin (20 µg/mL in 50 % acetonitrile and 1 % AA). Samples were sonicated for 5 minutes and 9.5 µL was added to two wells on a covered microwell plate and 5 µL of the sample was injected onto the column.

2.3.2.4. Biopsy Analysis

Biopsies were removed from the buttocks of skin that had been irradiated with 3 x MED, and also skin that had not been irradiated. Skin punch biopsies (with a diameter of 5 mm) were removed after volunteers had been injected at the site with lignocaine. Biopsies were immediately snap frozen in liquid nitrogen and then stored at -80 ºC until analysis.

To analyse the contents of the skin biopsy, the constituent cells needed to be broken open before homogenisation. Biopsies were removed from -80 ºC storage and initial weights were recorded using pre-weighed 2 mL eppendorf tubes. The procedure was conducted on ice and biopsies were only removed from -80 ºC storage when required (no mass preparation). Biopsies were held with sterilised tweezers over a waste beaker and flushed with ice cold hexane as many of the biopsies had blood present on the outer layer which may interfere with the results. After washing with hexane, a stainless steel rod and base (known as the bomb, a technique also referred to as cryo-pulverisation) were placed into a vat of liquid nitrogen for approximately one minute until effervescing ceased. The bomb was removed and using tweezers a biopsy was placed into the vat of liquid nitrogen until effervescing stopped. The biopsy was removed and then placed in between the rod and base and quickly hit with a hammer to pulverise the sample. Pulverisation

occurs as ice crystals are formed from the rapid freezing of the water content present within the biopsy, and on impact shattering can occur. The stainless steel retains the cold temperature and therefore the temperature of the sample is kept very low.

As can be seen in Figure 2-4, the biopsy became flat and flake-like, but did not become a fine powder (the water content of the biopsy caused the flakes to blend together to look like a paste when the temperature began to rise). The sample was immediately placed back into the 2 mL eppendorf tube using a scalpel and tweezers, and stored at -80 ºC until required. In between each biopsy bombing, the scalpel and tweezers were re-sterilised using 70 % ethanol and hot water.

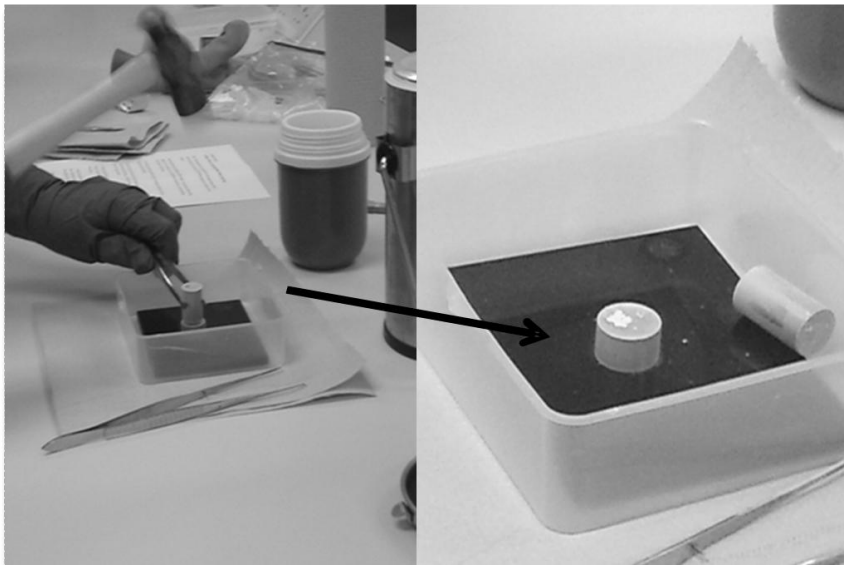


Figure 2-4. The stainless steel bomb used to break apart the skin biopsy structure as the first step in the homogenisation process. The bomb and biopsy were both placed in liquid nitrogen before the biopsy was struck with a hammer.

The second step of the homogenisation process involved using a micro homogeniser (Ultra Turrax, IKA T10 Basic, IKA Ltd, Cheshire, UK) on dry ice (to reduce the heat produced by the sheering process). To the 2 mL eppendorf containing the bombed biopsy sample, 100 µL ice cold chloroform (with 0.1 g/L butylated hydroxytoluene; Fisher Scientific Ltd, Leicestershire, UK) and 100 µL ice

cold sodium dithionite (0.3 M, with 0.08 µg EG; Sigma Aldrich, Dorset, UK) was added. The biopsy was homogenised by shearing of the samples between two very sharp blades on dry ice. After homogenisation (approximately 5 minutes), the sample was kept on dry ice for two minutes before centrifuging for 2 minutes at 17,000 g (4 ºC). The top aqueous layer was removed to a new eppendorf tube and 100 µL 0.3 M sodium dithionite (not containing EG) was added to the biopsy sample which was then vortexed for 1 minute. The sample was placed on dry ice for two minutes and then centrifuged again with the resulting top aqueous layer being combined with the previous.

500 µL ice cold ethyl acetate was added and the sample followed the same procedure as for urine, however 600 µL acetonitrile was used. The samples were reconstituted to 40 µL with water and 36 µL was added to the corresponding dried down ethyl acetate eppendorf, alongside 4 µL taxifolin (20 µg/mL in 50 % acetonitrile and 1 % AA). Samples were sonicated for 5 minutes and 18 µL was added to two wells on a covered microwell plate with 10 µL of the sample injected onto the column.

Chapter 3. Effect of Green Tea on Viability and Stress Response in the HaCaT Cell Line

3.1. Abstract

The response of the HaCaT (human keratinocyte) cell line to stress (starvation and oxidative) was assessed after treatment with green tea catechins, either present in an extract (GTE) or in a purified catechin mixture (GTC). Cell viability was monitored using the MTT uptake assay, and the cytotoxicity was explored using the LDH assay. For the first time, it was clear that HaCaT cells endured stress from starvation in the absence of 10 % fetal bovine serum (FBS) for 24 hours, apparent from a decrease in uptake and intracellular reduction of MTT. The presence of GTE or GTC during starvation improved the uptake of MTT, implying improved viability. LDH release into medium, a marker of cell death, was increased in cells during FBS starvation, and was reduced in the presence of GTE or GTC (by 97 and 79 %, respectively when compared with control treatment). Simultaneous treatment of HaCaT cells exposed to oxidative stress induced by extracellular hydrogen peroxide (<1 mM), increased uptake of MTT and reduced release of LDH into medium, suggesting improved viability and reduction in cell death when GTE or GTC were present during stress. Treatment with GTE or GTC reduced cell death, as apparent by reduced LDH release, after HaCaT cells were exposed to UVB. GTE or GTC treatment improved viability of cells exposed to a high dose of UVB (50 mJ/cm^2). In summary, treatment of HaCaT cells undergoing stress with green tea improved viability and reduced cell death, as monitored by MTT uptake and LDH presence.

3.2. Introduction

The two main assays commonly used to examine viability *in vitro* are the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake assay (viability) and lactate dehydrogenase (LDH) leakage assay (cytotoxicity). MTT is taken up by viable cells via passive diffusion due to the plasma membrane potential, and then is reduced intracellularly by NAD(P)H-dependent oxidoreductases and dehydrogenases (Berridge *et al*., 2005). The reduced MTT is monitored at 595 nm. The LDH assay assesses the presence of LDH within the medium. Upon cell membrane damage from necrosis, LDH is released from the cytoplasm into the medium. LDH is measured by conversion of lactate to pyruvate, followed by reduction of a tetrazolium salt monitored at 492 nm.

Assessing more than one assay allows for different cell mechanisms to be examined and for a more conclusive understanding of the effects that xenobiotics have on the cell line. One study explored the differences in using four separate assays on a mouse fibroblast cell line. The four assays explored were LDH release, MTT uptake, neutral red dye uptake and the presence of ATP in cells. Three assays represented cell viability but demonstrated different outcomes, highlighting that there are variations in sensitivity of each assay (Weyermann *et al*., 2005).

There are many tetrazolium salts available for analysis of cell viability. The most commonly used salt for analysis of green tea catechins *in vitro* is MTT. An alternative to MTT is the MTS salt which is water soluble. Wang *et al*. (2010b) analysed the difference in outcome of MTT and MTS assays after LNCAP and MCF-7 cells were treated with various concentrations of EGCG. There was no significant difference between the outcomes of the two tetrazolium salts. To impede interference of flavonoids with MTT or MTS reduction, the cells must be washed with HBSS or PBS before the salts are added to the cells (Bruggisser *et al*., 2002, Wang *et al*., 2010b).

A study by Slater *et al*. (1963) assessed the reduction of four tetrazolium salts in rat tissue suspensions and focused on reduction of the salts within the mitochondrial respiratory chain. This study indicated that MTT was reduced in the cytochrome C compartment of the chain. Many studies that utilise the MTT assay, relate cell viability to reduction of MTT by succinate dehydrogenase in mitochondria of living cells. This however is not the case, as many studies have since tested MTT reduction within different subcellular fractions (nuclear, mitochondrial, microsomal and cytosol) and have demonstrated reduction (Liu *et al*., 1997, Berridge and Tan, 1993).

The aim of this chapter is to investigate the effect of green tea extract and green tea catechins on the HaCaT cell line (immortalised human keratinocyte cells). Two assays were employed, one to assess viability (MTT uptake) of the cells and the other to assess cytotoxicity (LDH assay) in response to GTE or GTC at biologically relevant concentrations whilst the cells were exposed to different types of stress. Any alterations identified after green tea treatment could be related to interaction of green tea catechins with pathways that induce necrosis. The results obtained confirm that further assessment of the HaCaT cells by vitamin C uptake can be pursued.

3.3. Materials and Methods

The protocols used to perform viability and cytotoxicity analysis of the HaCaT cell samples are presented in section 2.2.

3.4. Results

As mentioned previously, the MTT assay is widely accepted as a viability assay that monitors the uptake and subsequent reduction of the tetrazolium salt by succinate dehydrogenase present within mitochondria of active cells. Many studies have confirmed the reduction of MTT in other subcellular fractions after uptake.

Therefore all results will be interpreted as uptake and reduction of MTT by viable cells, rather than mitochondrial activity.

3.4.1. Viability of HaCaT Cells after 24 hours Green Tea Treatment

When performing *in vitro* experiments, the MTT assay is used to confirm that tested compounds do not affect the viability of the cell line (i.e. toxic concentrations). HaCaT cells were treated with green tea extract (equivalent of 10 µM EGCG) for up to 24 hours in serum free medium. As FBS binds to polyphenols it is usually removed from experiments performed *in vitro*.

Over the 24 hours, cells treated with GTE were more viable in comparison to un-treated control cells as signified by a higher uptake of MTT (Figure 3-1). When medium was supplemented with 10 % FBS, there were significantly more viable cells present in comparison to the serum free cells. When cells were simultaneously treated with FBS and GTE, the uptake of MTT was increased by 16 % in comparison to cells treated with only FBS ($p<0.0001$). It is possible that FBS is binding to catechins present within GTE, and the effect on viability of any unbound catechins remaining occurs by 24 hours.

It is clear that over the 24 hour time course, viability is reduced when cells are experiencing starvation (absence of FBS). Viability is improved with the addition of FBS to the medium. With the addition of GTE to serum free medium, the viability of cells is maintained over the 24 hours (74 % increase in MTT uptake in cells at 24 hours in comparison to control; $p<0.005$).

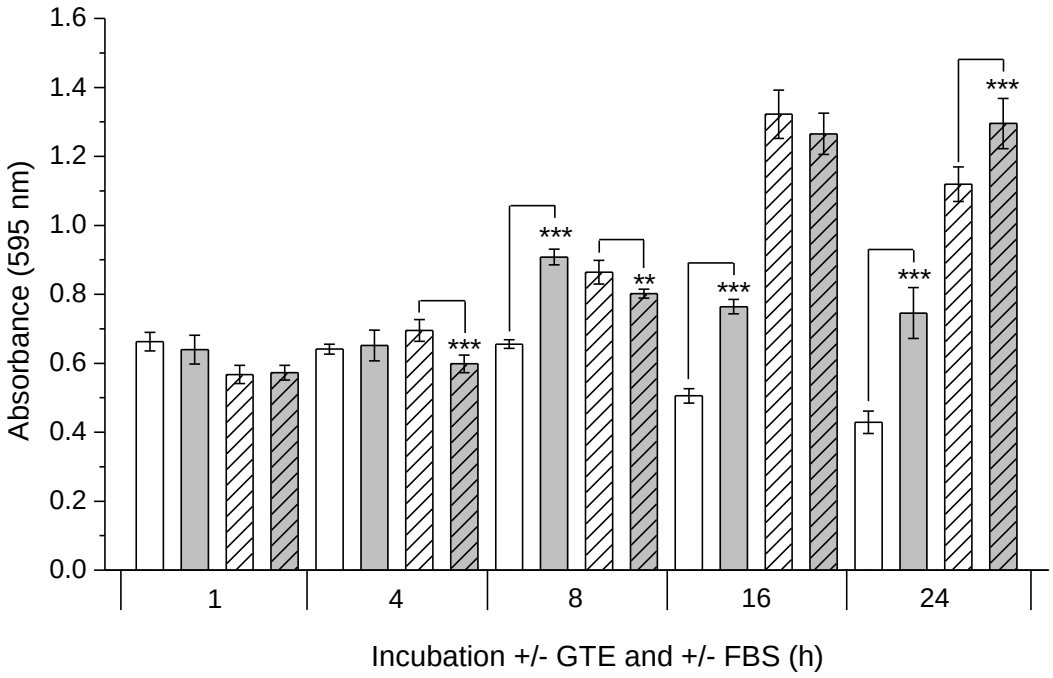


Figure 3-1. Uptake of MTT into HaCaT cells treated for up to 24 hours with or without GTE and also with or without FBS. GTE (*grey bars*) and control samples (*white bars*), with FBS (*stripes*) or without FBS (*no stripes*) were assessed using a spectrophotometer at 595 nm after cells were incubated for 2 hours with MTT. Experiments were performed in triplicate twice, and then technical triplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with or without GTE is shown for with and without FBS treatments; $^{**}p<0.001$, $^{***}p<0.0001$; average+/-S.E.

3.4.2. Cytotoxicity of HaCaT Cells after 24 hours Green Tea Treatment

To assess any cytotoxic effect that GTE may have had on HaCaT cells, medium was removed from between 1 and 24 hours after GTE treatment and then analysed for presence of LDH. LDH is released into medium surrounding the cells upon cellular damage, through damaged plasma membranes. All treatments had appropriate blanks that were subtracted before analysis of results. Control and GTE medium (without cells) was assessed after 24 hours incubation at 37 ºC, and there was no difference in the absorbance at 492 nm, confirming that green tea does not interfere with the assay by inhibiting reduction of lactate to pyruvate (data not shown).

The effect of FBS on the presence of LDH within medium was explored. Cell death was more apparent in control cells undergoing starvation (Figure 3-2). Without FBS present in the medium, the growth of cells may have been affected as growth factors and hormones that are usually supplemented in medium by the addition of FBS have been removed. This may have caused the increase in cell death resulting in release of LDH.

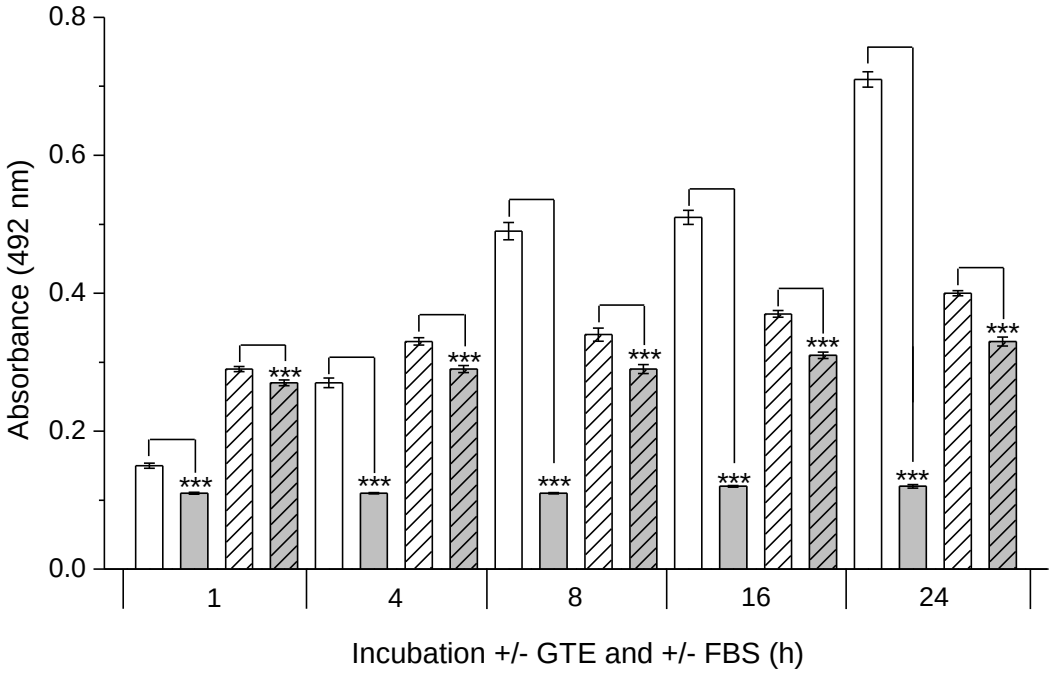


Figure 3-2. Release of LDH from HaCaT cells into the medium after treatment of cells for up to 24 hours with or without GTE and also with or without FBS. GTE (*grey bars*) and control samples (*white bars*), with FBS (*stripes*) or without FBS (*no stripes*) were monitored using a spectrophotometer at 492 nm. Experiments were performed in triplicate twice, and then technical duplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with or without GTE is shown for with FBS and without FBS treatments; $^{***}p<0.0001$; average+/-S.E.

Cells treated with GTE in the absence of FBS had a consistent level of LDH present within the extracellular medium (Figure 3-2). Cell death was increased in

cells starved of FBS and in the absence of GTE, over the 24 hour time period ($p<0.0001$), in comparison to cells treated with GTE. For example, by 24 hours there was a reduction in cell death by 83 % in GTE treated and serum free cells. GTE also significantly reduced the release of LDH into medium of cells simultaneously treated with FBS, but to a lesser extent than cells experiencing FBS starvation.

3.4.3. Involvement of Catechins on Viability and Cytotoxicity Assays Performed after 24 hours Green Tea Treatment in HaCaT Cells

To understand the extent of the role that the catechins present in GTE have on viability and cell death, HaCaT cells were treated for 24 hours with GTC or GTE in the absence of FBS. The GTC mixture (10 µM EGCG, 10 µM EGC and 1.9 µM EC) had a similar effect on the increase of MTT uptake and subsequent reduction by viable cells when compared to GTE (33 % and 44 % increase in MTT uptake for GTC and GTE treated cells, respectively; Figure 3-3A).

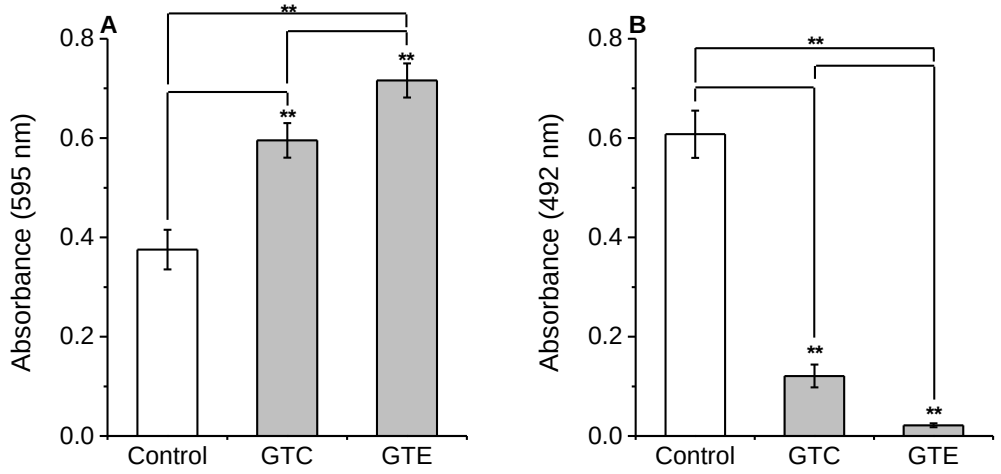


Figure 3-3. Uptake of MTT into HaCaT cells (A) and release of LDH into medium (B) of cells treated with GTE or GTC for 24 hours. GTE or GTC (*grey bars*) and control samples (*white bars*) were assessed using a spectrophotometer at 595 nm after cells were incubated for 2 hours with MTT, or at 492 nm for analysis of LDH. Experiments were performed in triplicate twice, and then technical triplicates were performed for each (Student's t-test; two tailed, two sample; $^{**}p<0.001$; average+/-S.E.).

A significant reduction of cell death, as indicated by a reduction of LDH in medium, was apparent for HaCaT cells treated with GTC, but not to the same extent as GTE (reduction in cell death by 79 % and 97 % for GTC and GTE, respectively; Figure 3-3B). Other components present in the GTE (possibly L-theanine or caffeine) are responsible for the remaining effect on the increase in MTT uptake and reduction of MTT, and reduction in LDH release into the medium.

The effect of concentration of individual catechins (EGCG, EGC and EC) and GTE was tested on viability and cytotoxicity, as monitored by the uptake and subsequent reduction of MTT, and the presence of LDH in medium. The viability of cells, as assessed by MTT uptake, was increased significantly in HaCaT cells treated with 10 µM GTE and 10 µM EGCG (44 and 54 %, respectively) when related to control (un-treated) cells ($p<0.0001$; Figure 3-4).

GTE and EGCG at 10 µM were the two treatments and the only concentration that also produced a significant reduction in cell death (Figure 3-5). In comparison to the respective control cells, LDH release was significantly reduced ($p<0.001$) by 55 % and 70 % (EGCG and GTE, respectively). This indicates that the catechin which influences viability and partially influences the reduction in cell death is EGCG (10 µM).

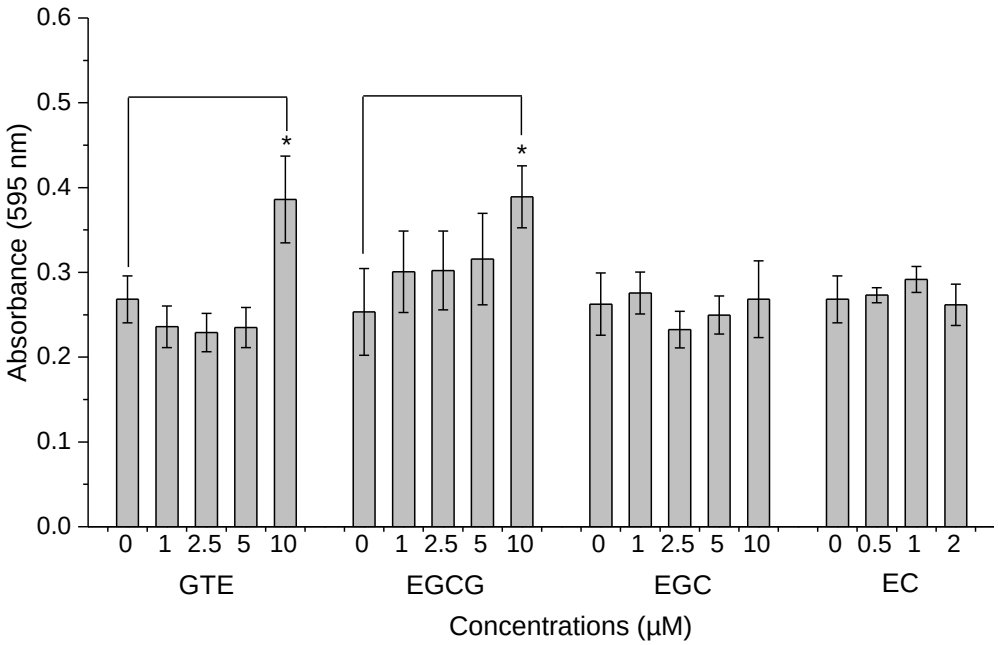


Figure 3-4. Uptake of MTT into HaCaT cells treated with GTE and individual catechins for 24 hours. Samples were assessed at 595 nm after cells were incubated for 2 hours with MTT. Experiments were performed in duplicate three times, and then technical duplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with catechins against control cells are shown; $^{*}p<0.05$; average+/-S.E.

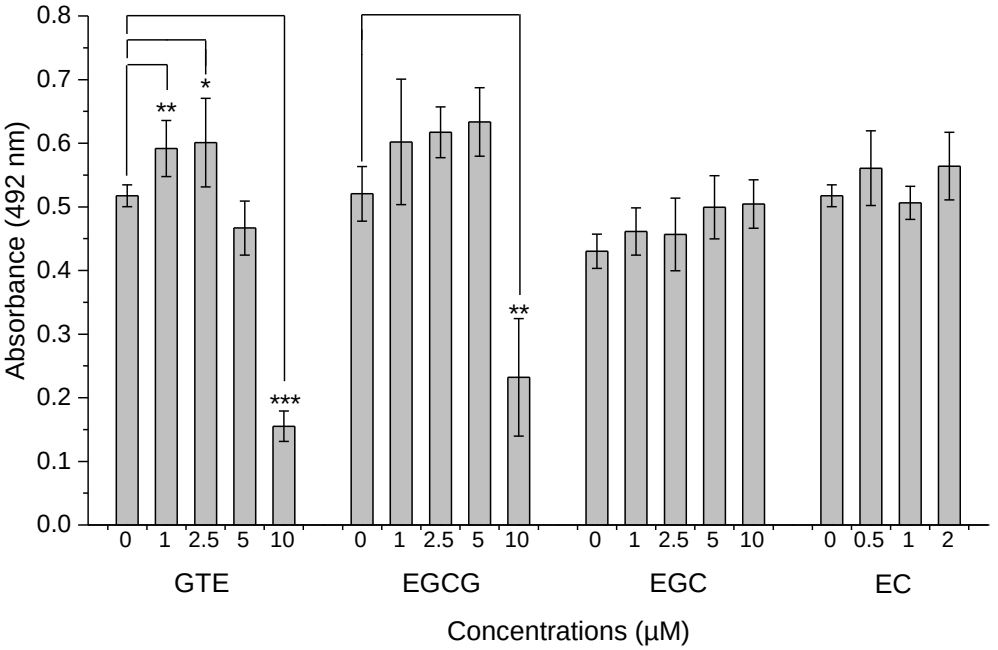


Figure 3-5. Release of LDH into the medium after HaCaT cells were treated with GTE and individual catechins for 24 hours. Samples were assessed at 492 nm. Experiments were performed in duplicate twice, and then technical duplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with catechins against control cells are shown; $^{*}p<0.05$; average+/-S.E.

3.4.4. Viability and Cytotoxicity of HaCaT Cells after Treatment with Hydrogen Peroxide and Green Tea

HaCaT cells were treated for 24 hours with GTE before treatment with various concentrations of hydrogen peroxide (<10 mM) for a further 24 hours. Above 1 mM hydrogen peroxide, the viability of cells, as assessed with MTT uptake, was substantially reduced in comparison to cells that were treated with no hydrogen peroxide ($p<0.0001$; Figure 3-6). Viability of HaCaT cells treated with 0.5 and 1 mM was significantly increased in comparison to control cells (no hydrogen peroxide; $p<0.0001$). Hydrogen peroxide at these concentrations might be simulating hormesis (activation of repair mechanisms by exposure of cells to toxins, and simulation of a beneficial effect at a low dose in comparison to high dose resulting in a biphasic dose response; Mattson, 2008). To assess a broad range of stress on the HaCaT cells, 0.1 and 1 mM hydrogen peroxide were used to examine vitamin C uptake (Chapter 4).

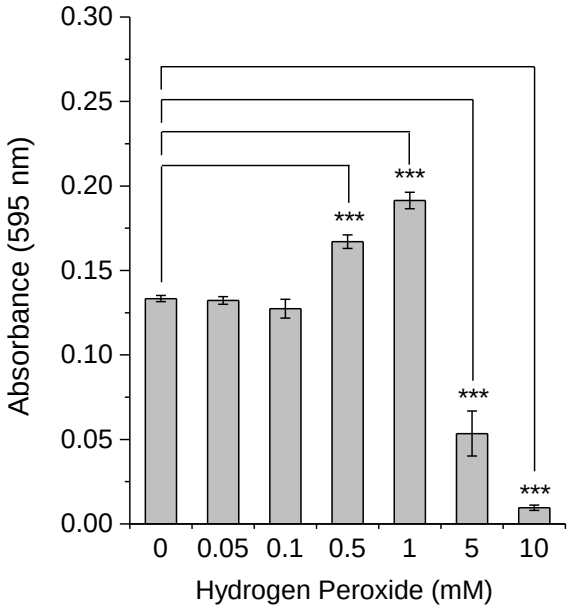


Figure 3-6. Uptake of MTT into HaCaT cells treated with GTE for 24 hours and then a range of hydrogen peroxide concentrations for 24 hours. Samples were assessed at 595 nm after cells were incubated for 2 hours with MTT. Experiments were performed in duplicate three times, and then technical triplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with hydrogen peroxide against un-treated control cells are shown; $^{***}p<0.0001$; average+/-S.E.

Simultaneous treatment of HaCaT cells with GTE or GTC and 0 or 0.1 mM hydrogen peroxide, identified that there was no clear difference between treatment with GTE or GTC (Figure 3-7). In comparison to untreated cells (no GTE or GTC), the presence of GTE significantly increased the uptake of MTT under all hydrogen peroxide conditions ($p<0.05$). The increase was only significant for GTC treatment for 0 and 0.1 mM hydrogen peroxide.

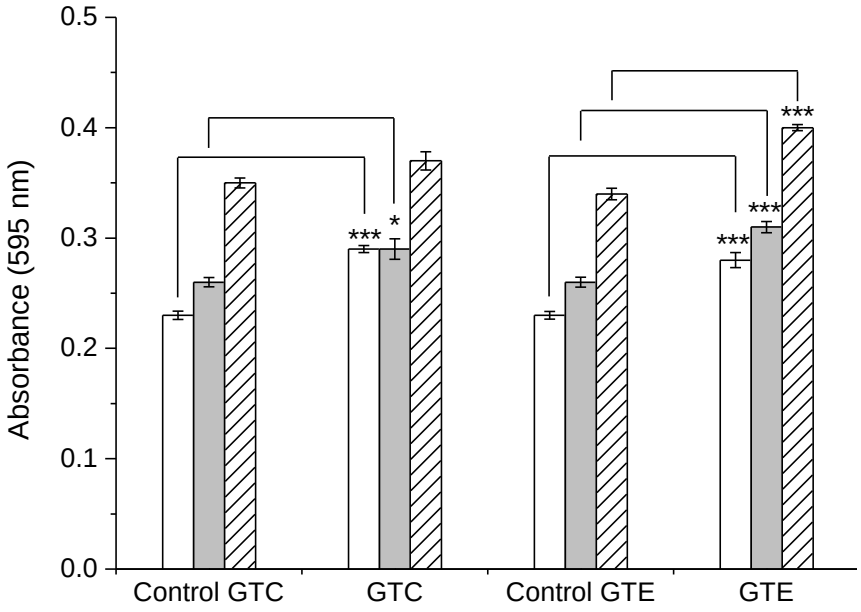


Figure 3-7. Uptake of MTT into HaCaT cells treated with GTE or GTC for 24 hours and then 0 (*white bars*), 0.1 (*grey bars*) or 1 mM (*striped bars*) hydrogen peroxide simultaneously with or without GTC or GTE for a further 24 hours. Samples were assessed at 595 nm after cells were incubated for 2 hours with MTT. Experiments were performed in duplicate three times, and then technical triplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with or without GTE is shown for each hydrogen peroxide condition; $^{*}p<0.05$, $^{***}p<0.0001$; average+/-S.E.

Treatment of HaCaT cells with GTE or GTC simultaneously with hydrogen peroxide, significantly reduced cell death, as was apparent by the release of LDH into the medium in comparison to the control cells. HaCaT cells treated with 0, 0.1 and 1 mM hydrogen peroxide treatment had a fold change of 81 %, 74 % and 57 % for GTC treatment and 96 %, 95 % and 90 % for GTE treatment, respectively, in comparison to cells not treated with green tea; Figure 3-8. The reduction by GTE

was more prominent, implying that components other than the catechins present within the extract could be contributing to the reduction in cell death. There was a reduction in cell death with increasing hydrogen peroxide concentration in control cells (not treated with GTE or GTC), again possibly due to hormesis.

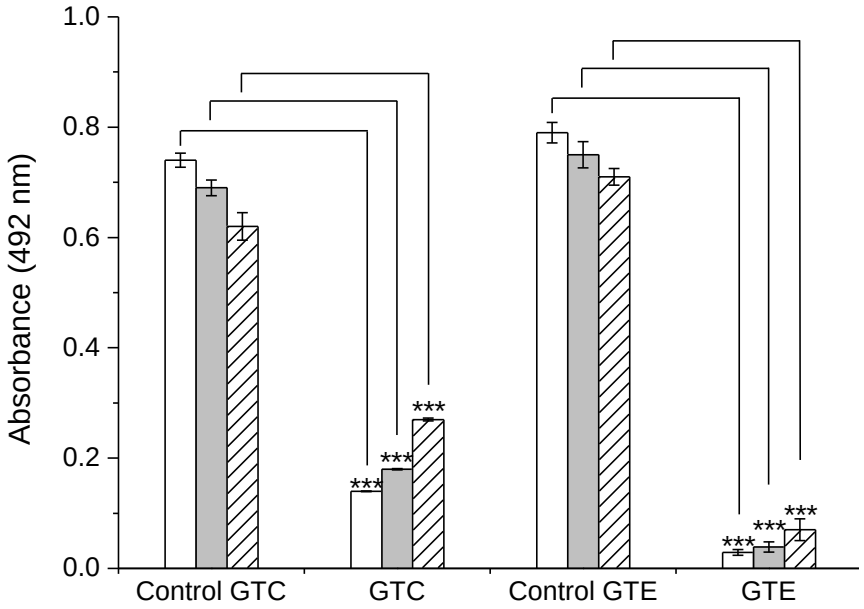


Figure 3-8. Release of LDH into the medium after HaCaT cells were treated with GTE or GTC for 24 hours and then 0 (*white bars*), 0.1 (*grey bars*) or 1 mM (*striped bars*) hydrogen peroxide simultaneously with or without GTC or GTE for a further 24 hours. Samples were assessed using a spectrophotometer at 492 nm. Experiments were performed in duplicate three times, and then technical duplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with or without GTE is shown for each hydrogen peroxide condition; $^{***}p<0.0001$; average+/-S.E.

3.4.5. Viability and Cytotoxicity of HaCaT Cells after Treatment with UVB and Green Tea

A UVB dose response indicated that increasing UVB corresponded with an increase in MTT uptake and reduction intracellularly (Figure 3-9). The increase in uptake was highest after exposure to 30 mJ/cm^2, when cells were treated without GTE and also with GTE, followed by a reduction in uptake of MTT for the

subsequent doses (Figure 3-9). After irradiation with 50 mJ/cm^2, the uptake of MTT in control cells was 83 % in comparison to un-irradiated cells; this dose was chosen as the high UVB dose.

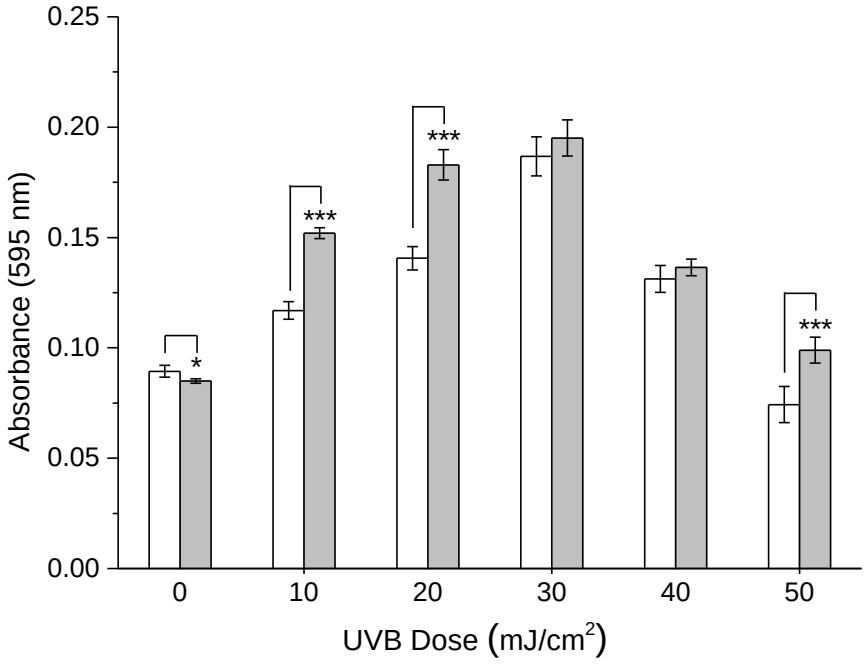


Figure 3-9. Uptake of MTT into HaCaT cells treated with GTE for 24 hours, followed by irradiation of cells by a range of UVB doses and then further treatment with GTE (*grey bars*) or without GTE (*white bars*) for another 24 hours. Samples were assessed at 595 nm after cells were incubated for 2 hours with MTT. Experiments were performed in duplicate three times, and then technical triplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with or without GTE is shown for each UVB dose; $^{*}p<0.05$, $^{***}p<0.0001$; average+/-S.E.

The extent of the protection that the catechins present within the GTE had on viability and cell death, after irradiation was also examined (Figures 3-10 and 3-11). HaCaT cells treated with GTE, but not GTC, and subjected to 10 mJ/cm^2, had an increase in uptake of MTT when compared to control cells (Figure 3-10). Control HaCaT cells irradiated with 50 mJ/cm^2, had an apparent reduction in viable cells as MTT uptake was reduced in comparison to un-irradiated cells. This reduction in

uptake did not occur for cells treated with GTE or GTC, suggesting that catechins inhibit UVB induced cell death.

There was a significant decrease in cell death, as was apparent from a reduction in LDH in the medium, for HaCaT cells treated with GTE and GTC, except for a significant increase of LDH release in GTC treated cells irradiated with 50 mJ/cm^2, in comparison to control treated cells (Figure 3-11). There was also a significance difference in LDH release between GTE and GTC treated cells as cell death was more prominent for GTE treatment than GTC, indicating that the catechins are involved in reducing UVB induced cell death, but that other components present within the extract are also involved.

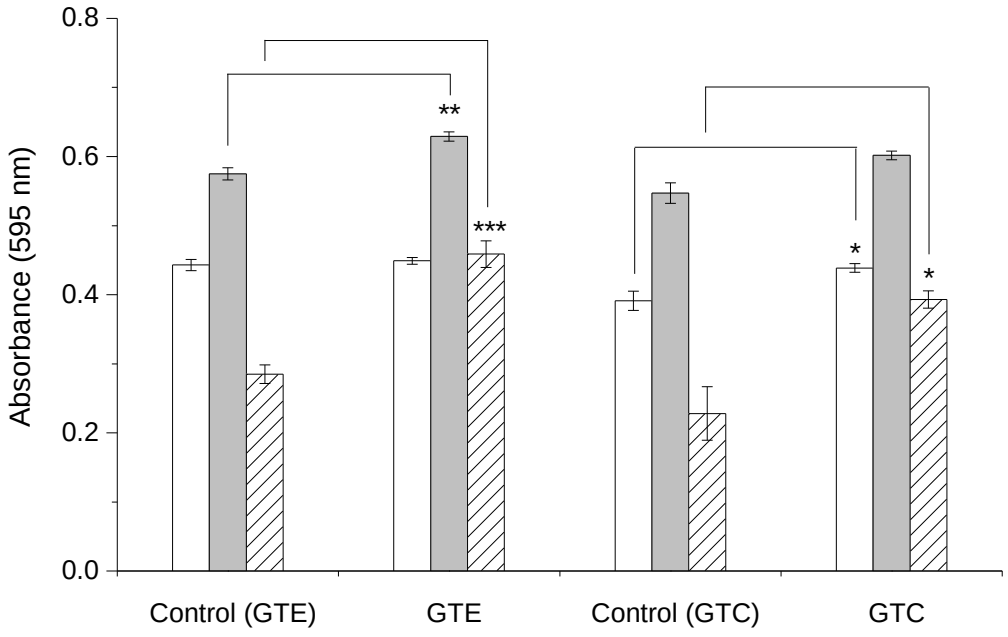


Figure 3-10. Uptake of MTT into HaCaT cells treated with GTE or GTC for 24 hours, followed by irradiation of cells by 0 (*white bars*), 10 (*grey bars*) or 50 mJ/cm^2 (*striped bars*) of UVB and then further treatment with or without GTE or GTC for another 24 hours. Samples were assessed at 595 nm after cells were incubated for 2 hours with MTT. Experiments were performed in duplicate twice, and then technical triplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with or without GTE is shown for each UVB dose; $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$; average+/-S.E.

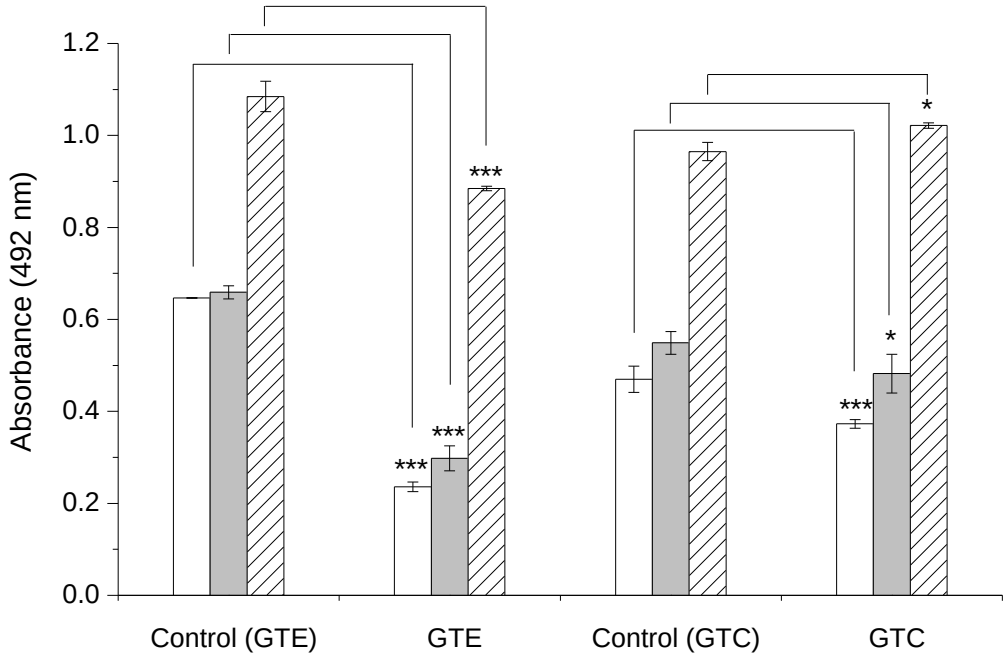


Figure 3-11. Release of LDH into the medium after HaCaT cells were treated with GTE or GTC for 24 hours, followed by irradiation of cells by 0 (*white bars*), 10 (*grey bars*) or 50 mJ/cm^2 (*striped bars*) of UVB and then further treatment with or without GTE or GTC for another 24 hours. Samples were assessed at 492 nm. Experiments were performed in duplicate three times, and then technical duplicates were performed for each (Student's t-test; two tailed, two sample). Significance between cells treated with or without GTE is shown for each UVB dose; $^{*}p<0.05$, $^{***}p<0.0001$; average+/-S.E.

3.4.6. Up-regulation of an Extracellular Matrix Protein in HaCaT Cells after Green Tea Treatment

The uptake of vitamin C into HaCaT cells was normalised to the amount of protein present within the sample. This accounts for any discrepancies that may occur during sample lysis (Chapter 4). After treatment of HaCaT cells for 24 hours with GTE and GTC, the amount of protein present within the sample was increased by 1.3 fold (section 4.4.4), which could be the result of an increase in protein synthesis within HaCaT cells. Trypan blue was another viability assay employed, but due to complications could not be used. The trypan blue assay is an exclusion assay that determines cell viability. Cells with an intact membrane do not allow

permeation of trypan blue and therefore blue stained cells are no longer viable. Before assessment with trypan blue, cells are lysed from the well plate using trypsin which acts on extracellular matrix proteins. After treatment with GTE and GTC, the activity of trypsin was inhibited. It is possible that the activity was inhibited by breakdown products present after 24 hour treatment (as catechins are degraded at neutral pH and are below the LOD for LC-MS and HPLC analysis), or by an increase in extracellular matrix proteins.

As the amount of protein present within the HaCaT cell samples after GTE or GTC treatment was increased, the regulation of an extracellular matrix protein (ECM) was assessed. Laminin is a basement membrane protein that is involved in adhesion, and is the main ECM that is secreted by epithelial cells (Freshney, 2005).

