A Human Study on the Intra- and Interindividual Variation in Absorption and Metabolism of Coffee Chlorogenic Acids and Effects on Biomarkers of Health in Humans

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Let food be thy medicine and medicine thy food.

Hippocrates, 460-370 BC

The candidate confirms that the work submitted is his 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.

Paper

da Encarnação, Joana Amarante; Farrell, Tracy L.; Ryder, Alexandra; Kraut, Nicolai U. and Williamson, Gary, 2014. *In vitro* enzymic hydrolysis of chlorogenic acids in coffee. *Molecular Nutrition & Food Research*, 10-1002/mnfr201400498

Conference presentations

Kraut, Nicolai U.; Kerimi, Asimina and Williamson, Gary. Quantitation of novel glycine conjugates of chlorogenic acid from coffee after consumption by volunteers, Oral presentation, *VI International Conference on Polyphenols and Health*, Buenos Aires, Argentina, 2013

Kraut, Nicolai U.; Kerimi, Asimina; Poquet, Laure and Williamson, Gary. Urinary markers of coffee consumption detected by LC-MS, Poster presentation, *V International Conference on Polyphenols and Health*, Sitges, Spain, 2011

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Abstract

Coffee is a rich source of caffeoylquinic acids, esters of caffeic or ferulic acid and quinic acid and its consumption is associated with various health benefits. However, upon ingestion of coffee, caffeoylquinic acids are abundantly absorbed, widely metabolised and extensively excreted in humans.

The first part of this thesis addresses the synthesis and subsequent analysis of several glycine conjugates of hydroxycinnamic acid in urine collected by six participants of a pilot human study. For the first time vanilloylglycine has been quantified in urine for the first time after coffee consumption in similar amounts to feruloylglycine, whereas 3,4-dimethoxycinnamoylglycine, 3,4-dimethoxydihydrocinnamoylglycine, 3,4-dimethoxybenzoylglycine and dihydroferuloylglycine have only been detected in trace amounts.

The second part of this thesis describes the chemical synthesis of several hundred milligrams of dihydrocaffeic acid-3-*O*-sulfate, ferulic acid-4-*O*-sulfate, and dihydroferulic acid-4-*O*-sulfate, the development of a rapid LC-MS method for the analysis of the five most excreted urinary metabolites of caffeoylquinic acids, the design and performance of a human study investigating the intra- and interindividual absorption of caffeoylquinic acids in humans and linking these results to biomarkers of health and food intake.

Among 62 participants, an 8-fold variation in excretion of the total amount of the five most abundant, urinary metabolites excreted over 36 hours after coffee consumption was calculated and the intraindividual variation between repeated visits was the highest for colonic metabolites. A moderate, negative correlation of the absorption of caffeoylquinic acids with the weekly consumption of coffee was established. The data suggests a strong impact of colonic catabolism on the absorption and metabolism of caffeoylquinic acids and a reducing effect of heavy coffee consumption on the absorption of caffeoylquinic acids in humans.

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List of Abbreviations

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BMI	Body mass index
bp	Boiling point
CGA	Chlorogenic acid
CoA	Coenzyme A
CQA	Caffeoylquinic acid
CQAL	Caffeoylquinic acid lactone
DHCA	Dihydrocaffeic acid
DHFA	Dihydroferulic acid
DHiFA	Dihydroisoferulic acid
diFQA	Diferuloylquinic acid
DMC	Dimethoxycinnamoyl-
DMCQA	Dimethoxycinnamoylquinic acid
EC	Enzyme classification
ESI	Electrospray ionisation
EU	European Union
FA	Ferulic acid
FQA	Feruloylquinic acid
GlucA	Glucuronic acid
HDL	High-density lipoprotein
HPLC	High pressure liquid chromatography
iFA	Isoferulic acid
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry

LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantitation
MCT	Monocarboxylate transporter
mp	Melting point
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS^2	Tandem mass spectrometry
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
OAT	Organic anion transporter
pH	Negative logarithm of hydronium ion concentration
PTFE	Polytetrafluoroethylene
RT	Retention time
S.A.	Société anonyme
SRM	Single reaction monitoring
TIC	Total ion chromatogram
UK	United Kingdom
USA	United States of America
VA	Vanillic acid

Chapter 1: Introduction

1.1 Coffee

Coffee is the world's third most consumed beverage after water and tea, and is prepared from roasted coffee beans grown on shrubs of the Rubiaceae family [1] in the latitude of the tropics and subtropics. The species *Coffea Arabica L.* and *Coffea canephora*, commonly referred to as Arabica and Robusta coffee, are of main economical interest. Less known species are *Coffea liberica* (Liberica coffee) and *Coffea dewevrei* (Excelsa coffee) [2]. The Arabica coffee plant was first botanically described by Linnaeus in 1753, although coffee beans have been cultivated, harvested and used to brew coffee in the Middle East since the 15th century.

After flowering it takes nine to eleven months to grow the ripe, deep-red fruit known as the coffee cherry [3]. As coffee is grown in mountainous areas, coffee cherries are still mainly hand-picked, a very labor-intensive work. A coffee picker can harvest 100-200 pounds of coffee fruits a day, yielding up to 20 pounds of coffee beans a day [3]. There are two coffee beans located side by side to each other covered by a silver skin, an endocarp, a mesocarp and a red exocarp. Prior to processing, in order to yield roasted coffee beans for sale, the fruit needs to be dried. The drying process is mainly performed underneath the sun, by spreading the coffee beans on cotton cloth or just on stone or concrete patios. The drying process can take up to 4 weeks during which the beans are turned by raking [4].

In 2009, Brazil (2,432,904 t), Vietnam (1,176,000 t) and Colombia (887,661 t) were the world's largest producers of green, unroasted coffee beans [5]. Green coffee beans are shipped, mainly to Western countries, for large-scale, industrial coffee roasting. Various roasting processes yield the roasted coffee beans needed to prepare several types of coffee brews ranging from mixtures with herbs, such as cardamom, known as Arabic-style coffee, Italian-invented espresso to paper-filtered or percolated coffee [3]. Green coffee beans contain up to 14% (dry weight) of phenolic compounds and roasting reduces the phenolic compound content significantly [6].

Coffee consumption varies considerably from several cups of coffee a day in Scandinavian countries down to two cups per year in China [3].

1.2 Caffeoylquinic acid and its derivatives in coffee

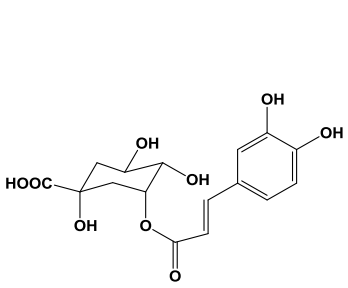
Coffee contains various plant-derived compounds, generally known as plant secondary metabolites of which some are known to exert certain beneficial effects to health.

Beside caffeine, melanoidins, and the diterpenes (cafestol and kahweol), caffeoylquinic acid and its derivatives are some of the plant bioactive compounds found in coffee beans [1].

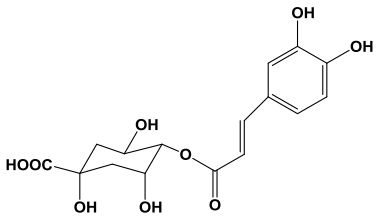
Chlorogenic acids (CGA) are a group of compounds consisting of various esters of *trans*-caffeic and/or *trans*-ferulic acid with quinic acid. Stalmach *et al.* detected 13 caffeoylquinic acid and its derivatives in instant coffee, which are illustrated in Figure 1.1, consisting of 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 3-*O*-feruloylquinic acid, 4-*O*-feruloylquinic acid, 5-*O*-feruloylquinic acid 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid, 3-*O*-caffeoyl-1,5-quinide, 4-*O*-caffeoyl-1,5-quinide and 4-*O*-*p*-coumaroylquinic acid and 5-*O*-*p*-coumaroylquinic acid [7]. All chemical structures are depicted according to IUPAC numbering [8].

5-Caffeoylquinic acid is most abundant with 25-30% molar of the total caffeoylquinic acid and its derivatives content, whereas other derivatives have relative abundances of 4-CQA > 3-CQA > 5-FQA > 4-FQA > 3-FQA > diCQAs [9]. The detrimental effect of roasting, accompanied by pyrolysis and maillard reactions, on the total caffeoylquinic acid and its derivatives content was demonstrated by Mills *et al.* [9].

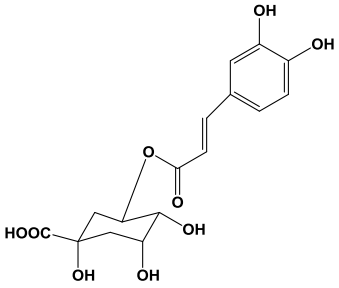
Figure 1.1: Caffeoylquinic acid and its derivatives detected in coffee according to Stalmach *et al.*, 2009



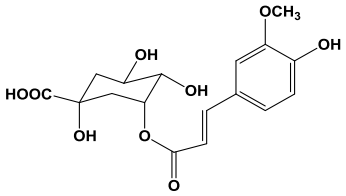
3-*O*-caffeoylquinic acid



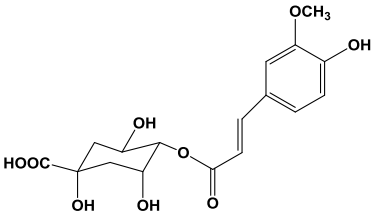
4-*O*-caffeoylquinic acid



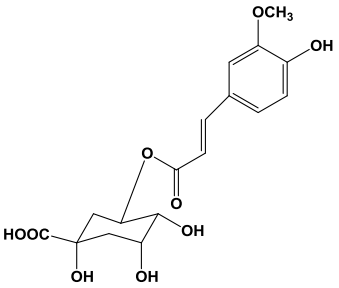
5-*O*-caffeoylquinic acid



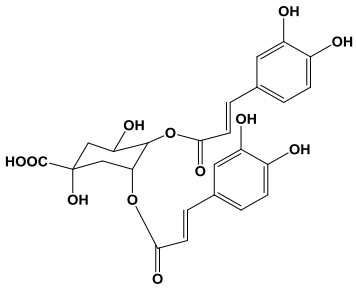
3-*O*-feruloylquinic acid



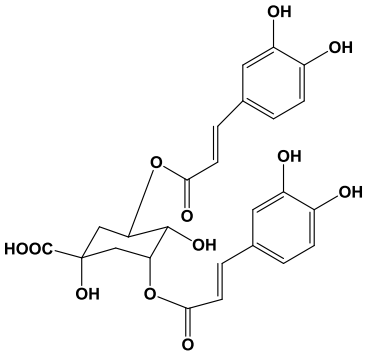
4-*O*-feruloylquinic acid



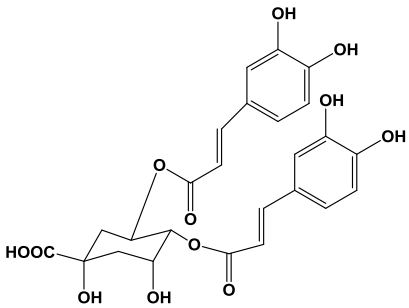
5-*O*-feruloylquinic acid



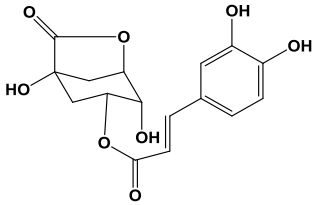
3,4-*O*-dicaffeoylquinic acid



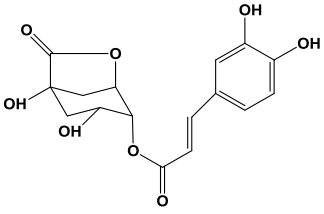
3,5-*O*-dicaffeoylquinic acid



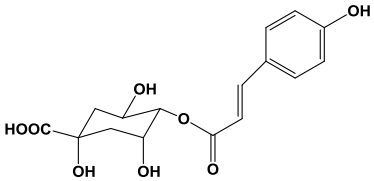
4,5-*O*-dicaffeoylquinic acid



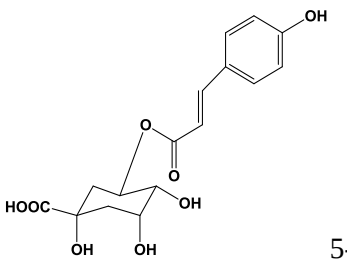
3-*O*-caffeoyl-1,5-quinide



4-*O*-caffeoyl-1,5-quinide



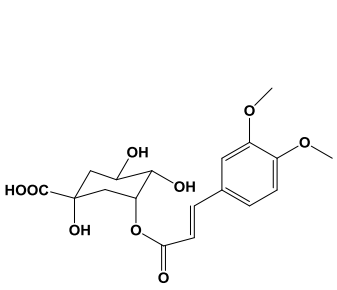
4-*O*-*p*-coumaroylquinic acid



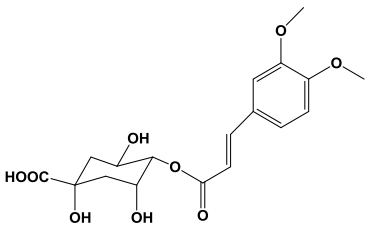
5-*O*-*p*-coumaroylquinic acid

Clifford *et al.* reported of twelve new derivatives of caffeoylquinic acid found in green coffee beans including 3-*O*-dimethoxycinnamoylquinic acid; 4-*O*-dimethoxycinnamoylquinic acid; 5-*O*-dimethoxycinnamoylquinic acid; 3-*O*-dimethoxycinnamoyl, 4-*O*-caffeoylquinic acid; 3-*O*-dimethoxycinnamoyl, 5-*O*-caffeoylquinic acid; 4-*O*-dimethoxycinnamoyl, 5-*O*-caffeoylquinic acid; 3,4-*O*-diferuloylquinic acid, 3,5-*O*-diferuloylquinic acid; 4,5-*O*-diferuloylquinic acid; 3-*O*-dimethoxycinnamoyl, 4-*O*-feruloylquinic acid; 3-*O*-dimethoxycinnamoyl, 5-*O*-feruloylquinic acid; 4-*O*-dimethoxycinnamoyl, 5-*O*-feruloylquinic acid [10] which are depicted in Figure 1.2.

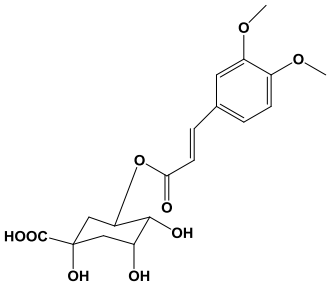
Figure 1.2: Minor derivatives of caffeoylquinic acid detected in green coffee beans according to Clifford *et al.*, 2006



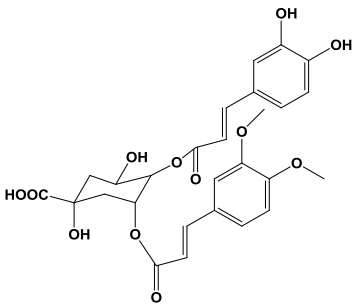
3-*O*-DMCQA



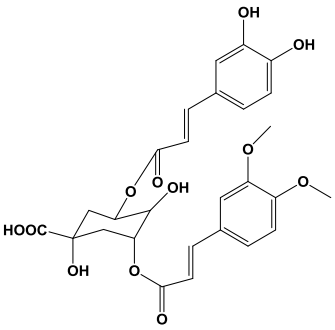
4-*O*-DMCQA



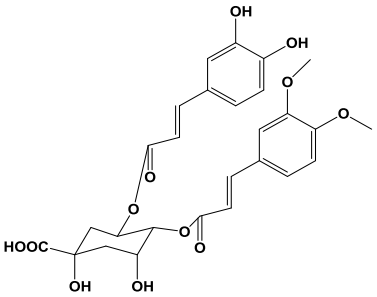
5-*O*-DMCQA



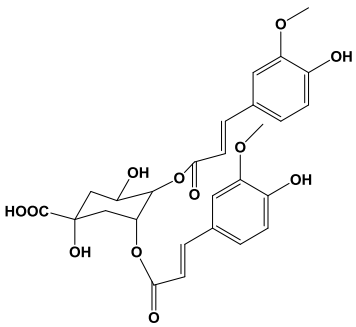
3-*O*-DMC, 4-*O*-CQA



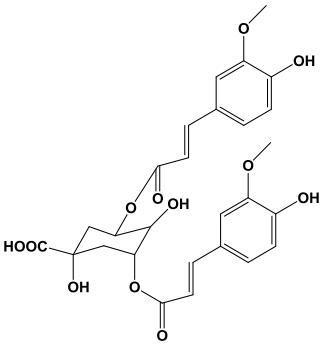
3-*O*-DMC, 5-*O*-CQA



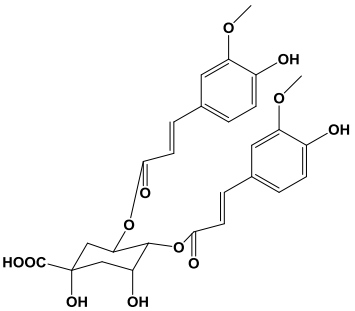
4-*O*-DMC, 5-*O*-CQA



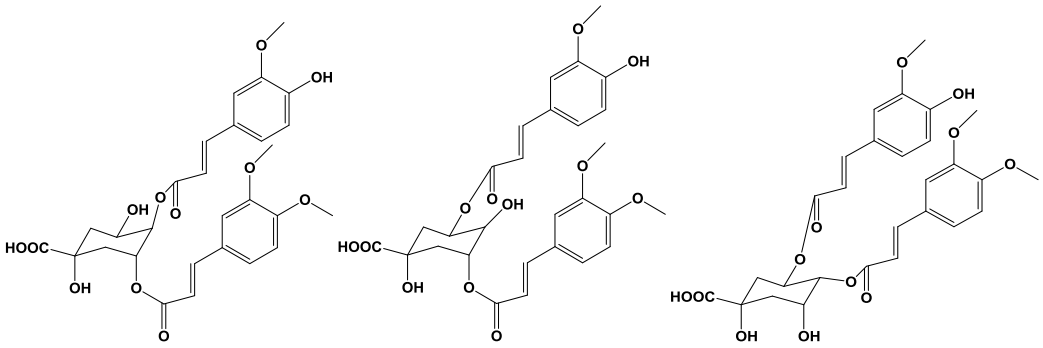
3,4-*O*-diFQA



3,5-*O*-diFQA



4,5-*O*-diFQA



3-*O*-DMC, 4-*O*-FQA 3-*O*-DMC, 5-*O*-FQA 4-*O*-DMC, 5-*O*-FQA

According to Clifford [11] a cup of coffee contains 70-350 mg of caffeoylquinic acid and its derivatives, thus coffee consumers in the UK ingest up to 1 g of caffeoylquinic acid and its derivatives per day. However, the content of caffeoylquinic acid and its derivatives in espresso coffee varies substantially, by 18-fold (24 to 422 mg/cup), between different coffee shops according to Crozier *et al.* [12]. Similar findings were reported by Ludwig *et al* [13] who quantified caffeoylquinic acid and its derivatives ranging from 6-188 mg per cup of coffee. Nonetheless, for habitual coffee drinkers, coffee is the major source of caffeoylquinic acid and its derivatives.

Recently, Nestle S.A. developed and launched an instant coffee, the commercially available *Nescafé Green Blend* [14], which is manufactured from a blend of green and roasted coffee beans in order to increase the caffeoylquinic acid and its derivatives content, which was independently analysed by Mills *et al.* [9]. In comparison with other Nescafé instant coffees, *Nescafé Green Blend*, contains about a 2.4-fold higher amount of caffeoylquinic acid and its derivatives.

1.3 Absorption, metabolism and excretion

Following ingestion of coffee, caffeoylquinic acid and its derivatives are abundantly absorbed, extensively metabolized, and widely excreted in urine.

1.3.1 Absorption

Several previous studies investigated the absorption and subsequent metabolism of caffeoylquinic acid and its derivatives in humans [7, 15-21]. Co-consumption of milk and coffee did not delay or reduce the absorption of caffeoylquinic acid and its derivatives in contrast to non-dairy creamer according to Renouf *et al.* [22].

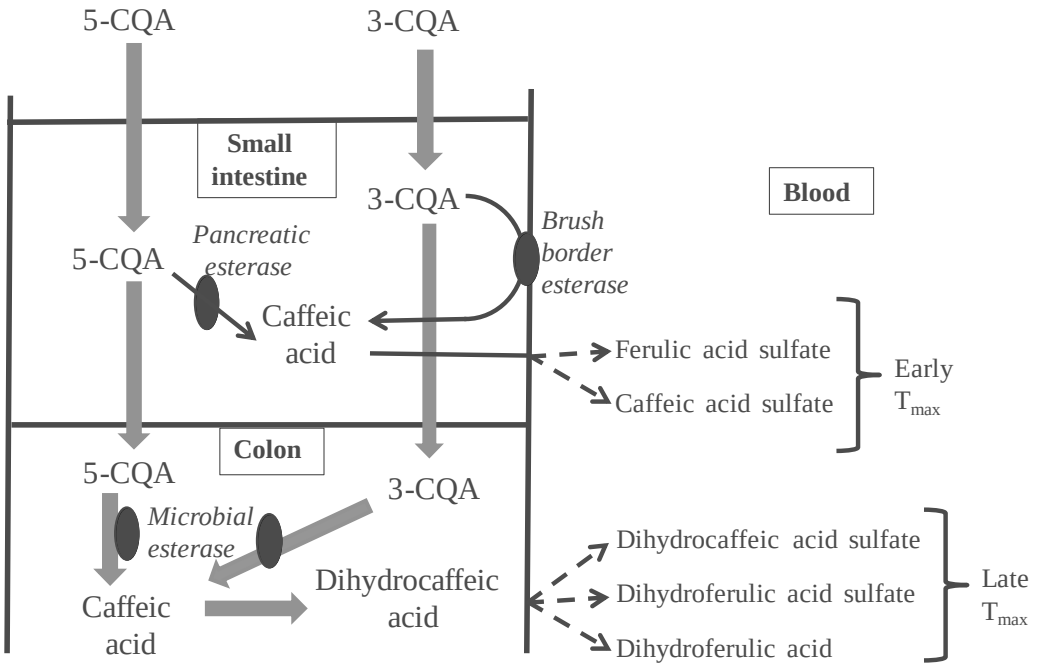
Within half an hour, minor amounts of intact 5-caffeoylquinic acid reach the circulatory system and show a T_{max} of 1h according to Stalmach *et al.* [7]. However, most early detected caffoylquinic acid and its derivatives derived compounds were found to be in their metabolised form, i.e. the ester bond between caffeic or ferulic acid and quinic acid has been cleaved and the free hydroxycinnamic acid has been conjugated with, e.g. sulphate. In fact, caffeic acid-3-*O*-sulphate and ferulic acid-4-*O*-sulphate are the first metabolites of caffeoylquinic acid and its derivatives to be detected in substantial concentrations in plasma after the ingestion of coffee. Prior to the absorption of caffeoylquinic acid and its derivatives, the cleavage of the ester bond between caffeic or ferulic acid and quinic acid is attributed to pancreatic and brush border esterases in the small intestine [15] and microbial esterases in the colon [17].

After passing through the stomach, the first important site of absorption is the small intestine where the free hydroxycinnamic acids are absorbed through the intestinal epithelium. The mechanism of transport is somewhat controversial. According to Konishi and Kobayashi [23] caffeic acid and 5-caffeoylquinic acid pass through the intestinal epithelium through paracellular diffusion and to a lesser extend through active transport via monocarboxylic acid transporters (MCT) in the Caco-2 cell monolayer absorption model, whereas Poquet *et al.* reported that ferulic acid is mainly transported through the intestinal epithelium by passive transcellular diffusion rather than via MCT [24].

Studies with ileostomists, participants who underwent surgical removal of the colon, showed that only 8-10 % of the ingested dose of caffeoylquinic acid and its derivatives are excreted in urine collected up to 24 hours after coffee consumption [15] and 76.2 % of the ingested dose of caffeoylquinic acid and its derivatives were detected in the ilea fluid [19]. Additionally, Erk *et al*. found that due to the high pH in the upper gastrointestinal tract (pH ~ 6.8), the hydroxycinnamic acids are present in their ionic form ($-COO^-$) and therefore not well absorbed [19]. In contrast, studies including healthy participants showed that the colon is the key site for absorption, since the highest plasma concentrations of metabolites of caffeoylquinic acid and its derivatives were found between 4.5-5.2 hours after coffee consumption [7, 16].

Figure 1.3 depicts the main sites for cleavage of the ester bond in the gastrointestinal tract, the absorption and subsequent release into the systemic circulation of humans derived from da Encarnação *et al* [25].

Figure 1.3: Main sites of cleavage of ester bond of 5-caffeoylquinic acid and 5-feruloylquinic acid and absorption into the systemic circulation, derived from da Encarnação *et al.*, 2014



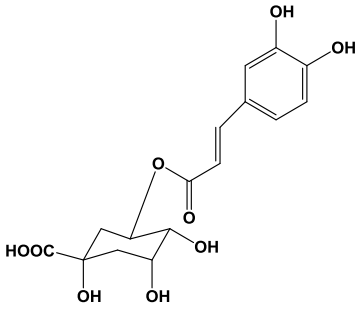
1.3.2 Metabolism

In addition to the cleavage of the ester bond between caffeic acid or ferulic acid and quinic acid, several other phase II conjugation reactions and reduction of the aliphatic double bond occur in the gastrointestinal tract after consumption of caffeoylquinic acid and its derivatives.

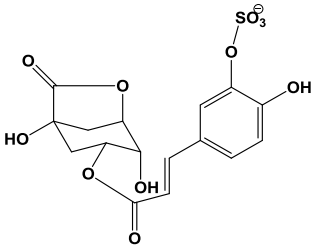
After coffee consumption, the ileal fluid of ileostomists has been collected and several phase II metabolites have been found, e.g. inter alia ferulic acid-4-*O*-sulfate, caffeic acid-3-*O*-sulfate, caffeic acid and ferulic acid [15]. Moreover, *in vitro* incubation of coffee with fecal slurries over six hours at 37ºC yielded metabolites including caffeic acid and ferulic acid with peak concentrations at 1 hour, and dihydrocaffeic acid and dihydroferulic acid with peak concentrations at 6 hours [26]. Additionally, phenolic acids with shorter side chains have been detected. These include 3,4-dihydroxybenzoic acid, 3-phenylpropionic acid, 3-(4'-hydroxyphenyl)propionic acid, benzoic acid, 3,4-dihydroxybenzoic acid and phenylacetic acid. These results indicated the presence of microbial esterases, reductases, dehydrogenases, demethoxyesterases and CoA-mediated decarboxylation.

Upon absorption, hydroxycinnamic acids, and to a certain extent, caffeoylquinic acid and their derivatives pass into the liver through the hepatic portal vein. Xenobiotics undergo the first pass effect before they enter the systemic circulation. During the first pass effect several phase II conjugations occur e.g. sulphation (sulfuryl-*O*-transferase), glucuronidation (UDP-glucuronyltransferase), methylation (catechol-*O*-methyltransferase) and addition of glycine (CoA mediated, glycine *N*-acyltransferase). Wong *et al.* [27] performed several *in vitro* and *in vivo* experiments to test the effect of liver S9 and intestinal S9 homogenates on hydroxycinnamic acids. Sulphation of hydroxycinnamic acids is favoured over glucuronidation. After the incubation of caffeic acid with liver S9 and intestinal S9 homogenates caffeic acid-3-*O*-sulfate and caffeic acid-4-*O*-sulphate were detected. When incubating ferulic acid, isoferulic acid, dihydrocaffeic acid and dihydroferulic acid with liver S9 and intestinal S9 homogenates, ferulic acid-4-*O*-sulphate, isoferulic acid-3-*O*-sulphate, dihydrocaffeic acid-3-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate, respectively, were analysed.

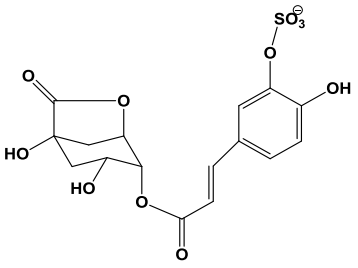
Figure 1.4: Metabolites of caffeoylquinic acid and its derivatives detected in human plasma after consumption of coffee by Stalmach *et al.*, 2009



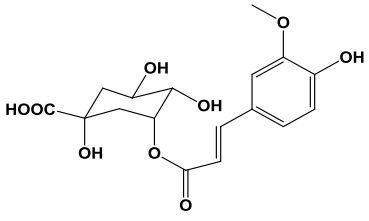
5-*O*-Caffeoylquinic acid



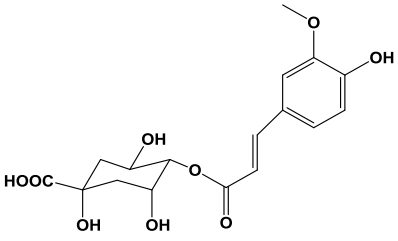
3-*O*-CQAL-*O*-Sulph



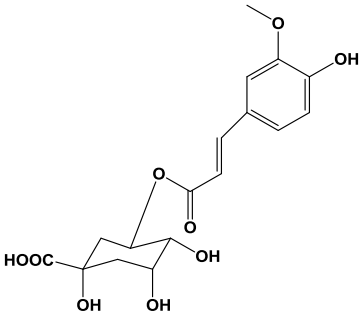
4-*O*-CQAL-*O*-Sulph



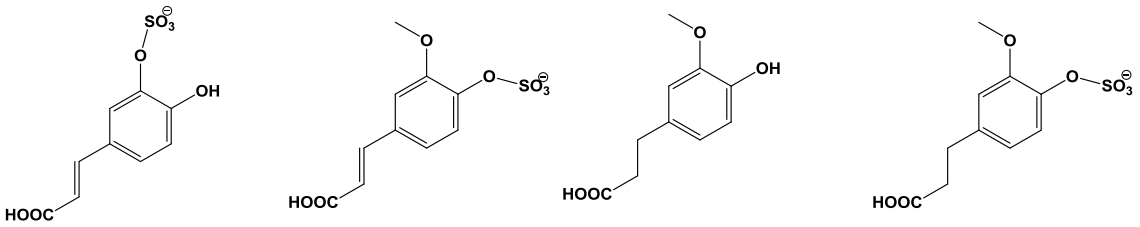
3-*O*-Feruloylquinic acid



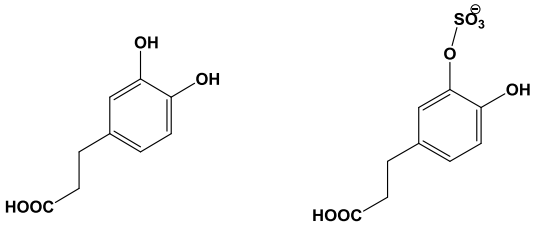
4-*O*-Feruloylquinic acid



5-*O*-Feruloylquinic acid



Caffeic acid-3-*O*-Sulph Ferulic acid-4-*O*-Sulph Dihydroferulic acid Dihydroferulic-4-*O*-Sulph



Dihydrocaffeic acid Dihydrocaffeic acid-3-*O*-Sulph

Stalmach *et al.* administered coffee containing 412 µmol caffeoylquinic acid and its derivatives to eleven, healthy volunteers and collected plasma samples at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 24 hours and urine samples from 0-2, 2-5, 5-8, and 8-24 hours [7]. Figure 1.4 depicts all 12 plasma metabolites. The first eight metabolites had a maximum plasma concentration within the first hour after coffee consumption, whereas the peak concentrations of the four dihydro forms were between 4.5-5.2 hours. The time to reach maximum plasma concentration indicates the site of absorption, thus the first eight metabolites are absorbed in the stomach and upper part of the small intestine, whereas the dihydro forms are absorbed in the colon. The only metabolite which showed a biphasic behavior was ferulic acid-4-*O*-sulfate with a T_{max} at 0.6 and 4.3 hours. The major plasma metabolites were dihydroferulic acid, dihydrocaffeic acid-3-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate, caffeic acid-3-*O*-sulphate, and ferulic acid-4-*O*-sulphate with a C_{max} of 385, 325, 145, 92, and 76 nM, respectively. In addition, Nagy *et al.* [28] detected 3,4-dimethoxycinnamic acid (T_{max} 60 minutes, C_{max} ~380 nM) and 3,4-dimethoxy-dihydrocinnamic acid (T_{max} 600 minutes, C_{max} ~80 nM) in plasma post coffee consumption. The data suggests that caffeoylquinic acid and its derivatives are highly absorbed and extensively metabolized within the human body.

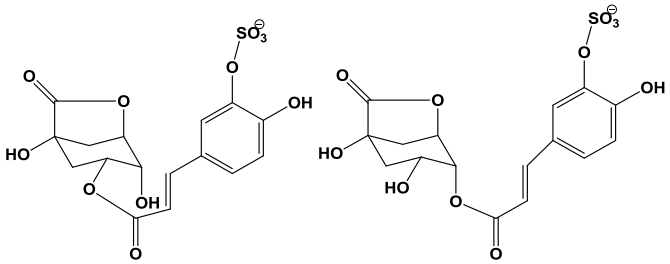
1.3.3 Excretion

Once metabolites of caffeoylquinic acid and its derivatives circulate in the vascular system they will be cleared via the renal system.

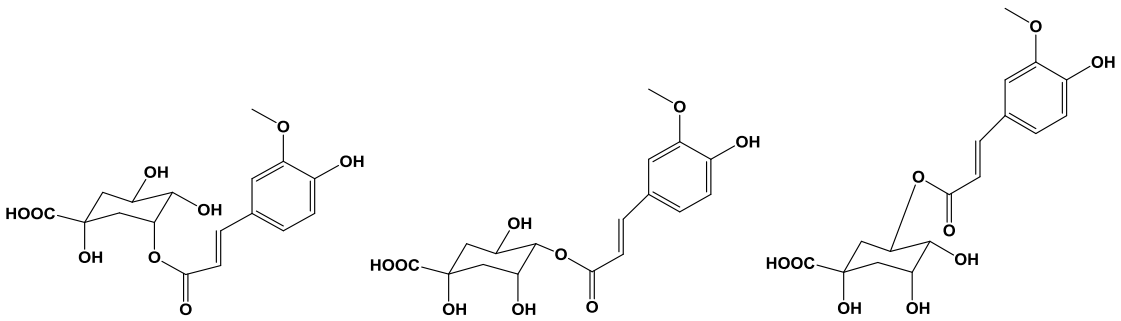
Using *in vitro* digestion, Wong *et al.* [29] demonstrated that organic anion transporters, OAT1, OAT3 and OAT4, but not ATP-binding cassette transporters, are responsible for the uptake of hydroxycinnamic acid conjugates, in particular dihydroferulic acid-4-*O*-sulphate, dihydrocaffeic acid-3-*O*-sulphate, ferulic acid-4-*O*-sulphate and caffeic acid-3-*O*-sulphate, into the renal system.

Stalmach *et al.* [7] quantified metabolites accounting for 29% of the ingested dose of caffeoylquinic acid and its derivatives in urine collected up to 24 hours after coffee consumption in healthy volunteers. Seventeen urinary metabolites were detected in urine which are shown in Figure 1.5.

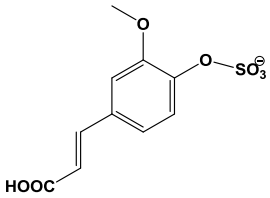
Figure 1.5: Metabolites of caffeoylquinic acid and its derivatives detected in urine after consumption of coffee by Stalmach *et al.*, 2009



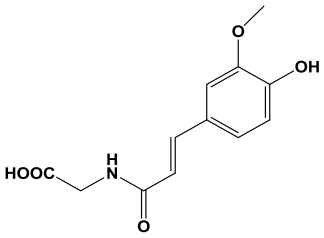
3-*O*-CQAL-*O*-Sulph 4-*O*-CQAL-*O*-Sulph



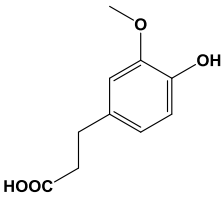
3-*O*-FQA 4-*O*-FQA 5-*O*-FQA



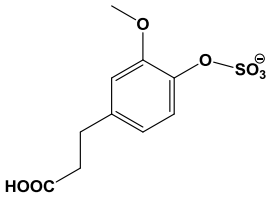
FA-4-*O*-Sulph



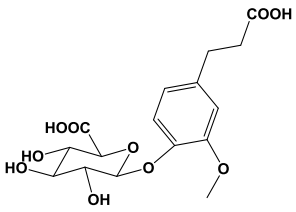
FA-Gly



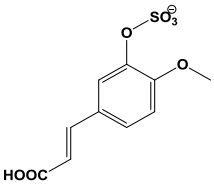
DHFA



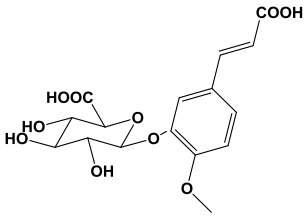
DHFA-4-*O*-Sulph



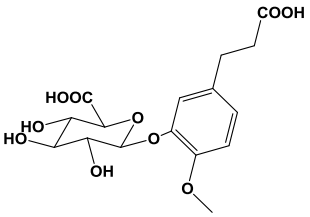
DHFA-4-*O*-GlucA



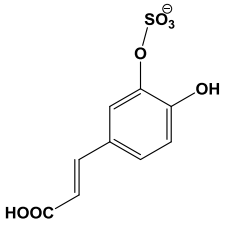
iFA-3-*O*-Sulph



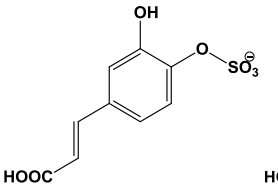
iFA-3-*O*-GlucA



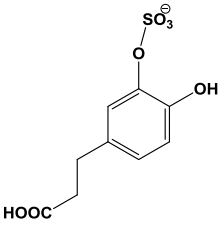
DHiFA-3-*O*-GlucA



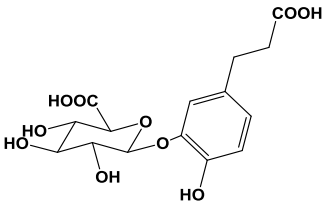
CA-3-*O*-Sulph



CA-4-*O*-Sulph



DHCA-3-*O*-Sulph



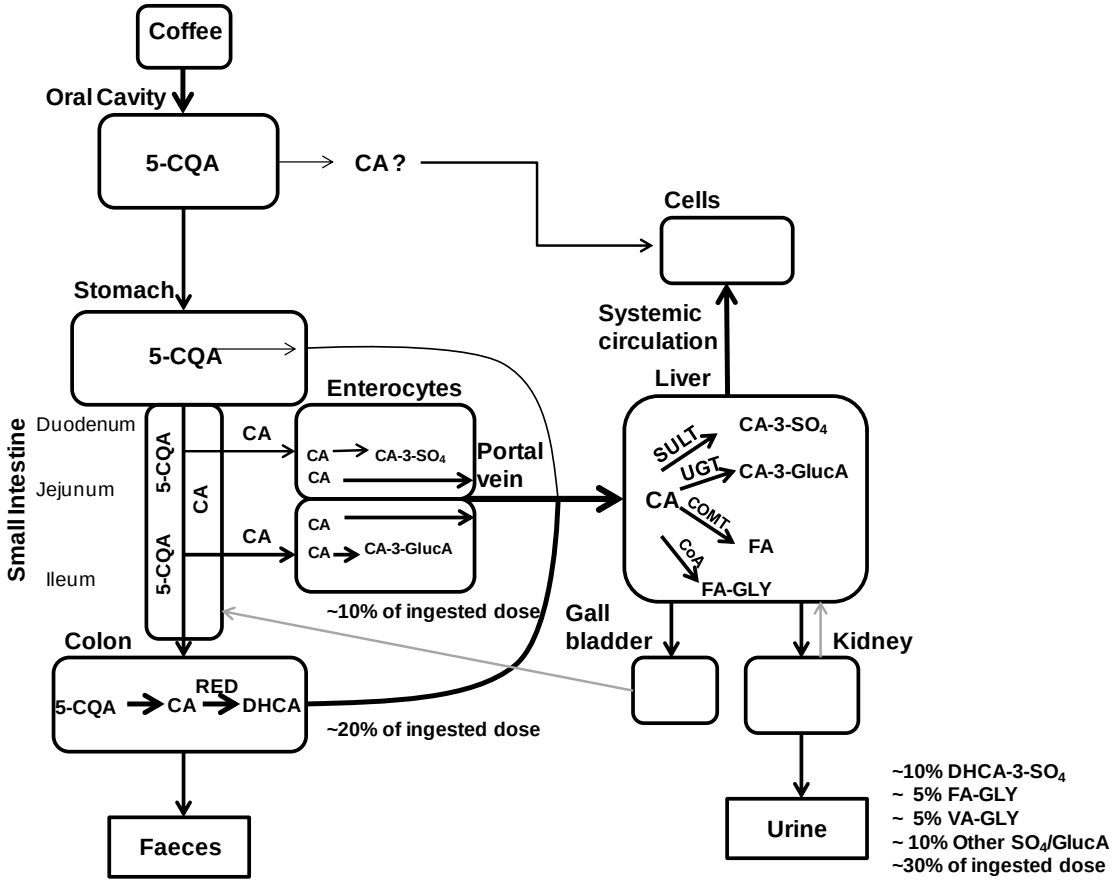
DHCA-3-*O*-GlucA

In contrast, only 8% of the ingested dose of caffeoylquinic and its derivatives were found in 0-24 hour urine of ileostomists [15].Two metabolites of caffeoylquinic acid and its derivatives were uniquely detected in urine, and not in plasma, i.e. feruloylglycine and dihydroisoferulicacid-3-*O*-glucuronide. This finding indicates that the conjugation with glycine and glucuronide occurs along with the excretion in the kidney and/or that these conjugates are immediately cleared from the systemic circulation and therefore cannot be detected in plasma. The data demonstrates that caffeoylquinic acid and its derivatives are well absorbed, extensively metabolised and widely excreted in urine post coffee consumption. A major pathway of excretion of caffeoylquinic acid and its derivatives is via the renal system [7].

Recent scientific advances, including the synthesis of sulphate and glucuronide conjugates of hydroxycinnamic acid [30], subsequent development and validation of LC-MS based analytical methods for biological fluids such as urine and plasma [16-19], have improved the quality of the analytical and therefore absorption, metabolism and excretion data substantially.

Figure 1.6 provides a general overview of the fate of 5-caffeoylquinic acid after ingestion.

Figure 1.6: Fate of 5-caffeoylquinic acid in the human body. Derived and adapted from Spencer, 2003



1.4 Bioavailability

To this date, several studies have been performed to assess the absorption and overall bioavailability of caffeoylquinic acid and its derivates from coffee in humans. In 2002, Naradini *et al.* performed a study including ten human volunteers drinking a single dose of coffee containing 96 mg of caffeoylquinic acid and its derivates [31]. A maximum plasma concentration of caffeic acid of 505 nmol/L was analysed. However, venepuncture, to obtain plasma, was only performed at 1 and 2 hours after coffee consumption, thus no precise calculation of the overall bioavailability could have been undertaken [31]. Rechner *et al.* conducted a human study on five participants who consumed two cups of coffee three times: at the beginning of the study, at one hour and two hours. Overall, 898 mg of caffeoylquinic acid and its derivatives were ingested and urine was collected up to 24 hours after the first consumption. Only 5.9 % of the ingested dose was excreted via urine [32].

Monteiro *et al.* published a human study with six subjects. In 2007, the subjects ingested 190 mL of freshly brewed coffee containing 1.202 g of caffeoylquinic acid and its derivatives. Plasma and urine were collected over four hours after ingestion and two plasma peaks were detected, the first one at 0.5-1.0 hours and the second one at 1.5-4.0 hours. The first peak indicates absorption in the small intestine and the second peak in the colon, probably promoted through colonic fermentation, however the overall bioavailability was not calculated [21]. In 2008, Farah *et al.* reported on a human study where ten participants consumed green coffee extracts and blood and urine were collected for up to eight hours post coffee intake. The ingested caffeoylquinic acid and its derivatives content was standardized to 170 mg and the bioavailability determined to be 33.1% in plasma [20].

In a more detailed study, Stalmach *et al.* reported various biomarkers of coffee consumption [7]. The study comprised eleven volunteers who consumed a 200 mL cup of coffee containing 412 µmol caffeoylquinic acid and its derivatives. Plasma and urine samples were collected over the next 24 hours without enzymatic hydrolysis of plasma and urinary metabolites of caffeoylquinic acid and its derivatives, thus providing a very detailed profile of the absorption, metabolism, and excretion. About 29% of the ingested dose of caffeoylquinic acid and its derivatives was recovered in urine. Caffeoylquinic acid and its derivatives were found in small amounts in

plasma with a T_{max} of 0.5-1 hours. Caffeic acid 3-*O*-sulfate and ferulic acid-4-*O*-sulfate were detected in up to 10-fold higher concentrations compared to unhydrolised caffeoylquinic acid and its derivatives, however, with a similar T_{max}. Reduced dihydro forms of hydroxycinnamic acids showed a T_{max} of 4.3-5.2 hours, indicating absorption after microbial catabolism in the colon. The C_{max} of these compounds was up to 100-fold higher compared to unhydrolysed caffeoylquinic acid and its derivatives. At 24 hours most metabolites of hydroxycinnamic acid had returned to baseline level. In urine mainly conjugates and hydrolysed forms of caffeoylquinic acid and its derivatives were detected. The main urinary metabolites were dihydrocaffeic acid-3-*O*-sulfate, feruloylglycine, dihydroferulic acid-4-*O*-sulfate, ferulic acid-4-*O*-sulfate, dihydroferulic acid, dihydroferulic acid-4-*O*-glucuronide, caffeic acid-3-*O*-sulfate and isoferulic acid-3-*O*-sulfate.

In 2009, Renouf *et al.* reported on quantitative, LC-MS based analysis of hydroxycinnamic acid conjugates in plasma post coffee consumption. Nine human volunteers consumed a coffee containing 335 mg caffeoylquinic acid and its derivatives and blood samples were collected up to 12 hours thereafter and at 24 hours [33]. All metabolites of caffeoylquinic acid and its derivatives were enzymatically hydrolysed using chlorogenate esterase, glucuronidase and sulfatase. Renouf *et al.* confirmed previous results with plasma peak times for ferulic, caffeic and isoferulic acids at T_{max} 1-2 hours after coffee ingestion and for the dihydro forms at T_{max} 6-8 hours in plasma. Additionally, substantial interpersonal variations in C_{max} and T_{max} were described.

In 2011, Williamson *et al.* [34] critically assessed the quality of previous human studies with focus on the absorption of caffeoylquinic acid and its derivatives and their plasma appearance by normalising the C_{max} to the dose of intake. Nardini *et al.* [31] reported up to four times higher values for the normalised C_{max} for caffeic acid. Monteiro *et al.* [21] reported about 200 times higher values for the normalised C_{max} for intact caffeoylquinic acid and its derivatives in plasma.

Summarising the previous studies on the absorption and metabolism of caffeoylquinic acid and its derivatives from coffee, it can be concluded that several hydroxycinnamic acids of caffeoylquinic acid and its derivatives are absorbed in the small intestine at a T_{max} of 0.5-1.0 hours and only a very minor amount of caffeoylquinic acid and its derivatives, whereas in the colon caffeoylquinic acid and its derivatives are hydrolysed by colonic microflora derived esterases and the free hydroxycinnamic acids are reduced to their dihydro forms leading to a T_{max} of 5-7 hours.

However, data on the overall bioavailability of coffee caffeoylquinic acid and its derivatives is limited in humans. In order to assess the bioavailability, blood samples need to be drawn at several time points after coffee consumption for a detailed description of the plasma concentration curve of metabolites of caffeoylquinic acid and its derivatives and subsequent meticulous calculation of the area under the curve. In addition, all metabolites of caffeoylquinic acid and its metabolites need to be detected and ideally chemically synthesised for subsequent development and validation of LC-MS based quantitative analytical methods.

1.5 Coffee and health

Coffee has long been thought of as detrimental to consumer's health, however, recent research indicates potential health benefits including reduced risk of cancer, cardiovascular disease, and diabetes [35-37].

1.5.1 Cardiovascular health

Globally cardiovascular diseases (CVD) are the main cause of death with over 17.1 million deaths in 2004 – 29% of all deaths. About 7.2 million deaths were attributed to coronary heart disease and 5.7 million were associated with stroke. CVDs will be the cause of 23.6 million deaths by 2030 [38]. CVDs are initiated by intermediate reasons such as a lack of physical activity, overweight and obesity, diabetes mellitus type II, tobacco use, unhealthy diet leading to high blood glucose levels, high blood lipid levels, sodium-rich diet and therefore high blood pressure [38].

Despite reporting of cholesterol raising effects of coffee consumption independent of the brewing method (paper filtered vs unfiltered), via randomized control trials [39, 40], caffeoylquinic acid demonstrated an inhibitory effect on LDL cholesterol oxidation *ex vivo* [41] and a single dose of coffee can increase plasma antioxidant activity by 5.5% according to Natella *et al.* [42]. Additionally, the ingestion of a single cup of coffee ameliorated endothelial function measured by flow-mediated dilation according Buscemi *et al.* [43], whereas a another study found that habitual, boiled coffee consumption has a beneficial effect on flow-mediated dilation in elderly participants [44]. In another randomized control trial, chronic coffee consumption was associated with a rise in blood pressure, however, the increase of the systolic and diastolic blood pressure was minor only 2.04 mmHg and 0.73 mmHg, respectively [45]. A paper reported the inverse correlation of coffee, not green tea consumption, on arterial stiffness, assessed using brachial-ankle pulse wave velocity, in Japanese men [46]. Moreover, Andersen *et al.* presented an epidemiological study including more than 40,000 post-menopausal women linking coffee consumption to improved anti-inflammatory status and protection against cardiovascular diseases reducing death incidence [47]. The current data suggests that moderate

coffee consumption as part of a healthy and balanced life style maybe associated with improved cardiovascular health.

1.5.2 Diabetes mellitus type II

Worldwide, 387 million people have been diagnosed with diabetes, indicating a prevalence rate of 8.6%, whereas an estimated 46.3% of cases have not been diagnosed according to the International Diabetes Foundation [48]. In the UK, numbers are similar with a prevalence rate of 6.0% indicating that 3.2 million British citizen suffer from diabetes [49]. Physical inactivity, overweight/obesity and a diet high in sugar and fat is considered to trigger diabetes mellitus type II [48, 49].

In a follow-up study of 126,210 health professionals in the UK over twelve years, a beneficial effect of long-term coffee consumption on the risk of diabetes mellitus type II was reported by Salazar-Martinez *et al.* in 2004 [50] and Ranheim *et al.* concluded from a literature study that there is a reduced risk of diabetes mellitus type II associated with the consumption of CGA [51]. In a Chinese study population of 2,332 participants, habitual coffee drinkers had a 38-46 % lower risk of diabetes mellitus type II compared to non-drinkers [52]. In 2009, Pimentel *et al.* reviewed several longitudinal studies and found that long-term, moderate coffee consumption (~ 4 cups per day) is associated with a reduced risk of diabetes [37]. A Norwegian study found that consumption of caffeoylquinic acid enriched coffee reduces glucose absorption by 6.9%, compared to a control, and elicits a reduction of the body mass in a randomized, double-blind, 12-week study including twelve obese participants [53]. In a prospective study including 18,417 men and 39,740 women, performed by Lopez-Garcia *et al.* [54] from 1986 to 1998, increases of caffeine intake of 213 and 143 mg/d for men and women was negatively associated with long-term weight gain with -0.43 kg and -0.35 kg for men and women, respectively. Additionally, in 2011, a German group published results from a prospective cohort study including 30 subjects, compared the effects of dark and light roast coffee, differing in their caffeoylquinic acid and its derivatives and *N*-methylpyridinium content, indicating that the dark roast coffee reduced the body weight significantly and improved the antioxidant status of erythrocytes, i.e. superoxide

dismutase and glutathione peroxidase, by 5.8 and 15%, respectively, and increased plasma tocopherol and glutathione concentrations by 41 and 14%, respectively [55].

In conclusion it can be stated that the current data indicates a beneficial effect of coffee consumption on insulin sensitivity and prevalence of diabetes. However, the relationship between the plant secondary metabolites in coffee and health remains unclear and further investigation on the *in vivo* bioactive properties of these compounds is required.

1.5.3 Other beneficial effects of coffee caffeoylquinic acid and its derivatives

In vitro antioxidant effects of coffee were shown using several tests including oxygen radical absorbance capacity (ORAC) [56], fluorescence recovery after photobleaching (FRAP) [57] and trolox equivalence antioxidant capacity (TEAC) [58]. However, in 2012, the US Department of Agriculture removed all data of the ORAC database on food, since no *in vivo* effects of the *in vitro* data could be established [59].

Strong antibacterial effects of coffee extracts on enterobacteria *in vitro* were demonstrated by Almeida *et al.* [60]. Santos *et al.* proved anti-inflammatory effect of caffeoylquinic acid and its derivatives in rats, however, the mechanism of action is not fully understood and requires further studies [61]. Andersen *et al.* reported the anti-inflammatory effects of coffee consumption assessed by the Iowa Women's Health Study including 41,836 post-menopausal women [62]. A review of 500 papers summarized the outcome and reported a protective effect of coffee consumption on hepatocellular, endometrial and colorectal cancer, whereas heavy coffee consumption was associated with bladder cancer [35]. Another review confirms the hepatoprotective effects and additionally the protective effect against liver cirrhosis [63]. The data suggests that further studies concerning the *in vivo* effect of metabolites of caffeoylquinic acid and its derivatives are needed in order to draw conclusions on the bioactivity.

1.6 Aims

Numerous studies have been performed to assess the *in vitro* and *in vivo* effect of coffee, coffee-derived potentially bioactive compounds or a mixture of both. However, to our knowledge, no human study has been performed to assess the connection between absorption, metabolism and excretion of coffee caffeoylquinic acid and its derivatives, biomarkers of health, i.e. cardiovascular health status, diabetes and food intake, i.e. coffee consumption, tea consumption and fruit and vegetable intake.

The specific aims of the studies of this thesis were:

1) Synthesis and subsequent detection and quantitation of novel, urinary glycine conjugates of hydroxycinnamic acids, especially of the recently described plasma 3,4-dimethoxycinnamic acid and 3,4-dimethoxy-dihydrocinnamic acid [28], in urine after coffee consumption

2) Synthesis of major urinary metabolites of caffeoylquinic acid and its derivatives in urine, e.g. dihydrocaffeic acid-3-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and ferulic acid-4-*O*-sulphate and subsequent development and validation of a quick, quantitative method based on LC-MS

3) Design and performance of a human study linking the absorption of caffeoylquinic acid and its derivatives, by means of assessing the total urinary excretion, to biomarkers of health, e.g. plasma thiols, uric acid, fasting insulin and glucose, and to food intake, e.g. coffee consumption, tea consumption, fruit and vegetable intake

4) Data analysis including correlation of absorption of caffeoylquinic acid and its derivatives to biomarkers of health and food intake

Hypothesis: Coffee consumption and absorption of coffee caffeoylquinic acid and its derivatives have a positive impact on biomarkers of health and are correlated with food intake.

Chapter 2: Chemical synthesis and analysis of novel glycine conjugates in urine after coffee consumption

2.1 Abstract

Chapter 2 describes the chemical synthesis of novel glycine conjugates of cinnamic acid derivatives, the performance of a human study and the qualitative and quantitative LC-MS analysis of six glycine conjugates, e.g. 3,4-dimethoxycinnamoylglycine, 3-(3,4-dimethoxyphenyl)propionylglycine, vanilloylglycine, 3,4-dimethoxybenzoylglycine, feruloylglycine and dihydroferuloylglycine. Beside the previously reported feruloylglycine, vanilloylglycine (33 µmol, equivalent to 4.5% of the ingested dose) was detected and quantified in urine after coffee consumption for the first time in similar, high amounts as feruloylglycine (39 µmol, equivalent to 5.3% of the ingested dose). Other glycine conjugates were only detected in trace amounts suggesting that these must be further modified by phase II metabolism or undergo another metabolic pathway than conjugation with glycine.

2.2 Introduction

In the mid 19th century Friedrich Woehler, a German chemist, was the first scientist to analyse organic constituents of urine. In 1841 Alexander Ure, a Scottish physician performed the first metabolism study in humans. He administered benzoic acid to a human subject and analysed hippuric acid in urine. Later, in 1845, Dessaignes demonstrated that hippuric acid is a glycine conjugate of benzoic acid [64].

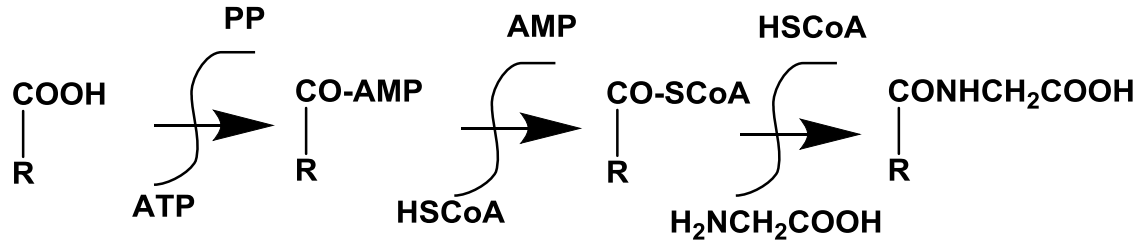
More than a century later, in 1957, Booth *et al.*[3] reported the finding of p-hydroxyhippuric acid in human urine after consumption of coffee. Moreover he also performed studies with individual compound namely caffeic acid and chlorogenic acid which he administered to humans and analysed their urine. After consumption of caffeic acid they detected at least nine metabolites in urine e.g. feruloylglycine and dihydroferulic acid. After ingestion of 5-caffeoylquinic acid they found caffeic acid, *m*-hydroxyhippuric acid, *m*-coumaric acid conjugated with glucoronic acid and dihydroferulic acid. They also suggested a complex

pathway of metabolism including conjugation, e.g. methylation and glucuronidation and chain shortening due to β-oxidation.

In the next two decades several animal studies, mainly on rats, were performed. In 1963 Dirscherl *et al.* [65] perfused rat liver with vanillic acid and found vanilloylglycine (3-methoxy-4-hydroxy hippuric acid) in the perfusate. Similar results were later presented by Strand *et al.* [66] when they found vanilloylglycine and isovanilloylglycine (3-hydroxy-4-methoxy hippuric acid) as urinary metabolites of vanillin in rats. In 1975, Solheim *et al.* demonstrated that 3,4-dimethoxycinnamic acid is metabolised to 3,4-dimethoxycinnamoylglycine and 3,4-dimethoxybenzoylglycine in rats and excreted in urine collected up to 24 hours after administration. 3,4-dimethoxycinnamoylglycine together with m-methoxyhippuric acid and hippuric acid was also found in urine collected for 24 hours after the consumption of garlic and onions in humans [32].

The mechanism of glycine conjugation is associated with beta-oxidation and therefore located in the mitochondria in particular in liver and kidney cells [64]. The following scheme (Figure 2.1) depicts the mechanism of glycine conjugation, starting with the activation through ATP, the addition of CoASH and the conjugation of glycine mediated by the enzyme glycine *N*-acyltransferase (EC 2.3.1.13) which was first isolated and described by Mawal *et al.* in 1994 [67].

Figure 2.1: Glycine conjugation via activation with ATP followed by addition of CoA and finally conjugation with glycine catalysed by glycine *N*-acyltransferase (EC 2.3.1.13) in mitochondria, derived and adapted from Knights *et al.*, 2007



More recently, Stalmach *et al.* detected feruloylglycine in human urine post coffee consumption. Feruloylglycine is the second most abundant metabolite of coffee caffeoylquinic acid in urine, but it is also the only glycine conjugate detected by Stalmach *et al.* [7].

Recently, 3,4-dimethoxycinnamoylquinic acid was determined in roasted coffee beans [10]. Since the 3,4-positions of the compound are blocked for phase II modification, e.g. sulphate and glucuronide addition, therefore it is hypothesised that the compound is prone to glycine conjugation on the carboxylic function. In order to tested this hypothesis, a six subject human study was performed where participants consumed a cup of coffee with known content of caffeoylquinic acid and its derivatives after a 36 hours washout, thereafter urine was collected for 36 hours in time periods of 0-4, 4-8, 8-12, 12-24 and 24-36 hours and several potential and chemically synthesised derivatives of cinnamic acid and benzoic acid conjugated with glycine were qualitatively and quantitatively analysed, e.g. vanilloylglycine, feruloylglycine, dihydroferuloylglycine, 3,4-dimethoxybenzoylglycine, 3,4-dimethoxybenzoylglycine and 3,4-dihydrodimethoxybenzoylglycine.

2.3 Materials and methods

2.3.1 Materials

3,4-Dimethoxycinnamic acid (99%), 3-(3,4-dimethoxyphenyl)propionic acid (99%), glycine methyl ester hydrochloride and ethyl acetate were purchased from Fisher Scientific Ltd. (Loughborough, UK). 3,4-Dimethoxybenzoylchloride, 3,5-dimethoxy-4-hydroxycinnamic acid, vanillic acid, 3-(4-hydroxy-3-methoxyphenyl)propionic acid, 4-methylmorpholine, 1-hydroxybenzotriazole hydrate, *O*-(benzotriazole-1-yl)-*N*,*N*,*N*`,*N*`-tetramethyluronium tetrafluoroborate, acetic acid anhydride, pyridine and thionyl chloride were acquired from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (LC-MS grade) was delivered by VWR International (Lutterworth, UK). Alfa Aesar (Ward Hill, MA, USA) provided anhydrous N,N-dimethylformamide (packed under Argon atmosphere). Water was purified using a Millipore Elix system (Millipore, MA, USA). 3-Caffeoylquinic acid was purchased from Purify Chengdu Biopurify Phytochemicals, Chengdu, China, 4-caffeoylquinic acid was purchased from HWI Analytik, Rülzheim, Germany and 5-caffeoylquinic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA). Nestle Green Blend™ was purchased from a local supermarket.

Feruloylglycine was a generous gift from Prof Alan Crozier, University of Glasgow, UK.

2.3.2 Methods

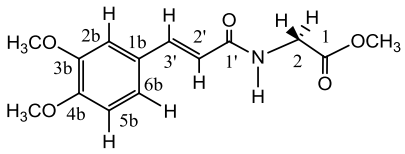
The chemical synthesis was performed in collaboration with Prof Philip Kocienski in the iPDR laboratory at the Department of Chemistry at the University of Leeds, UK. The structure of all intermediates and final compounds was determined by ^{1}H-NMR.

2.3.2.1 Synthesis of 3,4-dimethoxycinnamoylglycine and 3-(3,4-dimethoxyphenyl)-propionylglycine

1.07 g of 3,4-dimethoxycinnamic acid (5 mmol, 1.0 equiv.) or 1.05 g of 3-(3,4-dimethoxyphenyl)propionic acid were weighed in together with 0.65g (5 mmol, 1.0 equiv.) of glycine methyl ester hydrochloride, 0.69 g of 1-hydroxybenzotriazole (5 mmol, 1.0 equiv.), 1.62

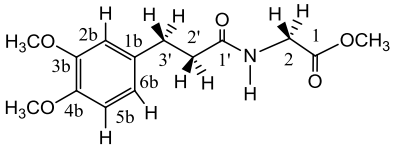
g *O*-(benzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluoroborate (5 mmol, 1.0 equiv.) and were dissolved in 18 mL of anhydrous *N,N*-dimethylformamide under nitrogen atmosphere. To start the reaction, 1.65 mL (15 mmol, 3.0 equiv.) of 4-methylmorpholine were added dropwise through a septum under stirring. After 24 hours the reaction mix was poured onto 150 mL of purified water and extracted three times with a small amount of ethyl acetate. Combined organic phases were washed with 1 M HCl, purified water, saturated sodium hydrogen carbonate solution, purified water and brine. Prior to reduction to dryness in *vacuo* ethyl acetate was dried over sodium sulphate.

Methyl (*E*)-2-(3-(3,4-dimethoxyphenyl)acrylamido)acetate



^{1}H-NMR (500 MHz, $CDCl_3$, ppm): δ 3.76 (3H, *s*, *methoxy* OCH_3-1), 3.87 (3H, *s*, *methoxy* OCH_3 -3b or 4b), 3.88 (3H, *s*, *methoxy* OCH_3 -3b or 4b), 4.18 (2H, *d*, *J*=5.3 Hz, CH_2-2), 6.39 (1H, *d*, *Jtrans* =15.6 Hz, *olefinic* CH=C-2'), 6.56 (1H, *bs*, NH), 6.82 (1H, *d*, *Jortho*=8.3 Hz, C_{ar}H-5b), 7.00 (1H, *d*, J_{meta}=1.8 Hz, C_{ar}H-2b), 7.05 (1H, *dd*, J_1=8.3 Hz, J_2=1.8 Hz, C_{ar}H-6b), 8.53 (1H, *d*, J_{trans} =15.64 Hz, *olefinic* CH=C-3')

Methyl 2-(3-(3,4-dimethoxyphenyl)propanamido)acetate

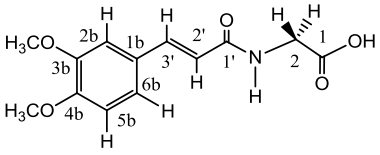


^{1}H-NMR (300 MHz, $CDCl_3$, ppm): δ 2.55 (2H, *t*, *J*=7.4 Hz, CH_2-3'), 2.95 (2H, *t*, *J*=7.4 Hz, CH_2-2'), 3.78 (3H, *s*, *methoxy* OCH_3-1), 3.87 (3H, *s*, *methoxy* OCH_3-3b or 4b), 3.88 (3H, *s*, *methoxy* OCH_3 -3b or 4b), 4.04 (2H, *d*, *J*=5.1 Hz, CH_2-2), 5.97 (1H, *br s*, NH), 6.84-6.73 (3H, m)

The intermediate was dissolved in 30 mL methanol and 6 mL 1 M NaOH and left at room temperature overnight. The solution was concentrated in vacuo and acidified with 25 mL of 1 M HCl to pH 2. The solution was extracted four times with ethyl acetate. Combined organic phases

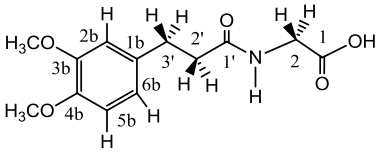
were washed with water and dried over sodium sulphate prior to reduction to dryness to yield fine crystalline powder. The solid was recrystallised from acetronitrile. The overall yields were 18.1% and 17.1% and the melting points 192-193°C and 114-117°C for 3,4-dimethoxycinnamoylglycine and 3-(3,4-dimethoxyphenyl)propionylglycine, respectively.

(*E*)-2-(3-(3,4-Dimethoxyphenyl)acrylamido)acetic acid



^{1}H-NMR (300 MHz, d6-DMSO, ppm): δ 3.78 (3H, *s*, *methoxy* OCH_3 -3b or 4b), 3.80 (3H, *s*, *methoxy* OCH_3 -3b or 4b), 3.89 (2H, *d*, *J*=5.85 Hz, CH_2-2), 6.61 (1H, *d*, J_{trans} =15.7 Hz, *olefinic* CH=C-2'), 6.99 (1H, *d*, J_{ortho}=8.3 Hz, C_{ar}H-5b), 7.14 (1H, *dd*, J_1=8.3 Hz, J_2=1.7 Hz, C_{ar}H-6b), 7.19 (1H, *d*, J_{meta}=1.6 Hz, C_{ar}H-2b), 7.38 (1H, *d*, J_{trans} =15.64 Hz, *olefinic* CH=C-3'), 8.29 (1H, *t*, *J*=5.9 Hz, NH), 12.6 (1H, *bs*, *carboxylic* CO_2H)

2-(3-(3,4-Dimethoxyphenyl)propanamido)acetic acid



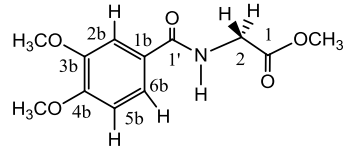
^{1}H-NMR (500 MHz, CD_3OD, ppm): δ 2.55 (2H, *t*, *J*=8.0 Hz, CH_2-3'), 2.89 (2H, *t*, *J*=8.0 Hz, CH_2-2'), 3.82 (3H, *s*, *methoxy* OCH_3 -3b or 4b), 3.84 (3H, *s*, *methoxy* OCH_3-3b or 4b), 3.91 (2H, *s*, CH_2-2), 6.79 (1H, *dd*, J_1=5.3 Hz, J_2=2.0 Hz, C_{ar}H-6b), 6.86 (1H, *bs*, C_{ar}H-2b), 6.87 (1H, *d*, J_{ortho}=5.3 Hz, C_{ar}H-5b)

2.3.2.2 Synthesis of 3,4-dimethoxybenzoylglycine

1.00 g of 3,4-dimethoxybenzoylchloride (5 mmol, 1.0 equiv.) and 0.933 g of glycine methyl ester hydrochloride (7.5 mmol, 1.5 equiv.) were dissolved in 20 mL of dichloromethane. 1.1 mL of 4-methylmorpholine (10 mmol, 2.0 equiv.) were added dropwise and the solution was left for 24 hours.

The reaction mixture was poured onto 100 mL of purified water and acidified slightly prior to triplicate extraction with ethyl acetate. Combined organic phases were neutralised over sodium hydrogencarbonate and dried over sodium sulphate prior to recrystallisation from ethyl acetate.

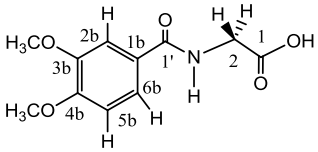
Methyl 2-(3,4-dimethoxybenzamido)acetate



^{1}H-NMR (500 MHz, $CDCl_3$, ppm): δ 3.79 (3H, *s*, *methoxy* OCH_3-1), 3.91 (6H, 2 x *s*, *methoxy* OCH_3-3b and 4b), 4.22 (2H, *d*, *J*=5.3 Hz, CH_2-2), 5.51 (1H, *d*, J_{ortho}=8.35 Hz, C_{ar}H-5b), 6.80 (1H, *dd*, J_1=8.3 Hz, J_2=2.1 Hz, C_{ar}H-6b), 7.64 (1H, *d*, J_{meta}=2.0 Hz, C_{ar}H-2b

The dried product was dissolved in 30 mL of methanol containing 8 mL of 1 M NaOH and left at room temperature overnight. The solution was acidified with 1 M HCl to pH 2 and extracted four times with ethyl acetate. The compound was recrystallised from water to yield fine, shiny plates. The yield was 28.3% and the melting point 190-195°C.

2-(3,4-Dimethoxybenzamido)acetic acid



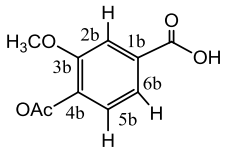
^{1}H-NMR (500 MHz, CD_3OD, ppm): δ 3.33 (1H, *s*, *fine splitting*, NH), 3.91 (6H, 2 x *s*, *methoxy* OCH_3-3b and 4b), 4.11 (2H, *s*, CH_2-2), 4.89 (1H, *bs*, NH), 7.04 (1H, *d*, J_{ortho}=8.4 Hz, C_{ar}H-5b), 7.5 (1H, *d*, J_{meta}=2.0 Hz, C_{ar}H-2b), 7.52 (1H, *dd*, J_1=8.35 Hz, J_2=2.0 Hz, C_{ar}H-6b)

2.3.2.3 Synthesis of vanilloylglycine

Under stirring acetic acid anhydride (83 mmol, 2.8 equiv) was added to a solution of vanillic acid (29.7 mmol) in pyridine (10 mL). The mixture was stirred for 3 h at room temperature and poured onto ice water (150 mL). The viscous product was extracted with ethyl acetate (100 mL). The solution was washed with 2 M HCl (100 mL) and water (50 mL). The extract was

dried using sodium sulphate and concentrated in *vacuo*. The pale yellow solid was recrystallised from ethyl acetate and methyl-tert-butylether yielding a beige solid (19.5 mmol, 65%). The melting point was 146-149°C.

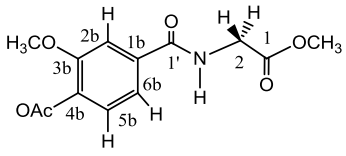
4-Acetoxy-3-methoxybenzoic acid



^{1}H-NMR (500 MHz, $CDCl_3$, ppm): δ 2.37 (3H, *s*), 3.95 (3H, *s*, *methoxy* OCH_3-3b), 7.17 (1H, *d*, J_{ortho}=8.2 Hz, C_{ar}H-5b), 7.74 (1H, *d*, J_{meta}=1.8 Hz, C_{ar}H-2b), 7.78 (1H, *dd*, J_1=8.2 Hz, J_2=1.8 Hz, C_{ar}H-6b)

The above product was dissolved in chloroform (25 mL) at ambient temperature with a small amount of dimethylformamide. Thionyl chloride (20 mmol, 1.2. equiv.) was added and heated under reflux. After half an hour the solvent was removed *in vacuo*. The yellow, oily product was dissolved in pyridine (10 mL) in an ice bath and glycine methyl ester hydrochloride (20 mmol, 1.2 equiv.) was added under stirring. After 1 hour the mixture was poured into water (150 mL) and the product extracted with ethyl acetate (75 mL). Combined organic layers were washed with 2 M HCl (100 mL), water (50 mL), saturated aqueous sodium hydrogen carbonate (25 mL) and brine (25 mL). The organic layer was dried over sodium sulphate and concentrated in vacuo. The yellow-orange oil was purified chromatographically using silica gel and ethyl acetate and petrol. The yield was 10.7 mmol equivalent to 63% yield.

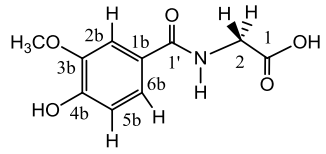
Methyl 2-(4-acetoxy-3-methoxybenzamido)acetate



^{1}H-NMR (500 MHz, CDCl3, ppm): δ 2.33 (3H, *s*, *methoxy* OCH_3-1), 3.79 (3H, *s*, *methoxy* OCH_3-4b), 3.86 (3H, *s*, *methoxy* OCH_3-3b), 4.20 (2H, *d*, *J*=5.2 Hz, C_2H2), 6.92 (1H, *br t*, *J*=4.7 Hz, NH), 7.07 (1H, *d*, *J*=8.2 Hz, C_{ar}H-5b), 7.32 (1H, *dd*, J_1=8.2 Hz, J_2=1.7 Hz, C_{ar}H-6b), 7.50 (1H, *d*, *J*=1.7 Hz, C_{ar}H-2b)

The estrified product (10.7 mmol) was solved in methanol (15 mL) and potassium hydroxide (30 mmol, 2.8 equiv.) was added dropwise in 2 mL of water. The solution was left at room temperature for 24 hours. Methanol was evaporated in *vacuo* and 6 M HCl (6 mL) were added prior to extraction with ethyl acetate (100 mL). Combined organic layers were washed with water (2 x 10 mL), dried over sodium sulphate and the solvent removed in vacuo. Methyl-tert-butyl ether was added to the yellow oil for solidification. The colour was removed by solving the product in methanol (20 mL) which was run over a charcoal filter. Methanol was removed in vacuo and the solid was dissolved in ethyl acetate and methyl-tert-butyl ether (20 mL 1:1). Overnight the product was kept at -20ºC which induced the formation of white fine crystals. The product was dried under vacuum at 80ºC. The yield was 4.9 mmol equivalent to 46%. The melting point was 165-168ºC.

2-(4-Hydroxy-3-methoxybenzoamido)acetic acid



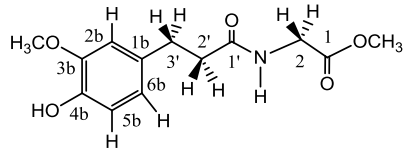
^{1}H-NMR (500 MHz, DMSO-d_6, ppm): δ 3.82 (3H, *s*, *methoxy* OCH_3), 3.91 (2H, *d*, *J*=5.85 Hz, $C2H_2$), 6.83 (1H, *dd*, J_1=8.2 Hz, J_2=1.0 Hz, $C^{ar}H$-6b), 7.37 (1H, *d*, *J*=8.55 Hz, $C^{ar}H$-5b), 7.44 (1H, *app s*, $C^{ar}H$-2b), 8.62 (1H, *br t*, *J*=4.9 Hz, NH), 9.60 (1H, *br s*, OH) 12.54 (1H, *br s*, COOH)

2.3.2.4 Synthesis of dihydroferuloylglycine

0.98 g of 3-(4-hydroxy-3-methoxyphenyl)propionic acid (5 mmol, 1.0 equiv.) was weighed along with 0.65g (5 mmol, 1.0 equiv.) of glycine methyl ester hydrochloride, 0.69 g of 1-hydroxybenzotriazole (5 mmol, 1.0 equiv.), 1.62 g *O*-(benzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluoroborate (5 mmol, 1.0 equiv.) and were solved in 18 mL of anhydrous N,N-dimethylformamide in a nitrogen atmosphere. To start the reaction, 1.65 mL (15 mmol, 3.0 equiv.) of 4-methylmorpholine was added dropwise through a septum whilst magnetically stirring. After 24 hours the reaction mix was poured onto 50 mL of purified water

and extracted with ethyl acetate (5 x 15 mL). Combined organic phases were washed with 2 M HCl, saturated sodium hydrogen carbonate solution, purified water and brine. Prior to evaporation to dryness in *vacuo* ethyl acetate was dried over sodium sulphate.

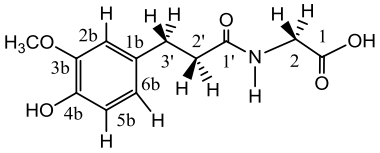
Methyl 2-(3-(4-hydroxy-3methoxyphenyl)propanamido)acetate



^{1}H-NMR (500 MHz, $CDCl_3$, ppm): δ 2.45 (2H, *t*, *J*=7.5 Hz, CH_2-3'*)*, 2.84 (2H, *t*, *J*=7.5 Hz, CH_2-2'), 3.68 (3H, *s*, *methoxy* OCH_3), 3.79 (3H, *s*, *methoxy* OCH_3), 3.96 (2H, *d*, *J*=5.2 Hz, CH_2-2), 5.88 (1H, *broad singlet*), 6.62 (1H, *dd*, J_1= 8.8 Hz, J_2= 1.9 Hz, C^{ar}H-6b), 6.65 (1H, *d*, J_{meta}= 1.8 Hz, C_{ar}H-2b), 6.76 (1H, *d*, J_{ortho}=8.8 Hz, C_{ar}H-5b)

The ester (4.6 mmol) was solved in methanol (10 mL) and 5 M sodium hydroxide solution (2 mL) were added. The solution was kept at ambient temperature for 24 hours, thereafter the solvent was removed *in vacuo* and the pH was adjusted to 2-3 with 5 M HCl. The product was extracted with ethyl acetate (5 x 5 mL). Combined organic phases were dried over sodium sulfate and the solvent evaporated in *vacuo*. The product crystallised at -20°C and was recrystallised from ethyl acetate and purified with a charcoal filter. The yield was 38% with a melting point of 148-150°C.

2-(3-(4-Hydroxy-3-methoxyphenyl)propanamido)acetic acid



^{1}H-NMR (500 MHz, CD_3OD, ppm): δ 2.54 (2H, *t*, *J*=8.1 Hz, CH_2-3'), 2.87 (2H, *t*, *J*=8.1 Hz, CH_2-2'), 3.86 (3H, *s*, *methoxy* OCH_3-1), 3.91 (3H, *s*, *methoxy* OCH_3-3b), 6.67 (1H, *dd*, J_1=8.0 Hz, J_2= 1.9 Hz, C_{ar}H-6b), 6.72 (1H, *d*, J_{ortho}=8.0 Hz, C_{ar}H-5b), 6.82 (1H, *d*, J_{meta}=1.7 Hz, C_{ar}H-2b

2.3.2.5 Study design

Six subjects (n=6), three female and three male, age 21 to 54 years, body mass index 22.9 +/- 2.4 kg/m^2, gave their written informed consent prior to the beginning of the human study. They were all in good health, non-smokers and did not take any medication as assessed by a health questionnaire (Appendix D: Health assessment and food frequency questionnaire).

Volunteers were asked to abstain from polyphenol-rich food for 36 hours prior to the study (wash-out) and 36 hours during the study. After an overnight fast for 10 hours, participants consumed 4 g of instant coffee solved in 200 mL of hot, but not boiling, water with a standardised breakfast consisting of two slices of toast with margarine and cheese. Water was allowed *ad libitum*.

The study design was approved by the University of Leeds Joint Faculty Ethics Committee (MEEC 10-035).

2.3.2.6 Sample collection and storage

Six subjects collected a baseline urine sample prior to the consumption of the coffee. Thereafter all urine was collected in time increments of 0-4, 4-8, 8-12, 12-24 and 24-36 hours in urine collection containers containing 3 g of ascorbic acid. Urine was centrifuged at 800 g and 4°C for 10 minutes within 48 hours after collection. The supernatant was stored at -20°C in aliquots of 13 mL containing 130 µL of a 10% aqueous sodium azide solution.

2.3.2.7 Sample preparation

Instant coffee (2 g) was dissolved in 200 mL demineralised water (90-95ºC). The coffee solution was diluted 1:5 with mobile phase A (95% demineralised water, 5% acetonitrile, 0.1% formic acid) and chilled at 4°C. Aliquots were centrifuged at 17,000 g and 4°C for 10 minutes. The supernatant was filtered through 0.2 µm syringe-driven PTFE filters. Three stock solutions of coffee were prepared and two aliquots were taken for the analysis.

Urine samples were thawed at 4°C and two aliquots of 990 µL were taken for each time point. 10 µL of internal standard solution (dihydrodimethoxycinnamoyl glycine, final concentration 10

µM), 10 µL of 50% formic acid and 1 mL of ethyl acetate were added prior to vortexing the samples for 1 minute. The samples were left for 15 minutes, vortexed for 1 minute and centrifuged at 2000 g at 4°C for 1 minute. 750 µL of the organic layer was taken. The extraction was repeated a second time. Combined organic layers were dried at ambient temperature under a gentle stream of nitrogen. The sample was reconstituted in 100 µL mobile phase A (95% ultrapure water, 5% acetonitrile and 0.1% formic acid) by sonication for 5 minutes.

Samples were prepared to determine the extraction efficiencies of six glycine conjugates. Therefore pooled urine collected from six participants collected after a 36 hours washout period, which is described in detail in chapter 4, was used for spiking. Urine (950 µL) and 50 µL of the standard solution to yield concentrations of 1, 5 and 10 µM was mixed. All samples were prepared in triplicate and processed as described above.

2.3.2.8 High performance liquid chromatography-mass spectrometry

The high performance liquid chromatography system was the Agilent 1200 series (Waldbronn, Germany) with a degasser, binary pump, temperature controlled autosampler and column compartment, and a diode array detector.

The Agilent 1200 series HPLC was coupled through a divert valve and a heated electrospray ionisation source to an Agilent 6410 A triple quadrupole mass spectrometer (Wokingham, UK).

The drying gas was nitrogen generated by a PEAK Scientific nitrogen generator (Inchinnan, UK).

Chromatographic settings for caffeoylquinic acid were used from a previously published method by T. Farrell *et al.*, 2011[68]. In brief, chromatographic separation was achieved on an Agilent ZORBAX Eclipse Plus Rapid Resolution C18 100 mm x 2.1 mm i.d., 1.8 µm using a reverse phase method consisting of mobile phase A (95% demineralised water, 5% acetonitrile, 0.1% formic acid) and mobile phase B (95% acetonitrile, 5% demineralised water, 0.1% formic acid) at a flow rate of 0.26 mL/min at 30°C. Elution was achieved using a gradient of 0% B for the first 17.3 minutes, up to 51.0 minutes B was increased to 25%, from 51.1 to 56.0 minutes the gradient was kept at 100% B for washing the column, prior to reequilibration from 56.1 to 65.0 minutes at 0% B. Samples were kept at 4°C and 5 µL injected onto the column in triplicate.

Chromatographic separation of glycine conjugates was achieved on an Agilent ZORBAX Eclipse Plus Rapid Resolution C18 100 mm x 2.1 mm i.d., 1.8 µm using a reverse phase method consisting of mobile phase A (95% demineralised water, 5% acetonitrile, 0.1% formic acid) and mobile phase B (95% acetonitrile, 5% demineralised water, 0.1% formic acid) at a flow rate of 0.3 mL/min at 30°C. Elution was achieved using a gradient from 0-35% B in 3 minutes, 35 % B from 3 to 6 minutes, 100% B from 6-11 minutes and reequilibration at 0% B form 11-17 minutes. Samples were kept at 4°C and 10 µL injected onto the column in triplicate.

2.3.2.9 Mass spectrometry

For the ionisation of caffeoylquinic acids the heated electrospray ionisation source was operated in negative mode with the gas flow set to 10 L/min, a nebuliser pressure of 60 psi at a temperature of 350°C. Multiple reaction monitoring (MRM) settings were optimised using commercially available standards at a concentration of 10 µg/mL. The following table (Table 2.1) shows the optimised settings for the individual compounds.

Table 2.1: Multiple reaction monitoring settings optimized for the individual caffeoylquinic acid

Compound	Retention time (min)	Precursor ion (m/z)	Fragmentor energy	Product ion (m/z)	Collision energy
3-Caffeoylquinic acid	6.2	353	120	191 179	20 20
5-Caffeoylquinic acid	14.1	353	120	191 179	10 20
4-Caffeoylquinic acid	19.4	353	120	179 173	15 15

The first four minutes of the LC flow were sent to waste, from 4-8 minutes the settings for 3-CQA, from 8-16 minutes the settings for 5-CQA and from 16 to 55 minutes the settings for 4-CQA were used, respectively. After 55 minutes the polarity was switched to positive to neutralise a potential charge accumulation in the ion capillary.

The heated electrospray ionisation source was operated in negative mode at optimised settings for feruloylglycine with the gas flow set to 13 L/min, a nebuliser pressure of 60 psi at a temperature of 350°C. Multiple reaction monitoring (MRM) settings were optimised using synthesised standards at a concentration of 10 µg/mL. The following table (Table 2.2) shows the optimised settings for the individual compounds.

Table 2.2: Multiple reaction monitoring settings optimised for the individual glycine conjugate

Compound	Retention time (min)	Precursor ion (m/z)	Fragmentor energy (V)	Product ion (m/z)	Collision energy (V)
Vanilloylglycine	4.2	224	130	100 123	6 10
Dihydroferuloylglycine	4.6	252	110	74 121	12 28
3,4-Dimethoxybenzoylglycine	5.0	238	110	194 122	5 25
Feruloylglycine	5.0	250	100	206 134	6 16
3-(3,4-Dimethoxyphenyl)propionyl-glycine	5.4	266	160	74 149	12 18
3,4-Dimethoxycinnamoylglycine	5.8	264	160	220 103	5 15

2.3.2.10 Quantitative analysis caffeoylquinic acids

External calibration was used to quantify the three caffeoylquinic acids. Calibration curves were run on day 1 and day 3 including nine calibrants (0.5, 1, 2.5, 5, 10, 25, 50, 100 and 200 µM) prepared on the individual days. The linarity was $R^2 \geq 0.99$. The limit of detection (LOD) and the limit of quantitation (LOQ) are calculated by LOD=(3.3*intercept)/slope and LOQ=(10*intercept)/slope, respectively. The values are presented in Table 2.3.

Table 2.3: Summary of limit of detection and limit of quantitation of 3-, 4-, and 5-caffeoylquinic acid in pmol

Compound	Limit of detection (pmol)	Limit of quantitation (pmol)
3-Caffeoylquinic acid	3.0	9.1
4-Caffeoylquinic acid	2.0	5.9
5-Caffeoylquinic acid	14.8	44.7

Three coffee samples were prepared and two aliquots taken for each samples which were injected in triplicate on day 2.

2.3.2.11 Quantitative analysis glycine conjugates

Calibration curves were prepared on the day of the analysis and included nine calibrants (0.1, 0.5, 1, 5, 10, 20, 40, 60, 80 and 100 µM). 10 µL of each calibrant was injected in triplicate. The calibration curves were run on three different days (Day 1, 3 and 5) with a linearity of $R^2 \geq 0.99$. The limit of detection (LOD) and the limit of quantitation (LOQ) are calculated by LOD=(3.3*intercept)/slope and LOQ=(10*intercept)/slope, respectively. The values are presented in Table 2.4.

Table 2.4: Summary of limit of detection and limit of quantitation of vanilloylglycine, dihydroferuloylglycine, 3,4-dimethoxybenzoylglycine, feruloylglycine, 3-(3,4-dimethoxyphenyl)propionylglycine and 3,4-dimethoxycinnamoylglycine in pmol

Compound	Limit of detection (pmol)	Limit of quantitation (pmol)
Vanilloylglycine	6.6	20.0
Dihydroferuloylglycine	37.2	112.6
3,4-Dimethoxybenzoylglycine	128.9	390.7
Feruloylglycine	5.1	15.4
3-(3,4-Dimethoxyphenyl)propionyl-glycine	2.6	7.9
3,4-Dimethoxycinnamoylglycine	74.0	224.2

Urine samples were prepared in duplicate and 10 µL of each sample was injected in triplicate on two different days (Day 2 and 4).

2.4 Results

2.4.1 Synthesis

Several hundred milligrams of 3,4-dimethoxycinnamoylglycine, 3-(3,4-dimethoxyphenyl)propionylglycine, 3,4-dimethoxybenzoylglycine, dihydroferuloylglycine and vanilloylglycine were chemically synthesised and characterised by ^{1}H-NMR and MS/MS with a purity of ≥95% derived from the ^{1}H-NMR spectra. According to our knowledge 3-(3,4-dimethoxyphenyl)propionylglycine and dihydroferuloylglycine are new compounds and have never been described in literature.

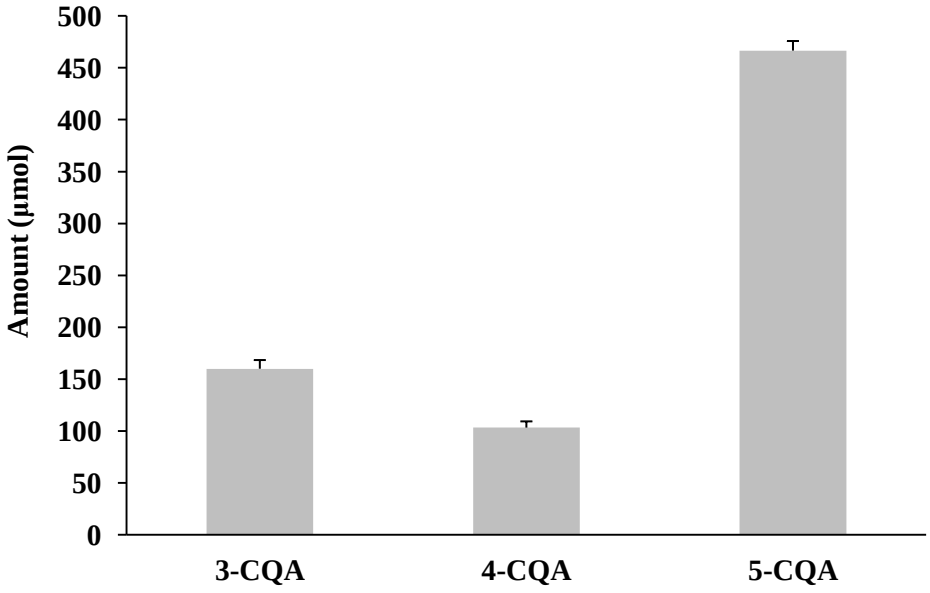
3,4-dimethoxycinnamoylglycine, 3,4-dimethoxybenzoylglycine, dihydroferuloylglycine, feruloylglycine and vanilloylglycine were used as standards for the preparation of calibration curves for external calibration and quantitation. The amounts synthesised are practical for weighing, i.e. several milligram (2-3 mg), providing high accuracy.

2.4.2 Caffeoylquinic acid content in instant coffee

3-, 4- and 5-caffeoylquinic acids were quantitatively analysed in instant coffee.

Figure 2.2 depicts the individual amounts of caffeoylquinic acid in 4 g of instant coffee. The amount of 3-, 4- and 5-caffeoylquinic acids is 160 ± 8.4, 103 ± 6.0 and 467 ± 9.2 μmol, respectively. Participants ingested a total amount of caffeoylquinic acids of 730 ± 23.7 μmol. The quantified amounts of caffeoylquinic acids are in line with previous findings [16].

Figure 2.2: Caffeoylquinic acid content in µmol in 4g instant coffee equivalent to one cup of coffee which was consumed by the participant, n=18 (three samples, two aliquots, three injections), mean ± standard deviation

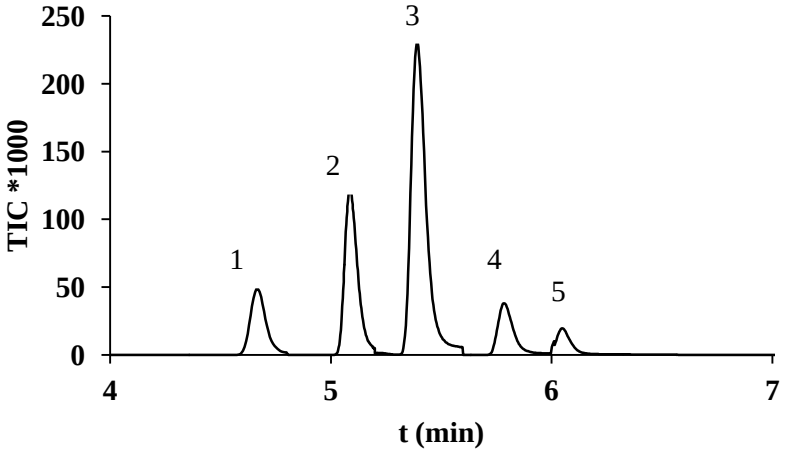


2.4.3 Novel glycine conjugates in urine post coffee consumption

The synthesised glycine conjugates serve as standards for unequivocal qualitative and quantitative analysis of caffeoylquinic acid metabolites in urine post coffee consumption. Therefore the compounds were used to determine the retention times, optimise multiple reaction monitorings and for quantitation by external calibration.

Figure 2.3 depicts the chromatographic separation of the total ion chromatogram.

Figure 2.3: Total ion chromatogram for six glycine conjugates including the internal standard, 10 µL injected of a solution of 1 µmol/L of vanilloylglycine (1), dihydroferuloylglycine (2), 3,4-dimethoxybenzoylglycine (3), feruloylglycine (3), 3,4-dimethoxycinnamoylglycine (4), and 3-(3,4-dimethoxyphenyl)propionylglycine (5, internal standard).



The total ion chromatogram shows five peaks, however, six compounds were analysed. 3,4-dimethoxybenzoylglycine and feruloylglycine coelute, but can be distinguished using their specific multiple reaction monitoring which are $(M-H)^-$ m/z 238-194 and 250-134, respectively.

Figure 2.4 depicts the single reaction monitoring of vanilloylglycine m/z 224-100 which was used as the quantifier transition since it yielded the highest intensity transition.

Figure 2.4: Single reaction monitoring for vanilloylglycine $(M-H)^-$ m/z 224-100, 10 µL injected of a solution of 1 µmol/L

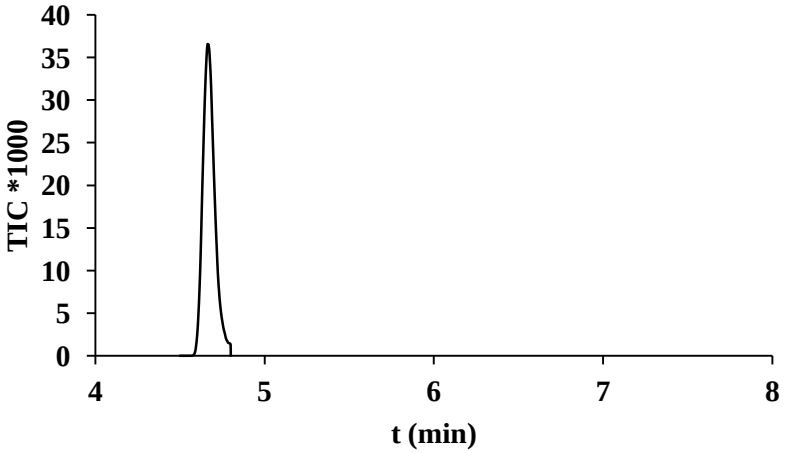


Figure 2.5 depicts the product ion spectrum of vanilloylglycine. The peak at m/z 100 is the glycine with the acid-amide bond fragment, the peak m/z 123 is the phenol moiety with a methoxy group. The single reaction monitoring m/z 224-123 is used as the qualifier transition. The minor peaks could not have been identified.