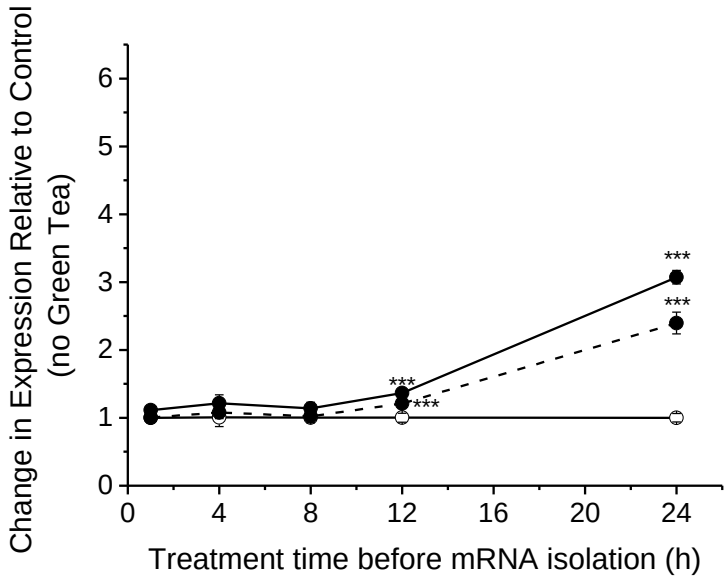


Figure 3-12. Change in expression of laminin in HaCaT cells after treatment for up to 24 hours with GTE or GTC. Expression of laminin in control cells (*white circles*) was normalised to 1 and GTE (*black circle*) and GTC (*black circle, dashed line*) were related to controls at the various time points. Experiments were performed as biological triplicates, and technical triplicates were performed for each biological replicate for analysis by qRT-PCR (Student's t-test; two tailed, two sample). Significance between cells treated with or without GTE or GTC is shown for each time point; $^{***}p<0.0001$; average+/-S.E.

The up-regulation of laminin expression was increased further in HaCaT cells treated with GTE in comparison to GTC (fold change of GTC is 77 % of fold change for GTE) and other components of the extract need to be present for a further increase in up-regulation (Figure 3-12 and Table 3-1). By 12 hours there is a significant fold change of 1.37+/-0.05 for GTE and 1.21+/-0.03 for GTC, and this increased to 3.1+/-0.1 for GTE and 2.4+/-0.2 for GTC by 24 hours treatment. The change in expression suggests an increase in laminin synthesis by green tea treated cells.

Table 3-1. C_T (threshold cycle) values for laminin and GAPDH corresponding to each time point in HaCaT cells treated with GTE or GTC.

C_T Values	1 h	4 h	8 h	12 h	24 h
Control					
GAPDH	18.68+/-0.09	18.9+/-0.2	18.9+/-0.1	19.1+/-0.1	19.8+/-0.4
Laminin	23.25+/-0.06	22.8+/-0.2	22.4+/-0.2	22.4+/-0.1	23.1+/-0.4
GTE					
GAPDH	18.68+/-0.09	19.2+/-0.5	19.2+/-0.1	19.2+/-0.1	19.46+/-0.02
Laminin	23.10+/-0.07	22.9+/-0.6	22.54+/-0.09	22.0+/-0.2	21.16+/-0.07
GTC					
GAPDH	18.66+/-0.09	18.7+/-0.2	19.08+/-0.09	19.2+/-0.1	19.7+/-0.2
Laminin	23.23+/-0.05	22.6+/-0.3	22.58+/-0.6	22.2+/-0.1	21.8+/-0.2

Values are an average of three technical repeats of three biological replicates; average+/-S.E.

3.5. Discussion

FBS is added to culture medium to promote cell adhesion and proliferation as it contains growth factors and hormones. It was apparent that the removal of FBS from the culture medium created starvation stress, as was clear from an increase in LDH release and a reduction in MTT uptake. This occurrence has also been observed in HepG2 cells treated in the presence or absence of FBS for 48 hours, with a significant reduction in cell viability by 60 % (Bai and Cederbaum, 2006). The viability of HepG2 cells undergoing stress induced by FBS starvation was monitored through use of the trypan blue exclusion assay, and observations in cell morphology

changes by light microscopy. The protection of FBS against stress induced from silver nitrate and hydrogen peroxide has been assessed previously in HaCaT cells, however the viability of cells not undergoing induced stress whilst in the absence on FBS were not assessed, and therefore this is the first study to examine this in HaCaT cells (Sun *et al*., 2006).

With the addition of green tea catechins or extract to serum free medium for 24 hours, the uptake of MTT appeared to be maintained when samples were analysed over the 24 hour period, and release of LDH was significantly reduced implying a reduction in cytotoxicity. FBS contains hormones and growth factors (including epidermal growth factor, fibronectin and thyroxine) that are essential for maintaining cell growth during differentiation. It is possible that green tea catechins are interacting with growth factor signalling pathways that maintain cell cycle. Treatment of aged primary keratinocytes with EGCG were demonstrated to undergo differentiation, as shown by significant increases in differentiation markers in comparison to control treated cells, including keratin 1, filaggrin and transglutaminase (Hsu *et al*., 2003).

Investigating starvation stress further could be of benefit *in vivo*. For example, diabetes can cause vitamin C starvation as a reduction in vitamin C (dehydroascorbic acid) has been observed as certain GLUT transporters (mainly GLUT4) require insulin for translocation to the membrane (Wilson, 2002). It may be possible that green tea catechins could have a role in reducing stress induced by vitamin C starvation in diabetic patients.

When directly comparing between HaCaT cells treated with green tea extract or a purified mixture of green tea catechins (EGCG, EGC and EC), it was clear that the maintenance of MTT uptake and reduction of LDH release was more prominent in cells treated with the green tea extract, and that when treated individually, EGCG (10 µM) displayed similar outputs to green tea extract treatment. Fibroblast cells treated with EGCG at a concentration of 10 µM have been

previously shown to reduce LDH release in medium (Hung *et al*., 2005). As EGCG is not present at high concentrations in plasma, for example 80 nM EGCG has been reported (Del Rio *et al*., 2010a) after consumption of green tea *in vivo*, and as at a neutral pH undergoes degradation (Chen *et al*., 1998), the effect would only be replicated *in vivo* if EGCG reached the skin and was localised at a similar, or possibly higher, concentration to 10 µM.

It appears that other components present within the extract (caffeine and the amino acid, L-theanine) are required to reduce cell death further, possibly synergistically. L-theanine increased the viability of ethanol induced cytotoxicity in a human hepatic cell line, as monitored by a reduction in LDH in cell medium (Li *et al*., 2012). The analysis of LDH leakage after renal epithelial cells were treated with a green tea extract, and also caffeine and L-theanine individually, showed a reduction in cell death, by a reduction in LDH release, after treatment with the extract and L-theanine but not caffeine (Yokozawa *et al*., 1997). Therefore, it is possible that L-theanine is the component involved synergistically with the green tea catechins, in reducing cell death as monitored by LDH leakage into the medium.

Green tea extract and catechin mixture were also able to increase the uptake of MTT and reduce the release of LDH in comparison to control cells, when stress was induced upon hydrogen peroxide exposure and also UVB exposure. It is possible that catechins, or more notably possible synergistic interactions between catechins and L-theanine within the extract, were able to interact with ROS formed after exposure of HaCaT cells to hydrogen peroxide or UVB, and therefore reduce cellular damage which would be of benefit *in vivo*, particularly after exposure of skin cells to the sun.

When assessing viability of HaCaT cells with the trypan blue exclusion assay, it was apparent that cells treated with green tea extract or green tea catechin mixture could not be lysed from the well plate with trypsin. The activity of trypsin could have been inhibited by green tea catechins, or breakdown products present,

however cells were washed twice with HBSS which would have potentially reduced the presence of these compounds. Therefore, an extracellular matrix protein, laminin, was evaluated after green tea catechin and extract treatment by qRT-PCR. After 24 hours treatment there was an up-regulation in laminin expression which could translate to an increase in laminin synthesis. ECM provide structural support to the cells, and it is known that matrix metalloproteinases are induced after exposure of skin cells to UV, and can degrade ECM eventually resulting in photo-aging (Quan *et al*., 2009). An increase in ECM by green tea catechins could potentially maintain the structural support of cells *in vivo*, and reduce photo-damage.

Taken together, it is appears possible that green tea catechins are able to reduce stress induced by exposure of keratinocytes to FBS starvation, hydrogen peroxide and UVB, and also increase ECM *in vitro*. In relation to skin cells *in vivo*, it appears that green tea catechins could provide protection against various stress environments, and reduce the incidence of photo-damage.

Chapter 4. Uptake of Vitamin C in HaCaT Cells after Green Tea Treatment

4.1. Abstract

One of the main roles of vitamin C within the body is maintenance of skin health. Vitamin C is depleted in skin after exposure to ultraviolet radiation in animal models, and as vitamin C has a role in photo protection, we tested whether green tea catechins could increase uptake and help replenish intracellular vitamin C. The HaCaT (human keratinocyte) cell line was employed for this study, and cells were exposed to green tea catechins, present in an extract (GTE) or in a purified catechin mixture (GTC), under different stress conditions (fetal bovine serum (FBS) starvation, hydrogen peroxide and UVB). Analysis of uptake was supported by using ^{14}C-radiolabelled vitamin C, and expression of vitamin C transporter mRNA was assessed using qRT-PCR; SVCT1 and SVCT2 (ascorbic acid, AA), and GLUT1, GLUT3 and GLUT4 (dehydroascorbic acid, DHAA). Cells undergoing starvation stress from an absence of 10 % FBS resulted in an apparent increase in expression of GLUT3 by 4 fold, and an increase in uptake of ^{14}C-DHAA by 2 fold after treatment with GTE. Catechins were responsible for the majority of ^{14}C-DHAA uptake. After induction of oxidative stress by extracellular exposure to hydrogen peroxide (<1 mM), GTE, and not GTC, enhanced the uptake of ^{14}C-DHAA. The uptake of vitamin C after exposure to UVB (10 and 50 mJ/cm^2) was not modified by treatment of cells with GTC. In summary, treatment with GTE increased uptake of ^{14}C-DHAA, but not ^{14}C-AA, into HaCaT cells that had experienced FBS starvation and hydrogen peroxide-induced oxidative stress.

4.2. Introduction

The skin is the largest organ of the human body, and it protects internal organs from the external environment. Skin is frequently exposed to sunlight, and UV radiation (UVR) has been linked with photo-aging. UVR causes damage to skin cells via many UV-induced mechanisms, namely production of reactive oxygen species (Lehmann *et al*., 1998, Pfeifer *et al*., 2005).

Vitamin C has been associated with protection of skin cells from inflammation induced by ultraviolet radiation both *in vitro* and *in vivo*. The majority of *in vivo* studies that highlight protection of skin cells from UV by vitamin C utilise topical application. Oral consumption has only been positively associated with UV protection (reduction in minimal erythema dose) when vitamin C was ingested alongside vitamin E (Placzek *et al*., 2005). The human studies affiliated with this thesis investigated the protection of a combination of vitamin C and green tea against UV-induced inflammation. The effect that has been explored here is the uptake of vitamin C in skin cells pre-treated with green tea.

As mentioned above, AA is involved in reducing UV-induced inflammation. Weber *et al*. (1999) highlighted the reduction of AA levels in the stratum corneum of mice after exposure to ozone in comparison to un-exposed skin. AA is involved in scavenging free radicals formed within the skin after exposure to ozone, and results in a depletion of AA by 60 %. Shindo *et al*. (1993) observed a significant reduction of AA by 62 % and DHAA by 44 % in the epidermis and 68 % reduction of AA and DHAA in the dermis of mice exposed to UV. Again, this is potentially due, at least in part, to the involvement of vitamin C in reducing reactive oxygen species (ROS) generated by UVR exposure.

In two studies by Cao *et al.* (Cao *et al*., 2007, Cao *et al*., 2008), the effects of flavonoids on glucose metabolism was examined. The first study in which rats were fed GTE for 6 weeks resulted in an up-regulation in mRNA levels of GLUT1 and GLUT4 in liver compared to rats fed the control (Cao *et al*., 2007). The second study

tested the effects of a polyphenolic fraction of cinnamon on mouse macrophages *in vitro*. After two hours incubation there was a significant up-regulation of GLUT1 and GLUT3 (Cao *et al*., 2008). DHAA enters cells via facilitative diffusion through glucose transporters (GLUT); GLUT1, GLUT3 and GLUT4. The two studies demonstrate that GTE is able to influence changes in mRNA levels of the GLUT transporters. It is possible that GTE could up-regulate the GLUT transporters and consequently increase the uptake of vitamin C (DHAA, which is rapidly converted to AA inside the cell; May *et al*., 1996). As it is known that AA levels within the different layers of skin are depleted after exposure to UV, it is possible that an up-regulation of GLUT1, GLUT3 or GLUT4 could lead to an increase in synthesis of the transporters and more efficient uptake of DHAA under an oxidative stress environment which would replenish levels of AA.

The aim of this chapter is to investigate the hypothesis that treatment of the HaCaT cell line (immortalised human keratinocyte cells) with GTE and GTC under different stress conditions (hydrogen peroxide and UVB), could induce expression of the five known vitamin C transporter mRNA levels, and enhance vitamin C uptake. This is the first time that the influence of flavonoids on vitamin C uptake in skin cells has been assessed.

4.3. Materials and Methods

For the protocols used to perform analysis of the HaCaT cell samples with regards to vitamin C uptake after treatment with green tea, refer to section 2.2.

4.4. Results

4.4.1. Synthesis of ^{14}C-DHAA

As ^{14}C-radio-labelled DHAA is not available commercially, it was synthesised by incubation of ^{14}C-AA with ascorbate oxidase (AO, from *Cucurbita sp.*). To confirm the conversion of AA to DHAA, methanol derivates of DHAA were monitored using a

spectrophotometer at 346 nm (Badrakhan *et al*., 2004). This method analysed the conversion of AA to DHAA in water with a resulting yield of between 93 and 104 % (Figure 4-1). For cell culture conditions, the conversion was performed in glucose free RPMI-1640 at 37 ºC, which resulted in 101 % conversion. Before each DHAA transport experiment, ^{14}C-DHAA was freshly synthesised to ensure the same conditions for each experiment and to avoid possible degradation.

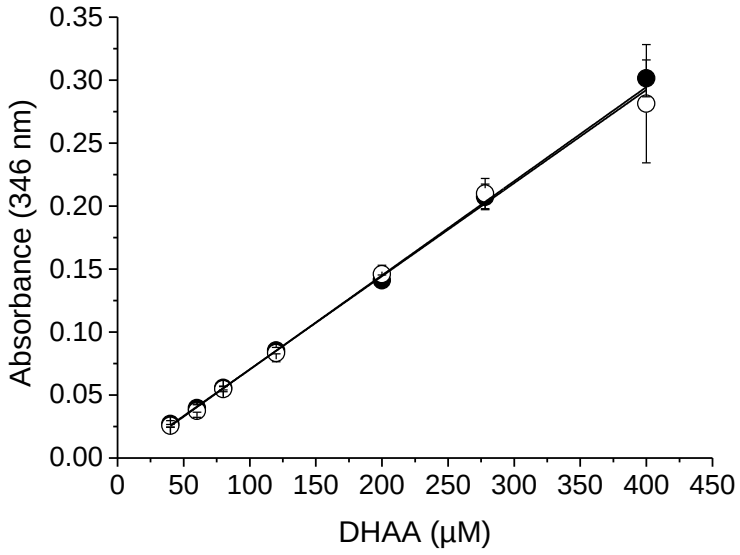


Figure 4-1. Conversion of AA to DHAA in water by AO (0.8 U in 50 % glycerol). A range of 40 to 400 µM AA was incubated for 5 min with AO before incubation for 30 min with methanol at 37 ºC. Analysis of DHAA standard (*black circles*) and DHAA synthesised from AA and AO (*white circles*) was monitored at 346 nm; average+/-S.E.

4.4.2. Positive Control for Vitamin C Transport

Initial cell culture experiments were performed using a positive control identified from the literature. This ensured the validity of the HaCaT cell uptake model as the results were comparable to published data. Cobalt chloride and iron chloride increased glucose uptake under hypoxia conditions in rat liver cells, fibroblasts and rat astrocytes and neurons after up-regulation of GLUT1 and GLUT3 (Behrooz and Ismail-Beigi, 1997, Hwang and Ismail-Beigi, 2002, Yu *et al*., 2008). The effects of

iron chloride and cobalt chloride on GLUT expression and DHAA uptake in HaCaT cells were tested.

The uptake of ^{14}C-DHAA into HaCaT cells was increased in comparison to control cells by 1.4 fold and 1.3 fold after 24 hour treatment with iron chloride and cobalt chloride, respectively (Figure 4-2A). GLUT1 and GLUT3 were up-regulated after 24 hour treatment with iron chloride in comparison to the un-treated control cells (Figure 4-2B), which could potentially lead to an increase in protein transporter synthesis, responsible for the increase in ^{14}C-DHAA uptake (Figure 4-2A). Gene expression could not be assessed for samples that underwent cobalt chloride treatment as mRNA from HaCaT cells was detected at very low levels in comparison to control, implying that treatment affected the viability of the cells.

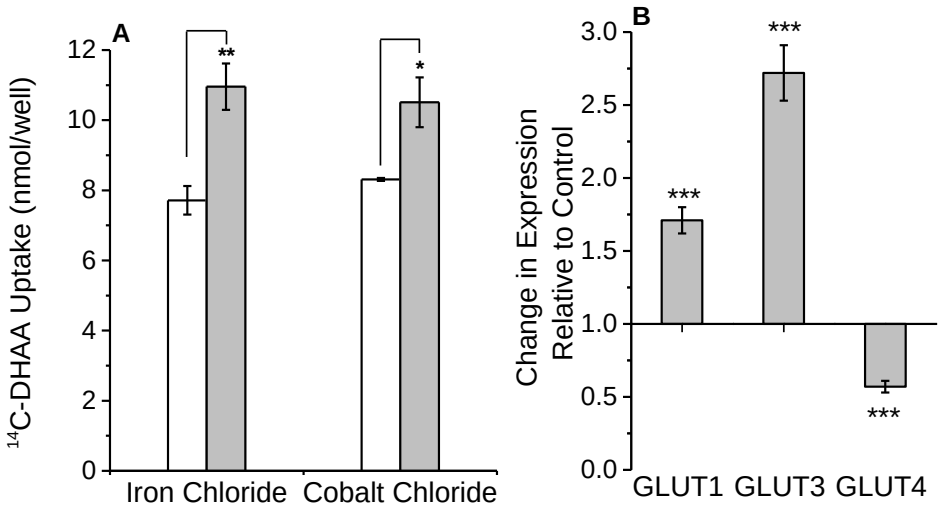


Figure 4-2. Uptake of 250 µM ^{14}C-DHAA in HaCaT cells (A) and regulation of glucose transporter expression (B) after pre-treatment for 24 h with iron chloride (100 µM) or cobalt chloride (250 µM). (A) Uptake of ^{14}C-DHAA (10 min incubation) in control (*white bars*) and treated cells (*grey bars*) was analysed using a scintillation counter. (B) After 24 h treatment with 100 µM iron cobalt, expression of GLUT transporter mRNA was normalised to 1 in the control samples. Samples treated with iron chloride were compared to normalised control samples. Uptake experiments were performed in triplicate twice, and mRNA isolation was performed in triplicate and three technical replicates were used for qRT-PCR of each biological sample ($^{*}p<0.01$, $^{**}p<0.001$, $^{***}p<0.0001$, Student's t-test; one tailed, two sample; average+/-S.E.).

Table 4-1. C_T values for GLUT1, GLUT3 and GLUT4, and GAPDH corresponding to each GLUT, in the HaCaT cell line treated with 100 µM iron chloride or control for 24 hours.

C_T Values	GLUT1	GAPDH	GLUT3	GAPDH	GLUT4	GAPDH
Control	20.8+/-0.2	15.8+/-0.1	31.0+/-0.2	15.7+/-0.2	31.5+/-0.2	18.3+/-0.1
Iron Chloride	20.3+/-0.4	16.0+/-0.4	29.7+/-0.5	15.9+/-0.5	32.5+/-0.3	18.5+/-0.3

Values are an average of three technical repeats of three biological replicates; average+/-S.E.

4.4.3. Presence of Catechins in GTE and GTC

As the green tea extract (GTE) is 38 % (w/w) catechins, HaCaT cells were treated with a purified mixture of catechins (GTC) using HPLC grade standards, in equivalent amounts to GTE to explore the effects of GTC treatment on vitamin C uptake under stress. GTE also contains caffeine, L-theanine, protein, fibre and other components. The composition of GTE and GTC treatment was 0.4 µM catechin, 1.8 µM ECG, 1.9 µM EC, 10 µM EGC and 10 µM EGCG. HPLC analysis (Figure 4-3) confirmed that the catechins present in the mixture had the same composition of the catechins present within the extract (Figure 4-4).

EGCG is very unstable in the medium (Figure 4-5), as has been shown previously in a sodium phosphate buffer at pH 7.4 (Chen *et al*., 1998). After 2 hours, EGCG could not be detected in GTE, as concentrations had fallen below the detection limit, and was detected at a very low concentration in GTC. EGC and EC in GTE and GTC were stable over the first two hours of incubation, but by 24 hours all catechins had fallen below the limit of detection.

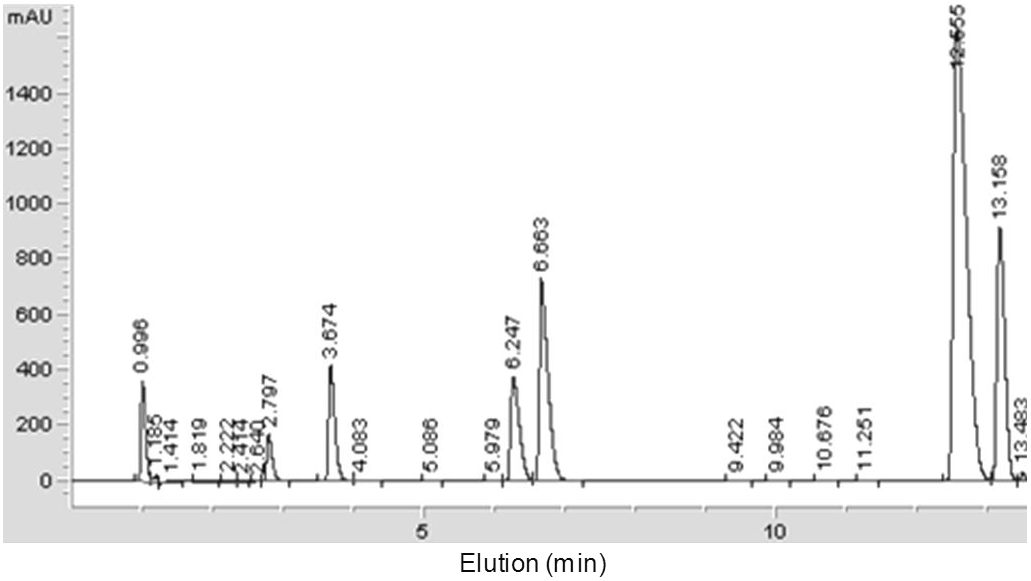


Figure 4-3. Elution order of catechin standards following separation by HPLC analysis. HPLC grade catechin standards (purity of >95 %) were dissolved in 0.1 % AA and 5 % ACN before injection; EGC (2.8 min), C (3.7 min), EC (6.2 min), EGCG (6.7 min), Taxifolin (12.6 min), ECG (13.2 min).

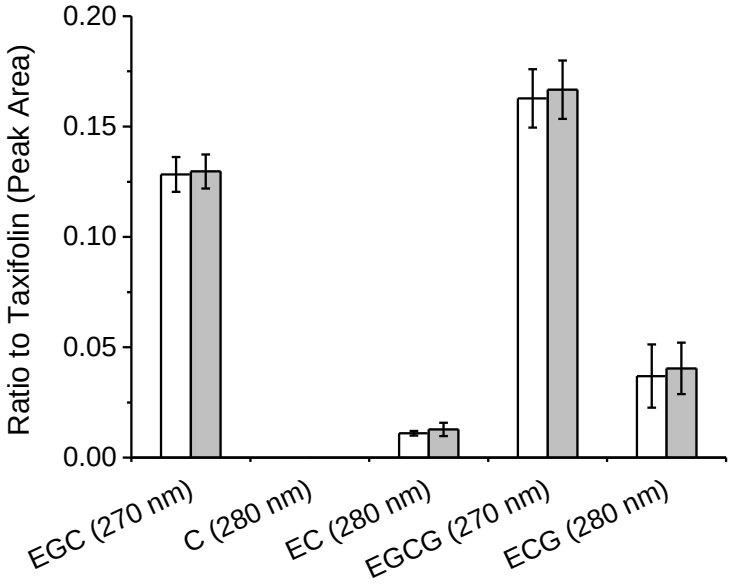


Figure 4-4. Comparison of the GTE and GTC used for treatment of the HaCaT cells. Individual catechins present in the GTC (*white bars*) or GTE (*grey bars*) were analysed at 270 nm or 280 nm, following separation by HPLC and were reported as ratios to the internal standard, taxifolin. Experiments were performed in triplicate; average+/-S.E.

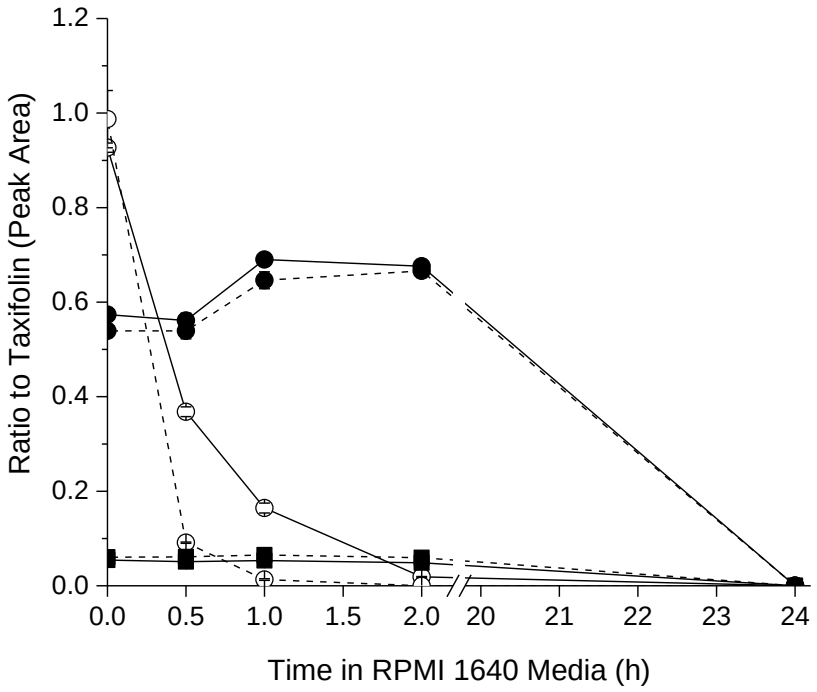


Figure 4-5. Degradation of the catechins present in the GTE or GTC under experimental conditions (glucose free RPMI-1640 medium at 37 ºC and pH 7.2) over 2 hours and at 24 hours. Individual catechins in GTE (*dashed lines*) or GTC (*solid lines*) were monitored using HPLC analysis and are presented relative to taxifolin; EC (black squares), EGC (*black circles*) and EGCG (*white circles*). Experiments were performed in triplicate; average+/-S.E.

4.4.4. Uptake of Vitamin C in HaCaT Cells after GTE and GTC Treatment

HaCaT cells were pre-treated with GTE (at an equivalent of 10 µM EGCG) for 1 to 24 hours followed by incubation with either ^{14}C-AA or ^{14}C-DHAA for up to 15 minutes, under FBS induced starvation stress. Without FBS present in the medium, cells were undergoing stress from starvation of hormones and growth factors, as was apparent after assessment of MTT uptake and LDH release (Chapter 3, section 3.4.1 and 3.4.2). No difference in uptake of ^{14}C-AA occurred between un-treated control cells and GTE treated cells after 1, 4, 8, 16 or 24 hours pre-treatment (uptake after 24 hours treatment is displayed in Figure 4-6A).

After 24 hour pre-treatment with GTE in serum free medium, there was a significant increase in ^{14}C-DHAA uptake into HaCaT cells incubated with 100 and

250 µM ^{14}C-DHAA (1.5 to 1.8, and 1.8 to 2.3 fold increase over the 5 to 15 minute incubation period, respectively), in comparison to un-treated control cells (Figure 4-6B). When directly comparing uptake of ^{14}C-AA against ^{14}C-DHAA, the uptake of ^{14}C-DHAA was higher than uptake of ^{14}C-AA under the same conditions. For the subsequent experiments, ten minutes was chosen as a suitable incubation time as the rate was linear up to the point (Figure 4-6).

To calculate the maximum rate of vitamin C uptake (V_{max}) and the binding affinity (K_m), HaCaT cells were pre-treated with or without GTE for 24 hours and then were incubated with a range of ^{14}C-AA or ^{14}C-DHAA for 10 minutes (Figure 4-7). Using these results, lineweaver burk plots were created for ^{14}C-AA and ^{14}C-DHAA uptake (Figure 4-8). There was no significant difference between uptake of ^{14}C-AA in control and GTE treated HaCaT cells, and so the K_m and V_{max} were calculated for control HaCaT cells; 25 µM and 502 nmol/mg protein/minute, respectively. A linear line of best fit could not be applied to the lineweaver burk plot for ^{14}C-DHAA (Figure 4-8B), implying complex kinetics due to multiple transporters or allostery.

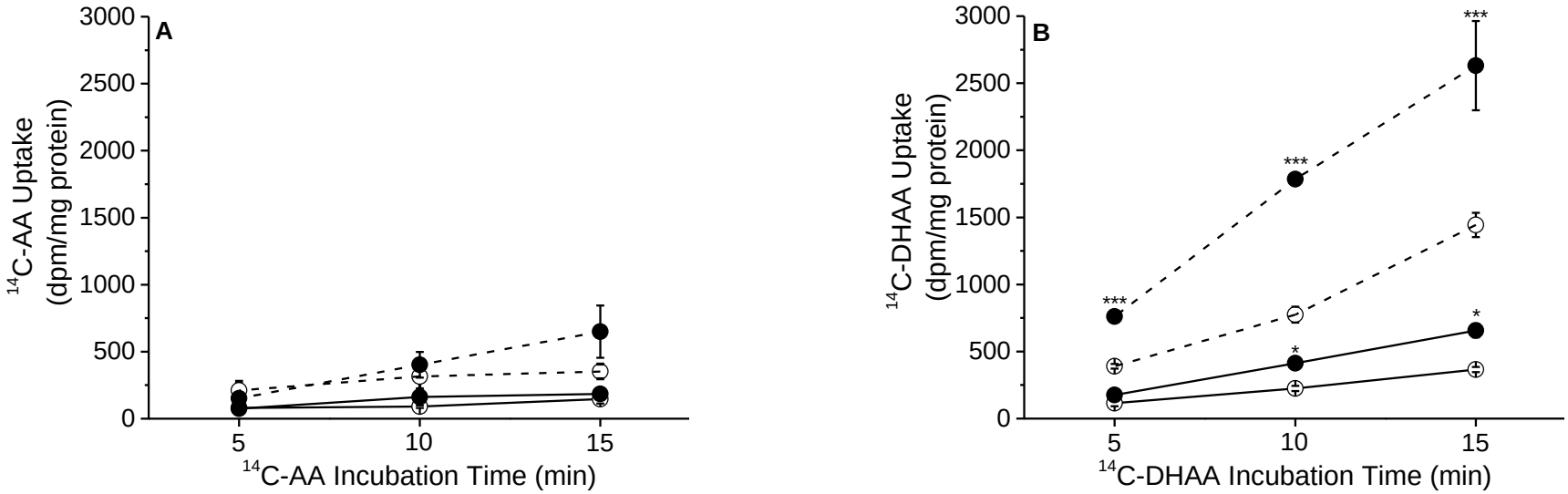


Figure 4-6. Uptake of ^{14}C-vitamin C in HaCaT cells after treatment for 24 h with GTE. HaCaT cells were incubated with 100 µM (*solid line*) or 250 µM (*dashed line*) of ^{14}C-AA (A) or ^{14}C-DHAA (B) for up to 15 minutes after treatment with control (*white circles*) or GTE (*black circles*). Uptake was monitored using a scintillation counter and results were normalised to mg protein present (Bradford assay). Experiments were performed in triplicate. No significance was detected between uptake in control and treated HaCaT cells. Significance is shown between control and GTE uptake at each time point (using student's t-test; two tailed, two sample; $^{*}p<0.05$, $^{***}p<0.0001$; average+/-S.E.).

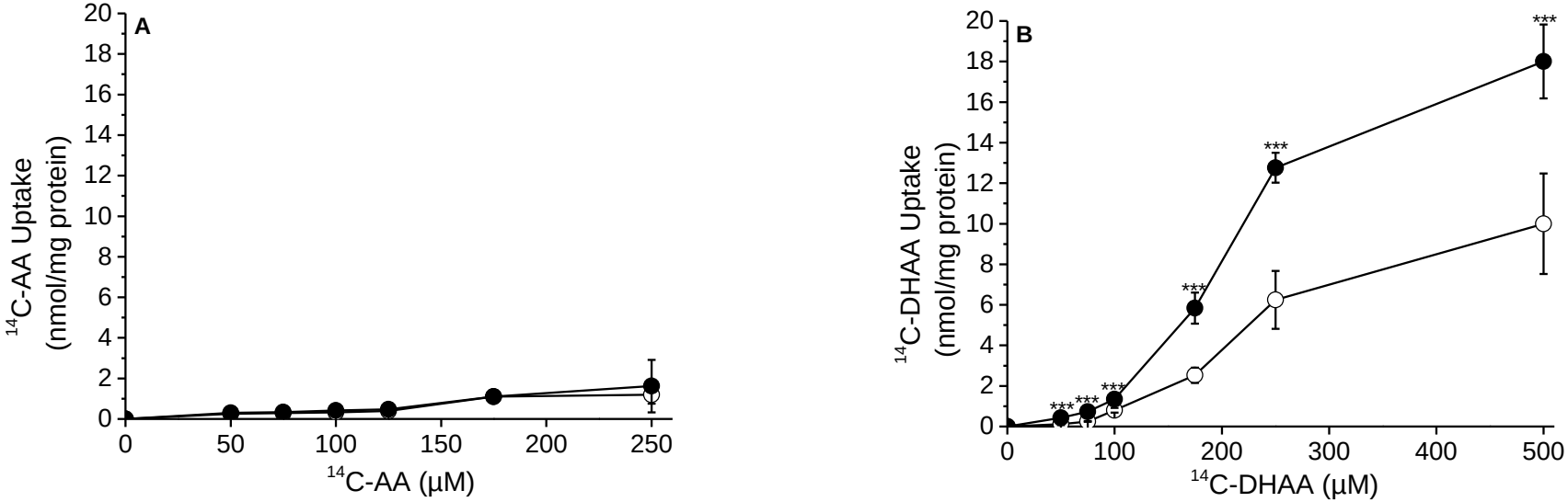


Figure 4-7. Uptake of ^{14}C-vitamin C after incubation of HaCaT cells with a range of ^{14}C-AA (A) or ^{14}C-DHAA (B) concentrations for 10 minutes, after 24 h pre-treatment with or without GTE. Uptake was assessed using a scintillation counter and normalised to protein for un-treated control cells (*white circles*) and GTE treated cells (*black circles*). Experiments were performed in duplicate twice and significance was assessed between control and green tea treatment for uptake of each ^{14}C-vitamin C concentration with the Student's t-test; one tailed, two sample, $^{***}p<0.0001$.

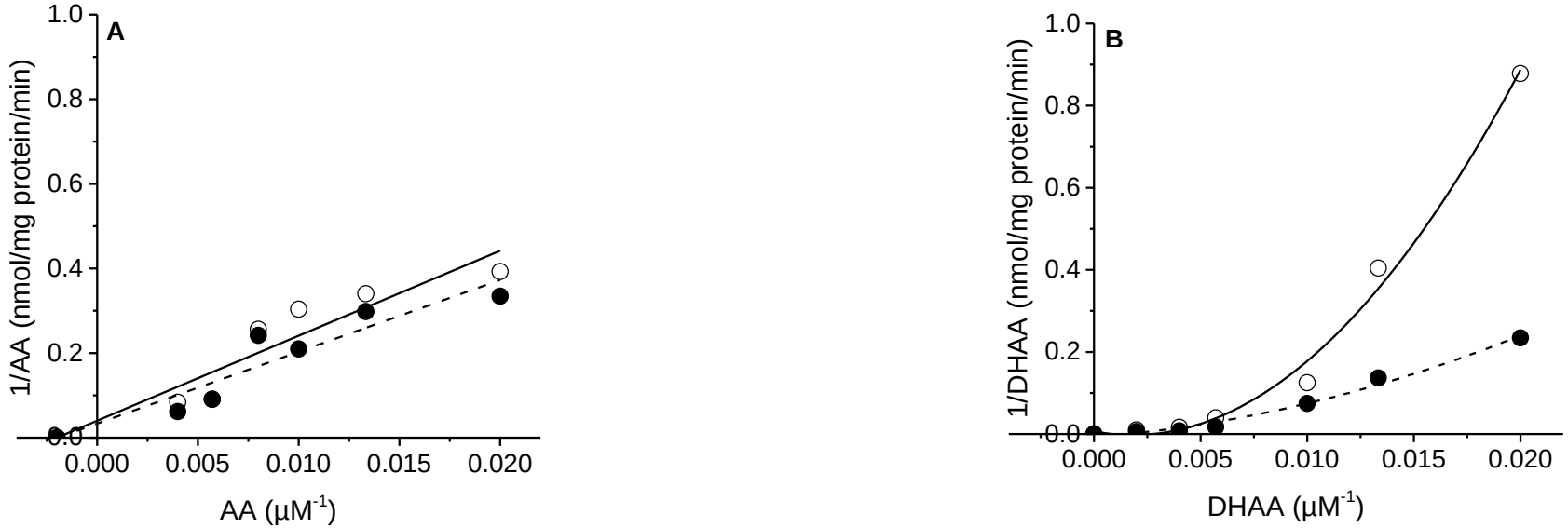


Figure 4-8. Lineweaver burk plots for ^{14}C-AA (A) and ^{14}C-DHAA (B) uptake in HaCaT cells, corresponding to results in Figure 4-7. Lines of best fit were plotted for un-treated control cells (*white circles, solid line*) and GTE (*black circles, dashed line*).

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To understand the role of the catechins present within the extract in increasing the uptake of ^{14}C-DHAA in HaCaT cells undergoing FBS induced starvation stress, cells were pre-treated with individual catechins and a mixture of catechins at concentrations relative to GTE (0.4 µM C, 1.9 µM EC, 1.8 µM ECG, 10 µM EGC and 10 µM EGCG) for 24 hours (Figure 4-9). The uptake of ^{14}C-DHAA was not influenced by catechins individually, but a significant increase in uptake occurred when HaCaT cells were treated with a mixture of EC, EGC and EGCG, and also when 4 or more catechins were present, in comparison to control treated cells.

The catechins chosen for analysis of vitamin C uptake after induced stress (from FBS starvation and stress induced from hydrogen peroxide and UVB exposure) were EC, EGC and EGCG (GTC). This is because C and ECG were present in the extract in very small quantities and there was no significant increase in uptake when C or ECG were added to the mixture. EC, EGC and EGCG are the main green tea catechins investigated in *in vitro* experiments, and have been highlighted as present in biological samples (plasma and urine) in human trials post-green tea consumption.

After incubating HaCaT cells with GTE or GTC for 24 hours under FBS induced starvation stress, there was an increase in ^{14}C-DHAA uptake (Figure 4-10), suggesting that the uptake of DHAA is enhanced to replenish intracellular AA. For the lower concentration of ^{14}C-DHAA, there was no difference in uptake of ^{14}C-DHAA between GTE or GTC treatment. However, there is a significant increase in uptake of 250 µM ^{14}C-DHAA after pre-treatment with GTE, in comparison to GTC, suggesting that other components within the GTE are responsible for a synergistic effect with the catechins. The results imply that uptake of ^{14}C-DHAA is increased in HaCaT cells pre-treated with GTE, with catechins influencing the majority of this effect, as is evident after pre-treatment with GTC.

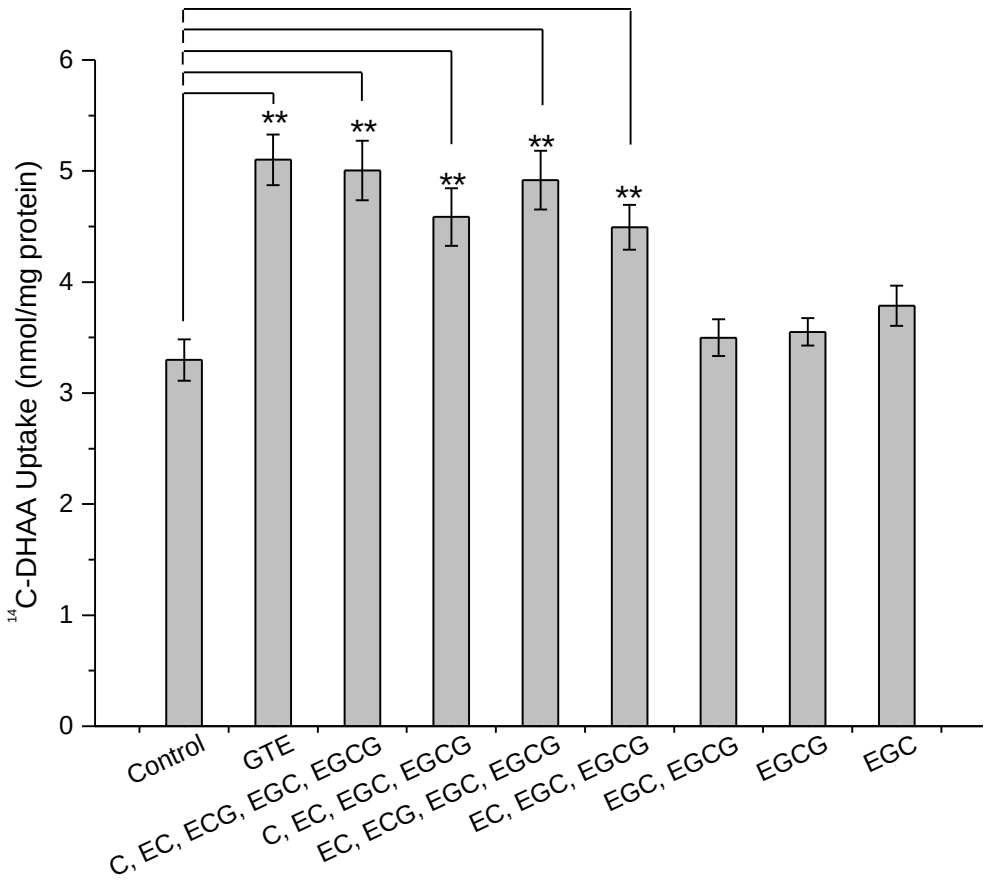


Figure 4-9. Uptake of 250 µM ^{14}C-DHAA after incubation for 10 minutes post 24 h pre-treatment with GTE and different mixtures of catechins. Experiments were performed in duplicate twice (**p<0.001 in comparison to control cells, Student's t-test; two tailed, two sample; average+/-S.E.).

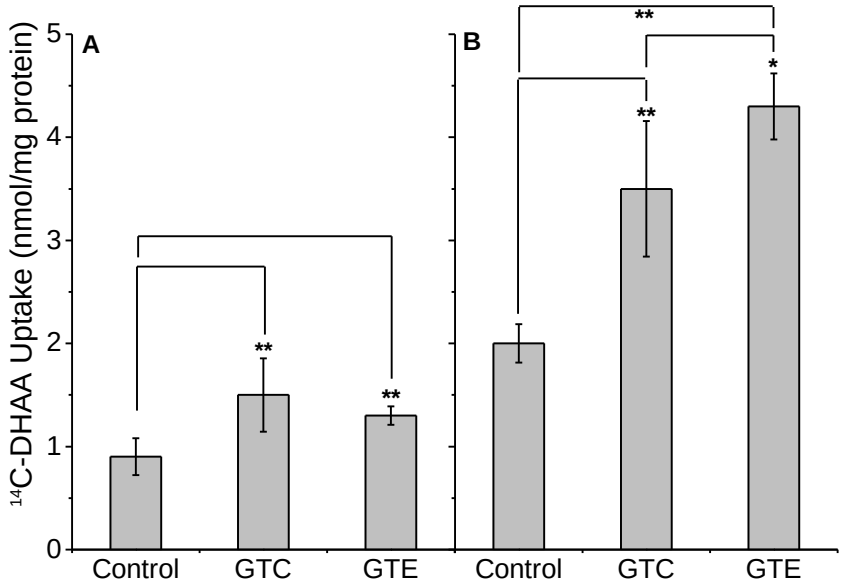


Figure 4-10. Uptake of ^{14}C-DHAA after 10 minutes incubation with 100 µM (A) or 250 µM (B) ^{14}C-DHAA after pre-treatment for 24 hours with green tea extract (GTE) or the green tea

catechin (GTC) mixture. Uptake was monitored using a scintillation counter and results were normalised to protein content. Experiments were performed in triplicate twice ($^{*}p<0.05$, $^{**}p<0.005$, Student's t-test; one tailed, two sample; average+/-S.E.).