Figure 2.5: Product ion spectrum of vanilloylglycine showing the precursor peak at $(M-H)^-$ m/z 224 and the two product ions at m/z 123 and 100, respectively, at a collision energy of 5 V

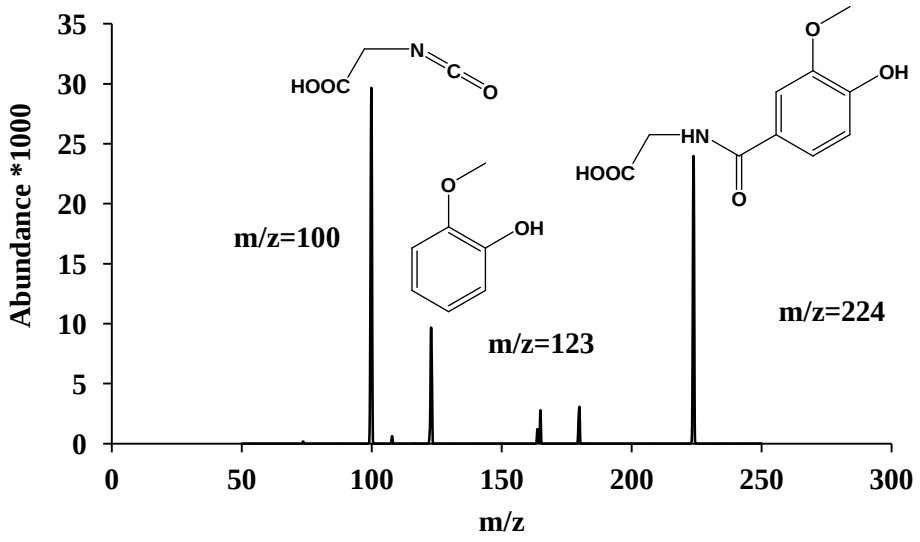
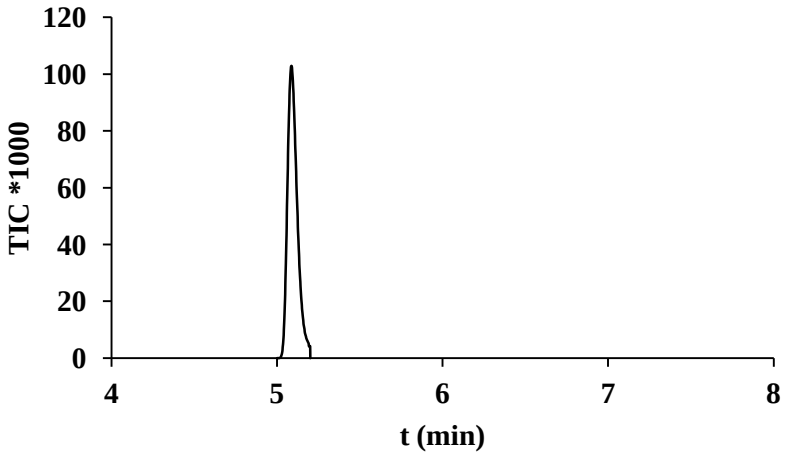


Figure 2.6 shows the single reaction monitoring of dihydroferulic acid m/z 252-74 which was used as the quantifier transition.

Figure 2.6: Single reaction monitoring for dihydroferuloylglycine $(M-H)^-$ m/z 252-74



In Figure 2.7 the product ion spectrum of dihydroferulic acid is depicted. The peak at m/z 74 is the glycine moiety. The underlying structure of peak at m/z 121 has not been identified, but is used as the qualifier transition.

Figure 2.8 depicts the single reaction monitoring of dimethoxybenzoylglycine m/z 238-194 which was used as the quantifier transition.

Figure 2.7: Product ion spectrum of dihydroferuloylglycine depicting the precursor peak at $(M-H)^-$ m/z 252 and two product ions at m/z 121 and 74, respectively, at a collision energy of 5 V

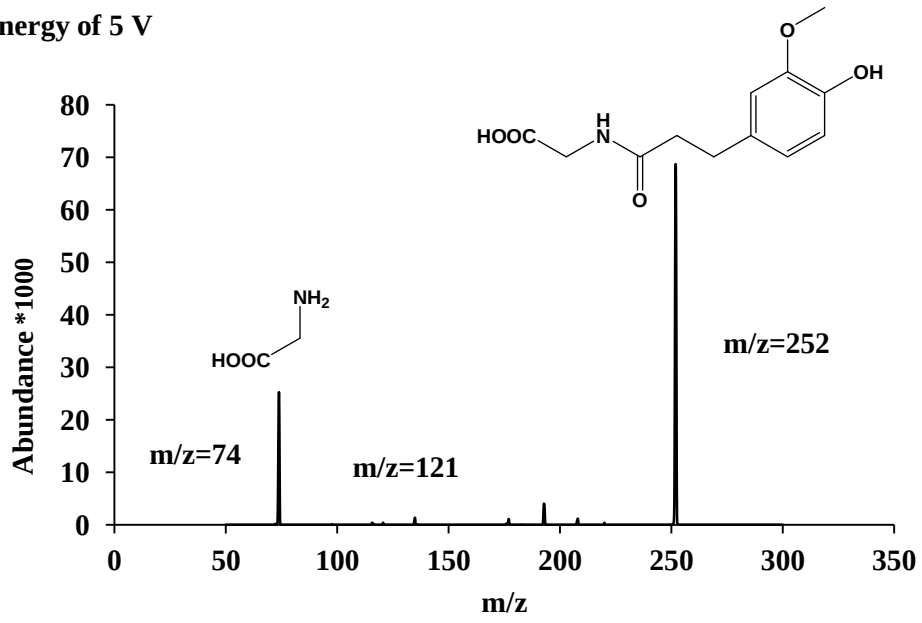
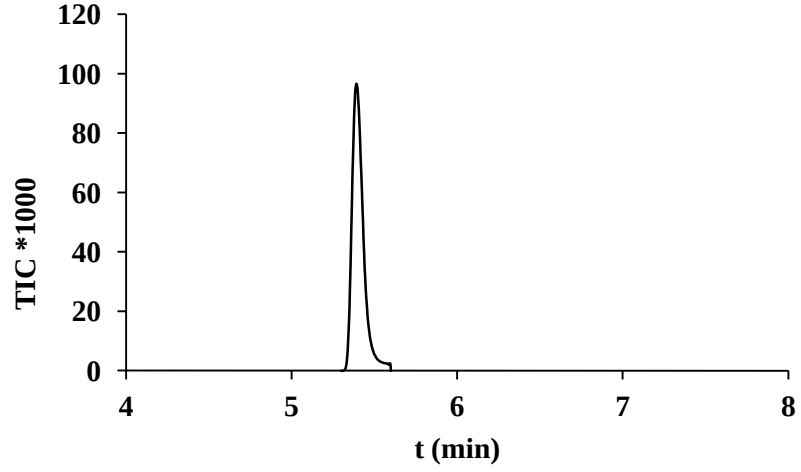


Figure 2.8: Single reaction monitoring for dimethoxybenzoylglycine $(M-H)^-$ m/z 238-194



In Figure 2.9 the product ion spectrum of dimethoxybenzoylglycine is depicted. The peak at m/z 194 is the precursor molecule minus the m/z 44 which indicates decarboxylation. The qualifier peak at m/z 122 was not identified.

Figure 2.9: Product ion spectrum of dimethoxybenzoylglycine depicting the precursor peak at $(M-H)^-$ m/z 238 and two product ions at m/z 194 and 122, respectively, at a collision energy of 5 V

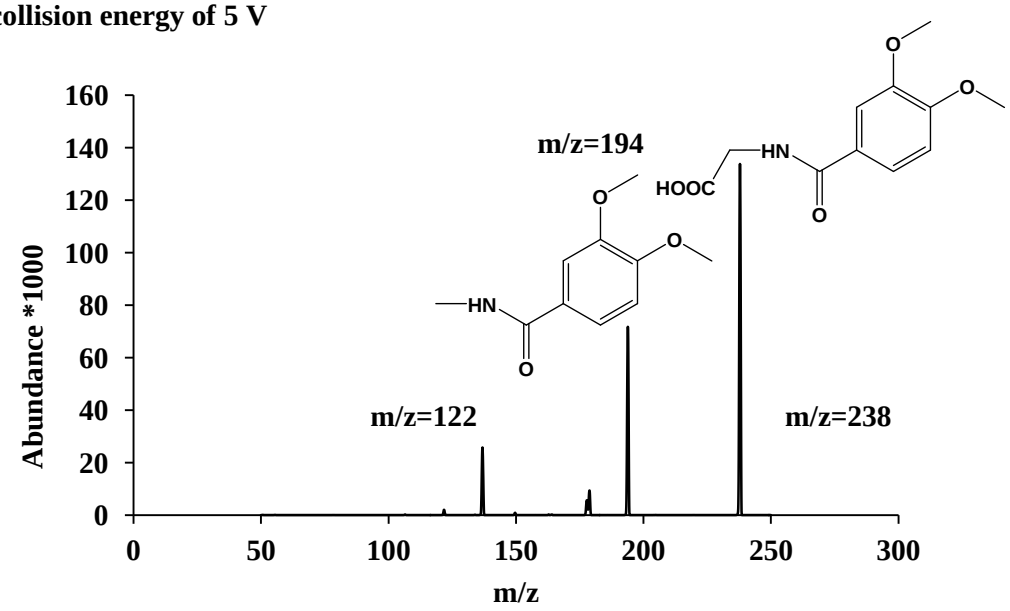


Figure 2.10 shows the single reaction monitoring of feruloylglycine m/z 250-134, the quantifier transition. The retention time is the same as of dimethoxybenzoylglycine. As the the peaks coelute there is a need to distinguish them, based on the different single reaction monitoring.

Figure 2.10: Single reaction monitoring of feruloylglycine $(M-H)^-$ m/z 250-134

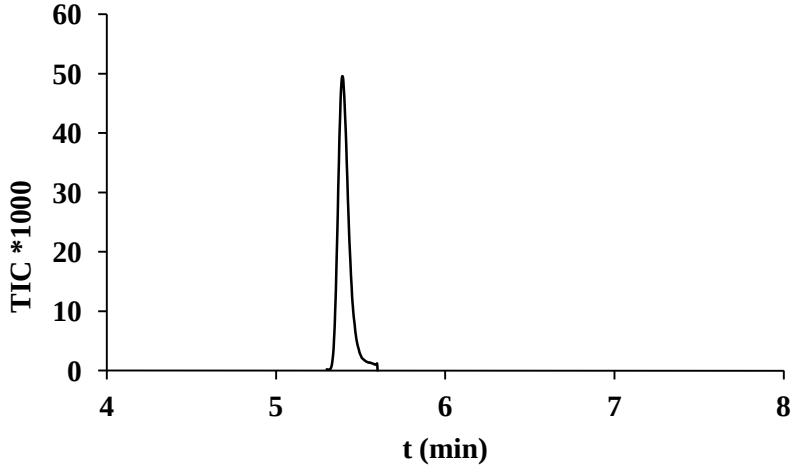
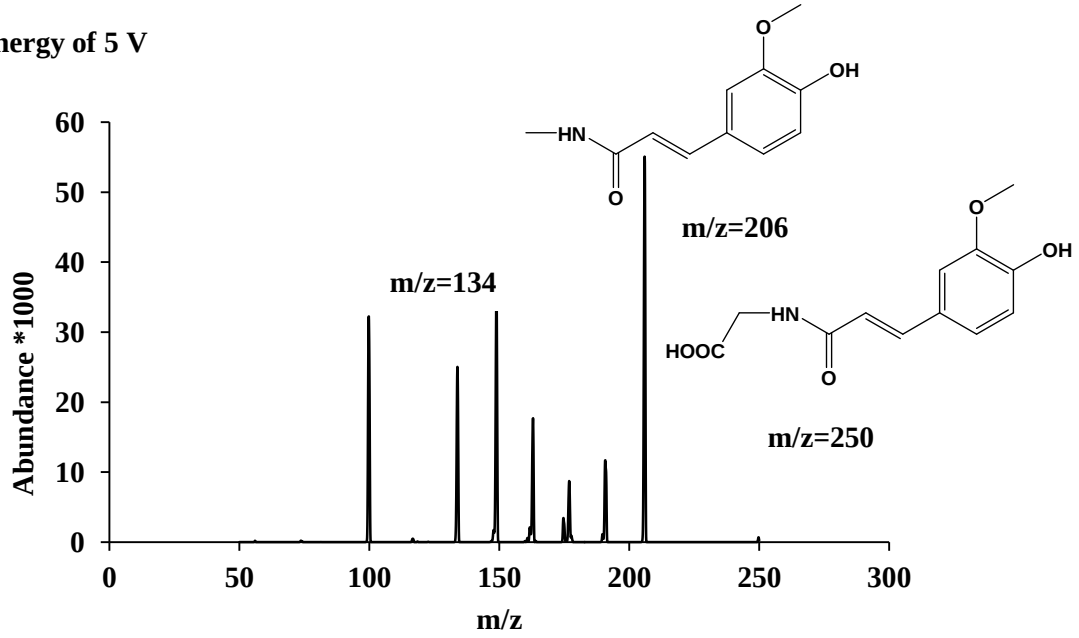


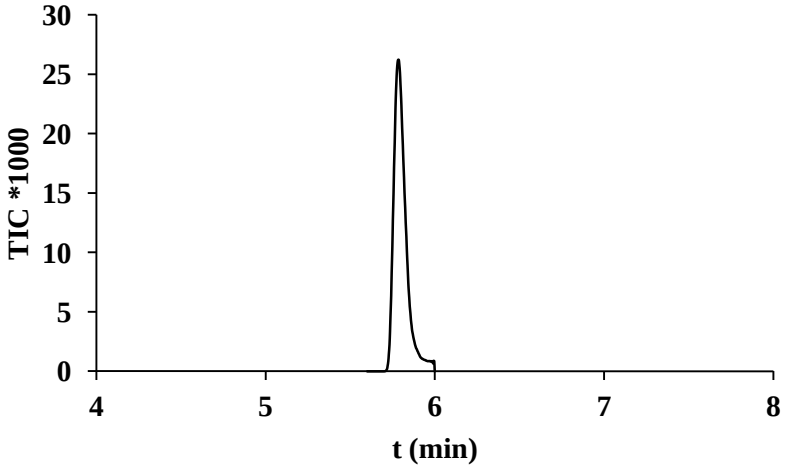
Figure 2.11 shows the product ion spectrum of feruloylglycine with the quantifier peak at m/z 206 and the qualifier peak at m/z 134. The peak at m/z 206 is the precursor structure which has been decarboxylated (-CO_2), the qualifier peak's structure has not been identified.

Figure 2.11: Product ion spectrum of feruloylglycine with a completely depleted precursor ion at $(M-H)^-$ m/z 250 and two product ions at m/z 206 and 134, respectively, at a collision energy of 5 V



The following figure shows the single reaction monitoring of the 3-(3,4-dimethoxyphenyl)propionyl glycine, the dihydro form of 3,4-dimethoxycinnamoyl glycine with m/z 266-74.

Figure 2.12: Single reaction monitoring of 3-(3,4-dimethoxyphenyl)propionylglycine $(M-H)^-$ m/z 266-74



The product ion spectrum (Figure 2.13) depicts the two major fragment peaks at m/z 149 and m/z 74. The latter is the quantifier peak at m/z 74 which is the glycine fragment. The qualifier peak at m/z 149 is the phenol ring with the C3-tail.

Figure 2.13: Product ion spectrum of 3-(3,4-dimethoxyphenyl)propionylglycine depicting the precursor peak at $(M-H)^-$ m/z 266 and two product peaks at m/z 149 and 74, respectively, at a collision energy of 5 V

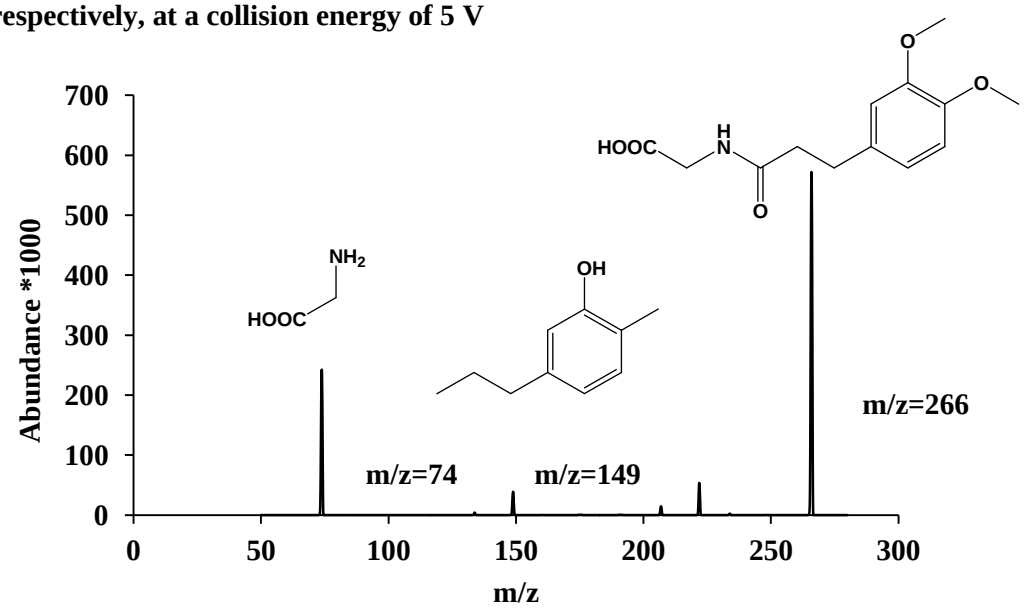


Figure 2.14 shows the single reaction monitoring of 3,4-dimethoxycinnamoylglycine m/z 264-220, the quantifier transition.

Figure 2.14: Single reaction monitoring for 3,4-dimethoxycinnamoylglycine $(M-H)^-$ m/z 264-220

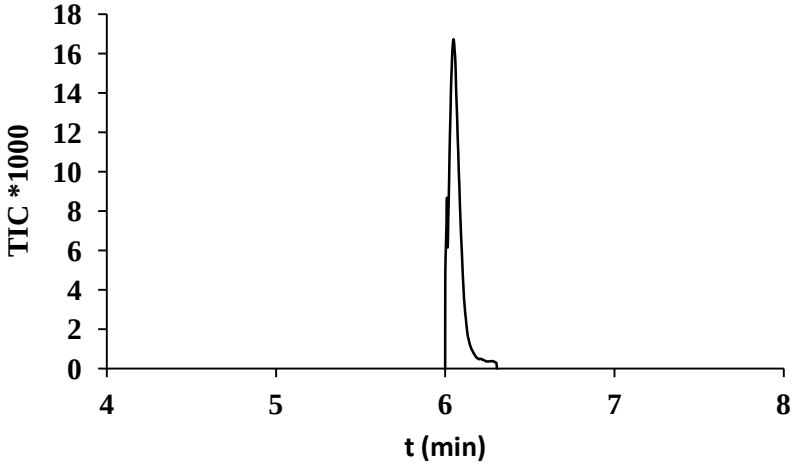
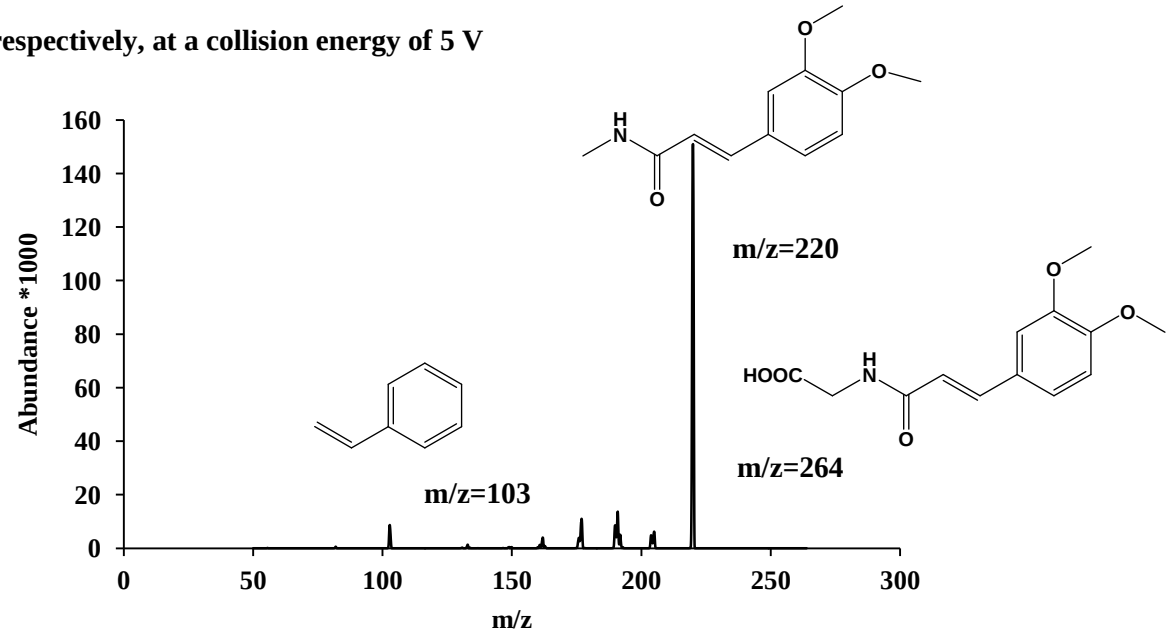


Figure 2.15 shows the product ion spectrum of 3,4-dimethoxycinnamoyl glycine. The quantifier peak at m/z 220 and the qualifier peak at m/z 103. The first peak is the decarboxylated form of the precursor peak and the latter is the benzene ring with a C2 tail. The precursor peak (m/z=264) is completely depleted.

Figure 2.15: Product ion spectrum of 3,4-dimethoxycinnamoylglycine with a completely depleted precursor ion at $(M-H)^-$ m/z 264 and two product ions at m/z 220 and 103, respectively, at a collision energy of 5 V



2.4.4 Glycine conjugates in urine

The recovery of six glycine conjugated urinary metabolites of caffeoylquinic acid and its derivatives were assessed and the results are depicted in Table 2.5.

Table 2.5: Recovery (%) of six urinary metabolites and internal standard at 1, 5, 10 µM, n=9 (three samples, three injections), mean ± standard deviation

	VA-GLY	DHFA-GLY	DMB-GLY	FA-GLY	DHDM-GLY	DMCA-GLY
1 µM	71.4 ± 1.4	67.3 ± 1.4	67.5 ± 1.5	112.5 ± 1.3	65.2 ± 2.3	117.2 ± 3.6
5 µM	63.2 ± 1.5	62.3 ± 2.1	72.5 ± 1.9	115.8 ± 1.8	72.3 ± 1.5	109.8 ± 2.7
10 µM	67.4 ± 1.5	75.4 ± 2.0	79.6 ± 2.2	114.1 ± 1.5	81.1 ± 2.5	115.9 ± 3.1

There is an overall tendency that the recovery rates are below the spiked amount, which could be due to insufficient extraction or ion suppression effects in the electrospray ionisation source. However, the recovery for feruloylglycine and dimethoxycinnamoylglycine are overall higher and exceed the spiked amount in the sample. This could be due to ion enhancement effects in the electrospray ionisation source.

Five of six participants completed the human study. One participant was excluded due to non-compliance with the study guidelines since its excretion exceeded 100% of the ingested dose of caffeoylquinic acids. Vanilloylglycine and feruloylglycine were detected and quantified as the only glycine metabolites. Dihydroferuloylglycine, 3,4-dimethoxybenzoylglycine, and 3,4-dimethoxycinnamoylglycine were detected, but not quantified as their concentrations were below the limit of quantitation. Feruloylglycine is the highest metabolite followed by vanilloylglycine. Vanilloylglycine has been reported for the first time as an urinary metabolite of coffee consumption or caffeoylacid intake.

Figure 2.16: Urinary excretion of vanilloylglycine (µmol) for individual subject (n=5) for 0-4, 4-8, 8-12, 12-24, and 24-36 hours post coffee consumption, n=6 (two biological replicates, triplicate injections), mean ± standard deviation

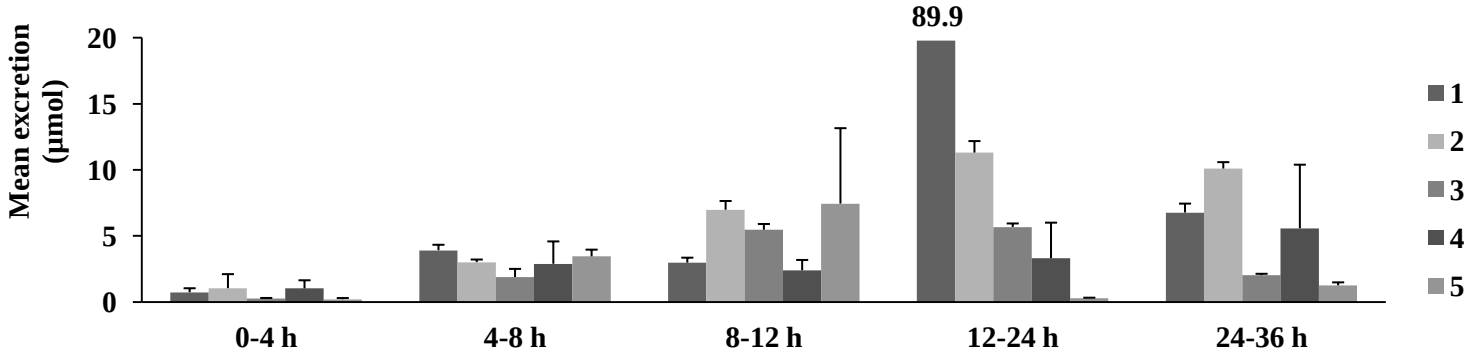
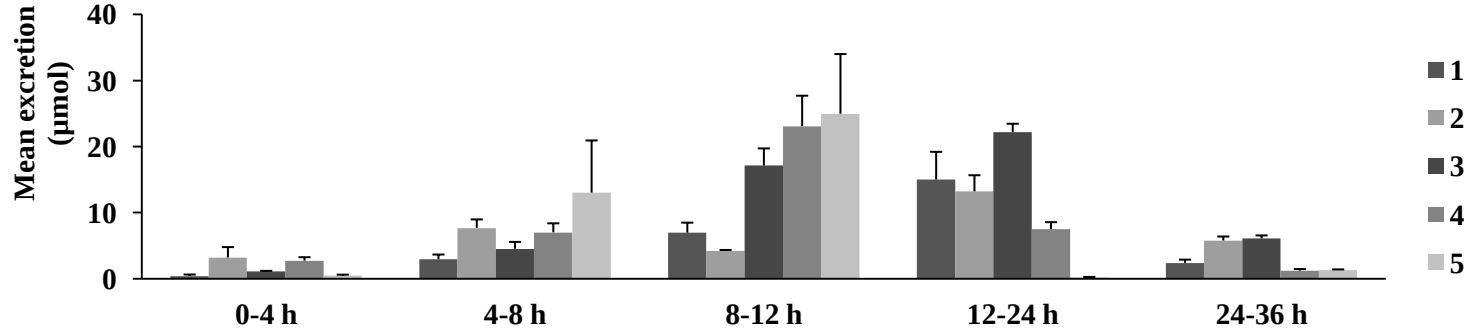


Figure 2.17: Urinary excretion of feruloylglycine (µmol) for individual subject (n=5) for 0-4, 4-8, 8-12, 12-24, and 24-36 hours post coffee Consumption, n=6 (two biological replicates, triplicate injections), mean ± standard deviation



Graph (Figure 2.16) depicts the vanilloylglycine excretion in urine per collection interval per subject. Most vanilloylglycine was excreted between 12-24 hours post coffee consumption, however, substantial amounts have also been excreted between 8-12 and 24-36 hours. Hypothetically, more vanilloylglycine would be excreted after 36 hours, however, only small amounts of vanilloylglycince have been detected in the baseline urine sample. The graph also depicts the interindividual variation of the amounts excreted.

Graph (Figure 2.17) shows the feruloylglycine excretion in urine per collection interval per subject. Most feruloylglycine was excreted between 8-12 hours post coffee consumption, however, substantial amounts were also excreted between 12-24 hours.

Graph (Figure 2.18) shows the cumulated vanilloylglycine for the five subjects for the individual urine collection intervals. The average total amount excreted for the five subjects is 33.0 ± 32.4 μmol for 0-36 hours post coffee consumption. The amount accounts for 4.5% molar of the ingested dose.

Graph (Figure 2.19) depicts the cumulated feruloylglycine for the five subjects for the individual urine collection intervals. The average cumulated amount excreted for the five subjects is 38.8 ± 8.7 μmol for 0-36 hours post coffee consumption. The amount accounts for 5.3% of the ingested dose.

The total amount of glycine conjugates excreted for 0-36 hours post coffee consumption account for 9.8% of the dose ingested.

Figure 2.18: Cumulated, mean excretion of vanilloylglycine (µmol) for individual subject (n=5) for 0-4, 4-8, 8-12, 12-24, and 24-36 hours post coffee consumption, n=6 (two biological replicates, triplicate injections), mean

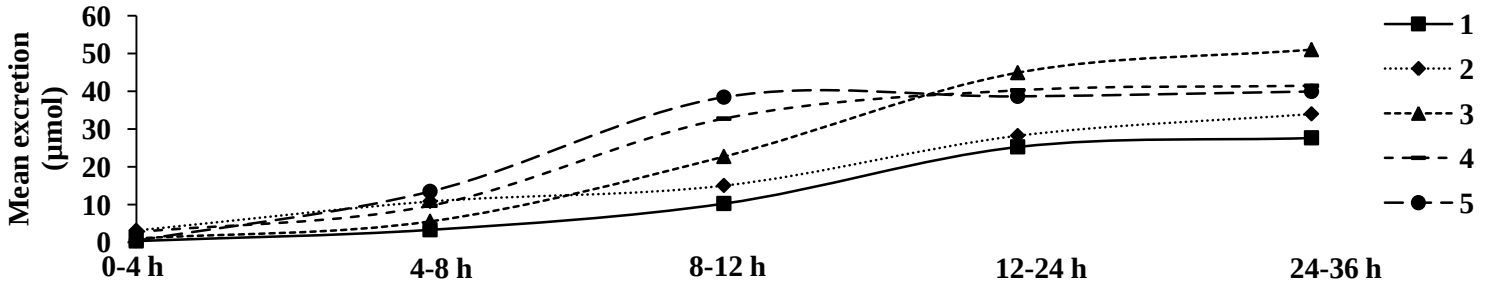
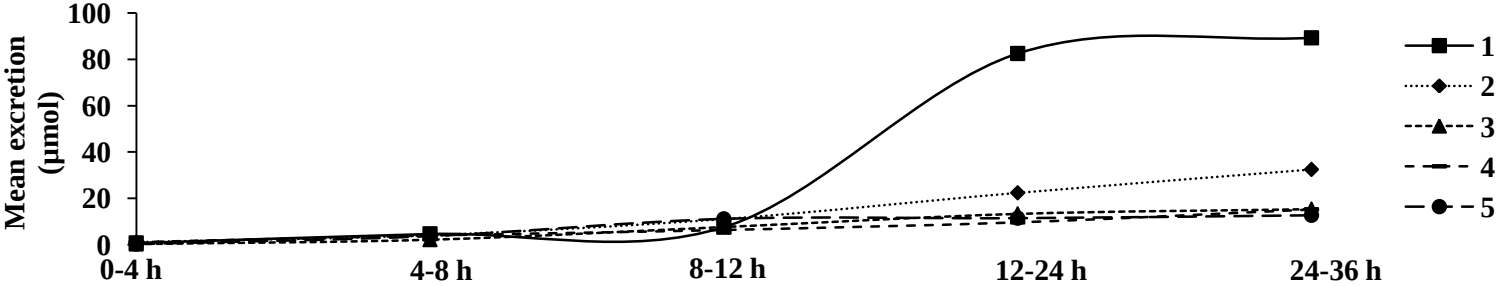


Figure 2.19: Cumulated, mean excretion of feruloylglycine (µmol) for individual subject (n=5) for 0-4, 4-8, 8-12, 12-24, and 24-36 hours post coffee consumption, n=6 (two biological replicates, triplicate injections), mean



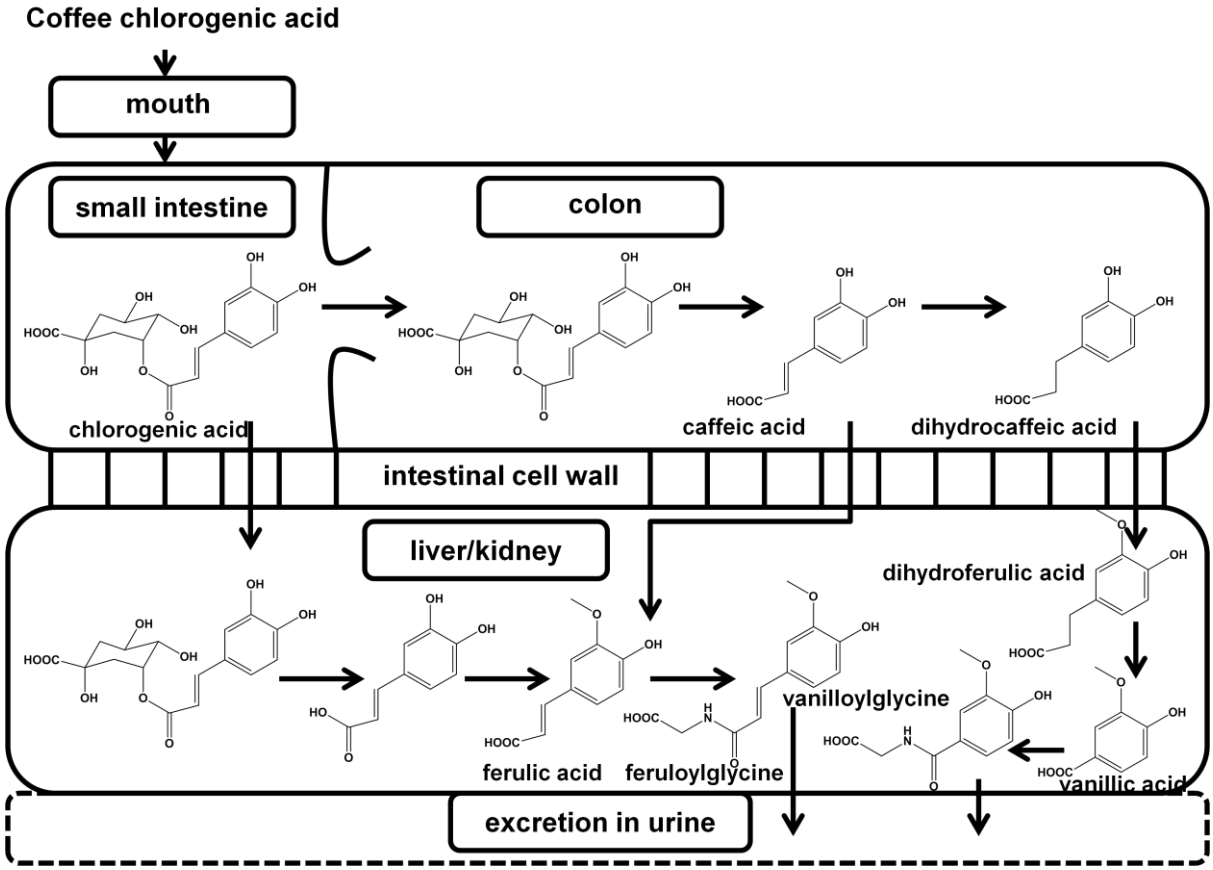
2.5 Discussion

Several new cinnamic acid and phenolic acid glycine conjugates were chemically synthesised, their structures confirmed by ^{1}H-NMR and MS^{2} and their physicochemical properties determined. The glycine conjugates were identified and quantitatively analysed in urine post coffee consumption by comparison of their specific retention time and multiple reaction monitoring on a high pressure liquid chromatography coupled via electrospray ionisation to a triple quadrupole mass spectrometer with the synthesised standards. Two of the synthesised glycine conjugates have never been described in literature – dihydroferuloylglycine and 3-(3,4-dimethoxyphenyl)propionylglycine.

After coffee consumption only feruloylglycine was previously reported as a urinary metabolite of chlorogenic acids. The urinary excretion of feruloylglycine accounted for 5.0% of the ingested dose according to Stalmach *et al*. [7]. It was the second most excreted metabolite after dihydrocaffeic acid-3-*O*-sulphate which accounted for 9.0 % of the ingested dose. However, for the first time vanilloylglycine has been detected and quantified as a urinary metabolite of coffee chlorogenic acids in humans and it accounts for 4.5% of the ingested dose. It is one of the most excreted metabolites of coffee chlorogenic acids in urine and of importance when calculating the absorption of coffee chlorogenic acids via the excretion rate in the human body.

Vanilloylglycine differs from feruloylglycine by a C2-unit. The chain shortening is attributed to the β-oxidation process in the hepatic mitochondria described for the first time in greater detail in Dr A. Kerimi's doctoral thesis, 2011 [69]. Prior to conjugation with glycine the hydroxycinnamic acid derivative is introduced into the β-oxidation process. During the β-oxidation the propionyl tail is shortened by two carbon atoms and either two or four hydrogen atoms depending on the oxidation stage of the compound. The loss of molecular weight facilitates the urinary excretion which is further enhanced by the subsequent conjugation with glycine. Both catalysed reactions are located in the mitochondrial matrix of liver and kidney cells [64]. A suggested metabolic pathway is shown in Figure 2.20.

Figure 2.20: Suggested metabolic pathway of coffee chlorogenic acids in the body with focus on glycine conjugation. Sulphation and glucuronidation are not shown. Derived and adapted from Olthof *et al*., 2003



3,4-dimethoxycinnamic acid linked to quinic acid via an ester bond has been reported as a coffee constituent [10] and 3,4-dimethoxycinnamic acid was found in plasma [28], recently. As the 3- and 4- positions are protected by a methoxy group, no functional group, e.g. sulfate or glucuronide can be attached during phase II metabolism. Therefore we hypothesised that the compound is prone to conjugation with glycine at the carboxylic tail and thus excreted in urine. However, we only found trace amounts of 3,4-dimethoxycinnamoyl glycine in urine. This leads to the assumption that the 4-position is demethylated during metabolism and feruloylglycine is the main metabolite. The demethylation step would also explain the occurrence of only trace amounts of 3,4-dimethoxybenzoylglycine, but substantial amounts of vanilloylglycine (4-hydroxy-3-methoxy-benzoylglycine) in urine.

Alternatively, 3,4-dimethoxycinnamic acid could undergo conjugation with glutathione by glutathione-*S*-transferase and thereafter it might enter the mercapturic acid pathway implying further modifications to the tripeptide tail including loss of glutamic acid and glycine, followed by acetylation of the remaining cysteine [70]. Conjugation with glutathione has also been reported during the incubation of ferulic acid with rat hepatocytes [71]. Further studies should investigate the presence of 3,4-dimethoxycinnamoyl-*N*-acetylcysteine which is potentially excreted in urine after coffee consumption. The conjugate with *N*-acetylcysteine of sulforaphane, an isothiocyanate present in cruciferous vegetables, was found in urine after ingestion of broccoli sprouts for example [72]. The dihydro form of feruloylglycine has only been detected in trace amounts and no dihydroform of 3,4-dimethoxycinnamoylglycine was found. The reduction of the double bond occurs in the colon and is attributed to microbial reductases [15].

We have shown that hydroxycinnamic acid, the hydrolysis products of chlorogenic acids, is decarboxylated and subsequently undergoes conjugation with glycine associated with the β-oxidation in mitochondria. Vanilloylglycine, differing by the loss of C_2H_2 compared to feruloylglycine, and was found to be the second most excreted urinary metabolite of caffeoylquinic acid and its derivatives.

Chapter 3: Synthesis of hydroxycinnamic acid sulphates, development of a LC-MS method for highest urinary metabolites of caffeoylquinic acid and its derivatives, analysis of caffeoylquinic acid and its derivatives in coffee, and digestion of coffee and caffeoylquinic acid and its derivatives with porcine pancreatin

3.1 Abstract

This chapter summarises the synthesis chemical standards, the subsequent development and validation of a LC-MS method for the analysis of major urinary metabolites of caffeoylquinic acid and its derivatives, the analysis of caffeoylquinic acid and its derivatives in a standardised coffee beverage and the digestion of coffee and caffeoylquinic acid and its derivatives by porcine pancreatin.

The first part describes the synthesis of three hydroxycinnamic acid sulphates, e.g. dihydrocaffeic acid-3-*O*-sulphate, ferulic acid-4-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate in hundred milligram amounts for use as standards for LC-MS based quantitation via external calibration. Additionally, a rapid sample procedure and LC-MS method was developed for the quantitative analysis of five major urinary metabolites of caffeoylquinic acid and its derivatives, i.e., vanilloylglycine, dihydrocaffeic acid-3-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate, ferulic acid-4-*O*-sulphate, and dihydroferulic acid. The method was used to assess the absorption of caffeoylquinic acid and its derivatives in urine obtained from volunteers in a human study described in detail in chapter 4. Moreover, caffeoylquinic acid and its derivatives, e.g. 3-, 4-, 5-caffeoylquinic acid, 3-, 4-, 5-feruloylquinic acid and 3,4-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid were quantified in coffee which is consumed by the participants in the human study described in chapter 4. Furthermore, coffee, 3-, 4- and 5-caffeoylquinic acid and 3-,4- and 5-feruloylquinic acid have been incubated individually over 60 minutes with porcine pancreatin, a mixture of digestive enzymes released by the pancreas into the duodenum. Caffeic acid concentration increased the most during the incubation of coffee, however, a small increase in ferulic acid concentration was also observed during the incubation of 3-,4- and 5-caffeoylquinic acid. No release of ferulic acid was observed when 3-, 4- and 5-feruloylquinic acid have been incubated with porcine pancreatin. The data indicate that pancreatin has a

substantial contribution to the hydrolysis of the ester bond between caffeic acid and the quinic acid moiety in caffeoylquinic acids, in particular on 5-caffeoylquinic acid, in the small intestine.

3.2 Introduction

Coffee caffeoylquinic acid and its derivatives are well absorbed, extensively metabolised and abundantly excreted in urine post coffee consumption. The major urinary metabolites are dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, dihydroferulic acid-4-*O*-sulphate, ferulic acid-4-*O*-sulphate and dihydroferulic acid according to Stalmach *et al.* [7]. In chapter 2, vanilloylglycine was reported for the first time as a metabolite of caffeoylquinic acid and its derivatives, excreted in similar amounts to feruloylglycine in urine.

Previously, Fumeaux *et al.* [30] described the synthesis of several hydroxycinnamic acid phase II metabolites including dihydrocaffeic acid-3-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and ferulic acid-4-*O*-sulphate. In order to assess the absorption of coffee caffeoylquinic acids and its derivatives, the excretion of urinary metabolites can be quantified after coffee consumption. Therefore dihydrocaffeic acid-3-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and ferulic acid-4-*O*-sulphate were synthesised using a modified protocol of Fumeaux *et al.* [30] to obtain practical amounts of these chemical standards for the LC-MS based quantitation of the major urinary metabolites via external calibration.

Earlier, several analytical methods for the quantitation of urinary metabolites of caffeoylquinic acid and its derivatives have been reported [7, 15, 17]. These methods aimed to quantify all the known urinary metabolites of caffeoylquinic acid and its derivatives and run over a long time of sixty minutes and are therefore not suitable for the screening of excretion of a larger study populations (>10). Therefore a new and quick method of sample preparation and LC-MS based quantitation using external calibration for the major urinary metabolites was developed and is described in this chapter.

Several caffeoylquinic acids and its derivatives have been quantitatively analysed in coffee [7, 9, 15, 17, 73]. The major ones are 3-, 4-, and 5-caffeoylquinic acid, 3-, 4-, and 5-feruloylquinic acid and 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. For

the assessment of the absorption of caffeoylquinic acid and its derivatives their amount was quantified in commercially available instant coffee which was administered in the human study described in chapter 4. The method for the quantitation was previously described by Farrell *et al.* [74].

Previous studies assessed the absorption, metabolism and excretion of caffeoylquinic acid and its derivatives in ileostomists, patients who underwent removal of their colon due to medical conditions [15, 17, 19]. The absorption of caffeoylquinic acids and its derivatives in ileostomists is attenuated compared to healthy people. Ileostomists only excreted 8 ± 1% of the ingested dose of caffeoylquinic acids and its derivatives [15] whereas healthy subjects excreted 29 ± 4%. The cleavage of the ester bond between caffeic acid and quinic acid in caffeoylquinic acid is partially located in the small intestine [15, 19], however, only a moderate hydrolysis has been observed in the upper gastrointestinal tract and major esterases were located in the colon [19]. Pancreatin, a complex mixture of various digestive enzymes including lipases, trypsin, peptidases, proteases, RNAses, DNAses, amylases and unspecific carboxylesterases is excreted by the pancreas into the duodenum, the upper part of the small intestine. In order to better understand the hydrolyzing effects of pancreatin on coffee, caffeoylquinic acid and its derivatives were incubated at 37ºC for 60 minutes with porcine pancreatin and caffeic acid and ferulic acid, respectively, and were quantified at 0, 10, 20, 40 and 60 minutes of incubation.

3.3 Materials and methods

3.3.1 Materials

Vanilloylglycine was synthesised as described in chapter 2. Feruloylglycine was generously donated by Prof Alan Crozier, University of Glasgow, UK. 3,4-dicaffeoylquinic acid was a gift from Dr Thomas Erk, University of Kaiserslautern, Germany and 3,5-dicaffeoylquinic acid was kindly provided by Prof Mike Clifford, University of Surrey, UK. 3-Caffeoylquinic acid was purchased from Purify Chengdu Biopurify Phytochemicals, Chengdu, China and 4-caffeoylquinic acid was purchased from HWI Analytik, Rülzheim, Germany. 3-Feruloyquinic acid and 4-feruloylquinic acid were purchased from Purify Chengdu Biopurify Phytochemicals, Chengdu, China.

All other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA. Chemicals for the synthesis were reagent grade, chemicals which served as standards for LC-MS quantitation or enzymatic digestion were LC-MS grade or of HPLC grade ≥ 99%.

Demineralised water was prepared using a Millipore water purification system, Millipore Corporation, Billerica, MA, USA.

The MPLC (Thomson Single Step, 16.5 g C18 Flash Column) was purchased from Biotage, Uppsala, Sweden.

3.3.2 Methods

The chemical synthesis was performed in collaboration with Prof Philip Kocienski in the iPDR laboratory at the Department of Chemistry at the University of Leeds, UK. The structure of all intermediates and final compounds was determined by ^{1}H-NMR, ^{13}C-NMR, and IR.

3.3.2.1 Synthesis of dihydrocaffeic acid-3-*O*-sulphate

The synthesis of dihydrocaffeic acid-3-*O*-sulphate consists of five individual synthesis steps. The first step introduced a benzyl group as a protecting group at the 4-position of 3,4-

dihydrobenzaldehyde. The subsequent step inserted a neopentyl sulphate group at the 3-position.

At the third step the aldehyde tail was extended by addition of a CH-COOEt group, thereafter the protecting benzyl group is removed by reduction with gaseous hydrogen, prior to the removal of the protecting neopentyl group and formation of the sodium salt. In the following the individual procedures are described in detail.

4-(Benzoyloxy)-3-hydroxybenzaldehyde (2) was prepared according to a procedure of Couladorous *et al.*[75]. 3,4-dihydroxybenzaldehyde (1) (10.0 g, 72.4 mmol, 1.0 equiv) and anhydrous potassium carbonate (9.0 g, 65.2 mmol, 0.9 equiv) were suspended in 85 mL dimethylformamide and heated at 60ºC for 4 h. The reaction mixture was allowed to cool to room temperature, benzyl bromide (9.09 mL, 76.0 mmol, 1.05 equiv) added and the resulting dark suspension stirred at room temperature for 18 h. Ethyl acetate (150 mL) and water (150 mL) were added and the layers allowed to separate and the aqueous layer extracted with ethyl acetate (100 mL). The combined organic layers were washed with ammonium chloride (saturated aqueous solution, 150 mL) and brine (150 mL). Activated charcoal was used to decolourise the solution which was passed through a plug of silica/celite subsequently and the filtrate concentrated *in vacuo* to give an orange/brown oily solid. Recrystallisation from ethyl acetate/petrol gave the title compound as an off-white solid (9.89 g, 43.3 mmol, 60%) with a mp 108-109 ºC. Spectroscopic data [^{1}H NMR (500 MHz, $CDCl_3$), ^{13}C NMR (75 MHz, $CDCl_3$), and IR] are in accordance with those reported [75].

^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.83 (1H, s, C3H), 7.48–7.34 (7H, m, C5H/C9H/ArH), 7.04 (1H, d, J = 8.2, C6H), 5.83 (1H, s, OH), 5.20 (2H, s, $PhCH_2O$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 191.3 (C3), 151.2 (C7), 146.4 (C8), 135.3 (C_{Ar}), 130.8 (C4), 129.0 (2C, $C_{Ar}H$), 128.8 ($C_{Ar}H$), 128.0 (2C, $C_{Ar}H$), 124.6 (C5H), 114.5 (C9H), 111.6 (C6H), 71.30 ($PhCH_2O$).

IR (solid): ν = 3600–2400 br s, 1745 m, 1673 s, 1603 s, 1513 s, 1454 s cm^{-1}.

Neopentyl chlorosulfate (4) was prepared from neopentyl alcohol (3) using a modification of Widlanski's procedure [76]. A solution of commercial neopentyl alcohol (3) (25.0 g, 284 mmol,

1.0 equiv) and pyridine (22.9 mL, 284 mmol, 1.0 equiv) in diethylether (60 mL) was added dropwise to a solution of sulfuryl chloride (23.0 mL, 284 mmol, 1.0 equiv) in diethyl ether (115 mL) at –78°C. The cooling bath was removed and the reaction mixture allowed to warm to room temperature over 20 minutes and then stirred at room temperature for 2 h. The white suspension was filtered, the white solid washed with diethyl ether (2 × 100 mL) and the filtrate concentrated *in vacuo* to give a colourless liquid. Distillation gave the title compound (50.0 g, 268 mmol, 94%) as a colorless liquid with a bp of 26–28°C at 0.3 mmHg. Spectroscopic data [^{1}H NMR (500 MHz, $CDCl_3$), ^{13}C NMR (75 MHz, $CDCl_3$), and IR] are in accordance with those reported [76] .

^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.17 (2H, s, C1H_2), 1.05 (9H, s, C2(CH_3)$_3$.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 85.6 (C1H_2), 32.0 C2), 26.0 (3C, C2(*C*H_3)$_3$).

IR (solid): ν = 2968 s, 2875 w, 1480 m, 1411 s, 1371 m, 1189 s, 950 s, 936 s, 855 s cm^{-1}.

2-(Benzyloxy)-5-formylphenyl neopentyl sulphate (5) was prepared using a modification of the procedure of Fumeaux *et al.* [30]. Neopentyl chlorosulphate (4) (9.81 g, 52.6 mmol, 1.2 equiv) in dichloromethane (10 mL) was added dropwise to a yellow solution of phenol (2) (10.0 g, 43.8 mmol, 1.0 equiv) and diazabicycloundecene (7.86 mL, 52.6 mmol, 1.2 equiv) in dichloromethane (90 mL) at room temperature and the resulting yellow/orange solution stirred for 21 h. The reaction mixture was washed with 1 M aqueous hydrochloric acid (150 mL), sodium hydrocarbonate (saturated aqueous, 150 mL) and brine (150 mL), dried over sodium sulphate, filtered and concentrated *in vacuo* to give an off-white solid. The crude product was passed through a plug of silicate, eluted with dichloromethane and crystallised from methyltertbutyl ether–petrol to give the title compound (14.7 g, 38.8 mmol, 89%) as a white solid, with a mp 58–59°C. Spectroscopic data [^{1}H NMR (500 MHz, $CDCl_3$), ^{13}C NMR (75 MHz, $CDCl_3$), and IR] are in accordance with those reported [30].

^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.88 (1H, s, C3H), 7.91 (1H, d, *J* = 2.0, C9H), 7.80 (1H, dd, *J* = 8.5, 2.0, C5H), 7.50–7.32 (5H, m, ArH), 7.18 (1H, d, *J* = 8.5, C6H), 5.22 (2H, s, PhCH_2O), 4.06 (2H, s, OC*H*$_2$C(CH_3)$_3$), 0.89 (9H, s, OCH_2C(C*H*$_3$)$_3$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 189.6 (C3), 155.7 (C7), 139.5 (C8), 135.1 (C_{Ar}), 130.8 (C5H), 130.1 (C4), 129.0 (2C, $C_{Ar}H$), 128.8 ($C_{Ar}H$), 127.8 (2C, $C_{Ar}H$), 124.0 (C9H), 114.1 (C6H), 84.0 ($OCH_2C(CH_3)_3$), 71.43 ($PhCH_2O$), 31.9 ($OCH_2C(CH_3)_3$), 25.9 (3C, $OCH_2C(CH_3)_3$).

IR (solid): ν = 3376 w, 3070 m, 2961 s, 2872, s, 2815 s, 2717 s, 1698 s, 1606 s, 1576 s, 1509 s, 1465 s, 1430 s, 1380 s, 1298 s cm^{-1}.

(*E*)-Ethyl 3-(4-(benzyloxy)-3-(((neopentyloxy)sulfonyl)oxy)phenyl)acrylate (7) was prepared using the procedure of Fumeaux *et al.* [30]. Ethyl 2-(triphenylphosphoranylidene)acetate (6) (20.1 g, 57.7 mmol, 1.0 equiv) was added in a single portion to aldehyde (5) (21.8 g, 57.7 mmol, 1.0 equiv) in dichloromethane–tetrahydrofuran (1:1; 90 mL) at room temperature and the resulting pale yellow solution stirred for 2.5 h. The reaction mixture was concentrated *in vacuo* to give a white oily solid that was passed through a plug of silicate eluting with petrol/ethyl acetate (3:1) and crystallised from methyltertbutyl ether–petrol to give the title compound (23.7 g, 52.8 mmol, 92%) as colourless needles, with a mp 76–77°C. The *E*:*Z* ratio was assigned using ^{1}H NMR spectroscopy with no (*Z*)-isomer being observed. Spectroscopic data [^{1}H NMR (500 MHz, $CDCl_3$)] are in accordance with those reported [30].

^{1}H NMR (500 MHz, $CDCl_3$): δ =7.63–7.54 (2H, m, C3H/C9H), 7.48–7.43 (2H, m, C5H/ArH), 7.42–7.32 (4H, m, ArH), 7.05 (1H, d, *J* = 8.6, C6H), 6.32 (1H, d, *J* = 15.9, C2H), 5.15 (2H, s, $PhCH_2O$), 4.25 (2H, q, *J* = 7.1, OCH_2CH_3), 4.04 (2H, s, $OCH_2C(CH_3)_3$), 1.33 (t, *J* = 7.1, OCH_2CH_3), 0.87 (9H, s, $OCH_2C(CH_3)_3$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 166.9 (C1), 152.1 (C7), 142.8 (C3H), 139.4 (C8), 135.6 (C_{Ar}), 128.9 (2C, $C_{Ar}H$), 128.7 ($C_{Ar}H$), 128.6 (C5H), 128.1 (C4), 127.9 (2C, $C_{Ar}H$), 122.4 (C9H), 118.0 (C2H), 114.4 (C6H), 83.9 ($OCH_2C(CH_3)_3$), 71.2 ($PhCH_2O$), 60.7 (OCH_2CH_3), 31.8 ($OCH_2C(CH_3)_3$), 25.9 (3C, $OCH_2C(CH_3)_3$), 14.5 (OCH_2CH_3).

IR (solid): ν = 3047 w, 2968 m, 2903 m, 2874 m, 1697 s, 1632 s, 1603 s, 1515 s, 1478 m, 1463 m, 1420 m, 1398 s 1313 s, 1287 s cm^{-1}.

Ethyl 3-(4-hydroxy-3-(((neopentyloxy)sulfonyl)oxy)phenyl)propanoate (8) was prepared using a modification of Fumeaux *et al.*[30] method. A suspension of palladium on activated carbon (10% dispersion; 500 mg) and compound (7**)** in ethanol (150 mL) was degassed with gaseous

hydrogen five times and stirred at room temperature under an atmosphere of hydrogen for 4 h. The reaction mixture was filtered through a pad of celite, the dark residue washed with dichloromethane (2 × 100 mL) and the combined filtrate concentrated *in vacuo*. The residual yellow/orange oil was purified by column chromatography on silicate eluting with petrol/ethyl acetate (5:1) to give the title compound (7.91 g, 21.9 mmol, 98%) as a colourless oil that solidifies on storage at *ca* –20°C. Spectroscopic data [^{1}H NMR (500 MHz, $CDCl_3$), ^{13}C NMR (75 MHz, $CDCl_3$), and IR] are in accordance with those reported [30].

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.13 (1H, d, *J* = 2.1, C9H), 7.05 (1H, dd, *J* = 8.3, 2.1, C5H), 6.97 (1H, d, *J* = 8.4, C6H), 4.13 (2H, s, $OCH_2C(CH_3)_3$), 4.12 (2H, q, J = 7.2, OCH_2CH_3), 2.89 (2H, t, *J* = 7.6, $C3H_2$), 2.59 (t, *J* = 7.7 Hz, $C2H_2$), 1.23 (t, *J* = 7.2 Hz, OCH_2CH_3), 1.01 (9H, s, $OCH_2C(CH_3)_3$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.2 (C1), 146.2 (C7), 137.6 (C8), 133.5 (C4), 128.5 (C5H), 122.2 (C6H), 118.3 (C9H), 84.3 ($OCH_2C(CH_3)_3$), 60.9 (OCH_2CH_3), 36.0 ($C2H_2$), 32.0 ($OCH_2C(CH_3)_3$), 30.0 ($C3H_2$), 25.9 (3C, $OCH_2C(CH_3)_3$), 14.3 (OCH_2CH_3).

IR (solid): ν = 3900–2900 br s, 2965 s, 2873 s, 1708 br s, 1624 m, 1594 m, 1519 s, 1398 s, 1297 s cm^{-1}.

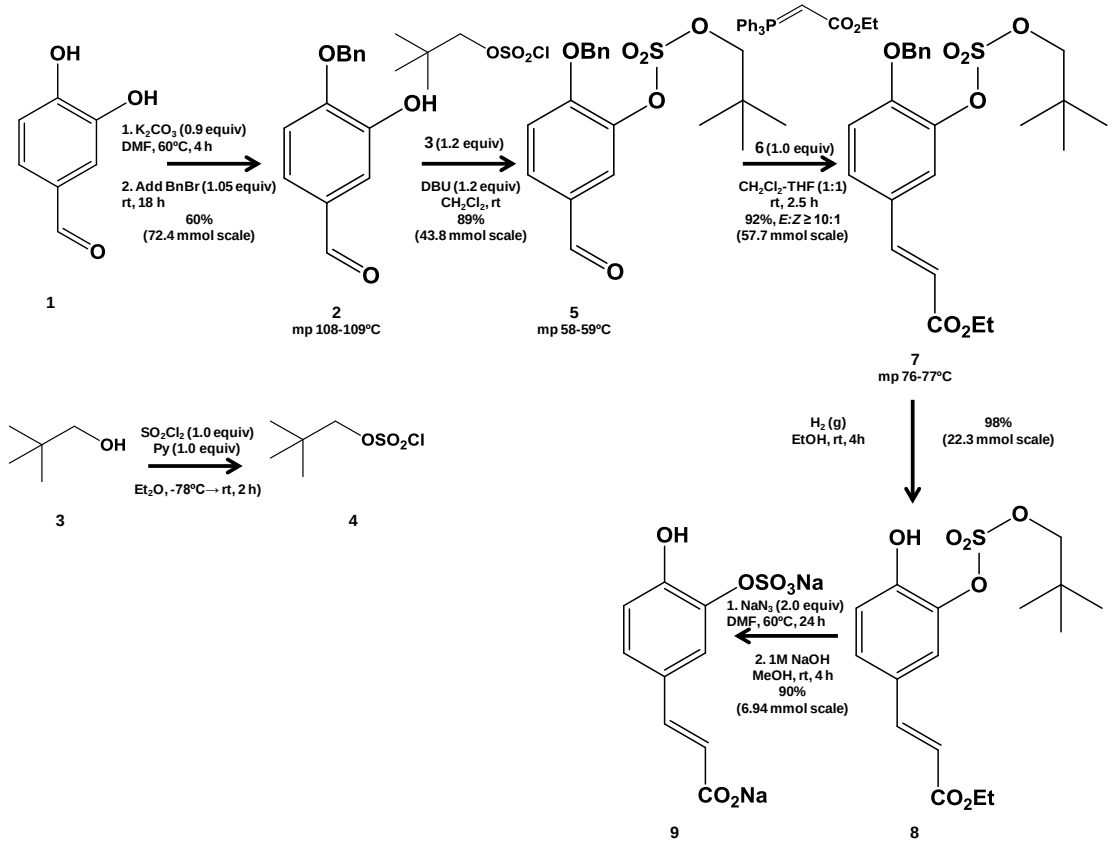
Dihydrocaffeic acid 3-*O*-sulphate (9) was prepared using a modified procedure of Fumeaux *et al.*[30]. A solution of phenol (8) (2.5 g, 6.94 mmol, 1.0 equiv) and sodium azide (902 mg, 13.9 mmol, 2.0 equiv) in dimethylformamide (21 mL) was heated at 60°C for 24 h. The reaction mixture was allowed to cool to room temperature and the solvent removed using distillation (*ca* 40°C at *ca* 1 mmHg) to give a off-white gummy residue that was dissolved in methanol, dry loaded onto silicate and passed through a plug of silicate eluting with dichloromethane/ethanol (5:1) to give a pale yellow oil. Methanol (14 mL) and 1 M aqueous sodium hydroxide (28.0 mL, 28.0 mmol, 4.0 equiv) was added and the resulting solution stirred at room temperature for 4 h. Amberlite CG-50 (3.0 g) was added to the reaction mixture and stirred at room temperature for 30 min. Filtration and concentration *in vacuo* gave an off-white solid that was purified using Biotage MPLC (Thomson Single Step, 16.5 g C18 Flash Column) using a gradient of methanol in water as eluent to give the title compound (1.92 g, 6.27 mmol, 90%) as a hydroscopic off-

white solid. Spectroscopic data [^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (75 MHz, DMSO-d_6)] are in accordance with those reported [30].

^{1}H NMR (500 MHz, DMSO-d_6): δ = 6.93 (1H, d, J = 2.2, C9H), 6.78 (1H, dd, J = 8.2, 2.2, C5H), 6.69 (1H, d, J = 8.1, C6H), 2.65 (2H, t, J = 7.6, C3H$_2$), 2.17 (2H, t, J = 7.6, C2H$_2$).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 177.3 (C1), 147.1 (C7), 140.4 (C8), 133.9 (C4), 124.6 (C5H), 122.9 (C9H), 116.9 (C6H), 39.8 (C2H$_2$), 31.6 (C3H$_2$).

Figure 3.1: Synthesis of dihydrocaffeic acid-3-*O*-sulphate



3.3.2.2 Synthesis of ferulic acid-4-*O*-sulphate

The synthesis of ferulic acid-4-*O*-sulphate consists of three individual synthesis steps. In the first step the carboxylic group of ferulic acid is protected by the formation of a methyl ester. In the second step neopentyl sulphate is introduced at the 4-position of the phenyl ring which is followed by the last step where the neopentyl unit split off due to hydrolysis followed by the addition of a sodium cation. In the following the individual synthesis steps are described in experimental detail.

(*E*)-Methyl 3-(4-hydroxy-3-methoxyphenyl)acrylate (11) was prepared using a modification of the procedure of Voisin-Chiret *et al.*[77]. Trimethyl orthoformate (38.0 mL, 348 mmol, 1.35 equiv) followed by concentrated sulfuric acid (1.38 mL, 25.8 mmol, 10 mol%) in methanol (40 mL) was added to a solution of ferulic acid (10) (50.0 g, 258 mmol, 1.0 equiv) in methanol (215 mL) at room temperature and the resulting yellow solution heated under reflux for 20 h. The reaction mixture was allowed to cool to room temperature, pyridine (6.26 mL, 77.4 mmol, 30 mol%) added and the mixture concentrated *in vacuo*. Methyltertbutyl ether (250 mL) was added, the solution washed with 1 M aqueous hydrochloric acid (2 × 150 mL), sodium bicarbonate (saturated aqueous, 100 mL) and brine (100 mL), dried over sodium sulfate, filtered and concentrated *in vacuo* to give a colourless oil. The title compound (43.8 g, 210 mmol, 82%) was crystallised as a white solid by the addition of diethylether (50 mL) and the addition of petrol (*ca* 50 mL) until cloud point was observed with stirring at 400 rpm for 20 h, with a melting point at (diethylether/petrol) 59–60 °C; Lit.[77] melting point of 68°C. Spectroscopic data [^{1}H NMR (500 MHz, $CDCl_3$), ^{13}C NMR (75 MHz, $CDCl_3$), and IR] are in accordance with those reported [77].

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.62 (1H, d, *J* = 15.9, C3H), 7.07 (1H, dd, *J* = 8.2, 2.0, C5H), 7.02 (1H, d, *J* = 1.8, C9H), 6.92 (1H, d, *J* = 8.1, C6H), 6.29 (1H, d, *J* = 15.8, C2H), 5.91 (1H, s, OH), 3.92 (3H, s, OCH_3), 3.80 (3H, s, OCH_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 167.9 (C1), 148.1 (C8), 146.9 (C7), 145.1 (C3H), 126.9 (C4), 123.1 (C5H), 115.1 (C2H), 114.9 (C6H), 109.5 (C9H), 56.0 (OCH_3). 51.8 (OCH_3).

IR (solid): ν = 3700–2700 br s, 3074 s, 2944 s, 2846 s, 1730 br m, 1605 m, 1512 m, 1227 m cm^{-1}.

(*E*)-Methyl 3-(3-methoxy-4-(((neopentyloxy)sulfonyl)oxy)phenyl)acrylate (12) was prepared using Fumeaux *et al.* modified procedure [30]. Neopentyl chlorosulphate (4) (20.0 g, 107 mmol, 1.2 equiv) in dichloromethane (20 mL) was added dropwise to a yellow solution of ester (11) (18.6 g, 89.3 mmol, 1.0 equiv) and diazabicycloundecene (16.0 mL, 107 mmol, 1.2 equiv) in dichloromethane (180 mL) at room temperature and the resulting yellow/orange solution stirred for 21 h. The reaction mixture was washed with 1 M aqueous hydrochloric acid (200 mL),

sodium bicarbonate (saturated aqueous, 200 mL) and brine (200 mL), dried over sodium sulphate, filtered and concentrated *in vacuo* to give an off-white solid. The crude product was passed through a plug of silicate, eluted with dichloromethane and crystallised from methyltertbutyl ether to give the title compound (28.7 g, 80.1 mmol, 90%) as a white solid, with a melting point of 105–106 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.64 (1H, d, *J* = 16.0, C3H), 7.38 (1H, d, *J* = 8.4, C6H), 7.14 (1H, d, *J* = 2.0, C9H), 7.13–7.10 (1H, m, C5H), 6.41 (1H, d, *J* = 15.9, C2H), 4.17 (2H, s, $OC\mathit{H}_2C(CH_3)_3$), 3.92 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 1.01 (9H, s, $OCH_2C(C\mathit{H}_3)_3$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 167.1 (C1), 151.6 (C8), 143.6 (C3H), 140.4 (C7), 134.6 (C4), 123.3 (C6H), 121.1 (C5H), 119.2 (C2H), 111.9 (C9H), 83.7 ($O\mathit{C}H_2C(CH_3)_3$), 56.1 (OCH_3), 52.0 (OCH_3), 32.0 ($OCH_2\mathit{C}(CH_3)_3$), 26.1 (3C, $OCH_2C(\mathit{C}H_3)_3$).

IR (solid): ν = 3081 w, 2970 s, 2872 m, 1708 s, 1645 m, 1634 m, 1599 m, 1505 s, 1464 m, 1449 m, 1409 s, 1370 m cm^{-1}.

Ferulic acid-4'-*O*-sulphate (13) was prepared using a modified procedure of Fumeaux *et al.*[30]. A solution of ester (12) (2.5 g, 6.98 mmol, 1.0 equiv) and sodium azide (907 mg, 14.0 mmol, 2.0 equiv) in dimethylformamide (21 mL) was heated at 60°C for 24 h. The reaction mixture was allowed to cool to room temperature and the solvent removed using distillation (*ca* 40°C at *ca* 1 mmHg) to give a off-white gummy residue that was dissolved in methanol, dry loaded onto silicate and passed through a plug of silicate eluting with dichloromethane/ethanol (5:1) to give an off-white solid. Methanol (14 mL) and 1 M aqueous sodium hydroxide (28.0 mL, 28.0 mmol, 4.0 equiv) was added and the resulting solution stirred at room temperature for 4 h. Amberlite CG-50 (3.0 g) was added to the reaction mixture and stirred at room temperature for 30 min. Filtration and concentration *in vacuo* gave an off-white solid that was purified using Biotage MPLC (Thomson Single Step, 16.5 g C18 Flash Column) using a gradient of methanol in water as eluent to give the title compound (2.0 g, 6.29 mmol, 90%) as a hydroscopic white solid. Spectroscopic data [^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (75 MHz, DMSO-d_6)] are in accordance with those reported [30].

^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.42 (1H, d, *J* = 8.3, C6H), 7.14 (1H, d, *J* = 15.9, C3H), 7.09 (1H, d, *J* = 1.9, C9H), 6.96 (1H, dd, *J* = 8.4, 2.0, C5H), 6.35 (1H, d, *J* = 15.8, C2H), 3.77 (3H, s, OCH_3).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 171.6 (C1), 150.5 (C8), 143.0 (C7), 136.9 (C3H), 131.7 (C4), 127.4 (C5H), 120.8 (C6H), 119.6 (C2H), 110.6 (C9H), 55.6 (OCH_3).

3.3.2.3 Synthesis of dihydroferulic acid-4-*O*-sulphate

The synthesis of dihydroferulic acid-4-*O*-sulphate consists of four individual synthesis steps. In the first step the carboxylic group of ferulic acid is protected by the formation of a methyl ester. In the second step neopentyl sulphate is introduced at the 4-position of the phenyl ring which is followed by reduction of the double bond with hydrogen, prior to the last step where the neopentyl unit is split off due to hydrolysis followed by the addition of a sodium cation.
In the following the individual synthesis steps are described in experimental detail.

Methyl 3-(3-methoxy-4-(((neopentyloxy)sulfonyl)oxy)phenyl)propanoate (14) was prepared using a modification of the procedure of Fumeaux *et al.*[30]. A suspension of palladium on activated carbon (10% dispersion; 500 mg) and compound (12) in methanol (150 mL) was degassed with gaseous hydrogen five times and stirred at room temperature under an atmosphere of hydrogen for 4 h. The reaction mixture was filtered through a pad of celite, the dark residue washed with dichloromethane (2 × 100 mL) and the combined filtrate concentrated *in vacuo* to give the title compound (9.86 g, 27.3 mmol, 98%) as a colourless oil that solidifies on storage at *ca* –20°C and was used in the subsequent step with no further purification.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.26 (1H, d, *J* = 8.2, C6H), 6.84 (1H, d, *J* = 2.0, C9H), 6.78 (1H, dd, *J* = 8.2, 2.0, C5H), 4.15 (2H, s, $OCH_2C(CH_3)_3$), 3.87 (3H, s, OCH_3), 3.68 (3H, s, OCH_3), 2.94 (2H, t, *J* = 7.7, $C3H_2$), 2.63 (2H, t, *J* = 7.7, $C2H_2$) 1.00 (9H, s, $OCH_2C(CH_3)_3$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.0 (C1), 151.0 (C8), 141.2 (C7), 137.5 (C4), 122.7 (C5H), 120.4 (C6H), 113.1 (C9H), 83.3 ($OCH_2C(CH_3)_3$), 55.9 (OCH_3), 51.7 (OCH_3), 35.4 ($C2H_2$), 31.8 ($OCH_2C(CH_3)_3$), 30.7 ($C3H_2$), 25.9 ($OCH_2C(CH_3)_3$).

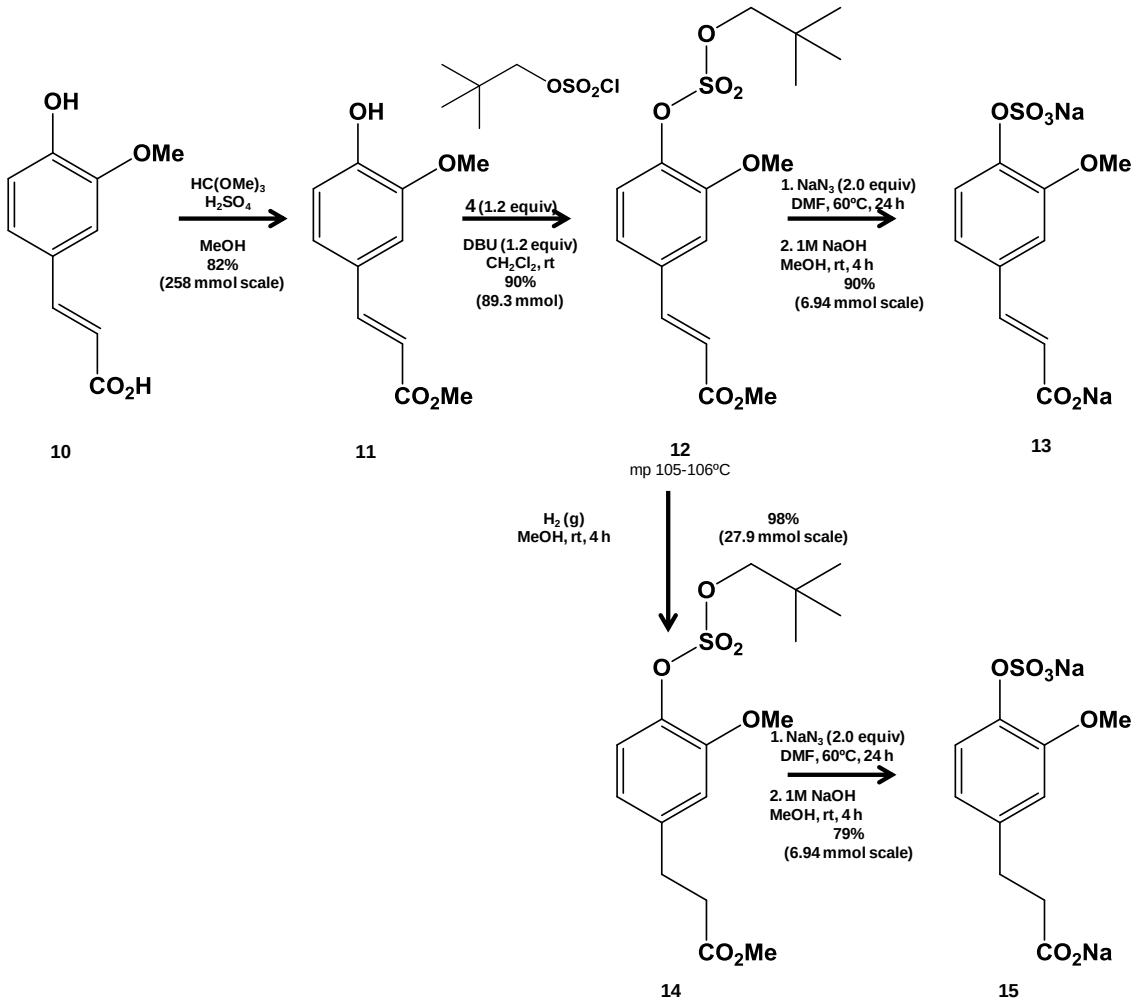
IR (solid): ν = 2962 s, 2873 m, 1738 s, 1604 m, 1509 s, 1437 m, 1399 s, 1369 s, 1271 s, 1207 s, 1183 s cm^{-1}.

Dihydroferulic acid-4-*O*-sulphate (15) was prepared using a modification of the procedure of Fumeaux *et al.*[30]. A solution of ester (14) (2.5 g, 6.94 mmol, 1.0 equiv) and sodium azide (902 mg, 13.9 mmol, 2.0 equiv) in dimethylformamide (21 mL) was heated at 60°C for 24 h. The reaction mixture was allowed to cool to room temperature and the solvent removed using distillation (*ca* 40°C at *ca* 1 mmHg) to give a off-white gummy residue that was dissolved in methanol, dry loaded onto silicate and passed through a plug of silicate, eluted with dichloroemethane/ethanol (5:1) to give a pale yellow oil. Methanol (14 mL) and 1 M aqueous sodium hydroxide (28.0 mL, 28.0 mmol, 4.0 equiv) was added and the resulting solution stirred at room temperature for 4 h. Amberlite CG-50 (3.0 g) was added to the reaction mixture and stirred at room temperature for 30 min. Filtration and concentration *in vacuo* yielded an off-white solid that was purified using Biotage MPLC (Thomson Single Step, 16.5 g C18 Flash Column) using a gradient of methanol in water as eluent to give the title compound (1.76 g, 5.50 mmol, 79%) as a hydroscopic white solid. Spectroscopic data [^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (75 MHz, DMSO-d_6)] are in accordance with those reported [30].

^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.28 (1H, d, *J* = 8.1 Hz, C6H), 6.79 (1H, d, *J* = 2.0 Hz, C9H), 6.64 (1H, dd, *J* = 8.3, 2.0 Hz, C5H), 3.70 (3H, s, OCH_3), 2.71 (2H, t, *J* = 7.9 Hz, $C3H_2$), 2.23 (2H, t, *J* = 7.9, $C2H_2$).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 177.1 (C1), 150.4 (C8), 140.3 (C7), 138.5 (C4), 121.1 (C5H), 119.6 (C6H), 112.8 (C9H), 55.6 (OCH_3), 39.7 ($C2H_2$), 32.2 ($C3H_2$).

Figure 3.2: Synthesis of ferulic acid-4-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate



3.3.2.4 Sample Preparation

Instant coffee (Nestle Green Blend™) was purchased from one batch at a local supermarket in Leeds, UK. The coffee was kept sealed until the analysis and stored in a dark, cool place.

Instant coffee (5 g) was taken from three different jars and mixed by stirring prior to weighing and dissolution.

500 mg of instant coffee mixture was weighed and 50 mL of demineralised water (90-95ºC) was added and stirred. The coffee was immediately chilled on ice and diluted 1:5 with mobile phase A consisting of 95% demineralised water, 5% acetonitrile (LC-MS grade) and 0.1% fromic acid (LC-MS grade) containing 1 mM ascorbic acid. The solution was centrifuged at 17,000 g for 10 minutes and the supernatant filtered through 0.22 µm PTFE. The sample was prepared in triplicate and 5 µL of each sample was injected onto the column in triplicate.

Urine samples were collected into sterilised unisex urine collection containers containing 1 g ascorbic acid for 0, 0-4, 4-8 and 8-12 h and 3 g ascorbic acid for 12-24 and 24-36 h, respectively. Within 48 h after collection the urine volume was determined by weight, an aliquote of 45 mL centrifuged at 4ºC for 10 minutes and 12 mL of the supernatant taken and an aqueous solution of sodium azide added with a final concentration of 0.1% prior to freezing the sample at -20ºC.

Urine samples were prepared according to a modification of a procedure described by Stalmach *et al.* [7].

Prior to LC-MS analysis urine samples were defrosted in a fridge overnight and one aliquot of 900 µL from each sample taken, 100 µL 100 µM sinapic acid in mobile phase A containing 1 mM ascorbic acid was added as an internal standard, vortexed for ten seconds, inverted twice, and centrifuged at 20,000 g at 4ºC for 10 minutes. The supernatant was filtered through 0.22 µm PTFE into amber vials.

Samples were prepared to determine the recovery rates of the six urinary metabolites. Therefore pooled urine collected from six participants collected after a 36 hours washout period, which is described in detail in chapter 4, was used for spiking. Urine (850 µL) and 50 µL of the standard solution to yield concentrations of 2, 5, and 50 µM, and 100 µL of 100 µM sinapic acid as an internal standard. Additionally, 900 µL of blank urine were mixed with 100 µL of 100 µM sinapic acid. All samples were prepared in triplicate and processed as previously described.

For the pancreatic digestion, 100 mg porcine pancreatin was suspended in 1 mL 20 mM potassium phosphate buffer pH 7.4. The suspension was briefly vortexed, and centrifuged at 20,000 g, prior to separating the supernatant. The protein content of the pancreatic solution was determined using a Bradford protein assay kit including a 96 well plate which was inserted into a PHERAstar, BMG LABTECH (Ortenberg, Germany) operated at 595 nm.

3-, 4-, and 5-caffeoylquinic acid and 3-, 4-, and 5-feruloylquinic acid were individually dissolved in potassium phosphate buffer to give 100 µM stock solutions. 1 g instant coffee was dissolved in 50 mL hot, but not boiling demineralised water. Prior to the incubation for the pancreatic digestion all solutions were heated at 37ºC. Potassium phosphate buffer (0.4 mL, 20

mM) was mixed with either 0.5 mL of coffee, 100 µM 3-, 4-, 5-caffeoylquinic acid, or 3-, 4-, 5-feruloylquinic acid and 0.1 mL pancreatin solution. Each solution was prepared in duplicate and one control with heat-deactivated pancreatic solution.

The samples were incubated at 37ºC for 60 minutes in an electrothermal incubator (Eppendorf, Hamburg, Germany). Aliquots of 100 µL were taken at the beginning, at 10, 20, 40 and 60 minutes and acidified with 10 µL 10 % aqueous hydrochloric acid, vortexed and cooled on ice, prior to be centrifuged at 20,000 g at 4ºC for 10 minutes.

3.3.2.5 High pressure liquid chromatography

The high performance liquid chromatography system was an Agilent 1200 series (Waldbronn, Germany) with a degasser, binary pump, temperature controlled autosampler and column compartment, and a diode array detector fitted with an Agilent ZORBAX Eclipse Plus Rapid Resolution C18 100 mm x 2.1 mm i.d., 1.8 µm.

Mobile phase A consisted of 95% demineralised water, 5% acetonitrile (LC-MS grade) and 0.1% formic acid (LC-MS grade), mobile phase B of 95% acetonitrile (LC-MS grade), 5% demineralised water and 0.1% formic acid (LC-MS grade). Both solvents were degassed using an ultrasonic bath (Clifton, Nickel Electro Ltd., Weston-super-Mare, UK).

For the liquid chromatography of coffee, chromatographic settings were derived and adapted from a method developed by Farrell *et al.* [68]. In brief, coffee samples were kept at 4ºC and 5 µL were injected in duplicate. The flow rate was set to 0.26 mL/min for a total run time of 65.1 minutes. The gradient started with 0% mobile phase B for 17.3 minutes, was increased to 25% up to 51.0 minutes, further increased to 100% from 51.1 to 56.0 minutes and dropped to 0% from 56.1 minutes for ten minutes for reequilibration. The column was thermostated at 30ºC.

Urine samples were kept at 4ºC and 10 µL were injected in duplicate. Chromatographic settings were optimized using synthesised (vanilloylglycine, feruloylglycine, dihydrocaffeic acid-3-*O*-sulphate, ferulic acid-4-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate) and commercially available standards (dihydroferulic acid and sinapic acid as an internal standard).

The flow rate was set to 0.4 mL/min, total run time of the method was 25 minutes with a gradient kept at 0% mobile phase B for the first 12 minutes, increased to 40% up to 16 minutes, further increased up to 100% at 16.5 minutes until 19.5 minutes and dropped to 0% from 20.0 minutes for 5 minutes for reequilibration. The column was thermostated at 35ºC.

Pancreatic digestion samples were kept at 4ºC and 5 µL injected in duplicate. The flow rate was set to 0.3 mL/min and separation with a 30 minutes gradient starting with 0% mobile phase B, increased to 8% over 14 minutes, increased to 19% up to 14.1 minutes and kept until 20.0 minutes, increased to 100% from 20.1 to 24.0 minutes and dropped to 0% at 24.1 minutes for 6 minutes for reequilibration. The column was thermostated at 30ºC.

3.3.2.6 Mass spectrometry

The Agilent 1200 series HPLC (Waldbronn, Germany) was coupled through a divert valve and a heated electrospray ionisation source to an Agilent 6410 A triple quadrupole mass spectrometer (Wokingham, UK).

For the mass spectrometric analysis of coffee caffeoylquinic acid and its derivatives, the heated electrospray ionisation source was operated in negative mode with a source temperature set to 350ºC, a nebulizer pressure of 10 L/min and a pressure of 60 psi. Multiple reaction monitoring (MRM) settings were optimised using commercially available standards at a concentration of 10 µg/mL. Table 3.1 shows the optimised settings for the individual compounds. The flow of the first 4 minutes was diverted into waste.

Table 3.1: Chromatographic settings and multiple reaction monitoring of caffeoylquinic acid and its derivatives

Compound	Time window (min)	Retention time (min)	Precursor ion (m/z)	Fragmentor energy (V)	Product ion (m/z)	Collision energy (V)
3-Caffeoylquinic acid	4.0-8.0	6.2	353	120	191 179	20 20
5-Caffeoylquinic acid	8.0-16.0	14.1	353	120	191 179	10 20
3-Feruloylquinic acid	16.0-23.0	16.4	367	120	193 134	20 20
4-Caffeoylquinic acid		19.4	353	120	179 173	15 15
5-Feruloylquinic acid	28.0-39.0	30.8	367	120	191 134	15 20
4-Feruloylquinic acid		31.8	367	120	193 173	10 10
3,4-Dicaffeoylquinic acid	39.0-55.0	41.6	515	150	353 173	20 20
3,5-Dicaffeoylquinic acid		42.2	515	150	191 134	20 20

After 55 minutes the polarity was switched to positive to neutralise a potential charge accumulation in the ion capillary.

For the mass spectrometric analysis of urinary metabolites of caffeoylquinic acid and its derivatives, the heated electrospray ionisation source was operated in negative mode with a source temperature set to 350ºC, a nebulizer nitrogen flow of 13 L/min and a pressure of 60 psi. Multiple reaction monitoring (MRM) settings were optimised using synthesised (vanilloylglycine, feruloylglycine, dihydrocaffeic acid-3-*O*-sulphate, ferulic acid-4-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate) and commercially available standards (dihydroferulic acid and sinapic acid as an internal standard) at a concentration of 10 µg/mL. Table 3.2 shows the optimised settings for the individual compounds. The eluent flow of the first 3 minutes was diverted into waste.

Table 3.2: Chromatographic settings and multiple reaction monitoring of vanilloylglycine, dihydrocaffeic acid-3-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate, ferulic acid-4-*O*-sulphate, feruloylglycine, dihydroferulic acid and sinapic acid (internal standard)

Compound	Time window (min)	Retention time (min)	Precursor ion (m/z)	Fragmentor energy (V)	Product ion (m/z)	Collision energy (V)
Vanilloylglycine	3.0-4.5	4.2	224	90	100 123 108	6 10 22
Dihydrocaffeic acid-3-*O*-sulphate	4.5-7.0	5.8	261	90	181 137 109	12 18 26
Dihydroferulic acid-4-*O*-sulphate	7.0-15.0	8.6	275	100	195 136 80	10 16 14
Ferulic acid-4-*O*-sulphate		10.7	273	90	193 178 134	8 16 26
Feruloylglycine	15.0-16.0	15.6	250	90	206 163 134	6 20 18
Dihydroferulic acid	16.0-16.5	16.2	195	90	136 121 93	12 26 30
Sinapic acid (internal standard)	16.5-19.5	16.7	223	90	208 193 164	6 16 10

After 19.5 minutes the polarity was switched to positive mode to neutralise a potential charge accumulation in the ion capillary.

For the mass spectrometric analysis of the pancreatic digestion samples, the heated electrospray ionisation source was operated in negative mode with a source temperature set to 350ºC, a nebulizer nitrogen flow of 13 L/min and a pressure of 60 psi. Multiple reaction monitoring settings were optimised using commercially available standards at a concentration of 10 µg/mL. The following table shows the optimised settings for the individual compounds. The eluent flow of the first 3 minutes was diverted into waste.

Table 3.3: Chromatographic settings and multiple reaction monitoring of caffeic acid and ferulic acid

Compound	Retention time (min)	Precursor ion (m/z)	Fragmentor energy (V)	Product ion (m/z)	Collision energy (V)
Caffeic acid	9.9	179	80	135	15
				89	30
Ferulic acid	17.6	193	90	134	8
				178	12

After 25.0 minutes the polarity was switched to positive mode to neutralise a potential charge accumulation in the ion capillary.

3.3.2.7 Quantitation through external calibration

Caffeoylquinic acids and its derivatives in coffee were quantified using external calibration with eight calibrants including 0.5, 1, 2, 5, 10, 20, 50 and 100 µM for each individual compound. The calibrants were prepared in mobile phase A containing 1 mM ascorbic acid. The linearity was $R^2 \geq 0.99$ over the entire range. The limit of detection (LOD) and the limit of quantitation (LOQ) are calculated by LOD=(3.3*intercept)/slope and LOQ=(10*intercept)/slope, respectively. The values are presented in Table 3.4.

Table 3.4: Summary of the limit of detection and limit of quantitation caffeoylquinic acid and its derivatives, 5 µL injected

Compound	Limit of detection (µM)	Limit of quantitation (µM)
3-Caffeoylquinic acid	5.8	17.5
4-Caffeoylquinic acid	1.4	4.3
5-Caffeoylquinic acid	6.3	19.2
3-Feruloylquinic acid	0.6	1.9
4-Feruloylquinic acid	1.1	3.3
5-Feruloylquinic acid	4.8	14.8
3,4-Dicaffeoylquinic acid	0.45	1.4
3,5-Dicaffeoylquinic acid	0.1	0.2

Urinary metabolites of caffeoylquinic acid and its derivatives were quantified using external calibration. The first calibration curves with twelve calibrants including 10, 20, 50, 500 nM, 1, 2, 4, 6, 8, 10, 20 and 50 µM. Further calibration curves were run, including five calibrants 0.5, 1, 5, 10 and 50 µM. The calibrants were prepared in mobile phase A containing 1 mM ascorbic acid. The linearity for the first and following calibration curves were $R^2 \geq 0.99$ and $R^2 \geq 0.999$,

respectively. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated by LOD=(3.3*intercept)/slope and LOQ=(10*intercept)/slope, respectively. The values are presented in Table 3.5.

Table 3.5: Summary of the limit of detection and limit of quantitation of urinary metabolites of caffeoylquinic acid and its derivatives, 10 µL injected

Compound	Limit of detection (µM)	Limit of quantitation (µM)
Vanilloylglycine	0.4	1.2
Dihydrocaffeic acid-3-*O*-sulphate	0.6	1.7
Dihydroferulic acid-4-*O*-sulphate	0.4	1.3
Ferulic acid-4-*O*-sulphate	0.5	1.6
Feruloylglycine	0.1	0.2
Dihydroferulic acid	0.5	1.5
Sinapic acid	0.3	0.8

The first calibration curve was run on day 1 thereafter samples of six subjects were run and a calibration curve with five calibrants followed by samples of six subjects. The cycle was repeated for 11, 8 and 7 times for the first visit (n=62), the second visit (n=47) and the third visit (n=40), respectively. Urinary concentration and subsequent total amounts excreted, have been corrected using the recovery factor of the internal standard.

In the pancreatic digestion samples, caffeic acid and ferulic acid were quantified through external calibration. Calibration curves included eight calibrants, e.g. 10, 250, 500 nM, 1, 2, 5, 10 and 20 µM with a linearity of $R^2 \geq 0.999$. The limit of detection (LOD) and the limit of quantitation (LOQ) are calculated by LOD=(3.3*intercept)/slope and LOQ=(10*intercept)/slope, respectively. Table 3.6 presents the values. The calibrants have been prepared in 20 mM potassium phosphate buffer (pH=7.4).

Table 3.6: Summary of the limit of detection and limit of quantitation of caffeic acid and ferulic acid, 10 µL injected

Compound	Limit of detection (µM)	Limit of quantitation (µM)
Caffeic acid	0.01	0.04
Ferulic acid	0.04	0.13

3.4 Results

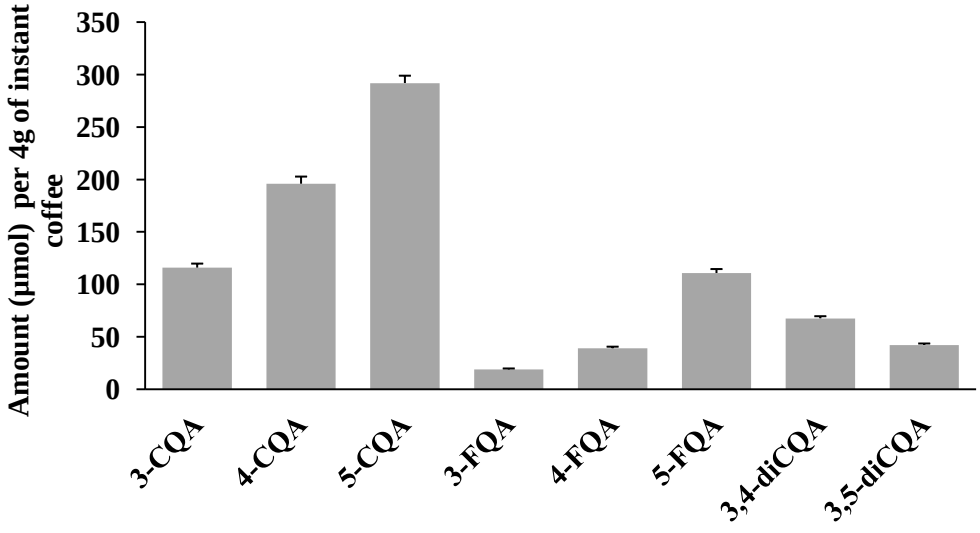
3.4.1 Synthesis of hydroxycinnamic acid sulphates

Several hundred milligrams of dihydrocaffeic acid-3-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and ferulic acid-4-*O*-sulphate were chemically synthesised and their quality and purity confirmed by NMR, IR and LC-MS. The compounds served as analytical standards for the quantitation of urinary metabolites of caffeoylquinic acid and its derivatives. For the first time these sulphates have been synthesised in amounts visible to the human eye. Subsequently, their appearance was described and their melting points determined.

3.4.2 Coffee caffeoylquinic acid and its derivatives content

Eight caffeoylquinic acid and its derivatives have been analysed in commercially available instant coffee. The following graph (Figure 3.3) shows the amounts of the individual compound in 4 g instant coffee, the amount which was ingested in a subsequent study described in 4.3.3.

Figure 3.3: Caffeoylquinic acid and its derivatives content in µmol in 4g instant coffee, n=9 (three biological samples, three injections), mean ± standard deviation



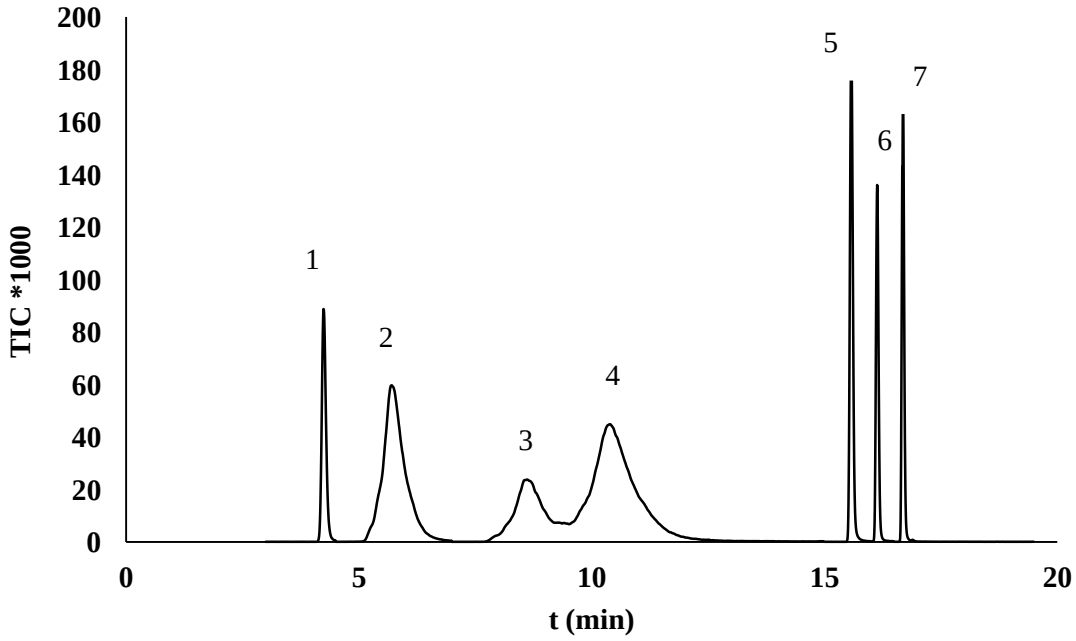
The total amount of caffeoylquinic acid and its derivatives is 881.8 +/- 27.2 µmol and were about two-fold higher when compared with other publications [7, 20, 31], however, green, unroasted coffee beans were intentionally used to prepare the coffee by the manufacturer.