To understand the modification of vitamin C uptake, the expression of the vitamin C transporters was monitored over a 24 hour period (C_T (threshold cycle) values shown in Tables 4-2 and 4-3). As is clear in Figure 4-11, GLUT3 was up-regulated by 4 fold after 4 hours treatment with GTE, when compared to un-treated control cells, whereas GLUT1 and GLUT4 were down-regulated; GLUT3 could be responsible for the increase in ^{14}C-DHAA. It is known that GLUT4 is translocated to the cell membrane after stimulation by insulin. As there was no insulin present in the medium used, and because the C_T values for GLUT4 were very high, indicating low expression, analysis of GLUT4 was not performed in the subsequent experiments.

A concentration dependence was explored for GLUT3 and SVCT1 as both had clear changes in expression after 4 hours treatment with GTE (Figure 4-11). There was no significant change in expression (up-regulation for GLUT3 and down-regulation for SVCT1) below 5 µM GTE treatment (equivalent of 5 µM EGCG, 5 µM EGC and 0.95 µM EC; Figure 4-12). The role of the catechins present within GTE on GLUT3 expression was also explored. GTC treatment up-regulated GLUT3 mRNA expression by 3 fold in comparison to un-treated control cells. This increase corresponded to 75 % of GLUT3 up-regulation after GTE treatment, suggesting that other components in GTE (L-theanine or caffeine) are possibly required to synergistically increase the up-regulation of GLUT3 by 4 fold.

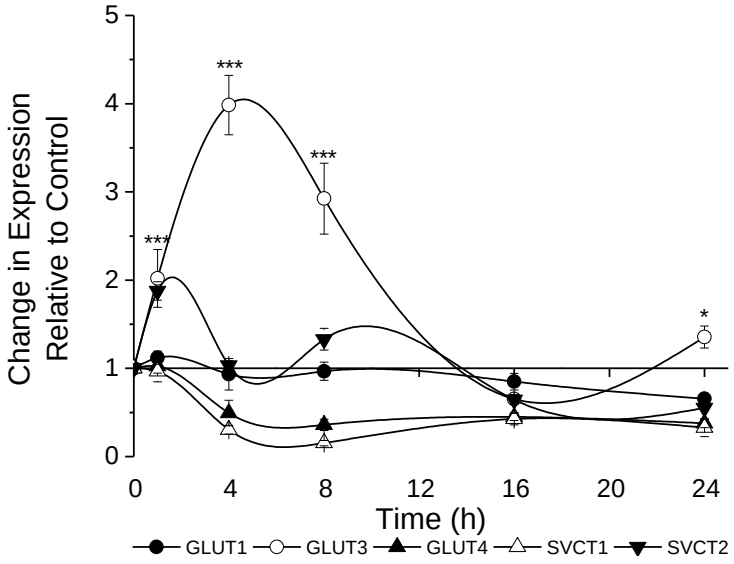


Figure 4-11. Change in expression of the five vitamin C transporters (GLUT1, GLUT3 and GLUT4 for DHAA transport, and SVCT1 and SVCT2 for AA transport) after treatment with GTE for up to 24 hours, relative to un-treated control cells. Expression was normalised to 1 in the control samples, and samples treated with GTE were compared to these. Experiments were performed in triplicate and three technical replicates were used for qRT-PCR of each biological sample (Student's t-test; two tailed, two sample). Significance is shown for GTE treatment on GLUT3 expression; $^{*}p<0.05$, $^{***}p<0.0001$; average+/-S.E.

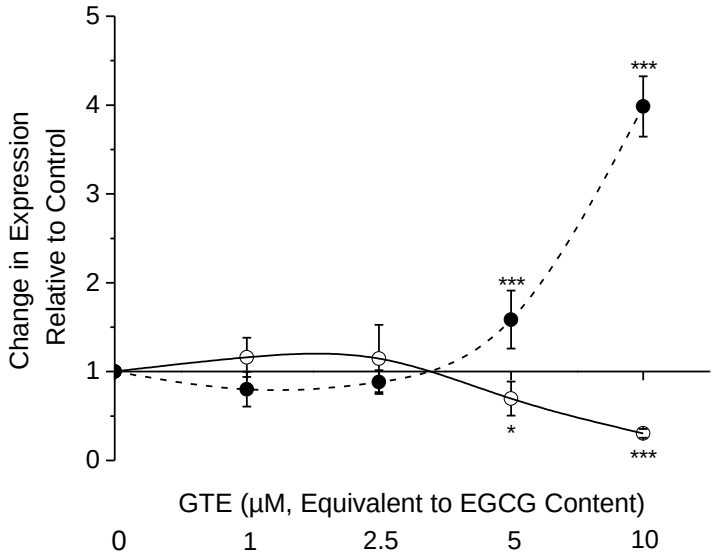


Figure 4-12. Dose dependency changes in expression of GLUT3 (*black circles, dashed line*) and SVCT1 (*white circles, solid line*) transporters when HaCaT cells were treated with GTE for 4 hours. Expression was normalised to 1 in the control samples, and samples treated with GTE were compared to these. Experiments were performed in triplicate and three technical replicates were used for qRT-PCR of each biological sample (Student's t-test; two tailed, two sample, $^{*}p<0.05$, $^{***}p<0.0001$).

Table 4-2. C_T values for vitamin C transporters (GLUT1, GLUT3, GLUT4, SVCT1 and SVCT2) after treatment with or without GTE for up to 24 hours.

	GLUT1		GLUT3		GLUT4		SVCT1		SVCT2	
Time (h)	No GTE	GTE	No GTE	GTE	No GTE	GTE	No GTE	GTE	No GTE	GTE
1	24.3+/-0.3	24.5+/-0.1	30.7+/-0.3	30.0+/-0.2	30.1+/-0.4	30.4+/-0.1	33.7+/-0.3	34.1+/-0.1	27.5+/-0.3	26.8+/-0.2
4	24.1+/-0.4	24.1+/-0.3	33.3+/-0.4	31.2+/-0.2	30.6+/-0.4	31.5+/-0.3	32.8+/-0.4	34.4+/-0.3	26.7+/-0.4	26.4+/-0.3
8	24.3+/-0.2	24.0+/-0.1	33.3+/-0.1	31.5+/-0.1	31.3+/-0.2	32.4+/-0.1	33.3+/-0.2	35.5+/-0.2	27.1+/-0.2	26.4+/-0.2
16	24.7+/-0.1	24.7+/-0.2	32.5+/-0.1	32.9+/-0.1	31.5+/-0.2	32.5+/-0.2	34.4+/-0.2	35.4+/-0.2	26.9+/-0.1	27.4+/-0.2
24	24.7+/-0.2	25.2+/-0.5	32.6+/-0.2	32.0+/-0.5	31.7+/-0.2	33.0+/-0.5	34.5+/-0.2	36.0+/-0.5	27.2+/-0.2	28.0+/-0.5

Values are an average of three technical repeats of three biological replicates; average+/-S.E.

Table 4-3. C_T values for GAPDH in the vitamin C transporter PCR samples after treatment with or without GTE for up to 24 hours.

	GLUT1		GLUT3		GLUT4		SVCT1		SVCT2	
Time (h)	No GTE	GTE	No GTE	GTE	No GTE	GTE	No GTE	GTE	No GTE	GTE
1	17.7+/-0.3	17.9+/-0.1	17.6+/-0.3	17.9+/-0.2	17.6+/-0.4	17.9+/-0.1	17.5+/-0.3	17.9+/-0.1	17.6+/-0.3	17.8+/-0.2
4	18.3+/-0.4	18.2+/-0.3	18.0+/-0.4	17.8+/-0.2	17.3+/-0.4	17.2+/-0.3	17.5+/-0.4	17.4+/-0.3	17.8+/-0.4	17.6+/-0.3
8	18.5+/-0.2	18.1+/-0.1	18.2+/-0.1	17.9+/-0.1	17.8+/-0.2	17.3+/-0.1	17.7+/-0.2	17.2+/-0.2	18.0+/-0.2	17.7+/-0.2
16	16.6+/-0.1	16.3+/-0.2	16.1+/-0.1	15.8+/-0.1	17.6+/-0.2	17.5+/-0.2	18.2+/-0.2	18.0+/-0.2	18.4+/-0.1	18.2+/-0.2
24	16.7+/-0.2	16.5+/-0.5	16.3+/-0.2	16.1+/-0.5	17.6+/-0.2	17.4+/-0.5	18.4+/-0.2	18.2+/-0.5	18.6+/-0.2	18.5+/-0.5

Values are an average of three technical repeats of three biological replicates; average+/-S.E.

The C_T values for GLUT3 are shown in Figure 4-13. It appears that GTE is maintaining expression, as opposed to up-regulation (Figure 4-11). Up until 8 hours treatment, the C_T value for GTE treatment was maintained at approximately 31 (Table 4-2 and Figure 4-13), whereas the cells not treated with GTE had an increase in C_T value over the 24 hour treatment, corresponding to less transporter mRNA present within the sample.

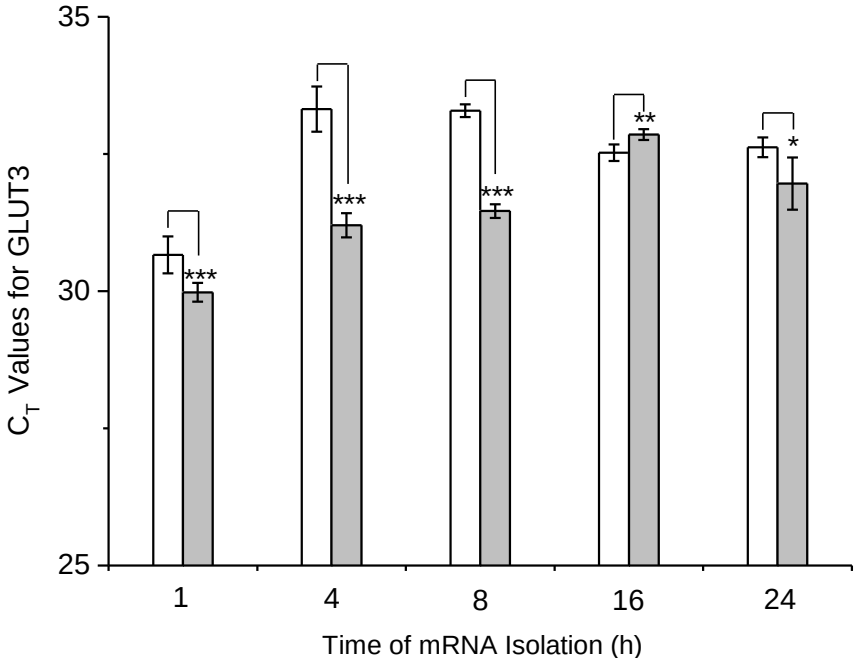


Figure 4-13. C_T values for GLUT3 over the 24 hour time course of HaCaT cells treated with GTE (un-treated control cells, *white bars* and GTE treated cells, *grey bars*). Experiments were performed in triplicate and three technical replicates were used for qRT-PCR of each biological sample. (Student's t-test; two tailed, two sample, $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$; average+/-S.E.).

4.4.5. Uptake of Vitamin C after Treatment with Hydrogen Peroxide and GTE or GTC

HaCaT cells were subjected to oxidative stress as induced by extracellular hydrogen peroxide. Hydrogen peroxide is present within skin cells after exposure to UV *in vivo*, so therefore these experiments represent exposure to external oxidative stress, and are not a direct simulation of UV stress. HaCaT cells were first treated with a range of hydrogen peroxide concentrations. Cytotoxicity and viability assays

were employed to identify two suitable concentrations of hydrogen peroxide (Chapter 3 section 3.4.4).

The MTT and LDH assays previously indicated that without FBS in the medium for 24 hours, un-treated control cells experienced stress induced by FBS starvation, to a greater extent than cells treated with GTE. To alleviate this, the hydrogen peroxide treatments were performed with a 24 hour GTE or GTC pre-treatment, followed with or without GTE or GTC simultaneously with or without hydrogen peroxide for a further 24 hours (Figure 2-1). After this 48 hour period, the HaCaT cells were incubated with 100 or 250 µM ^{14}C-AA or ^{14}C-DHAA for 10 minutes to assess vitamin C uptake.

Table 4-4. Uptake of ^{14}C-DHAA after 10 minute incubation with 100 or 250 µM ^{14}C-DHAA, after cells were treated with hydrogen peroxide and GTE or GTC for 24 hours.

	^{14}C-DHAA Uptake (nmol/mg protein)		
	Hydrogen Peroxide (mM)		
	0	0.1	1
100 µM ^{14}C-DHAA			
No GTC	0.7+/-0.2	0.9+/-0.1	1.2+/-0.2
GTC	1.0+/-0.2*	1.1+/-0.4	1.4+/-0.4
No GTE	0.7+/-0.2	0.8+/-0.1	1.1+/-0.2
GTE	1.5+/-0.3***	1.8+/-0.5***	2.7+/-0.5***
250 µM ^{14}C-DHAA			
No GTC	1.9+/-0.2	2.7+/-0.6	3.9+/-0.5
GTC	2.8+/-0.7**	3.8+/-0.7**	4.0+/-0.7
No GTE	2.3+/-0.6	2.3+/-0.2	3.6+/-0.3
GTE	5+/-2***	5.5+/-0.9***	9+/-2***

All cells were pre-treated before hydrogen peroxide exposure with either GTE or GTC for 24 hours. Uptake was monitored using a scintillation counter and results were normalised to mg protein present (Bradford assay). Experiments were performed in duplicate five times (Student's t-test; two tailed, two samples, $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$; average+/-S.E.).

There was no change in ^{14}C-AA uptake post hydrogen peroxide stress between cells treated with or without GTE (performed four times in duplicate, data not shown). Therefore the effect of GTC treatment on ^{14}C-AA uptake was not

explored. However, there was a significant increase in uptake of ^{14}C-DHAA (100 and 250 µM) in cells treated simultaneously with GTE for all hydrogen peroxide conditions (Table 4-4), suggesting that cells favour the uptake of DHAA to replenish the intracellular AA stock during oxidative stress.

Under all conditions, the uptake of ^{14}C-DHAA (250 µM) was at least doubled in GTE treated cells when compared with un-treated control cells. GTC treatment also significantly increased the uptake of ^{14}C-DHAA (250 µM) after simultaneous treatment with 0 and 0.1 mM hydrogen peroxide, but not to the same extent as for GTE treatment, implying that other components (L-theanine or caffeine) are potentially involved in enhancing ^{14}C-DHAA uptake. As only ^{14}C-DHAA transport was altered, and uptake was more significant in GTE treated cells, GLUT1 and GLUT3 mRNA expression was assessed by qRT-PCR after simultaneous treatment of GTE and hydrogen peroxide (Figure 4-14).

COX2 is a marker of inflammation, and treatment of HaCaT cells with hydrogen peroxide induces the expression of COX2 (Isoherranen *et al*., 1999). To validate that stress is occurring within this cell model, COX2 expression in HaCaT cells was explored post-hydrogen peroxide treatment. After 4 hours incubation with hydrogen peroxide, COX2 expression was up-regulated in HaCaT cells, implying that cells were undergoing stress (Figure 4-14A). Simultaneous treatment of hydrogen peroxide with GTE for 24 hours down-regulated COX2 expression (Figure 4-14B).

The effect of hydrogen peroxide and GTE treatment on the expression of GLUT1 and GLUT3 was also investigated. In comparison to cells not exposed to hydrogen peroxide, treatment of HaCaT cells with 0.1 mM had no effect on expression of GLUT1 or GLUT3, however treatment with 1 mM hydrogen peroxide for 4 hours resulted in a 10+/-2 fold increase in GLUT3 (Figures 4-14C and E).

When GTE was simultaneously present with 0.1 mM hydrogen peroxide, the expression of GLUT1 and GLUT3 mRNA was slightly up-regulated after 4 hours

(Figures 4-14D and F). GLUT1 expression was down-regulated after exposure with 1 mM hydrogen peroxide whereas expression of GLUT3 was slightly increased after 24 hours exposure of GTE and hydrogen peroxide. It is difficult to confirm which transporter has the most efficient role in ^{14}C-DHAA uptake into HaCaT cells exposed to GTE and hydrogen peroxide, and therefore the transcriptional changes of both GLUT1 and GLUT3 should be assessed to understand the extent of protein synthesis.

The initial C_T values were also assessed. Housekeeping genes are expressed in all cells at a consistent level as they maintain basic cellular function. They are used in PCR experiments to normalise data between samples as the expression should be consistent for all treatments. As can be seen in Table 4-5, there was no difference in expression of GAPDH, the housekeeping gene assessed, for GTE treated or control samples for individual experiments for 1 hour and 4 hours samples. In the 24 hour samples, the expression of GAPDH is higher for all GTE conditions suggesting that GTE is altering the expression of GAPDH; these results should be interpreted with caution.

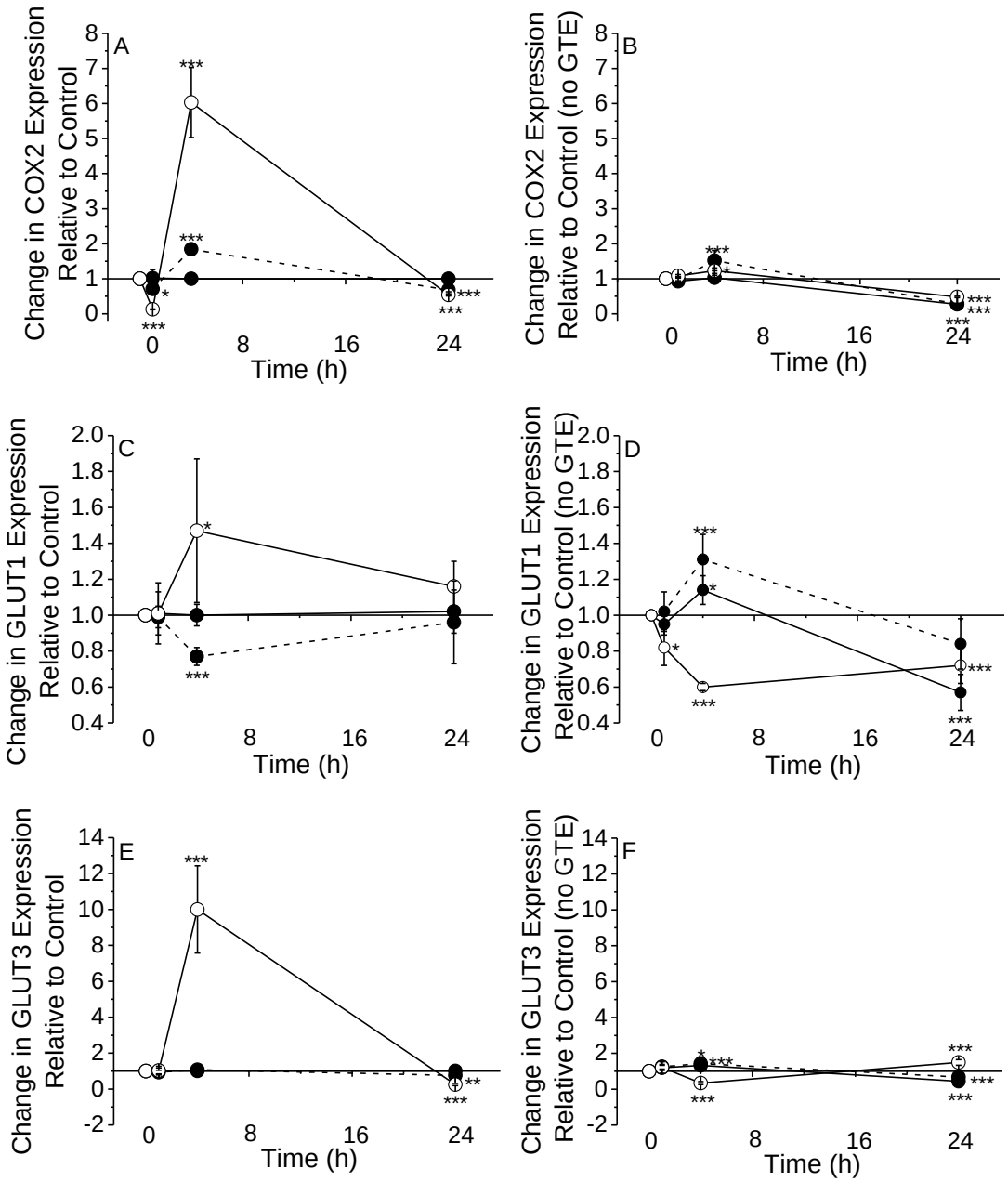


Figure 4-14. Change in expression of COX2, GLUT1 and GLUT3 in HaCaT cells following treatment with hydrogen peroxide or simultaneous treatment with hydrogen peroxide and GTE over a 24 hour period. COX2 (A), GLUT1 (C) and GLUT3 (E) expression following incubation with hydrogen peroxide for 24 hours (subsequent to pre-treatment for 24 hours with GTE) were relative to control cells (no hydrogen peroxide treatment), normalised to 1. COX2 (B), GLUT1 (D) and GLUT3 (F) expression following simultaneous incubation with GTE and hydrogen peroxide for 24 hours (subsequent to pre-treatment for 24 hours with GTE) were relative to control cells (no GTE treatment), normalised to 1. The hydrogen peroxide concentrations assessed were 0 mM (black circles), 0.1 mM (black circles and dashed line), 1 mM (white circles). Experiments were performed in biological triplicate, and technical triplicates were performed for each biological replicate for analysis by qRT-PCR. (Student's t-test; two tailed, two sample, $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$; average+/-S.E.).

Table 4-5. C_T values for COX2, GLUT1 and GLUT3 and corresponding GAPDH for PCR samples after treatment with or without GTE and with or without hydrogen peroxide for up to 24 hours.

		Hydrogen Peroxide (mM)			Hydrogen Peroxide (mM)		
		0	0.1	1	0	0.1	1
Incubation (h)	Treatment	COX2			GAPDH		
1	No GTE	21.9+/-0.2	21.76+/-0.06	24.9+/-0.13	18.5+/-0.2	17.8+/-0.2	18.49+/-0.07
	GTE	21.8+/-0.2	21.7+/-0.2	24.9+/-0.3	18.2+/-0.2	17.8+/-0.2	18.6+/-0.2
4	No GTE	27.67+/-0.08	27.2+/-0.1	25.18+/-0.08	18.93+/-0.03	19.3+/-0.2	19.0+/-0.2
	GTE	27.2+/-0.1	26.6+/-0.3	25.2+/-0.3	18.48+/-0.07	19.4+/-0.1	19.4+/-0.2
24	No GTE	26.6+/-0.2	26.7+/-0.2	27.5+/-0.2	19.6+/-0.3	19.1+/-0.2	19.6+/-0.2
	GTE	27.9+/-0.4	27.2+/-0.2	27.5+/-0.5	18.9+/-0.4	17.8+/-0.1	18.5+/-0.4
		GLUT1			GAPDH		
1	No GTE	22.7+/-0.2	22.7+/-0.2	22.8+/-0.1	18.6+/-0.3	18.7+/-0.2	18.8+/-0.3
	GTE	22.6+/-0.1	22.55+/-0.08	22.9+/-0.1	18.52+/-0.09	18.5+/-0.2	18.6+/-0.2
4	No GTE	25.0+/-0.1	25.1+/-0.2	23.8+/-0.7	19.01+/-0.04	18.7+/-0.1	18.3+/-0.3
	GTE	24.4+/-0.2	24.6+/-0.1	25.1+/-0.4	18.59+/-0.08	18.6+/-0.2	18.8+/-0.4
24	No GTE	23.5+/-0.2	23.5+/-0.4	22.96+/-0.07	21.4+/-0.5	21.3+/-0.4	21.1+/-0.2
	GTE	23.2+/-0.1	22.4+/-0.3	22.2+/-0.5	20.4+/-0.3	19.9+/-0.3	19.8+/-0.4
		GLUT3			GAPDH		
1	No GTE	35.9+/-0.7	36.1+/-0.6	36.0+/-0.6	20.9+/-0.4	21.1+/-0.4	21.1+/-0.3
	GTE	35.2+/-0.2	35.4+/-0.4	35.5+/-0.6	20.6+/-0.2	20.7+/-0.3	20.8+/-0.3
4	No GTE	34.8+/-0.2	34.3+/-0.4	30.6+/-0.5	19.3+/-0.1	18.8+/-0.2	18.4+/-0.3
	GTE	34.0+/-0.2	33.7+/-0.5	32.6+/-0.6	18.89+/-0.07	18.8+/-0.1	18.8+/-0.2
24	No GTE	35.1+/-0.6	35.6+/-0.4	36.5+/-0.2	22.2+/-0.5	22.2+/-0.4	21.5+/-0.4
	GTE	35.3+/-0.3	35+/-1	34.6+/-0.5	21.2+/-0.4	20.8+/-0.4	20.2+/-0.5

Samples were all initially treated with GTE for 24 hours before hydrogen peroxide treatment. Values are an average of three technical repeats of three biological replicates; average+/-S.E.

4.4.6. Uptake of Vitamin C after Irradiation with UVB and Treatment with GTE

HaCaT cells were exposed to UVB irradiation, which simulates exposure of skin to the sun. The uptake of ^{14}C-DHAA into HaCaT cells incubated with 250 µM ^{14}C-DHAA for 10 minutes was 4.9+/-0.3 and 8.0+/-0.7 nmol/mg for control and GTE treatment, respectively (data not shown). GTC had no influence on ^{14}C-DHAA uptake when compared to un-treated control cells (Figure 4-15B), whereas GTE reduced the uptake of 100 µM ^{14}C-DHAA in comparison to control cells treated with 10 and 50 mJ/cm^2, but for the other conditions no change in uptake occurred. There was also a significant change in ^{14}C-DHAA uptake between pre-UVB treatment of cells for 24 hours with GTC and GTE for some, but not all, conditions. GTC pre-UVB treatment of cells significantly increased the uptake of ^{14}C-DHAA in comparison to cells pre-UVB treated with GTE.

The uptake of ^{14}C-AA was also not influenced when cells were treated with GTC (Figure 4-15A). However, treatment of GTE reduced the uptake of ^{14}C-AA (50 and 250 µM), in comparison to un-treated control cells exposed to 10 mJ/cm^2 UVB. There was also a significant increase in uptake of 100 µM^{14}C-AA in cells treated with GTE and exposed to 50 mJ/cm^2. The uptake of DHAA was significantly higher in comparison to AA after exposure of HaCaT cells to UVB, suggesting that HaCaT cells favour the uptake of DHAA by facilitated diffusion in comparison to active transport of AA (Figure 4-15). Although significant changes in uptake of vitamin C have been shown under certain conditions, the fold changes between control and treatment is small and therefore it can be concluded that GTE does not enhance the uptake of vitamin C after HaCaT cells have been exposed to UVB.

EC-*O*-sulphate and EGC-*O*-glucuronide are two conjugated metabolites of green tea catechins that are found at significantly higher levels post-green tea supplement consumption (Chapter 5, section 5.4.2). To understand the role of green tea catechin metabolite conjugates on ^{14}C-DHAA uptake in HaCaT cells after UVB exposure, the two conjugates were synthesised and cells were treated with

conjugates for 24 hours. As the synthesised compounds were not pure and the concentration used for treatment of HaCaT cells was unknown, the results attained serve as an indication of potential changes in vitamin C uptake.

Control samples were identical to conjugate treatment samples, except the enzymes, sulphotransferase (SULT) and uridine 5'-diphospho-glucuronosyltransferase (UGT), were exchanged for water. As the enzymes used for conjugation were from cytosolic and microsomal fractions of pig liver, other components present within the fractions (including mitochondria and ribosomes) might also have an influence on vitamin C uptake and therefore the enzymes (without EC or EGC present) were also analysed (Table 4-6).

There was no difference in uptake of ^{14}C-DHAA in HaCaT cells exposed to 10 mJ/cm^2, and treated with or without EC-*O*-sulphate or SULT, or exposed to 50 mJ/cm^2, and treated with or without EGC-*O*-glucuronide or UGT (Table 4-6). However, uptake appeared to be increased in cells treated post-10 mJ/cm^2 UVB exposure without EC-*O*-sulphate, and post-50 mJ/cm^2 UVB exposure without EGC-*O*-glucuronide, in comparison to cells treated with EC-*O*-sulphate or EGC-*O*-glucuronide, respectively. When assessing the uptake of ^{14}C-DHAA after enzyme treatment, it is clear from Table 4-6 that the uptake is similar to ^{14}C-DHAA uptake after conjugate treatment, suggesting that there is a constituent present within both that is influencing ^{14}C-DHAA uptake to a similar extent. Without using a pure form of EC-*O*-sulphate or EGC-*O*-glucuronide, it is difficult to gauge if vitamin C uptake can be enhanced after UVB exposure from these results.

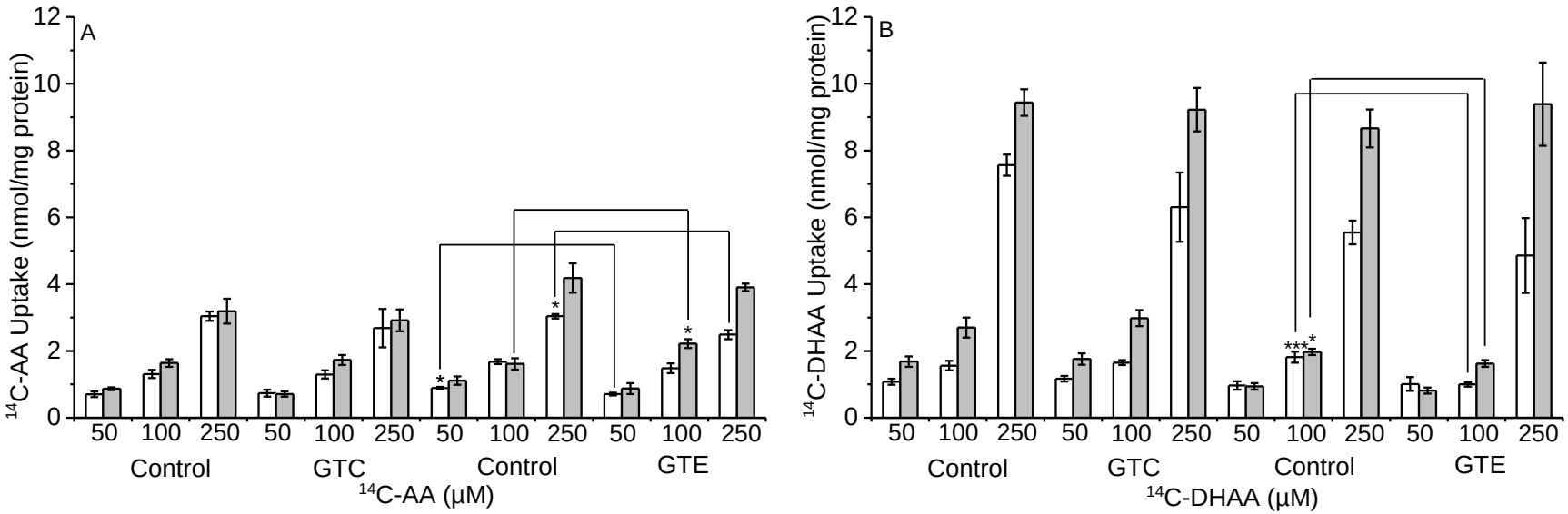


Figure 4-15. Incubation of HaCaT cells with ^{14}C-vitamin C for 10 minutes after cells were irradiation with 10 (*white bars*) or 50 (*grey bars*) mJ/cm^2 UVB. Cells were treated with or without GTE or GTC for 24 hours after UVB exposure (and 24 hours pre-UVB with GTE or GTC), before incubation for 10 minutes with ^{14}C-AA (A) and ^{14}C-DHAA (B). Uptake was monitored using a scintillation counter and results were normalised to mg protein present; Bradford assay. Experiments were performed in duplicate three times for ^{14}C-DHAA uptake and twice for ^{14}C-AA (Student's t-test; two tailed, two sample, $^{*}p<0.05$; average+/-S.E.).

Table 4-6. Incubation of HaCaT cells for 10 minutes with ^{14}C-DHAA after cells were irradiated with 10 or 50 mJ/cm^2 UVB.

		^{14}C-DHAA (250 µM) Uptake (nmol/mg protein)	
Pre-UVB 24 hours	Post-UVB 24 hours	10 mJ/cm^2	50 mJ/cm^2
No EC-*O*-Sulphate	No EC-*O*-Sulphate	6.1+/-0.2	7.6+/-0.4*
	EC-*O*-Sulphate	5.8+/-0.1	5.5+/-0.1
EC-*O*-Sulphate	No EC-*O*-Sulphate	6.9+/-0.3	9.3+/-0.4**†
	EC-*O*-Sulphate	5.9+/-0.1	5.5+/-0.1
SULT	SULT	5.45+/-0.07	6.07+/-0.07
No EGC-*O*-Glucuronide	No EGC-*O*-Glucuronide	8.7+/-0.5†	6.5+/-0.5
	EGC-*O*-Glucuronide	6.64+/-0.09††	5.5+/-0.2
EGC-*O*-Glucuronide	No EGC-*O*-Glucuronide	9.3+/-0.6*†	5.8+/-0.2
	EGC-*O*-Glucuronide	4.8+/-0.3	5.8+/-0.1
UGT	UGT	4.68+/-0.09	5.5+/-0.1

Cells were treated with or without EC-*O*-sulphate or SULT, or with or without EGC-*O*-glucuronide or UGT, for 24 hours pre-UVB and also 24 hours post-UVB. Uptake was monitored using a scintillation counter and results were normalised to mg protein present; Bradford assay. Experiments were performed in triplicate twice (Student's t-test; two tailed, two sample, $^{*}p<0.05$, $^{**}p<0.001$ between conjugates and no treatment, and $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.001$ between conjugates and no treatment, and enzymes; average+/-S.E.).

4.5. Discussion

Ascorbic acid levels within skin are depleted after exposure to UV *in vivo*. An increase in vitamin C transport by a food bioactive within a skin model subjected to stress has been investigated here. Before vitamin C transport was explored, the cytotoxicity of green tea catechins was investigated to confirm that concentrations used for treatment were not toxic. When HaCaT cells were treated with green tea extract (GTE) or a purified green tea catechin mixture (GTC) for 24 hours it was clear that control cells were undergoing stress from FBS starvation, as indicated by a reduction in MTT uptake and an increase in LDH release (Chapter 3, sections 3.4.1 and 3.4.2).

During FBS starvation, GTE was able to maintain the expression of the GLUT3 transporter (compared to GLUT3 expression in control cells after 1 hour

incubation) and the uptake of 100 and 250 µM ^{14}C-DHAA was significantly higher than in control cells. As the C_T values for GLUT3 indicate a low level of expression in the HaCaT cells, the presence of the GLUT3 transporter within the cell membrane should be carefully examined by western blotting or ELISA. GLUT1 and GLUT3 could also be over-expressed in HaCaT cells to identify the affinities of these two transporters for DHAA uptake.

Treatment with GTC was able to replicate the uptake of ^{14}C-DHAA by approximately 80 % of the uptake after GTE treatment. The uptake of ^{14}C-AA was also explored but there was no change after treatment with GTE or GTC in comparison to control cells. In comparison to uptake of ^{14}C-DHAA, the uptake of ^{14}C-AA was much lower. This has been reported in other studies, and it has been suggested that this is due to the movement of DHAA via facilitated diffusion, whereas AA requires two sodium ions for transport of one molecule (Savini *et al*., 2000, Savini *et al*., 2002).

As removal of FBS from HaCaT cells appeared to cause starvation induced stress, HaCaT cells were pre-treated with GTE or GTC for 24 hours prior to hydrogen peroxide or UVB exposure as in a potential *in vivo* situation it would be likely that the catechins were already present within skin cells, or surrounding skin cells, prior to exposure from stress. It was clear that the uptake of ^{14}C-DHAA was increased in GTE treated cells (simultaneously treated with hydrogen peroxide) when compared with control cells post-hydrogen peroxide exposure, but no change occurred in ^{14}C-DHAA uptake in GTE treated cells post-UVB exposure.

The increase in ^{14}C-DHAA uptake post-GTE however, did not occur post-treatment with green tea catechins, implying that catechins were either working synergistically with other components present within the extract, or that the other components (L-theanine and caffeine) had a greater involvement in enhancing ^{14}C-DHAA uptake during hydrogen peroxide-induced stress. It is also possible that during hydrogen peroxide-induced stress, the catechins are involved in interactions

outside of the cell that are not related to ^{14}C-DHAA uptake, for example reducing ROS formation post-hydrogen peroxide exposure (Katiyar *et al*., 2001, Huang *et al*., 2007).

As mentioned, there was no change in uptake of ^{14}C-DHAA after HaCaT cells were exposed to UVB and treated with GTE. This contradicts the proposed hypothesis that after exposure to UVB and treatment with green tea, intracellular AA will be depleted and GTE will enhance the uptake of ^{14}C-DHAA during stress to replenish the intracellular stores of AA. The identification of conjugated derivatives of green tea catechins and metabolites in urine and blood samples post-consumption of green tea have been widely reported. Therefore, future research should focus on the conjugated derivatives, but as they are not commercially available this proves difficult. EC-*O*-sulphate and EGC-*O*-glucuronide are two commonly researched metabolites present in urine and plasma samples post-consumption of green tea. To access the effect of conjugated green tea catechins on ^{14}C-DHAA uptake, the metabolites were first synthesised. The metabolites were synthesised using enzymes from cytosolic and microsomal fractions of pig liver, and although a protein precipitation step was included (addition of acetonitrile), the purity of the two metabolites was unknown and it is possible that other components from the fractions still remained. The synthesis was performed with the enzyme and water instead of EC and EGC to test the extent of the enzyme fraction on the uptake of vitamin C. The uptake activity of ^{14}C-DHAA in HaCaT cells treated with the metabolites was not significantly different to treatment with the enzyme fraction, and therefore these results may suggest that EC-*O*-sulphate and EGC-*O*-glucuronide treatment had no effect, or that because the purity and concentration were unknown, the results are inconclusive. Once pure standards of a known amount have been synthesised, the UVB experiments should be performed again to understand if there is any influence on DHAA uptake after treatment of HaCaT cells with metabolites from green tea catechins.

In summary, catechins present within the green tea extract were able to maintain the expression of GLUT3 and increase DHAA uptake into HaCaT cells that were undergoing stress from FBS starvation. It is possible that other components present within the extract (L-theanine or caffeine) were involved in the uptake of DHAA after HaCaT cells undergo stress induced by hydrogen peroxide. Under these conditions it is possible that DHAA uptake is increased to possibly replace AA that has been depleted during free radical scavenging of ROS produced by hydrogen peroxide. ROS are also formed after skin cells are exposed to UVB, however, there was no difference in DHAA uptake when GTE treated cells were compared with control post-UVB exposure. In relation to *in vivo* interactions, green tea catechins could potentially improve the uptake of DHAA during starvation conditions, for example in a diabetic patient (Wilson, 2002), or whilst cells are undergoing oxidative stress induced from hydrogen peroxide.

Chapter 5. Analysis of Urine from the Green Tea Human Studies by LC-MS-MS

5.1. Abstract

After consumption of green tea, bioavailability of free-form (unconjugated with sulphate esters or glucuronic acid) catechins (GTC) and metabolites can be assessed in urine. Two 3 month human intervention studies were completed in which volunteers consumed a daily supplement containing green tea and vitamin C at a low or high dose (equivalent of 2 or 5 cups of green tea, respectively). Urine was collected over 24 hours at four separate time points throughout the intervention, and the presence of GTC and metabolites were identified by LC-MS-MS analysis. Urine excreted during the low dose intervention (pilot study) was assessed after enzyme deconjugation, and of the 36 metabolites identified, 20 significantly increased in week 12 urine when compared to baseline (pre-supplementation), with many compounds only present post-consumption of green tea supplements. These compounds were used as a foundation for analysis of free-form, methyl, glucuronide and sulphate conjugated green tea catechin metabolites present during the high dose supplementation, double-blind randomised controlled trial using a combination method (acetonitrile precipitation and ethyl acetate extraction) that efficiently isolated both free-form and conjugated metabolites. The main conjugates present in week 12 urine after high dose supplementation were EC-*O*-sulphate, *O*-methyl-EC-*O*-sulphate, *O*-methyl-EGC-*O*-glucuronide, *O*-methyl-M4-*O*-sulphate, M4-*O*-sulphate, M6/M6'-*O*-glucuronide and M6/M6'-*O*-sulphate. For the first time, glucuronide and sulphate derivatives of 3-hydroxybenzoic acid, benzoic acid, hippuric acid and syringic acid were identified in urine post green tea consumption. In summary, the majority of green tea catechin metabolites excreted into urine collected for 24 hours are conjugated with methyl esters, sulphate esters and glucuronide acid, and a combination method allowed for identification of both free-form (unconjugated) and conjugated derivates of GTC.

5.2. Introduction

Following consumption of green tea, catechins enter into the digestive system and can undergo phase II metabolism by conjugation (addition of methyl esters, sulphate esters and glucuronic acid by catechol-*O*-methyltransferase (COMT), sulphotransferase (SULT) and uridine 5'-diphospho-glucuronosyltransferase (UGT), respectively), and can also be further metabolised to break down products, phenolic acids, by microbial metabolism in the colon. Conjugation can occur at many sites during digestion, but in particular, the occurrence of green tea catechin and associated metabolite conjugation has been identified in the small intestine, liver and colon (Williamson, 2004).

Biological samples (urine and plasma) collected from human intervention studies post-consumption of green tea, have employed deconjugation of urine and plasma samples with sulphatase and β-glucuronidase prior to LC-MS analysis (Van Amelsvoort *et al*., 2001, Lee *et al*., 2002, Zimmermann *et al*., 2009, Renouf *et al*., 2010). However, due to inconsistencies in hydrolysis by sulphatase (Saha *et al*., 2012), and a growing interest in improving the knowledge of the form that green tea catechins and metabolites are present in, current research involves the analysis of conjugated forms of green tea catechin metabolites (methyl, glucuronide and sulphate) relevant to free-form standards (Stalmach *et al*., 2009, Del Rio *et al*., 2010a). Free-form standards are usually spiked into blank biological samples and standards curves are generated for quantification of conjugates relative to free-forms, as conjugated derivatives of green tea catechins and metabolites are currently not commercially available.

The majority of recent human intervention studies analysing biological samples post-consumption of green tea catechins have focused on identification of metabolites utilising LC-MS. LC-MS has higher sensitivity than HPLC analysis, and multiple conjugate derivatives can be monitored in a single LC-MS method by

identifying transition changes during fragmentation of the sample, which aids identification of conjugated derivatives that are commercially unavailable.

The 3 month human intervention studies conducted during this thesis provided urine samples for evaluation of green tea catechin and metabolites excreted from the body post-consumption, and allowed for compliance testing to be performed. The aim of this chapter is to identify metabolites present within urine samples post-consumption of a low and high dose, and to also confirm compliance of volunteers to the pilot study (low dose, 2 cups of green tea) and RCT (high dose, 5 cups of green tea). A combination method of acetonitrile (for protein precipitation) and ethyl acetate extractions (to isolate free-form catechin derivatives) was employed, which has not been utilised before in analysis of biological samples post-green tea consumption.

5.3 Materials and Methods

For the protocols used to perform analysis of the pilot study urine and compliance of the RCT urine, see sections 2.3.1.1 and 2.3.1.2, respectively.

5.4. Results

Green tea catechins and green tea catechin metabolites that were not attached to a sulphate ester or glucuronic acid moieties will be referred to throughout as the free-form. This can relate to catechin metabolites that did not undergo phase II metabolism, or the chemical structures remaining after removal of the moieties by sulphatase and β–glucuronidase treatment.

As the positions of the conjugate moieties (methyl ester, sulphate ester and glucuronic acid) of green tea catechin metabolites are unknown in the work presented in this thesis, they will be referred to as *O*-methyl-catechin-*O*-glucuronide or -*O*-sulphate, instead of x-*O*-methyl-catechin-x-*O*-glucuronide or x-*O*-sulphate.

5.4.1. Green Tea Pilot Study, Urinary Data

The green tea human intervention pilot study provided samples for method optimisation and assessment of biological samples (biopsy and blister fluid by TD, urine by KC) at a low dose (equivalent of 2 cups of green tea). Enzymatic deconjugation was employed as sulphate and glucuronide standards for green tea catechins were not commercially available. The free-form green tea catechins and free-form metabolites present within biological samples were examined.