3-, 4-, and 5-Caffeoylquinic acid account for 68.5% with 5-CQA accounting for 33.0% of the total caffeoylquinic acid and its derivatives content on a molar basis.

3.4.3 Urine

Six major urinary metabolites of caffeoylquinic acids were separated with a newly developed reverse phase, gradient method. Baseline separation was achieved for most compounds within 17 minutes depicted in Figure 3.4. However, there is a slight overlap of dihydroferulic acid-4-*O*-sulphate and ferulic acid-4-*O*-sulphate. These compounds can be distinguished using their specific multiple reaction monitoring.

Figure 3.4: Total ion chromatogram of six major urinary CQA metabolites and the internal standard, vanilloylglycine (1), dihydrocaffeic acid-3-*O*-sulphate (2), dihydroferulic acid-4-*O*-sulphate (3), ferulic acid-4-*O*-sulphate (4), feruloylglycine (5), dihydroferulic acid (6) and sinapic acid (7)



Sulphate conjugates tend to yield broader peaks which could be due to incomplete protonisation [68], however, the pH cannot be dropped further due to incompatibility with the column, the HPLC and the mass spectrometer.

The recovery of six urinary metabolites of caffeoylquinic acid and its derivatives were assessed and the results are depicted in Table 3.7.

Table 3.7: Recovery (%) of six urinary metabolites and internal standard at 2, 5, 50 µM, n=6 (three samples, two injections), mean ± standard deviation

	VA-GLY	**DHCA-3-*O*-S**	**DHFA-4-*O*-S**	**FA-4-*O*-S**	**FA-GLY**	**DHFA**	**SA**
2 µM	84.4 ± 1.2	76.7 ± 0.8	65.2 ± 3.2	48.5 ± 0.3	80.4 ± 0.9	62.1 ± 0.6	30.1 ± 11.9
5 µM	105.8 ± 1.4	126.9 ± 1.2	115.4 ± 2.4	96.8 ± 1.8	102.1 ± 1.5	93.6 ± 1.2	101.6 ± 1.9
50 µM	94.9 ± 0.4	111.9 ± 0.9	103.4 ± 1.0	99.7 ± 0.5	95.4 ± 1.3	92.0 ± 0.9	86.6 ± 1.1

There is an overall tendency that the recovery rates of 2 µM concentration are very low, whereas the recovery for 5 and 50 µM are overall higher and closer to the amount spiked in the sample. However, the recovery for the sulphates, especially the dihydro forms at 5 µM concentration tend to be above the amount spiked in urine. This could be due to ion enhancement effects in the electrospray ionisation source [68].

3.4.4 Pancreatic digestion of coffee, caffeoylquinic acid and its derivatives

The presented data has been published by da Encarnaçao *et al.*, 2014 [25].

Incubation of the coffee solution with porcine pancreatin at 8.5 mg protein/mL led to hydrolysis of caffeoylquinic acid and its derivatives present in the coffee to give free caffeic acid, with a rate of 203 pmol/mg protein/min as shown in Figure 3.5. Pure caffeoylquinic acids (50 µM each) were tested as pancreatin substrates. Linear rates over 60 min were obtained, equivalent to 1.6, 2.4 and 5.4 pmol/mg protein/min for 3-CQA, 4-CQA and 5-CQA, respectively, depicted in Figure 3.6, Figure 3.7, and Figure 3.8. No significant increase in ferulic acid was observed after pancreatin treatment of coffee, and also no ferulic acid was detected after incubation of pancreatin with 50 µM 3-FQA, 4-FQA and 5-FQA. The data suggests that CQAs, and in

particular 5-CQA, are better substrates for pancreatin, a complex mixture of various lipases, peptidases, amylases and non-specific carboxylesterases. No digestion was observed when the coffee solution and each of the caffeoylquinic acids were incubated with a heat-inactivated pancreatin solution.

Figure 3.5: Pancreatic digestion of instant coffee with measument of caffeic acid concentration over sixty minutes with samples taken at 0, 10, 20, 40, and 60 minutes, n=4 (two biological samples, two injections), mean ± standard deviation. The enzymatic rate is 203 pmol/mg protein/min

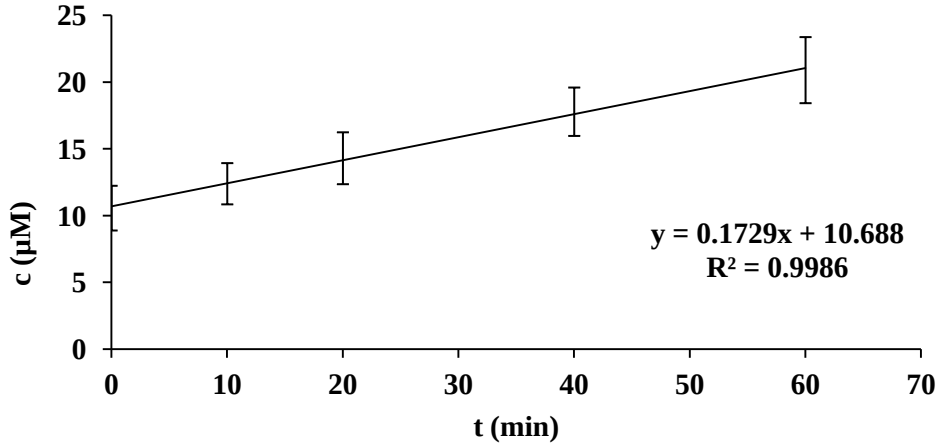


Figure 3.6: Pancreatic digestion of 3-CQA with measurement of caffeic acid concentration over sixty minutes with samples taken at 0, 10, 20, 40, and 60 minutes, n=4 (two biological samples, two injections), mean ± standard deviation. The enzymatic rate is 1.6 pmol/mg protein/min

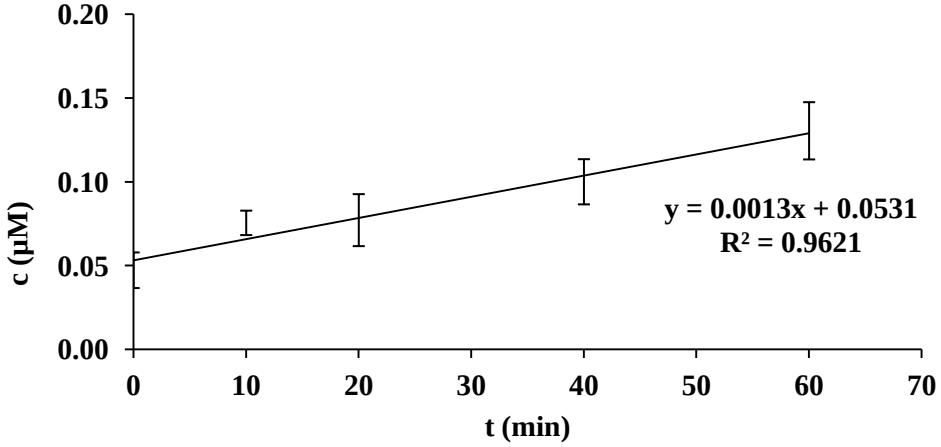


Figure 3.7: Pancreatic digestion of 4-CQA with measurement of caffeic acid concentration over sixty minutes with samples taken at 0, 10, 20, 40, and 60 minutes, n=4 (two biological samples, two injections), mean ± SD. The enzymatic rate is 2.4 pmol/mg protein/min

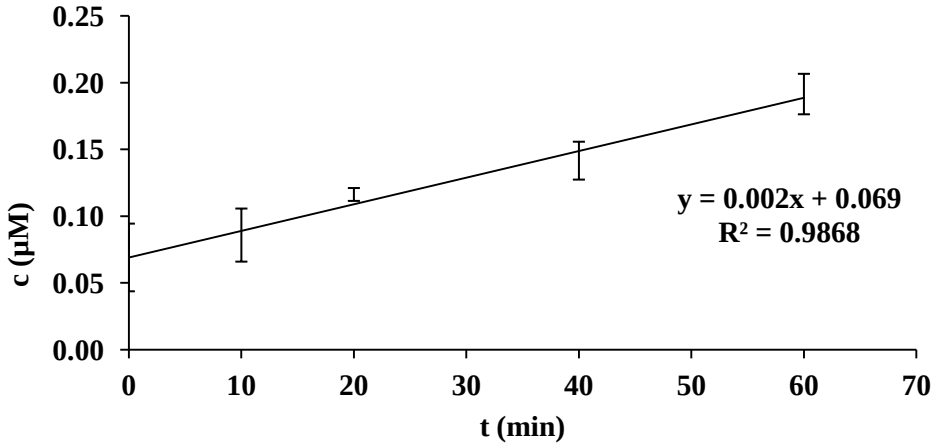
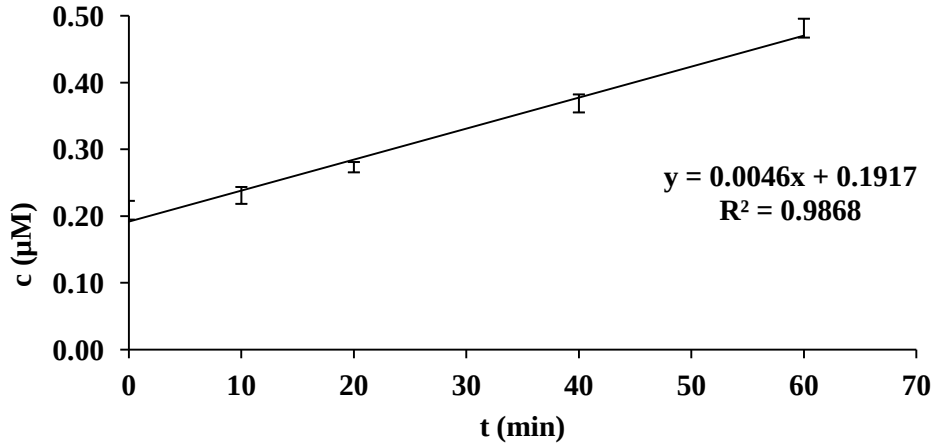


Figure 3.8: Pancreatic digestion of 5-CQA with measurement of caffeic acid concentration over sixty minutes with samples taken at 0, 10, 20, 40, and 60 minutes, n=4 (two biological samples, two injections), mean ± standard deviation. The enzymatic rate is 5.4 pmol/mg protein/min



3.5 Discussion

Dihydrocaffeic acid-3-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and ferulic acid-4-*O*-sulphate have been synthesised in hundreds of milligram amounts, purity confirmed by MS^2, ^{1}H-NMR, ^{13}C-NMR and IR, and their physicochemical properties determined, which are in line with a previous publication of Fumeaux *et al.* [30]. For the first time these sulphates have been synthesised in practical amounts. The synthesised amounts of these non-commercially available standards allowed us to develop and validate a new, quick method for the quantitative LC-MS analysis of the six highest urinary metabolites of caffeoylquinic acids and its derivatives, e.g. vanilloylglycine, feruloylglycine, dihydrocaffeic acid-3-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate, ferulic acid-4-*O*-sulphate and dihydroferulic acid. Previous methods developed by Stalmach *et al.* [7] and Farrell *et al.* [68] exceeded a run time of 60 minutes and were therefore not suitable for the analysis of several hundred urine samples in a reasonable time period. The method consists of a quick sample preparation step followed by a chromatographic method which fully separates the six major urinary metabolites of caffeoylquinic acid and its derivatives over 17 minutes and takes 30 minutes to finish. The LC-MS method has been validated including linearity, limit of detection and quantitation and recovery rates from spiked samples and is therefore fit for purpose. The limit of detection and quantitation of the developed LC-MS method were up to a factor of 20 higher compared to Farrell *et al.* [68], however, Farrell *et al* based her calculation on the signal-to-noise-ratio whereas the slope and intercept of the equation of the calibration curves to calculate LOD and LOQ, respectively, were used here. The recovery rates are very close to the spiked amount for 5 and 50 µM (100 ± 15%) and reasonable for 2 µM. The method was used to analyse urine samples of participants in a study described in chapter 4, where the results are also presented.

Numerous previous studies described the detection and quantification of caffeoylquinic acid and its derivatives in coffee beverages [10, 12, 17, 19, 20, 78]. The major compounds are 3-, 4- and 5-caffeoylquinic acid whereas 5-caffeoylquinic acid is by far the most abundant [7, 15, 19]. In this study, Nescafe Green Blend™, a commercially available instant coffee, was analysed for caffeoylquinic acid and its derivatives, e.g. 3-, 4-, and 5-caffeoylquinic acid, 3-, 4-, and 5-

feruloylquinic acid, 3,4-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid. However, 4,5-dicaffeoylquinic acid was not analysed since the chemical standard was not available.

With 68.5% (molar ratio), 3-, 4-, and 5-caffeoylquinic acid were the major constituents, whereas 3-, 4-, and 5-feruloylquinic acid accounted for only 19.1% (molar ratio) and 3,4-diCQA and 3,5-diCQA for only 12.4% (molar ratio) of the total amount of 881.8 µmol of caffeoylquinic acid and its derivatives. The ratio of the various caffeoylquinic acid and its derivatives is in accordance with previous publications [7, 15], however, the total amount of caffeoylquinic acid and its derivatives is more than two-fold higher compared with these publications. In contrast, by comparison with Erk *et al's* publication [17], the caffeoylquinic acid content in this chapter is on the lower end (881.8 µmol vs 1053, 2219 and 4525 µmol).

Over the past five years, three studies by independent groups have been published on the absorption, metabolism and excretion of caffeoylquinic acid and its derivatives in humans who underwent ileostomy, removal or by-pass of the colon. The studies were performed to assess the role of the stomach and small intestine on the absorption and metabolism of caffeoylquinic acid and its derivatives excluding the effect of colonic catabolism. The studies found that the absorption is attenuated, as measured as the percentage of ingestion (8 ± 1% [15] and 15 ± 7% [17]) in 24 hour urine and the metabolites detected included mainly non-reduced caffeic and ferulic acid phase II metabolites. The following reactions are attributed to the small intestine cleavage of the ester bond in caffeoylquinic acid and its derivatives, sulphation of the free hydroxycinnamic acids and the methylation of caffeic acid yielding isoferulic acid [15]. Whereas in the colon the cleavage of the ester bond in caffeoylquinic acid and its derivatives is attributed to colonic microbial esterases [26], the cleavage in the small intestine has not yet been investigated in detail. Pancreatin, a complex mixture of various digestive enzymes including lipases, trypsin, peptidases, proteases, RNAses, DNAses, amylases and unspecific carboxylesterases excreted by the pancreas into the duodenum, the upper part of the small intestine, is supposed to have a hydrolysing effect on the ester bond of caffeoylquinic acid and its derivatives ascribed to the carboxylesterases. The hydrolysing effect of pancreatin was tested on coffee, 3-, 4-, and 5-caffeoylquinic acid and 3-, 4-, and 5-feruloylquinic acid. Coffee was the best substrate concerning the rate of hydrolysis of caffeoylquinic acid and its derivatives (203

pmol/mg protein/min). Results from caffeoylquinic acid and feruloylquinic acid treatment by porcine pancreatin indicated that 5-caffeoylquinic acid is the highest substrate with a rate of hydrolysis (5.4 pmol/mg protein/min) up to 5-fold higher when compared to 3-CQA (1.6 pmol/mg protein/min) and 4-CQA (2.4 pmol/mg protein/min). No hydrolysing effect of porcine pancreatin was observed on feruloylquinic acids. This indicates that the early plasma appearance of ferulic acid phase II metabolites [15, 33] is probably due to the methylation of the 3-position of caffeic acid after cleavage of the ester bond and further phase II conjugation reactions which is in line with the data of Stalmach *et al.*[15]. The data indicates that pancreatin has a substantial contribution to the hydrolysis of the ester bond between caffeic acid and the quinic acid moiety in caffeoylquinic acids, in particular on 5-caffeoylquinic acid, in the small intestine.

Chapter 4: Design and performance of human study and subsequent analysis of highest urinary metabolites of caffeoylquinic acid and its derivatives

4.1 Abstract

This chapter reports on the design and performance of the human study, the procedure of the urine sample analysis and presents the data of the urinary excretion of metabolites of caffeoylquinic acid and its derivatives.

The study aimed to investigate the intra- and interindividual variation of absorption and metabolism of caffeoylquinic acid and its derivatives and the linkage to some biomarkers of health. The intra- and interindividual variation of absorption and metabolism of caffeoylquinic acid and its derivatives after coffee consumption is described in detail in chapter 4, whereas the correlation to biomarkers of health is described in chapter 5.

The study consisted of three phases and included 63, 47 and 40 participants. During each phase participants underwent a wash-out of 36 hours prior to the beginning of the study, consumed a cup of standardised coffee (881.8 µmol of caffeoylquinic acid and its derivatives), followed by a low polyphenol diet during the 36 hours of urine collection thereafter. A baseline urine sample was collected and all urine was collected in time periods of 0-4, 4-8, 8-12, 12-24 and 24-36 hours. The urine was analysed by LC-MS for the six major metabolites of caffeoylquinic acid and its derivatives. The highest excreted metabolites are dihydrocaffeic acid-3-*O*-sulphate (60.2 µmol), feruloylglycine (51.7 µmol), vanilloylglycine (41.9 µmol), ferulic acid-4-*O*-sulphate (40.7 µmol), dihydroferulic acid-4-*O*-sulphate (27.0 µmol), and dihydroferulic acid (19.2 µmol) for phase 1. The total amount excreted was 240.6 µmol which is equivalent to 27.3% of the ingested dose.

The inter- and intraindividual variation is also described. In phase 1, phase 2 and phase 3 the summed amount of all five metabolites of caffeoylquinic acid and its derivatives excreted over 36 hours vary by up to 8-fold, 10-fold and 35-fold between the participants, respectively. The intraindividual variation for the individual participants who completed all three phases of the study was the highest for dihydrocaffeic acid-3-*O*-sulphate (52.3% CV), vanilloylglycine

(51.8% CV), dihydroferulic acid-4-*O*-sulphate (50.2% CV), dihydroferulic acid (46.1% CV), ferulic acid-4-*O*-sulphate (37.4% CV) and feruloylglycine (35.9% CV). The colonic microbial derived metabolites showed the highest coefficient of variation whereas the small intestinal metabolites were demonstrated to show a lower coefficient of variation. This leads us to the conclusion that the colonic microflora undergoes substantial changes over a time period of six to eight weeks in terms of quantity and composition of bacterial strains and their metabolic competence might be due to changes.

4.2 Introduction

Several studies have been performed to investigate the absorption, metabolism and excretion of caffeoylquinic acid and its derivatives in healthy humans and ileostomists [7, 15-18, 21, 22, 28, 30, 31, 33, 73, 79, 80]. Some studies showed the extensive metabolism caffeoylquinic acid and its derivatives undergo, when passing through the human body [7, 17]. However, the first studies investigating the absorption, metabolism and excretion of caffeoylquinic acid and its derivatives in humans used either enzymatic or alkaline hydrolysis to cleave the phase II functional groups, e.g. sulfate and glucuronide, to yield the aglycone form of caffeic or ferulic acid for quantification, and thus did not provide a full scheme of the potential bioactive forms within the circulatory system of the human body. Stalmach *et al.* were the first to quantify plasma and urinary metabolites of caffeoylquinic acid and its derivatives using some chemically synthesised standards, e.g. feruloylglycine and isoferuloylglycine for precise LC-MS based quantitation [7] in 2009. A year later, Fumeaux *et al.* chemically synthesised several sulfates and glucuronides of caffeic and ferulic acid, the aglycone forms of caffeoylquinic acid and its derivatives, and quantified these in urine and plasma post coffee consumption [30]. Moreover, a study was performed to investigate the absorption of caffeoylquinic acid and its derivatives in ileostomists demonstrating that two third of all ingested compounds reach the colon and only 8% are excreted as urinary metabolites over 24 hours after coffee consumption [15]. Additionally, further studies were performed to investigate the effect of dose of caffeoylquinic acid and its derivatives in healthy humans and in ileostomists indicating the reduction of absorption of higher doses of caffeoylquinic acid and its derivatives due to enzyme saturation,

limitation of substrates, limited transport capacities at the enterocyte (influx and efflux) or an influence of the gastrointestinal transit time [16, 17].

In addition to studies of absorption, metabolism and excretion of caffeoylquinic acid and its derivatives from coffee, several epidemiological studies have been performed. An inverse correlation was found between coffee consumption and diabetes mellitus type II [37, 52] and coffee consumption showed beneficial effects on the prevention of endometrial and hepatocellular cancer [35, 63]. Furthermore regular, moderate coffee consumption is indicated to prevent Alzheimer and related diseases [81].

Previous studies either investigated the absorption, metabolism and excretion of coffee caffeoylquinic acid and its derivatives in humans or investigated effects of isolated caffeoylquinic acid and its derivatives on cell models, however, until now no study has investigated the relationship between absorption of coffee caffeoylquinic acids and its derivatives and the health status of participants. The following study aims to link these two important factors together. The chosen biomarkers of health chosen are plasma thiols, e.g. cysteine, homocysteine, cysteinylglycine and glutathione for cardiovascular health and insulin as an indicator for the metabolic syndrome. In urine, the five highest metabolites of caffeoylquinic acid and its derivatives according to Stalmach *et al.* [7] including the newly detected and quantified vanilloylglycine, described in 2.4.4, were analysed, e.g. dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, dihydroferulic acid-4-*O*-sulphate, ferulic acid-4-*O*-sulphate and dihydroferulic acid.

This chapter describes the design and execution of the human study, the sample collection, storage and analysis of the urine and the presentation of urinary excretion of metabolites of caffeoylquinic acid and its derivatives including the display of inter- and intraindividual variation. The relationship between absorption of coffee caffeoylquinic acid and its derivatives in humans and the biomarkers of health is presented in chapter 5.

4.3 Methods

4.3.1 Samples size calculation

The calculation of the sample size takes the mean and standard deviation of a specific parameter of a population into account in order to provide meaningful, statistical data. The samples size calculation was performed using various biomarkers of health measured in baseline plasma, e.g. thiols (total cysteine, homocysteine, cysteinylglycine, glutathione) and insulin. The sample size was calculated using the following website http://www.statisticalsolutions.net/pss_calc.php. Table 4.1 depicts the measured mean and standard deviation of a pilot study including six subjects in column 2. The mean and standard deviation of previously published data is depicted in column 3. The probability of falsely calculating the null hypothesis was set to 0.05 and the probability of correctly rejecting the false null hypothesis was set to 0.8. The calculated sample size for the individual compound is shown in column 4 and the number of participants is the highest for homocysteine with 52, thus the aim was to recruit at least 52 participants. With 62 participants in phase 1, the target was met.

Table 4.1: Sample size calculation based on the measured biomarkers of health, e.g. thiols and insulin form a pilot study population (n=6). The measurement of homocysteine indicate a requirement of at least 52 participants in order to obtain, meaningful, statistically significant data, whereas for cysteine and insulin only 6 and 15 participants were required. Derived and adapted from Miss Joana Amarante Encarnacao's thesis, 2015

Biomarker	$\mu_0 \pm SD$	$\mu_1 \pm SD$	n (sample size)
Total cysteine	251 ± 10	289 ± 31	6
Total homocysteine	7.4 ± 2	8.1 ± 1.8	52
Total cysteinylglycine	37 ± 4	28 ± 2	1
Total glutathione	7.9 ± 0.6	3.71	1
Insulin	7.3 ± 1.9	9.6 ± 3.1	15

4.3.2 Recruitment and personal information

Healthy participants were recruited by poster advertisement on the campus of the University of Leeds and were asked to attend an information session where the course of the human study, the inclusion criteria and the risks were explained. Prior to the beginning of the study participants gave their written, informed consent and were informed of the possibility to withdraw from the study at any time. A subject code was assigned to every participant and their personal data kept apart from the data of the study. Thereafter they filled in a health questionnaire to assess their conformity with the inclusion criteria. Healthy participants were defined by inclusion criteria such as age (18-70 years), body mass index (18-29 kg/m^2), non-smoker, or weak smoker, alcohol consumption (no more than 4 UK units on average on a daily basis), physical health (did not suffer from diagnosed chronic diseases, haemophilia, or underwent previous gastrointestinal tract surgery), medication (no intake of long term prescription only medicine) and women were neither pregnant nor lactating. Additionally, information on physical activity and bowel movement was gathered. Participants who fulfilled these inclusion criteria were accepted into the study and filled in a food frequency questionnaire in order to assess their food and drink intake, covering among others coffee and tea consumption and type, fruit, vegetable and spice consumption.

4.3.3 Study design

After providing their written informed consent and assessment of conformity with the inclusion criteria, participants were invited to the first phase of the study and were given a food restriction list and a food diary. They were asked not to consume several types of food for 72 hours, 36 hours prior to their visit (washout) and 36 hours after their visit (urine collection). The following foods were included in the restriction list: any type of coffee, tea, chocolate, alcoholic beverages, all fruits and fruit-based products with the exception of banana and kiwi, all vegetables and vegetable-based products with the exception of mushrooms, cucumber, courgette, asparagus, lettuce, pumpkin and fruit or vegetable-based food supplements. The

participant's diet was documented in the food diary during the 72 hours to assure compliance with the food restriction.

The washout started on the evening of day 1 and participants fasted for at least 10 hours overnight on day 2. At the next morning (day 3), participants were allowed to void the morning urine as normal and drank at least 500 mL of water one hour prior to arriving at the School of Food Science and Nutrition between 7:00 and 9:30 am.

Anthropometric measurements of the height, weight, waist and hip circumference and the blood pressure were recorded. Two baseline blood samples of 6 mL were collected by venepuncture of the antecubital vein. The blood was immediately processed to yield plasma described in Miss Joana Encarnaçao's thesis, 2015.

Participants collected a baseline urine sample. Afterwards they drank a cup of coffee containing 882 µmol of caffeoylquinic acids and its derivatives as described in chapter 3 and were provided with a breakfast consisting of toast, hard and spread cheese and milk. They were allowed to add water or milk to their coffee according to their preference. They were instructed to collect all urine in time periods of 0-4, 4-8, 8-12, 12-24 and 24-36 hours post coffee consumption and to report any loss.

The urine collection finished in the evening of day 4. At the morning of day 5, urine was returned and immediately processed and subsequently analysed as described in chapter 3. The same procedure (phase 2 and 3) was repeated about six and twelve weeks after the completion of the first phase for the assessment of intrapersonal variation in absorption. Participants were remunerated with GBP 10 for each completed phase. The individual data is stored separately from the participants codes and is kept confidential. The human study was approved by the University of Leeds Joint Faculty Ethics Committee (MEEC 10-035) and was registered with the clinicaltrials.gov database (NCT01912144). All human study documents can be found in the appendix.

4.3.4 Analysis of human samples

Human urine samples were stored at –20ºC up to 12 months after the collection. The samples were processed and quantitatively analysed by LC-MS as described in detail in 3.3.2.

4.4 Results

4.4.1 Description of the study population

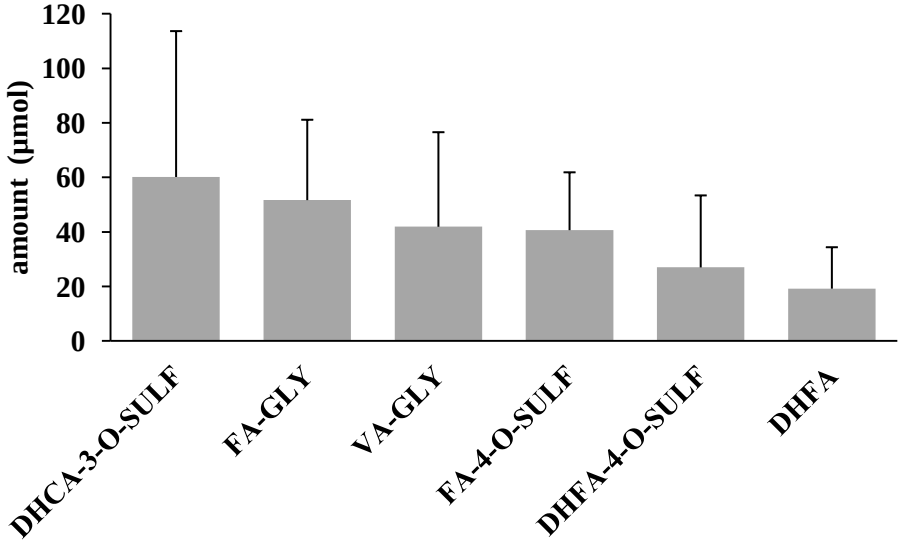
Initally, 63 participants were recruited for the human study, of which 62, 46, and 38 participants completed phase 1, phase 2 and phase 3, respectively. 41 female and 21 male volunteers participated in phase 1 with an average age of 28.3 years (18-53), an average body mass index of 23.3 kg/m^2 (18.0-29.6) and an average waist to hip ratio of 0.76 (0.67-0.94). Four participants were smokers and three former smokers. After completion of phase 1, some participants decided to quit the study or were not allowed to enter phase 2 due to time constraints. From the initial 62 participants, 46 completed phase 2. Participants of the phase 2 of the human study had an average age of 29.0 years (19-53), an average body mass index of 23.7 kg/m^2 (18.8-29.5) and an average waist to hip ratio of 0.77 (0.68-0.91). Three participants were smokers and three former smokers. Due to time constraints only 38 participants completed phase 3. Participants of phase 3 had an average age of 28.6 years (19-53), an average body mass index of 23.3 kg/m^2 (18.4-29.5) and an average waist to hip ratio of 0.77 (0.66-0.89). Three participants were smokers and three former smokers. None of the participants suffered from diagnosed chronic diseases, haemophilia, underwent previous gastrointestinal tract surgery or took prescription only medicine during the study.

4.4.2 Excretion of urinary metabolites of caffeoylquinic acid and its derivatives

The six major urinary metabolites (Figure 4.1) of caffeoylquinic acid and its derivatives were analysed in urine before and in time periods of 0-4, 4-8, 8-12, 12-24 and 24-36 hours post coffee consumption, e.g. dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid. Only small amounts of metabolites of caffeoylquinic acid and its derivatives of 0.8 ± 1.6 µmol dihydrocaffeic-acid-3-*O*-sulphate, 1.2 ± 2.2 µmol feruloylglycine, 0.8 ± 2.4 µmol vanilloylglycine, 0.9 ± 2.5 µmol ferulic acid-4-*O*-sulphate, 0.4 ± 1.0 µmol dihydroferulic acid-

4-*O*-sulphate and 0.2 ± 0.4 µmol dihydroferulic acid were detected in baseline urine. These amounts are negligible compared to the amounts excreted post coffee consumption.

Figure 4.1: Excretion of six major urinary metabolites for 36 hours after coffee consumption averaged for 62 participants in phase 1. Dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid account for 60.2 ± 53.5, 51.7 ± 29.5, 41.9 ± 34.7, 40.7 ± 21.2, 27.0 ± 26.4 and 19.2 ± 15.2 µmol, respectively, n=62, mean ± standard deviation



In the whole urine collected up to 36 hours after coffee consumption, averaged for all 62 participants, 60.2 ± 53.5 µmol dihydrocaffeic acid-3-*O*-sulphate, 51.7 ± 29.5 µmol feruloylglycine, 41.9 ± 34.7 µmol vanilloylglycine, 40.7 ± 21.1 µmol ferulic acid-4-*O*-sulphate, 27.0 ± 26.4 µmol dihydroferulic acid-4-*O*-sulphate and 19.2 ± 15.2 µmol dihydroferulic acid were quantitatively analysed. In accordance with a previous publication [7], dihydrocaffeic acid-3-*O*-sulphate is the highest excreted metabolite followed by feruloylglycine. The third most excreted metabolite is vanilloylglycine with 41.9 ± 34.7 µmol which was neither detected, nor quantified, in urine after consumption of coffee in any previous studies. In Stalmach's *et al.* [7] study dihydroferulic acid-4-*O*-sulphate was the third highest metabolite followed by ferulic-acid-3-*O*-sulfate. In our study ferulic acid-4-*O*-sulphate (40.7 ± 21.1 µmol) is the fourth highest metabolite followed by dihydroferulic acid-4-*O*-sulphate (27.0 ± 26.4 µmol). The lowest metabolite from the six metabolites analysed is dihydroferulic acid with 19.2 ± 15.2 µmol.

Adding all the metabolites excreted in the urine up to 36 hours, the amount equals to 240.6 ± 123.8 µmol which accounts for 27.3 % of the ingested dose of 881.8 µmol caffeoylquinic acid and its derivatives (compare chapter 3). Dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid accounted for 6.8, 5.9, 4.8, 4.6, 3.1 and 2.2 molar % of the ingested dose, respectively. The sulphated and glycine conjugate metabolites accounted for 14.5 and 10.6 molar % of the ingested dose, respectively.

Figure 4.2: Total excretion of six major urinary metabolites for 36 hours after coffee consumption for 62 participants of phase 1 in µmol. The lowest amount excreted is 82.9 µmol and the highest amount excreted is 693.2 µmol

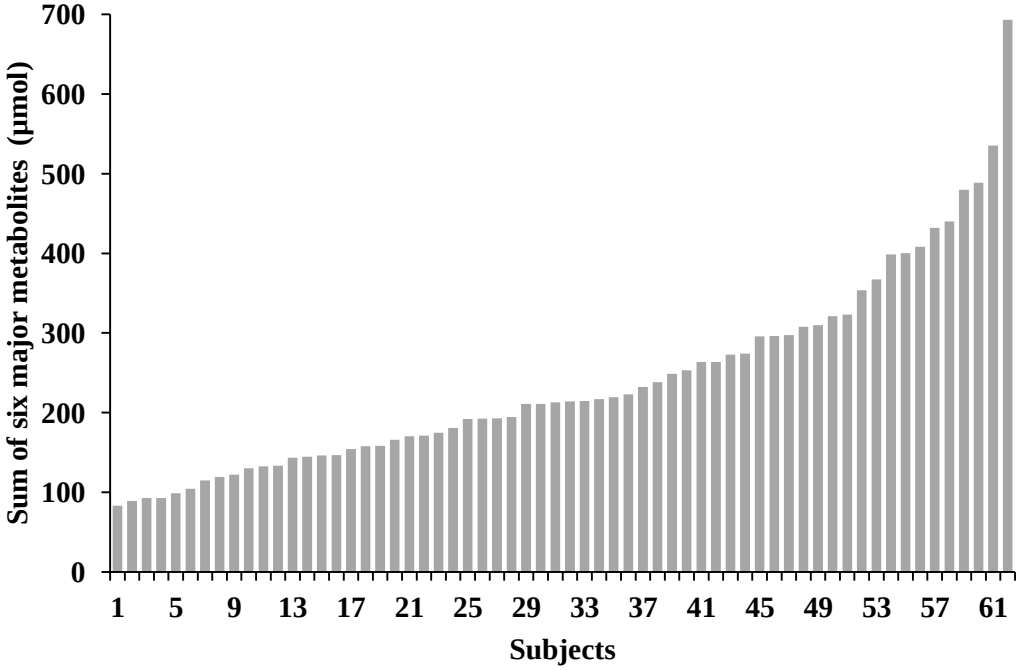


Figure 4.2 depicts the total amount of six major metabolites excreted for 36 hours after coffee consumption of 62 participants of phase 1. The lowest and highest amounts excreted are 82.9 and 693.2 µmol, respectively. Performing a Saphiro-Wilk test (p=0.000) indicates that the data is not normally distributed with a skew towards the upper end including two statistical outliers of 535.3 and 693.2 µmol. Since these values are equivalent to 60.7 and 78.6 % of the ingested dose of caffeoylquinic acid and its derivatives and therefore hypothetically possible, they were not excluded from the set of data for further analysis in chapter 5.

Since urine was collected in time periods of 0-4, 4-8, 8-12, 12-24 and 24-36 hours, each individual sample was quantitatively analysed. The summary of the excretion for dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid is depicted in Figure 4.3 and Figure 4.4. The excretion peak was at 12-24 hours for dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine and ferulic acid-4-*O*-sulphate, whereas the excretion peaks for dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid were at 8-12 hours. Only vanilloylglycine showed the excretion peak at 24-36 hours. Substantial amounts were of dihydrocaffeic acid-3-*O*-sulphate and feruloylglycine and were also excreted at 8-12 hours. Ferulic acid-4-*O*-sulphate showed biphasic behaviour with the second peak being at 4-8 hours. Dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid were also abundantly excreted at 12-24 hours, whereas substantial amounts for vanilloylglycine were excreted at 12-24 hours.

Figure 4.3: Excretion in µmol of dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine and vanilloylglycine for time periods of 0-4, 4-8, 8-12, 12-24, 24-36 hours after the consumption of coffee for 62 participants of phase 1, n=62, mean ± standard deviation

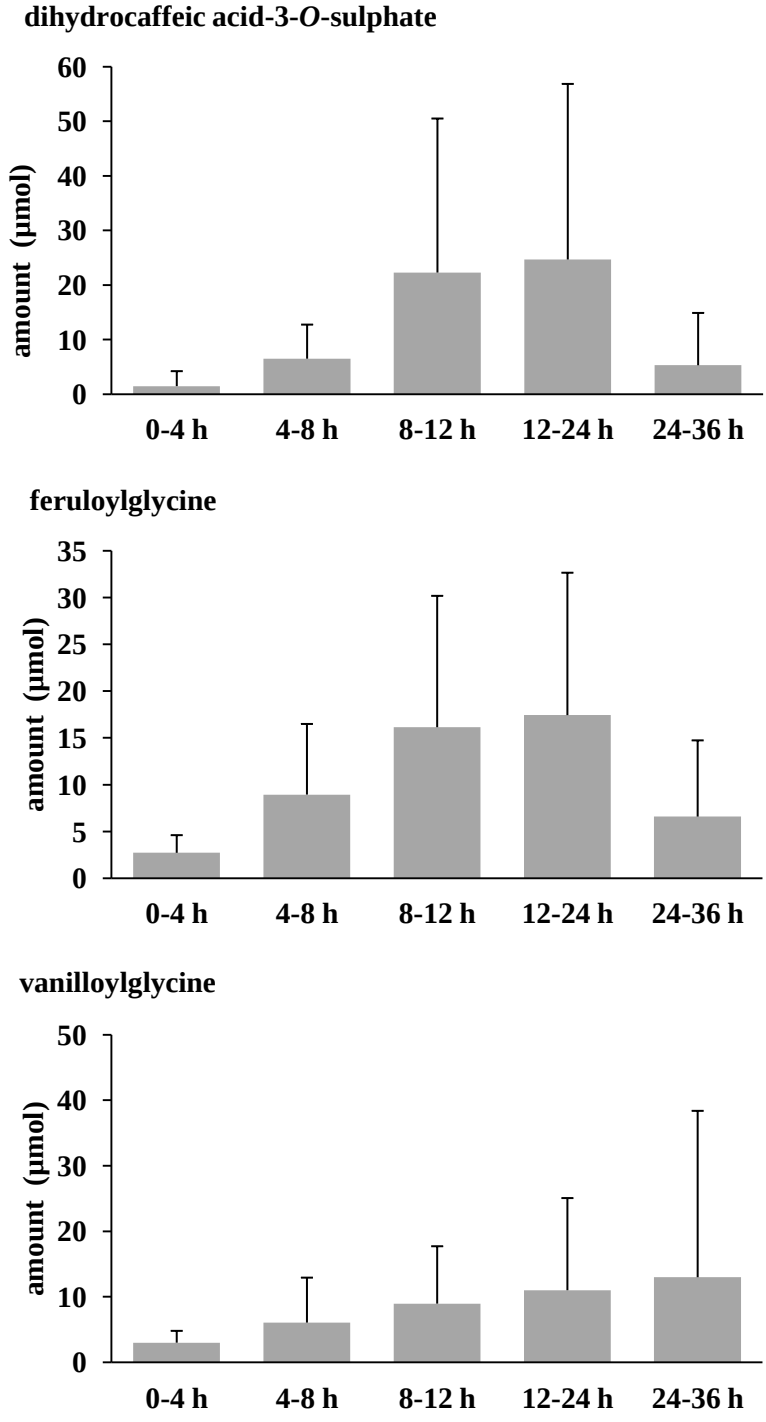


Figure 4.4: Excretion in µmol of ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for time periods of 0-4, 4-8, 8-12, 12-24, 24-36 hours after the consumption of coffee for 62 participants of phase 1, n=62, mean ± standard deviation

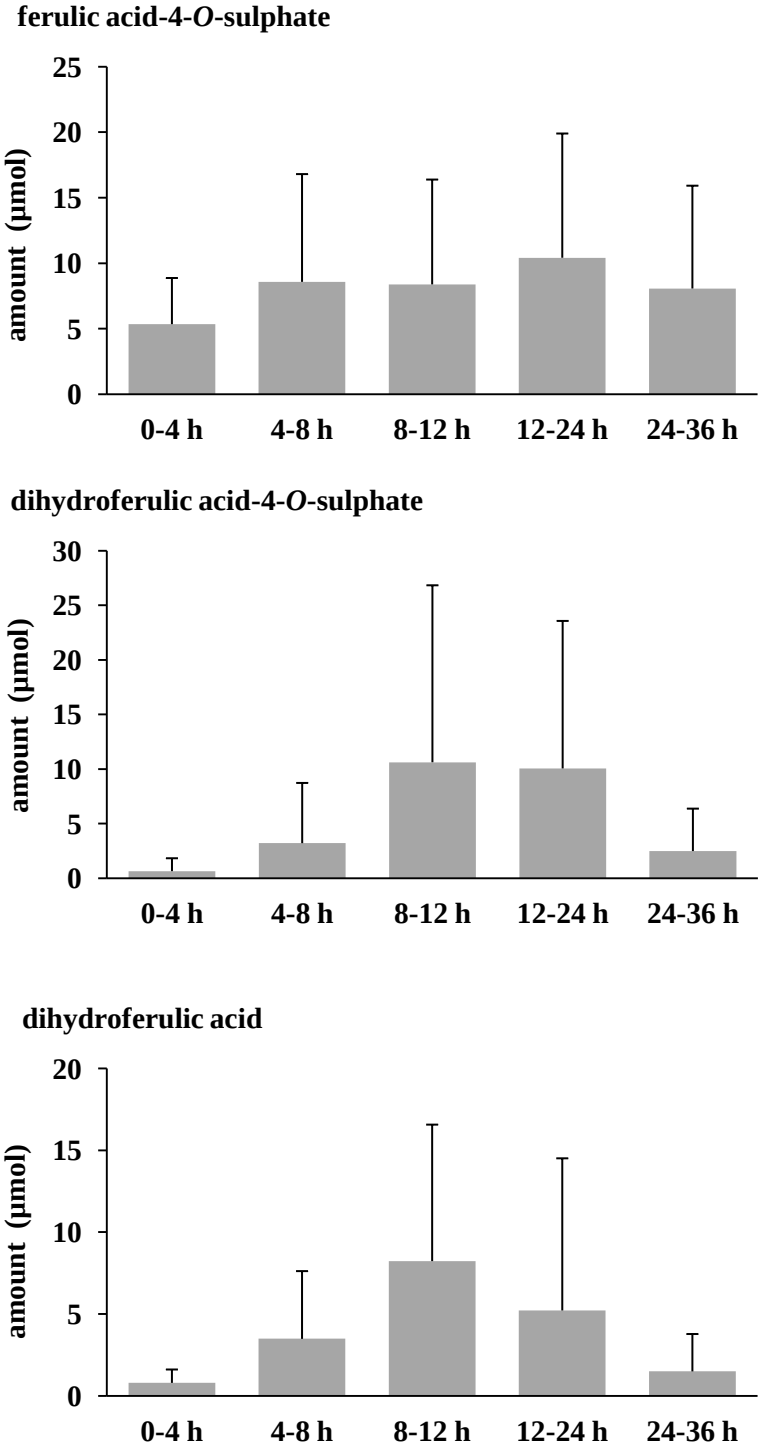
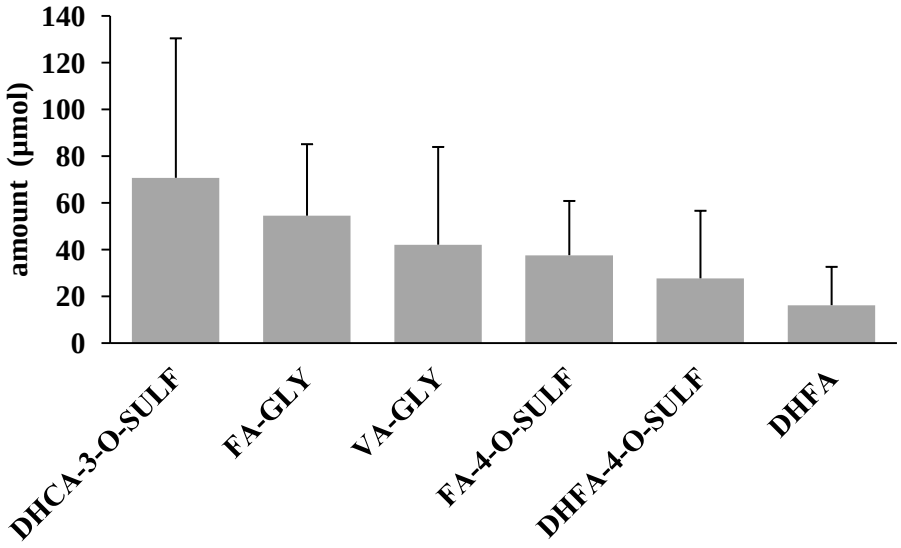


Figure 4.5: Excretion of six major urinary metabolites for 36 hours after coffee consumption averaged for 46 participants in phase 2. Dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulfate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid account for 70.7 ± 59.7, 54.5 ± 30.6, 42.1 ± 41.8, 37.6 ± 23.2, 27.7 ± 28.9 and 16.2 ± 16.4 µmol, respectively, n=46, mean ± standard deviation



Baseline urine of 47 participants was analysed for dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid and small amounts of 0.5 ± 0.0, 0.7 ± 0.9, 0.5 ± 0.0, 0.4 ± 0.7, 0.3 ± 0.6 and 0.1 ± 0.1 µmol, respectively. These amounts are negligible compared to the amounts excreted post coffee consumption.

In phase 2, urine of 47 participants (Figure 4.5) was collected up to 36 hours after coffee consumption and dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid quantitatively analysed with 70.7 ± 59.7, 54.5 ± 30.6, 42.1 ± 41.8, 37.6 ± 23.2, 27.7 ± 28.9 and 16.2 ± 16.4 µmol, respectively. The amounts excreted follow the same pattern as in phase 1 and a previously published study [7] with the exception of ferulic acid-4-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate. Again, ferulic acid-4-*O*-sulphate with 37.6 ± 23.2 µmol is the fourth highest metabolite followed by dihydroferulic acid-4-*O*-sulphate with 27.7 ± 28.9 µmol. Data of one participant is excluded from the set of data due to non-compliance with the study

guidelines, as the amount of metabolites of caffeoylquinic acid and its derivatives exceeded 100% of the ingested dose (131%).

All the metabolites excreted in urine over 36 hours after the consumption of coffee account for an amount of 248.7 ± 130.2 µmol which is equivalent to 28.2% of the ingested dose of 881.8 µmol caffeoylquinic acid and its derivatives. Dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid account for 8.0, 6.2, 4.8, 4.6, 4.3 and 1.8% of the ingested dose, respectively. The combined sulphate and glycine metabolites account for 15.4 and 11.0% of the ingested dose, respectively.

Figure 4.6: Total excretion of six major urinary metabolites for 36 hours after coffee consumption for 46 participants of phase 2 in µmol. The lowest amount excreted is 55.1 µmol and the highest amount excreted is 562.3 µmol

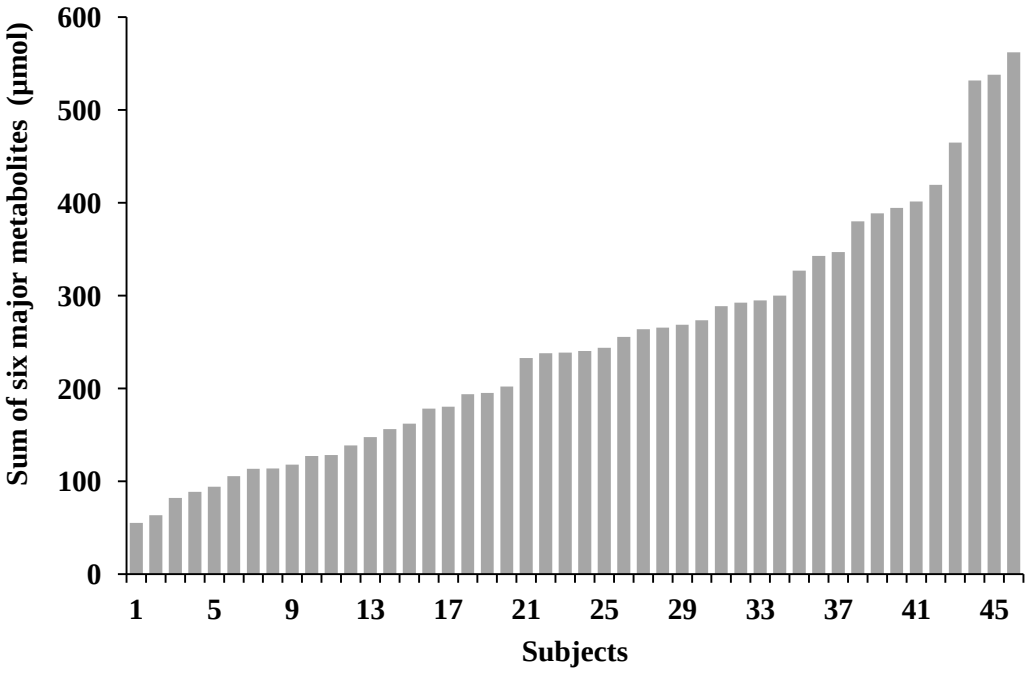


Figure 4.6 depicts the total amount of six major metabolites excreted for 36 hours after coffee consumption of individual participants of phase 2 expressed in µmol. The lowest and highest amounts excreted are 55.1 and 562.3 µmol, respectively. Performing a Saphiro-Wilk test (p=0.045) indicates that the data is not normally distributed with a light skew towards higher values, but without any statistical outliers.

The summary of the excretion for dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid is depicted in Figure 4.7 and Figure 4.8.

The excretion peak was at 12-24 hours for dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, ferulic acid-4-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate, whereas the excretion peak for dihydroferulic acid was at 8-12 hours. Only vanilloylglycine showed the excretion peak at 24-36 hours. Substantial amounts of dihydrocaffeic acid-3-*O*-sulfate and feruloylglycine were also excreted at 8-12 hours. In contrast to phase 1, ferulic acid-4-*O*-sulphate did not show biphasic behaviour with the second peak being at 4-8 hours. Dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid were also abundantly excreted at 12-24 hours, whereas substantial amounts of vanilloylglycine were excreted at 12-24 hours.

Figure 4.7: Excretion in µmol of dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine and vanilloylglycine for time periods of 0-4, 4-8, 8-12, 12-24, 24-36 hours after the consumption of coffee for 46 participants of phase 2, n=46, mean ± standard deviation

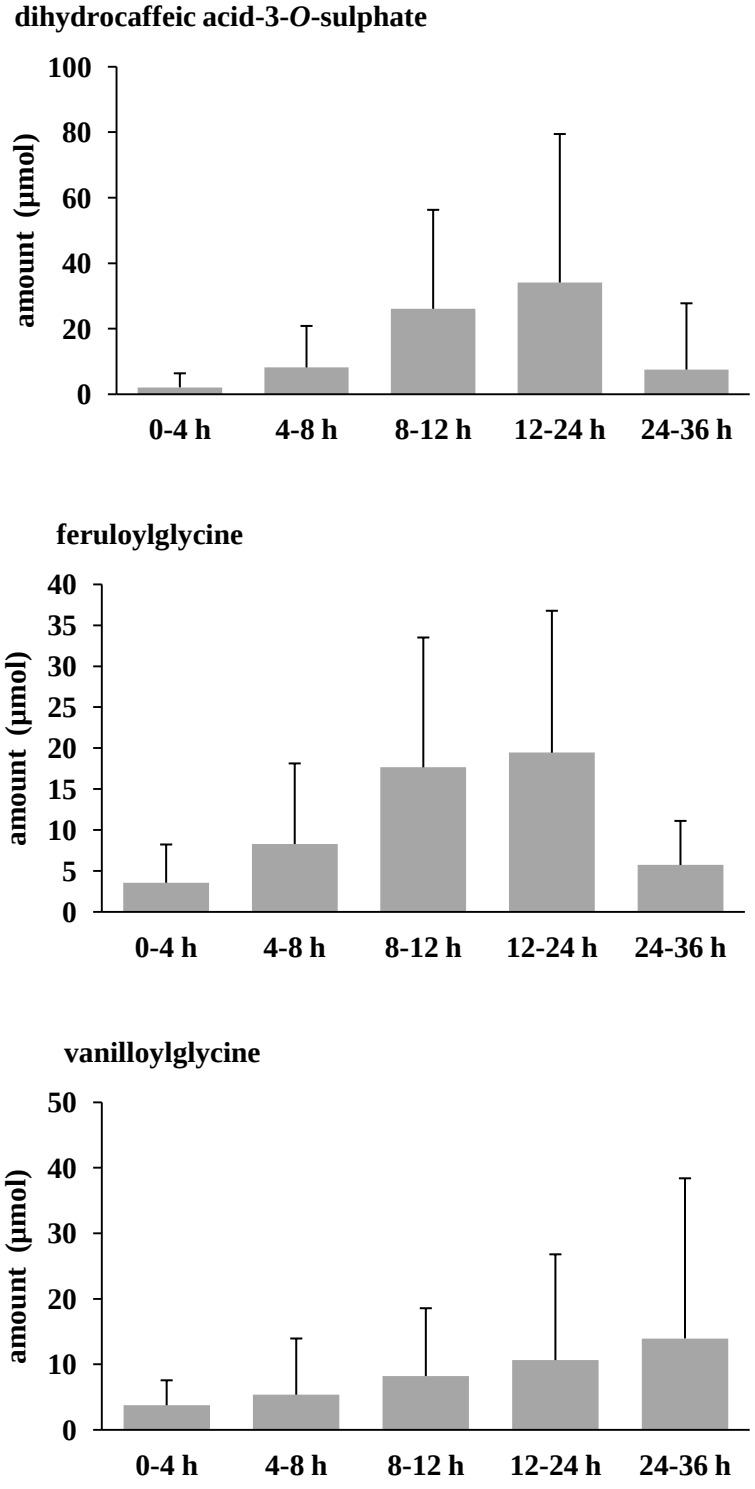


Figure 4.8: Excretion in µmol of ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for time periods of 0-4, 4-8, 8-12, 12-24, 24-36 hours after the consumption of coffee for 46 participants of phase 2, n=46, mean ± standard deviation

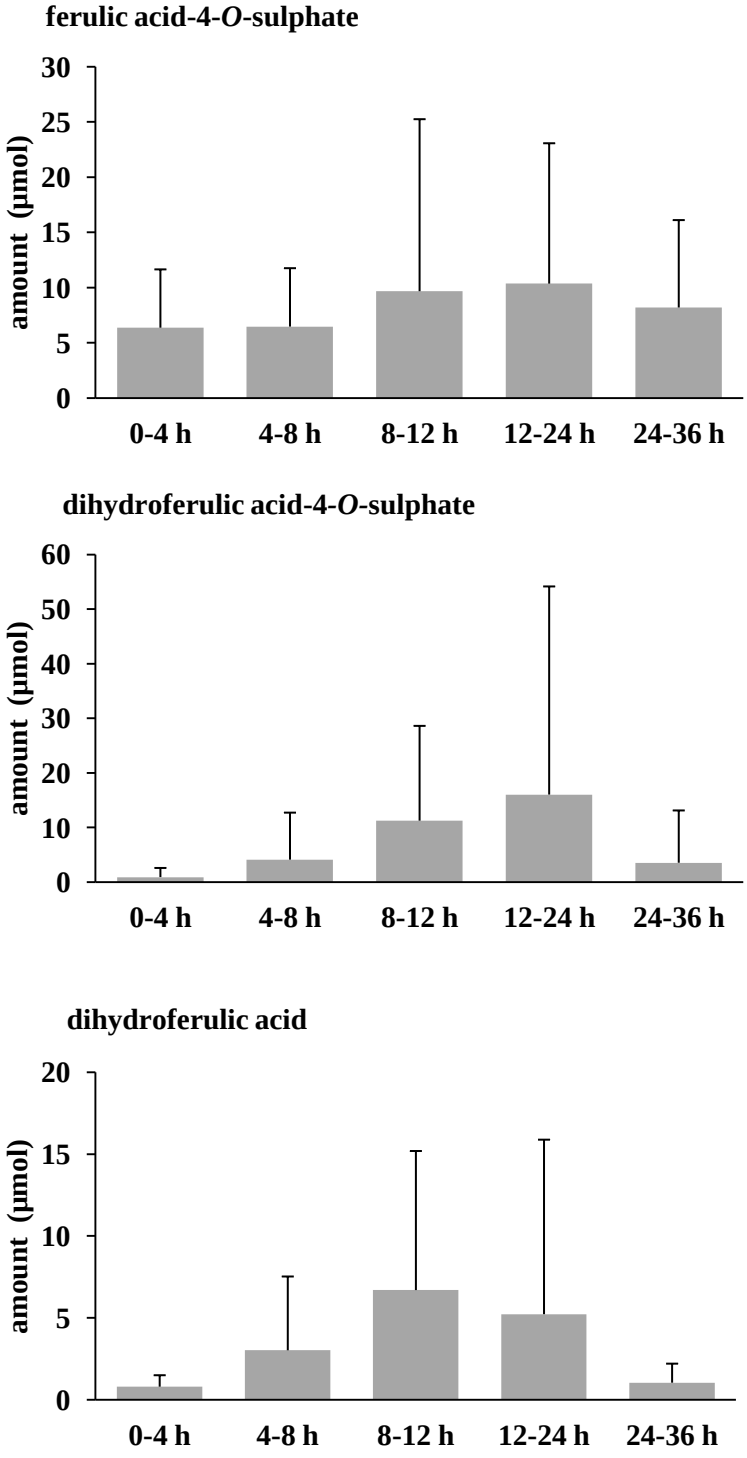
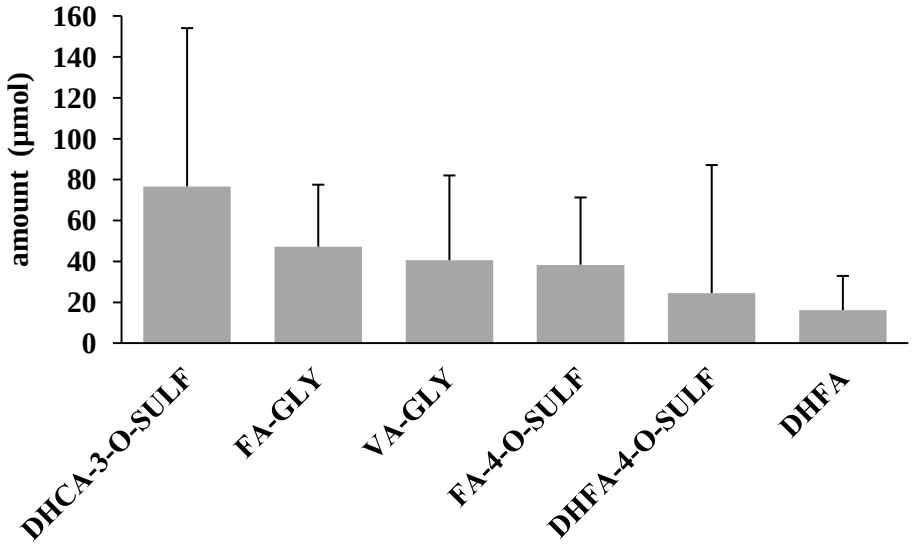


Figure 4.9: Excretion of six major urinary metabolites for 36 hours after coffee consumption averaged for 38 participants in phase 3. Dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid account for 76.6 ± 74.5, 47.1 ± 29.2, 40.6 ± 41.2, 38.3 ± 29.6, 24.5 ± 17.4 and 16.1 ± 16.2 µmol, respectively, n=38, mean ± standard deviation



In phase 3, urine of 38 participants was collected after a 36 hours washout and was analysed for dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid with 0.8 ± 2.1, 0.7 ± 1.2, 0.5 ± 1.0, 0.3 ± 0.6, 0.4 ± 1.0 and 0.1 ± 0.3 µmol, respectively. As in phase 1 and phase 2, the amounts excreted in the baseline urine are negligible compared to the amounts excreted post coffee consumption.

Figure 4.9 depicts the amounts of dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid which were analysed in all the urine of 38 participants of phase 3 collected for 36 hours post coffee consumption with 76.6 ± 74.5, 47.1 ± 29.2, 40.6 ± 41.2, 38.3 ± 29.6, 24.5 ± 17.4 and 16.1 ± 16.2 µmol, respectively.

All the metabolites excreted over 36 hours after the consumption of a cup of coffee averaged for 38 participants of phase 3 account for a total amount of 243.2 ± 169.2 µmol which is equivalent to 27.8 % of the ingested dose of caffeoylquinic acid and its derivatives.

Dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid account for 8.7, 5.3, 4.6, 4.3, 2.8 and 1.8% of the ingested dose. The combined sulphate and glycine metabolites account for 15.8 and 10.0% of the ingested dose, respectively.

Figure 4.10: Total excretion of six major urinary metabolites for 36 hours after coffee consumption for 38 participants of phase 3 in µmol. The lowest amount excreted is 17.9 µmol and the highest amount excreted is 631.9 µmol

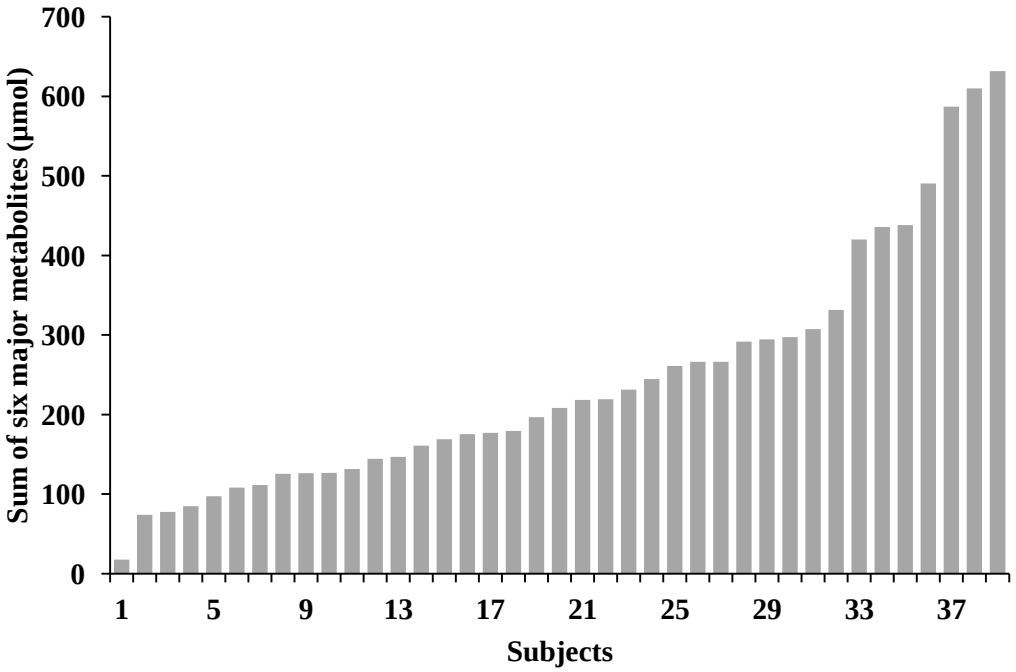


Figure 4.10 depicts the total amount of six major metabolites excreted for 36 hours after coffee consumption of 38 participants of phase 3 expressed in µmol. The lowest and highest amounts excreted are 17.9 and 631.9 µmol, respectively. Performing a Saphiro-Wilk test (p=0.002) indicates that the data is not normally distributed with a skew towards the upper end including three statistical outliers of 587.2, 609.9 and 631.9 µmol. Since these values are equivalent to 66.6, 69.2 and 71.7% of the ingested dose of caffeoylquinic acid and its derivates and therefore hypothetically possible, they were not excluded from the set of data for further analysis in chapter 5.

The summary of the excretion for dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid is depicted in Figure 4.11and Figure 4.12.

The excretion peak was at 12-24 hours for dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate, whereas the excretion peak for dihydroferulic acid was at 8-12 hours. Substantial amounts were of dihydrocaffeic acid-3-*O*-sulphate and feruloylglycine were also excreted at 8-12 hours. In contrast to phase 2, but in accordance with phase 1, ferulic acid-4-*O*-sulphate did show biphasic behaviour with the second peak being at 4-8 hours. Dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid were also abundantly excreted at 12-24 hours, whereas substantial amounts for vanilloylglycine were excreted at 12-24 hours.

Figure 4.11: Excretion in µmol of dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine and vanilloylglycine for time periods of 0-4, 4-8, 8-12, 12-24, 24-36 hours after the consumption of coffee for 38 participants of phase 3, n=38, mean ± standard deviation

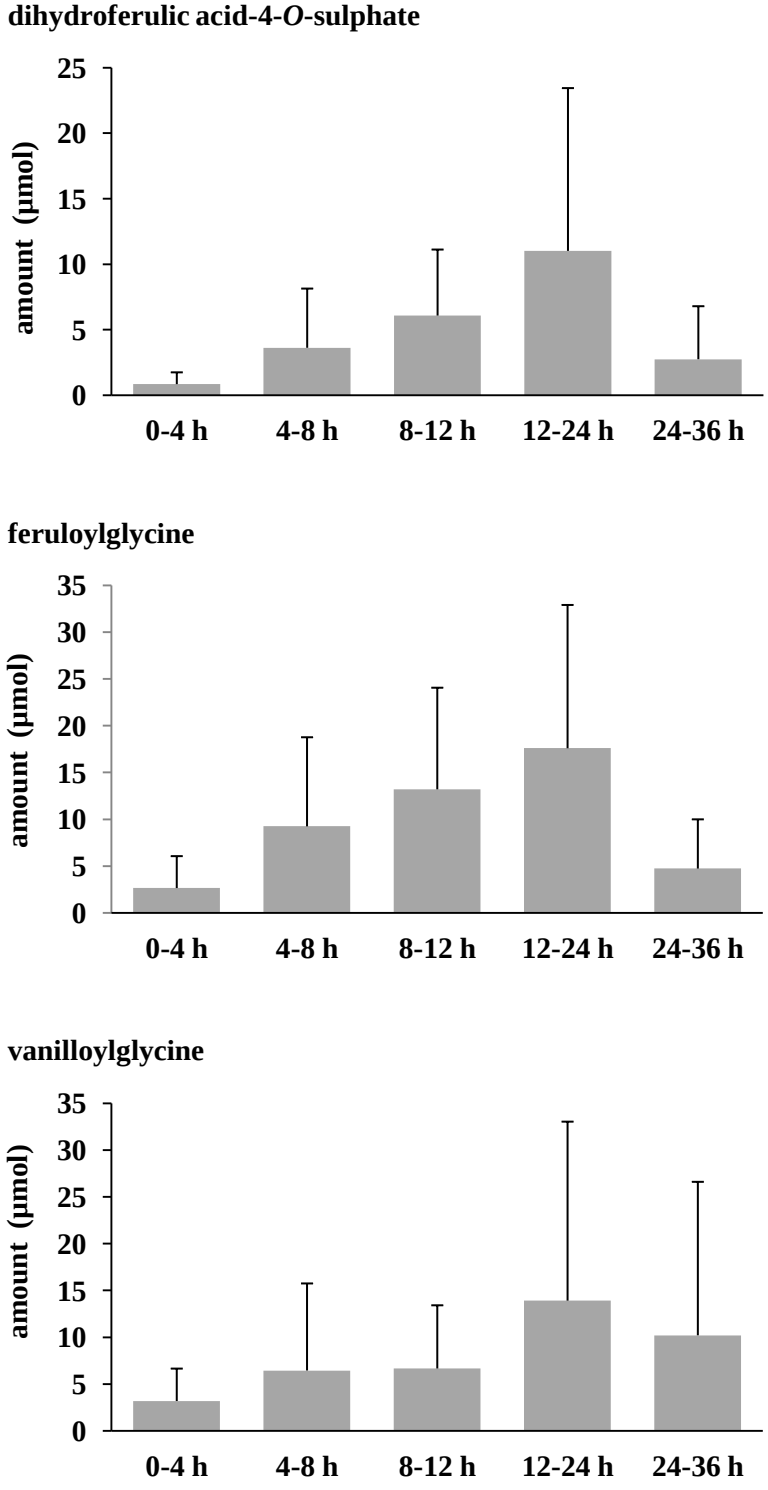
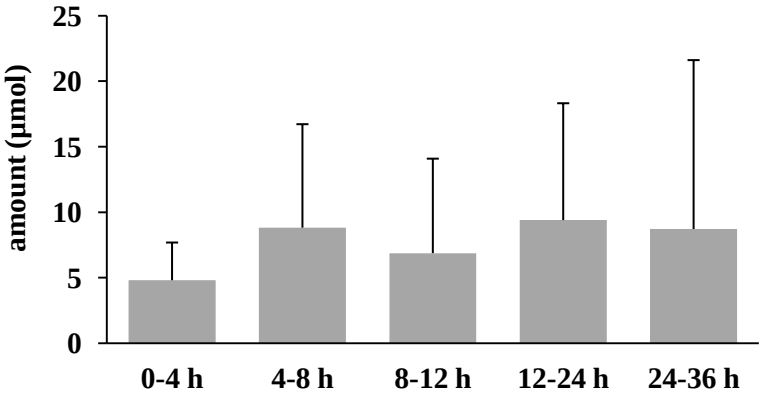
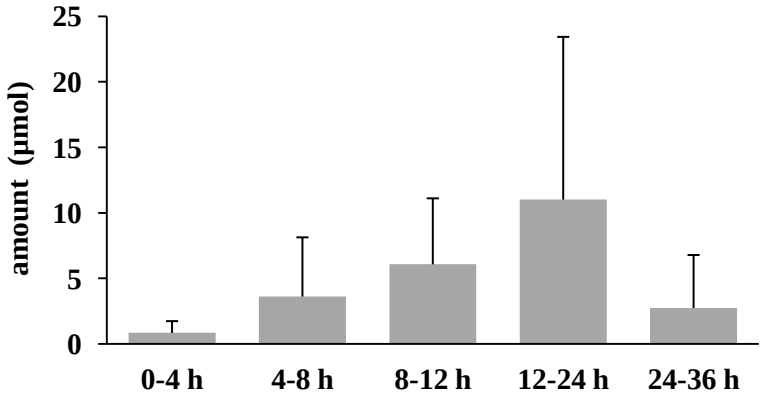


Figure 4.12: Excretion in µmol of ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for time periods of 0-4, 4-8, 8-12, 12-24, 24-36 hours after the consumption of coffee for 38 participants of phase 3, n=38, mean ± standard deviation

ferulic acid-4-*O*-sulphate



dihydroferulic acid-4-*O*-sulphate



dihydroferulic acid

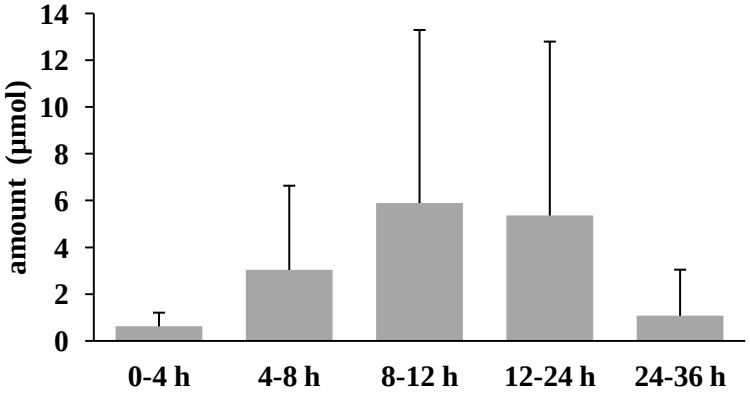


Figure 4.13: Excretion of six major urinary metabolites for 36 hours after coffee consumption of 36 participants for all three phases, n=36, data presented as mean values in µmol

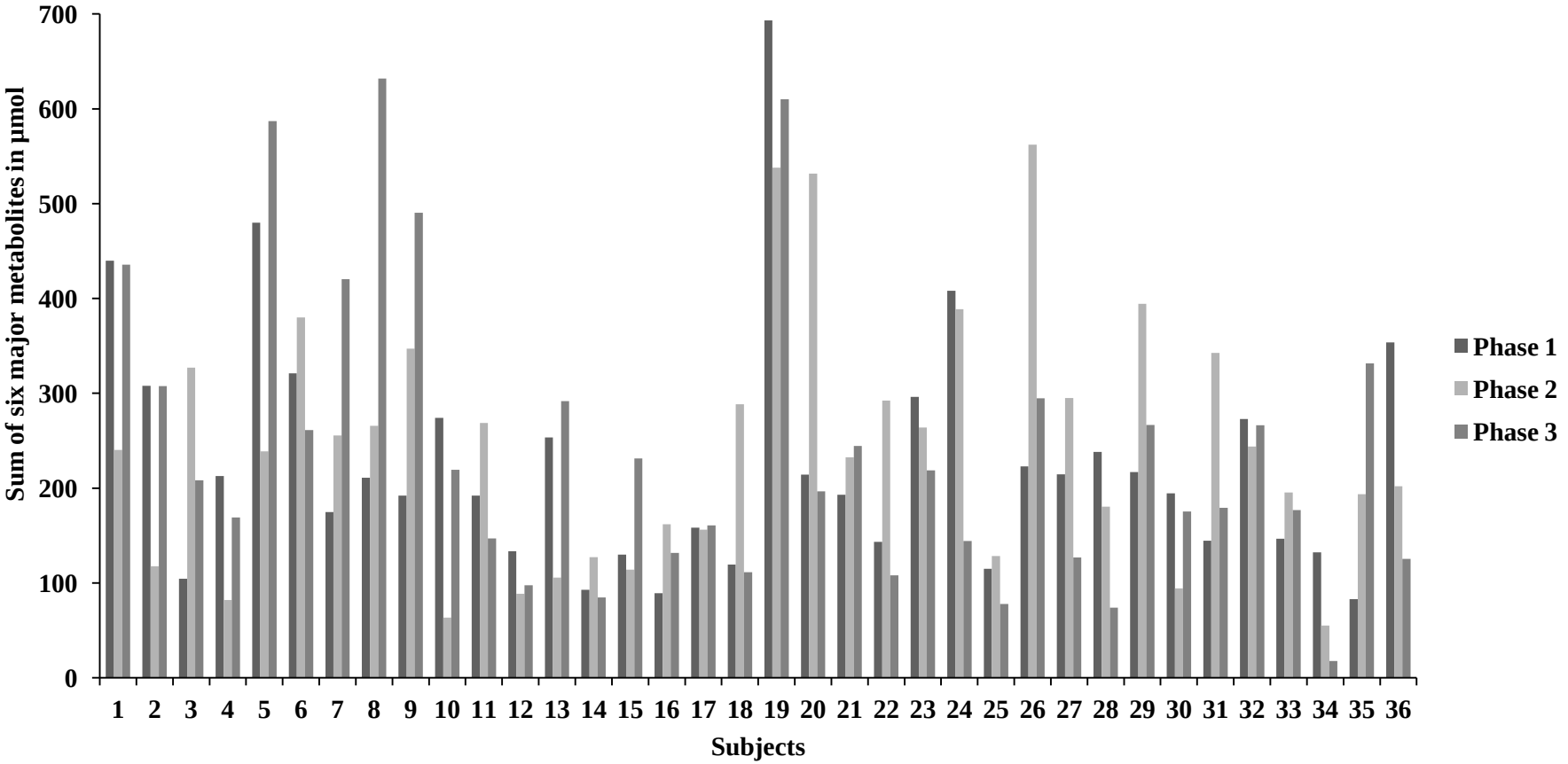


Figure 4.13 shows the excretion of six major urinary metabolites for 36 hours after coffee consumption of 36 particpants who completed all three phases of the study. The data is presented as mean values. The data of total urinary excretion of all six major urianry metabolites of caffeoylquinic acid and its derivatives shows variation of up to 3-fold between the three phases. In order to understand where the intraindividual variation derives from, the individual metabolites were also plotted for the three phases of the study in the following graphs (Figure 4.14, Figure 4.15, Figure 4.16, Figure 4.17, Figure 4.18, and Figure 4.19).

Figure 4.14: Excretion of dihydrocaffeic acid-3-*O*-sulfate for 36 hours after coffee consumption of 36 participants for all three phases, n=36, data presented as mean value in µmol

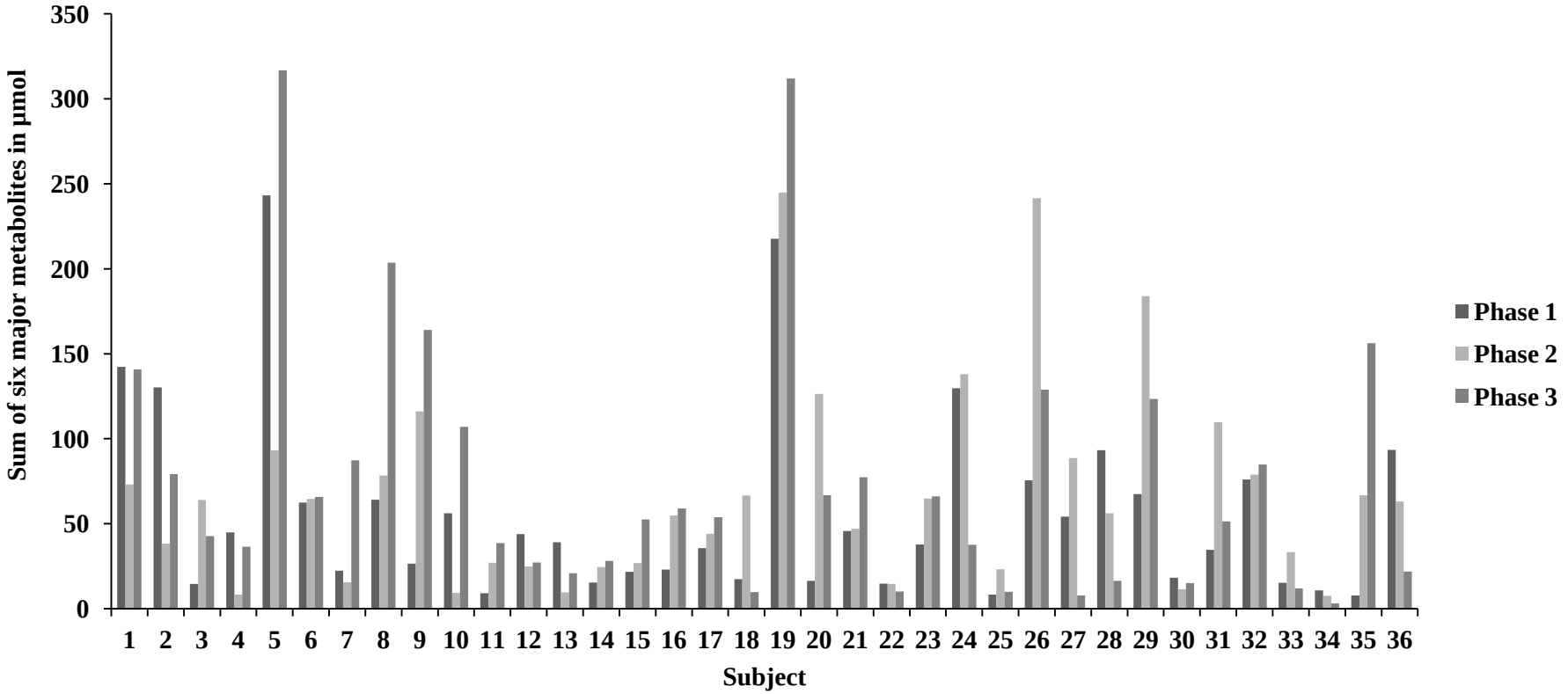


Figure 4.15: Excretion of feruloylglycine for 36 hours after coffee consumption of 36 participants for all three phases, n=36, data presented as mean value in µmol

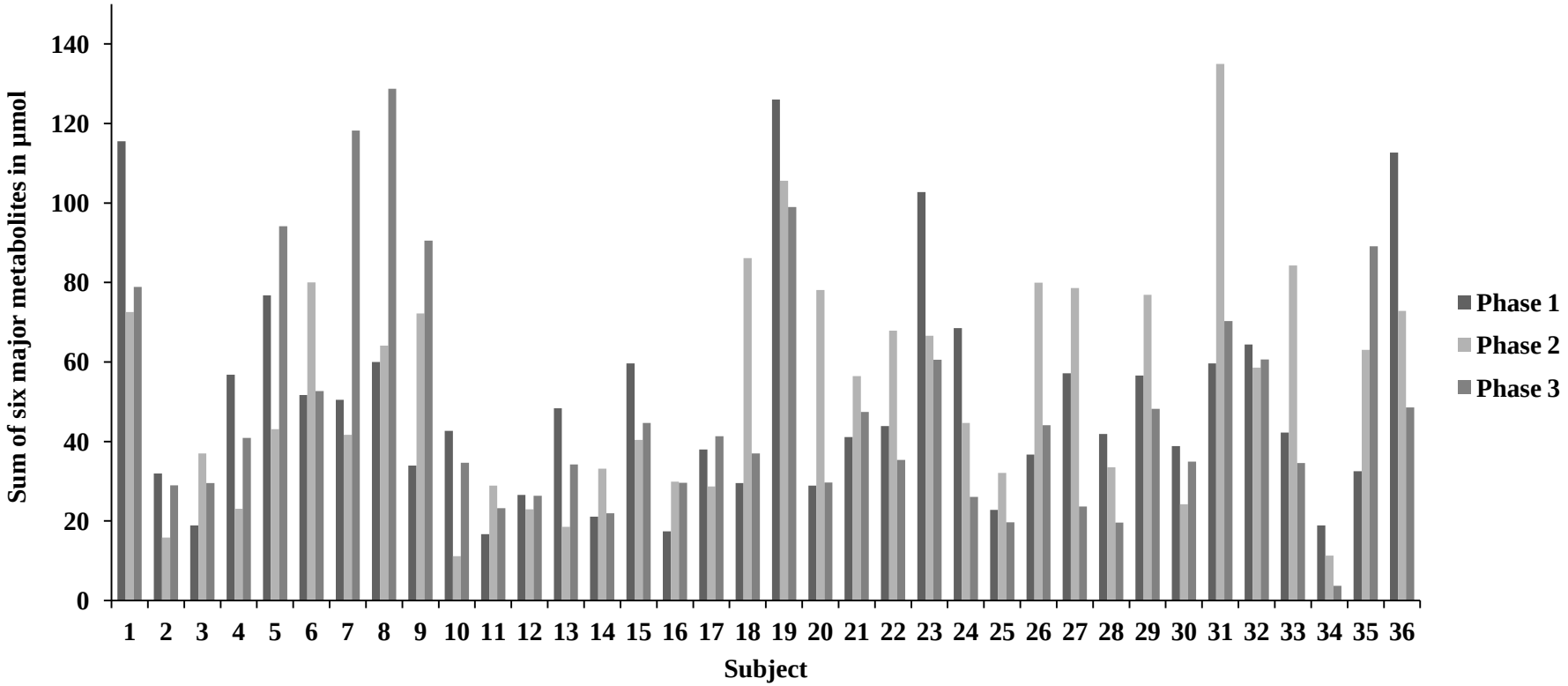


Figure 4.16: : Excretion of vanilloylglycine for 36 hours after coffee consumption of 36 participants for all three phases, n=36, data presented as mean value in µmol

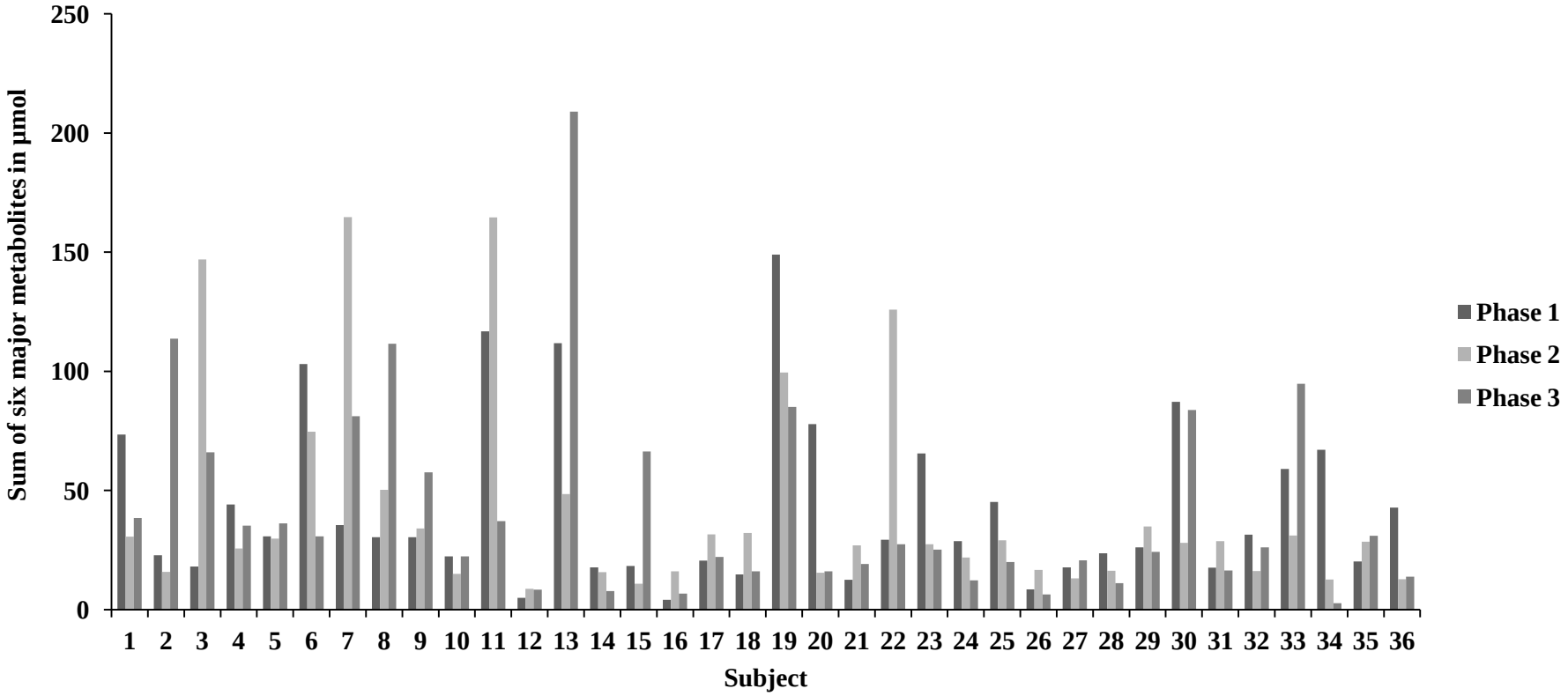


Figure 4.17: Excretion of ferulic acid-4-*O*-sulphate for 36 hours after coffee consumption of 36 participants for all three phases, n=36, data presented as mean value in µmol

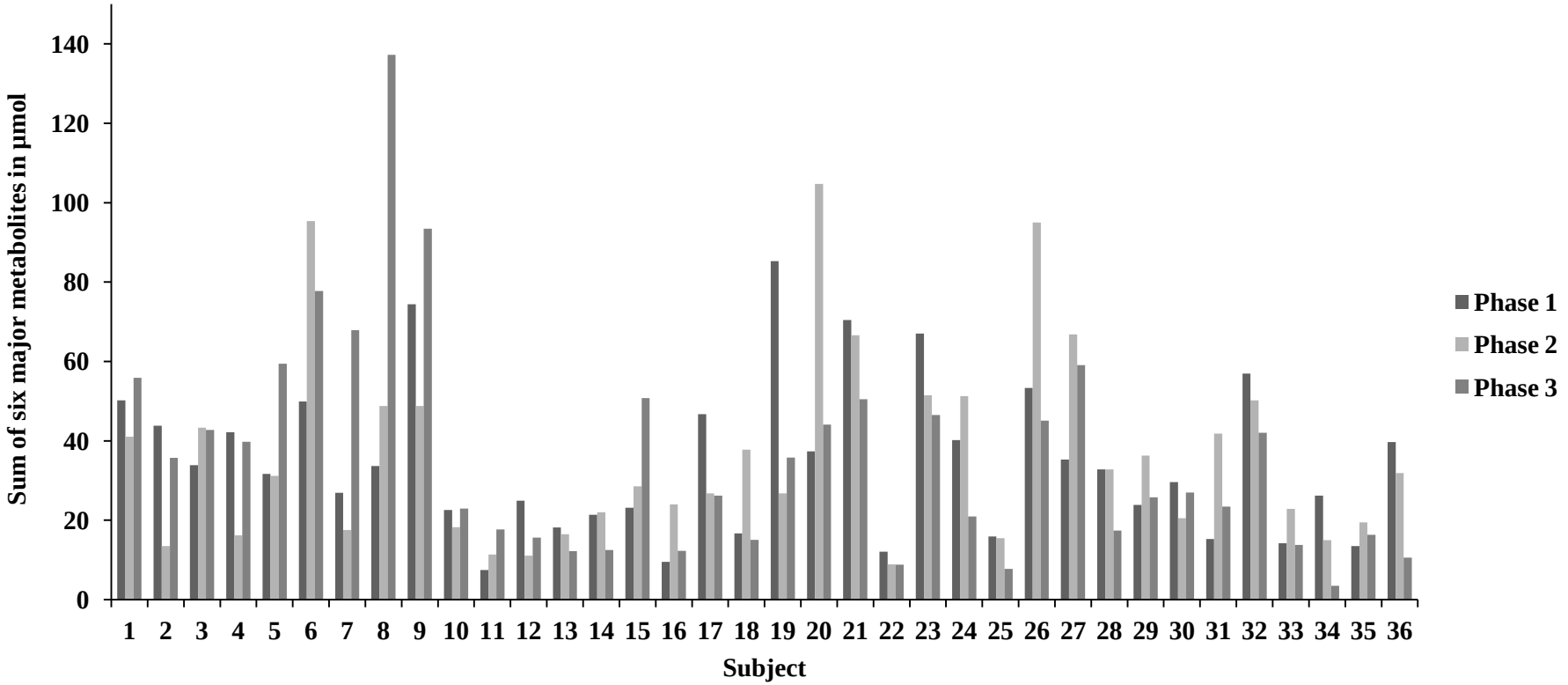


Figure 4.18: Excretion of dihydroferulic acid-4-*O*-sulphate for 36 hours after coffee consumption of 36 participants for three phases, n=36, data presented as mean value in µmol

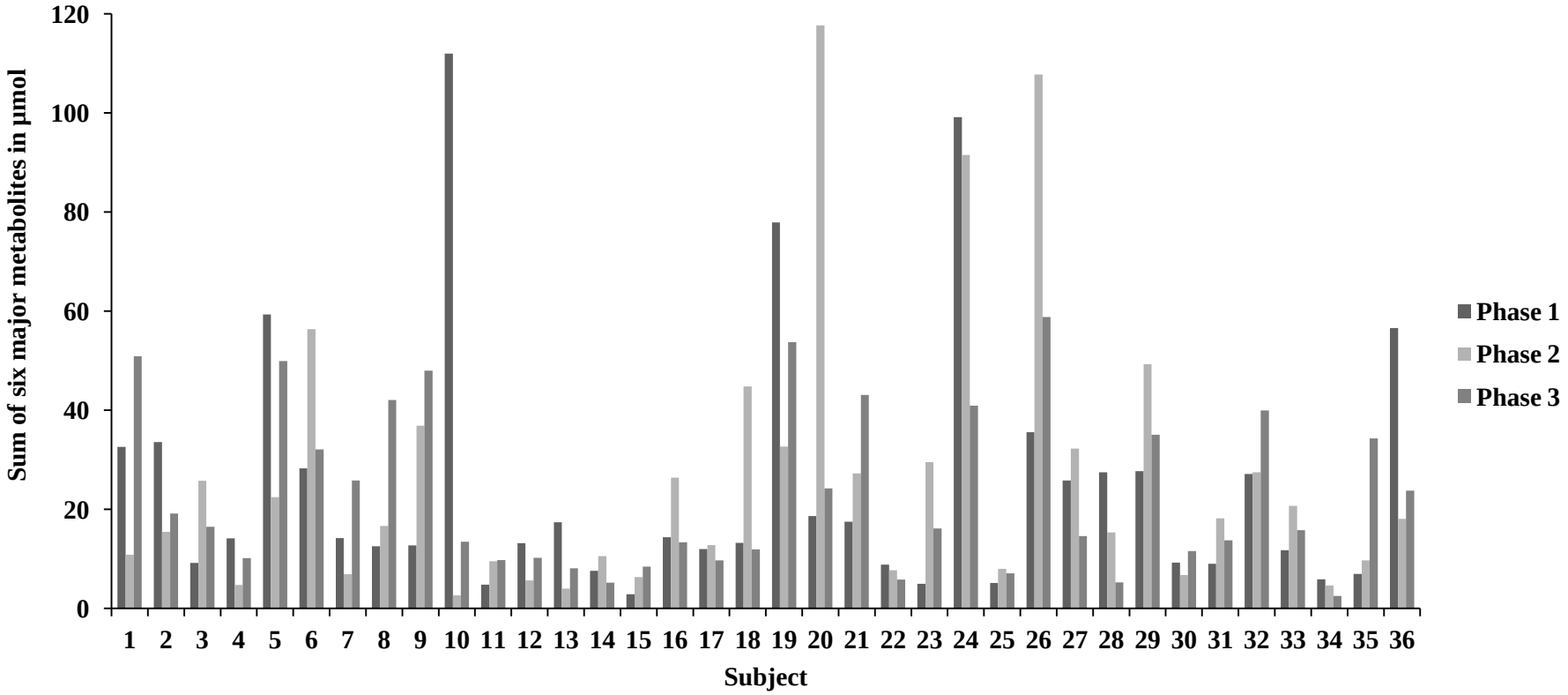
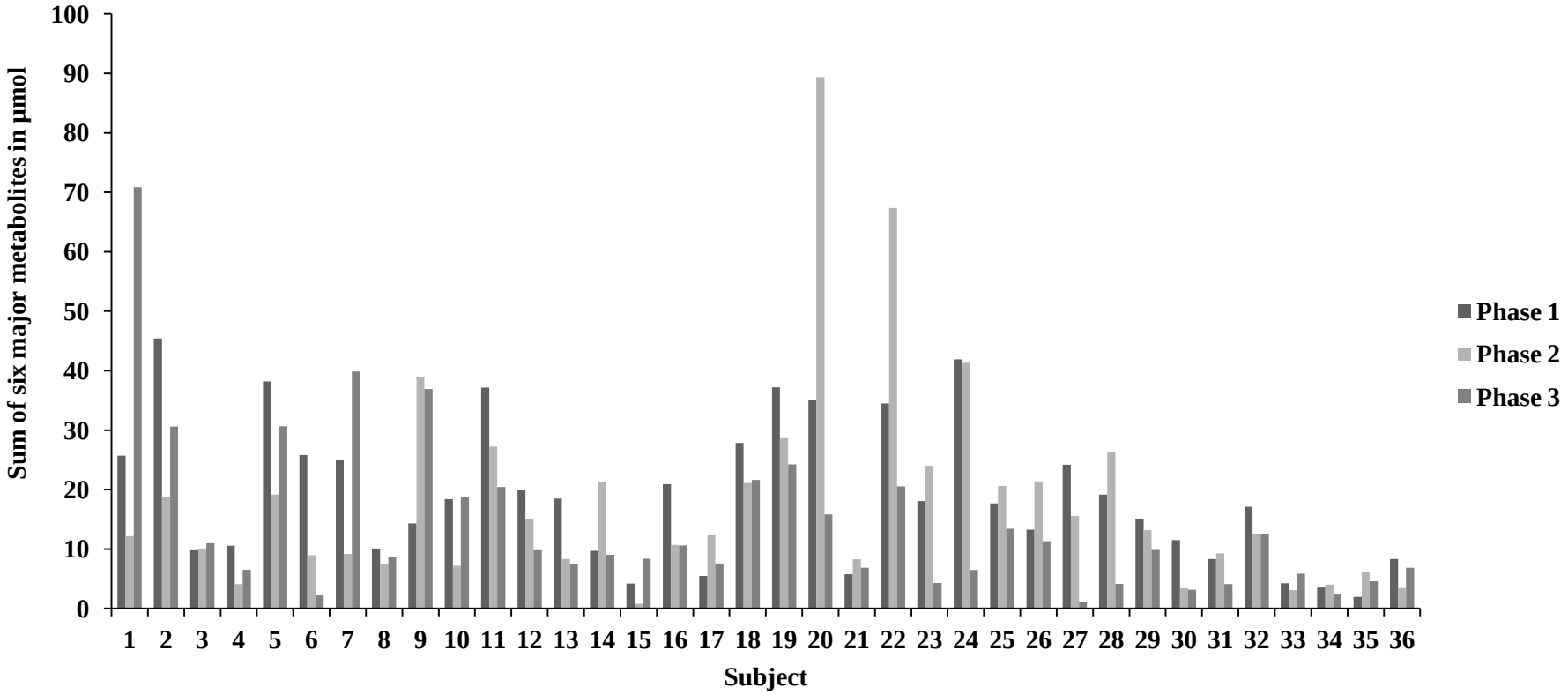


Figure 4.19: Excretion of dihydroferulic acid for 36 hours after coffee consumption of 36 participants for three phases, n=36, data presented as mean value in µmol



Figures (Figure 4.14 to Figure 4.19) depict the excretion of dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for 36 hours after coffee consumption for 36 particpants who completed all three phases of the study in µmol. The intraindividual variation of excretion, i.e. the variation of excretion between the three phases, varies by up to a factor of six. In order to better understand the intraindividual variation, the average coefficient of variation for each metabolite was calculated and is depicted in Table 4.2.