Conditions for enzymes were optimised (data not shown, TD) by altering the amount of enzyme and incubation time. The optimal condition for urine was incubation of 40 µL sample with 5 U sulphatase (Type VIII, Abalone entrails) and 200 U β-glucuronidase (Type X, E. coli) for 60 minutes at 37 ºC. During analysis of the pilot study samples, Type X β-glucuronidase was discontinued and replaced by Type IX (E. coli) as there was no difference in results obtained (Figure 5-1).

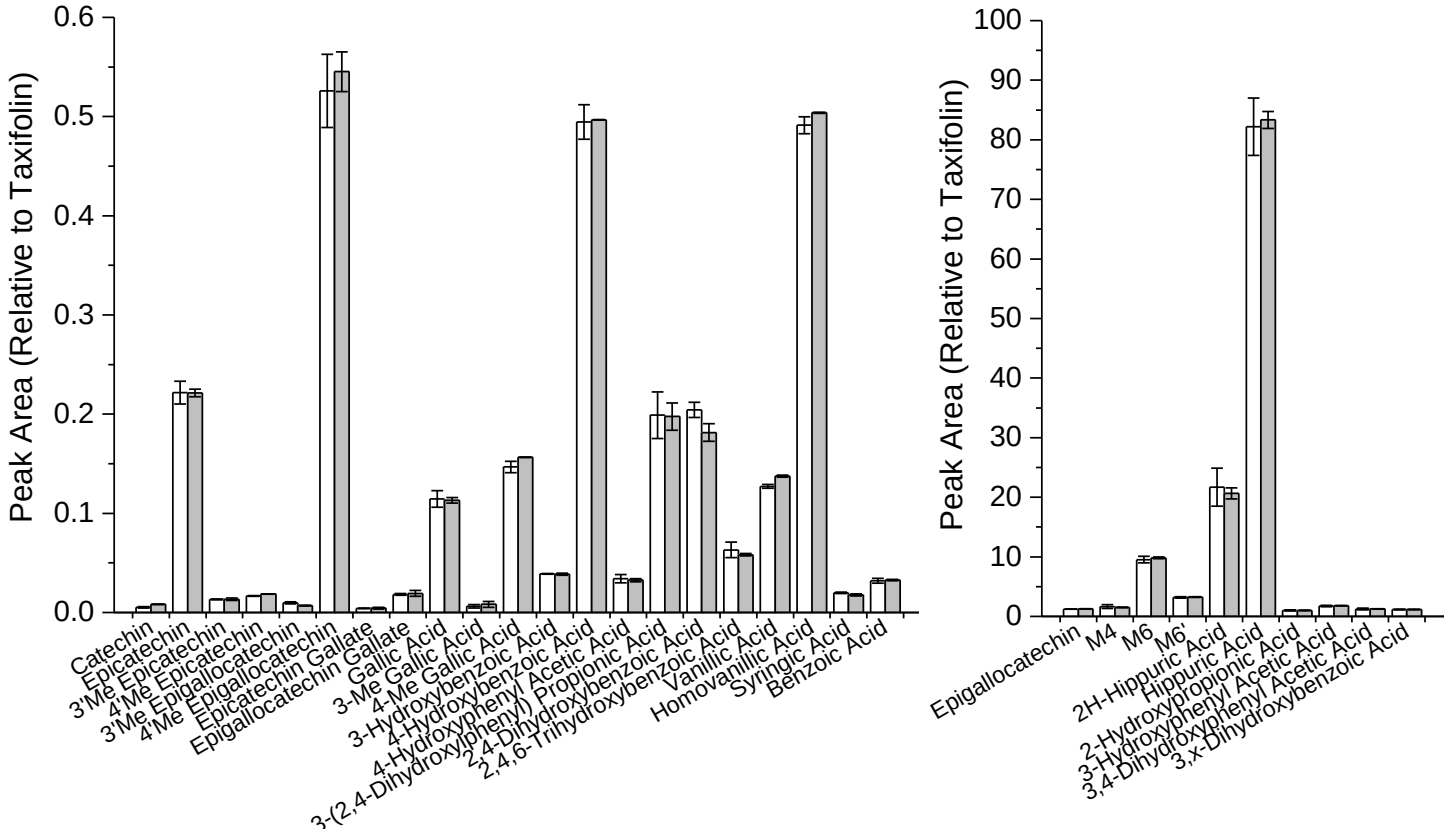


Figure 5-1. Validating the use of Type IX β–glucuronidase as a replacement for Type X. Week 12 urine (40 µL from the same volunteer) was incubated with 200 U β–glucuronidase for 60 minutes at 37 ºC. Experiments were performed in technical duplicate, and results for Type IX (*grey bars*) and Type X (*white bars*) β–glucuronidase incubation were relative to the internal standard, taxifolin; average+/-S.D.

The urine of 16 female volunteers was collected over 24 hours at four separate time points; one pre-supplementation (baseline) and three post-supplementation (day one, week 6 and week 12). Urine was tested for compliance to the study. As the diet was not restricted and as metabolites identified following green tea consumption are not all exclusive to green tea (cocoa and black tea also, but EGC is mainly exclusive to green tea), a significant increase of metabolites excreted in baseline urine to post-supplementation urine was the marker of compliance.

One volunteer withdrew from the study and another was removed as they were not compliant (no green tea catechins or metabolites present in the week 6 urine sample). In total, there were 14 compliant volunteers, but the volume for the total urinary excretion of one volunteer at week 12 was not recorded at the University of Manchester, and therefore the urine was analysed as n=13. This particular volunteer excreted 2600 mL at baseline, 2380 mL at day 1 and 2880 mL at week 6 so it was assumed that they would also excrete over 2 L at week 12. The week 12 urine was tested and EGC, 3' and 4'-*O*-methyl-EGC, 3' and 4'-*O*-methyl-EC, 5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone (M4), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (M6) and 5-(3',5'-dihydroxyphenyl)-γ-valerolactone (M6') were present. The presence of EGC in urine can be representative of green tea consumption as it is only found in small or trace amounts in other dietary sources (black tea, strawberries, blackberries and wine), whereas EC is present in higher amounts in cocoa in comparison to green tea.

Out of 36 metabolites tested, 20 were significantly increased between baseline and week 12 (Table 5-1 and 5-2). As can be seen in Table 5-3, 7 of the 20 metabolites significantly increased for all 13 volunteers post-green tea supplement consumption when the individual baseline samples were compared with day 1, week 6 and week 12 samples. These were EC and 3' and 4'-*O*-methyl-EC,

EGC and 3' and 4'-*O*-methyl-EGC and M4 valerolactone. All except for 4'-*O*-methyl-EGC were present in some of the baseline samples (Table 5-3).

Table 5-1. The 36 green tea catechin metabolites that were identified in urine and the 20 that were significant in volunteers at the 12 week stage in comparison to baseline urine (pre-supplementation).

Increase of Green Tea Catechin Metabolites at Week 12 Compared to Baseline	
Significant	Not Significant
Epicatechin	4-*O*-Me-Gallic Acid
3'-*O*-Me-Epicatechin	2,4-Dihydroxybenzoic Acid
4'-*O*-Me-Epicatechin	3,x-Dihydroxybenzoic Acid
Epicatechin Gallate	2,4,6-Trihydroxybenzoic Acid
Epigallocatechin	2-Hydroxypropionic Acid
3'-*O*-Me-Epigallocatechin	3-Hydroxyphenyl Acetic Acid
4'-*O*-Me-Epigallocatechin	4-Hydroxyphenyl Acetic Acid
Epigallocatechin Gallate	3,4-Dihydroxyphenyl Acetic Acid
Catechin	3-(3-Hydroxylphenyl) Propionic Acid
Gallocatechin	3-(4-Hydroxylphenyl) Propionic Acid
Gallocatechin Gallate	3-(2,4-Dihydroxylphenyl) Propionic Acid
Gallic Acid	4-Hydroxybenzoic Acid
3-*O*-Me-Gallic Acid	Mandelic Acid
3-Hydroxybenzoic Acid	Vanillic Acid
M4 Valerolactone	Homovanillic Acid
M6 Valerolactone	2H Hippuric Acid
M6' Valerolactone	
Syringic Acid	
Benzoic Acid	
Hippuric Acid	

Metabolites are presented in the free-form as glucuronide and sulphate moieties were cleaved. Significance was determined using Student's t-test; two-tailed, paired.

Table 5-2. Amount of free-form green tea catechin metabolites excreted in week 12 urine that were significantly increased in comparison to baseline urine, after volunteers consumed daily supplements equating to 2 cups of green tea.

	Mean +/- SD Amount Excreted in Urine (µmol) Collected for 24 hours			
Compound	Baseline	Day One	Week 6	Week 12
Epicatechin†	0.3+/-0.4	7+/-4*	5+/-4*	7+/-5*
3'-*O*-Me-Epicatechin†	0.06+/-0.08	0.6+/-0.3*	0.5+/-0.4*	0.6+/-0.3*
4'-*O*-Me-Epicatechin†	0.04+/-0.05	0.2+/-0.2**	0.2+/-0.2**	0.3+/-0.2*
Epicatechin Gallate	0.000+/-0.002	0.01+/-0.01*	0.02+/-0.01*	0.01+/-0.01**
Epigallocatechin†	0.2+/-0.4	22+/-13*	20+/-16*	25+/-20*
3'-*O*-Me-Epigallocatechin†	0.01+/-0.04	0.2+/-0.1*	0.2+/-0.2*	0.2+/-0.2*
4'-*O*-Me-Epigallocatechin†	0+/-0	8+/-8*	8+/-9**	9+/-8**
Epigallocatechin Gallate	0.00+/-0.01	0.06+/-0.05*	0.06+/-0.04*	0.08+/-0.09**
Catechin	0.01+/-0.02	0.2+/-0.1*	0.1+/-0.1*	0.2+/-0.2*
Gallocatechin	0+/-0	0.4+/-0.5**	0.3+/-0.5***	0.6+/-0.6**
Gallocatechin Gallate	0+/-0	0.003+/-0.009	0+/-0	0.01+/-0.02*
Gallic Acid	0.6+/-0.7	1+/-1	0.7+/-0.5***	1+/-1**
3-*O*-Me-Gallic Acid	0.6+/-0.6	1+/-1	0.9+/-0.8	1+/-1***
3-Hydroxybenzoic Acid	1+/-1	2+/-2	2+/-3	4+/-4***
M4 Valerolactone†	0.3+/-0.4	30+/-27**	18+/-25***	21+/-21**
M6 Valerolactone	10+/-12	33+/-25**	27+/-28***	31+/-24***
M6' Valerolactone	0.5+/-0.7	18+/-16**	12+/-13**	15+/-15**
Syringic Acid	2+/-1	4+/-5	3+/-2	4+/-4***
Benzoic Acid	81+/-83	95+/-60	100+/-130	140+/-120***
Hippuric Acid	4000+/-2200	5100+/-2500	4300+/-1900	5300+/-1700***

Urine was collected for 24 hours at baseline and week 12. Samples were performed in technical duplicates. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test; two tailed, paired) and †significant increase from baseline of metabolite in week 12 urine for all volunteers; average+/-S.D.

Table 5-3. Presence of green tea catechin metabolites excreted in the urine of volunteers.

		Increase from Baseline (Number of Volunteers)		
Compound	Baseline (Number of Volunteers)	Day One	Week 6	Week 12
Epicatechin	9	All	All	All
3'-*O*-Me-Epicatechin	5	All	All	All
4'-*O*-Me-Epicatechin	6	All	All	All
Epicatechin Gallate	2	9	11	9
Epigallocatechin	5	All	All	All
3'-*O*-Me-Epigallocatechin	2	All	All	All
4'-*O*-Me-Epigallocatechin	0	All	All	All
Epigallocatechin Gallate	1	11	All	12
Catechin	1	9	11	11
Gallocatechin	0	6	4	7
Gallocatechin Gallate	0	1	0	3
Gallic Acid	10	11	11	11
3-*O*-Me-Gallic Acid	11	7	9	9
3-Hydroxybenzoic Acid	11	9	9	11
M4 Valerolactone	11	All	All	All
M6 Valerolactone	12	10	10	10
M6' Valerolactone	11	12	12	All
Syringic Acid	All	11	8	9
Benzoic Acid	All	8	6	11
Hippuric Acid	All	8	7	10

Urine was collected for 24 hours at baseline (pre-supplementation), day one, week 6 and week 12. Results are expressed as number of volunteers with a significant increase from baseline urine to the post-supplementation time points. Baseline results are expressed as number of volunteers that excreted the particular metabolite, as green tea catechin metabolites are not all exclusive to green tea consumption.

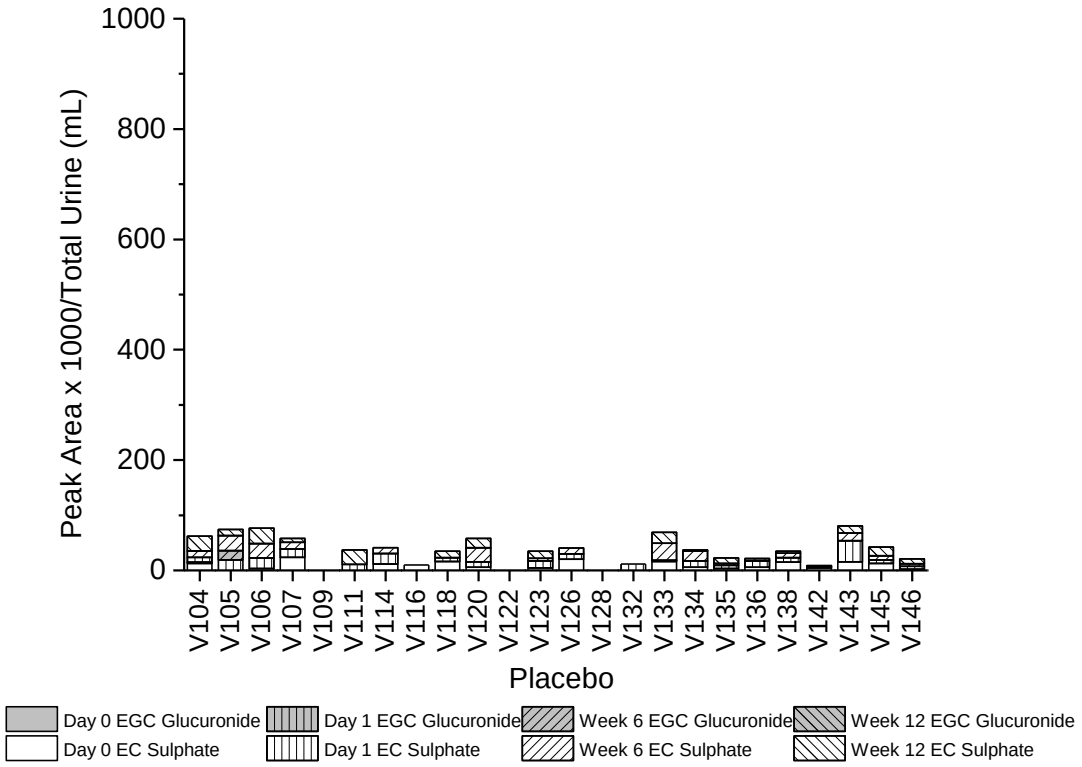
5.4.2. Deciphering between Control and Active RCT Volunteers, and Testing for Compliance

A 3 month randomised controlled trial (RCT) was performed using the high dose green tea supplements (equivalent to 5 cups of green tea). The RCT was designed to test the hypothesis that consumption of green tea supplements offered protection from UVR induced changes within skin as assessed using immunohistochemistry (changes in number of neutrophils, $CD3^+$ cells and $CD8^+$ cells). The urine was used to identify the volunteers who had consumed the

placebo or the green tea supplement (and subsequently test for compliance). A short LC-MS method (19.2 mins) analysed two conjugates that have been reviewed in the literature as being adequate biomarkers of green tea consumption (Wang *et al*., 2008). As commercial standards were not available, EGC-*O*-glucuronide and EC-*O*-sulphate were synthesised using enzymes and then spiked into blank baseline urine to identify the retention time of these two conjugates (4.7 and 5.3 minutes, respectively; for synthesis see section 2.3.1.2.1.).

For confirmation that volunteers were consuming green tea supplements, and for compliance to the intervention, both conjugates (EC-*O*-sulphate and EGC-*O*-glucuronide) were identified in urine from day 1, week 6 and week 12. As can be seen in Figure 5-2, there was a signify cant difference between subjects who were consuming the placebo or the green tea supplement daily for 3 months. Excretion of EC-*O*-sulphate and EGC-*O*-glucuronide was clearly increased in comparison to baseline following daily consumption of green tea supplements for 3 months.

Volunteers in the green tea supplementation group with a ‡ symbol appeared to have not complied with the intervention (Figure 5-2). These volunteers provided a week 12 urine sample which was analysed and contained EC-*O*-sulphate and EGC-*O*-glucuronide (corresponding peak areas were large in comparison to baseline implying compliance), but as the total urine volume was not recorded (at the University of Manchester) the concentration could not be determined. Therefore, week 12 samples were not included in Figure 5-2, and this resulted in smaller cumulative concentrations of EC-*O*-sulphate and EGC-*O*-glucuronide in comparison to other consumers of the green tea supplement.



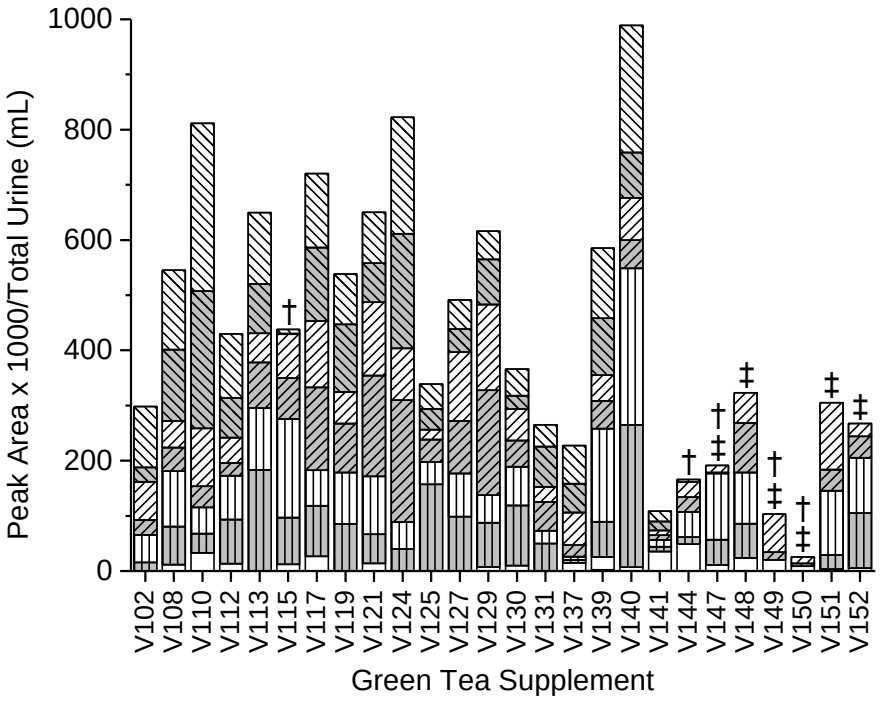


Figure 5-2. Validation of volunteers consuming green tea or placebo supplements from identification of both EC-*O*-sulphate and EGC-*O*-glucuronide in post-supplementation urine, collected for 24 hours. † = lack of compliance to active supplementation, ‡ = data missing.

Many volunteers consuming the placebo excreted EC-*O*-sulphate as the diet was not controlled, however post-green tea supplementation resulted in a 9 times higher excretion of EC-*O*-sulphate in comparison to placebo consumption. As well as green tea (7.9 mg/100 g), EC is also found in other dietary sources including cocoa (14.6 mg/100 g milk chocolate, 70.4 mg/100 g dark chocolate), black tea (3.9 mg/100 g) and some fruits including berries. In comparison, EGC (19.7 mg/100 mL green tea) has only been identified in black tea (7.2 mg/100 mL) and in trace amounts in wine and berries (Phenol Explorer, 2013). EGC-*O*-glucuronide was only occasionally excreted by volunteers consuming the placebo, but was excreted by all consumers of the green tea supplement, and can therefore be considered a biomarker of green tea consumption.

In summary, volunteers 115, 144, 147, 149 and 150 were in the green tea supplement group, but they did not comply fully for the 3 months and so their samples were not included in analysis. In total, there were 24 volunteers on the placebo and 26 on the green tea supplement, with only 21 compliant (Figure 5-2).

5.4.3. Differences between Low and High Dose Supplementation

As aliquots from the pilot study were still available, a direct analysis between low and high dose supplementation was performed to understand the difference between metabolism of a low (equivalent of 2 cups) and high (5 cups) dose of green tea. The pilot study was performed to provide biological samples for method development (urine, blister fluid, biopsies), and for assessment of metabolites present post-consumption of the low dose. As the pilot study was performed separate to the RCT, the volunteers were not the same. The urine of 13 volunteers was assessed for the low dose consumption, and 21 volunteers were used for the high dose.

For the baseline samples, EGC-*O*-glucuronide was only present in one volunteer for both the low and high dose supplements, whereas EC-*O*-sulphate was

present in 12 volunteers for the low dose and 15 for the high dose. It is clear in Figure 5-3, that the presence of both conjugates of interest significantly increased post-consumption for both doses. In relation to the two doses, both EGC-*O*-glucuronide and EC-*O*-sulphate were excreted in significantly higher concentrations after high dose consumption (equivalent of 5 cups of green tea; Table 5-4). The results were represented as peak area as the internal standards (taxifolin and ethyl gallate) spiked into the urine had a poor recovery; the internal standards were possibly retained in urine during protein precipitation with acetonitrile and were therefore not present in the acetonitrile fraction.

Table 5-4. P values for significance of excretion of EC-*O*-sulphate and EGC-*O*-glucuronide in urine collected for 24 hours after consumption of low dose (2 cups) or high dose (5 cups) green tea supplements daily for 3 months (Student's t-test; one tail, two samples).

	P Values for Low vs High Dose			
Compound	Baseline	Day One	Week 6	Week 12
EGC-*O*-Glucuronide	-	0.03	0.03	0.01
EC-*O*-Sulphate	0.4	0.07	0.03	0.01

Table 5-5. P values for significance of EC-*O*-sulphate and EGC-*O*-glucuronide excretion in urine collected for 24 hours, between different time points analysed; baseline (0), day 1 (1), week 6 (6) and week 12 (12) (Student's t-test; one-tail, paired).

Dose	Compound	0 vs 1	0 vs 6	0 vs 12	1 vs 6	1 vs 12	6 vs 12
Low	EGC-*O*-Glucuronide	-	-	-	0.7	0.5	0.9
	EC-*O*-Sulphate	0.00001	0.01	0.0001	0.9	0.9	0.8
High	EGC-*O*-Glucuronide	-	-	-	0.9	0.5	0.4
	EC-*O*-Sulphate	0.00002	0.000008	0.00004	0.3	0.2	0.06

As EGC-*O*-glucuronide was only present in baseline urine for one volunteer, student's t-test between baseline and other time points could not be performed (-).

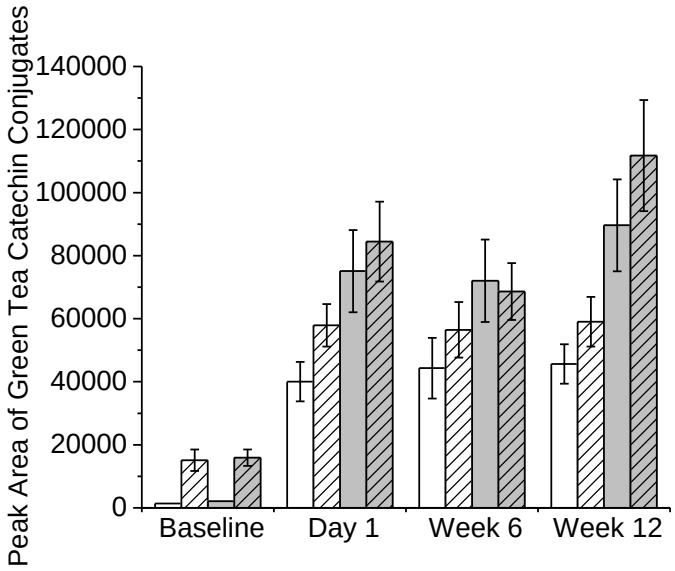
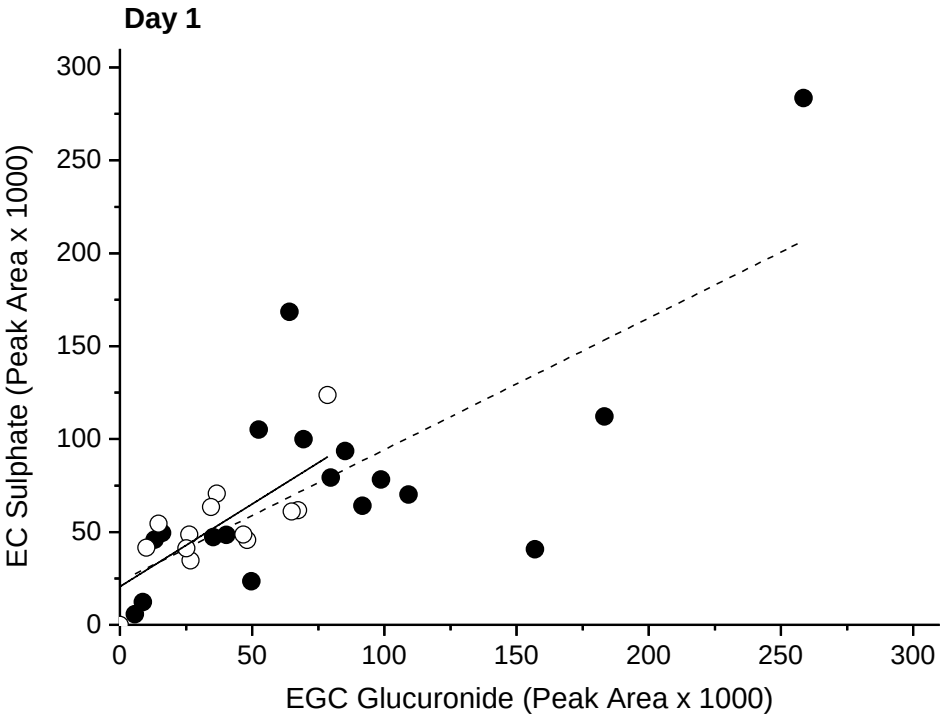
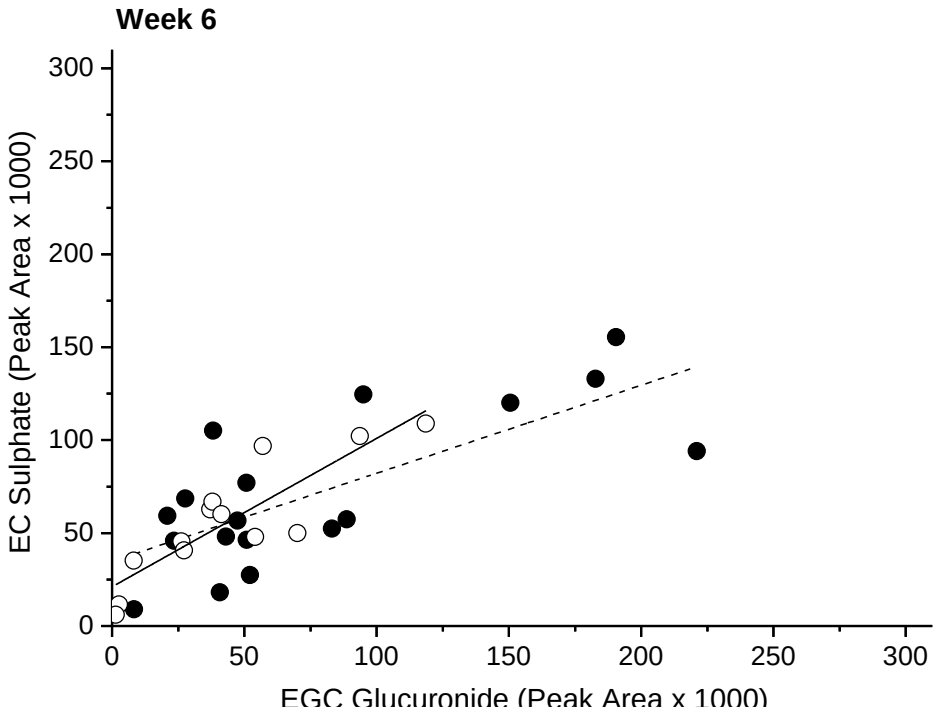


Figure 5-3. Excretion of EGC-*O*-glucuronide and EC-*O*-sulphate post-consumption of the low and high dose supplements, equivalent of 2 or 5 cups respectively. Low dose is represented by *white bars*, high dose by *grey bars*, and for EC-*O*-sulphate excretion bars have *stripes*, whereas EGC-*O*-glucuronide is represented by *no stripes*. Samples were prepared in technical duplicates; average+/-S.D.

As can be seen in Table 5-5, all EC-*O*-sulphate concentrations were significantly increased ($p<0.05$) between baseline and post-supplementation urine excreted after consumption of the low and high dose, and there was no difference between excretion of EC-*O*-sulphate and EGC-*O*-glucuronide in post-supplementation samples.

As there was a large inter-individual variation between the excretion of EC-*O*-sulphate (CV of 0.5 and 0.7 for low and high dose, respectively) and EGC-*O*-glucuronide (CV of 0.6 and 0.8 for low and high dose, respectively), the data was assessed to identify if there was a relationship between volunteers that excreted low concentrations of the two conjugates or high concentrations. There was a weak positive association ($R^2<0.8$) between excretion of EC-*O*-sulphate and EGC-*O*-glucuronide after low and high dose supplementation over 3 months, and for the high dose it appeared that the association became stronger over the 3 months (Figure 5-4).


Day 1
EC Sulphate (Peak Area x 1000)
EGC Glucuronide (Peak Area x 1000)
0
50
100
150
200
250
300


Week 6
EC Sulphate (Peak Area x 1000)
EGC Glucuronide (Peak Area x 1000)
0
50
100
150
200
250
300

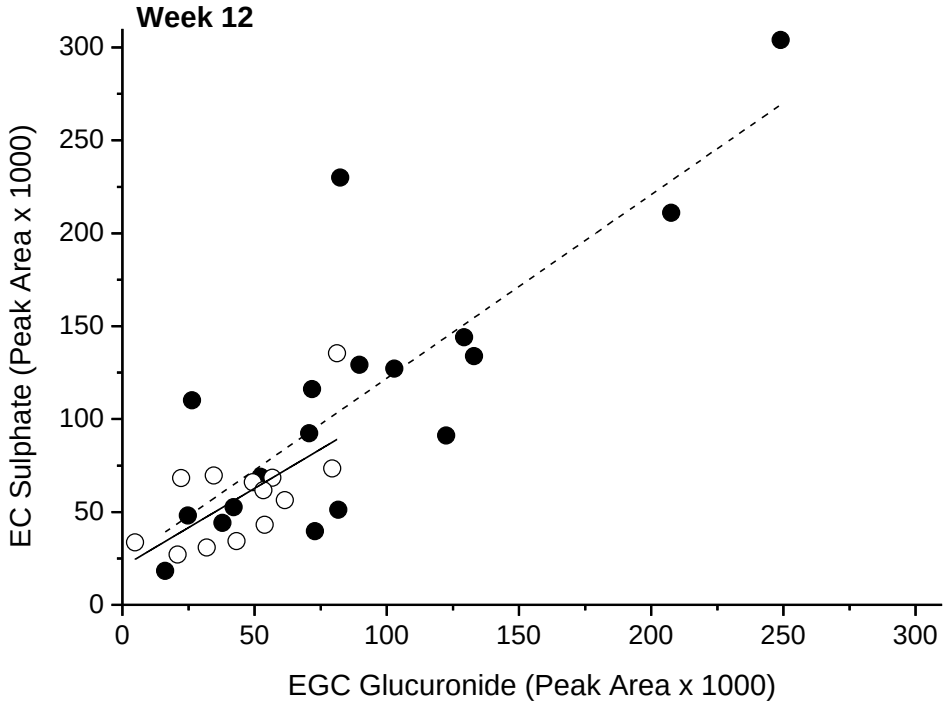


Figure 5-4. Correlations between excretion of EC-*O*-sulphate and EGC-*O*-glucuronide in urine collected for 24 hours post-supplementation of low dose (2 cups, *white circles, black line*) or high dose (5 cups, *black circles, dashed line*). Linear trendlines were allocated to each time point; R^2=0.5382 (low, day 1), 0.4997 (high, day 1), 0.7399 (low, week 6), 0.5151 (high, week 6), 0.3947 (low, week 12) and 0.6454 (high, week 12).

5.4.4. Analysing Conjugates

To understand in more depth the metabolism of the green tea catechins, the phase II green tea catechin metabolites and conjugates (methyl, sulphate and glucuronide) were investigated. As the conjugated forms of the green tea catechin metabolites are not currently commercially available and due to time constraints, the synthesis of all of the conjugates could not be performed, so therefore a review of the literature was used to understand possible transitions that occur during fragmentation of compounds when analysed using mass spectrometry (Table 5-6).

Table 5-6. Review of green tea catechin metabolite conjugate (sulphate and glucuronide) transitions found in the literature, and presence of conjugates in urine and plasma.

Compound	M-H (m/z)	MS^2 (m/z)	Paper	Urine	Plasma
EC-*O*-Glucuronide	465	289	Calani	U	NA
			Del Rio	U	P
			Mullen	U	P
			Sang	U	NA
			Stalmach	U	P
EC-*O*-Sulphate	369	289	Calani	U	NA
			Del Rio	U	P
			Mullen	U	P
			Sang	U	NA
			Stalmach	U	P
O-Me-EC-*O*-Sulphate	383	303	Calani	U	NA
			Del Rio	U	P
			Mullen	U	P
			Sang	U	NA
			Stalmach	U	P
O-Me-EC-*O*-Glucuronide	479	303	Del Rio	U	ND
EGC-*O*-Glucuronide	481	305	Calani	U	NA
			Del Rio	U	P
			Mullen	U	P
			Sang	U	NA
			Stalmach	U	P
EGC-*O*-Sulphate	385	305	Calani	U	NA
			Del Rio	U	ND
			Sang	U	NA
			Stalmach	U	ND
O-Me-EGC-*O*-Glucuronide	495	319	Calani	U	NA
			Del Rio	U	P
			Mullen	U	P
			Sang	U	NA
			Stalmach	U	P
O-Me-EGC-*O*-Sulphate	399	319	Calani	U	NA
			Del Rio	U	P
			Mullen	U	P
			Sang	U	NA
			Stalmach	U	P
EGCG-*O*-Sulphate	537	457	Stalmach	ND	P
ECG-*O*-Sulphate	521	441	Stalmach	ND	P
O-Me-Gallic Acid-*O*-Sulphate	263	183	Mullen	U	ND
M4-*O*-Glucuronide	399	223	Calani	U	NA
			Del Rio	U	ND
			Sang	U	NA
M4-*O*-Sulphate	303	223	Calani	U	NA
			Del Rio	U	ND
			Sang	U	NA
O-Me-M4-*O*-Glucuronide	413	237	Calani	U	NA
O-Me-M4-*O*-Sulphate	317	237	Calani	U	NA
			Del Rio	U	ND
			Sang	U	NA
M6/M6'-*O*-Glucuronide	383	207	Calani	U	NA
			Del Rio	U	ND
			Sang	U	NA
M6/M6'-*O*-Sulphate	287	207	Calani	U	NA
			Del Rio	U	ND
			Sang	U	NA
O-Me-M6/M6'-*O*-Glucuronide	397	221	Calani	U	NA

Review of Calani *et al*., 2012, Del Rio *et al*., 2010a, Mullen *et al*., 2010, Sang *et al*., 2008, Stalmach *et al*., 2009. Urine (U), plasma (P), not detected (ND), not analysed for (NA).

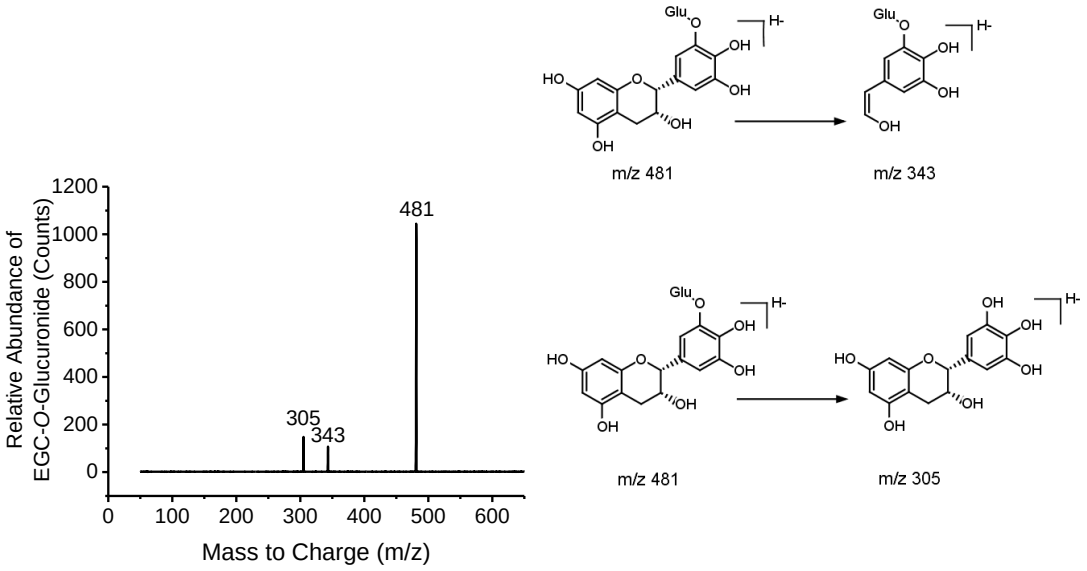
5.4.4.1 Fragmentation Patterns

During the method development, various collision energies (between 0 and 30 eV) were applied to the parent mass to produce multiple “daughter” masses. The largest (or in some cases, only) daughter peak was used for quantification purposes (relating to the free-form catechin metabolite standard curves) and the second largest peak was used for qualitative analysis (i.e. when both quantifier and qualifier peaks are present at the same time this confirms that the quantifier peak correlates to the mass of the compound of interest).

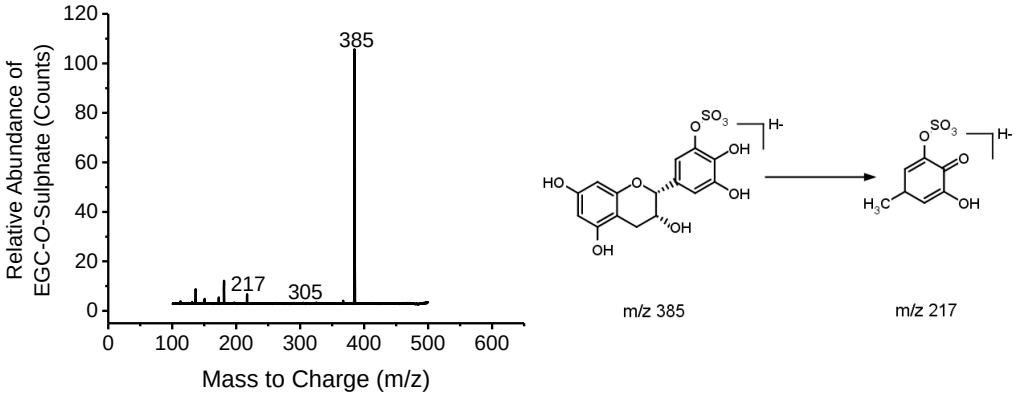
Table 2-2 is a list of the compounds of interest and the transitions that were identified for analysis. The loss of 80 atomic mass units (amu) occurred from the removal of the sulphate moiety; M4-*O*-sulphate (303>223), *O*-methyl-M4-*O*-sulphate (317>237), M6/M6'-*O*-sulphate (287>207), EC-*O*-sulphate (369>289), *O*-methyl-EC-*O*-sulphate (383>303), EGC-*O*-sulphate (385>305), *O*-methyl-EGC-*O*-sulphate (399>319), 3-hydroxybenzoic acid-*O*-sulphate (217>137), benzoic acid-*O*-sulphate (201>121), gallic acid-*O*-sulphate (249>169), hippuric acid-*O*-sulphate (258>178) and syringic acid-*O*-sulphate (277>197). The change in transition by a 176 amu loss occurs after the removal of the glucuronide moiety; M4-*O*-glucuronide (399>223), EC-*O*-glucuronide (465>289), EGC-*O*-glucuronide (481>305), *O*-methyl-EGC-*O*-glucuronide (495>319), 3-hydroxybenzoic acid-*O*-glucuronide (313>137), hippuric acid-*O*-glucuronide (354>178), *O*-methyl-gallic acid-*O*-glucuronide (359>183) and syringic acid-*O*-glucuronide (373>179). Any losses of 14 amu result because of the loss of the methyl group.

The other transitions evident after different collision energies were applied are displayed in Figure 5-5. The potential fragmentation patterns are also displayed in Figure 5-5, although if the transition is the removal of the conjugate moiety this is not displayed.

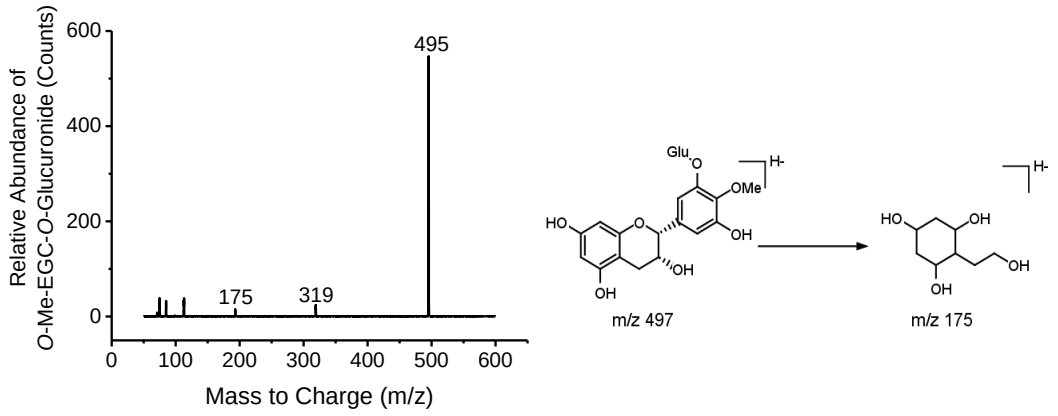
Sang *et al*. (2008) displayed images of the relative abundance for some of the catechin metabolites after collision energies were applied. The same transitions were found in this study after collision energies were applied, for EGC-*O*-glucuronide (481>305 and 481>343), EGC-*O*-sulphate (383>305), *O*-methyl-EGC-*O*-glucuronide (497>319 and 497>175), *O*-methyl-EGC-*O*-sulphate (399>319 and 399>217), EC-*O*-sulphate (369>289 and 369>245), *O*-methyl-EC-*O*-sulphate (383>303 and 383>137) M4-*O*-glucuronide (399>223 and 399>179), M4-*O*-sulphate (303>223 and 303>179) and M6/M6'-*O*-sulphate (287>207 and 287>163) (Figure 5-5).



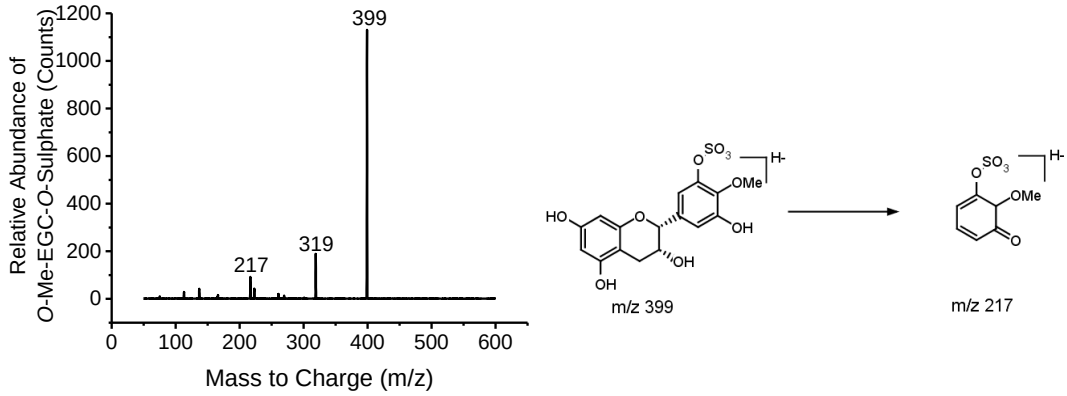
EGC-*O*-Glucuronide



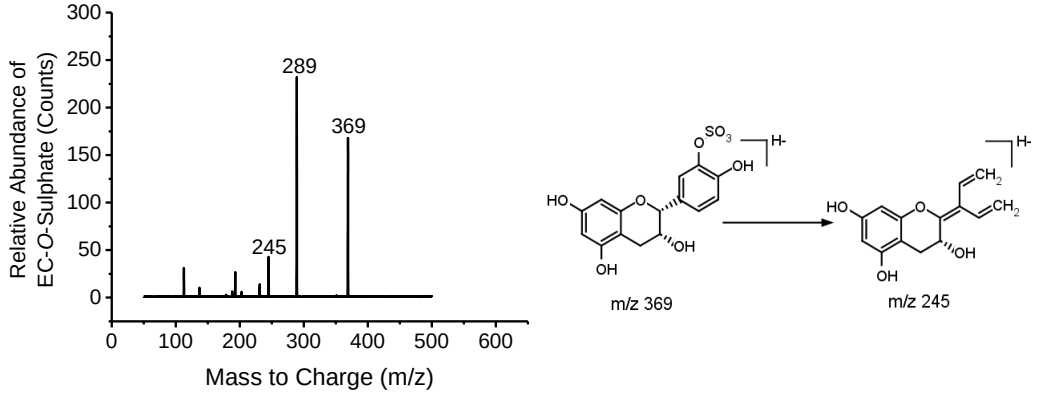
EGC-*O*-Sulphate



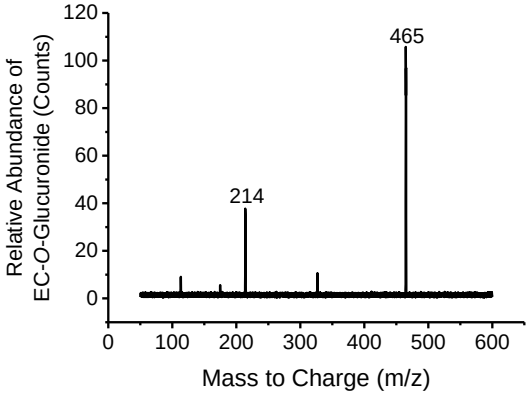
O-Methyl-EGC-*O*-Glucuronide

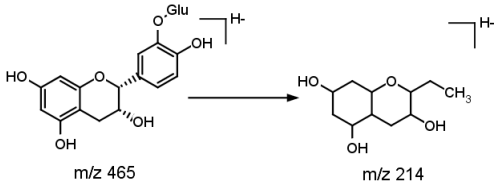


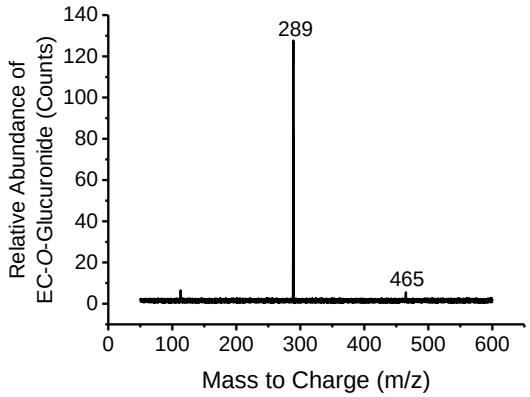
O-Methyl-EGC-*O*-Sulphate



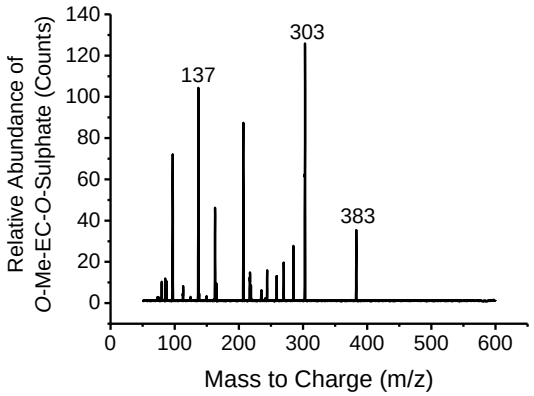
EC-*O*-Sulphate

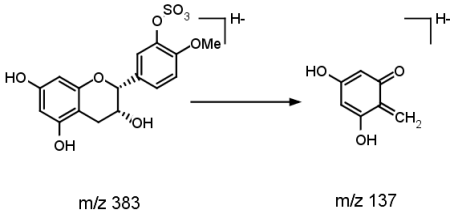




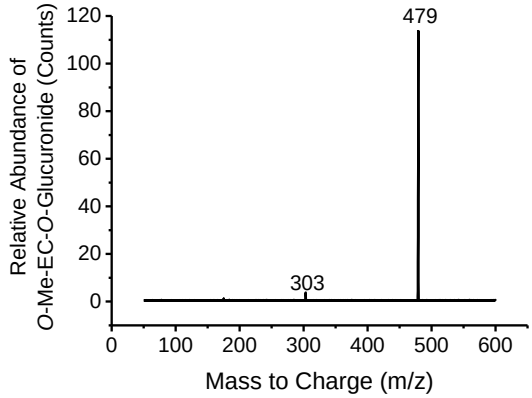


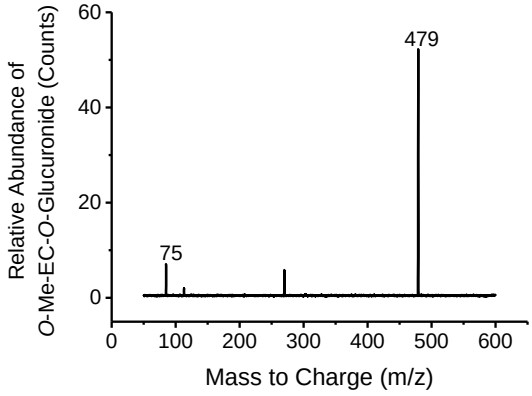
EC-*O*-Glucuronide

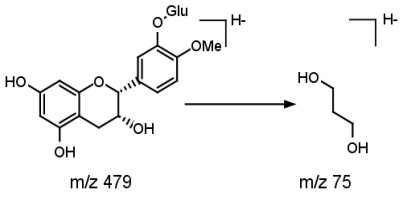




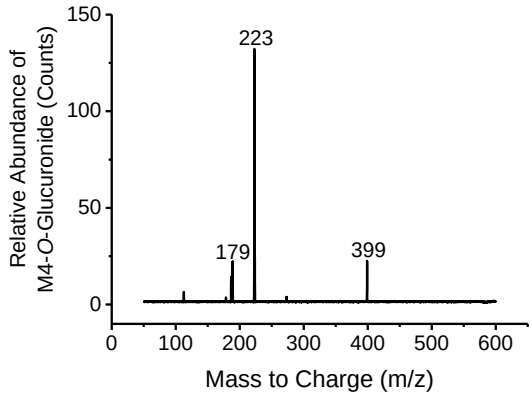
O-Methyl-EC-*O*-Sulphate

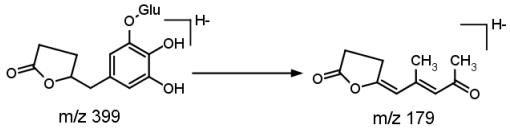




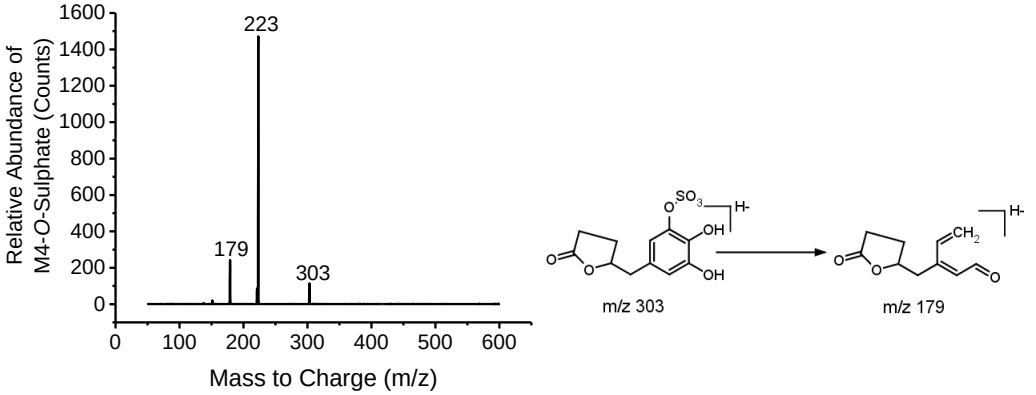


O-Methyl-EC-*O*-Glucuronide

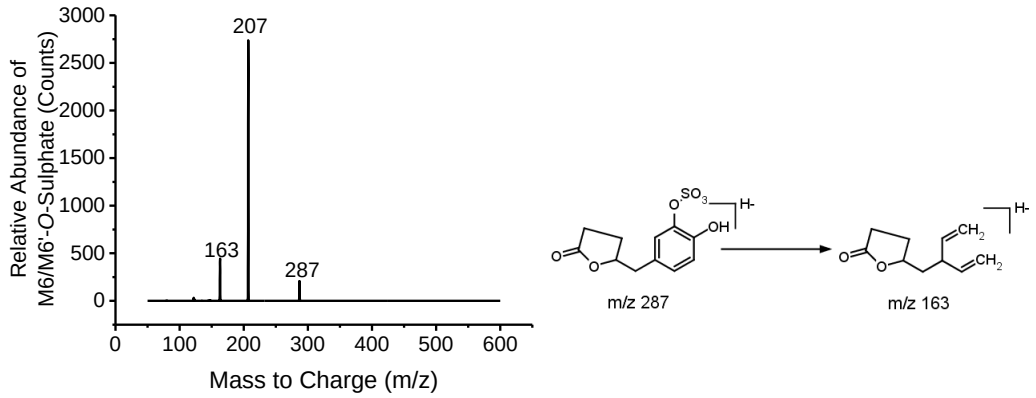




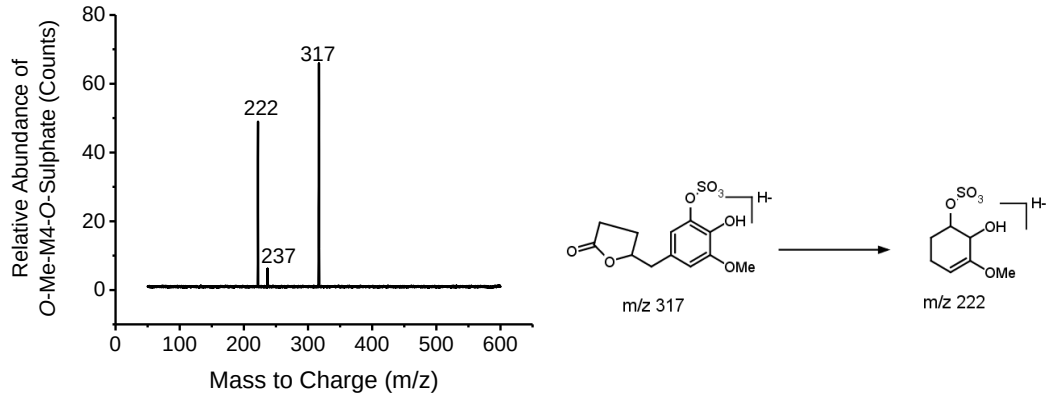
M4-*O*-Glucuronide



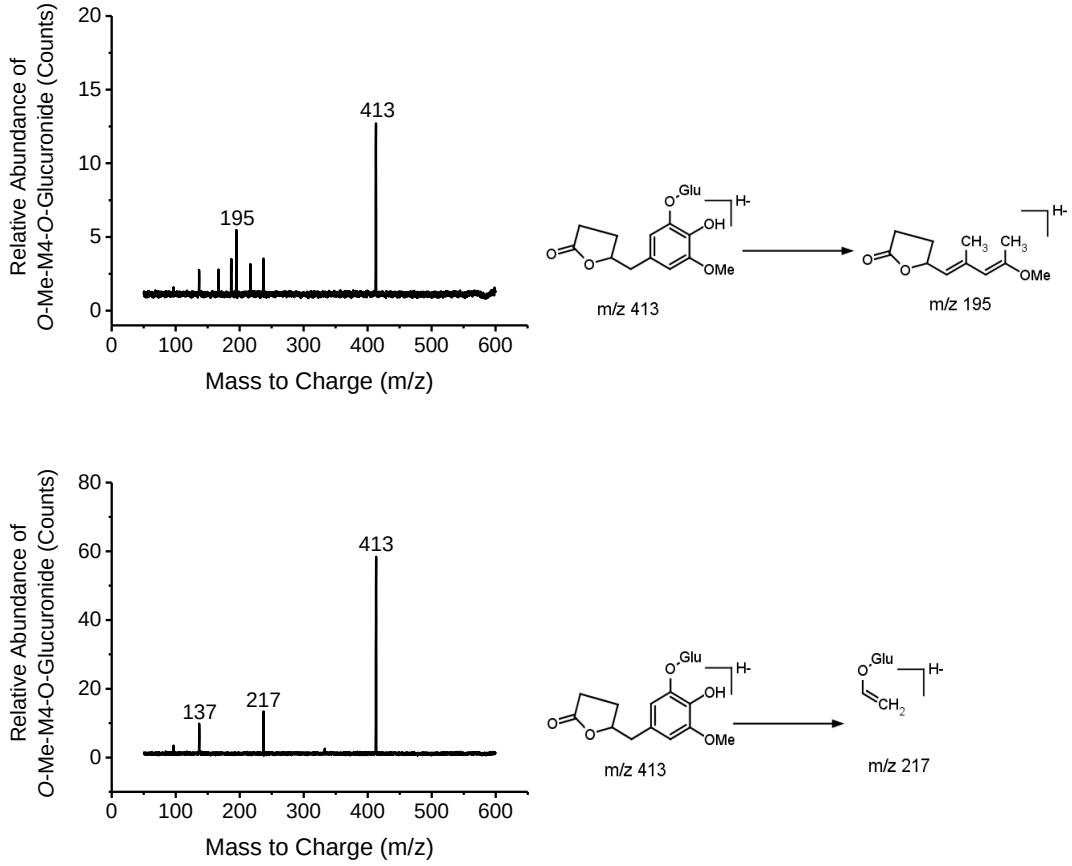
M4-*O*-Sulphate



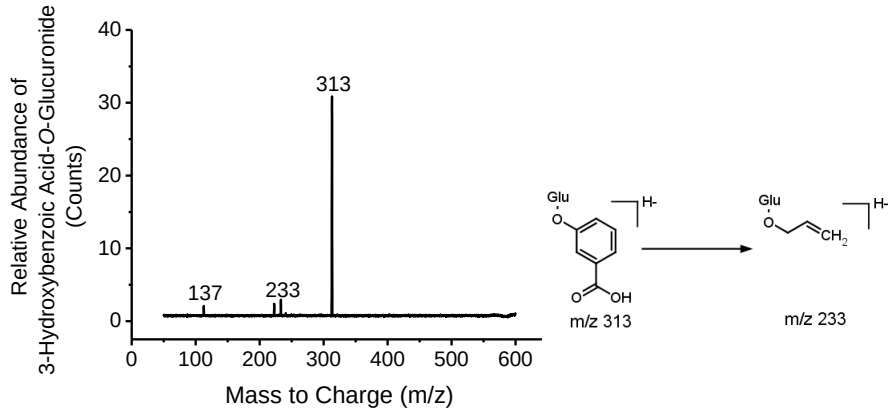
M6/M6'-*O*-Sulphate



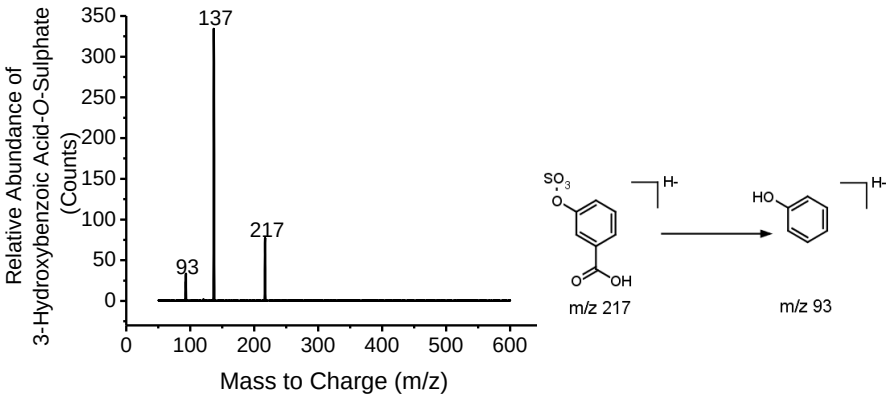
O-Methyl-M4-*O*-Sulphate



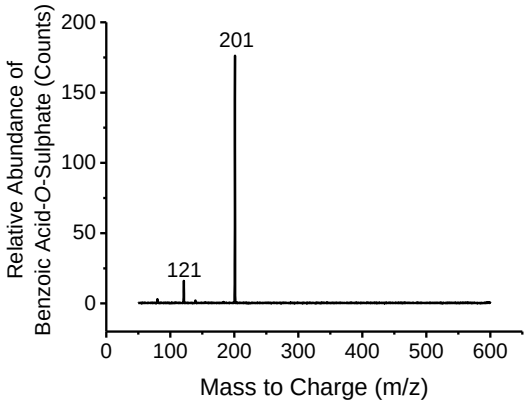
O-Methyl-M4-*O*-Glucuronide



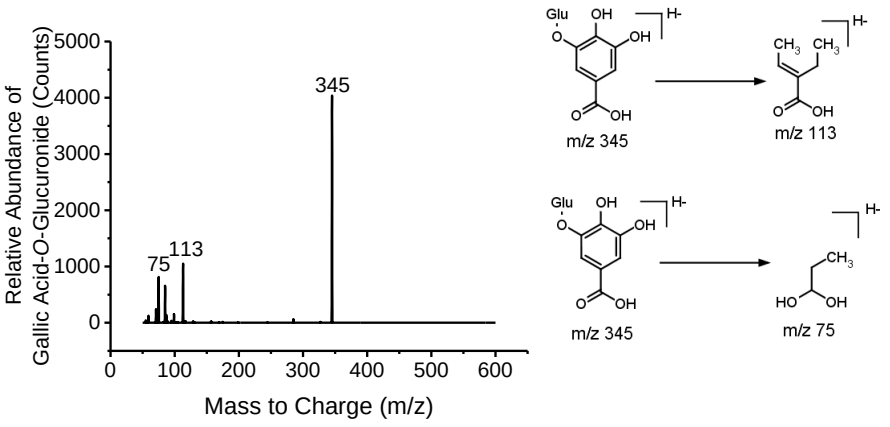
3-Hydroxybenzoic Acid-*O*-Glucuronide



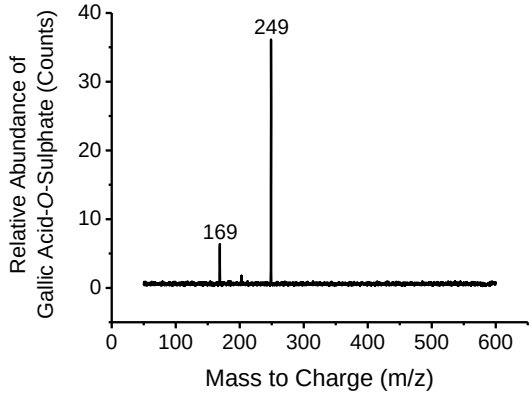
3-Hydroxybenzoic Acid-*O*-Sulphate



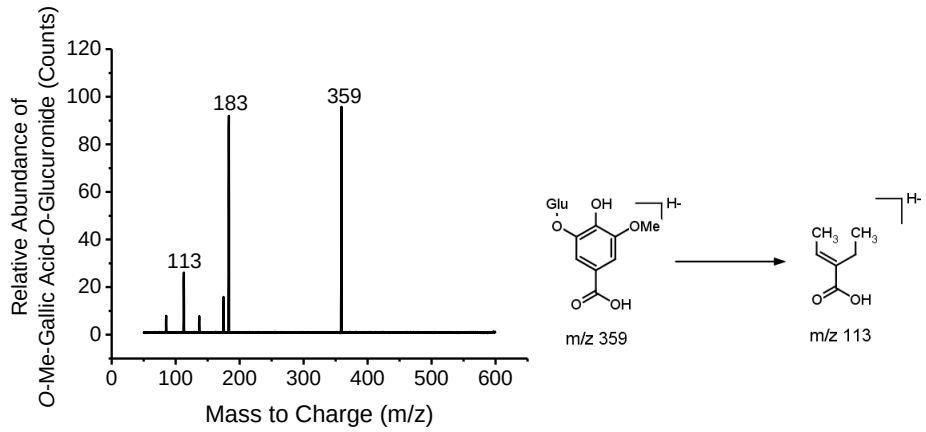
Benzoic Acid-*O*-Sulphate



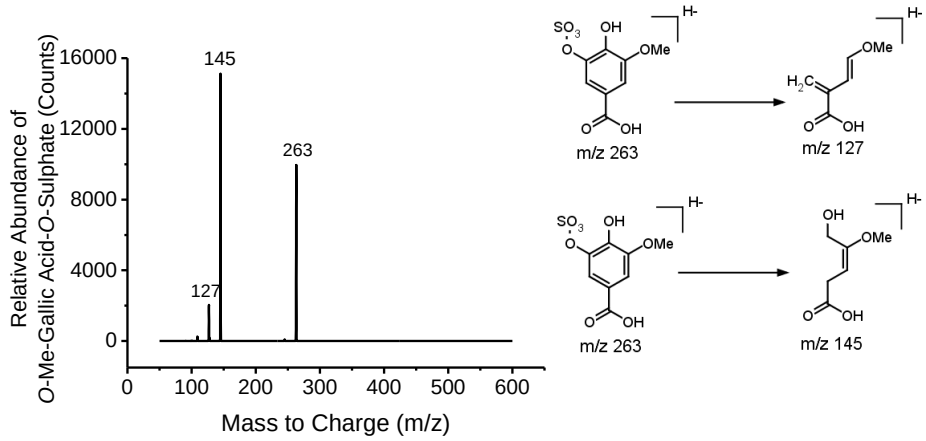
Gallic Acid-*O*-Glucuronide



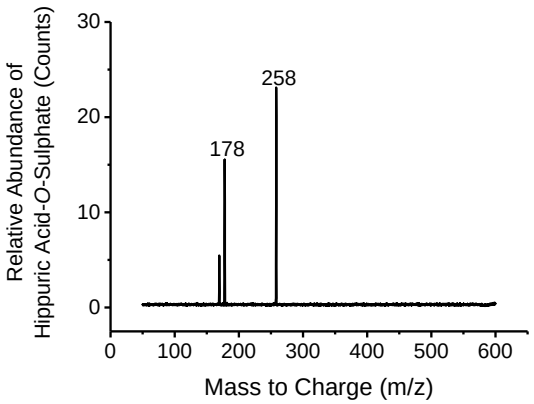
Gallic Acid-*O*-Sulphate



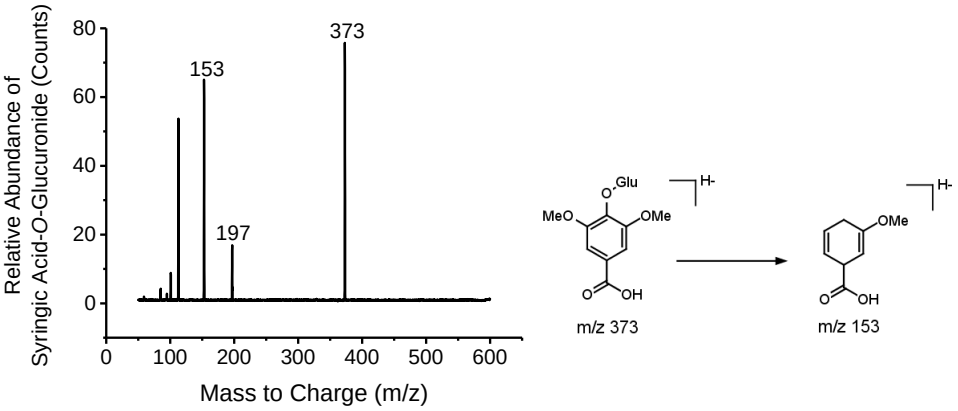
O-Methyl-Gallic Acid-*O*-Glucuronide



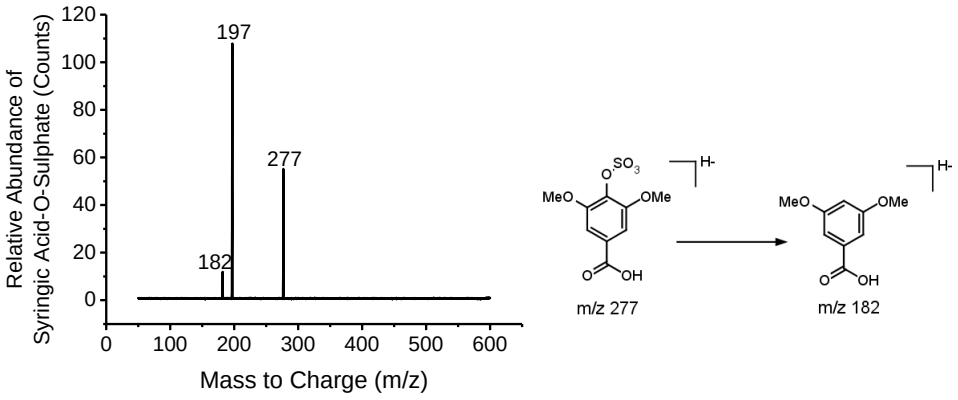
O-Methyl-Gallic Acid-*O*-Sulphate



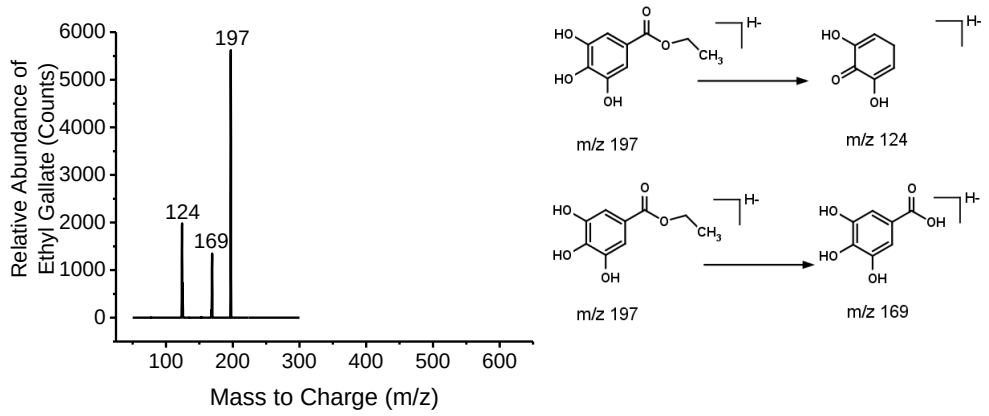
Hippuric Acid-*O*-Sulphate



Syringic Acid-*O*-Glucuronide



Syringic Acid-*O*-Sulphate



Ethyl Gallate

Figure 5-5. Transitions and feasible fragmentation patterns for conjugates listed in Table 2-2. Fragmentation patterns are only displayed when the transition was not the removal of the conjugated moiety.

After collision energies and fragmentation values had been determined, the relative retention times for the conjugates were noted (Table 5-7) and used as a reference for the urine samples (pilot and RCT) bioavailability study samples (urine, plasma, skin biopsy and blister fluid). Chromatograms for the conjugates that were present in week 12 urine samples are displayed in Figure 5-6. The majority of the peaks were very sharp and clear, except for EGC-*O*-sulphate and quercetin-*O*-glucuronide. Both were identified by the presence of the qualifier peaks at the same retention times, and were only used if the signal to noise ratio was above 10, limit of quantification (LOQ).

Table 5-7. Relative retention times of green tea catechin metabolites; conjugated and free-forms.

Compound	Retention Time (min)
3-*O*-Me-Gallic Acid	5.8
M6/M6'-*O*-Glucuronide	7.3
Epigallocatechin	9.1
Hippuric Acid	9.4
3-Hydroxybenzoic Acid	10.2
O-Me-Gallic Acid-*O*-Sulphate	10.9
Epigallocatechin-*O*-Glucuronide	11.1
O-Me-Epigallocatechin-*O*-Glucuronide	11.7
Syringic Acid	11.9
M4-*O*-Glucuronide	12.1
Epicatechin-*O*-Glucuronide	12.2
M6	12.2
Epicatechin	12.3
3'-*O*-Me-Epigallocatechin	12.5
4'-*O*-Me-Epigallocatechin	12.5
Epigallocatechin Gallate	12.8
Gallic Acid	12.8
Epigallocatechin-*O*-Glucuronide	12.9
Ethyl Gallate	12.9
Syringic Acid-*O*-Glucuronide	13.3
Epicatechin-*O*-Glucuronide	13.4
O-Me-Gallic Acid-*O*-Glucuronide	13.5
4'-*O*-Me-Epicatechin	13.8
Epicatechin Gallate	13.9
Catechin	13.9
Taxifolin	14.0
Benzoic Acid	14.1
O-Me-Epigallocatechin-*O*-Glucuronide	14.4
O-Me-Epigallocatechin-*O*-Glucuronide	15.3
Gallic Acid-*O*-Glucuronide	15.3
Quercetin-*O*-Glucuronide	15.8
Hippuric Acid-*O*-Sulphate	16.1
O-Me-M6/M6'-*O*-Sulphate	16.2
Quercetin	16.3
M6/M6'-*O*-Sulphate	16.6
Benzoic Acid-*O*-Sulphate	17.4
O-Me-Epigallocatechin-*O*-Sulphate	17.6
O-Me-M4-*O*-Sulphate	17.9
M6/M6'-*O*-Sulphate	18.4
O-Me-Epicatechin-*O*-Sulphate	18.7
Epigallocatechin-*O*-Sulphate	18.7
O-Me-M4-*O*-Glucuronide	19.2

Retention times correlate with urine processed using the method in section 2.3.2.1.

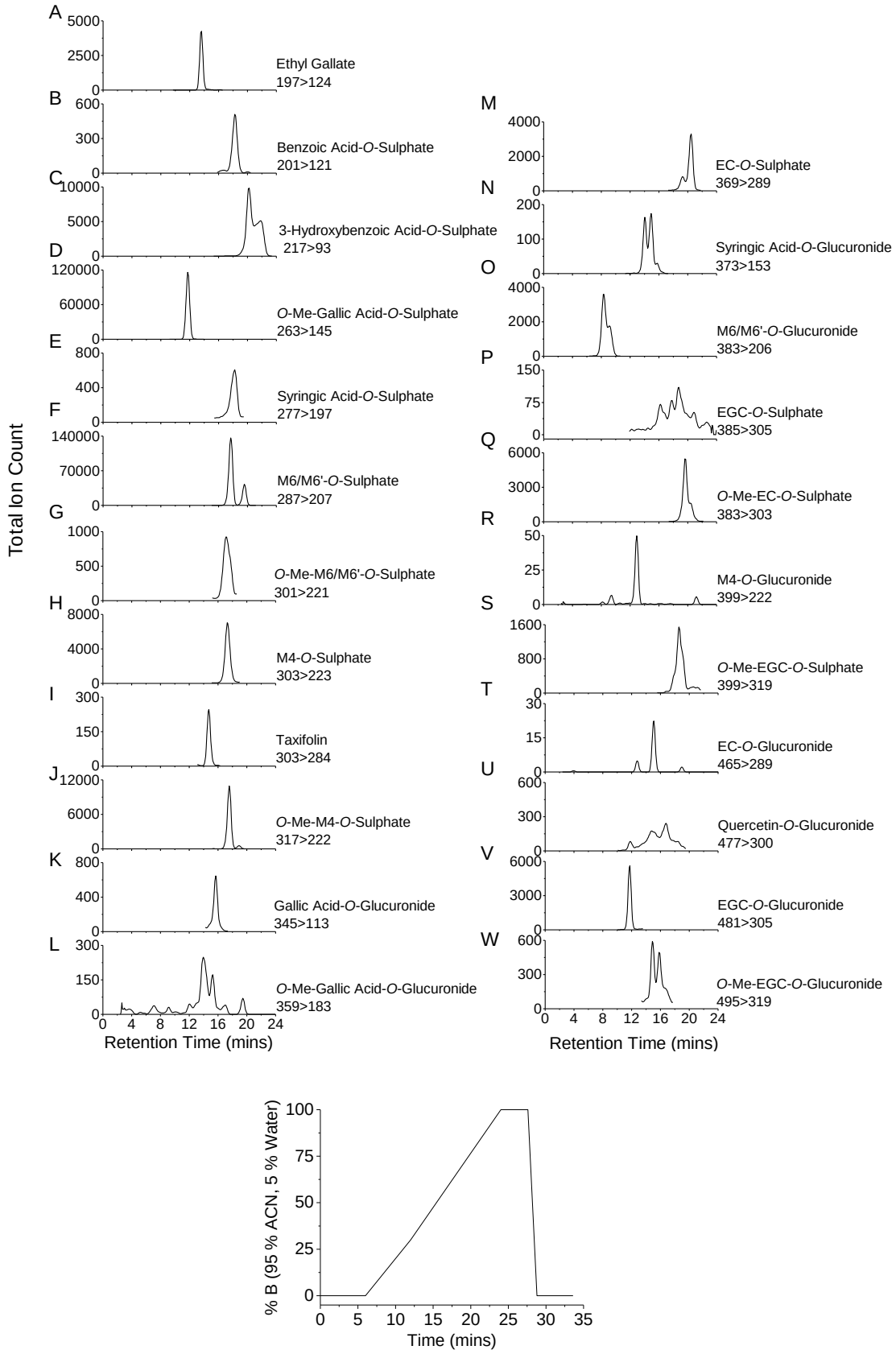


Figure 5-6. Chromatograms of green tea catechin metabolites (conjugated and free-forms) and internal standards, and the gradient used for separation. Chromatograms were isolated from a week 12 urine sample; initially 200 µL, reconstituted to 100 µL after processing using

the method in section 2.3.2.1. (A) Ethyl gallate, (B) benzoic acid-*O*-sulphate, (C) 3-hydroxybenzoic acid-*O*-sulphate, (D) *O*-methyl-gallic acid-*O*-sulphate, (E) syringic acid-*O*-sulphate, (F) M6/M6'-*O*-sulphate, (G) *O*-methyl-M6/M6'-*O*-sulphate, (H) M4-*O*-sulphate, (I) taxifolin, (J) *O*-methyl-M4-*O*-sulphate, (K) gallic acid-*O*-glucuronide, (L) *O*-methyl-gallic acid-*O*-glucuronide, (M) EC-*O*-sulphate, (N) syringic acid-*O*-glucuronide, (O) M6/M6'-*O*-glucuronide, (P) EGC-*O*-sulphate, (Q) *O*-methyl-EC-*O*-sulphate, (R) M4-*O*-glucuronide, (S) *O*-methyl-EGC-*O*-sulphate, (T) EC-*O*-glucuronide, (U) quercetin-*O*-glucuronide, (V) EGC-*O*-glucuronide, (W) *O*-methyl-EGC-*O*-glucuronide.

5.4.5. Green Tea Catechin Free-form and Metabolite Conjugates Present in Urine

In total, 55 compounds were investigated post-daily consumption of either the low or high dose green tea supplements for 3 months. The compounds were free-form, free-form conjugates and metabolite conjugates (listed in Table 2-2). Of the 55 compounds investigated, 31 were present in at least one volunteer, post-consumption of the green tea supplement (Table 5-8). *O*-Methyl-M4-*O*-glucuronide was the only compound not present in both low and high dose urine samples, possibly as it was below the limit of detection (LOD) in urine excreted post-low dose supplementation. The isomers, and corresponding conjugates, of EC, EGC, EGCG (C, GC and GCG) and M6 or M6' could not be distinguished between using this LC-MS method. EGCG and ECG were also not detected in any of the samples which indicated that these compounds were below the LOD or were not excreted.

More compounds were significantly increased in urine post-consumption of the high dose supplement for 3 months, in comparison to the low dose (Table 5-9). This is partly because the conjugates were present in more volunteers (as there were the samples of the 21 volunteers analysed for the high dose study, compared with 13 for the low dose pilot study) and partly because the amounts excreted were higher in relation to baseline. For both low and high dose, there was a significant

increase when compared to baseline for EC-*O*-sulphate, *O*-methyl-EC-*O*-sulphate, *O*-methyl-EGC-*O*-glucuronide, *O*-methyl-M4-*O*-sulphate, M4-*O*-sulphate, M6/M6'-*O*-glucuronide and M6/M6'-*O*-sulphate. For the low dose, there was also a significant increase from baseline to day 1 for M4-*O*-glucuronide. For the high dose, significant increases were also observed for EC-*O*-glucuronide, EGC-*O*-glucuronide (not present at baseline in enough low dose samples to perform statistical analysis), *O*-methyl-EGC-*O*-sulphate and from baseline to day 1 for hippuric acid and 3-hydroxybenzoic acid-*O*-sulphate.

An increase in excretion of 20 metabolites after high dose supplement consumption was evident in comparison to excretion post-low dose supplementation. These included EC-*O*-sulphate, EC-*O*-glucuronide, *O*-methyl-EC-*O*-sulphate, EGC-*O*-glucuronide, EGC-*O*-sulphate, -*O*-methyl-EGC-*O*-glucuronide, *O*-methyl-EGC-*O*-sulphate, gallic acid-*O*-glucuronide, M4-*O*-sulphate, M6/M6'-*O*-glucuronide, M6/M6'-*O*-sulphate, *O*-methyl-M6/M6'-*O*-sulphate, 3-hydroxybenzoic acid, 3-hydroxybenzoic acid-*O*-sulphate and hippuric acid (Table 5-10).

Table 5-8. Green tea catechin metabolites (free-form and conjugates) excreted in urine collected for 24 hours, from the 13 volunteers consuming low dose supplementation (2 cups of green tea) or the 21 volunteers consuming high dose supplementation (5 cups of green tea) over the 3 month intervention.

Low Dose	Compound (µmol)/Total Urine Excreted							
Compound	Baseline	n=	Day One	n=	Week 6	n=	Week 12	n=
Epicatechin-*O*-Glucuronide	3.9+/-0.4	11	4.3+/-0.8	13	4.2+/-0.9	12	7+/-2	13
Epicatechin-*O*-Sulphate	2+/-1	7	20+/-8	11	10+/-3	11	16+/-4	13
O-Me-Epicatechin-*O*-Sulphate	50+/-10	9	200+/-50	11	130+/-40	11	300+/-100	13
Epigallocatechin-*O*-Glucuronide	4	1	3+/-1	13	2.6+/-0.6	12	5+/-2	13
Epigallocatechin-*O*-Sulphate	0	0	2+/-1	3	0.2+/-0.3	3	0.8+/-0.4	4
O-Me-Epigallocatechin-*O*-Glucuronide	70+/-20	9	30+/-10	13	20+/-10	12	70+/-30	13
O-Me-Epigallocatechin-*O*-Sulphate	10	1	40+/-10	12	40+/-10	11	60+/-20	13
Quercetin-*O*-Glucuronide	15+/-4	4	9	1	7+/-3	3	20+/-10	5
Quercetin-*O*-Sulphate	0	0	10	1	0	0	0	0
Kaempferol-*O*-Glucuronide	0	0	32	1	0	0	0	0
Kaempferol-*O*-Sulphate	3+/-1	2	6+/-4	2	0.1+/-0.1	1	3+/-1	3
Gallic Acid-*O*-Glucuronide	130+/-40	12	70+/-30	12	80+/-30	12	200+/-100	13
3-*O*-Me Gallic Acid	0.07+/-0.01	7	0.07+/-0.03	11	0.05+/-0.02	10	0.2+/-0.1	11
O-Me-Gallic Acid-*O*-Glucuronide	0.4+/-0.1	9	0.32+/-0.07	11	0.25+/-0.08	10	0.6+/-0.3	10
O-Me-Gallic Acid-*O*-Sulphate	50+/-10	13	32+/-6	13	50+/-20	12	60+/-20	13
M4-*O*-Glucuronide	0.65+/-0.01	2	3+/-1	12	3+/-1	10	10+/-8	13
M4-*O*-Sulphate	4+/-2	8	30+/-10	12	20+/-10	12	20+/-10	13
O-Me-M4-*O*-Glucuronide	0	0	0	0	0	0	0	0
O-Me-M4-*O*-Sulphate	2+/-1	2	50+/-20	12	40+/-30	10	40+/-20	12
M6/M6'-*O*-Glucuronide	1.3+/-0.5	6	10+/-3	12	6+/-2	12	11+/-2	13
M6/M6'-*O*-Sulphate	20+/-10	12	300+/-100	13	200+/-100	12	200+/-100	13
O-Me-M6/M6'-*O*-Sulphate	0	0	4+/-2	9	5+/-3	5	3+/-1	7
3-Hydroxybenzoic Acid	0.26+/-0.06	10	0.16+/-0.04	12	0.17+/-0.05	11	0.5+/-0.2	11
3-Hydroxybenzoic Acid-*O*-Sulphate	18+/-5	13	16+/-4	13	15+/-3	12	30+/-20	13
Benzoic Acid-*O*-Sulphate	70+/-10	6	50+/-20	9	60+/-10	8	40+/-10	8
Syringic Acid-*O*-Glucuronide	2.8+/-0.6	12	3+/-1	13	2.0+/-0.5	12	2.8+/-0.9	13
Syringic Acid-*O*-Sulphate	14+/-7	2	30+/-10	6	10+/-4	8	60+/-50	5
Hippuric Acid	14000+/-4000	13	11000+/-2000	13	18000+/-8000	12	17000+/-6000	13
Hippuric Acid-*O*-Sulphate	1	1	0.3	1	1	1	2+/-2	3

High Dose	Compound (µmol)/Total Urine Excreted							
Compound	Baseline	n=	Day One	n=	Week 6	n=	Week 12	n=
Epicatechin-*O*-Glucuronide	4.7+/-0.7	19	14+/-2	20	8+/-1	20	10+/-2	17
Epicatechin-*O*-Sulphate	3.4+/-0.6	14	40+/-10	21	30+/-10	21	35+/-5	18
O-Me-Epicatechin-*O*-Sulphate	60+/-10	13	500+/-100	21	400+/-100	21	500+/-100	18
Epigallocatechin-*O*-Glucuronide	0.3+/-0.2	4	7+/-1	21	6+/-2	21	8+/-1	18
Epigallocatechin-*O*-Sulphate	0	2	1.7+/-0.6	9	1.1+/-0.3	7	1.9+/-0.5	8
O-Me-Epigallocatechin-*O*-Glucuronide	20+/-6	14	50+/-10	19	40+/-10	20	40+/-20	17
O-Me-Epigallocatechin-*O*-Sulphate	1.2+/-0.6	3	110+/-20	21	90+/-10	21	150+/-40	18
Quercetin-*O*-Glucuronide	12+/-4	8	20+/-10	5	5+/-1	5	3+/-3	2
Quercetin-*O*-Sulphate	20+/-20	2	8	1	3	1	0	2
Kaempferol-*O*-Glucuronide	0	2	0	0	33	1	42	1
Kaempferol-*O*-Sulphate	3+/-3	2	6+/-1	4	13+/-6	8	10+/-5	4
Gallic Acid-*O*-Glucuronide	180+/-80	19	300+/-100	21	150+/-30	21	140+/-50	17
3-*O*-Me Gallic Acid	0.18+/-0.05	10	0.19+/-0.05	17	0.6+/-0.3	18	0.11+/-0.02	17
O-Me-Gallic Acid-*O*-Glucuronide	0.5+/-0.2	9	0.9+/-0.3	10	0.5+/-0.2	9	0.6+/-0.3	8
O-Me-Gallic Acid-*O*-Sulphate	60+/-10	21	80+/-10	21	80+/-20	21	60+/-10	18
M4-*O*-Glucuronide	0.9	1	12+/-4	18	13+/-5	20	8+/-2	16
M4-*O*-Sulphate	5+/-2	15	110+/-50	19	70+/-30	20	50+/-10	18
O-Me-M4-*O*-Glucuronide	0.5+/-0.4	3	0	0	0.8+/-0.8	2	0.7+/-0.6	3
O-Me-M4-*O*-Sulphate	1.2+/-0.5	6	150+/-60	20	140+/-50	21	80+/-20	18
M6/M6'-*O*-Glucuronide	2.4+/-0.6	8	22+/-7	20	90+/-60	21	20+/-4	18
M6/M6'-*O*-Sulphate	30+/-10	21	800+/-300	20	600+/-200	21	500+/-100	18
O-Me-M6/M6'-*O*-Sulphate	0	2	20+/-20	20	10+/-3	18	9+/-2	16
3-Hydroxybenzoic Acid	0.8+/-0.2	20	0.6+/-0.1	20	0.53+/-0.09	21	0.5+/-0.1	18
3-Hydroxybenzoic Acid-*O*-Sulphate	17+/-4	18	30+/-10	21	22+/-6	21	18+/-3	17
Benzoic Acid-*O*-Sulphate	100+/-30	11	130+/-60	7	50+/-20	6	50+/-10	4
Syringic Acid-*O*-Glucuronide	3.0+/-0.7	18	4+/-1	17	3.0+/-0.8	18	2.9+/-0.7	14
Syringic Acid-*O*-Sulphate	80+/-50	10	110+/-90	13	40+/-10	13	30+/-10	13
Hippuric Acid	13000+/-3000	21	23000+/-4000	21	20000+/-5000	21	19000+/-3000	18
Hippuric Acid-*O*-Sulphate	9+/-7	4	6+/-2	7	7+/-3	7	3+/-2	3

For week 6 low dose urine samples, analysis was for 12 volunteers as the urine aliquot for one volunteer had finished. Samples were analysed in technical duplicate; average+/-S.E.

Table 5-9. Significance of green tea catechin metabolite excretion between different time points in urine collected for 24 hours, after low dose (2 cups of green tea) or high dose (5 cups) supplementation.

Low Dose	P Values						Frequency (Volunteers/Time Point)			
Compound	0 vs 1	0 vs 6	0 vs 12	1 vs6	1 vs 12	6 vs 12	0	1	6	12
Epicatechin-*O*-Glucuronide	0.3	0.4	0.1	0.5	0.1	0.1	11	13	12	13
Epicatechin-*O*-Sulphate	0.02*	0.004**	0.003**	0.1	0.4	0.1	7	11	11	13
O-Me-Epicatechin-*O*-Sulphate	0.01**	0.009**	0.02*	0.2	0.3	0.1	9	11	11	13
Epigallocatechin-*O*-Glucuronide	-	-	-	0.3	0.2	0.1	1	13	12	13
Epigallocatechin-*O*-Sulphate	-	-	-	0.01**	0.2	0.1	0	3	3	4
O-Me-Epigallocatechin-*O*-Glucuronide	0.03*	0.02*	0.5	0.4	0.1	0.1	9	13	12	13
O-Me-Epigallocatechin-*O*-Sulphate	-	-	-	0.4	0.2	0.1	1	12	11	13
Quercetin-*O*-Glucuronide	-	0.1	0.3	-	-	0.2	4	1	3	5
Quercetin-*O*-Sulphate	-	-	-	-	-	-	0	1	0	0
Kaempferol-*O*-Glucuronide	-	-	-	-	-	-	0	1	0	0
Kaempferol-*O*-Sulphate	0.3	-	0.4	-	0.2	-	2	2	1	3
Gallic Acid-*O*-Glucuronide	0.1	0.1	0.3	0.5	0.1	0.1	12	12	12	13
3-*O*-Me Gallic Acid	0.5	0.2	0.1	0.3	0.1	0.1	7	11	10	11
O-Me-Gallic Acid-*O*-Glucuronide	0.3	0.2	0.2	0.2	0.1	0.1	9	11	10	10
O-Me-Gallic Acid-*O*-Sulphate	0.1	0.4	0.4	0.2	0.2	0.4	13	13	12	13
M4-*O*-Glucuronide	0.02*	0.1	0.1	0.4	0.2	0.2	2	12	10	13
M4-*O*-Sulphate	0.03*	0.1	0.02*	0.3	0.3	0.5	8	12	12	13
O-Me-M4-*O*-Glucuronide	-	-	-	-	-	-	0	0	0	0
O-Me-M4-*O*-Sulphate	0.02*	0.1	0.02*	0.4	0.4	0.5	2	12	10	12
M6/M6'-*O*-Glucuronide	0.01**	0.007**	0.0001***	0.1	0.5	0.04*	6	12	12	13
M6/M6'-*O*-Sulphate	0.01**	0.01**	0.001**	0.2	0.3	0.3	12	13	12	13
O-Me-M6/M6'-*O*-Sulphate	-	-	-	0.4	0.3	0.3	0	9	5	7
3-Hydroxybenzoic Acid	0.1	0.1	0.2	0.4	0.1	0.1	10	12	11	11
3-Hydroxybenzoic Acid-*O*-Sulphate	0.4	0.3	0.2	0.4	0.2	0.2	13	13	12	13
Benzoic Acid-*O*-Sulphate	0.3	0.3	0.1	0.4	0.3	0.2	6	9	8	8
Syringic Acid-*O*-Glucuronide	0.5	0.2	0.5	0.3	0.5	0.2	12	13	12	13
Syringic Acid-*O*-Sulphate	0.1	0.4	0.2	0.1	0.3	0.2	2	6	8	5
Hippuric Acid	0.2	0.3	0.3	0.2	0.2	0.5	13	13	12	13
Hippuric Acid-*O*-Sulphate	-	-	-	-	-	-	1	1	1	3

High Dose	P Values						Frequency (Volunteers/Time Point)			
Compound	0 vs 1	0 vs 6	0 vs 12	1 vs 6	1 vs 12	6 vs 12	0	1	6	12
Epicatechin-*O*-Glucuronide	0.001***	0.02*	0.002**	0.03*	0.1	0.2	19	20	20	17
Epicatechin-*O*-Sulphate	0.00003***	0.001***	0.00001***	0.4	0.4	0.4	14	21	21	18
O-Me-Epicatechin-*O*-Sulphate	0.000002***	0.0001***	0.000004***	0.1	0.4	0.1	13	21	21	18
Epigallocatechin-*O*-Glucuronide	0.00002***	0.001***	0.00001***	0.3	0.3	0.2	4	21	21	18
Epigallocatechin-*O*-Sulphate	-	-	-	0.2	0.4	0.1	2	9	7	8
O-Me-Epigallocatechin-*O*-Glucuronide	0.005**	0.1	0.1	0.3	0.3	0.5	14	19	20	17
O-Me-Epigallocatechin-*O*-Sulphate	0.000004***	0.000001***	0.001***	0.2	0.2	0.1	3	21	21	18
Quercetin-*O*-Glucuronide	0.3	0.05*	0.04*	0.1	0.1	0.3	8	5	5	2
Quercetin-*O*-Sulphate	-	-	-	-	-	-	2	1	1	2
Kaempferol-*O*-Glucuronide	-	-	-	-	-	-	2	0	1	1
Kaempferol-*O*-Sulphate	0.3	0.1	0.2	0.1	0.2	0.3	2	4	8	4
Gallic Acid-*O*-Glucuronide	0.2	0.3	0.3	0.1	0.1	0.4	19	21	21	17
3-*O*-Me Gallic Acid	0.4	0.2	0.2	0.2	0.1	0.1	10	17	18	17
O-Me-Gallic Acid-*O*-Glucuronide	0.2	0.5	0.4	0.2	0.3	0.4	9	10	9	8
O-Me-Gallic Acid-*O*-Sulphate	0.1	0.2	0.4	0.5	0.2	0.2	21	21	21	18
M4-*O*-Glucuronide	-	-	-	0.4	0.3	0.2	1	18	20	16
M4-*O*-Sulphate	0.02*	0.01**	0.0004***	0.3	0.1	0.2	15	19	20	18
O-Me-M4-*O*-Glucuronide	-	0.4	0.4	-	-	0.5	3	0	2	3
O-Me-M4-*O*-Sulphate	0.01**	0.005**	0.0004***	0.5	0.2	0.1	6	20	21	18
M6/M6'-*O*-Glucuronide	0.01**	0.1	0.0003***	0.2	0.4	0.1	8	20	21	18
M6/M6'-*O*-Sulphate	0.01**	0.0003***	0.0001***	0.3	0.1	0.2	21	20	21	18
O-Me-M6/M6'-*O*-Sulphate	-	-	-	0.2	0.2	0.4	2	20	18	16
3-Hydroxybenzoic Acid	0.3	0.2	0.2	0.3	0.3	0.4	20	20	21	18
3-Hydroxybenzoic Acid-*O*-Sulphate	0.03*	0.2	0.4	0.2	0.04*	0.3	18	21	21	17
Benzoic Acid-*O*-Sulphate	0.3	0.1	0.1	0.1	0.1	0.5	11	7	6	4
Syringic Acid-*O*-Glucuronide	0.2	0.5	0.5	0.2	0.2	0.5	18	17	18	14
Syringic Acid-*O*-Sulphate	0.4	0.2	0.1	0.2	0.2	0.2	10	13	13	13
Hippuric Acid	0.03*	0.1	0.1	0.4	0.2	0.4	21	21	21	18
Hippuric Acid-*O*-Sulphate	0.4	0.4	0.2	0.4	0.1	0.1	4	7	7	3

Analysis was performed to examine changes from baseline (0) to day 1 (1), week 6 (6) or week 12 (12), and also between the post-supplement samples; Student's t-test, one tailed, paired, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Table 5-10. Significant differences between urinary excretion of free-form and conjugated green tea catechin metabolites after low and high dose green tea supplementation for 3 months.

Low vs High Dose	P Values			
Compound	Baseline	Day 1	Week 6	Week 12
Epicatechin-*O*-Glucuronide	0.2	0.001***	0.01**	0.1
Epicatechin-*O*-Sulphate	0.03*	0.04*	0.01**	0.003**
O-Me-Epicatechin-*O*-Sulphate	0.2	0.001**	0.001**	0.03*
Epigallocatechin-*O*-Glucuronide	-	0.02*	0.02*	0.1
Epigallocatechin-*O*-Sulphate	-	0.5	0.01**	0.07
O-Me-Epigallocatechin-*O*-Glucuronide	0.02*	0.02*	0.1	0.2
O-Me-Epigallocatechin-*O*-Sulphate	-	0.001**	0.002**	0.03*
Quercetin-*O*-Glucuronide	0.3	-	0.3	0.1
Quercetin-*O*-Sulphate	-	-	-	-
Kaempferol-*O*-Glucuronide	-	-	-	-
Kaempferol-*O*-Sulphate	0.5	0.5	-	0.1
Gallic Acid-*O*-Glucuronide	0.3	0.02*	0.1	0.3
3-*O*-Me Gallic Acid	0.08	0.05*	0.1	0.2
O-Me-Gallic Acid-*O*-Glucuronide	0.2	0.1	0.1	0.5
O-Me-Gallic Acid-*O*-Sulphate	0.2	0.002**	0.2	0.4
M4-*O*-Glucuronide	-	0.04*	0.04*	0.4
M4-*O*-Sulphate	0.4	0.1	0.04*	0.02*
O-Me-M4-*O*-Glucuronide	-	-	-	-
O-Me-M4-*O*-Sulphate	0.3	0.1	0.03*	0.1
M6/M6'-*O*-Glucuronide	0.1	0.1	0.1	0.02*
M6/M6'-*O*-Sulphate	0.3	0.05	0.01**	0.02*
O-Me-M6/M6'-*O*-Sulphate	-	0.1	0.1	0.002**
3-Hydroxybenzoic Acid	0.02*	0.0004***	0.001**	0.5
3-Hydroxybenzoic Acid-*O*-Sulphate	0.4	0.02*	0.2	0.3
Benzoic Acid-*O*-Sulphate	0.2	0.1	0.4	0.3
Syringic Acid-*O*-Glucuronide	0.4	0.2	0.1	0.5
Syringic Acid-*O*-Sulphate	0.1	0.2	0.04*	0.3
Hippuric Acid	0.4	0.01**	0.4	0.4
Hippuric Acid-*O*-Sulphate	-	-	-	0.4

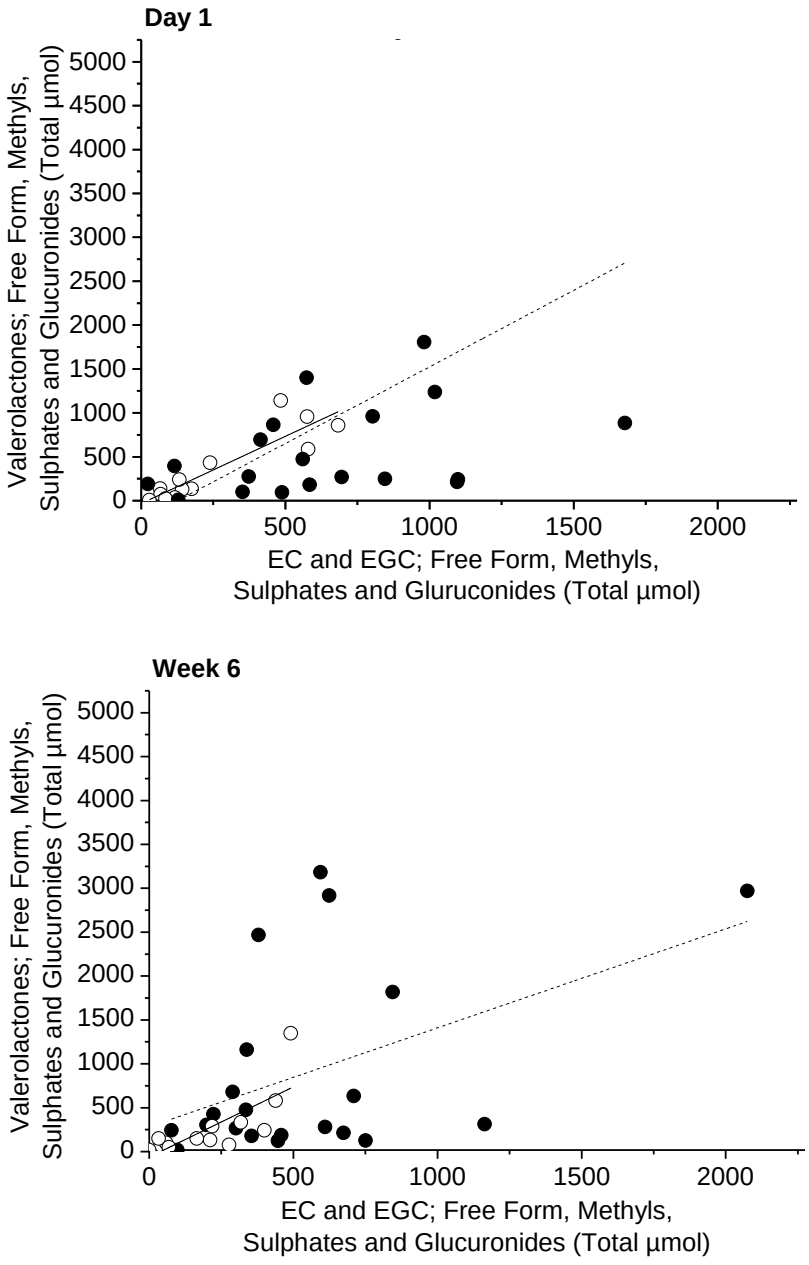
Student's t-test; one tailed, two sample, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.4.6. Relationship between Excretion of EC and EGC Conjugates and Valerolactone Conjugates in Urine

It was clear on analysis of green tea catechins and metabolite conjugates that there was a large range in excretion of EC-*O*-sulphate and EGC-*O*-glucuronides. As it is known that M4, M6 and M6' are microbial metabolites of EC and EGC (Figure 1-3), and that intestinal microbial flora differs between individuals (Eckburg *et al*., 2005), a relationship was explored to consider whether

individuals who excreted low levels of EC and EGC conjugates might excrete high levels of valerolactone conjugates, and vice versa.

As can be seen in Figure 5-7, there was a weak positive association in low excretion of EC, EGC and conjugates and high excretion of valerolactones and conjugates after low or high dose supplementation for 3 months. Trying to understand the relationship in the excretion of EC and EGC conjugates against their complete metabolic fate is very difficult to achieve.



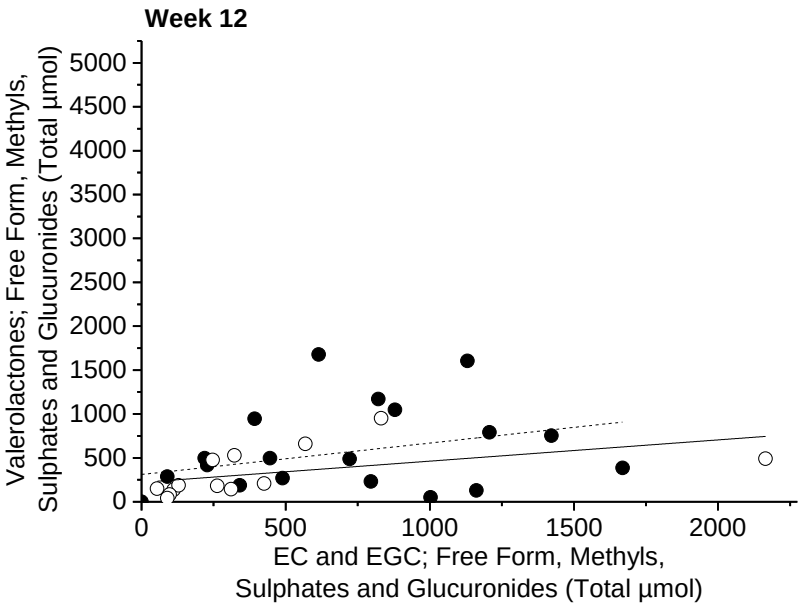


Figure 5-7. Correlations between total excretion of EC, EGC, *O*-methyl-EC and *O*-methyl-EGC and corresponding conjugates with excretion of valerolactones, *O*-methyl-valerolactones and conjugates, in urine collected for 24 hours post-supplementation of low dose (2 cups, *white circles, black line*) or high dose (5 cups, *black circles, dashed line*) after 1 day, 6 weeks and 12 weeks of low and high green tea supplement consumption. Linear trendlines were allocated to each time point; R^2=0.7980 (low, day 1), 0.2253 (high, day 1), 0.5185 (low, week 6), 0.1727 (high, week 6), 0.1853 (low, week 12), 0.0770 (high, week 12).

5.5. Discussion

The urine samples collected for 24 hours at 4 time points (baseline, day 1, week 6 and week 12) throughout the 3 month human intervention studies were essentially accumulated for compliance testing to the daily supplementation. Whilst compliance was being confirmed, the samples also provided valuable information on the presence of green tea catechins and associated metabolites identified in urine samples post-consumption.

Firstly, the low dose (equivalent of 2 cups of green tea) pilot intervention study urine was assessed for compliance with enzyme deconjugation, and it was apparent that 20 compounds were present at significantly higher concentrations

than when compared to pre-supplementation baseline samples. These 20 compounds were explored in the high dose supplementation studies (equivalent of 5 cups of green tea), in which urine samples were assessed using a combination method which allowed efficient analysis of free-form (unconjugated) and conjugated derivatives of catechins and metabolites. A review of the literature highlighted that a combination method that allows for analysis of both free-form and conjugated derivatives of green tea catechins has not been identified. For the first time, conjugated (sulphate esters and glucuronic acid) derivatives of phenolic acids were identified in urine post-green tea consumption; including 3-hydroxybenzoic acid sulphate, benzoic acid sulphate, hippuric acid sulphate and syringic acid glucuronide. This method was developed for analysis of bioavailability samples in Chapter 6, as it is possible that free-form catechins could be present in skin biopsy samples post-consumption of green tea supplementation, and therefore an efficient method to assess both unconjugated and conjugated metabolites was required.

Initially, a short, high throughput method was created to analyse the urine of all RCT volunteers (and eventually bioavailability volunteers) to distinguish between those who had been supplementing their diet with a placebo for 3 months, and those consuming green tea (at the equivalent of 5 cups a day). Unfortunately, the week 12 urine for some volunteers was not recorded during the intervention performed at the University of Manchester, and these volunteers had to be removed from analysis. Of the 50 volunteers involved in the RCT, 24 appeared to be taking the placebo and 21 out of the 26 taking the supplement were compliant.

There was a clear dose response between volunteers consuming low or high dose supplements daily for 3 months, which is in line with other reported data (Yang *et al*., 1998, Chow *et al*., 2005, Luo *et al*., 2006, Wang *et al*., 2008, Renouf *et al*., 2013). However, this is the first study to assess the dose response of a range of green tea catechin and metabolite conjugate derivatives excreted into urine collected for 24 hours. Over the 3 months, there was a significant increase between

low and high dose excretion of EC-*O*-glucuronide, EC-*O*-sulphate, *O*-methyl-EC-*O*-sulphate, EGC-*O*-glucuronide, EGC-*O*-sulphate, *O*-methyl-EGC-*O*-sulphate, M4-*O*-sulphate and M6/M6'-*O*-sulphate into 24 hour urine.

It is known that flavonoids can modify particular gut microbiota (Dolara *et al*., 2005, Tzounis *et al*., 2008), and therefore there was a possibility that the gut microbiota may have changed during daily consumption of green tea supplements for 3 months. Gut microbiota are involved in the breakdown of catechins and metabolites to smaller products, phenolic acids. Therefore, changes in particular gut microflora would result in an increase or decrease in particular metabolites; for example *Escherichia coli*, *Bifidobacterium sp*., *Lactobacillus sp*., *Bacteroides sp*. and *Eubacterium ep*. have been associated with the breakdown of flavonoids (Cardona *et al*., 2013). If consumption of green tea were to increase the population of these microflora species within the colon, then the production of breakdown products would increase. However, analysis of changes in metabolites excreted into urine collected for 24 hours throughout the three month interventions revealed that a modification in gut microbiota had not occurred.

A relationship was also explored between excretion of EC and EGC verses valerolactones (known microbial metabolites of EC and EGC). This was to investigate if a relationship existed between excretion of a high concentration of valerolactones by volunteers, with excretion of low concentrations of EC and EGC. This would suggest that EC and EGC had been broken down further due to the presence of particular gut microbiota as it is known that there is a large inter-individual variation between gut microbiota (Eckburg *et al*., 2005). However, correlation of data indicated that a very weak positive relationship existed, implying that there is a very weak association between high excretion of EC and EGC with high excretion of valerolactones, and vice versa; the opposite of the hypothesis. This weak association suggests that volunteers who have the ability to metabolise

green tea catechins more efficiently, have a higher concentration of a range of green tea catechins and metabolites excreted into the urine.

A direct comparison with another study that quantified conjugated derivatives of EC and EGC excreted in urine for up to 24 hours after a green tea drink (49 µmol EC and 230 µmol EGC ingested) relevant to free-form standards, illustrated that the concentrations of EC metabolites assessed in this thesis appears to be higher (Stalmach *et al*., 2009). The excretion of EGC metabolites are relatively similar (EGC-*O*-glucuronide, 6.5 and 8 µmol; EGC-*O*-sulphate, 2.6 and 1.9 µmol for Stalmach *et al.* and the present study, respectively), however the difference in quantification of EC conjugates is vast (EC-*O*-glucuronide, 1.5 and 10 µmol; EC-*O*-sulphate, 6.7 and 35 µmol, *O*-methyl-EC-*O*-sulphate 10.9 and 500 µmol for Stalmach *et al.* and the present study, respectively). This comparison highlights the importance of using commercially available standards, as without, over-quantification can occur because a response factor has not been applied. This has been reported previously when hydroxycinnamic acid conjugates were expressed relevant to commercial standards and then relevant to free-form (unconjugated) derivatives (Farrell *et al*., 2011). Stalmach *et al*. (2009), also tuned the LC-MS with EC in the tuning mixture. This can maximise the response of EC and related compounds, which may result in smaller differences in response factors between EC and conjugated derivatives, minimising the chance of over-quantification.

The results obtained here indicate that green tea catechin metabolites excreted into urine collected for 24 hours are mainly present in the conjugated form, and that a combination method can allow for assessment of both free-form and conjugated derivatives of green tea catechin metabolites within one procedure.

Chapter 6. Bioavailability of Green Tea Catechin Metabolites in Skin

6.1. Abstract

The skin is the largest organ in the human body and it acts as a barrier protecting internal organs from the external environment, including UV radiation. UV radiation can penetrate the layers of the skin, causing photo-damage through induction of inflammation and generation of reactive oxygen species. Flavonoids are secondary metabolites produced by plants for numerous functions, including UV protection. Flavonoids are present in the diet and many studies have examined the application of topical green tea to the skin of mice and humans in association with reducing UV-induced inflammation. For the first time, bioavailability of green tea catechin metabolites in skin has been investigated using LC-MS-MS. A 3 month human intervention study was conducted in which volunteers consumed daily green tea supplements. It was apparent that 7 green tea catechin metabolites were identified in skin biopsy samples, and 20 metabolites were identified in interstitial blister fluid, both pre- and post- UV irradiation, and pre- and post- supplementation. The interstitial fluid was derived from plasma, and it was clear that 15 green tea catechin metabolites were present in both plasma and interstitial fluid samples collected 3 hours post-consumption of the green tea supplement. *O*-Methyl-gallic acid-*O*-sulphate and M6/M6'-*O*-sulphate were the two metabolites identified in skin biopsy, interstitial and plasma samples, of 6 and 3 volunteers, respectively. The results presented here imply that green tea catechin metabolites are bioavailable within skin.

6.2. Introduction

Regular consumption of green tea has been associated with protective health benefits, including reducing the incidence of cardiovascular disease, cancer and neurological disease (Scalbert *et al*., 2005). It is possible that green tea catechins could influence a reduction in the incidence of UV-induced inflammation in skin, as has been highlighted in many *in vitro* studies using epidermal keratinocyte cell lines, (including Afaq *et al*., 2003, Song *et al*., 2006, Wu *et al*., 2009), and *in vivo* studies in mice and humans (Wang *et al*., 1991, Katiyar *et al*., 2001, Heinrich *et al*., 2011).

As mentioned, it is possible that green tea catechins could provide protection in skin from UV radiation. A study in mice by Wang *et al*. (1991) compared oral consumption of green tea with topical application, and it was clear that the incidence of tumour formation post-UV irradiation was reduced after oral consumption. A recent human oral intervention study also suggested that protection could potentially occur, as was seen in a reduction in erythema (measured as 1.25x minimal erythemal dose; Heinrich *et al*., 2011).

Numerous studies have investigated the bioavailability of green tea catechins and associated metabolites in plasma and urine samples with LC-MS following consumption of a dietary source in the form of a beverage or supplement. Most recent research of biological samples involves the analysis of conjugate derivates, relevant to standard curves created from free-form standards (Stalmach *et al*., 2009, Del Rio *et al*., 2010a).

The analysis of skin tissue samples after consumption of green tea by humans, for assessment of conjugated forms of green tea catechins and metabolites (sulphate and glucuronide moieties), has never been conducted. The aim of this chapter is to improve the knowledge of green tea catechins and metabolites, assessed as both free-form and conjugated forms (methyl, sulphate and glucuronide), in biological samples collected after a 3 month human intervention

study in which volunteers consumed green tea supplements daily. In particular, the bioavailability of green tea catechin metabolites in skin biopsies and interstitial (blister) fluid surrounding skin cells is assessed for the first time, with the aim to identify metabolites that could potentially be involved in reducing the inflammation and photo-damage caused by exposure of skin to UV radiation.

6.3. Materials and Methods

For the protocols used to perform analysis of the samples from the 3 month human green tea supplement bioavailability study, see section 2.3.2. The biological samples assessed were plasma, interstitial blister fluid, skin biopsies and urine.

6.4. Results

As the positions of the conjugate moieties (methyl ester, sulphate ester and glucuronic acid) of green tea catechin metabolites are unknown in the results presented in this thesis, they will be referred to as *O*-methyl-catechin-*O*-glucuronide or -*O*-sulphate, instead of x-*O*-methyl-catechin-x-*O*-glucuronide or x-*O*-sulphate.

6.4.1. Recovery of Green Tea Catechin Conjugates from Different Matrices

Before analysis of biological samples (urine, plasma, blister fluid, skin biopsy) from the human intervention green tea supplement bioavailability study by mass spectrometry, the recoveries of EC, EGC, EC-*O*-sulphate and EGC-*O*-glucuronide were confirmed using blank baseline urine samples so that a procedure could be identified that allowed for good recovery of free-form and conjugated derivatives.

The RCT urine samples were analysed by removing protein with the addition of acetonitrile, before injection onto the LC-MS. Acetonitrile was removed by centrifugal evaporation (at 40 ºC). Assessment of the recovery of EC and EGC

standards spiked into blank baseline urine, identified a poor recovery (3+/-2 % and 13+/-6 %, respectively, data not shown) from this matrix when using the protein precipitation method. However, EC-*O*-sulphate and EGC-*O*-glucuronide synthesised enzymatically had recoveries from urine of 79+/-13 % and 38+/-1%, respectively. As the concentrations of spiked EC-*O*-sulphate and EGC-*O*-glucuronide were unknown, an equivalent volume was directly analysed by LC-MS, and peak areas corresponding to the conjugates in spiked urine were related to the direct analysis. The recovery of EC and EGC from urine using the pilot study method (ethyl acetate extractions of urine instead of acetonitrile), resulted in a better recovery; 91+/-5% and 88+/-9 %, respectively. Therefore, it is clear that the recovery of the free-form catechins is hindered by the change in organic solvent from ethyl acetate to acetonitrile.

To improve the recovery of the free-form catechins from the green tea supplement bioavailability study, a combination method was employed. Biological samples (urine, plasma, interstitial blister fluid and skin biopsies) were first extracted with ethyl acetate, and the protein was precipitated from the remaining sample with acetonitrile. The solvents were removed and the dried down ethyl acetate fraction was reconstituted and combined with the dried down acetonitrile fraction, before analysis by LC-MS. For the combination method, plasma and urine samples were processed on ice, until centrifugal evaporation at room temperature. To reduce deconjugation activity from the presence of sulfatase or β-glucuronidase in the skin biopsies, and possibly in blister fluid, these samples were handled on dry ice, at -78 ºC.

The recovery of catechins and conjugates assessed in urine was improved using the combination method (EC, 95+/-6%; EGC, 111+/-5 %; EC-*O*-sulphate, 91+/-7 %; and EGC-*O*-glucuronide, 94+/-6 %). The combination method allowed for isolation of both free-form and conjugated catechins, and was used for analysis of all bioavailability samples; urine, plasma, interstitial blister fluid and skin biopsies

(Table 6-1). Limit of quantification (LOQ, signal to noise ratio above 10) was determined after analysis of all bioavailability samples, for the free-form catechins related to the metabolites present in each of the biological samples (Table 6-2).

Table 6-1. Recovery of compounds spiked into blank biological samples; plasma, blister fluid and biopsy solution.

	Recovery of Compounds		
Compound	Plasma (%)	Blister Fluid (%)	Biopsy Solution (%)
Epicatechin	107+/-14	90+/-2	102+/-27
Epigallocatechin	103+/-3	75+/-8	105+/-6
Epicatechin-*O*-Sulphate	117+/-2	86+/-2	108+/-15
Epigallocatechin-*O*-Glucuronide	97+/-2	32+/-5	62+/-13
Ethyl Gallate	93+/-1	104+/-13	96+/-4

Recoveries were performed in technical triplicate at a minimum of three concentrations. Percentage recovery was relative to compounds not spiked into biological samples; average+/-S.E.

Table 6-2. Limit of quantification for the free-form green tea catechin metabolites (related to sulphate or glucuronide forms) for each biological sample; plasma, biopsy or blister fluid.

	Limit of Quantification (pmole)		
Compound	Plasma	Blister	Biopsy
Epicatechin	2	20	-
3'-*O*-Methyl Epicatechin	1	20	-
4'-*O*-Methyl Epicatechin	0.1	-	1
Epigallocatechin	0.2	10	-
3'-*O*-Methyl Epigallocatechin	1	-	-
4'-*O*-Methyl Epigallocatechin	0.6	230	-
Epicatechin Gallate	0.1	-	-
Epigallocatechin Gallate	0.1	-	-
Gallic Acid	40	110	-
3-*O*-Methyl Gallic Acid	2	10	10
Quercetin	20	60	30
Kaempferol	110	-	-
M6	3	30	9
Syringic Acid	20	40	-
3-Hydroxybenzoic Acid	5	30	-
Benzoic Acid	50	590	-
Hippuric Acid	0.4	40	-

LOQ were performed in technical duplicate.

6.4.2. Reproducibility of Samples during Preparation

All analyses of plasma samples were performed in technical duplicates to assess experimental error. Before centrifugal evaporation, the acetonitrile fractions from the plasma samples were placed in pre-weighed eppendorf tubes. The aqueous fraction remaining after centrifugal evaporation was calculated by re-weighing the eppendorf tube. On average, the remaining aqueous fraction of the plasma samples was 68+/-68 µL. To account for this variation, samples were reconstituted to 100 µL with Millipore water (or a dilution factor was applied for samples above 100 µL) and 10 µL injections onto the LC-MS were performed in triplicate.

During the recovery experiments it was noted that a repeat injection of taxifolin from certain plasma samples resulted in a wide variability (CV = 1.1 for one sample), whereas for other plasma samples the variability was small (CV = 0.1 for another volunteer). The variability initially indicated that the plasma samples were not homogeneous, but as this occurrence only happened for taxifolin (internal standard), and no other compounds during the development stage, this theory was disregarded. It is possible that the taxifolin in some samples was being suppressed by a component present within the plasma of some volunteers. Ethyl gallate was not observed to be suppressed in plasma samples analysed for recovery, and therefore was used as the internal standard for all biological samples for the bioavailability study. The CV for the recovery of spiked catechins and conjugates from plasma samples ranged from 0.01 to 0.1, implying triplicate injections of the plasma samples processed in duplicate resulted in good reproducibility.

A pilot study using blister fluid was performed by a co-worker (TD). This method was published (Rhodes *et al*., 2013) and has been adapted here for the bioavailability study (3 month human intervention study, daily consumption of high dose green tea supplements; equivalent of 5 cups of green tea). On average, the total volume of blister fluid for volunteers was 101 +/- 44 µL. The minimum volume

used for analysis was 40 µL and technical duplicates were performed, with duplicate injections onto the LC-MS. The CV for the recovery of spiked catechins and conjugates from blank blister fluid ranged between 0.03 and 0.2, suggesting duplicate injections of duplicate sample processing allows for good reproducibility.

Optimisation of the biopsy protocol was difficult as only a few blank (pre-green tea supplement consumption) biopsies were available for analysis. As there was only one blank biopsy per volunteer (samples remaining from the pilot study) direct comparison of two methods using one biological sample could not occur. The CV for the recovery of spiked catechins and conjugates from blank skin biopsies ranged from 0.1 to 0.2, suggesting duplicate injections of the skin biopsy sample post homogenisation and processing resulted in good reproducibility.

Although the reproducibility was good, only a small number of free-form catechins and metabolites were identified in pilot study biopsies post green tea supplementation, processed during the optimisation stage with the combination method. This was possibly because the concentrations of the catechins were too small for detection by LC-MS. After centrifugal evaporation there was no aqueous fraction remaining for the blister samples, however 134+/-19 µL remained after centrifugal evaporation of the processed biopsy samples, potentially due to the addition of the antioxidant, sodium dithionite. The time that the biopsy samples were subjected to centrifugal evaporation was increased, but the aqueous fraction remaining was not reduced. Centrifugal evaporation was performed at room temperature as it was clear that the addition of heat during centrifugal evaporation (40 ºC) reduced the recovery of free-form catechins and metabolites in urine, and although it is possible that heat would remove the aqueous fraction, this was not examined. The aqueous fraction remaining after the processing of the skin biopsies was also subjected to freeze drying, however the aqueous fraction was not significantly reduced and therefore freeze drying was not employed.

6.4.3. Bioavailability of Green Tea Catechin Metabolites Present in Plasma after Daily Supplementation for Three Months

As green tea catechin conjugate metabolites (sulphate and glucuronide) are not commercially available, the biological samples were analysed using peak area, relative to ethyl gallate (internal standard), so as not to influence the results by over or underestimation of conjugates present as each compound has a specific response factor for LC-MS analysis (Farrell *et al*., 2011).

After three months daily supplementation with a high dose of green tea (equivalent to 5 cups), there were 26 green tea catechin metabolites in plasma three hour post-supplementation, compared with 19 metabolites after the first ingestion of the supplement on day 1 (Table 6-3). The additional metabolites after 3 months supplementation were mainly valerolactones (M4-*O*-sulphate, M4-*O*-glucuronide and M6/M6'-*O*-glucuronide), which were present due to ongoing metabolism of the supplement ingested on the previous day (valerolactones present in urine 48 hours after supplement ingestion; Calani *et al*., 2012). After three months daily supplementation, the highest peak area corresponded to M6/M6'-*O*-sulphate, present in all 11 bioavailability volunteers, and the highest peaks corresponding to conjugated forms of M4, EC and EGC were *O*-methyl-M4 (9 volunteers), EC-*O*-sulphate and *O*-methyl-EC-*O*-sulphate, and *O*-methyl-EGC-*O*-sulphate (all 11 volunteers; Table 6-3).

This is the first time that quercetin and kaempferol conjugates have been identified in plasma following ingestion of green tea in humans; most human intervention studies utilise enzyme deconjugation with β-glucuronidase and sulfatase. The peaks corresponding to quercetin-*O*-sulphate (in 3 volunteers) and kaempferol-*O*-glucuronide (1 volunteer only), were very small, but were all above a signal to noise ratio of 10 (LOQ). This is also the first time that conjugates of particular microbial metabolites have been reported in plasma (syringic acid-*O*-sulphate, 3-hydroxybenzoic acid-*O*-sulphate and benzoic acid-*O*-sulphate).

Hippuric acid was also present in all 11 volunteers, with a large corresponding peak area. The results here illustrate that after 3 months daily supplementation of green tea, there is an increase in green tea catechin metabolites identified in plasma samples three hours post-supplement consumption, resulting from microbial metabolites derived from the previous days' supplementation.

Table 6-3. Average peak area (relative to ethyl gallate) of green tea catechin metabolites present in plasma 3 hours post ingestion of a green tea supplement, after 3 months supplementation.

	Peak Area Ratio (x 10^{-2})	
Compound	3 h, Day 1	3 h, 3 Months
Epicatechin-*O*-Sulphate	0.14+/-0.02	0.23+/-0.04*
Epicatechin-*O*-Glucuronide	0.04+/-0.01	0.05+/-0.01
O-Methyl-Epicatechin-*O*-Sulphate	0.13+/-0.02	0.21+/-0.04
Epigallocatechin	0.01	0.0122+/-0.0006
Epigallocatechin-*O*-Sulphate	0.04+/-0.01	0.033+/-0.007
Epigallocatechin-*O*-Glucuronide	0.059+/-0.007	0.08+/-0.02
O-Methyl-Epigallocatechin-*O*-Sulphate	0.10+/-0.02	0.12+/-0.02
O-Methyl-Epigallocatechin-*O*-Glucuronide	0.018+/-0.005	0.019+/-0.004
Epicatechin Gallate	0.006	0.018+/-0.003
Epicatechin Gallate-*O*-Sulphate		0.03
Epigallocatechin Gallate	0.016+/-0.003	0.04+/-0.01
Gallic Acid-*O*-Sulphate		0.04+/-0.01
O-Methyl-Gallic Acid-*O*-Sulphate	0.40+/-0.07	0.50+/-0.09
Quercetin-*O*-Sulphate		0.025+/-0.004
Kaempferol-*O*-Glucuronide		0.03
M4-*O*-Sulphate		0.10+/-0.02
M4-*O*-Glucuronide		0.020+/-0.007
O-Methyl-M4-*O*-Sulphate	0.01	0.38+/-0.08
M6/M6'-*O*-Sulphate	0.10+/-0.04	0.6+/-0.2*
M6/M6'-*O*-Glucuronide		0.05+/-0.02
O-Methyl-M6/M6'-*O*-Sulphate	0.04+/-0.02	0.056+/-0.004
Benzoic Acid-*O*-Sulphate	0.19+/-0.06	0.14+/-0.03
3-Hydroxybenzoic Acid	0.04+/-0.01	0.09+/-0.03
3-Hydroxybenzoic Acid-*O*-Sulphate	0.01	0.06+/-0.03
Syringic Acid-*O*-Sulphate	0.07+/-0.06	0.03+/-0.01
Hippuric Acid	0.4+/-0.1	0.46+/-0.06

Number of volunteers each conjugate was present in was also recorded; total number of volunteers was 11. Samples were analysed in biological duplicate and technical triplicate (Student's t-test; one tailed, paired *$p<0.05$; average +/- S.E.).

6.4.4. Bioavailability of Green Tea Catechin Metabolites in Blister Fluid

Of the 55 metabolites investigated, 20 metabolites were identified in blister fluid. Compounds identified in blister fluid were analysed as peak area relative to ethyl gallate, as standards for green tea catechin metabolite conjugates were not commercially available (Table 6-4). As can be seen in Table 6-4, the majority of metabolites were identified post-green tea supplementation; 3 month samples. EC-*O*-sulphate and *O*-methyl-EC-*O*-sulphate were the most prominent conjugated forms of EC, and for EGC this was *O*-methyl-EGC-*O*-sulphate.

Table 6-4. Number of volunteers each green tea catechin metabolite was detected in blister fluid; pre- and post- supplementation and also with and without UV irradiation.

	Compounds Identified in Blister Fluid Samples (Number of Volunteers)			
Compound	Pre, No UV	Pre, UV	Post, No UV	Post UV
Epicatechin-*O*-Sulphate			10	8
Epicatechin-*O*-Glucuronide				3
O-Methyl-Epicatechin-*O*-Sulphate			8	10
Epigallocatechin-*O*-Glucuronide			2	2
O-Methyl-Epigallocatechin-*O*-Sulphate			6	7
O-Methyl-Epigallocatechin-*O*-Glucuronide			1	1
Epigallocatechin Gallate	1	1	1	
Gallic Acid-*O*-Glucuronide	2	1		
O-Methyl-Gallic Acid-*O*-Sulphate	11	11	11	11
M4-*O*-Sulphate			4	4
M4-*O*-Glucuronide			2	1
O-Methyl-M4-*O*-Sulphate			6	7
M6/M6'-*O*-Sulphate	3	2	10	9
M6/M6'-*O*-Glucuronide			4	4
Hippuric Acid	11	11	11	11
Benzoic Acid	1	1		
Benzoic Acid-*O*-Sulphate			1	3
Syringic Acid-*O*-Sulphate		1		
Syringic Acid-*O*-Glucuronide		1		
3-Hydroxybenzoic Acid-*O*-Sulphate			1	3

For the valerolactones, M6/M6'-*O*-sulphate and *O*-methyl-M4-*O*-sulphate had the highest corresponding peak areas in blister fluid after 3 months supplementation with green tea (Table 6-5). When two green tea catechin

metabolites were assessed relative to free-form standards spiked into pre-supplement (blank) blister fluid, they were detected as pmole/total blister volume (µL, which could be converted to µM). These were EC-O-sulphate (7+/-4 µM) and EGC-O-glucuronide (5+/-4 µM).

Table 6-5. Green tea catechin metabolites in blister fluid measured as peak area, relative to internal standard (ethyl gallate), in total blister fluid extracted.

	Compound in Blister Fluid/Total µL Collected (Ratio to IS, $x10^{-6}$)			
Compound	Pre, No UV	Pre, UV	Post, No UV	Post, UV
Epicatechin-*O*-Sulphate			5+/-3	9+/-5
Epicatechin-*O*-Glucuronide				3+/-2
O-Methyl-Epicatechin-*O*-Sulphate			4+/-2	5+/-3
Epigallocatechin-*O*-Glucuronide			2+/-2	3.3+/-0.2
O-Methyl-Epigallocatechin-*O*-Sulphate			3+/-2	2+/-2
O-Methyl-Epigallocatechin-*O*-Glucuronide			1	2
Epigallocatechin Gallate	3	2	2	
Gallic Acid-*O*-Glucuronide	1+/-1	12		
O-Methyl-Gallic Acid-*O*-Sulphate	57+/-37	90+/-73	46+/-28	55+/-38
M4-*O*-Sulphate			2+/-1	5+/-3
M4-*O*-Glucuronide			0.7+/-0.4	4
O-Methyl-M4-*O*-Sulphate			9+/-6	14+/-8
M6/M6'-*O*-Sulphate	7+/-4	15+/-16	46+/-47	78+/-88
M6/M6'-*O*-Glucuronide			3+/-3	6+/-5
Hippuric Acid	31+/-35	67+/-72	21+/-14	33+/-30
Benzoic Acid	1	2		
Benzoic Acid-*O*-Sulphate			2	4+/-4
Syringic Acid-*O*-Sulphate		2		
Syringic Acid-*O*-Glucuronide		2		
3-Hydroxybenzoic Acid-*O*-Sulphate			8	8+/-6

Samples were analysed pre and post supplementation and with or without UV irradiation. Samples were analysed in biological duplicate and technical duplicate; average+/-S.E.

6.4.5. Bioavailability of Green Tea Catechin Metabolites in Biopsy Samples

The biopsy samples were much more difficult to handle than the other biological samples from the bioavailability study as they were not liquid and had to be homogenised. Ethical approval states that volunteers can only provide four biopsy samples for skin studies (Montastler *et al*., 2010), and so as there were no extra samples for method development, the remaining pilot study samples were

used. However, there were not many samples remaining, and so therefore optimisation by analysis of green tea catechin recoveries was limited. It was also limited because there were no identical post-supplementation samples to compare different homogenisation methods.

Many challenges were faced when trying to homogenise the skin tissue and isolate green tea catechin metabolites. A number of techniques were evaluated, including crushing the biopsy with a pestle and mortar whilst in liquid nitrogen, homogenising with a sheer disperser, using a dounce homogeniser on ice and "bombing" the sample. The techniques utilised were sheer dispersion and bombing, as the pestle and mortar technique and use of a dounce homogeniser did not result in a homogenised sample. Bombing involves freezing the biopsy in liquid nitrogen and subjecting the frozen biopsy to force from a hammer whilst the biopsy was placed between a stainless steel rod and base that were also in liquid nitrogen (Figure 2-3). The biopsy was first homogenised by bombing as this reduced the size of the biopsy (formation of flakes), followed by further homogenisation using sheer dispersion on dry ice (with an UltraTurrax).

M6, a metabolite of EC consumption, was present in each sample from one volunteer, suggesting the consumption of chocolate, black tea or wine, as M6 was present in baseline samples (pre-green tea consumption) as well as post-supplementation. Apart from M6, all other metabolites were identified post-supplementation (Tables 6-6 and 6-7). As the values were higher in the pre-supplement samples than the post-supplement samples, this would imply that the M6 present in these samples is not from green tea consumption. For this particular volunteer, M6/M6'-*O*-sulphate was present in blister fluid with peak area ratios (to ethyl gallate) of 3×10^{-6} (pre-supplement, no UV) and 11×10^{-6} (pre-supplement, UV), and 89×10^{-6} (post-supplement, no UV) and 82×10^{-6} (post-supplement, UV), and M6/M6'-*O*-glucuronide was present post-supplement with UV irradiation only, with a peak area ratio of 3×10^{-6}. In the plasma sample of

this volunteer, M6/M6'-*O*-glucuronide had a peak area ratio of 4×10^{-2}, and M6/M6'-*O*-sulphate had a peak area ratio of 87×10^{-2}. Enzymes within the skin may possibly have deconjugated M6/M6'-*O*-sulphate and M6/M6'-*O*-glucuronide to M6.

Table 6-6. Number of volunteers each green tea catechin metabolite was detected in biopsy samples; pre- and post- supplementation and also with and without UV irradiation.

	Compounds Identified in Biopsy Samples (Number)			
Compound	Pre, No UV	Pre, UV	Post, No UV	Post UV
O-Methyl-Epicatechin-*O*-Sulphate			1	
Gallic Acid-*O*-Glucuronide			1	1
O-Methyl-Gallic Acid-*O*-Sulphate				1
O-Methyl-Gallic Acid-*O*-Glucuronide				1
Quercetin			1	
M6	1	1	1	1
M6/M6'-*O*-Sulphate			2	3

Table 6-7. Green tea catechin metabolites in biopsies measured as peak area, relative to internal standard (ethyl gallate), standardised to the initial weight of the biopsy.

	Average Ratio ($x10^{-6}$)/mg Biopsy			
Compound	Pre, No UV	Pre, UV	Post, No UV	Post, UV
O-Methyl-Epicatechin-*O*-Sulphate			2	
Gallic Acid-*O*-Glucuronide			0.8	0.9
O-Methyl-Gallic Acid-*O*-Sulphate				0.6
O-Methyl-Gallic Acid-*O*-Glucuronide				1.4
Quercetin			1	
M6	7	10	6	2
M6/M6'-*O*-Sulphate			0.9+/-0.5	0.8+/-0.2

Samples were analysed pre- and post- supplementation and with or without UV irradiation. Samples were analysed in technical duplicate; average+/-S.E.

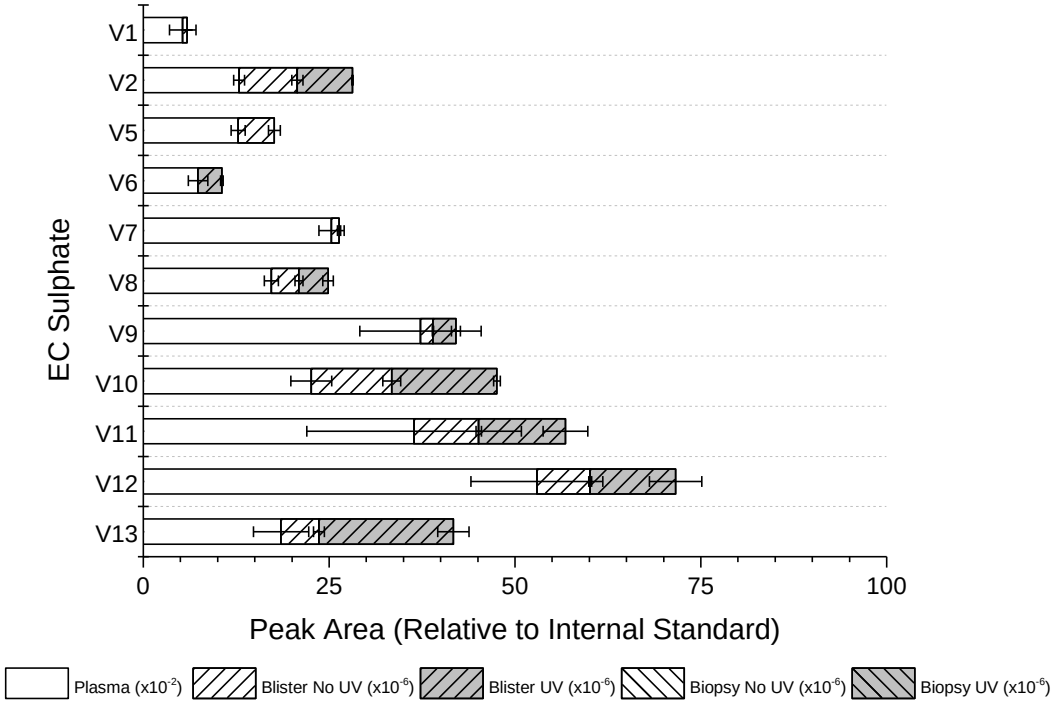
Assessment of green tea catechin metabolites relative to free-form standards highlighted that the majority of metabolites were detected as pmole/mg biopsy (data not shown). However, as mentioned, it is possible that over-quantification due to differences in LC-MS response factors (Farrell *et al*., 2011) could occur, and therefore samples were assessed as peak areas relative to

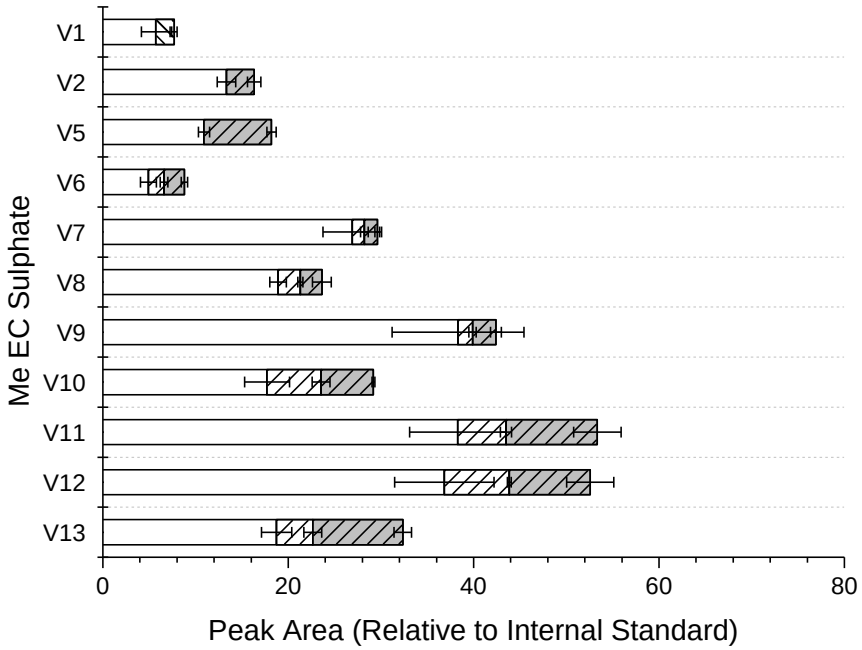
ethyl gallate. It is possible that more metabolites were present within the biopsy tissue, but the samples were too diluted for detection of other metabolites (Table 6-7), as the average aqueous fraction remaining after centrifugal evaporation was 134+/-19 µL.

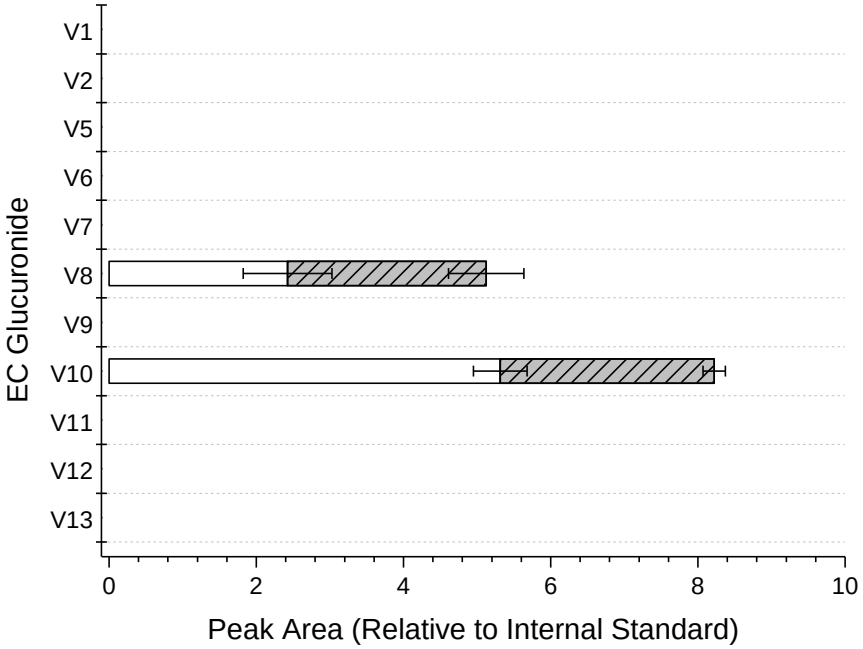
6.4.6. Relationship between Green Tea Catechin Metabolites Present in Bioavailability Samples

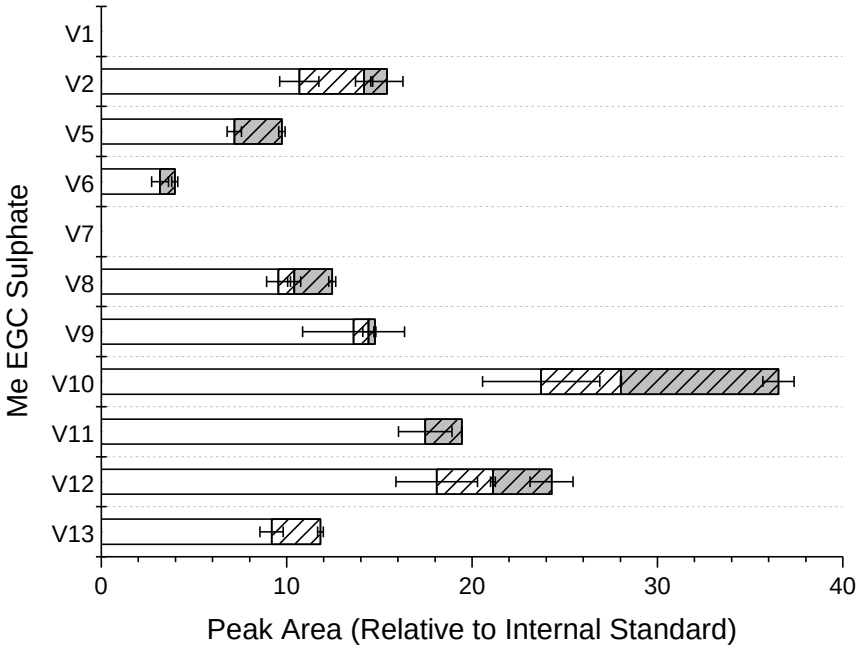
The purpose of the bioavailability study was to investigate the localisation of the green tea catechin metabolites in biological samples; chiefly the identification of green tea catechin metabolites in skin samples. Blister interstitial fluid surrounds skin cells, and metabolites present are derived from plasma. Therefore, plasma and blister fluid can be directly compared, and as blister fluid is the extracellular fluid surrounding skin, it is feasible that green tea catechin metabolites present within biopsy samples can be related to interstitial fluid, and consequently plasma samples.

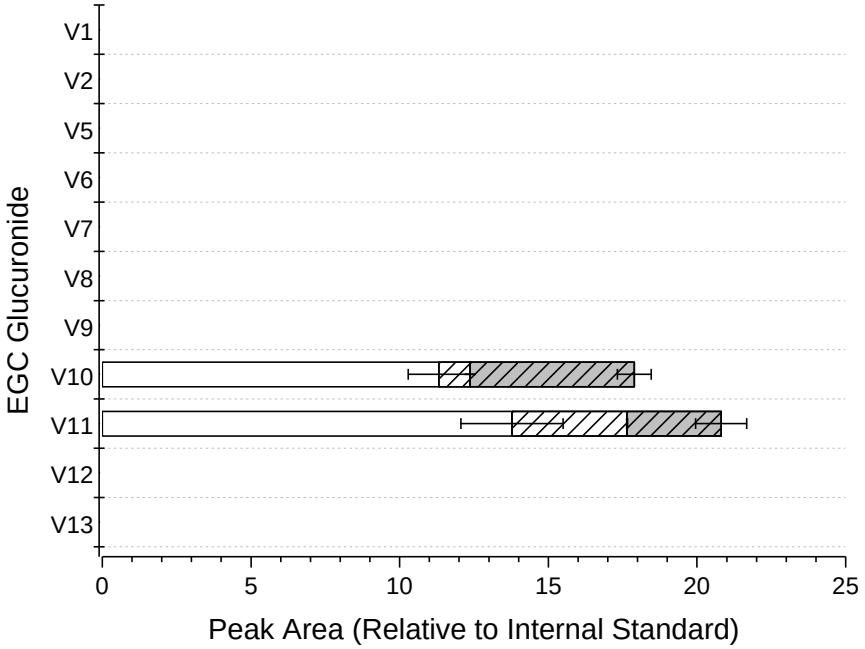
In total, there were 15 green tea catechin metabolites that were present in at least two biological samples (plasma, blister fluid and biopsy; Figure 6-1). The responses of metabolites in plasma by LC-MS analysis were much higher than the interstitial blister and skin biopsy samples, and therefore the ratios were analysed as 10^{-6} for blister and biopsy samples, and 10^{-2} for plasma samples. The 15 metabolites identified in at least two of the three sample types were EC-*O*-sulphate, *O*-methyl-EC-*O*-sulphate, EGC-*O*-glucuronide, *O*-methyl-EGC-*O*-sulphate, EGCG, *O*-methyl-gallic acid-*O*-sulphate, M4-*O*-sulphate, M4-*O*-glucuronide, *O*-methyl-M4-*O*-sulphate, M6/M6'-*O*-sulphate, M6/M6'-*O*-glucuronide, benzoic acid-*O*-sulphate, 3-hydroxybenzoic acid-*O*-sulphate and hippuric acid.

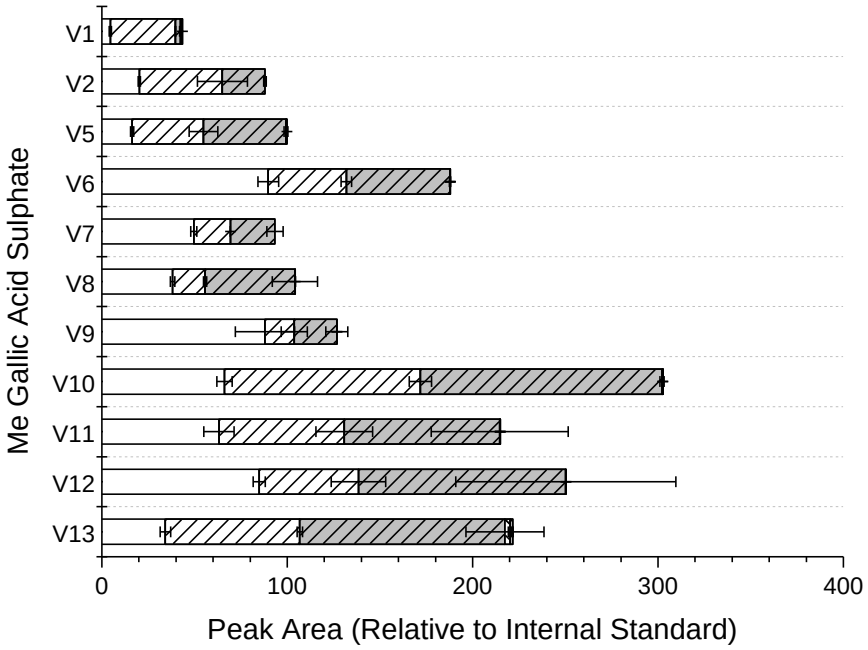

EC Sulphate
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
25
50
75
100
Peak Area (Relative to Internal Standard)
Plasma (x10-2)
Blister No UV (x10-6)
Blister UV (x10-6)
Biopsy No UV (x10-6)
Biopsy UV (x10-6)

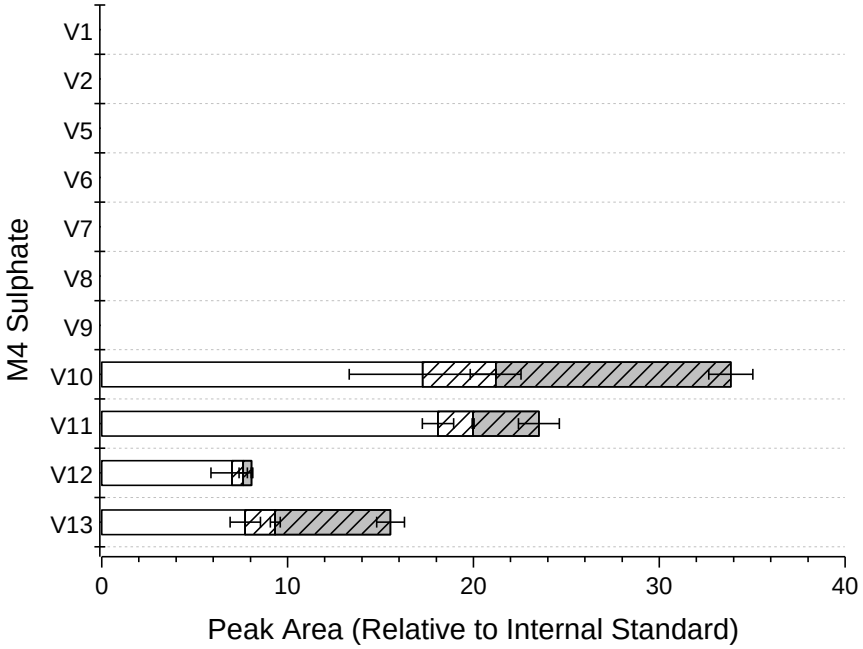

Me EC Sulphate
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
20
40
60
80
Peak Area (Relative to Internal Standard)

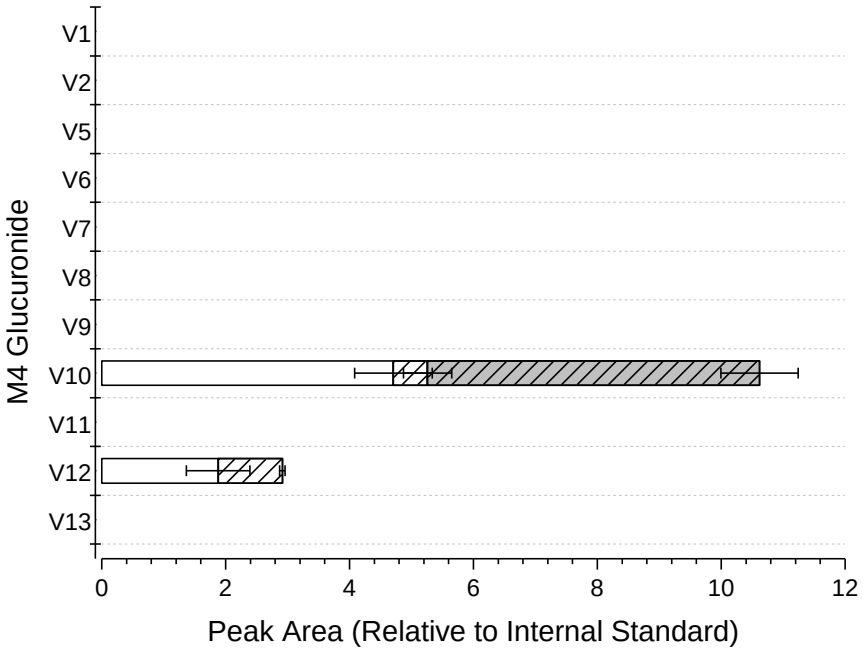

EC Glucuronide
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
2
4
6
8
10
Peak Area (Relative to Internal Standard)

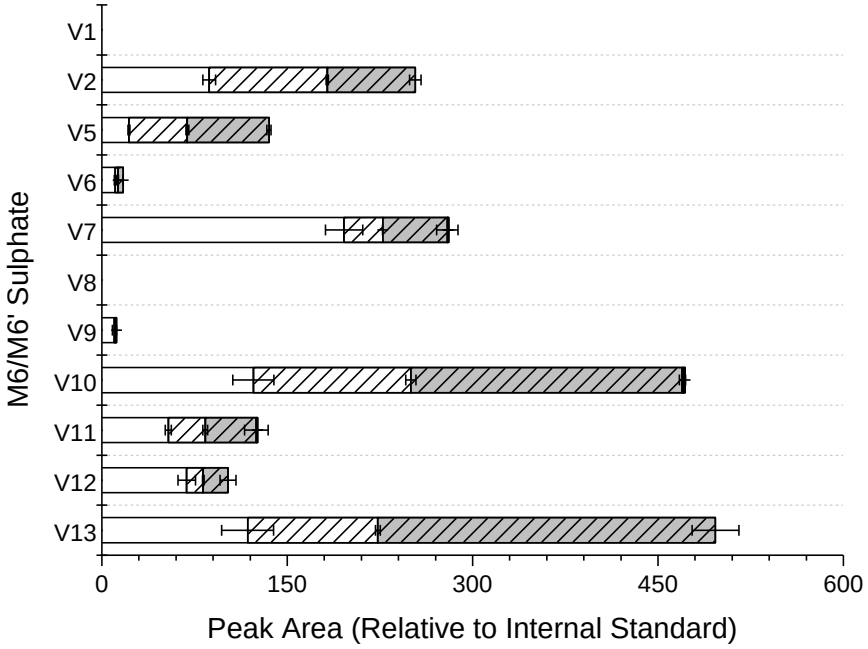

Me EGC Sulphate
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
10
20
30
40
Peak Area (Relative to Internal Standard)

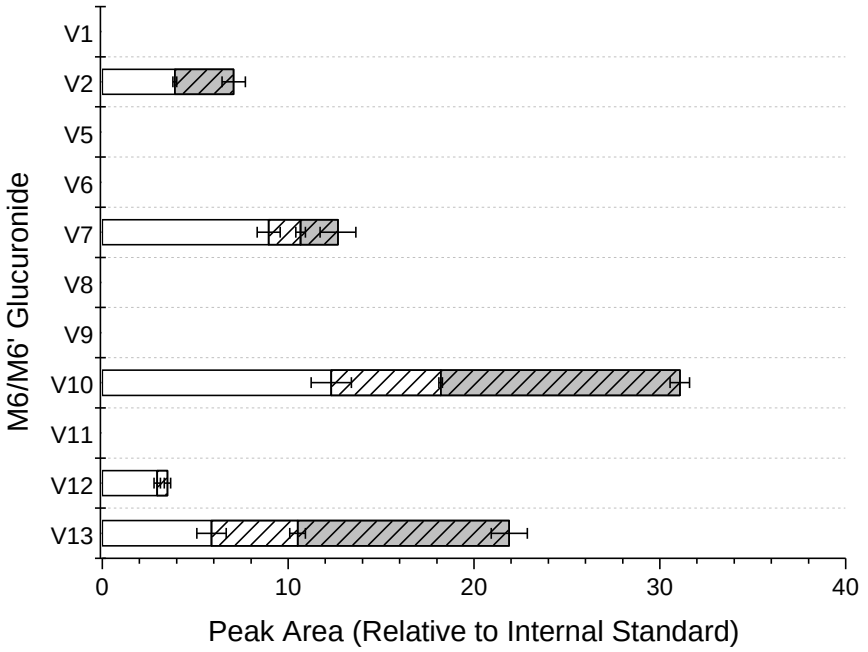

EGC Glucuronide
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
5
10
15
20
25
Peak Area (Relative to Internal Standard)


Me Gallic Acid Sulphate
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
100
200
300
400
Peak Area (Relative to Internal Standard)


M4 Sulphate
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
10
20
30
40
Peak Area (Relative to Internal Standard)


M4 Glucuronide
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
2
4
6
8
10
12
Peak Area (Relative to Internal Standard)


M6/M6' Sulphate
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
150
300
450
600
Peak Area (Relative to Internal Standard)


M6/M6' Glucuronide
V1
V2
V5
V6
V7
V8
V9
V10
V11
V12
V13
0
10
20
30
40
Peak Area (Relative to Internal Standard)

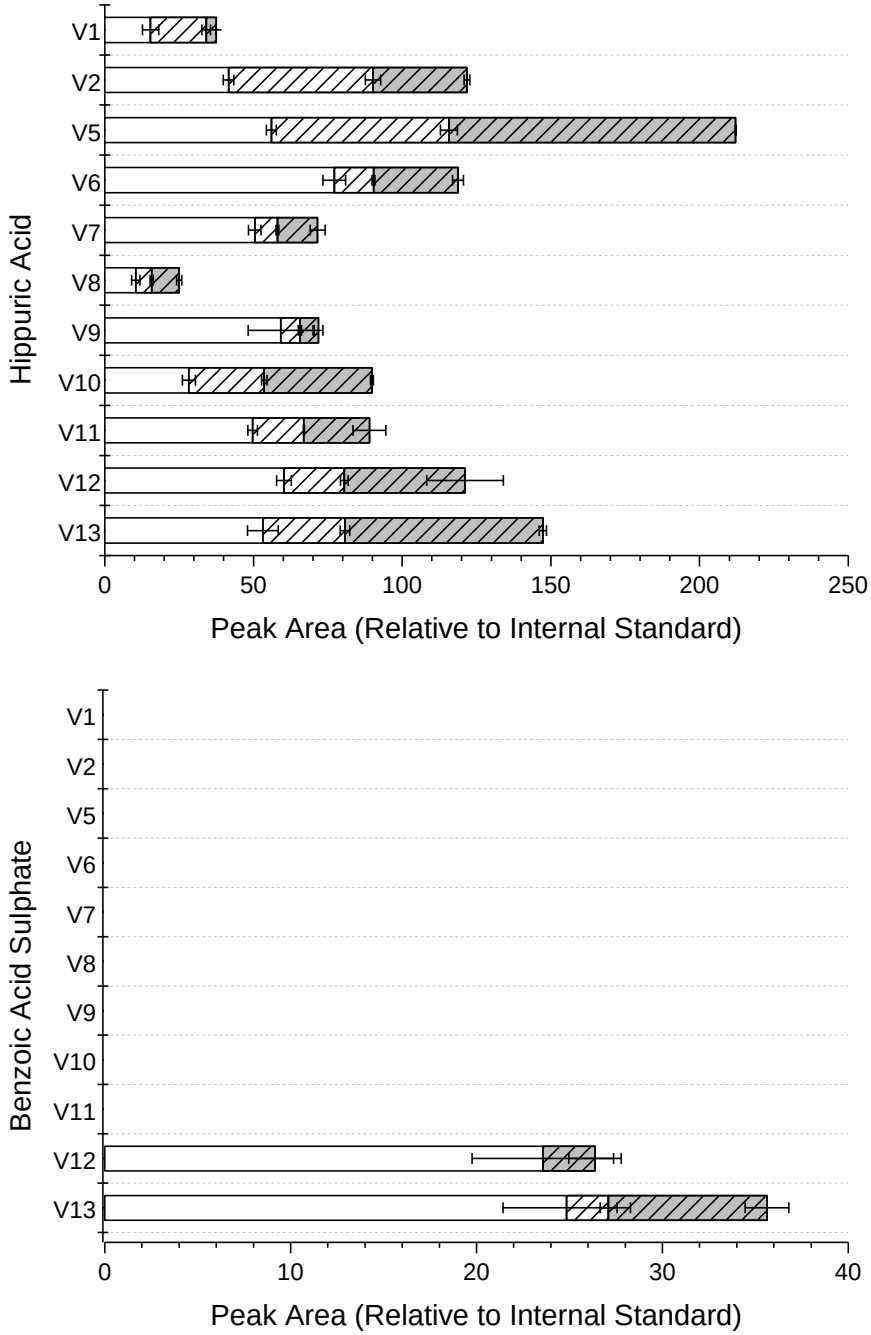


Figure 6-1. Identification of green tea catechin metabolites for individual volunteers, in plasma, blister fluid and biopsy samples post-supplementation, with or without UV irradiation. As peak areas in plasma were larger, plasma was analysed as peak area $x10^{-2}$; blister and biopsy analysis was peak area $x10^{-6}$. Metabolites identified in volunteers for more than one biological sample were; EC-*O*-sulphate, *O*-methyl-EC-*O*-sulphate, EC-*O*-glucuronide, *O*-methyl-EGC-*O*-sulphate, EGC-*O*-glucuronide, *O*-methyl-gallic acid-*O*-sulphate, M4-*O*-sulphate, *O*-methyl-M4-*O*-sulphate, M4-*O*-glucuronide, M6/M6'-*O*-sulphate, M6/M6'-*O*-glucuronide, hippuric acid and benzoic acid-*O*-sulphate. EGCG and 3-hydroxybenzoic acid sulphate were also identified in more than one biological sample for one volunteer only.

The majority of green tea catechin metabolites identified in more than one bioavailability sample occurred between plasma and blister fluid only (urine was collected for 24 hours after 3 months supplementation, not only after 3 h consumption and was therefore excluded from direct analysis). As mentioned before, this could be due to the biopsy samples being too diluted, or because the metabolites do not enter the skin, so any potential biological function the metabolites have is from the interstitial fluid surrounding the skin. There was a large range between the number of comparable metabolites for each volunteer suggesting a large inter-individual variation (Figure 6-1). Only 2 metabolites were identified in all three biological samples; plasma, blister fluid and biopsy samples. These were *O*-methyl-gallic acid-*O*-sulphate (6 volunteers) and M6/M6'-*O*-sulphate (3 volunteers).

6.4.7. Plasma Pharmacokinetics of Green Tea Catechin Metabolites

Plasma was collected from volunteers pre- and post- supplementation on day 1 of the human intervention. Volunteers were following their normal diet, therefore the baseline (pre-supplementation) samples identified green tea catechin metabolites that were normally present within the volunteers' daily diet. Plasma was collected at baseline (pre-supplement, time 0h) and then 0.5, 1, 1.5, 2, 3, 4 and 6 hours.

The maximum concentration (C_{max}) was assessed as both concentration and peak area (Table 6-8). As mentioned previously, when assessing green tea catechin metabolites relevant to available free-form standards, response factors can result in overestimation. For example, when normalising data to a 50 mg dose of EGCG consumed within the green tea supplement, *O*-methyl-EC-*O*-sulphate is identified at a concentration of 1.2 µM. This is almost 5 times higher than concentrations measured in other green tea intervention studies (Williamson *et al*., 2011b).

Therefore the pharmacokinetic plasma samples were analysed as peak area, relevant to the internal standard.

Table 6-8. T_{max} and C_{max} for green tea catechin metabolites identified in plasma after consumption of green tea supplementation on day 1.

Compound	T_{max} (h)	C_{max} (µM)	C_{max} (Peak Area Ratio to IS)
Epicatechin-*O*-Sulphate	4+/-1	9+/-3	0.2+/-0.1
Epicatechin-*O*-Glucuronide	3.1+/-0.9	2+-/1	0.04+-/0.03
O-Methyl-Epicatechin-*O*-Sulphate	3+/-1	13+/-5	0.2+/-0.1
Epigallocatechin	3+/-2	1+/-2	0.1+/-0.1
Epigallocatechin-*O*-Sulphate	3.4+/-0.9	0.7+/-0.3	0.04+/-0.02
Epigallocatechin-*O*-Glucuronide	3+/-1	1.9+/-0.9	0.09+/-0.05
O-Methyl-Epigallocatechin-*O*-Sulphate	4+/-1	3+/-2	0.1+/-0.1
O-Methyl-Epigallocatechin-*O*-Glucuronide	3+/-2	0.9+/-0.5	0.03+/-0.02

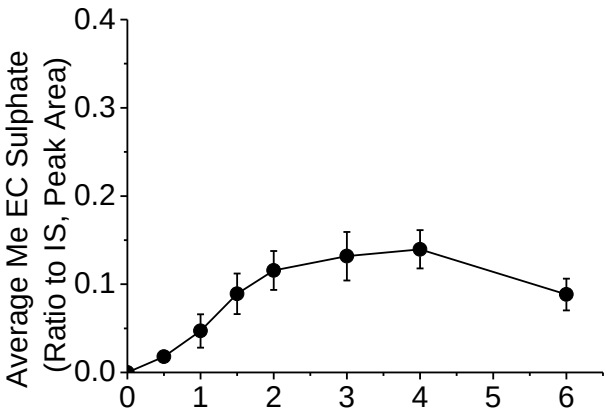
Samples were analysed from baseline to 6 hours post-supplementation. Maximum concentration was assessed as both µM and peak area relevant to internal standard (ethyl gallate), to highlight elevated responses to green tea consumption in plasma in comparison to the literature; average+/-S.E.

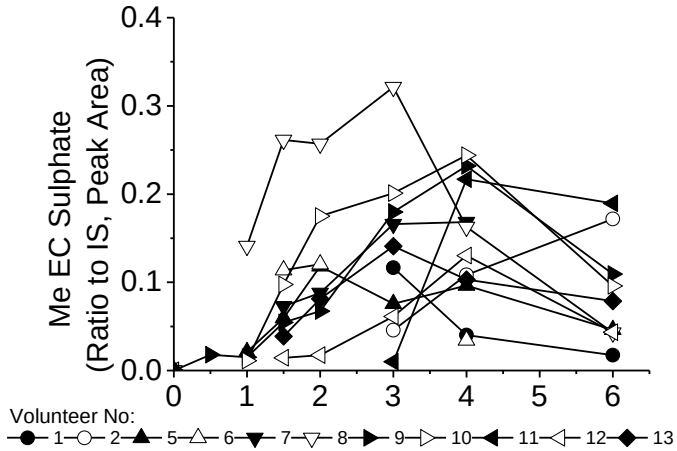
The data for the 11 volunteers was collated and presented in two graphs for each green tea catechin metabolite identified (Figure 6-2). The first graph displays the average appearance of the compound post-green tea consumption over the 6 hour time course, whereas the second graph is the data for the individual volunteers (highlighting variability between individuals).

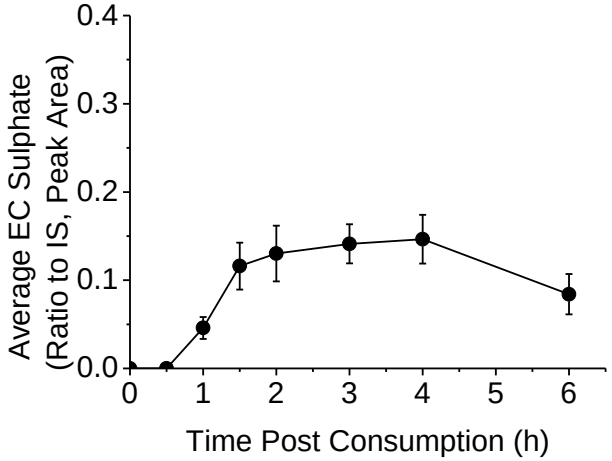
The pharmacokinetics of the catechins is similar to reports in the literature. In the baseline sample *O*-methyl-EC-*O*-sulphate, EC-*O*-sulphate, EGC-*O*-glucuronide, *O*-methyl-EGC-*O*-sulphate and EC-*O*-glucuronide were not present. The green tea catechin metabolites were detected after 1 hour, except for EC-*O*-glucuronide which was first detected 1.5 hours post supplement consumption (Figure 6-2).

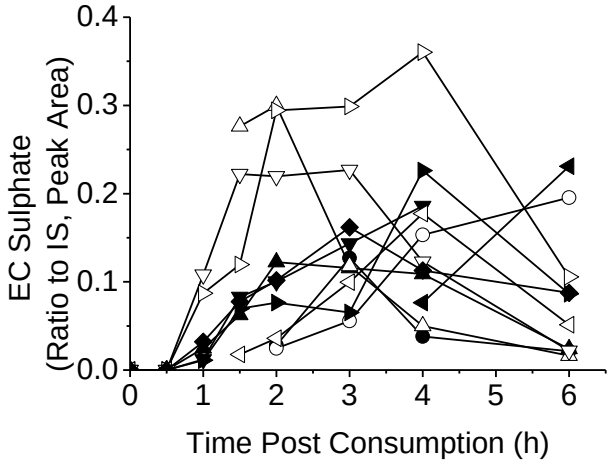
Five green tea catechin metabolites were detected in baseline samples, 3 of which are known endogenous metabolites; hippuric acid, benzoic acid-*O*-sulphate

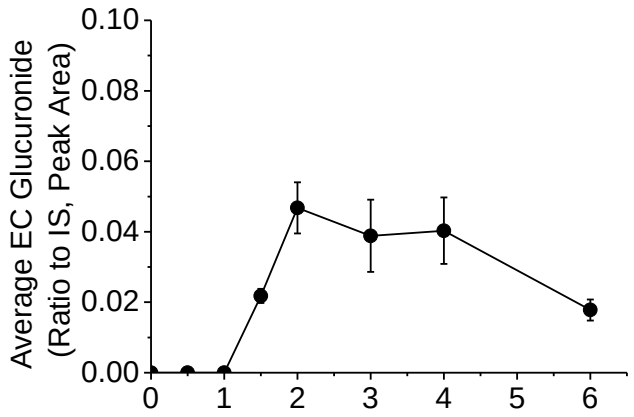
and 3-hydroxybenzoic acid. These metabolites have also been reported post-consumption of black tea, coffee and dark chocolate. *O*-Methyl-gallic acid-*O*-sulphate and M6/M6'-*O*-sulphate were also detected, possibly from metabolism of dietary consumption from the previous day. Gallic acid is in ale, vinegar, black tea, apple juice, blackberries and red wine, and M6, derived from epicatechin, has been identified in plasma samples after consumption of dark chocolate (Phenol Explorer, 2013).

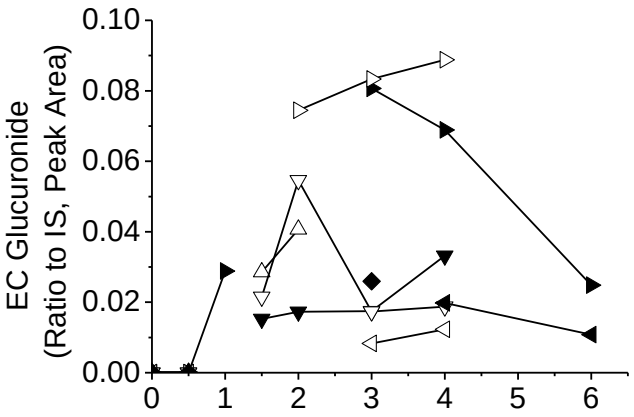

Average Me EC Sulphate
(Ratio to IS, Peak Area)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6

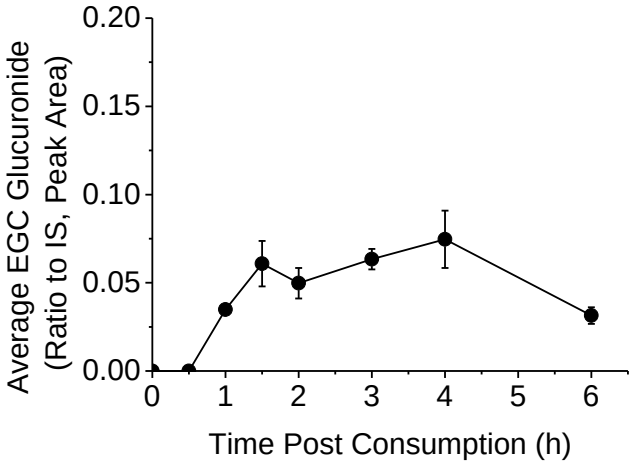

Me EC Sulphate
(Ratio to IS, Peak Area)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6
Volunteer No:
1
2
5
6
7
8
9
10
11
12
13


Average EC Sulphate
(Ratio to IS, Peak Area)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6
Time Post Consumption (h)


EC Sulphate
(Ratio to IS, Peak Area)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6
Time Post Consumption (h)


Average EC Glucuronide
(Ratio to IS, Peak Area)
0.10
0.08
0.06
0.04
0.02
0.00
0
1
2
3
4
5
6


EC Glucuronide
(Ratio to IS, Peak Area)
0.10
0.08
0.06
0.04
0.02
0.00
0
1
2
3
4
5
6


Average EGC Glucuronide
(Ratio to IS, Peak Area)
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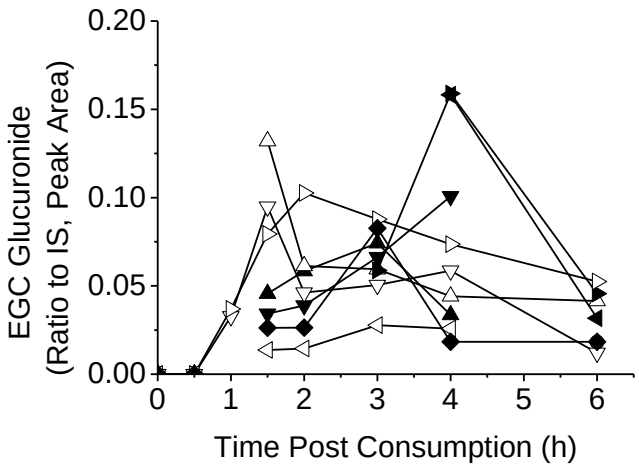
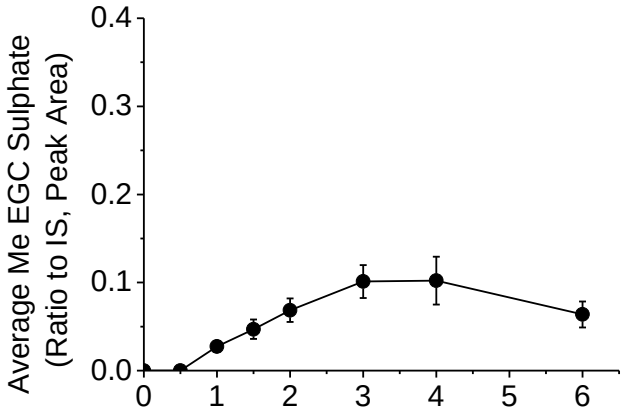
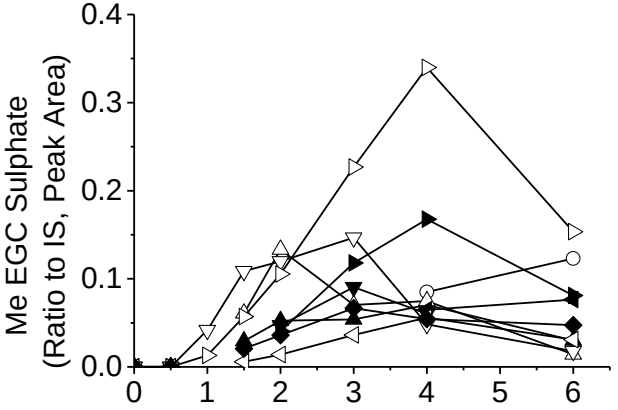
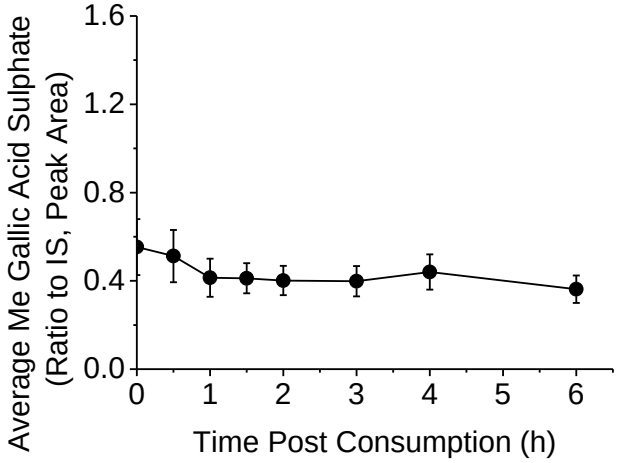
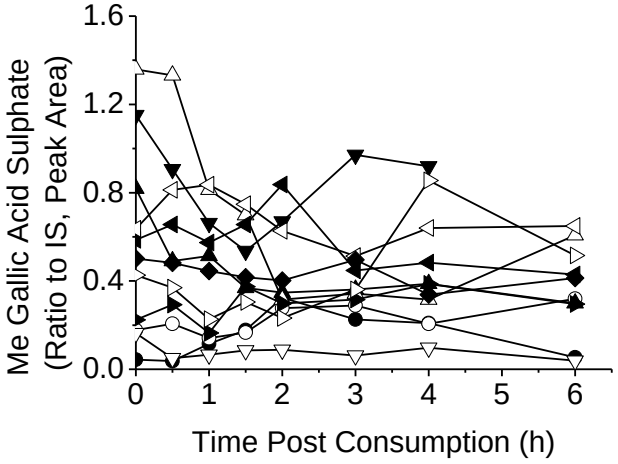
0.15
0.10
0.05
0.00
0
1
2
3
4
5
6
Time Post Consumption (h)

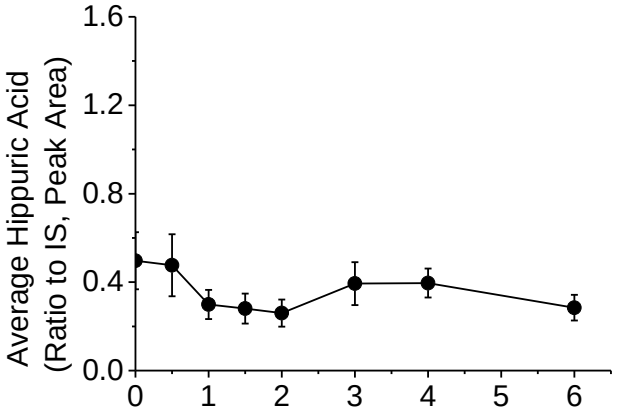

EGC Glucuronide
(Ratio to IS, Peak Area)
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
6
Time Post Consumption (h)

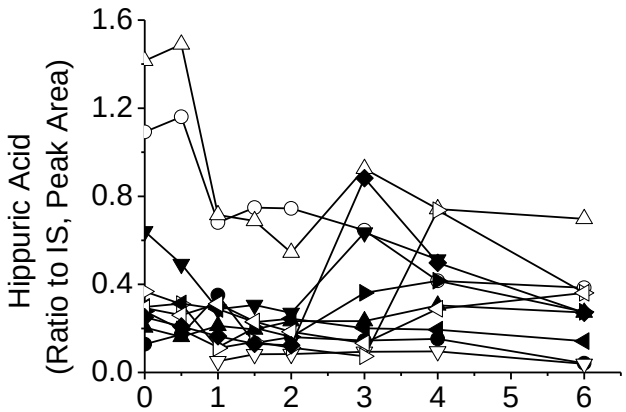

Average Me EGC Sulphate
(Ratio to IS, Peak Area)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6

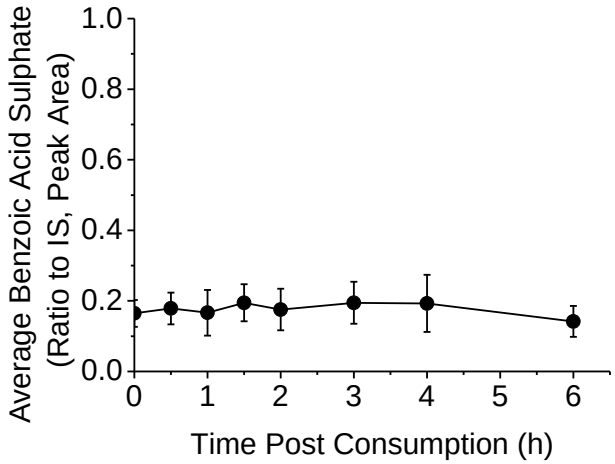

Me EGC Sulphate
(Ratio to IS, Peak Area)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6

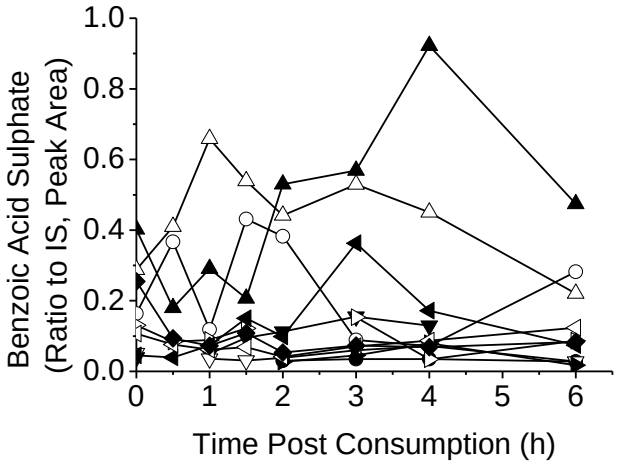

Average Me Gallic Acid Sulphate
(Ratio to IS, Peak Area)
0.0
0.4
0.8
1.2
1.6
0
1
2
3
4
5
6
Time Post Consumption (h)


Me Gallic Acid Sulphate
(Ratio to IS, Peak Area)
0.0
0.4
0.8
1.2
1.6
0
1
2
3
4
5
6
Time Post Consumption (h)


Average Hippuric Acid
(Ratio to IS, Peak Area)
1.6
1.2
0.8
0.4
0.0
0
1
2
3
4
5
6


Hippuric Acid
(Ratio to IS, Peak Area)
1.6
1.2
0.8
0.4
0.0
0
1
2
3
4
5
6


Average Benzoic Acid Sulphate
(Ratio to IS, Peak Area)
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Time Post Consumption (h)


Benzoic Acid Sulphate
(Ratio to IS, Peak Area)
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Time Post Consumption (h)

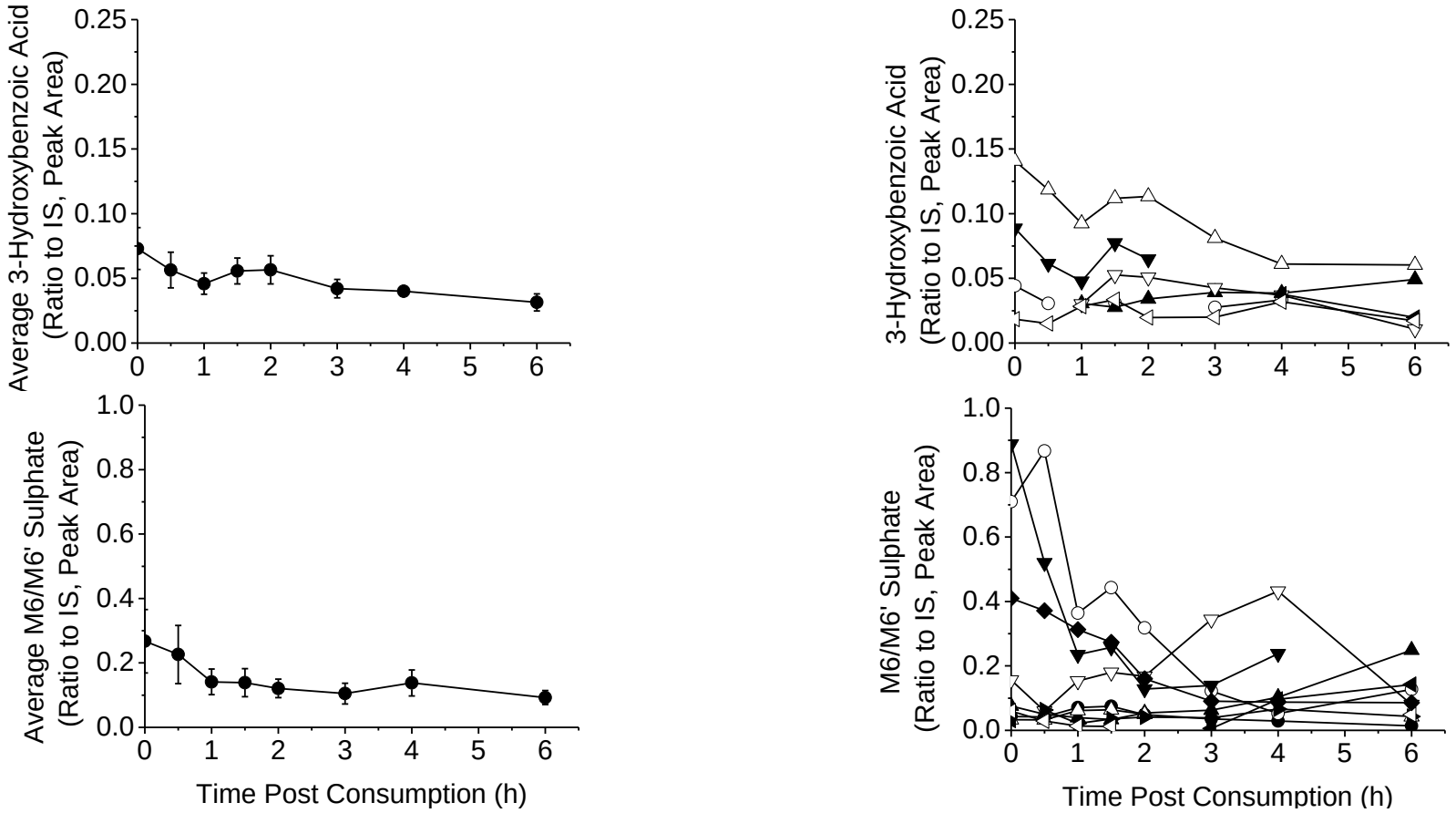


Figure 6-2. Average and individual pharmacokinetics for green tea catechin metabolites in the plasma of each volunteer post-consumption of green tea supplementation. Metabolites detected included *O*-methyl-EC-*O*-sulphate, EC-*O*-sulphate, EGC-*O*-glucuronide, *O*-methyl-EGC-*O*-sulphate, EC-*O*-glucuronide, *O*-methyl-gallic acid-*O*-sulphate, hippuric acid, benzoic acid-*O*-sulphate, 3-hydroxybenzoic acid and M6/M6'-*O*-sulphate (assessed as peak area, relative to internal standard; average +/- S.E.).

6.4.8. Bioavailability of Green Tea Catechin Metabolites in Urine

For compliance to the bioavailability study, urine was collected for 24 hours at four separate time points throughout the 3 month green tea supplement intervention; baseline, day 1, week 6 and week 12. Compliance was assessed using a short LC-MS method that identified EC-*O*-sulphate and EGC-*O*-glucuronide post consumption (data not shown). Urine samples were also analysed for identification of green tea catechin metabolites investigated in the bioavailability samples; plasma, interstitial blister fluid and skin biopsies. Post-consumption of green tea supplements there was clearly an increase in excretion (measured in 24 h urine collected) of EC-*O*-glucuronide, EC-*O*-sulphate, *O*-methyl-EC-*O*-sulphate, EGC-*O*-glucuronide, EGC-*O*-sulphate, *O*-methyl-EGC-*O*-sulphate, M4-*O*-sulphate, *O*-methyl-M4-*O*-sulphate, M6/M6'-*O*-glucuronide and M6/M6'-*O*-sulphate (Table 6-9).

Table 6-9. Excretion of green tea catechin metabolites in urine collected for 24 hours over the 3 month supplementation period.

	Green Tea Catechin Metabolite (µmol)/Total Urine Excreted			
Compound	Baseline	Day 1	Week 6	Week 12
Epicatechin-*O*-Glucuronide	4+/-1	9+/-2**	10+/-3**	10+/-3**
Epicatechin-*O*-Sulphate	3+/-1	26+/-6**	34+/-10**	26+/-5***
O-Methyl-Epicatechin-*O*-Sulphate	31+/-8	180+/-30***	300+/-80*	230+/-40***
Epigallocatechin-*O*-Glucuronide	0	6+/-2	5+/-1	6+/-1
Epigallocatechin-*O*-Sulphate	0.003	0.07+/-0.04	0.3+/-0.2	0.2+/-0.1
O-Methyl-Epigallocatechin-*O*-Glucuronide	17+/-12	27+/-10	50+/-20	30+/-10
O-Methyl-Epigallocatechin-*O*-Sulphate	0	70+/-10	140+/-60	80+/-20
Quercetin-*O*-Glucuronide	4+/-3	3+/-3	2+/-2	7+/-5
Quercetin-*O*-Sulphate	5+/-3	3+/-3	2+/-1	14+/-12
Gallic Acid-*O*-Glucuronide	120+/-50	190+/-100	70+/-30	140+/-60
3-*O*-Methyl Gallic Acid	0.013+/-0.009	0.026+/-0.008	0.06+/-0.04	0.05+/-0.01
O-Methyl-Gallic Acid-*O*-Glucuronide	0.06	0.4+/-0.2	0.05+/-0.03	0.2+/-0.1
O-Methyl-Gallic Acid-*O*-Sulphate	24+/-5	27+/-7	40+/-10	23+/-4
M4-*O*-Glucuronide	0.009	2.8+/-0.9	6+/-2	5+/-2
M4-*O*-Sulphate	1.7+/-0.7	15+/-4**	30+/-10**	30+/-10*
O-Methyl-M4-*O*-Glucuronide	0	0	0.012+/-0.008	0
O-Methyl-M4-*O*-Sulphate	0.3+/-0.2	35+/-10**	60+/-20**	65+/-25**
M6/M6' Glucuronide	0.4+/-0.3	7+/-3	15+/-4**	13+/-4**
M6/M6'-*O*-Sulphate	20+/-10	170+/-70*	360+/-100**	270+/-80***
O-Methyl-M6/M6'-*O*-Sulphate	0.05	1.8+/-0.5	3+/-1	4+/-2
3-Hydroxybenzoic Acid	0.14+/-0.04	0.4+/-0.2	0.19+/-0.06	0.4+/-0.1
3-Hydroxybenzoic Acid-*O*-Sulphate	7+/-2	8+/-2	11+/-6	9+/-3
Benzoic Acid-*O*-Sulphate	40+/-30	60+/-30	40+/-20	40+/-30
Syringic Acid-*O*-Glucuronide	2.1+/-0.5	3+/-1	1.9+/-0.4	2.8+/-0.7
Syringic Acid-*O*-Sulphate	3+/-2	6+/-5	9+/-9	2+/-1
Hippuric Acid	7000+/-2000	8000+/-2000	14000+/-5000	9000+/-2000
Hippuric Acid-*O*-Sulphate	0.03	0	0.06	0

Samples were prepared in biological duplicate (Student's t-test; one tailed, paired, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; average+/-S.E).

6.5. Discussion

The purpose of the green tea bioavailability study was to determine if green tea catechins and metabolites could be identified in skin samples (interstitial blister fluid and biopsies), and to establish if there was a correlation between the presence of the metabolites in the skin with metabolites in the plasma, as it is known that capillaries provide the skin with the required essential nutrients. In total, there were 15 metabolites that were identified in at least two of the three bioavailability samples collected 3 hours post-green tea supplementation on day 1. These were EC-*O*-sulphate, EC-*O*-glucuronide, *O*-methyl-EC-*O*-sulphate, EGC-*O*-glucuronide, *O*-methyl-EGC-*O*-sulphate, EGCG, *O*-methyl-gallic acid-*O*-sulphate, M4-*O*-sulphate, M4-*O*-glucuronide, *O*-methyl-M4-*O*-sulphate, M6/M6'-*O*-sulphate, M6/M6'-*O*-glucuronide, benzoic acid-*O*-sulphate, 3-hydroxybenzoic acid-*O*-sulphate and hippuric acid.

Only two metabolites were identified in all three samples (plasma, interstitial fluid and biopsies) and these were *O*-methyl-gallic acid-*O*-sulphate and M6/M6'-*O*-sulphate. The majority of the metabolites identified in more than one bioavailability sample were present within interstitial fluid and plasma; interstitial fluid is the extracellular fluid surrounding cells and it is derived from plasma. Of the 55 metabolites investigated, 7 were identified in biopsy samples, 20 were identified in interstitial blister fluid and 26 were identified in plasma. The majority of metabolites identified in blister fluid were present only post-green tea supplementation, and there was no difference in the peak area between pre- or post- UV irradiation samples.

As mentioned previously, over-estimation during quantification can occur as response factors for conjugated derivatives are unknown, and as has been reported previously, the sulphate and glucuronide forms can be associated with response factors that are up to 10 times higher than response factors for the free-forms (unconjugated) compounds (Farrell *et al*., 2011). Therefore, the biological samples

assessed during the bioavailability study were reported as peak areas relative to ethyl gallate. However, standard curves were created by spiking standards into blank, pre-supplement samples. A direct comparison between the concentration of EC-*O*-sulphate and EGC-*O*-glucuronide in blister and plasma samples demonstrated that the concentration in the extracellular interstitial blister fluid surrounding the skin cells is similar to the concentration in plasma; 9+/-3 µM and 7+/-4 µM for EC-*O*-sulphate, and 1.9+/-0.9 µM and 5+/-4 µM for EGC-*O*-glucuronide, plasma and blister fluid respectively. As it is known that blister fliud is derived from the plasma, the concentrations confirm that the content of both biological fluids is similar for these two green tea catechin metabolites, and possibly others. This information may be helpful for future *in vitro* studies investigating flavonoid treatment in skin, which could concentrate on using concentrations that have been reported in the plasma previously.

The fewer metabolites identified in skin biopsies could be due to a number of reasons, including the procedure for isolating catechin metabolites in biopsies was not efficient, metabolites did not enter skin cells or that metabolites present within the skin biopsy samples were bound to proteins present intracellularly. The efficiency of the procedure for assessing the biopsy samples was difficult to perform as there was only one biopsy per condition, and therefore multiple methods in homogenisation could not be directly compared. Samples were analysed by a combination of bombing the samples in liquid nitrogen, and sheer homogenisation on dry ice. There was only one catechin identified in a biopsy; *O*-methyl-EC-*O*-sulphate, and for one particular volunteer M6 was identified in each biopsy sample (pre- and post-green tea supplement consumption and pre- and post-UV irradiation) suggesting that this metabolite was from consumption of another source of EC, and not from green tea supplement consumption.

In the pilot study (Rhodes *et al*., 2013), there were 8 metabolites identified in skin biopsy samples. The free-form (unconjugated) derivatives of metabolites

present after consumption of green tea supplements were assessed by enzyme deconjugation, and therefore a direct comparison cannot be achieved as response factors for conjugated derivatives are unknown. As mentioned, only one catechin was identified in a biopsy for the bioavailability study, whereas EC, EGC, 4'-*O*-methyl-EGC and EGCG were identified in the pilot study. This could possibly be due to the accumulation of free-form derivatives after enzyme deconjugation, which may result in a higher response factor in comparison to lower concentrations of individual conjugates for a particular catechin.

In the plasma samples collected 3 hours post-green tea supplement consumption after 3 months daily consumption, there were 26 green tea catechins and metabolites detected. The metabolite with the largest response, as monitored as peak area relevant to ethyl gallate, was M6/M6'-*O*-sulphate. Valerolactones were present in the three hour samples due to ongoing metabolism of the supplement ingested on the previous day (valerolactones have been reported in urine 48 hours after supplement ingestion; Calani *et al*., 2012). The other metabolites identified in the plasma with large peak areas in comparison to other metabolites were *O*-methyl-EGC-*O*-sulphate, EGC-*O*-glucuronide, *O*-methyl-EC-*O*-sulphate and EC-*O*-sulphate, which are in line with other studies (including Del Rio *et al*., 2010a). For the first time, quercetin-*O*-sulphate, kaempferol-*O*-glucuronide, syringic acid-*O*-sulphate, 3-hydroxybenzoic acid-*O*-sulphate and benzoic acid-*O*-sulphate were identified in plasma samples post-green tea consumption. The identification of quercetin and kaempferol after consumption of green tea has been assessed previously in plasma, but relevant to free-form derivatives post enzyme deconjugation (Hollman *et al*., 2001, Jin *et al*., 2004).

On the first day of green tea supplementation, plasma was collected over 6 hours post-consumption. The baseline sample, collected pre-supplementation, identified green tea catechin metabolites that can also be consumed from other dietary sources. These mainly included phenolic acids, breakdown products

reported after consumption of black tea, cocoa and red wine. The highest responses, as assessed by peak area, over the 6 hour period post-green tea supplement consumption were peaks corresponding to EC-*O*-sulphate, *O*-methyl-EC-*O*-sulphate, EGC-*O*-glucuronide and *O*-methyl-EGC-*O*-glucuronide, with catechins first identified in plasma an hour after consumption.

When the pharmacokinetic samples were assessed relevant to standard curves created using free-form (unconjugated) derivatives it was clear that over-estimation of concentrations for green tea catechin conjugates had possibly occurred, as has been mentioned previously (Farrell *et al*., 2011). The C_{max} of EGCG was 45 nM when normalised to a 50 mg dose of EGCG in the green tea supplements, which is in line with previous studies; range of between 19 and 262 nM/50 mg dose (reviewed by Williamson *et al*., 2011). EGCG has been reported to have a dose reponse and therefore normalising to a standard dose amount consumed can be used to evaluate concentrations between different studies (Ullmann *et al*., 2003, Chow *et al*., 2005, Renouf *et al*., 2013). However, EC and EGC are known to not follow a dose response and therefore normalising data to a single dose can not occur (Chow *et al*., 2005, Renouf *et al*., 2013).

The review by Williamson *et al*., (2011) also normalised *O*-methyl-EC-*O*-sulphate data from two studies to 58 and 265 nM/50 mg EGCG (amount in green tea). For this thesis, the C_{max} of *O*-methyl-EC-*O*-sulphate was 1.2 µM/50 mg dose, and therefore results have been presented as peak area relevant to ethyl gallate to minimise possible over-quantification, however EC does not necessarily follow a dose response pattern and therefore normalising the data to a set amount consumed is difficult.

In summary, the results here suggest that green tea catechin metabolites can reach the skin post-consumption in the unconjugated (free) form and as conjugated derivates; assessed using the combination method to efficiently identify both (Chapter 5). As expected, the majority were present in the conjugated form.

There was a clear overlap between metabolites present in plasma samples collected 3 hours post-consumption, and interstitial blister fluid. The extracellular interstitial blister fluid derives from plasma, as it receives nutrients, water and oxygen from the blood capillaries present within the skin layers.

Any reductions observed in UV inflammatory markers, (including number of neutrophil cells, and cyclooxygenase and lipoxygenase (LOX) regulation) currently undergoing analysis at the University of Manchester and University of Bradford, subsequent to 3 months consumption of a high dose of green tea supplements (equivalent to 5 cups of green tea) could potentially be associated with the metabolites reported to be present here, as an association between green tea supplement consumption in the pilot study (low dose, equivalent to 2 cups of green tea) was reported to reduce 12-hydroxyeicosatetraenoic acid, a mediator of UV inflammation synthesised by LOX (Rhodes *et al*., 2013).

Chapter 7. Discussion and Future Work

7.1. Purpose of Investigation and Novelty

Each year approximately 100,000 cases of non melanoma skin cancer are diagnosed in the UK, deeming it the most commonly diagnosed type of cancer. The incidence of malignant melanoma has quadrupled since the 1980s, and in 2010 it was the fifth highest cancer form diagnosed (Cancer Research, 2012). A more robust method is required to protect human skin against ultraviolet radiation.

Polyphenols are secondary metabolites produced by plants to protect against the external environment, such as UV protection, and to provide colour and sensory characteristics (Gould and Lister, 2005). The treatment of skin cells with dietary flavonoids both *in vitro* and *in vivo* has been extensively investigated. Research for the protection of green tea catechins, in particular against UV-induced inflammation, is abundant *in vitro*. However, only limited studies in humans have been performed and as a protective effect may occur this warrants the exploration of green tea catechin consumption in association with UV protection. Authors of published studies have primarily reported the topical application of a treatment containing green tea catechins to the skin, and have demonstrated a reduction in markers of UV-induced inflammation.

When oral consumption of green tea catechins was compared against topical application in mice, it was clear that skin tumour formation was further reduced after exposure to UVR in mice that had orally consumed the green tea catechins as opposed to topical application (Wang *et al*., 1991). The assessment of skin bioavailability in humans after oral consumption of green tea catechins was investigated in this thesis (Chapter 6), and will be discussed in relation to protection against UV-induced inflammation.

Vitamin C, a dietary component well documented for its antioxidant activity and protection against scurvy, is also strongly linked to providing protection against UV-induced inflammation. Similar to green tea catechins, numerous studies have

investigated the *in vitro* and *in vivo* effects of vitamin C in relation to preventing UV-induced damage within skin. The depletion of the antioxidant system within skin cells has also been noted in the skin of mice after exposure to UV (Shindo *et al*., 1993). For the first time, the influence of the uptake of an antioxidant required in the skins' defence system against UV-induced inflammation, by flavonoids has been researched (Chapter 4). The reduction of stress induced from fetal bovine serum (FBS) starvation, hydrogen peroxide exposure and UVB irradiation, within the HaCaT (immortal human keratinocyte) cell model following pre-treatment with green tea catechins and green tea extract was also investigated, and for the first time green tea catechin protection against stress induced from the absence of FBS was evident in HaCaT cells (Chapter 3).

Green tea catechins were selected as the food bioactives to be investigated. In each of the human intervention studies (Chapters 5 and 6), a supplement containing green tea catechins and ascorbic acid was consumed for 3 months. Ascorbic acid was added to improve stability of catechins in the intestine (Chen *et al*., 1998), and thus improving the bioavailability; so potentially both ascorbic acid and catechins from green tea may reach the skin after oral administration.

Many bioavailability studies have assessed the metabolites present in urine and plasma after consumption of green tea catechins. The majority of green tea catechin metabolites were conjugated with methyl esters, sulphate esters and/or glucuronic acid by catechol-*O*-methyltransferase (COMT), sulphotransferase (SULT) and uridine 5'-diphospho-glucuronosyltransferase (UGT), with a limited concentration of catechin metabolites present in the free-form. For the first time a procedure to efficiently analyse both unconjugated (free-form) and conjugated metabolites of catechins using one method was employed for assessment of biological samples (plasma, urine, interstitial blister fluid and skin biopsies; Chapters 5 and 6). This was particularly important for assessment of skin biopsy samples as it is known that SULT, UGT and COMT are present within the skin (Dooley *et al*.,

2000, Ritter, 2000, Poquet *et al*., 2008), as are β-glucuronidase (Montagna, 1957) and sulfatase (Olson *et al*., 1968), and therefore depending on activity, it is possible that the green tea catechins and metabolites could have been in the free- or conjugated form.

7.2. Discussion of Outcomes

The removal of FBS, serum containing hormones and growth factors, from RPMI-1640 medium for 24 hours caused a reduction in uptake of MTT into HaCaT cells and also an increase in release of LDH from the cytoplasm of cells, across the damaged membrane, suggesting that viability was reduced and cytotoxicity was increased (Chapter 3). Treatment of cells with green tea extract (at an equivalent of 10 µM EGCG) reduced the incidence of stress induced by FBS starvation. It is possible that this was the result of green tea catechin treatment, or breakdown products present after 24 hour incubation. Future research could focus on the interactions between green tea catechins and growth factors and hormones that are present within FBS, including epidermal growth factor, insulin growth factor I and II, fibronectin growth factor and platelet derived growth factor (Freshney, 2005).

Polyphenols have been extensively demonstrated to induce apoptosis specifically in cancerous cells only *in vitro* (Giovannini and Masella, 2012). Green tea catechins (in particular EGCG) and quercetin have been reported to induce cell cycle arrest and increased anti-proliferation activity in cancerous cells (Ahmad *et al*., 1997, Thakur *et al*., 2012, Wang *et al*., 2012), but it is possible that in non-tumourgenic cells (such as HaCaTs) the growth cycle could be sustained by catechins whilst cells are undergoing starvation stress. Research into this area is limited. One study has shown beneficial effects of EGCG (50 µM; a high concentration for catechins) on aged primary keratinocytes by increased activity of

keratin 1, filaggrin and transglutaminase, and induction of p57/KIP2; markers of differentiation and proliferation in keratinocytes (Hsu *et al*., 2003).

The protection of FBS against stress induced in a skin cell model has been assessed previously using HaCaT cells. The presence of FBS reduced the stress caused by incubation of HaCaT cells with silver nitrate or hydrogen peroxide, in comparison to HaCaT cells treated with the stressors but in the absence of FBS (Sun *et al*., 2006). However, unlike the results obtained in this thesis, the removal of FBS from the medium without the addition of other stress was not assessed and therefore these results are the first to highlight FBS induced starvation stress in keratinocyte cells. Starvation induced stress caused by the absence of FBS has also been observed in HepG2 cells (Bai and Cederbaum, 2006).

Other studies have investigated the importance of L-theanine, an amino acid present in green tea, in protecting cells against stress *in vitro*. L-theanine reduced the presence of LDH in medium after cytotoxicity was induced in a human hepatic cell line (Li *et al*., 2012), so it is possible that L-theanine and catechins synergistically reduced the stress induced by the absence of FBS in HaCaT cells. To confirm the importance of L-theanine present within green tea for skin health during starvation (and also for vitamin C uptake), the effect of L-theanine treatment at the concentration present within the extract used (equivalent of 10 µM EGCG) on HaCaT cells undergoing stress should be investigated.

Hydrogen peroxide-induced oxidative stress in cells has been widely acknowledged, and can occur after exposure of skin cells (both *in vitro* and *in vivo*) to UV (Masaki *et al*., 1995). Hydrogen peroxide can produce reactive oxygen species, and can induce the activation of protein kinases (p38, JNK and ERK) resulting in the translocation of MAPKs to the nucleus where transcription factors (including c-Jun, ATF-2, CHOPP, GADD and Elk-1) can be activated leading to proinflammatory cytokine synthesis and apoptosis (Peus *et al*., 1999).

The uptake of MTT into HaCaT cells was increased in cells treated with hydrogen peroxide (<1 mM) and green tea extract, in comparison to cells treated with hydrogen peroxide alone, and the release of LDH from cytoplasm through damaged cell membranes was reduced. Again, it appeared that L-theanine might have synergistically interacted with the green tea catechins to protect again stress induced by extracellular hydrogen peroxide treatment. This was potentially due to scavenging of reactive oxygen species produced, or by inhibition of MAPK signalling pathway induction.

Similar results were obtained after HaCaT cells were exposed to UVB, and therefore green tea catechins and L-theanine appeared to protect against UVB-induced inflammation. This has been previously reported by others; Wu *et al*. (2009) demonstrated an increase in viability of HaCaT cells after UVB exposure with green tea catechin treatment, and many others have monitored the reduction of various markers of UVB induced stress in cells treated with green tea catechins (Table 1-5).

LDH release gives a clear indication of cytotoxicity, as LDH is only present after it has entered into the extracellular medium through a damaged cell membrane. It is difficult to confirm that MTT uptake is an exclusive marker of cell viability; it could possibly represent metabolic activity within cells. MTT is a tetrazolium salt with a net positive charge, taken up by cells via the plasma membrane potential (Berridge *et al*., 2005). It is possible that green tea catechins enhanced the uptake of MTT into HaCaT cells by increasing synthesis and translocation of ion channels and pumps involved in MTT uptake. Green tea catechins may also have increased the synthesis of NAD(P)H-dependent oxidoreductases and dehydrogenases present within the cell that are involved in reduction of MTT, which again would lead to confusion in terms of increased viability.

Cells treated with green tea catechins could not be lysed from the well plates after incubation with trypsin, and therefore the number of cells could not be counted. This would have confirmed if the cell count was higher after green tea catechin treatment, in comparison to the cells undergoing FBS starvation. An increase in cell density would have potentially also demonstrated an increase in uptake and reduction of MTT (Hsu *et al*., 2003), and not necessarily viability. A more appropriate measure of cell viability that could be used in future research is the assessment of incorporation of fluorescent signals into DNA during DNA replication. This would avoid uncertainties involving enhanced uptake, or increased reduction of MTT by reductases and dehydrogenases that may be present extracellularly after cell membrane damage or in higher concentrations intracellularly, leading to false positives.

As mentioned, it was also observed that green tea catechin treated HaCaT cells could not be lysed from well plates with trypsin. It is possible that the activity of trypsin on the adhesion proteins was inhibited by green tea catechins or breakdown products (not analysed for) still present in the medium or within cells. However, catechins were below the limit of detection (HPLC analysis) after 24 hour treatment and other studies have demonstrated the rapid degradation of catechins at a neutral pH and physiological temperature (Chen *et al*., 1998).

Laminin is the main extracellular matrix protein secreted by epithelial cells for adhesion, and therefore the modulation of laminin expression by green tea catechins was explored. An up-regulation of laminin expression was identified, and it is therefore possible that an increase in laminin protein was responsible for increasing the adhesion of the HaCaT cells to the well plate. This phenomenon could be assessed by gene knockout, and the effect of green tea extract and catechin treatment on cellular adhesion could be investigated further.

Extracellular matrix proteins are important in providing structural support to cells, and induction of matrix metalloproteinase (MMP) by exposure of skin cells to

UV has resulted in degradation of these proteins leading to photo-aging (Quan *et al*., 2009). Reducing degradation of extracellular matrix proteins and inhibition of MMP activity by green tea catechins and proanthocyanidins from grape seeds in prevention of cancer initiation has been suggested previously (Katiyar, 2006).

As mentioned, antioxidants within the defence system of the skin are depleted *in vivo* after exposure to UV (Shindo *et al*., 1993). As green tea catechins have been strongly linked to protection against UV, and also modulation of dehydroascorbic acid (oxidised form of vitamin C) transporters (GLUTs) in a rat model and mouse macrophage cells, the catechins were explored as a viable option for enhancing the uptake of vitamin C into skin cells after exposure of UV. All five known vitamin C transporters have been identified in human skin and their modulation accompanied by assessment of vitamin C under various conditions of stress was explored in this thesis (Chapter 4).

GLUT1 was the highest expressed vitamin C transporter within the HaCaT cell system (C_T approximately 24), which is also true *in vivo* (Gherzi *et al*., 1992, Nestlé Research Centre, unpublished data). However, it appeared that during FBS starvation, GLUT3 was the main transporter involved in increased uptake of DHAA after cells were treated with green tea extract. Green tea extract treatment maintained the expression of GLUT3 mRNA in comparison to a decrease in expression observed for untreated FBS starved HaCaT cells.

After HaCaT cells were subjected to hydrogen peroxide stress (<1 mM), the uptake of ^{14}C-DHAA was again increased in cells treated with green tea extract. It appeared that other components present within the extract (caffeine and L-theanine) were required for a synergistic interaction with catechins (or breakdown products present during the 24 hour treatment) to enhance the uptake of ^{14}C-DHAA after hydrogen peroxide exposure. The influence of other components within the extract on ^{14}C-DHAA uptake should be investigated further. It is possible that the stress induced by hydrogen peroxide in HaCaT cells (Chapter 3) was minimised by the

simultaneous presence of green tea extract by inhibition of hydrogen peroxide activated MAPK pathways and possibly reduction in ROS formation, which could maintain the uptake of ^{14}C-DHAA into HaCaT cells. GLUT1 and GLUT3 were up-regulated over a 24 hour period post simultaneous exposure of HaCaT cells to hydrogen peroxide and green tea extract, implying that both transporters were involved in supporting the uptake of ^{14}C-DHAA after hydrogen peroxide exposure.

Treatment of HaCaT cells exposed to UVB with green tea extract did not result in an increase in uptake of vitamin C, as was anticipated. This is the first attempt to improve uptake of an antioxidant required by the skin in the defence against UV-induced inflammation by a flavonoid. Replenishment of the intracellular antioxidants would result in improved protection against UV-induced damage, and should therefore reduce the incidence of melanoma initiation. It is possible that any influence that green tea catechins possess *in vivo* on vitamin C uptake could be the result of the conjugated (methyl, sulphate or glucuronide) forms present. For example, 3'-*O*-methyl-EC provided protection against hydrogen peroxide-induced damage in dermal fibroblasts, but a mixture of EC-*O*-glucuronides had no effect (Spencer *et al*., 2001). As reviewed by Ritter (2000), the activity of some glucuronides has been shown to be equal or enhanced in comparison to the parent free-form, including morphine 6-*O*-glucuronide and glucuronides of all-trans retinol and retinoic acid. This has also been demonstrated by some glucuronide conjugates of quercetin in relation to inhibition of xanthine oxidase and lipoxygenase (Day *et al*., 2000a).

The evaluation of urine excreted over 24 hours at 4 separate time points during the 3 month green tea supplement interventions allowed assessment of conjugate (methyl, sulphate or glucuronide) and free-form green tea catechin metabolites present. The evaluation utilised a combination procedure that analysed both free-form (present in ethyl acetate extractions) and conjugated derivatives (present after precipitation of proteins using acetonitrile) that were combined after

evaporation of solvents (Chapter 5). This is the first time a combination method has been applied for assessment of biological samples post-green tea consumption.

As the conjugated (methyl, sulphate or glucuronide) derivatives of green tea catechins and associated metabolites were not available commercially, the conjugates were assessed relative to free-forms (standard curves performed in urine). This technique can result in both over-estimation of the compound present due to differences in response factors when assessed with LC-MS. When hydroxycinnamic acid conjugates were estimated relative to the free-form, there was a vast range in response factor ratios with some conjugates responding up to 10 times higher than the free-form, resulting in over-quantification (Farrell *et al*., 2011).

The metabolites excreted in urine after consumption of green tea supplements (equivalent to either 2 or 5 cups of green tea per day) were similar to other studies (Sang *et al.,* 2008, Stalmach *et al.,* 2009, Del Rio *et al.,* 2010a, Mullen *et al.,* 2010, Stalmach *et al.,* 2010 and Calani *et al.,* 2012), with the identification of conjugated forms of microbial breakdown products (but not exclusive for catechin consumption) for the first time; hippuric acid-*O*-sulphate, 3-hydroxybenzoic acid-*O*-sulphate, benzoic acid-*O*-sulphate, syringic acid-*O*-sulphate and syringic acid-*O*-glucuronide. It was clear that methyl derivatives were also conjugated with either glucuronic acid or a sulphate ester. To validate identification of these conjugates, they should be synthesised, or future biological samples post-green tea consumption should be assessed using LC-MS^3, to compare fragmentation patterns of conjugated derivatives against the unconjugated free-form standards.

In comparison to amounts of metabolites excreted in urine, there are slight differences with other studies. The major catechin metabolite excreted after consumption of green tea supplements for 12 weeks was *O*-methyl-EC-*O*-sulphate, followed by M6/M6'-*O*-sulphate; similar to results obtained by Calani *et al*., (2012) and Del Rio *et al*., (2010a). However, Stalmach *et al*., (2009) identified 4'-*O*-methyl-EGC-*O*-sulphate as the main metabolite excreted, followed by

O-methyl-EC-*O*-sulphate. Stalmach *et al*., (2009) and Del Rio *et al*., (2010a) assessed the excretion of urinary metabolites relative to EC and EGC standards. In both studies the LC-MS was tuned with EC in the tuning mixture, maximising the signal achieved by EC and similar compounds. This, and a difference in experimental procedure in comparison to other studies (polyphenol free diet, short term intervention, amount of catechins consumed), could explain the reason for the amounts of green tea catechin metabolites excreted and reported in this thesis being higher in comparison to other studies.

Knowledge of catechin bioavailability to target sites and identification of the structural forms present could help to identify the compounds involved in the putative health benefits ascribed to green tea consumption. Bioavailability of catechins (as free-form and conjugate derivatives) in skin was investigated for the first time in this thesis (Chapter 6), with the aim to associate any protective effects observed (analysis at the University of Bradford and the University of Manchester) with the presence of green tea catechins within skin (biopsy samples) or surrounding skin cells (interstitial fluid).

In biopsy samples there were 7 metabolites identified (all post-consumption of green tea only, excluding M6 which was also present pre-supplementation in one volunteer, with no significant difference between the samples). In interstitial fluid there were 20 metabolites identified; the majority were conjugated with methyl esters, sulphate esters or glucuronic acid with only EGCG, quercetin, hippuric acid and benzoic acid present in the free-form. Any beneficial effects observed after green tea consumption and UV irradiation of skin could be attributed to induction of intracellular signalling pathways activated extracellularly from binding of green tea catechins and metabolites present in the interstitial blister fluid to membrane receptors.

As mentioned, the studies by Stalmach *et al*., (2009) and Del Rio *et al*., (2010a) assessed the green tea catechin metabolites excreted in urine and plasma

relative to EC and EGC standards, after the LC-MS was tuned with EC in the tuning mixture. When the plasma samples in this thesis were assessed relative to the free-form standards, the concentrations observed were much higher (µM in comparison to nM) than published data (Stalmach *et al*., 2009, Del Rio *et al*., 2010a). Therefore the peak areas of the compounds present in the plasma sample were assessed, and only when a standard was available, was the actual concentration calculated and presented.

Direct comparison between free-form catechins and catechin metabolites identified in plasma with published data can only occur when enzyme deconjugation was not used. For example, the main plasma green tea catechin or metabolite observed by Del Rio *et al*. (2010a) was EGCG (80 nM), followed by EGC-*O*-glucuronide (41 nM) and then *O*-methyl-*O*-EGC sulphate (38 nM). The volunteers consumed 188 mg EGCG/400 mL. The range of EGCG observed over the 6 hour analysis of plasma samples in this thesis post-green tea supplementation (544 mg EGCG) was 80 nM to 490 nM (C_{max} of 226 nM). A direct comparison of nM in plasma per mg consumed is 0.4 nM/mg for both. A dose response for EGCG consumption has been reported previously (Ullmann *et al*., 2003, Chow *et al*., 2005, Renouf *et al*., 2013) and therefore EGCG can be normalised to a set amount consumed. However, a dose reponse for EC has only been reported in one study (Renouf *et al*., 2013) and by contrast, has been reported as having no dose response in another (Chow *et al*., 2005). In both of these studies EGC was reported to have no dose response, which was accounted to the high inter-individual variation.

When normalised to a 50 mg dose of EGCG consumed, the C_{max} was 45 nM which is within the range (19 to 262 nM) normalised in the review by Williamson *et al.* (2011). However, when analysing *O*-methyl-EC-*O*-sulphate normalised to a 50 mg dose by Williamson *et al.* (2011), relative to *O*-methyl-EC in this thesis, the C_{max} was much higher than other studies, indicating over-estimation of this

metabolite when relative to the standard; 1.2 µM/50 mg dose compared with two studies normalised to 58 and 265 nM/50 mg dose. However, as mentioned the evidence for a dose response following EC consumption is conflicting so it is possible that the concentration in this thesis following consumption of 94 mg EC is higher and not over-estimated.

The main green tea catechin metabolites of plasma samples, assessed as peak area relative to internal standard, post-consumption of a high dose green tea supplement (equivalent to 5 cups of green tea) were EC-*O*-sulphate and *O*-methyl-EC-*O*-sulphate, followed by *O*-methyl-EGC-*O*-sulphate and EGC-*O*-glucuronide. These are also the main metabolites identfied in plasma by Del Rio *et al.* (2010) and Stalmach *et al*. (2010). The inter-individual variation of metabolites identified in plasma was large, which has been reported previously (Renouf *et al.,* 2013).

7.3. Overall Conclusions and Future Work

In summary, the results reported in this thesis provide evidence that supports the experimental hypotheses. Green tea catechins and metabolites are bioavailable in skin and proposed protective effects against UV may potentially occur extracellularly (interstitial fluid). Green tea catechins (or breakdown products present) are able to increase the uptake of DHAA in an *in vitro* keratinocyte cell line (HaCaT cells) after cells undergo FBS induced starvation and also hydrogen peroxide-induced stress, possibly due to a maintained expression of the GLUT3 transporter in comparison to control cells. Catechins were not able to increase vitamin C uptake after HaCaT cells were exposed to UVB, but were able to reduce UV-induced stress as demonstrated by a reduction in LDH release. The HaCaT cell line is utilised in numerous studies *in vitro* as it is accepted that it maintains the full epidermal differentiation capacity as *in vivo* keratinocytes (Boukamp *et al*., 1988).

The uptake of vitamin C after green tea catechin pre-treatment in a melanoma cell line could be investigated to examine the potential for green tea catechin enhancement of vitamin C (creating a high dose concentration) in cancer therapy (Ohno *et al*., 2009).

Further *in vitro* investigations should focus on the bioactivity of green tea catechin metabolites and conjugates within skin (methyl, sulphate, glucuronide), and the effect that they have on vitamin C transport post-UVB exposure. The bioactivity could be explored by assessment of the interactions between green tea catechins and metabolites with membrane receptors. But first, conjugate derivatives of green tea catechins and associated metabolites should be chemically synthesised to allow for analysis of pure compounds within *in vitro* models. Enhanced uptake of other antioxidants depleted within the skins' defence system (e.g. vitamin E, recycled by vitamin C) by food bioactives, namely flavonoids, could also be investigated.

A more efficient method of analysis of green tea catechins and metabolites (or other flavonoids) within skin biopsies also warrants investigation. As a maximum of four biopsies can be removed per volunteer, different homogenisation techniques could be evaluated post-flavonoid consumption, and efficiency of extractions compared.

Until the markers of inflammation within skin post-UV irradiation (neutrophils, $CD3^+$ cells and $CD8^+$ cells, University of Manchester; cyclooxygenase, lipoxygenase prostaglandins and arachidonic acid, University of Bradford) have been analysed and related to green tea catechin consumption, a relationship between green tea catechins and reduction in UV-induced damage cannot be formed. However, if the markers are reduced an association may be formed between green tea and vitamin C consumption and UV protection; potentially leading to industrial production of oral supplements that provide internal protection against photo-damage as opposed to the topical products that are currently available.

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