Table 4.2: Coefficient of variation (%) of the excretion of dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for 36 hours after coffee consumption of 36 participants for all three phases, n=36, mean coefficient of variation in per cent ± standard deviation

	DHCA-3-*O*-SO_4	FA-GLY	VA-GLY	FA-4-*O*-SO_4	DHFA-4-*O*-SO_4	DHFA
Mean CV % ±SD	**52.3 ±24.5**	**35.9 ± 15.5**	**51.8 ± 27.7**	**37.4 ± 18.1**	**50.2 ± 25.4**	**46.1 ± 23.8**

The mean coefficient of variation was the highest for dihydrocaffeic acid-3-*O*-sulphate, vanilloylglycine and dihydroferulic acid-4-*O*-sulphate with 52.3, 51.8 and 50.2%, respectively. The dihydroforms of the hydroxycinnamic acids are formed by reductases derived from microbiota in the colon according to Stalmach *et al.* 2010 [15]. The mean coefficient of variation for feruloylglycine and ferulic acid-4-*O*-sulphate is 35.9 and 37.4%, respectively. These metabolites are not associated with the metabolism of colonic microbiota [7, 15].

4.5 Discussion

Assessing the absorption of coffee caffeoylquinic acid and its derivatives in human subjects can be performed by the non-invasive measurement of metabolites in urine collected after coffee consumption. Thus the excretion ratio, a quotient of the amount excreted in urine and the amount ingested, is determined which is suitable for the assessment of the minimum absorption. However, ingestion of xenobiotic compounds is followed by phase I and phase II metabolism in order to facilitate excretion from the human body. Coffee caffeoylquinic acids and its derivatives are metabolised in a complex pathway of colonic and hepatic metabolites [7]. Stalmach *et al.* [7] quantified 12 and 17 metabolites of caffeoylquinic acid and its derivatives in plasma and urine, respectively. The five highest urinary metabolites were dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, dihydroferulic acid-4-*O*-sulphate, ferulic acid-4-*O*-sulphate and dihydroferulic acid with 37.1, 20.7, 12.4, 11.1 and 9.7 µmol, respectively. Additionally, other twelve metabolites were quantified, e.g. dihydroferulic acid-4-*O*-glucuronide, caffeic acid-3-*O*-sulphate, isoferulic acid-3-*O*-glucuronide, dihydroisoferulic acid-3-*O*-glucuronide, 3-*O*-ferulic acid, 4-*O*-ferulic acid, 3-*O*-caffeoylquinic acid lactone-*O*-sulphate, 5-*O*-ferulic acid, 4-*O*-caffeoylquinic acid lactone-*O*-sulphate, dihydrocaffeic acid-3-*O*-glucuronide, caffeic acid-3-*O*-sulphate and isoferulic acid-3-*O*-sulphate. Stalmach *et al.* determined the excretion rate with 29.1% of the dose of caffeoylquinic acid and its metabolites ingested in urine collected for 24 hours thereafter. Another publication reported similar ranges varying from 16 to 25% depending on the dose ingested [16]. Eleven participants ingested three different doses, e.g. 412, 635, 795 µmol of caffeoylquinic acid and its derivatives and excreted 101, 160 and 125 µmol of metabolites equivalent to 24, 25 and 16%. When the higher dose of 795 µmol caffeoylquinic acid and its derivatives is ingested, the excretion is about a third lower compared to the intermediate dose. This phenomenon could be due to enzyme saturation, limitation of substrates, limited transport capacities at the enterocytes (influx and efflux) or an influence on the gastrointestinal transit times according to Erk *et al.*[17].

The study presented here consisted of three phases, in order to investigate the intraindividual variation of absorption and metabolism of caffeoylquinic acid and its derivatives, where participants had to follow a washout procedure, consumed a cup of coffee and collected all their

urine for 36 hours thereafter in time periods of 0-4, 4-8, 8-12, 12-24 and 24-36 hours. Table 4.3 depicts the amounts of the individual metabolites excreted, the percentage of the dose ingested of the study presented here and the data from two studies performed by Stalmach *et al.* published in 2009 and 2014 with different doses.

Table 4.3: Comparison of urinary metabolites in absolute excretion in µmol and as a percentage of the dose ingested of three studies with different amounts of caffeoylquinic acid and its derivatives ingested. For better comparability, only urinary metabolites analysed in the study described in this thesis are compared to the studies performed by Stalmach *et al.* and values are not taken into consideration calculating the excretion ratio (percentage of dose ingested). Moreover, urine in the study described in this thesis was collected for 36 hours after the consumption of coffee in contrast to 24 hours for the studies performed by Stalmach *et al.* . n.a. = compound not analysed in this study

Compound	**Phase 1**		**Stalmach *et al.*, 2009 [7]**		**Stalmach *et al.*, 2014 [16]**	
	Amount in µmol	Percentage of ingested dose	Amount in µmol	Percentage of ingested dose	Amount in µmol	Percentage of ingested dose
CQAs ingested	881.8		412.0		795.0	
Dihydrocaffeic acid-3-*O*-sulphate	60.2	6.8	37.1	9.0	47.9	6.0
Feruloylglycine	51.7	5.9	20.7	5.0	27.2	3.4
Vanilloylglycine	41.9	4.8	n.a.	n.a.	n.a.	n.a.
Ferulic acid-4-*O*-sulphate	40.7	4.6	11.1	2.7	16.1	2.0
Dihydroferulic acid-4-*O*-sulphate	27.0	3.1	12.4	3.0	27.2	3.4
Dihydroferulic acid	19.2	2.2	9.7	2.4	10.6	1.3
All metabolites	240.6	27.3	91.0	22.1	129.0	16.2

The data presented in the previous table (Table 4.3) is conclusive. The most excreted urinary metabolites of ingested caffeoylquinic acid and its derivatives are dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for the study described in this thesis. In contrast, Stalmach *et al.* did not analyse vanilloylglycine and found dihydroferulic acid-4-*O*-sulphate to be more

highly excreted compared to ferulic acid-4-*O*-sulphate in her 2009 and 2014 study. Moreover, due to the fact that in the study described in this thesis, urine was collected for 36 hours instead of 24 hours post coffee consumption, the overall percentage of urinary excretion of the dose ingested is 1.7-fold higher compared to the data from Stalmach's study in 2014 [16]. Additionally, the studies performed by Stalmach included eleven participants, whereas the phase 1 of the study described in this thesis included 62 participants. Interestingly, the excretion ratios of the study described in this thesis show more similarity with the results from the excretion ratios of the 2009 study of Stalmach, however, the coffee beverage administered contained only 412 µmol compared to 882 µmol of caffeoylquinic acid and its derivatives.

The interindividual variation is substantial with a factor of 8.3, 10.2 and 35.3 between the total excretion rate of the lowest excreter and the highest excreter of the three phases (82.9-693.2, 55.1-562.3 and 17.9-631.9 µmol). These findings are, however, in accordance with previous publications [7, 15-17, 20].

The advantage of the study described in this thesis is the fact that participants underwent three phases where they were asked to repeat the same procedure after at least six and twelve weeks. The results demonstrate major variation of the intraindividual excretion between the three visits. The sum of the total excretion of metabolites of caffeoylquinic acid and its derivatives varies up to four-fold. In order to better understand and assess the variation the coefficient of variation was calculated for the individual compounds for 36 people who completed all three phases of the study. Table 4.2 depicts the coefficient of variation of excretion for all the six metabolites namely dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid. Metabolites which are known to be formed in the colon of humans [26], e.g. dihydrocaffeic acid-3-*O*-sulfate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid, showed the highest variation. This data leads to the suggestion that the colonic microbiota consisting of inter alia *E. coli*, *B. lactis*, and *L. gasseri*, change over the time range of several weeks. Previous studies investigated the effect of pre-/probiotics, antibiotic therapy and functional food consumption, leading to proliferation of intestinal microflora or hinderance of growth, respectively [82-84] and investigated the effect of either human faecal microbiota on polyphenols [85] or faecal slurries

on coffee caffeoylquinic acid and its derivatives [26]. Other studies quantitatively assessed food intake by means of food frequency questionnaires, detected and quantified the microbial strains faeces and correlated the results of both. A negative correlation was found between fibre intake, e.g. soluble pectin and *B. coccoides* and *C. leptum*, whereas a positive correlation was found between *Akkermansia* and *B. coccoides* with resistant starch. The only correlation analysed including a polyphenol was a negative correlation with orange-derived flavanone and *B. coccoides* and *C. leptum.* A longitudinal study over four weeks found a positive correlation between fructo-oligosaccharides intake and the quantity of bifidobacteria in healthy volunteers. Only a limited number of studies have been performed to investigate the longitudinal effect of tea polyphenols on gastrointestinal tract microbiota [86-88] , however, to our knowledge, neither a study has been performed to investigate the longitudinal changes of human faecal microbiota, nor has the intraindividual variation of absorption and excretion on coffee caffeoylquinic acid and its derivatives *in vivo* been described. Since participants were not requested to take a record of their diet in between the three phases, no effects of their diet on the absorption and excretion on coffee caffeoylquinic acid and its derivatives can be investigated further. Future, longitudinal studies on the intraindividual absorption and excretion, would require a detailed, quantitative recording of the food intake several weeks prior to and during the study.

Chapter 5: Correlation of urinary excretion of metabolites of caffeoylquinic acid and its derivatives, biomarkers of health, and food intake

5.1 Abstract

The following chapter links the data of urinary excretion of caffeoylquinic acid and its derivatives with biomarkers of health. The statistical analysis is described and is focused on the correlation between the excretion data presented in chapter 4, biomarker of health data, described in Ms Joana Amarante Encarnacao's thesis, and data on food intake recorded by the means of a food frequency questionnaire. Several correlations are apparent. The main correlations are between the 0-36 hours excretion of dihydrocaffeic acid-3-*O*-sulphate after coffee consumption and plasma fasting insulin (r_s=0.428, p=0.009), the 0-36 hours excretion of ferulic acid-4-*O*-sulphate after coffee consumption and plasma fasting insulin levels (r_s=0.448, p=0.006), the 0-36 hours excretion of vanilloylglycine after coffee consumption and DL-homocysteine (r_s=0.432, p=0.010) and between the 0-36 hours excretion of dihydrocaffeic acid-3-*O*-sulphate after coffee consumption with both the consumption of cups of coffee per week (r_s=-0.434, p=0.008) and also the related coffee level, a measure of consumption of cups of coffee per week multiplied by the strength of the coffee (r_s=-0.461, p=0.005).

5.2 Introduction

Over the past decade, the absorption, metabolism and excretion of coffee caffeoylquinic acid and its derivatives in humans have been intensively investigated. Several papers have presented data on absorption either assessed by pharmacokinetic studies [20, 21, 89], via the measurement of urinary excretion, or both [7, 16]. Chemically synthesised compounds and subsequently developed and validated analytical methods have improved the quality of the quantitative data substantially [30].

Moreover, multiple epidemiological or meta-analysis papers have reported on the several health benefits of habitual, moderate coffee consumption including a reduced risk of diabetes type II [37], a lower risk of stroke and heart failure [90], a lower risk of coronary heart diseases in

women [91], and protective effects on hepatocellular and endometrial cancer [35]. Additionally, some studies reported acute health effects such as a reduced LDL oxidation in humans [42] and an amelioration of plasma antioxidant capacity, potentially ascribed to a reduced plasma uric acid level [92] post a single dose of coffee. However, up to date, no human study has been performed linking absorption, metabolism and excretion to biomarkers of health. This is of particular importance, since Stalmach *et al.* [7] confirmed a substantial interindividual variation in absorption of coffee caffeloylquinic acid and its derivatives in humans. The following chapter presents the linkage of the data of excretion of urinary metabolites of caffeoylquinic acid and its derivatives, described in detail in chapter 4, to biomarkers of health, i.e. thiols (L-cysteine, DL-homocysteine, cysteinylglycine and L-glutathione), as an indicator of the plasma antioxidant status [93, 94], uric acid, primarily associated with gout [95], insulin and glucose, as markers for metabolic syndrome and type II diabetes [96]. The biochemical analysis was performed by Miss Joana Amarante Encarnacao and the data will be presented in her thesis in 2015.

5.3 Statistical analysis

IBM SPSS statistics 22 software (Armonk, NY, USA) was used for the statistical analysis of the data of urinary excretion of metabolites of caffeoylquinic acid and its derivatives and the correlation to the plasma biomarkers of health. Data was entered as mean values for the individual urinary metabolite of caffeoylquinic acid and its derivatives and coefficient of variation for the averaged sum of all urinary metabolites for participants who completed the three phases of the human study in order to take the intraindividual variation into account. Prior to correlation analysis by bivariate correlation testing, the normal distribution of the data was tested by means of a Shapiro-Wilk test. According to the outcome of the Shapiro-Wilk test, bivariate correlation is performed by a Pearson's bivariate correlation test for normally distributed data and by a Spearman's bivariate correlation test for the non-parametric data. Significant levels were defined as standard $p \leq 0.05$* and $p \leq 0.01$**.

5.3.1 Test for normal distribution

Firstly, the average sum of six urinary metabolites, dihydrocaffeic acid-3-*O*-sulfate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulfate, dihydroferulic acid-4-*O*-sulfate and dihydroferulic acid for each individual participant who completed all three phases were tested for normal distribution and the descriptive parameters including mean, standard deviation, and the confidence interval were determined.

Secondly, the average concentrations of plasma thiols (L-cysteine, DL-homocysteine, cysteinylglycine and L-glutathione), plasma uric acid, plasma insulin and glucose for each individual participant who completed all three phases were tested for normal distribution and the descriptive parameters including mean, standard deviation, and the confidence interval were determined.

Thirdly, the average intake of cups of coffee per week, the coffee level (cups of coffee per week multiplied by strength of coffee [weak=1, strong=3]), the intake of cups of black tea per week, portions of fruit per week, portions of vegetables per week, and portions of yoghurt per week for each individual participant who completed all three phases were tested for normal distribution and the descriptive parameters including mean, standard deviation, and the confidence interval were determined.

Finally, the coefficient of variation for the averaged sum of all urinary metabolites excreted between 0-36 hours after coffee consumption for participants who completed the three phases of the human study was tested for normal distribution.

5.3.2 Bivariate correlation of urinary excretion with biomarkers of health

The normally distributed data of feruloylglycine was correlated with the normally distributed data of L-cysteine, cysteinylglycine, L-glutathione, uric acid, glucose and insulin using bivariate Pearson's correlation.

The non-normally distributed data of the average sum of six urinary metabolites, dihydrocaffeic acid-3-*O*-sulphate, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid was correlated with the normally distributed data of L-cysteine,

cysteinylglycine, L-glutathione, uric acid, glucose and insulin and with the non-normally distributed data of DL-homocysteine by means of Spearman's bivariate correlation. The normally distributed data of feruloylglycine was correlated with the non-normally distributed data of DL-homocysteine using Spearman's bivariate correlation.

5.3.3 Bivariate correlation of urinary excretion with food intake

The normally distributed data of feruloylglycine was correlated with the normally distributed data of the intake of portions of fruit per week using the Pearson's bivariate correlation.

The other urinary excretion data was correlated with the intake of cups of coffee per week, coffee level, the intake of portions of vegetables per week and portions of yoghurt per week by the means of the Spearman's bivariate correlation.

5.3.4 Bivariate correlation of the coefficient of variation for the average sum of all urinary metabolites of caffeoylquinic acid and its derivatives with the biomarkers of health

The non-normally distributed data of the coefficient of variation (%) of the average sum of the excretion of all urinary metabolites for participants who completed three phases of the human study (n=36) was correlated with plasma L-cysteine, DL-homocysteine, cysteinylglycine, L-glutathione, uric acid, glucose and insulin using the Spearman's bivariate correlation.

5.3.5 Bivariate correlation of the coefficient of variation for the average sum of all urinary metabolites of caffeoylquinic acid and its derivatives with food intake

The non-normally distributed data of the coefficient of variation (%) of the average sum of the excretion all urinary metabolites for participants who completed three phases of the human study (n=36) was correlated with the intake of cups of coffee per week, the coffee level, the

intake of cups of tea per week, the portions of fruit per week, the portions of vegetables and the portions of yoghurt using the Spearman’s bivariate correlation.

5.4 Results

5.4.1 Test for normal distribution

Primarily, the average sum of six urinary metabolites, dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for each individual participants who completed all three phases and the average sum of the individual metabolite for each individual participants were tested for normal distribution. The descriptive statistical parameters are summarized in Table 5.1. The null hypothesis of feruloylglycine (p=0.115) has been accepted, thus the data is normally distributed, whereas the null hypothesis for the average sum of all metabolites (p=0.001), dihydrocaffeic acid-3-*O*-sulphate (p=0.000), vanilloylglycine (p=0.000), ferulic acid-4-*O*-sulphate (p=0.004), dihydroferulic acid-4-*O*-sulphate (p=0.000) and dihydroferulic acid (p=0.000) has been rejected, thus the data is not normally distributed.

Table 5.1: Summary of descriptive statistical parameters for averaged sum of all metabolites, dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for participants who completed three phases of the human study (n=36)

Compound	Mean (µmol)	Standard deviation	Confidence interval (95%) lower bound	upper bound	Shapiro-Wilk significance
Averaged sum of all metabolites	238.19	120.42	197.44	278.93	0.001
Dihydrocaffeic acid-3-*O*-sulphate	64.45	53.31	46.42	82.49	0.000
Feruloylglycine	49.58	22.68	41.91	57.26	0.115
Vanilloylglycine	40.43	30.76	30.02	50.83	0.000
Ferulic acid-4-*O*-sulphate	38.04	23.02	30.25	45.83	0.004
Dihydroferulic acid-4-*O*-sulphate	28.03	30.46	17.72	38.33	0.000
Dihydroferulic acid	17.66	13.68	13.03	22.28	0.000

Figure 5.1, Figure 5.2, Figure 5.3 depict the histograms for the average sum of six urinary metabolites, dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for each individual participants who completed all three phases.

Figure 5.1: Histogram of average sum of six urinary metabolites for participants who completed all three phases of the human study (n=36)

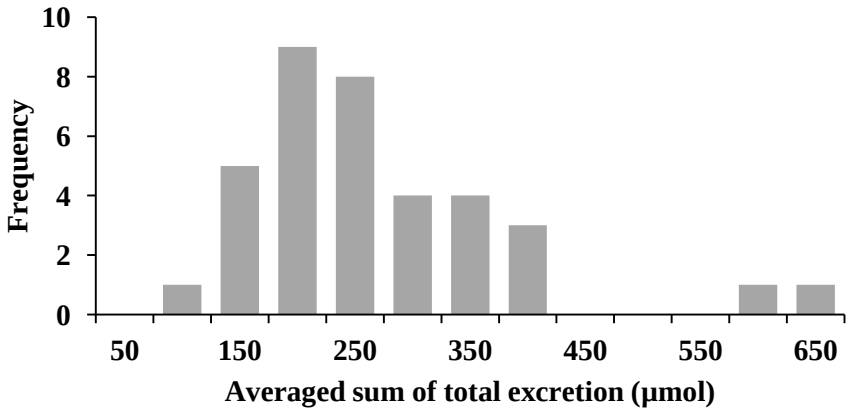
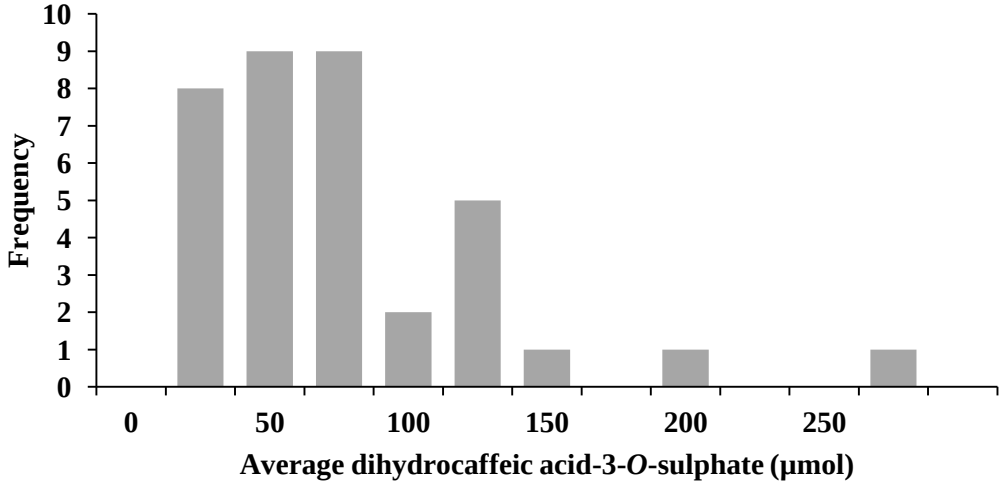
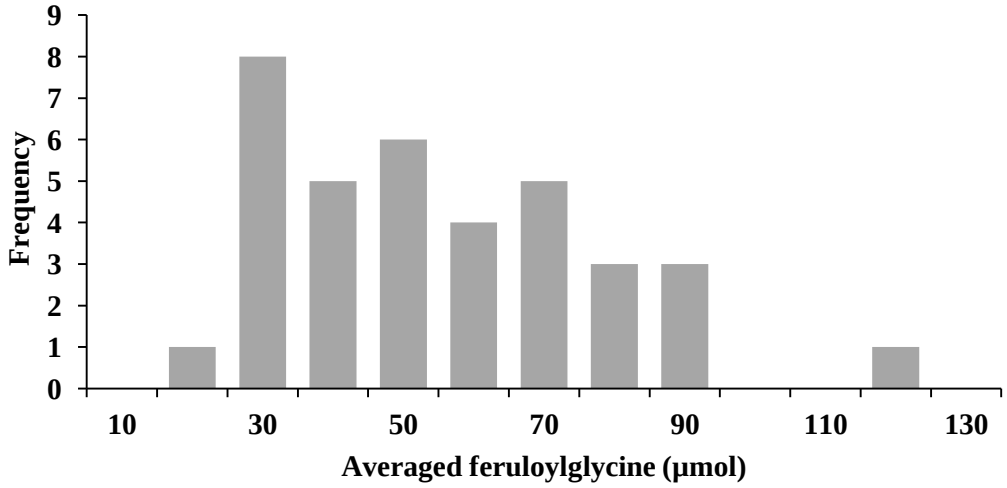


Figure 5.2: Histograms of average dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine and vanilloylglycine for participants who completed all three phases of the human study (n=36)





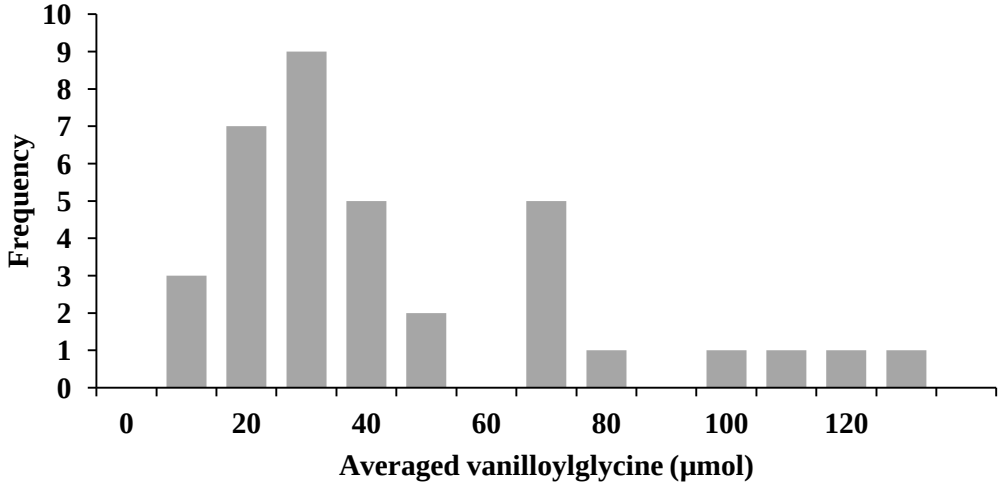
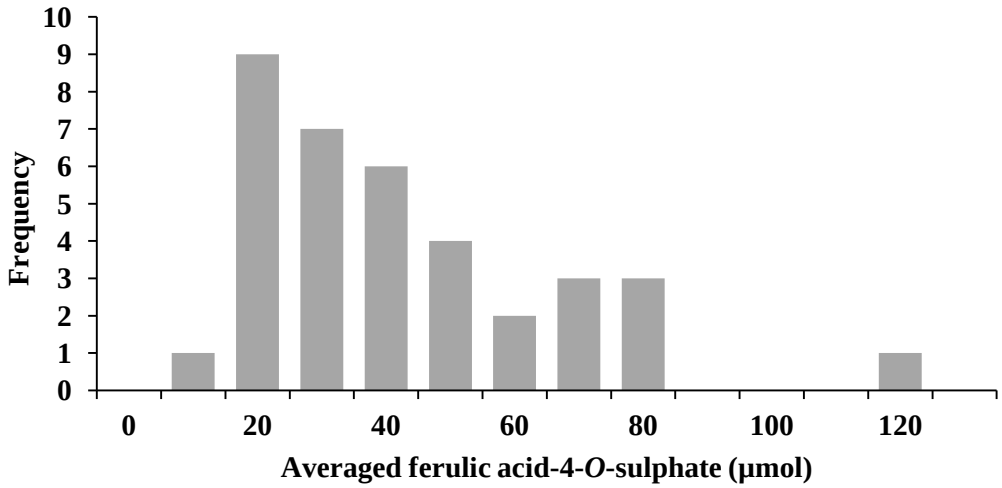
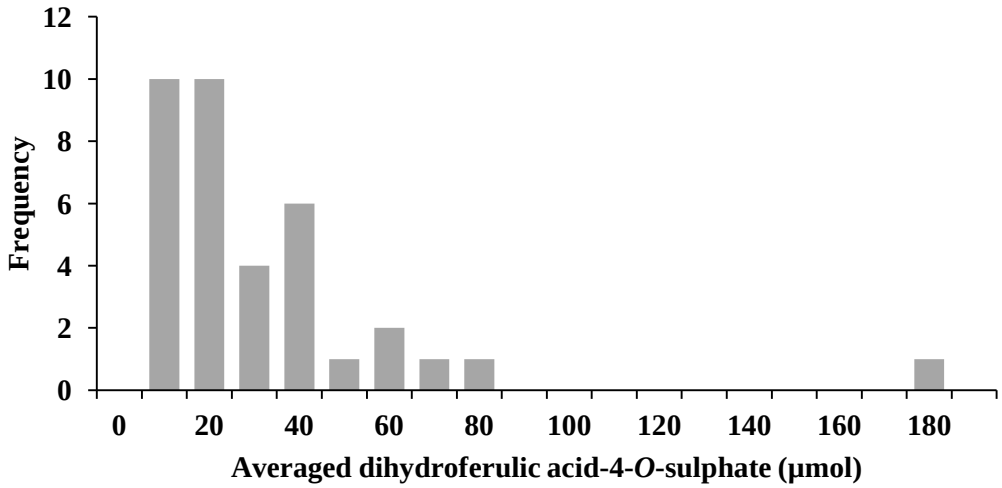
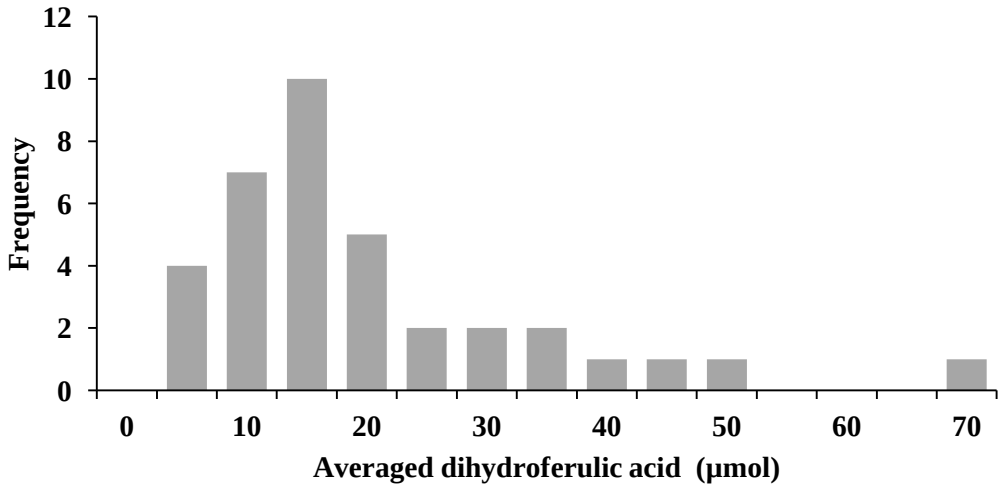


Figure 5.3: Histograms of average ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid for participants who completed all three phases of the human study (n=36).







In the histogram of the average sum of six urinary metabolites for 36 participants who completed the three phases of the human study (Figure 5.1), two statistical outliers of 593.4 and 613.8 µmol were detected by box plot analysis (data not shown). Since the amounts excreted were equivalent to 67.2 and 69.6% of the dose ingested, and were therefore hypothetically possible, the values were not excluded for the subsequent correlation analysis. In the following histograms of the average dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine and vanilloylglycine excretion for 36 participants who completed the three phases of the human study (Figure 5.2), three statistical outliers of 192.8, 217.7 and 258.2 µmol, one of 110.2 µmol and one of 123.11 µmol, respectively, were detected by box plot analysis (data not shown). In the histograms of ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and dihydroferulic acid excretion for 36 participants who completed the three phases of the human study (Figure 5.3) one statistical outlier of 111.8 µmol, three of 67.4, 77.2 and 172.7 µmol and one of 66.9 µmol, respectively, were detected by box plot analysis (data not shown). The outliers were not excluded due to the reason mentioned afore.

Secondly, L-cysteine, DL-homocysteine, cysteinylglycine, L-glutathione, uric acid, glucose and insulin for each individual participants who completed all three phases (n=36) were tested for normal distribution. The descriptive statistical parameters are summarised in Table 5.2. The null hypothesis of L-cysteine (p=0.385), cysteinylglycine (p=0.730), L-glutathione (p=0.123), uric acid (p=0.415), glucose (p=0.073) and insulin (p=0.118) has been accepted, thus the data is normally distributed, whereas the null hypothesis for the DL-homocysteine (p=0.000) has been rejected, thus the data is not normally distributed.

Table 5.2: Summary of descriptive statistical parameters for averaged L-cysteine, DL-homocysteine, cysteinylglycine, L-glutathione, uric acid, fasting glucose and insulin for participants who completed three phases of the human study (n=36).

Compound	Mean (µM)	Standard deviation	Confidence interval (95%) lower bound	upper bound	Shapiro-Wilk significance
L-cysteine	259.41	22.68	251.74	267.09	0.385
DL-homocysteine	9.51	4.08	8.13	10.89	0.000
Cysteinylglycine	30.38	5.30	28.59	32.14	0.730
L-glutathione	7.94	1.54	7.42	8.46	0.123
Uric acid (mM)	0.26	0.05	0.24	0.28	0.415
Glucose (mM)	4.76	0.32	4.66	4.87	0.073
Insulin (mU/L)	9.16	2.39	8.35	9.97	0.118

Figure 5.4 depicts the histograms of the average plasma L-cysteine, DL-homocysteine, cysteinylglycine, and L-glutathione, whereas Figure 5.5 shows the histograms of the average plasma uric acid, glucose and insulin for participants who completed all three phases of the human study (n=36).

Figure 5.4: Histograms of average plasma L-cysteine, DL-homocysteine, cysteinylglycine, and L-glutathione for participants who completed three phases of the human study (n=36).

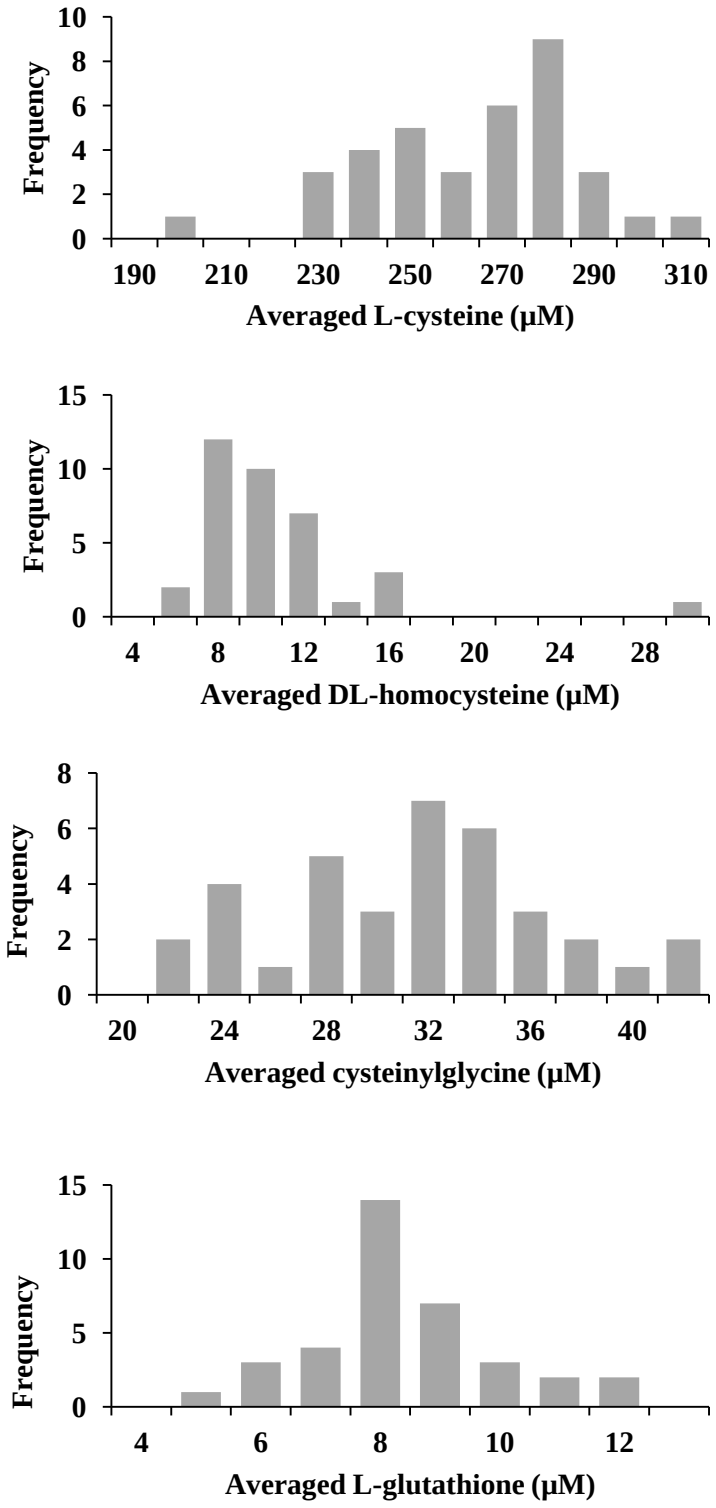
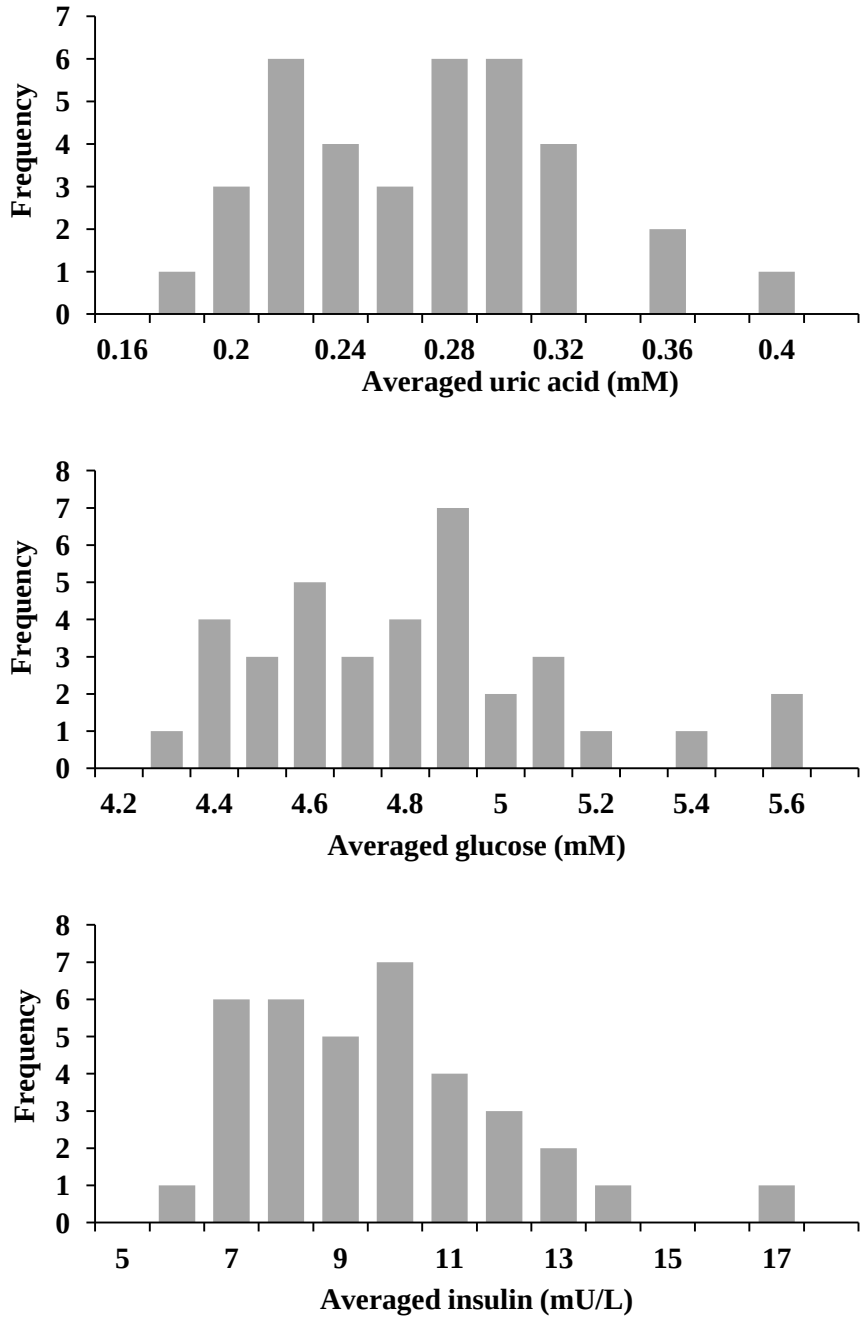


Figure 5.5: Histograms of average plasma uric acid, fasting glucose and insulin for participants who completed three phases of the human study (n=36).



In the histogram of average plasma DL-homocysteine for 36 participants who completed the three phases of the human study (Figure 5.4), one statistical outlier of 28.26 µM was detected by box plot analysis (data not shown). Additionally, in the histogram of average plasma L-glutathione for 36 participants who completed the three phases of the human study (Figure 5.4), three statistical outliers, two at the upper end of 11.13 and 11.17 µM and one at the lower end of 4.71 µM., were detected by box plot analysis (data not shown). Since the plasma concentrations

of the thiols were located outside the normal physiological ranges (DL-homocysteine: 6.5-11.9 µM and L-glutathione: 4.9-7.3 µM) according to Bald *et al.* and Turell *et al* [93, 94], the outlying values were excluded and the normal distribution reanalysed. For DL-homocysteine, the null hypothesis was rejected again ($p=0.018$, $n=35$) and the data did not follow a normal distribution and thus was used for the subsequent non-parametric correlation analysis, whereas for L-glutathione the null hypothesis was accepted ($p=0.265$, $n=33$) again, thus the data remained normally distributed.

In the histogram of plasma glucose and insulin for 36 participants who completed the three phases of the human study two statistical outliers of 5.50 and 5.59 mM, and 16.49 mU/L, respectively, were detected by box plot analysis (data not shown). The statistical outliers were not excluded, since these did not exceed healthy physiological ranges (glucose: ≤7.0 mM and insulin: ≥14 mU/L) according to Olokoba *et al.* and Weyer *et al.* [96, 97].

Thirdly, the intake of cups of coffee per week, the coffee level (cups of coffee per week * strength of coffee [weak=1, strong=3]), cups of tea per week, portions of fruit per week, portions of vegetables per week, portions of fruit and vegetables per week and the portions of yoghurt per week for each individual participants who completed all three phases were tested for normal distribution and the descriptive statistical parameters are summarised in Table 5.3.

Table 5.3: Summary of descriptive statistical parameters for the intake of cups of coffee per week, coffee level, cups of tea per week, portions of fruit per week, portions of vegetables per week, and portions of yoghurt per week for participants who completed three phases of the human study (n=36).

Compound	Mean (Cups or portions)	Standard deviation	Confidence interval (95%) lower bound	upper bound	Shapiro-Wilk significance
Cups of coffee per week	12.40	12.86	8.05	16.75	0.000
Coffee level	32.83	39.94	19.32	46.35	0.000
Cups of tea per week	7.10	11.91	3.07	11.13	0.000
Portions of fruit per week	22.79	13.09	18.36	27.21	0.178
Portions of vegetables per week	21.83	15.37	16.63	27.03	0.000
Portions of yoghurt per week	2.44	3.02	1.42	3.47	0.000

Figure 5.6 and Figure 5.7 depict the histograms of intake of cups of coffee per week, coffee level, cups of tea per week, portions of fruit per week, portions of vegetables per week, portions of fruit and vegetables per week and portions of yoghurt per week.

Figure 5.6: Histograms of intake of cups of coffee per week, coffee level, and cups of tea per week for participants who completed three phases of the human study (n=36).

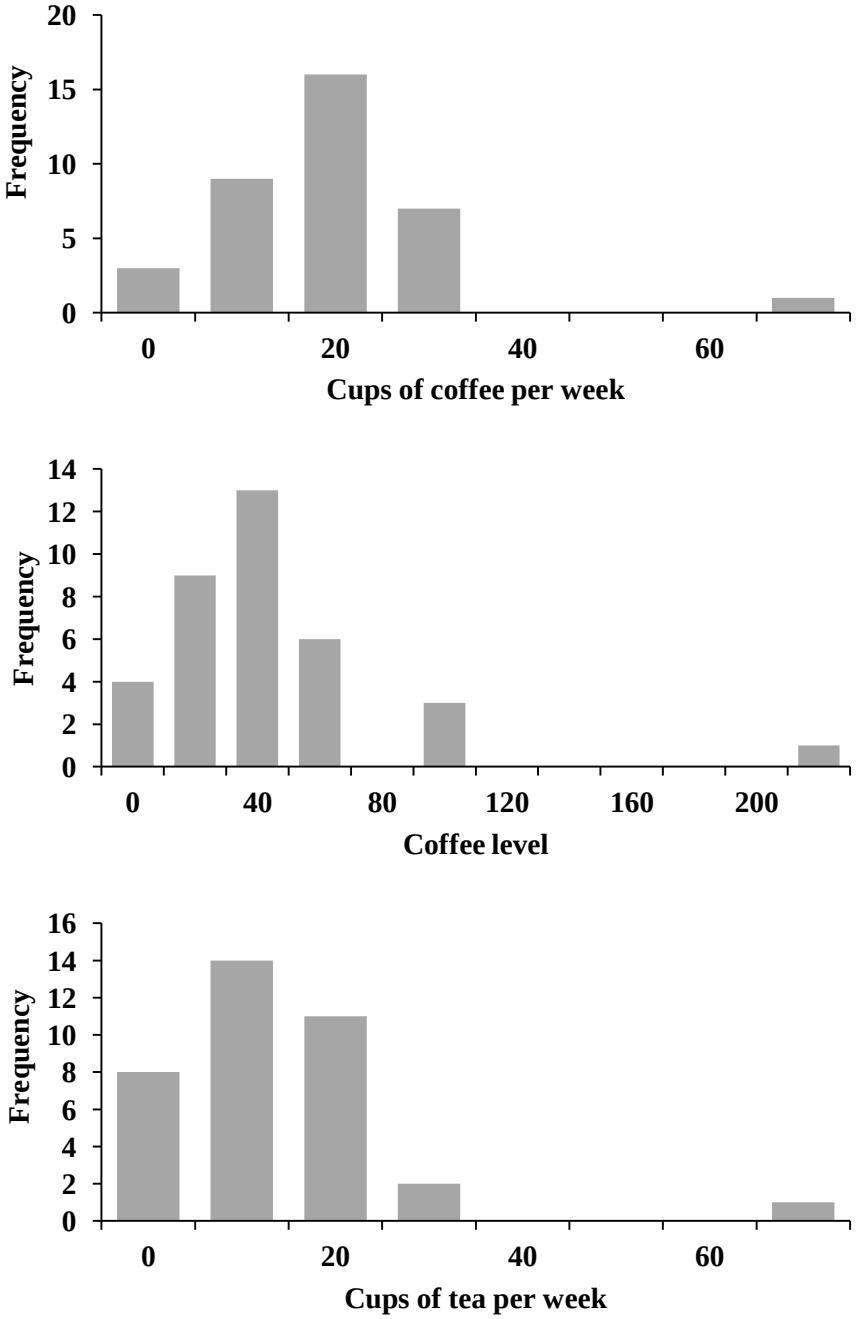
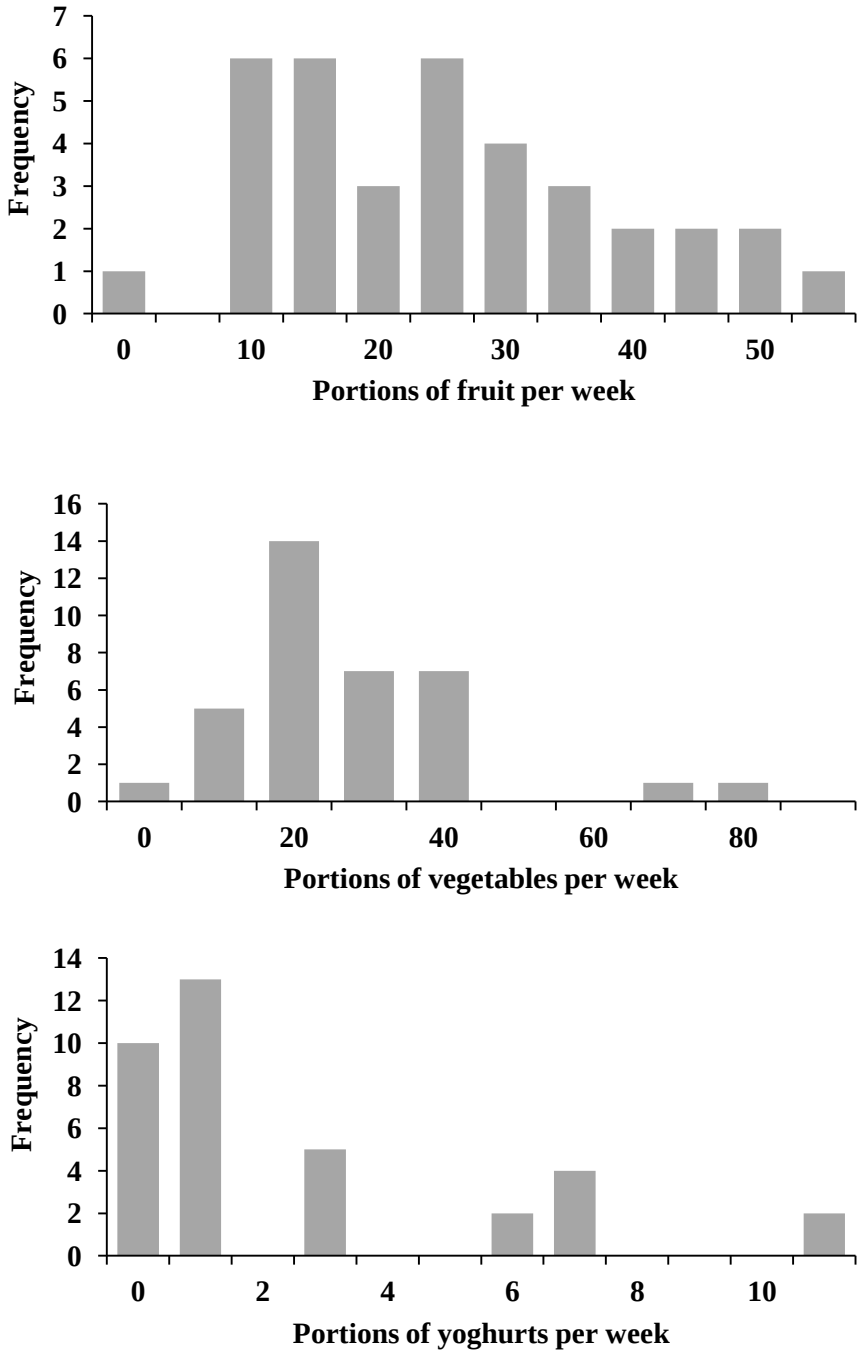


Figure 5.7: Histograms of intake of portions of fruit per week, portions of vegetables per week, and portions of yoghurt for participants who completed three phases of the human study (n=36).



The null hypotheses of the portions of fruit per week (p=0.178) was accepted, thus the data is normally distributed, whereas the null hypotheses of cups of coffee per week (p=0.000), coffee level (p=0.000), cups of tea per week (p=0.000), portions of vegetables per week (p=0.000), and portions of yoghurt per week (p=0.000) were rejected, thus the data is not normally distributed. However, no outliers were excluded as the food intake expectedly varies between participants.

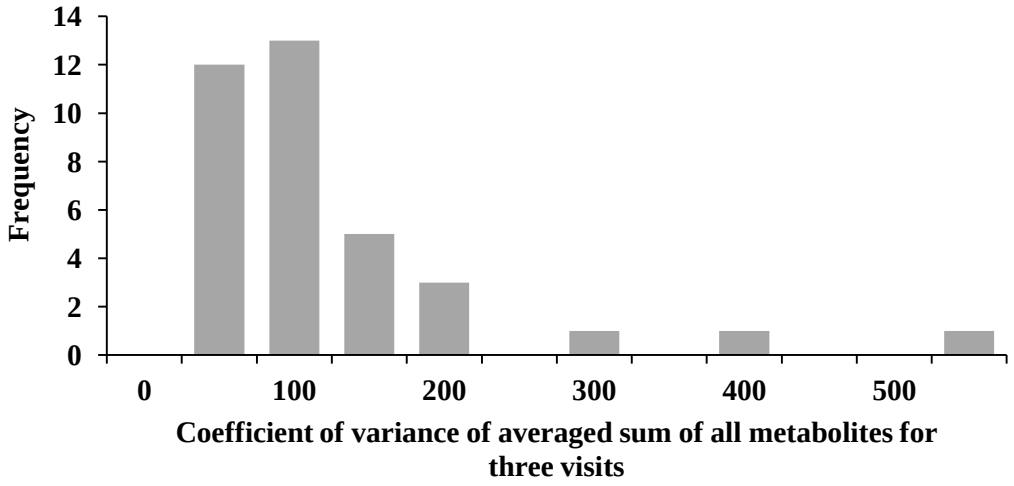
Finally, the normal distribution was tested for the coefficient of variation for the averaged sum of all urinary metabolites excreted between 0-36 hours for participants who completed the three phases of the human study. The descriptive statistical parameters are presented in the following Table 5.4.

Table 5.4: Summary of descriptive statistical parameters for coefficient of variation (%) of the average sum of all urinary metabolites for participants who completed three phases of the human study (n=36).

Parameter	Mean (%)	Standard deviation	Confidence interval (95%) lower bound	upper bound	Shapiro-Wilk significance
Coefficient of variation of the averaged sum of all urinary metabolites	96.92	102.46	62.25	131.89	0.000

The null hypothesis (p=0.000) of the Shapiro-Wilk test for normal distribution was rejected, thus the data is not normally distributed and the non-parametric Spearman's correlation test was applied for subsequent correlations. Three statistical outliers were detected in the histogram by the means of box plot analysis, however, these were not excluded due to the fact that the effect of the high variation in excretion was tested on the biomarkers of health and food frequency data.

Figure 5.8: Histogram of the coefficient of variation (%) of the average sum of all urinary metabolites for participants who completed three phases of the human study (n=36).



5.4.2 Bivariate correlation of urinary excretion with biomarkers of health

Urinary excretion data was correlated with biomarkers of health either by the use of Pearson's correlation for normally distributed data or by the means of Spearman's correlation for non-normally distributed data. The results are presented in Table 5.5. Weak correlations were defined with r ≥0.300 - <0.400 and moderate correlations were defined with r ≥0.400 - <0.500.

A weak, but statistically significant (p=0.032) positive correlation (r_s=0.358) was found between the averaged sum of all metabolites excreted over 36 hours and insulin. A weak, but statistically significant (p=0.047) negative correlation (r_s=-0.333) was found between the urinary excretion over 36 hours of dihydrocaffeic acid-3-*O*-sulphate and cysteinylglycine and a moderate, statistically significant (p=0.009) positive correlation (r_s=0.428) was detected between the urinary excretion over 36 hours of dihydrocaffeic acid-3-*O*-sulphate and insulin. A moderate, statistically significant (p=0.010) positive correlation (r_s=0.432) was analysed between the excretion over 36 hours of vanilloylglycine and DL-homocysteine. A moderate, statistically significant (p=0.006) positive correlation (r_s=0.448) was found between ferulic acid-4-*O*-sulfate excreted over 36 hours in urine and insulin. No other statistically significant correlations were detected.

Table 5.5: Summary of correlation coefficients, r_S: Spearman's coefficient, unless labeled by r_p: Pearson's coefficient, significant levels, p≤ 0.05*, p≤0.01**

		Averaged sum of all metabolites	DHCA-3-*O*-sulfate	FA-GLY	VA-GLY	FA-4-*O*-sulfate	DHFA-4-*O*-sulfate	DHFA
L-cysteine	r_s	-0.132	-0.266	r_p=-0.134	0.238	-0.163	-0.235	0.224
	Sig.	0.444	0.117	0.437	0.162	0.342	0.167	0.189
	N	36	36	36	36	36	36	36
DL-homocysteine	r_s	0.151	-0.015	0.150	**0.432	0.193	-0.007	0.118
	Sig.	0.387	0.933	0.390	0.010	0.266	0.967	0.498
	N	36	35	35	35	35	35	35
Cysteinylglycine	r_s	-0.294	*-0.333	r_p=-0.121	0.249	-0.195	-0.321	-0.122
	Sig.	0.082	0.047	0.480	0.143	0.254	0.056	0.479
	N	36	36	36	36	36	36	36
L-glutathione	r_s	-0.009	-0.026	r_p=-0.141	0.116	-0.074	0.097	-0.033
	Sig.	0.962	0.887	0.434	0.519	0.682	0.590	0.853
	N	33	33	33	33	33	33	33
Uric acid	r_s	0.141	0.115	r_p=0.083	0.016	0.228	0.268	-0.151
	Sig.	0.411	0.505	0.631	0.927	0.181	0.114	0.381
	N	36	36	36	36	36	36	36
Glucose	r_s	0.123	0.166	r_p=0.203	0.036	0.162	0.023	-0.006
	Sig.	0.476	0.334	0.235	0.835	0.345	0.894	0.971
	N	36	36	36	36	36	36	36
Insulin	r_s	*0.358	**0.428	r_p=0.321	-0.144	**0.448	0.309	0.254
	Sig.	0.032	0.009	0.056	0.401	0.006	0.067	0.135
	N	36	33	36	36	36	36	36

5.4.3 Bivariate correlation of urinary excretion with food intake

Urinary excretion data was correlated with food intake data by the means of Spearman's correlation for non-normally distributed data, whereas only the normally distributed excretion of feruloylglycine and the normally distributed data of the portions of fruit per day were correlated using a Pearson's correlation. The results are presented in Table 5.6. Weak correlations were defined with r ≥0.300 - <0.400 and moderate correlations were defined with r ≥0.400 - <0.500.

Several correlations were established between the excretion of urinary metabolites of caffeic acid and its derivatives and the weekly food intake. At a significance level of $p \leq 0.01$ a moderate negative correlation was detected between the 0-36 hours excretion of dihydrocaffeic acid-3-*O*-sulphate, the intake of cups of coffee per week (r_s=-0.434, p=0.008) and the 0-36 hours excretion of dihydrocaffeic acid-3-*O*-sulphate and the coffee level (r_s=-0.461, p=0.005), respectively.

Further weak, but statistically significant ($p \leq 0.05$), negative correlations were analysed between the averaged sum of all urinary metabolites of caffeoylquinic acid and its derivatives for 0-36 hours and the intake of portions of fruit per week (r_s=-0.341, p=0.042) and the averaged sum of all urinary metabolites of caffeoylquinic acid and its derivatives for 0-36 hours and the intake of yoghurt per week (r_s=-0.348, p=0.038) , respectively.

Additionally, at a significance level of $p \leq 0.05$, weak negative correlations were found between the 0-36 hours excretion of feruloylglycine and the intake of portions of fruit per week (r_p=-0.342, p=0.041) and a moderate negative correlation between the 0-36 hours excretion of feruloylglycine the intake of portions of yoghurt (r_s=-0.409, p=0.013), respectively.

Moreover, several weak, negative correlations at a significance level of $p \leq 0.05$ were established between the 0-36 hours urinary excretion of dihydroferulic acid-4-*O*-sulphate, the coffee level (rs=-0.379, p=0.023) and the intake of portions of yoghurt per week (rs=-0.363, p=0.029), respectively. No other statistically significant correlations were detected.

Table 5.6: Summary of correlation coefficients, r_S: Spearman's coefficient, unless labelled by r_p: Pearson's coefficient, significant levels, p≤ 0.05*, p≤0.01**

		Average sum of all metabolites	DHCA-3-*O*-sulfate	FA-GLY	VA-GLY	FA-4-*O*-sulfate	DHFA-4-*O*-sulfate	DHFA
Cups of coffee per week	r_s	-0.301	**-0.434	-0.227	0.269	-0.254	-0.327	-0.227
	Sig.	0.075	0.008	0.183	0.113	0.136	0.051	0.184
	N	36	36	36	36	36	36	36
Coffee level	r_s	-0.317	**-0.461	-0.230	0.285	-0.312	*-0.379	-0.207
	Sig.	0.060	0.005	0.177	0.092	0.064	0.023	0.225
	N	36	36	36	36	36	36	36
Cups of tea per week	r_s	-0.069	-0.015	-0.015	-0.037	-0.003	-0.011	-0.223
	Sig.	0.691	0.932	0.932	0.832	0.986	0.949	0.192
	N	36	36	36	36	36	36	36
Portions of fruit per week	r_s	*-0.341	-0.312	r_p=*-0.342	0.016	-0.231	-0.199	-0.238
	Sig.	0.042	0.064	0.041	0.925	0.174	0.244	0.161
	N	36	36	36	36	36	36	36
Portions of vegetables per week	r_s	-0.095	0.000	-0.248	-0.228	-0.079	0.053	-0.042
	Sig.	0.582	0.998	0.145	0.182	0.649	0.759	0.804
	N	36	36	36	36	36	36	36
Portions of yoghurt per week	r_s	*-0.348	-0.319	*-0.409	0.045	-0.238	*-0.363	-0.202
	Sig.	0.038	0.058	0.013	0.794	0.162	0.029	0.236
	N	36	36	33	386	36	36	36

5.4.4 Bivariate correlation of coefficient of variation of the average sum of all urinary metabolites of caffeoylquinic acid and its derivatives with the biomarkers of health

The non-normally distributed data of the coefficient of variation (%) of the average sum of the excretion of all urinary metabolites for participants who completed three phases of the human study (n=36) was correlated with plasma L-cysteine, DL-homocysteine, cysteinylglycine, L-glutathione, uric acid, glucose and insulin using the Spearman's bivariate correlation (Table 5.7).

A moderate, positive correlation at a significance level of $p \leq 0.01$ was detected between coefficient of variation of the average sum of all urinary metabolites of caffeoylquinic acid and its derivatives and L-cysteine (r_s=0.457, p=0.005) and a moderate, negative correlation at a significance level of $p \leq 0.01$ was detected between the coefficient of variation of the average sum of all urinary metabolites of caffeoylquinic acid and its derivatives and plasma uric acid (r_s=-0.443, p=0.007). No other statistically significant correlations were established.

Table 5.7: Summary of correlation coefficients, r_S: Spearman's coefficient, significant levels, p≤ 0.05*, p≤0.01**

		Coefficient of variation (%)
L-cysteine	r_s	**0.457
	Sig.	0.005
	N	36
DL-homocysteine	r_s	0.131
	Sig.	0.455
	N	35
Cysteinylglycine	r_s	0.142
	Sig.	0.409
	N	36
L-glutathione	r_s	-0.053
	Sig.	0.769
	N	33
Uric acid	r_s	**-0.443
	Sig.	0.007
	N	36
Glucose	r_s	-0.069
	Sig.	0.691
	N	36
Insulin	r_s	-0.082
	Sig.	0.635
	N	36

5.4.5 Bivariate correlation of coefficient of variation of the average sum of all urinary metabolites of caffeoylquinic acid and its derivatives with food intake

The non-normally distributed data of the coefficient of variation (%) of the average sum of the excretion all urinary metabolites for participants who completed three phases of the human study (n=36) was correlated with the intake of cups of coffee per week, the coffee level, the intake of cups of tea per week, the portions of fruit per week, the portions of vegetables and the portions of yoghurt using the Spearman's bivariate correlation (Table 5.8).

No statistically significant correlations were analysed between the coefficient of variation of the average sum of all urinary metabolites of caffeoylquinic acid and its derivatives with the intake of cups of coffee per week, the coffee level, the intake of cups of tea per week, the portions of fruit per week, the portions of vegetables per week and the portions of yoghurt per week.

Table 5.8: Summary of correlation coefficients, r_S: Spearman's coefficient, significant levels, p≤ 0.05*, p≤0.01**

		Coefficient of variation (%)
Cups of coffee per week	r_s	0.192
	Sig.	0.262
	N	36
Coffee level	r_s	0.231
	Sig.	0.176
	N	36
Cups of tea per week	r_s	-0.226
	Sig.	0.184
	N	36
Portions of fruit per week	r_s	0.026
	Sig.	0.882
	N	36
Portions of vegetables per week	r_s	-0.039
	Sig.	0.821
	N	36
Portions of yoghurt per week	r_s	0.230
	Sig.	0.177
	N	36

5.5 Discussion

In chapter 5, the data of urinary excretion of metabolites of caffeoylquinic acid and its derivatives over 36 hours after coffee consumption for participants who completed the three phases of the human study, presented in chapter 4, was linked to several baseline plasma biomarkers of health, analysed by Miss Joana Amarante Encarnacao and will be presented in her PhD thesis in 2015, and data of participant's food intake, collected by the means of semi-quantitative food frequency questionnaires (see appendix).

Prior to correlation analysis by either Pearson's correlation for normally distributed data or Spearman's for non-normally distributed data, the normal distribution of the data of urinary excretion of metabolites of caffeoylquinic acid and its derivatives over 36 hours after coffee consumption for participants who completed the three phases of the human study, i.e. average sum of all urinary metabolites, its coefficient of variation between the three phases, dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and ferulic acid, was tested. The data of all parameters, but not the excretion of feruloylgycine, is skewed towards the higher end, which is in line with data of previous publications which reported on the interindividual excretion of urinary metabolites of coffee caffeoylquinic acid and its derivatives [7, 16]. Additionally, the data of the biomarkers of health was also tested for normal distribution and the plasma level of DL-homocysteine was the only one which did not follow a normal distribution. Outliers were detected in the data of DL-homocysteine and L-glutathione. These outliers were excluded on the basis of their location beyond the normal physiological ranges. Further statistical outliers of the data of glucose and insulin were analysed, however, these were not excluded, as they lay within healthy physiological ranges. Furthermore the normal distribution for the data of food intake was investigated. All followed a non-normal distribution and were skewed towards the higher end, except for the portions of fruit per week. Several statistical outliers have been detected in the data of food intake, however, these have not been excluded due to the fact that the food intake depended on the participant's dietary habits.

Thereafter, the data of urinary excretion of metabolites of caffeoylquinic acid and its derivatives over 36 hours after coffee consumption for participants who completed the three phases of the

human study, i.e. average sum of all urinary metabolites, its coefficient of variation between the three phases, dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and ferulic acid was correlated with biomarkers of health, i.e. plasma L-cysteine, DL-homocysteine, cysteinylglycine, L-glutathione, uric acid, glucose and insulin. All together four weak and one moderate correlation were found. However, the samples size calculation based on DL-homocysteine, described in chapter 4, required 52 participants. The target of 52 participants has not been met, however, some of the results from the correlation between the urinary excretion of metabolites of caffeoylquinic acid and its derivatives over 36 hours after coffee consumption for participants who completed the three phases of the human study and biomarkers of health are statistically significant.

The correlation between the data on urinary excretion of metabolites of caffeoylquinic acid and its derivatives over 36 hours after coffee consumption for participants who completed the three phases of the human study, i.e. average sum of all urinary metabolites, its coefficient of variation between the three phases, dihydrocaffeic acid-3-*O*-sulphate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulphate, dihydroferulic acid-4-*O*-sulphate and ferulic acid, and the intake of cups of coffee per week, coffee level, the intake of cups of tea per week, the portions of fruit per week, the portions of vegetables per week and the portions of yoghurt per week showed some significant results. Both the cups of coffee per week consumed and the coffee level (cups of coffee per week multiplied by the strength of the coffee) showed weak or moderate negative correlations with the highest metabolite dihydrocaffeic acid-3-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate. This is in accordance with previously published data by Stalmach *et al.* [16], they demonstrated that a lower proportion of a high dose of coffee caffeoylquinic acid and its derivatives ingested, is excreted in 0-24 hour urine post coffee consumption and Erk *et al* [17] argue that this might be due to enzyme saturation, limitation of substrates, limited transport capacities at the enterocytes (influx and efflux) or an influence of the gastrointestinal tract transit time. The general notion from the data is that habitual consumption of several cups of coffee per day attenuates the absorption of coffee caffeoylquinic acid and its derivatives and therefore their excretion as urinary metabolites.

Chapter 6: Summary and perspective on future research

6.1 Summary

Several hydroxycinnamic acids, the hydrolysis product of caffeoylquinic acid and its derivatives, have been chemically conjugated with glycine yielding vanilloylglycine, 3,4-dimethoxybenzoylglycine, dihydroferuloylglycine, 3,4-dimethoxycinnamoylglycine, and 3,4-dimethoxydihydrocinnamoylglycine. These have been characterized using ^{1}H-NMR spectroscopy and MS^2 and their physicochemical properties were determined. Subsequently, a liquid-liquid extraction based sample preparation method followed by a rapid LC-MS method were developed and applied to quantitatively analyse urine samples collected at baseline, 0-4, 4-8, 8-12, 12-24 and 24-36 hours after ingestion of a standardised coffee beverage containing 730 µmol caffeoylquinic acid and its derivatives by six subjects. After the previously described feruloylglycine (39 µmol over 36 hours), vanilloylglycine (33 µmol over 36 hours) was also quantified in similar amounts in urine post coffee consumption, in contrast, the other glycine conjugates were only detected in trace amounts. The data suggests that hydroxycinnamic acids undergo beta-oxidation and subsequent conjugation with glycine linked to the beta-oxidation in mitochondria, whereas previously detected plasma 3,4-dimethoxycinnamic acid and 3,4-dimethoxydihydrocinnamic acid either undergo demethylation or are conjugated with other amino acids or peptides.

Practical quantities of dihydrocaffeic acid-3-*O*-sulphate, ferulic acid-4-*O*-sulphate and dihydroferulic acid-4-*O*-sulphate were chemically synthesized, characterized by ^{1}H-NMR, ^{13}C-NMR, IR and MS^2, and their physicochemical properties described. Subsequently these standards were used, along with feruloylglycine, vanilloylglycine, dihydroferulic acid and sinapic acid to develop a quick sample preparation method, followed by a LC-MS based quantitative method for urine samples. In addition, 3-, 4-, and 5-caffeoylquinic acid, 3-, 4-, and 5-feruloylquinic acid, 3,4-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid were quantitatively analysed in a standardised coffee beverage prior to administration to participants of a subsequent human study. Moreover, the *in vitro* effect of porcine pancreatin, a complex mixture of digestive enzymes including lipases, peptidases, amylases and non-specific

carboxylesterases secreted by the pancreas via the main pancreatic duct and the ampulla of Vater into the duodenum, on coffee, 3-, 4-, and 5-caffeoylquinic acid, and 3-, 4-, and 5-feruloylquinic acid. At 0, 10, 20, 40, and 60 minutes aliquots were taken and free caffeic acid and ferulic acid, respectively, quantified. The enzymic rates for caffeic acid were determined with 203, 1.6, 2.4 and 5.4 pmol/(mg protein*min) for coffee, 3-, 4-, and 5-caffeoylquinic acid, respectively. No free ferulic acid was detected in coffee nor in 3-, 4-, and 5-feruloylquinic acid digestion mixtures. The data indicate that pancreatin has a substantial contribution to the hydrolysis of the ester bond between caffeic acid and the quinic acid moiety in caffeoylquinic acids, in particular on 5-caffeoylquinic acid, in the small intestine.

Subsequently, a human study investigating the correlation of absorption of coffee caffeoylquinic acid and its derivatives, measured by means of quantitation of urinary metabolites over 36 hours after consumption of a standardised coffee beverage, to several biomarkers of health and food intake was undertaken. The study consisted of three phases and each phase included a 72 hour diet low in polyphenols, 36 hours prior to consumption of the coffee beverage (washout) and 36 hours thereafter, collection of a baseline plasma and urine sample, consumption of a standardized coffee beverage and a breakfast and collection of all urine up to 36 hours thereafter. In urine samples, collected from 0-4, 4-8, 8-12, 12-24 and 24-36 hours after coffee consumption the five most excreted metabolites of caffeoylquinic acid and its derivatives were quantified with dihydrocaffeic acid-3-*O*-sulphate (60.2 µmol), feruloylglycine (51.7 µmol), vanilloylglycine (41.9 µmol), ferulic acid-4-*O*-sulphate (40.7 µmol), dihydroferulic acid-4-*O*-sulphate (27.0 µmol), and dihydroferulic acid (19.2 µmol) adding up to 240.6 µmol which is equivalent to 27.3% of the ingested dose for 62 participants of phase 1. Similar results were found for phase 2 and 3 including 46 and 39 participants, respectively. Furthermore, the inter- and intraindividual variation was determined. In phase 1, phase 2 and phase 3 the total amount of metabolites of caffeoylquinic acid and its derivatives excreted over 36 hours vary interindividually by up to 8-fold, 10-fold and 35-fold, respectively, and for the participants who completed all three phases of the study the average intraindividual coefficient of variation was 52.3, 51.8, 50.2, 46.1, 37.4 and 35.9% for dihydrocaffeic acid-3-*O*-sulphate, vanilloylglycine, dihydroferulic acid-4-*O*-sulphate, dihydroferulic acid, ferulic acid-4-*O*-sulphate and

feruloylglycine, respectively. The data indicates that vanilloylglycine is the third most excreted metabolite after dihydrocaffeic acid-3-*O*-sulphate and feruloylglycine. The interindividual variation is in line with previous publications [7, 16], however, the data on the intraindividual variation is novel and, according to our knowledge, has not been previously reported. Metabolites of caffeoylquinic acid and its derivatives formed and absorbed in the colon showed the largest coefficient of variation between the three visits which leads us to the conclusion that the colonic microflora involved in the catabolism of the afore mentioned compounds is prone to changes in quality and quantity within six and twelve weeks between phase 1 and 2 and phase 2 and 3, respectively.

Finally, the data of excretion of caffeoylquinic acid and its derivatives, used as a surrogate for the absorption, is correlated with biomarkers of health, e.g. L-cysteine, DL-homocysteine, cysteinylglycine and L-glutathione, uric acid, insulin and glucose in plasma and the food intake, assessed by semi-quantitative food frequency questionnaires, e.g. intake of cups of coffee per week, the coffee level (cups of coffee per week multiplied by strength), intake of cups of tea per week, portions of fruit per week, portions of vegetables per week and portions of yoghurt per week using a bivariate correlation test, e.g. Pearson's or Spearman's correlation test for normally or non-normally distributed data, respectively. Moderate correlations were found between the 0-36 hours excretion of dihydrocaffeic acid-3-*O*-sulphate after coffee consumption and plasma insulin levels (r_s=0.428, p=0.009), the 0-36 hours excretion of vanilloylglycine after coffee consumption and DL-homocysteine levels (r_s=0.432, p=0.010), the 0-36 hours excretion of ferulic acid-4-*O*-sulphate after coffee consumption and plasma insulin levels (r_s=0.448, p=0.006), and between the 0-36 hours excretion of dihydrocaffeic acid-3-*O*-sulphate after coffee consumption and the consumption of cups of coffee per week (r_s=-0.434, p=0.008) and the coffee level, a measure of consumption of cups of coffee per week multiplied by the strength of the coffee (r_s=-0.461, p=0.005). It can be concluded that there is a reduced absorption of caffeoylquinic acid and its derivatives with increased, habitual coffee intake.

6.2 Perspective on future research

Previously it has been shown that 3,4-dimethoxycinnamic acid and 3,4-dimethoxydihydrocinnamic acid are readily absorbed after coffee consumption and circulate within the blood stream [28], however, their further fate is uncertain and only minor amounts were detected in urine. Recently, ferulic acid was incubated with hepatocytes and several metabolites of ferulic acid have been detected using high-resolution MS, *inter alia* a glutathione conjugate. This leads to the assumption that 3,4-dimethoxycinnamic acid and 3,4-dimethoxydihydrocinnamic acid might be conjugated with glutathione, undergo the mercapturic acid pathway and are excreted as 3,4-dimethoxycinnamic acid-*N*-acetylcysteine and 3,4-dimethoxydihydrocinnamic-*N*-acetylcysteine, respectively.

Catabolic metabolism of caffeoylquinic acid and its derivatives was assessed recently and several free hydroxycinnamic acids and their dihydroforms were detected after *in vitro* incubation of coffee with fecal slurries [26], however, incubation and subsequent measurements were only performed at a single time point. The novel finding of the intraindividual variation in particular of colonic metabolites of caffeoylquinic acid and its derivatives within twelve weeks leads to suggestion of performing the mentioned afore experiment with fecal slurries from participants taken at two or more time points over several weeks, incubate coffee with these and quantify the metabolites of caffeoylquinic acid and its derivatives. Additionally, performing a human study, as described in this thesis, with additional, quantitative recording of food intake and stool samples collected at each visit including quantitation of the fecal microflora, would help to elucidate the effect of diet on the colonic catabolism of caffeoylquinic acid and its derivatives.

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Appendix A: Advertisement sheet for human study

Appendix B: Participant's information sheet

Participant's information sheets

Research Project Title: ***Absorption of Coffee Antioxidants***

You are being invited in taking part in a research study. Please read carefully the information given and make sure you understand why the study is going to be done and what it will involve, before making any decision. Thank you for taking the time to read this.

- **What is the project's purpose?**

Phenolic acids are naturally-occurring constituents of plant-derived foods and beverages with antioxidant activity. They are abundantly consumed in a typical UK diet and for people who drink it, coffee is the major dietary source for them. In this study we will focus on phenolic acids from coffee, since recent studies have shown protective effects of coffee consumption on some human chronic diseases. Your participation in this study will help to understand the absorption of phenolic acids in people.

- **Am I a suitable candidate for this study?**

We are looking for **male** and **female** volunteers meeting the following criteria:

Inclusion criteria:

- Age 18-70 years
- Normal Body Mass Index (BMI) 18-29 kg/m^2
- Non-smoker, former or weak smoker (max. 5 cigarettes per day)
- No more than 4 alcoholic units as a regular and daily consumption
- No diagnosed chronic disease (e.g. pancreas, kidneys, liver, heart)
- No haemophilia
- No long term prescribed medication (contraceptive medication allowed)
- No previous GI (gastrointestinal) tract operation
- Not pregnant or breast feeding

To help us to assessing your suitability for this study, additional information will be sought from you before the study begins (e.g. dietary habits, relevant medical history, menstrual cycle for female participants).

- **Do I have to take part in the study?**

Taking part in this research study is entirely voluntary and if you do not feel comfortable anymore in participating, you have the right to withdraw from it at any time, without having to justify yourself. If you decide to take part in this study, you will be given a consent form to sign before starting the study. This will indicate you have understood the benefits and risks in taking part in this research.

- **What will happen to me if I take part? What are my responsibilities?**

It is important that you abstain from consuming any food and drinks which are high in phenolic acids (please refer to the document "food restriction list"), starting 36hrs before the day of the study (washout period) and during the study. You will be fasting from the previous night 10 pm, for the study days and you will be asked to keep an honest record of your meals and drinks during the same period. On the study day, before coming to the School of Food Science and Nutrition (FS&N), you will be able to go the toilet as usual and will drink at least one glass of water at least 1hour before your appointment at the school of FS&N.

In the morning of the study day, a few measurements will be taken (e.g. height, weight, waist and hip circumference, blood pressure) at the School of FS&N. We will collect, from your elbow pit, a little amount of your blood before you drink the coffee. You will then be given a cup (4g/200ml) of instant coffee (high in coffee antioxidants) along with a small breakfast and you will collect all your urine, during the following 36hrs (0hrs, 0-4hrs, 4-8hrs, 8-12hrs, 12-24hrs and 24-36hrs), into closed containers provided.

In order to check for the consistency of the results, we will recall you 2 and 4 months after your first visit.

⚠ It is very important that all the urine in the 36 hours mentioned is collected, however, if any is lost, it is not dramatic, and you will simply be asked to start again, including the 36-hour washout period.

In total, if you are willing to participate in each recall, you would only have to come 6 times (3 times for the study days, and the following day for bringing back the collected urine).

- **What foods and drinks am I allowed to consume?**

A list of food and drinks to avoid and allowed will be given to you along with meals suggestions (refer to the document named "food restriction list"). It is important that you are honest when you record your meals during the washout and study periods, as this will help us interpret the results. If you agree in participating in the study, you will be asked to follow a rigorous diet during the period mentioned.

- **What are the possible disadvantages and risks of taking part?**

The part in this study you might find of less comfort is the blood donation. However, the blood collection will be done by a trained phlebotomist.

- **What are the possible benefits of taking part?**

Although there are no immediate benefits for the volunteers participating in the project, it is hoped that this work will help understanding better the effects of coffee consumption, particularly its antioxidant components, on human health.

- **Will my information and data be kept safe? Will I be identifiable with my samples?**

Your participation in the study is strictly confidential. Before the beginning of the study, codes will be attributed to each volunteer so that no participant will be identified to the data. These codes and the results will be stored and kept safely from unauthorized persons.

- **What will happen to the results of the research project?**

The obtained and analysed results will be stored for 10 years after the end of the study. When any data is to be published, they will not be linked to any participant and no participant will be identifiable in any report or publication. If you wish so, we will inform you about the outcome of the results, you will not, however, be able to recognise yourself in any results.

- **How will I be compensated?**

All participants will receive £10 for each study visit for their contribution to the study. Absence at work will not be compensated.

- **Who is organising and funding the research?**

This research study will be done at the School of Food Science & Nutrition and is partially funded by Nestlé, Switzerland.

Thank you for considering joining this study.
You will be given a copy of this document and the signed consent form for your personal records.

Please contact one of the below research team member, for further information:

Mr Nicolai Kraut (main investigator, PhD student): fsnuk@leeds.ac.uk

Ms Joana Encarnação (main investigator, PhD student): fsje@leeds.ac.uk

Appendix C: Consent form

Coffee Study-2012

Participant Consent Form

Title of Research Project: *Absorption of Coffee Antioxidants*

Name of Researchers: Ms Joana Encarnação and Mr Nicolai Kraut

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

1 I confirm that I have read and understand the information sheet dated **08-07-2011** explaining the above research project and I have had the opportunity to ask questions about the project. ☐

2 I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without there being any negative consequences. In addition, should I not wish to answer any particular question or questions, I am free to decline. In this case I will contact either Ms Encarnação or Mr Kraut. ☐

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

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

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

6 I am willing to be recontacted if I am selected to participate in later stages of the study ☐

7 I am willing to come back if I am selected to participate in later stages of the study ☐

Name of participant	Date	Signature
Name of person taking consent (*if different from lead researcher*)	Date	Signature
Lead researcher	Date	Signature

To be signed and dated in presence of the participant

To be signed and dated in presence of the participant

Copies:

Once this has been signed by all parties the participant should receive a copy of the signed and dated participant consent form, the information sheet and any other written information provided to the participants. A copy of the signed and dated consent form should be kept with the project's main documents which must be kept in a secure location.

Appendix D: Health assessment and food frequency questionnaire

Health assessment & Dietary habits Questionnaires

July 2011

University of Leeds
School of Food Science & Nutrition

This section was made to allow us confirm whether you are or not suitable to become a participant in this research study (health assessment questionnaire). We would also like to see if there is any link between your normal dietary habits (excluding special events) or other daily habits and how well your body absorbs antioxidants which are present in coffee (dietary habits questionnaire).

This questionnaire is divided into two main sections, themselves divided into several sections:

1° Health assessment questionnaire:	General health Blood donation Smoking habits Drinking habits Physical activity routine Bowel movement
2° Dietary habits questionnaire:	Beverages (coffee, tea and alcohol) Snacks Main meals (breakfast, lunch, dinner) Spices and herbs

As the health assessment questionnaire will mainly allow us to confirm whether or not you will be suitable to participate in the study, we would like to ask you to answer all questions as honestly as possible, for accuracy.

Thank you.

PART I:
HEALTH ASSESSMENT QUESTIONNAIRE

Please fill in the following details: (please leave * marked in blank)

Subject's code*	
Date of birth	
Gender (please circle)	Female / Male
Weight	
Height	
Waist circumference*	
Hip circumference*	

Please remember to put a tick (✓) on every line

GENERAL HEALTH	No	Yes	Don't know	Other (please specify)
Do you consider yourself to be a generally healthy person?				
Do you take the contraceptive pill?				
Do you take any other prescription medicine currently? (If yes, please specify below)				
What kind of medication and what is the duration of the therapy?				
Have you ever undergone gastrointestinal tract surgery (e.g. stomach, small intestine or colon/large bowel?)				
Do you suffer from diabetes?				
Have you been diagnosed with any other chronic disease (heart, liver, kidney)? If yes, please specify: _____				
Do you suffer from haemophilia				
Are you pregnant or breast feeding?				
Do you suffer from any food allergy? If yes, please specify:_____				
Do you have difficulty swallowing?				

BLOOD DONATION	No	Yes	Maybe
Have you donated blood within the past 4 weeks or are you going to donate blood within the next 4 weeks?			

SMOKING HABITS	No	Yes	I Have given up	Other (please specify)
Do you smoke?				
	1-4	5-10	10-15	More than 15
If yes, how many cigarettes a day?				
How old were you when you started smoking? _____ years old				
If you are a former smoker, how long ago did you stop smoking? _____ months ago				

DRINKING HABITS	No	Yes	Sometimes	Always
Do you consume more than 4 alcoholic drinks a day?				

PHYSICAL ACTIVITY HABITS	never	Once a week	Twice a week	3 to 4 times a week	Once a day or more
How often do you exercise?					
How many hours of exercise do you do per week					Hrs/week
Light exercise (e.g. walking, yoga)					
Moderate exercise (e.g. fast walking, easy swimming, easy cycling)					
Strenuous exercise (e.g. running, vigorous swimming, high impact aerobics)					
Muscle gain					
Other (please specify): _____					

BOWEL MOVEMENT	> 1x once a day	1x a day	1x every 2 days	1x every 3 days or less
Please specify the normal frequency of your bowel movement?				

Do you feel there might be any reason why you could not participate in the study? (Please specify below)

All answers will be treated confidentially and no link between your personal details and answers given will be made.

Please confirm with your signature the truth of your given answer.

Participants Name	Date	Signature
_____	_____	_____

Thank you for taking the time to answer all questions and helping us with our research.

PART II:
DIETARY HABITS

It is important that you answer all questions and we would like to ask you to remember putting only one tick (✓) in all lines. If you are unsure of a brand, please put a "?" in the appropriate box.
Please only specify a product in the "other (please specify)" case, if this product is not mentioned in the options and is a product that you consume a lot.

Symbols and abbreviations used:

Symbol or abbreviation	Meaning
>	More than
<	Less than
Y/N	Yes or No
tsp	Tea spoon

Example of how to fill the questionnaire:

	Daily				1 - 2 cups in a week	NONE
	> 8 cups	6 - 8 cups	3 - 5 cups	1 - 2 cups		
Decaffeinated	✓					
Latte					✓	

A) **Beverages:** In this section several questions on your habits of consumption of coffee, tea, hot chocolate and alcoholic drinks are asked.

How long have you been drinking coffee? _____

HOW OFTEN DO YOU DRINK THE FOLLOWING TYPES OF COFFEE?
Please put a tick (✓) on every line

	Daily				1 - 2 cups in a week	NONE
	> 8 cups	6 - 8 cups	3 - 5 cups	1 - 2 cups		
Caffeinated						
Decaffeinated						
Strong						
Medium						
Weak						
Instant						
Filtered						
Espresso						
Cappuccino						
Latte						
Turkish						
Arabic						
Other (Please specify below)						

HOW DO YOU USUALLY HAVE YOUR COFFEE?
Please put a tick (✓) on every line

	A lot	Some	A little	NONE
Sugar				
Sweetener				
Milk				
Cream				
Non-dairy creamer				
little alcohol				
Other (Please specify):				

HOW OFTEN DO YOU DRINK THE FOLLOWING TYPES OF HOT CHOCOLATE? Please put a tick (✓) on every line						
	Daily				1 - 2 cups in a week	NONE
	> 8 cups	6 - 8 cups	3 - 5 cups	1 - 2 cups		
Dark chocolate						
Milk chocolate						
White chocolate						
Bitter chocolate (unsweetened cocoa)						
Other (Please specify below):						

HOW DO YOU USUALLY HAVE YOUR HOT CHOCOLATE? Please put a tick (✓) on every line				
	A lot	Some	A little	NONE
Sugar				
Cinnamon				
Cardamom				
Other (Please specify):				

How long have you been drinking tea? _____

HOW OFTEN DO YOU DRINK THE FOLLOWING TYPES OF TEA?
Please put a tick (✓) on every line

	Daily				1 - 2 cups in a week	NONE
	> 8 cups	6 - 8 cups	3 - 5 cups	1 - 2 cups		
Black						
Green						
Rooibos						
Chai tea (Chai latte)						
Herbal infusion						
Fruit infusion						
Other (Please specify)						

HOW DO YOU USUALLY HAVE YOUR BLACK TEA?
Please put a tick (✓) on every line

	Daily				1 - 2 cups in a week	NONE
	> 8 cups	6 - 8 cups	3 - 5 cups	1 - 2 cups		
Decaffeinated						
Instant						
Tea bag						
Tea leaves						
Iced tea (commercial)						

	A lot	Some	A little	NONE
With sugar				
With sweetener				
With milk				
With non-dairy creamer				
Black				
Other (Please specify):				

HOW OFTEN DO YOU DRINK THE FOLLOWING TYPES OF ALCOHOLIC DRINKS? Please put a tick (✓) on every line					
	Daily			1 - 2 units in a week	NONE
	> 4 units	3 - 4 units	1 - 2 units		
Beer (half a pint = 1 unit)					
Cider (half a pint = 1 unit)					
Wine (125ml glass = 1 unit)					
Spirits (small measure = 1 unit) (e.g. Whisky, Vodka)					
Alcopops (175ml bottle = 1 unit) (e.g. Bacardi breezer)					
Other (Please specify):					

B) Snacks:

HOW OFTEN DO YOU EAT THE FOLLOWING TYPES OF SNACKS? Please put a tick (✓) on every line							
	Daily			5 - 6x per week	2 - 4x per week	1x per week	NONE
	> 2x	1 - 2x	1x				
Fruit							
Yoghurt							
Dried fruits							
Nuts							
Crisps							
Chocolate							
Cake							
Crackers							
Other (Please specify):							

C. Main meals: (Cruciferous vegetables* include broccoli, cauliflower, cabbage, brussel sprouts, parsnips, kale, kohlrabi, bok choy, radish.)

IN GENERAL, HOW OFTEN DO YOU EAT THE FOLLOWING TYPES OF VEGETABLE?
Please put a tick (✓) on every line

	Daily				5 - 6x per week	2 - 4x per week	1x per week	< 1x per month	NONE
	> 5	4 - 5x	2 - 3x	1x					
Salad									
Cruciferous vegetables*									
Green beans / peas									
Carrot									
Sweet corn									
Tomato									
Potato (incl. Chips, fries, boiled, roasted, etc)									
Other (please specify):									

IN GENERAL, HOW MANY PIECES OF FRUIT DO YOU EAT?
(1 piece = what fits into the palm of your hand) Please put a tick (✓) on every line

	Daily				5 - 6 per week	2 - 4 per week	1 per week	< 1 per month	NONE
	> 5	4 - 5	2 - 3	1					
Berries (any)									
Citrus fruits*									
Kiwi									
Grapes									
Peaches / nectarines									
Apple									
Banana									
Dried fruits (any)									
Other (please specify):									

Citrus fruits* include orange, clementine, mandarin, pomelo, grapefruit, lime, lemon.

HOW OFTEN DO YOU HAVE THE FOLLOWING OPTIONS FOR BREAKFAST? Please put a tick (✓) on every line						
	Every Day	5 - 6x per week	2 - 4x per week	1x per week	< 1x per month	Never
Cereals						
Porridge						
Muesli						
White bread						
Wholemeal bread						
Yoghurt						
Fruit						
Dairy milk						
Soy milk						
Tea						
Coffee						
Eggs						
Bacon/sausage						
Other (Please specify):						

HOW OFTEN DO YOU HAVE THE FOLLOWING OPTIONS FOR LUNCH? Please put a tick (✓) on every line						
	Every Day	5 - 6x per week	2 - 4x per week	1x per week	< 1x per month	Never
Salad						
Soup						
Sandwich						
Pizza						
Pasta						
Burger / chips						
Meat / fish						
Rice / potatoes						
Curries						
Other (Please specify):						

HOW OFTEN DO YOU HAVE THE FOLLOWING OPTIONS FOR DINNER? Please put a tick (✓) on every line						
	Every Day	5 - 6x per week	2 - 4x per week	1x per week	< 1x per month	Never
Salad						
Soup						
Sandwich						
Pizza						
Pasta						
Burger /chips						
Meat/fish						
Rice/potatoes						
Curries						
Other (Please specify):						

c) **Spices and herbs:**

HOW OFTEN DO YOU ADD FOLLOWING SPICES AND HERBS TO YOUR MEALS? Please put a tick (✓) on every line						
SPICES	Every Day	5 - 6x per week	2 - 4x per week	1x per week	< 1x per month	Never or don't know
Pepper						
Chilli						
Cumin						
Saffron						
Ginger						
Cardamom						
Star anise						
Cinnamon						
Turmeric						
Cloves						
Other (please specify):						

HERBS	Every Day	5 - 6x per week	2 - 4x per week	1x per week	< 1x per month	Never
Mint						
Coriander						
Thyme						
Rosemary						
Other (please specify):						

D) Food supplements:

HOW OFTEN OF THE FOLLOWING FOOD SUPPLEMENT DO YOU CONSUMME? Please put a tick (✓) on every line						
HERBS	Every Day	5 - 6x per week	2 - 4x per week	1x per week	< 1x per month	Never
Berry (any)based supplement						
Seed (any) based supplement						
supplement						
Minerals and trace elements (e.g. iron, magnesium, calcium)						
Vitamins (any)						
Other (please specify):						

You have now completed the dietary habits questionnaire.
Thank you for taking the time to answer all questions and helping us with our research

Appendix E: Food restriction list and diary

Coffee study-2012

Food restriction list

FOODS TO AVOID
Coffee (any form: drink, sweets, cake, ...)
Any form of dark and milk chocolate (drinks, bars, cake, ...)
Teas and herbal teas
Any alcohol
Most fruits, including freshly pressed juices (except banana and kiwi)
Most vegetables and tomato-based products
Wholemeal wheat products
Fruit/vegetable-based food supplements (in any form, i.e., powder, capsule, etc.)

EXAMPLES OF FOODS ALLOWED
Any meat and fish
Rice, pasta, noodles, beans, eggs
Potato (e.g. boiled, roast, baked, mash, chips, fries, ...)
White bread
Non- wheat breakfast cereals (e.g. corn flakes, oats, rice krispies)
Some vegetables: mushrooms, cucumber, courgette, asparagus, lettuce, pumpkin and lentils
Milk and any other non-chocolate containing dairy product (e.g. butter, cream, cheese, yoghurts, ...)
Water, squash drinks and sodas (e.g. coke, sprite, etc.)
White chocolate (no dark or milk chocolate!)
Non-chocolate based sweets and cakes (e.g. sponge cake, cheesecake, lollipops, jelly, marshmallows, mints)
Seasoning: salt, pepper, olive oil and a little bit of aromatic herbs, salad dressing
Nuts (without their skins), crisps, nachos, etc.
Vitamins

MEALS SUGGESTION:

We will send you a notification of the study a few days in advance. Along with the reminder, we will suggest you a list of meals to help you.

Food diary

Date : ___________________ Participant's code : ______________________

Approx. time	Food details	Amount (please refer to the scale given)
Example : 8.15am	rice krispies	small 1 2 3 (X) 4 5 large
Example : 12.30pm	Chicken breast	1 2 (X) 3 4 5
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Have you consumed any food mentioned in the "foods to avoid" list? (If yes, please indicate which one and the amounts)?

_____
_____
_____
_____

Indication of portion sizes: