Bioavailability of coffee polyphenols: focus on dose- and structure response

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Für Julia und meine Eltern

"The doctor of the future will no longer treat the human frame with drugs, but rather will cure and prevent disease with nutrition."

Thomas Alva Edison

*1847 Milan, Ohio

†1931 West Orange, New Jersey

American Inventor

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List of abbreviations

ABC	ATP-binding casette
ACN	acetonitrile
amu	atomic mass unit
APS	ammonium persulfate
ATP	adenosine triphosphate
AUC	area under the curve
ax	axial
Bidist.	bidistilled
BMI	body mass index
BW	body weight
CA	caffeic acid
cf.	compare (Latin:"*confe*r")
cfu	colony forming units
CGA	chlorogenic acid
cm	centimeter
C_{max}	maximum concentration
CoA	coenzyme A
COMT	catechol-*O*-methyl transferase
CQA	caffeoylquinic acid
CQL	caffeoylquinide
d	doublet
DAD	diode – array - detection
DAR	dose-absorption relationship
dd	double doublet
ddd	triple doublet
DHCA	dihydrocaffeic acid
DHFA	dihydroferulic acid
diCQA	dicaffeoylquinic acid
DiMeDHCA	dimethoxydihydrocaffeic acid
DiMeCA	dimethoxycaffeic acid
DMSO	dimethyl sulfoxide

e.g.	*exempli gratia*
EDTA	ethylendiaminetetraacetic acid
eq	equatorial
ESI	electrospray ionization
EST	esterase
et al.	et alii
EtOH	ethanol
FA	ferulic acid
FQA	feruloylquinic acid
GI	gastrointestinal
GIT	gastrointestinal tract
HBSS	Hanks´ balanced salt solution
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
IgG-HRP	immunoglobulins - horseradish peroxidase
Ileo	Ileostomist
IS	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
J	coupling constant
Kcal	kilo calorie
L	litre
LOD	limit of detection
log D	logarithm of the distribution coefficient
LOQ	limit of quantification
M	$mol*L^{-1}$
m	multiplet
m/z	mass to charge ratio
MCT	monocarboxylic acid transporter
MDR	multidrug resistance protein
mg	milligram
MHz	megahertz
MRM	multiple reaction monitoring
MRP	multidrug resistance - associated protein
MS	mass spectrometer

mS	milli sievert
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
ng	nanogram
NMR	nuclear magnetic resonance
NRC	Nestlé Research Centre, Vers-chez-les-Blanc, Lausanne, Switzerland
p-gp	P-glycoprotein
pKa	ionization constant in water
PVDF	polyvinylidene fluoride
Q	quadrupole
QA	D-(-)-quinic acid
RA	reductase
RP	reversed phase
rpm	ration per minute
S/N	signal to noise
SAR	structure-absorption relationship
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	second
SPE	solid phase extraction
TEMED	tetraethylenediamine
SULT	sulfotransferase
TBS-T	tris buffered saline - tween
T_{max}	maximum amount at a certain time point
TT	transit time
UGT	UDP-glucuronosyltransferase
vs	*versus*
λ	wave length

Summary

A positive affection of human health by nutrition is of high interest, especially for food being daily consumed in high amounts. This is the case for coffee, one of the most consumed beverages worldwide. In epidemiological studies a reduced risk of type II diabetes, cognitive, cardiovascular and certain cancer disorders were associated with a lifelong daily intake. These positive health effects are partially associated with the high polyphenol content (especially chlorogenic acids) in coffee. Numerous *in vitro* studies using this class of polyphenols are also indicating an association. These chlorogenic acids (CGA), consisting essentially of a hydroxycinnamic acid esterified with D-(-)-quinic acid (QA), are therefore suggested to be *nutraceuticals*. However, so far their accurate bioefficacy with daily consumption of CGA by coffee is not well understood. The decisive basis for this is a precise knowledge of CGA bioavailability after oral application as it describes the amount of CGA which is available at the site of action.

The bioavailability of a molecule can be influenced by various factors. These can be widely classified by the dosage form, the proband´s physiology and the physico-chemical properties of the ingested molecule. Series of studies describes a somewhat controversial and inconsistent bioavailability data for CGA, especially in the upper GIT. Since different dosages in matrices and doses, with partially different CGA compositions and different analytical strategies were used, it is not possible to take these differences into account with overall bioavailability.

The objective of the present study was to investigate the influence of individual factors on bioavailability of CGA in the upper parts of the GIT. For that purpose we used two different study designs.

First we monitored **dose-response effects *in vivo*** for the total absorption, metabolic pattern in the upper gastro intestinal tract and colonic availability of CGA and QA after oral coffee application. Especially the effect of the ingested dose on the sites where absorption occurs within the GIT were here of interest. For this, we performed a **randomized, double-blinded, crossover study** with five probands with a terminal ileostomy. After a two day polyphenol free diet, the **ileostomists** consumed, in three separate occasions, **instant coffee** with **different CGA contents** (HIGH 4,525 µmol; MEDIUM 2,219 µmol; LOW 1,053 µmol) and different amounts of free QA (HIGH 2,457 µmol; MEDIUM 1,373 µmol, LOW 695 µmol). Plasma, urine and ileal fluid

samples were collected. Except for plasma samples, we used an analytical strategy for all samples without enzymatic cleavage of CGA conjugates. CGA and corresponding metabolites were identified and quantified by **HPLC-DAD**, **-ESI-MS** and **-ESI-MS/MS**. QA was quantified by **stable isotope dilution analysis**.

Additionally, the influence of **molecular properties** of CGA and QA from coffee on bioavailability was assessed (**structure-absorption relation**). Due to the extensive biotransformation of CGA in the human body such investigations were performed ***ex vivo*** with **pig jejunal mucosa** using the **Ussing chamber model**. For this purpose we used QA and individual CGA from coffee, namely: caffeoylquinic acids (CQA), feruloylquinic acids (FQA), caffeic acid (CA), dicaffeoylquinic acids (diCQA). These compounds were incubated using individual experiments at physiological concentrations from 0.2 up to 3.5 mM. We also investigated a possible **secretion** and the **mechanism of absorption** (dose-absorption relation) of the main CGA from coffee, 5-caffeoylquinic acid. The mucosa and individual supernatants of Ussing chamber halves were analyzed by **HPLC-DAD** and **-ESI-MS/MS.**

After the consumption of different, increasing doses of coffee the following $AUC_{0\text{-}8h}$ for CGA and metabolites were determined in plasma (after treatment with glucuronidase and sulfatase): 4,412 ± 751 $nM*h_{0\text{-}8}^{-1}$ (HIGH), 2,394 ± 637 $nM*h_{0\text{-}8}^{-1}$ (MEDIUM) and 1,782 ± 731 $nM*h_{0\text{-}8}^{-1}$ (LOW) respectively. The renal excretion accounted for CGA and metabolites 8.0 ± 4.9% (HIGH), 12.1 ± 6.7% (MEDIUM) and 14.6 ± 6.8% (LOW) of the ingested dose. About ⅔ of the CGA from coffee consumption was available in the colon dose independent: Therefore, we assumed the sites of absorption within the GIT were not influenced by dose. Higher ingested CGA doses lead to an accelerated ileal excretion. Interindividual difference in gastrointestinal transit times of coffee were probably related to the systemic availability of CGA. This is corroborated *ex vivo* by a direct proportional relationship of incubation time with absorbed CGA amounts. Consequently the interindividual differences of **proband´s physiology** are a **decisive bioavailability factor** for CGA uptake. **New metabolites** in ileal and renal excretion were detected and therefore a much more extensive metabolism of CGA than recently expected. For instance, CGA with a caffeic acid moiety were predominantly sulfated at position 3´ instead of CGA with ferulic acid moiety predominantly renally excreted as glucuronide. In the ileal

effluent samples, sulfation of both structural units dominated. However, glucuronidation of CGA slightly was increased with increasing consumed coffee dose.

We conclude that the consumption of high CGA doses via coffee might influence the gastrointestinal transit time and consequently affect CGA absorption *in vivo*. Nevertheless, the dose-response studies concluded that **different CGA and QA doses** from coffee have only **minor effects on absorption** in the upper GIT. This is also corroborated ***ex vivo*** by a **non-saturable absorption** (linear dose-flux relationship) of 5-CQA, indicating **passive diffusion** as major mechanism of absorption.

Due to the extensive biotransformation of CGA in the human body after consumption of coffee, the conclusion of influence of molecular properties on absorption is limited. However, no diCQA were determined *in vivo* but for the first time significant amounts of FQA, CQA and QA in plasma and urine were present. These observations were corroborated *ex vivo* in the Ussing chamber model. According to the subgroup of CGA different amounts were transferred through the jejunal pig mucosa in increasing order: diCQA, trace amounts; CQA, ≈ 1%; CA, ≈ 1.5%; FQA, ≈ 2%; and QA, ≈ 4%. No differences were observed within the CGA subgroups. Thus we conclude that the **mechanism** of **CGA absorption** is governed by its **physico-chemical properties**.

Furthermore, we identified an active efflux **secretion** for 5-CQA in the pig jejunum *ex vivo* and we are postulating an active efflux for hydrophilic CGA metabolites such as CQA-sulfates or FQA-glucuronides which might decrease total CGA bioavailability.

In the course of this PhD thesis the influence of several factors on bioavailability of CGA from coffee was shown. The increased consumption of CGA doses by coffee had a minor effect on oral bioavailability in ileostomists. This was approved *ex vivo* since CGA absorption is governed by diffusion. Consequently, the consumption of high amounts of CGA from coffee with the daily diet is not limiting its bioavailability. However, the systemic availability of CGA is associated with the different proband´s physiology, especially the interindividual gastrointestinal transit time which is possibly influenced by dose and the physico-chemical properties of CGA. Hence, bioavailability of CGA also depends on the CGA composition of the food consumed.

Zusammenfassung

Eine Verringerung des Erkrankungsrisikos von sogenannten Zivilisationskrankheiten durch die Ernährung ist in den industrialisierten Ländern von großer Bedeutung. Eine solche potentielle Wirkung wird dem Kaffee Getränk nachgesagt. Eines der weltweit meist konsumierten Getränke. Ein reduziertes Risiko für Type II Diabetes-, Kognitiver-, Herzkreislauf- und bestimmte Krebs- Erkrankungen werden in epidemiologischen Untersuchungen mit einem täglichen, lebenslangen Konsum in Zusammenhang gebracht. Die beschriebenen Effekte auf die Gesundheit werden zum Teil der hohen Polyphenol Menge im Kaffee (insbesondere den Chlorogensäuren) zugeschrieben. Zahlreiche *in vitro* Studien mit dieser Polyphenolklasse weisen ebenfalls auf einen derartigen Zusammenhang hin. Daher werden die Chlorogensäuren (CGA), bestehend aus einer Hydroxyzimtsäure verestert mit D-(-)-Chinasäuren (QA), auch als sogenannte "*Nutraceuticals*" bezeichnet. Allerdings ist bis jetzt deren exakte biologische Wirkung bei täglichem Verzehr von CGA durch Kaffee nicht eingehend erfasst. Die entscheidende Grundlage dafür ist ein präzises Verständnis der Bioverfügbarkeit nach oraler Applikation, da diese die Menge an CGA beschreibt, welche am Wirkort verfügbar ist. Die Bioverfügbarkeit eines Moleküls kann von verschiedenen Faktoren beeinflusst werden. Diese können in die Art der Dosis, die Physiologie des Probanden und der physikalisch-chemischen Eigenschafften des Moleküls unterteilt werden. Durch eine Vielzahl von Studien ist ein etwas kontroverser und unzureichender Kenntnisstand der Bioverfügbarkeit von CGA beschrieben, besonders bezüglich deren Absorption im oberen Gastrointestinaltrakt. Da unterschiedliche Dosierungen in Matrix und Menge, teilweise mit unterschiedlichen CGA Profil und verschiedenen analytischen Strategien (mit oder ohne enzymatischer Spaltung von CGA Metaboliten) verwendet wurden, ist die Bedeutung einzelner Parameter auf die Bioverfügbarkeit nicht erfassbar. Ziel dieser Arbeit war es, einen Einfluss individueller Parameter auf die Bioverfügbarkeit der CGA im oberen Gastrointestinaltrakt zu untersuchen. Dazu verwendeten wir zwei unterschiedliche Ansätze.

Zunächst überprüften wir ***in vivo*** den **Einfluss** der **applizierten Dosis** auf die Absorption und den Metabolismus im oberen Gastrointestinaltrakt und die Verfügbarkeit im Kolon von CGA und QA nach oraler Applikation von Kaffee (Dosis-Wirkungs Studie). Von besonderem Interesse war dabei der Einfluss der

aufgenommenen Dosis auf den Ort der Absorption innerhalb des Gastrointestinaltraktes. Dazu führten wir eine **randomisierte, crossover Doppelblindstudie** mit fünf Probanden mit einem Stoma des terminalen Ileums durch. Nach einer zwei Tage dauernden Polyphenol-freien Ernährung konsumierten die **Ileostomie**-**Probanden** an drei separaten Gelegenheiten, **Instant**-**Kaffee** mit **unterschiedlichen CGA-Gehalt** (HOCH 4.525 µmol; MITTEL 2.219 µmol, NIEDRIG 1.053 µmol) und unterschiedlichen Gehalten an freier QA (HOCH 2.457 µmol; MITTEL 1.373 µmol, NIEDRIG 695 µmol). Es wurden Plasma Proben, sowie der gesamte Urin und Stoma-ausfluss entnommen. Mit Ausnahme der Plasma Analyse wurde für alle Proben eine analytische Strategie gewählt, welche keinerlei enzymatische Spaltung von Metaboliten der CGA vorsah. CGA und deren Metabolite wurden mittels **HPLC**-**DAD**, -**ESI**-**MS** und -**ESI**-**MS/MS** identifiziert und quantifiziert. Für die Quantifizierung der QA wurde eine **Isotopenverdünnungs Analyse** angewandt.

Zusätzlich wurde die Bedeutung der **molekularen Eigenschaften** auf die Bioverfügbarkeit der CGA des Kaffees und der QA bestimmt (**Struktur**-**Absorptions**-**Beziehung**). Bedingt durch die intensive Biotransformation (der CGA) im menschlichen Körper wurden derartige Überlegungen ***ex vivo*** mittels der **Mukosa des Schweinejejunums** im **Ussing Kammer Model** durchgeführt. Dafür wurden QA und einzelne CGA des Kaffees verwendet; und zwar: Kaffeoylchinasäuren (CQA), Feruloylchinasäuren (FQA), Kaffeesäure (CA) und diKaffeoylchinasäuren (diCQA). Diese Verbindungen wurden in einzelnen Experimenten in physiologischen Konzentrationen von 0.2 bis 3.5 mM verwendet. Weiterhin wurde auch eine mögliche **Sekretion** und der **Mechanismus der Absorption** (Dosis-Absorptions Beziehung) anhand der bedeutendsten CGA aus Kaffee, der 5-Kaffeoylchinasäure, untersucht. Die Mukosa und die einzelnen Überstände der Ussing Kammer Hälften wurden analysiert mittels **HPLC-DAD** und **-ESI-MS/MS.**

Nach Verzehr unterschiedlicher Dosen Kaffee wurde folgende $AUC_{0\text{-}8h}$ für CGA und deren Metaboliten im Plasma bestimmt (nach Behandlung mit Glucuronidase und Sulfatase): 4412 ± 751 $nM*h_{0\text{-}8}^{-1}$ (HOCH), 2394 ± 637 $nM*h_{0\text{-}8}^{-1}$ (MITTEL) und 1782 ± 731 $nM*h_{0\text{-}8}^{-1}$ (GERING). Die renale Ausscheidung betrug 8,0 ± 4,9% (HOCH), 12,1 ± 6,7% (MITTEL) und 14,6 ± 6,8% (GERING) der konsumierten CGA Dosis. Etwa ⅔ der CGA des applizierten Kaffees waren im Kolon Dosis-unabhängig

verfügbar, somit scheint der Ort der Absorption innerhalb des Gastrointestinaltraktes nicht durch die Dosis beeinflussbar zu sein. Eine größere aufgenommene CGA Menge führte zu einer beschleunigten ilealen Exkretion. Durch interindividuelle Unterschiede der gastrointestinalen Transitzeit des Kaffees konnte ein wahrscheinlicher Zusammenhang zur systemisch verfügbaren CGA Menge hergestellt werden. Dies konnte, durch einen direkt proportionalen Zusammenhang von Inkubationszeit und absorbierter CGA Menge, *ex vivo* bestätigt werden. Folglich sind die interindividuellen Unterschiede in der **Physiologie** der **Probanden** ein **entscheidender Faktor** für die **Bioverfügbarkeit** der CGA. Es wurden **neuartige Metabolite** in den ilealen und renalen Ausscheidungen charakterisiert und somit ein intensiver Metabolismus der CGA. Beispielsweise wurden CGA mit Kaffeesäureresten bevorzugt an Position 3´ sulfatiert, während CGA mit Ferulasäureresten renal zu einem größeren Anteil als Glucuronid ausgeschieden wurden. In der ilealen Flüssigkeit dominierten Sulfate beider struktureller Einheiten. Allerdings gewann der Anteil der CGA-Glucuronide mit größerer, verzehrter Kaffee Dosis an Bedeutung.

Es wurde beobachtet, dass der Verzehr einer hohen Dosis CGA durch Kaffee die gastrointestinale Transitzeit beeinflussen kann und dadurch die CGA Absorption *in vivo* beeinflusst wird. Dennoch konnte durch Dosis-Wirkungs-Studien gezeigt werden, dass **unterschiedliche CGA**- **und QA**-**Dosen** aus Kaffee nur einen **geringen Einfluss auf die Absorption** im oberen Gastrointestinaltrakt haben. Dies konnte ebenfalls ***ex vivo***, durch eine **nicht sättigbare Absorption** (linearen Dosis-Fluss) von 5-CQA, welches auf eine **passive Diffusion** als Haupt-Mechanismus der Absorption hinweist, bestätigt werden.

Durch die intensive Biotransformation der CGA im menschlichen Körper, nach dem Verzehr von Kaffee, konnte nur bedingt eine Aussage über den Einfluss der molekularen Eigenschaften auf die Absorption getroffen werden. Allerdings konnte *in vivo* keine diCQA, aber zum ersten Mal signifikante Mengen von FQA, CQA und QA in Plasma und Urin erfasst werden. Diese Beobachtung wurde *ex vivo* im Ussing-Kammer-model bekräftigt. Die einzelnen Untergruppen der CGA wurden unterschiedlich durch die Mukosa des Schweine-jejunums transportiert, in aufsteigender Reihenfolge: diCQA, Spuren; CQA ≈ 1%; CA ≈ 1.5%; FQA ≈ 2%; und QA ≈ 4%. Innerhalb der CGA-Untergruppen wurden keine Unterschiede beobachtet. Somit kamen wir zu dem Schluss, dass die **Absorption** der CGA und QA durch

deren **physikalisch-chemischen Eigenschaften** bestimmt wurde. Weiterhin identifizierten wir eine aktive **Sekretion** der 5-CQA im Jejunum des Schweins *ex vivo* und postulieren eine aktive Sekretion für weitere CGA und deren hydrophilen Metabolite wie CQA-Sulfate oder FQA-Glucuronide, welche die gesamte Bioverfügbarkeit der CGA verringern könnte.

Im Verlauf dieser Arbeit konnte der Einfluss verschiedener Faktoren auf die Bioverfügbarkeit der CGA aus Kaffee aufgezeigt werden. Der Verzehr von zunehmenden Mengen CGA mittels Kaffee hatte geringen Einfluss auf die orale Bioverfügbarkeit in Ileostomie-Probanden. Dies wurde *ex vivo* durch eine passive Diffusion als bestimmenden Mechanismus der Absorption bestätigt. Folglich wirkt sich der Verzehr hoher Mengen CGA durch die tägliche Ernährung nicht limitierend auf deren Bioverfügbarkeit aus. Allerdings scheint die systemische Verfügbarkeit der CGA von den unterschiedlichen Physiologien der Probanden abzuhängen. Insbesondere der gastrointestinalen Transitzeit. Ebenso wie von den unterschiedlichen physikalisch-chemischen Eigenschaften der einzelnen CGA Verbindungen. Somit ergibt sich eine unterschiedliche Bioverfügbarkeit aus Lebensmitteln mit gleichem Gehalt an CGA aber einem unterschiedlichen CGA Profil.

1 Introduction and motivation

Coffee is a popular beverage especially in western countries and it is consumed for its stimulating effects and aromatic flavour. Furthermore, coffee is one of the most consumed beverages worldwide, with increasing consumption (IOC, 2012a). In 2011 up to 149 L per person was consumed in Germany (Kaffeeverband, 2012).

In the past coffee consumption was associated with harmful effects on human health whereas recent epidemiological investigations associated long-term effects of coffee consumption with a reduced risk of type II diabetes, neurodegenerative-, cardiovascular- and certain cancer disorders (Michels et al., 2005; Larsson and Wolk, 2007; Powers et al., 2008; Tunnicliffe and Shearer, 2008; Bravi et al., 2009; Eskelinen et al., 2009; Wu et al., 2009). Coffee is a complex mixture with more than 1000 compounds generating the unique taste and aroma (Hatzold, 2012a). Numerous bioactive compounds such as caffeine, CGA and coffee diterpenes may be associated with the described epidemiological health effects. As *in vitro* and *in vivo* studies observed positive acute effects of CGA such as antioxidant activity (Natella et al., 2002) and an activation of enzymes involved in detoxification and antioxidant defense (Boettler et al., 2011), this molecular class from coffee will be focused on here.

CGA are a subclass of polyphenols and are predominantly esters of D-(-)-quinic acid (QA) (**10a**[1]) with either ferulic acid (FA) (**7a**) forming feruloylquinic acids (FQA) or caffeic acid (CA) (**5a**) forming caffeoylquinic acids (CQA) (Manach et al., 2004). CGA shows a wide distribution among edible plants but coffee is the richest source of CGA in the western diet. A common coffee (200 mL) serves between 70 and 350 mg CGA (Clifford, 1999; Farah and Donangelo, 2006). As coffee drinkers are normally consuming coffee several times daily over a decade, coffee is an interesting mediator for the bioactive CGA. Taking the consumption of CGA from coffee into account with the reported health effects, the understanding of CGA concentration at the site of molecule action in the human body is of decisive relevance. In detail, CGA composition in consumed coffee and bioavailability data of CGA such as absorption

[1] **Bold numbers in brackets are chemical structures of CGA being of relevance in this thesis (see chapter 8).**

in the gastro intestinal mucosa, the subsequent metabolization and distribution in the body are of interest. Controversial data on the total bioavailability of these compounds has been generated in various human intervention studies with healthy volunteers or volunteers with an ileostomy (without a colon), mostly by single dose consumption with various study designs summarized by (Olthof et al., 2001; Kahle et al., 2007; Stalmach et al., 2010; Hagl et al., 2011; Williamson et al., 2011). The dosage form (such as different CGA composition from various food matrices) or dose were different in these studies. These are factors that can have an important influence on the oral bioavailability of molecules (Chereson, 1996; El-Kattan and Varma, 2012).

We pose the question if the total bioavailability in the upper GIT and colonic availability of CGA and QA from coffee is affected by consumption of different doses? Especially, QA bioavailability as this has not been considered up to now.

Moreover, an influence of different ingested CGA compositions from various food matrices on oral bioavailability might be possible since the physico-chemical properties of a molecule can influence its oral bioavailability (Lipinski et al., 1997). Due to the extensive metabolism, hydrolyzation and interesterification of CGA in the human body (Kahle et al., 2005; Stalmach et al., 2009), an influence of individual physico-chemical properties of CGA could not be detected *in vivo*. Up to now only a few *in vitro* and *ex vivo* CGA bioavailability studies have investigated such a structure-absorption relation (SAR) of CGA (Konishi and Kobayashi, 2004; Deußer, 2010; Farrell et al., 2011), but no SAR data of the major coffee CGA is available so far.

2 State of knowledge

2.1 Coffee

The seeds (beans) of the coffee tree which belongs to the Rubiaceae family, genus *Coffea*, are used to prepare a beverage known as coffee (Eisenbrand and Schreier, 2005; Farah, 2012). This beverage has been the most consumed one in the world for decades. Therefore, coffee is one of the most important primary products in world trade. Approximately eight million tons (representing 500 billion cups or 134 million coffee bags) were produced worldwide in 2010 (IOC, 2012a) with an increasing production over the last five years. Countries with the highest production are Brazil (48 million bags), Vietnam (18 million bags), Indonesia (9.5 million bags) followed by Colombia (9 million bags) (IOC, 2012a). The reasons for this popularity are the unique aromatic taste, stimulating effects and most recently the changed image of coffee as health benefits from long-term coffee consumption have been observed (see chapter 2.3.) (Hatzold, 2012a).

The production of coffee from the coffee fruit to cup is a complex task involving several steps (Hatzold, 2012b). First of all, the production of coffee starts with cultivation and harvesting the coffee fruits (coffee cherry) containing the coffee beans. In order to extract the green coffee beans from the cherries the pulp and skin has to be removed by a dry or wet extraction method (Crozier et al., 2009; Hatzold, 2012b). After extraction the green beans are ready for roasting, but as 10% of the produced coffee is for decaffeinated consumption a part of the produced green beans is treated with an organic solvent (such as dichloromethane or ethyl acetate) and water for caffeine removal.

During the roasting process at maximum temperatures of + 210 to + 240°C coffee beans change color to almost black and gain their characteristic aroma and flavor by chemical changes of bean constituents (see chapter 2.1.2 and 2.2.6.2). According to the roasting degree the color of coffee varies from light to very dark. The roasted coffee beans were ground and marketed or used for instant coffee production.

Typically instant coffee production follows a treatment of ground-roast coffee with hot water and pressure for extraction of water-soluble compounds. This kind of brew is then cooled, sometimes centrifuged and dried by a freeze-drying or spray-drying process.

For the brewing of coffee hot water is used normally at a temperature of + 90 to + 95°C with various brewing methods such as simple percolation, boiled coffee or espresso machine.

More than 80 coffee species have been identified, but only *Coffea Arabica* also known as Arabica coffee (approximately 70% of trading volume) and *Coffea canephora* also known as Robusta coffee (approximately 30% of trading volume) are economically important (IOC, 2012b).

2.1.1 Chemical composition of green coffee

The green coffee bean is the processed seed from the coffee cherry (Crozier et al., 2009). Its chemical composition is dominated by carbohydrates and fiber, proteins, lipids, minerals and chlorogenic acids (CGA) (see Table 2-1) (Farah, 2012). CGA and other minor components present in green coffee beans such as caffeine, trigonelline, soluble fiber and diterpenes (cafestol and kahweol from the lipid fraction) are probably bioactive compounds (see Table 2-1) (Farah, 2012).

Table 2-1: Chemical composition of green coffee [in g*100 g^{-1}] beans from *Coffea Arabica* and *Coffea Canephora*, copied from (Farah, 2012).

	Concentration [g*100 g^{-1}]	
Compounds	*Coffea arabica*	*Coffea canephora*
Carbohydrates/fiber		
Sucrose	6.0 - 9.0	0.9 - 4.0
Reducing sugars	0.1	0.4
Polysaccharides	34 - 44	45 - 55
Lignin	3.0	3.0
Pectin	2.0	2.0
Nitrogenous compounds		
Protein/peptides	10.0 - 11.0	11.0 - 15.0
Free amino acids	0.5	0.8 - 1.0
Caffeine	0.9 - 1.3	1.5 - 2.5
Trigonelline	0.6 - 2.0	0.6 - 0.7

Compounds	Concentration [g*100 g^{-1}] *Coffea arabica*	*Coffea canephora*
Lipids		
Coffee oil (triglycerides with unsaponifiables, sterols, tocopherols)	15.0 - 17.0	7.0- 10.0
Diterpenes (free and esterified)	0.5 - 1.2	0.2 - 0.8
Minerals	3.0 - 4.2	4.4 - 4.5
Acids and Esters		
Chlorogenic acids	4.1 -7.9	6.1 - 11.3
Aliphatic acids	1.0	1.0
Quinic acid	0.4	0.4

2.1.2 Chemical composition of roasted coffee and coffee brew

During coffee bean roasting the bean composition changes and new compounds and a complex aroma are formed due to pyrolysis, Strecker degradation and Maillard reactions. The roasting process is performed at a temperature of + 230°C for a few minutes or alternatively for + 180°C up to approximately 20 min (Crozier et al., 2009). The final composition of compounds depends on factors such as raw material, roasting degree, roasting type, time, temperature and air-flow speed in the roasting chamber (Farah, 2012). Melanoidins, proteins, lipids and reducing sugars such as arabinogalactan are the major non volatile compounds in the roasted coffee bean (see Table 2-2).

Table 2-2: Chemical composition of non volatile compounds in roasted coffee [in g*100 g^{-1}] beans from *Coffea Arabica* and *Coffea Canephora,* copied from (Farah, 2012).

	Concentration [g*100 g^{-1}]	
Compounds	*Coffea arabica*	*Coffea canephora*
Carbohydrates/fiber		
Sucrose	4.2	1.6
Reducing sugars	0.3	0.3
Polysaccharides (arabinogalactans, mannans, and glucans)	31 - 33	37
Lignin	3.0	3.0
Pectins	2.0	2.0
Free amino acids	ND	ND
Caffeine	1.1 - 1.3	2.4 - 2.5
Trigonelline	0.2 - 1.2	0.3 - 0.7
Nicotinic acid	0.016 - 0.026	0.014 - 0.025
Lipids		
Coffee oil (triglycerides with unsaponifiables)	17.0	11.0
Diterpene esters	0.9	0.2
Minerals	4.5	4.7
Acids and Esters		
Chlorogenic acids	1.9 - 2.5	3.3 - 3.8
Aliphatic acids	1.6	1.6
Quinic acid	0.8	1.0
Melanoidines	25	25

ND = not detected

More than 950 volatile compounds have been identified in roasted coffee. These volatile compounds can be divided into the following classes: furans and pyrans, pyrazines, pyrroles, ketones and phenols, hydocarbons, alcohols, aldehydes, acids

and anhydrides, esters, lactones, thiophenes, oxazoles, thiazoles, pyridines, amines and various sulfur and nitrogen compounds (Farah, 2012).

According to different coffee preparation conditions the amount of roasted been components in the coffee brew varies. These factors are: grid, brewing method, proportion of coffee to water, hardness and temperature of water, length of time coffee is in contact with water and the filter material (Farah, 2012). Several compounds pass into the brew such as CGA, caffeine, trigonelline, nicotinic acid, minerals and polysaccharides (predominantly galactomannans and type II arabinogalactans) (see Table 2-3). Whereas most of the lipid fraction remains in the solid material after brewing (Farah, 2012).

Table 2-3: Representive chemical composition of coffee brew in [mg*100 mL^{-1}], copied from (Farah, 2012).

Compounds	Concentration
Caffeine	50 - 380
Chlorogenic acid	35 - 500
Trigonelline	40 - 50
Soluble fiber	200 - 800
Protein	100
Lipids	0.8
Minerals	250 - 700
Niacin	10
Melanoidins	500 – 1,500
Volatiles	ND

ND = not detected

Due to inappropriate harvesting, weather conditions during primary processing, or improper storage of coffee, the brew can contain minor incidental constituents such as ochratoxin A (a mycotoxin), biogenic amines (*e.g.* putrescine, spermindine), *β*-carbolines (*e.g.* harman, norharman), acrylamide and polycyclic aromatic hydrocarbons (*e.g.* Benzo[*α*]pyrene) (Farah, 2012).

2.2 Chlorogenic acids (CGA), esters of hydroxycinnamic acids (HCA) and D-(-)-quinic acid (QA)

2.2.1 Chemical class and nomenclature

Chlorogenic acids (CGA) are part of the phenolic acid family which is a member of the widespread chemical structure of polyphenols (several hydroxyl groups on aromatic rings) (Manach et al., 2004). In the past CGA were recognized as an ester of D-(-)-quinic acid (QA) (**10a**) and the hydroxycinnamic acid (HCA) caffeic acid (CA) (Clifford, 1999). Up to now it is known that several cinnamic acids are conjugated with QA (Clifford, 1999). Classically, CGA is a family of esters formed of *trans*-cinnamic acid with one of the hydroxyl groups of QA (1L-1(OH),3,4,5-tetrahydroxycyclohexane carboxylic acid, according to IUPAC (IUPAC, 1976)), which has axial hydroxyls on carbons 1 and 3 and equatorial hydroxyls on carbons 4 and 5 (Clifford, 1999) (Figure 2-1).

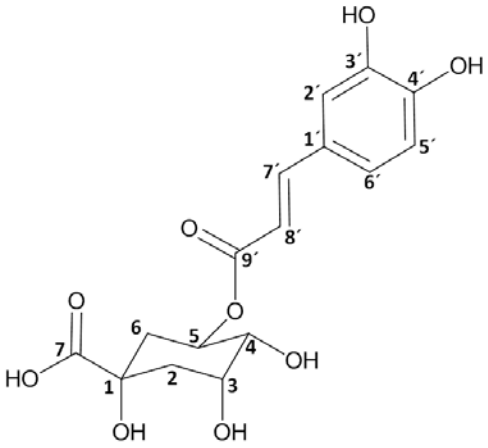


Figure 2-1: Chemical structure of 5-caffeoylquinic acid (1d), an ester of D-(-)-QA (10a) and the hydroxycinnamic acid caffeic acid (5a); with IUPAC numbering (IUPAC, 1976).

CGA can be divided into different subgroups depending on the number and chemical structure of cinnamic acids at the QA molecule. Clifford et al. is subdividing four groups of “classical” CGA (Clifford, 1999):

- mono esters of cinnamic acids, *e.g*. caffeoyl- (CQA (**1a-d**)), feruloyl- (FQA (**3a-c**)) and *p*-coumaroylquinic acid esters (*p*-CoQA)
- di-esters, tri-esters and tetra-esters, *e.g*. diCQA (**4a-c**), triCQA and tetraCQA
- mixed di-esters of caffeic- (CA) and ferulic acid (FA), *e.g*. CFQA

- mixed esters with varying number of CA and a dibasic aliphatic acid, *e.g.* succinic acid

Chemical structures of CGA being of relevance in our investigation are shown in chapter 8, Overview of chemical structures.

2.2.2 Physico-chemical properties of CGA

Several reactions of CGA depending on their physico-chemical properties are described in literature. The pH has a major influence on CGA properties.

For instance, an interesterification (acyl migration) is reported at a pH higher than 6 for mono esters or at the beginning of coffee roasting process. The cinnamic acid moiety is able to change its position in the space of the three hydroxyl positions, 3-, 4- and 5- at the QA molecule (see Figure 2-1) (Trugo and Macrae, 1984a; Farah et al., 2006).

Furthermore, the lipophilicity (log P) is affected by pH depending on the dissociated or non dissociated free carboxylic group of a quinic acid moiety or a free cinnamic acid. The microspecies distribution (dissociated-, non dissociated carboxylic group, according to Marvin-sketch 5.3.1) of the ionic or neutral form varies according to the pH (see Figure 2-2). For instance, log P of 5-CQA (**1d**) changes from - 0.27 in the neutral form (COOH) to - 3.8 in the dissociated ionic form (COO^-) (calculated with Marvin-sketch 5.3.1). This may play a pivotal role in a potential passive permeability of CGA across different GI compartments such as the stomach with a low pH in contrast to the small intestine with a higher pH (El-Kattan and Varma, 2012).

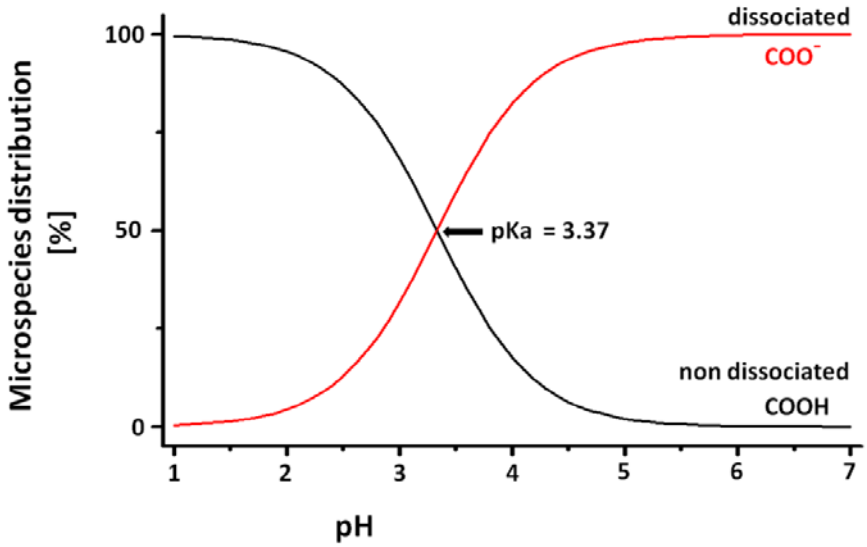


Figure 2-2: Graph of relationship between pH and the different microspecies (dissociated-, non dissociated carboxylic group (COOH)) of 5-caffeoylquinic acid (1d) (calculated with Marvin-sketch 5.3.1).

During processing of food total amount and chemical structure of CGA are affected. The high temperature of coffee roasting in particular leads to hydrolysis or formation of an intramolecular ester of CGA. While hydrolysis releases the esterified compounds (QA (**10a**) and cinnamic acid) (Scholz and Maier, 1990; Farah and Donangelo, 2006) lactonization forms an intramolecular ester of QA, the quinide (1,5-γ-quinolactone). This esterification takes place between the hydroxyl group at carbon 5 and the carboxylic group at carbon 1 of the QA molecule (see Figure 2-1) (Bennat et al., 1994; Farah et al., 2005a; Farah et al., 2005b).

During the roasting process CGA also contribute to the formation of polymeric compounds such as melanoidins in a covalent or non-covalent way (Nunes and Coimbra, 2010).

2.2.3 Biosynthesis of HCA and QA and its functions in plants

CGA are formed via the phenylpropanoid ($C_6 – C_3$ compounds) and shikimate ($C_6 – C_1$) pathway (Dixon et al., 2002; Crozier et al., 2009) as secondary metabolites induced by environmental stress. Stress factors could include mechanical wounding, infection by microbial pathogens, excessive UV or high visible light levels (Herrmann, 1995; Farah and Donangelo, 2006). Phenolic acids in higher plants are formed from L-phenylalanine and tyrosine via the shikimic acid pathway (Figure 2-3). Hereby

carbohydrate precursors, derived from glycolysis and pentose phosphate shunt (phospho-enolpyruvate and D-erythrose-4-phosphate) are synthesized into aromatic acids. A key enzyme in the biosynthesis of CGA is phenylalanine ammonialyase, which forms the parent *trans*-cinnamic acid from L-phenylalanine.

QA (**10a**) is formed by the shikimic acid pathway intermediate 3-dehydroquinate (Figure 2-3). Maybe on the basis of cinnamic acid hydroxylation or by the action of

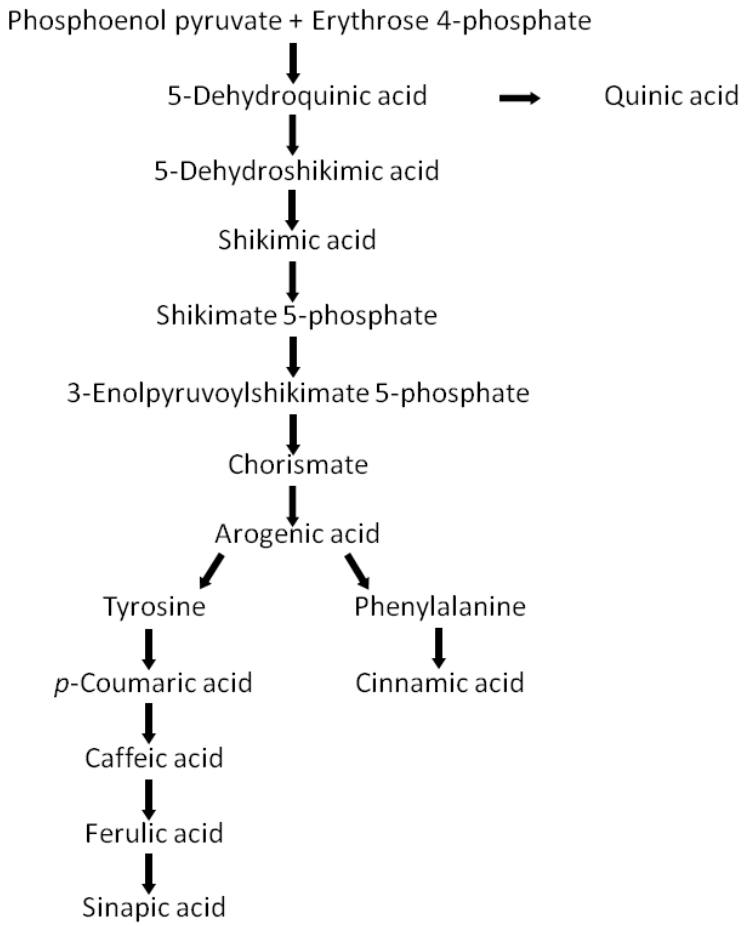


Figure 2-3: Biosynthesis of hydroxycinnamic acids (HCA) and quinic acid (QA) in plants via the shikimic acid pathway according to (Farah and Donangelo, 2006).

tyrosine aminolyase from tyrosine the hydroxycinnamic acids (CA (**5a**), FA (**7a**), *p*-CoA) are produced. Formation of the mono ester 5-CGA (5-CQA (**1d**), 5-FQA (**3c**), 5-*p*-CoQA) may start with binding of *trans*-cinnamic acid to coenzyme A (CoA) by a CoA lyase, followed by a transfer to quinic acid by cinnamoyl transferase (Gross, 1981; Farah and Donangelo, 2006). Cinnamic acids at positions 3- and 4- of the quinic acid moiety may be based on acyl migration (Gross, 1981; Farah and Donangelo, 2006). The functions of CGA in plants are numerous. Via specific

enzymes CGA can be transformed into polymerization products and lignin contributing to defense mechanisms and the synthesis of plant cell wall constituents (Mazzafera and Robinson, 2000; Takahama, 2004; Farah and Donangelo, 2006). High levels of CGA found in coffee seeds lead to the assumption that CGA have specific plant physiological functions (Farah and Donangelo, 2006). It may contribute to seed germination and cell growth, through regulation of indolacetic acid, a hormone being involved in plant growth (Clifford, 1985; Farah and Donangelo, 2006).

2.2.4 Occurrence in agricultural crops

HCA in the free form or esterified with QA (CGA) are extremely widespread in the plant kingdom (Clifford, 1999), summarized by (Neveu et al., 2010). A ranking of agricultural crops in terms of CGA and HCA content is difficult, due to different amounts in plants depending on seasonal influences (Manach et al., 2004).

Due to the positive health effects attributed to CGA consumption, plants which are consumed in large amounts and are rich in HCA and CGA are of high interest. For instance, a single cup of coffee may contain 70 – 500 mg CGA (Clifford, 1999; Farah, 2012). Plants such as fruits, vegetables and especially herbs are contributing in lower amounts to the daily CGA and HCA intake and are therefore of lower interest, despite the partially high HCA amounts (Crozier et al., 2009; Neveu et al., 2010). Fruits with the highest HCA contents are blueberries, kiwi, cherries, plum and apple with 0.5 - 2 g HCA per kg fresh weight. Amounts are summarized in Table 2-4.

Table 2-4: Hydroxycinnamic acid content (free or esterified compounds such as CGA) in selected foods, summarized by (Trugo and Macrae, 1984a; Clifford, 1999; Manach et al., 2004; Farah, 2012).

Source (serving size)	mg*kg^{-1} fresh wt (or mg*L^{-1})	mg per serving
Blueberry (100 g)	2,000 - 2,200	200 - 220
Kiwi (100 g)	600 - 1,000	60 - 100
Cherry (200 g)	180 - 1,150	36 - 230
Plum (200 g)	140 - 1,150	28 - 230
Eggplant (200 g)	600 - 660	120 - 132
Apple (200 g)	50 - 600	10 - 120
Pear (200 g)	15 - 600	3 - 120
Chicory (200 g)	200 - 500	40 - 100

Source (serving size)	mg*kg^{-1} fresh wt (or mg*L^{-1})	mg per serving
Artichoke (100 g)	450	45
Potato (200 g)	100 - 190	20 - 38
Corn flour (75 g)	310	23
Flour: wheat, rice, oat (75 g)	70 - 90	5 - 7
Cider (200 mL)	10 - 500	2 - 100
Coffee (200 mL)	350 - 1,750	70 - 500

Generally, HCA are bound as glycosylated derivatives or in esterified forms (quinic-, tartaric-, shikimic acid). Caffeic acid is the most abundant phenolic acid in free form as well as in esterified form, especially in coffee. Furthermore it represents 75% - 100% of the total HCA content of most fruits (Manach et al., 2004). CA can be found in all parts of fruits with the highest content in the outer parts of ripe fruits. The concentrations decrease during maturation, but total amounts increase with increasing size of fruit (Manach et al., 2004).

The main dietary source of ferulic acid (**7a**) is cereal grain with an estimated amount of ≈ 0.8 – 2 g per kg dry weight for wheat grain. This may represent up to 90% of total polyphenols (Sosulski et al., 1982; Lempereur et al., 1997; Manach et al., 2004). Rice and oat flours contain the same amount of FA (**7a**) as wheat flour (63 mg per kg), whereas the concentration of maize flour is three times as high (Shahidi and Naczk, 2003). Only 10% of ferulic acid is found in soluble free form in wheat bran, mostly esterified with arabinoxylans and hemicelluloses in the aleurone and pericarb (Lempereur et al., 1997).

2.2.5 Daily dietary burden of HCA and CGA

Due to missing biomarkers for HCA exposure an estimation of long-term intake is very difficult. Furthermore, data of HCA composition of commodities after processing, such as cooking, baking, etc. and consumption of these food throughout the world is inconsistent (Clifford, 1999; Manach et al., 2004).

Based on literature data and a seven day dietary protocol, Radtke et al. estimated a daily HCA intake of 211 mg in the Bavarian population (Radtke et al., 1998). In Japan dietary intake of HCA was estimated to be double for coffee drinkers (426 mg a day) (Fukushima et al., 2009). Different food content and especially different food preferences lead to high differences in HCA incorporation (Manach et al., 2004).

Thus, Clifford (Clifford, 1999) divided the UK population into three categories according to their food consumption. Coffee drinkers which have the highest dietary burden of CGA (500 – 800 mg), followed by people consuming bran and people consuming mainly citrus. Those who drink several cups of coffee, consume bran and consume citrus might have an intake of 1 g HCA a day, in contrast to people consuming none or low amounts of these foods. HCA intake of these people is estimated to be 25 mg a day (Clifford, 1999).

2.2.6 CGA profile and amount of coffee

As coffee is reported to be a major HCA dietary source being usually consumed daily and lifelong by coffee drinkers, the precise CGA profile and amount of this beverage is of high interest.

2.2.6.1 CGA composition of green coffee beans

In green coffee beans CGA are the dominating fraction of phenolic acids and have been studied for more than a century (Clifford, 1979; Trugo and Macrae, 1984b; Farah and Donangelo, 2006). The values of CGA on dry matter based (see Table 2-5) in green coffee beans is affected by many factors (species, cultivar, degree of maturation, agricultural practices, soil, climate) and varies from 4.0 to 8.4% for *Coffea Arabica* and 7.0 to 14.4% for *Coffea Canephora* (so-called, Robusta), summarized by (Farah and Donangelo, 2006).

The dominating part is the esterified HCA, especially the CGA: CQA (3-CQA (**1b**), 4-CQA (**1c**), 5-CQA (**1d**)); FQA (3-FQA (**3a**), 4-FQA (**3b**), 5-FQA (**3c**)); diCQA (3,4-diCQA (**4a**), 3,5-diCQA (**4b**), 4,5-diCQA (**4c**)), *p*-CoQA (3-*p*-CoQA, 4-*p*-CoQA, 5-*p*-CoQA) and six mixed diesters of CFQA (Clifford et al., 2003). The 5-CQA (**1d**) isomer is by far the dominating CGA accounting for 56 – 62% of the total (Farah and Donangelo, 2006). Additionally, the described CGA compounds were detected in Robusta coffee in trace amounts, *e.g*. diferuloylquinic-, dimethoxycinnamoylquinic-, caffeoyl-dimethoxycinnamoylquinic- and feruloyl-dimethoxycinnamoylquinic acid (Clifford et al., 2006).

Table 2-5: Chlorogenic acid (CGA) profiles and contents of green coffee beans expressed in [g % dry matter basis], summarized by (Farah and Donangelo, 2006). CQA = caffeoylquinic acid (1a-d); FQA = feruloylquinic acid (3a-c); diCQA = dicaffeoylquinic acid (4a-c).

Samples	CQA	FQA	diCQA	Total CGA	References
C. arabica					
C. arabica	5.76	0.25	0.87	6.88	(Trugo and Macrae, 1984b)
var. Caturra	4.63	0.33	0.66	5.62	(Clifford and Ramirez-Martinez, 1991)
Wild*	3.26	0.19	0.60	4.10	(Ky et al., 2001)
Angola	4.30	0.57	1.23	6.10	(Correia et al., 1995)
Brazil	4.20	0.28	0.77	5.25	(Farah et al., 2005b)
C. canephora					
cv. Robusta	6.82	0.60	1.37	8.80	(Trugo and Macrae, 1984b)
cv. Robusta	5.33	0.79	1.05	7.17	(Clifford and Ramirez-Martinez, 1991)
Wild*	7.66	1.43	2.31	11.3	(Ky et al., 2001)
cv. Robusta	3.43	0.54	1.20	6.08	(Correia et al., 1995)
var. Robusta	5.77	0.47	1.34	7.58	(Farah et al., 2005b)

*average

Besides the dominating cinnamoylquinic acid conjugates, a second group of HCA with amino acid conjugation such as tryptophan and tyrosine conjugates was identified in green coffee beans summarized by (Farah and Donangelo, 2006). Furthermore, a small part of phenolic glycosides were estimated in the past (Clifford, 1985). Additionally, the free HCA (caffeic- (**5a**), ferulic- (**7a**), *p*-coumaric acid) and quinic acid (**10a**) were found in only minor amounts in the green coffee beans (Clifford, 1985; Farah and Donangelo, 2006). Compounds different to CGA have also been identified in green coffee beans in small amounts: anthocyanidins (cyanidins, pelargonidines, and one peonidin) as a residue of the red skin fruit, lignans (secoisolariciresinol, lariciresinol, matairesinol, pinoresinol) as summarized by (Clifford, 1985; Farah and Donangelo, 2006).

2.2.6.2 CGA composition of roasted coffee and coffee beverages

During roasting of green coffee beans the amount and profile of CGA dramatically changes. With every 1% loss of coffee bean dry matter there is a simultaneous reduction of 8 - 10% of the total CGA amount (Crozier et al., 2009). Especially, interesterification (acyl-migration), epimerization, hydrolysis, lactonization and incorporation into melanoidins occur during roasting (see chapter 2.2.2). Total CGA content in commercial roasted coffees ranges from 0.5 up to 7% (Table 2-6), depending on the type of processing, degree of roasting and coffee blend (Farah and Donangelo, 2006).

Due to interesterifications during the roasting process, the amount of dominating 5-CQA (**1d**) decreases whereas the 3- (**1b**) and 4-CQA (**1c**) substitutes substantially increases (Trugo and Macrae, 1984b; Farah et al., 2005b).

A further consequence of the roasting process is the formation of lactones. About 7% of CGA in regular Arabica coffee and about 5.5% in Robusta coffee is transformed to a quinide (1,5-γ-quinolactone) (Table 2-6) (Farah and Donangelo, 2006). The major quinide formed is the 3-caffeoylquinide (3-CQL, (**2a**)) followed by the 4-caffeoylquinide (4-CQL, (**2b)**) (Farah et al., 2005b). Other quinides are present only in minor levels (Table 2-6). Via thermal syn-elimination further low molecular degradation products of CGA are formed, *e.g.*: catechol, 4-ethylcatechol, 4-methylcatechol, pyrogallol and hydroxyhydroquinone (Muller et al., 2006; Farah, 2012).

Table 2-6: Chlorogenic acid profiles of ground roasted and instant coffees expressed in [g % dry matter basis], summarized by (Farah and Donangelo, 2006). CQA = caffeoylquinic acid (1a-d); FQA = feruloylquinic acid (3a-c); diCQA = dicaffeoylquinic acid (4a-c); CQL = caffeoylquinide (2a-b); FQL = feruloylquinide; diCQL = dicaffeoylquinide; CoQL = Cumaroylquinide.

Coffee samples	CQA	FQA	diCQA	CQL	FQL	diCQL	CoQL	References
Ground roasted:								
Commercial	1.62			0.23				(Bennat et al., 1994)*
	2.26	0.21	0.19	0.31				(Schrader et al., 1996)*

Coffee samples	CQA	FQA	diCQA	CQL	FQL	diCQL	CoQL	References
	0.94	0.15	0.14					(Monteiro and Trugo, 2005)*
C. arabica[a] Bourbon	2.15	0.17	0.14	0.36	0.04	0.01	0.01	(Farah et al., 2005b)
C. arabica[a] Longberry	1.65	0.15	0.13	0.33	0.04	0.01	0.01	(Farah et al., 2005b)
C. arabica	3.23			0.32				(Bennat et al., 1994)
C. canephora[a] cv. robusta	2.76	0.34	0.23	0.39	0.03	0.03		(Farah et al., 2005b)
Instant coffee:								
Non-decaffeinated	3.00	0.61	0.28					(Trugo and Macrae, 1984b)*
	1.90	0.21	0.07					(Nogueira and Trugo, 2003)*
	6.40	< 1	< 1					(Renouf et al., 2013)
Decaffeinated	4.03	0.72	0.23					(Nogueira and Trugo, 2003)*

* average; [a] Laboratory roast (light medium)

Extraction of coffee polyphenols during coffee preparation is affected by numerous factors like grind of coffee, proportion of coffee to water relation, brewing method, water temperature and contact time with water. Nevertheless, significant amounts are transferred into domestic brews and commercial soluble coffee powders (Crozier et al., 2009). During domestic brewing of coffee normally 80 – 100% of total CGA is extracted, resulting in 70 – 350 mg CGA per 200 mL cup of coffee (Clifford, 1999; Farah and Donangelo, 2006).

2.3 Health effects associated with coffee consumption (focus on CGA)

The lifelong daily exposure of food containing CGA, especially coffee and its potentially beneficial effects on human health generates a growing interest in them. Despite the numerous compounds in coffee, it is caffeine, diterpenes and the polyphenol fraction that are suggested to contribute to health effects, depending on

coffee preparation (Bonita et al., 2007). *In vitro* and *in vivo* studies considered acute health effects such as increasing plasma antioxidative capacity and decreasing LDL oxidizability after coffee consumption (Natella et al., 2002; Yukawa et al., 2004).
The coffee constituent quinic acid was not supposed to be bioactive in the past. But QA supports the synthesis of tryptophan and nicotinamide in the GI. This increased production is linked to DNA repair enhancement and NF-κB inhibition (Pero et al., 2009; Pero and Lund, 2011). Hence, it is suggested that QA consumption is associated with health benefits.
Nevertheless, some coffee constituents such as acrylamide or furan are associated with potential harmful effects on human health. However, these potential harmful effects could not be confirmed by epidemiological data on long term ingestion. Moreover, epidemiological studies reviewed a reduced risk for certain cancer-, cardio-vascular-, neurodegenerative- and Type II diabetes diseases.

2.3.1 Increasing plasma antioxidant activity

Due to the presence of its *π*-electron system the catechol group of HCA shows the ability to scavenge reactive oxygen species. Thus, oxidative degradation processes at biological structures are decelerated or hindered (Lemańska et al., 2001; Eisenbrand and Schreier, 2005). Stalmach et al. identified caffeoylquinic acids as the most abundant antioxidants in roasted coffee by HPLC analysis and additionally supposed a contribution of Maillard reaction products to the coffee antioxidant activity (Stalmach et al., 2006). Also catabolites of CA (**5a**) such as DHCA (**6a**) showed an antioxidant capacity *in vitro*, summarized by (Bonita et al., 2007).
The *in vivo* antioxidant capacity of coffee was investigated after the consumption of a single dose of coffee showing a significant increase of plasma antioxidant activity (Natella et al., 2002) and in a short term study (5 cups a day) showing a significant increase of plasma glutathione (16%) (Esposito et al., 2003). Bakuradze and coworkers identified decreased DNA oxidative damage and an elevated glutathione level as well as glutathione reductase activity after consumption of a coffee rich in green bean and roasted coffee constituents (Bakuradze et al., 2011).
Nevertheless, low CGA levels reaching the circulatory system implies that its mechanisms of action might be beyond the modulation of oxidative stress (Crozier et al., 2009).

Potential molecular targets and corresponding cellular signaling pathways contributing to chemo preventive health effects have been recently identified *in vitro* and *in vivo* (Volz et al., 2012). More specifically, coffee constituents such as 5-CQA are activators of the NF erythroid 2-related factor (Nrf) protein (Boettler et al., 2011). This protein induces the antioxidant response element (ARE) mediated expression of Phase II enzymes involved in detoxification and antioxidant defense (Venugopal and Jaiswala, 1998).

2.3.2 Cardiovascular disease (CVD) and coffee consumption

An indication of positive effects on the cardiovascular system for coffee drinkers has long been suggested (Bidel and Tuomilehto, 2012). Nevertheless, existing literature is inconsistent. A protective heart mechanism was found in healthy Japanese males after coffee consumption (3 cups a day) as LDL oxidizability significantly decreased and returned to baseline after cessation of coffee (Yukawa et al., 2004). Moreover, Bonita et al. associated the coffee polyphenols with cardiovascular benefits via lowering blood pressure (Bonita et al., 2007). In a recent meta analysis of 21 prospective cohort studies the coronary heart disease risk by coffee consumption was tested and an increased long term risk, as previously assumed, was not observed (Wu et al., 2009). In addition, habitual coffee consumption was correlated with a risk decrease of 18% in female and 13% in male subjects. Positive effects on cardiovascular health were corroborated as well as by other epidemiological and cohort studies (Greenberg et al., 2008; Mukamal et al., 2009).

Regarding the compounds from coffee responsible for the described effects, caffeine and CGA are in focus. Caffeine has acute cardiovascular effects such as increasing blood pressure, circulating catecholamine levels, arterial stiffness and endothelium dependent vasodilation (Smits et al., 1985; Mahmud and Feely, 2001; Papamichael et al., 2005), but it is also associated with the development of CVD (Bidel and Tuomilehto, 2012). *In vitro* CA was shown to inhibit LDL oxidation (Nardini et al., 1995) and CGA stabilized membranes and improved the cell energy status in a study on cardiomyocytes (Chlopčíková et al., 2004).

2.3.3 Lower risk of Type II diabetes disease

Recent reviews highlighted a dose-response relationship between coffee consumption and a substantially lower risk of type 2 diabetes which is characterized by insulin resistance (Van Dam and Hu, 2005; Tunnicliffe and Shearer, 2008). Numerous physiological mechanisms are involved in this effect, such as a hindered glucose absorption in the gut and a reduced gluconeogenesis in the liver. It is assumed in epidemiological and *in vitro* studies that a coffee polyphenol fraction causes this anti diabetic effect (Tunnicliffe and Shearer, 2008). More specifically, the coffee quinides seemed to enhance the insulin activity in rats directly (Shearer et al., 2003; Shearer et al., 2007). 5-Caffeoylquinic acid is modulating carbohydrate absorption *in vitro* as inhibition of porcine *α*-amylase preparations (Stümpel et al., 2001; Rohn et al., 2002; Narita and Inouye, 2009; Matusheski et al., 2012) and inhibition of glucose-6-phosphatase (which might be important for carbohydrate absorption at the enterocyte) was observed.

2.3.4 Reduced cancer risk

Associations of coffee consumption with a reduced cancer risk is discussed as epidemiological studies suggest a reduced risk of liver, kidney, uterine, head, neck, breast and colorectal cancers (Michels et al., 2005; Larsson and Wolk, 2007; Bravi et al., 2009; Nkondjock, 2012). Several bioactive compounds from coffee can be associated with the lower cancer risk. The diterpenes from coffee namely cafestol and kahweol shows anticarcinogenic properties such as inducing phase II enzymes involved in carcinogen detoxification in animal and cell studies (Cavin et al., 2002; Huber et al., 2003) and inhibition of phase I enzymes responsible for carcinogen activation was observed in rat liver and primary hepatocytes (Cavin et al., 2008). The CGA fraction of coffee shows antioxidant effects (Rodriguez de Sotillo et al., 2006) and is associated with a lower risk of type II diabetes disease (see chapter above) which is a marker for certain cancer diseases (Renehan et al., 2008). Furthermore, CA inhibits DNA methylation in cultured human cancer cells (Vucic et al., 2008). DNA hypermethylation is a mechanism for silencing genes such as tumor-suppressor proteins, DNA-repair enzymes and receptors in tumor cells (Nkondjock, 2012).

2.3.5 Liver health

Beneficial effects on liver health has been observed according to coffee consumption with effects such as reducing blood levels of liver enzymes, potential benefits at risk for cirrhosis and hepatocellular carcinoma (La Vecchia, 2005; Muriel and Arauz, 2012). According to epidemiological data, coffee appears to have a moderate effect in reducing risk of cirrhosis as a relationship between coffee consumption and a reduced risk was observed (Gallus et al., 2002; Klatsky et al., 2006). The increase of plasma antioxidant capacity after coffee consumption as already described in chapter 2.3.1. may be responsible for a reduced risk of cirrhosis for coffee drinkers (Muriel and Arauz, 2012). Coffee is a complex mixture of compounds therefore, no definite evidence is available for an individual compound responsible for the described liver effects (Muriel and Arauz, 2012).

2.3.6 Neuroprotective effects of coffee consumption

Acute effects of coffee consumption on better cognitive function are well known, specially the effect of caffeine (Hatzold, 2012c). Furthermore, epidemiological evidence suggests that long term ingestion of coffee seems to have positive influences on a reduced risk of cognitive decline, dementia, and Alzheimer´s and Parkinson´s disease (Quintana et al., 2007; Tan et al., 2007; Powers et al., 2008; Eskelinen et al., 2009; Lindsay et al., 2012). Although several bioactive compounds are present in coffee, caffeine and CGA are specifically associated with the described effects (Lim and Tan, 2012; Lindsay et al., 2012).

2.3.7 Adverse health effects of coffee consumption

Some of the compounds from coffee, mostly formed during roasting such as furan and acrylamide, are also associated with potential harmful effects on human health. The total amount of these compounds is influenced by the type of roasting and preparation of the brew.

Furan in coffee is formed during roasting of beans (Guenther, 2012). Although the levels of furan are decreased during processing, coffee is an important source of furan for adults (Morehouse et al., 2008). In animal studies furan was shown to be carcinogenic to the liver of mice or rats (National Toxicology Program, 1993; Guenther, 2012), however, epidemiological studies revealed no evidence of a

possible carcinogenic effect in humans (Bakhiya and Appel, 2010) (see chapter 2.3.5.).

In 1994 acrylamide was classified as a group 2A carcinogen (“probably carcinogenic to humans”) based on data of genotoxictiy and carcinogenicity in rodents (IARC, 1994). Furthermore, Wilson and coworkers found an increased risk of endometrial cancer among high acrylamide consumers (Wilson et al., 2010). As well as furan the coffee contaminant acrylamide is also formed during roasting of coffee beans. The Maillard reaction is the major pathway for acrylamide formation during roasting with the precursor amino acid asparagine as a backbone of the acrylamide molecule (Stadler et al., 2002). Although the acrylamide levels drop approximately 2 min into roasting and during storage (Hoenicke and Gatermann, 2005) coffee is a rich source of acrylamide in the daily diet (World Health Organization, 2011).

Coffee is the most important source for exogenous heterocyclic ß-carboline alkaloids namely norharman and harman (Herraiz and Chaparro, 2006). About 4 to 20 µg per 100 mL were present in the coffee brew (Herraiz, 2002; Herraiz and Chaparro, 2006) since these compounds are formed during roasting by Pictet-Spengler condensation of indolethylamins and carbonylic compounds followed by oxidation and decarboxylation (Farah, 2012). An association of norharman and harman intake with effects on human health is inconclusive. However, adverse health effects have been suggested such as an involvement in addictions and *in vivo* genotoxicity (Herraiz, 2000; Farah, 2012).

Polycyclic aromatic hydrocarbons (PAH) are formed at high roasting temperatures. Hence, these compounds are mostly detected in very dark roasts (Orecchio et al., 2009; Farah, 2012). PAH such as benzo[α]pyrene or phenanthrene are carcinogens with low water solubility (Houessou et al., 2005). So the major parts present in roasted ground coffee are not present in the same amount (less than 35%) in the coffee brew (Houessou et al., 2007; Farah, 2012).

Not only the type of roasting but also the type of coffee preparation is important for potentially harmful components in the brew, because only boiled unfiltered coffee contains the diterpenes cafestol and kahweol in significant amounts. These diterpenes are associated with both positive effects (as already described above) and adverse effects. The diterpenes from coffee are also suggested to increase serum cholesterol and extracellular accumulation of LDL which promotes atherosclerosis (Bønaa et al., 1988; Rustan et al., 1997; Bonita et al., 2007).

2.4 Physiology of the gastrointestinal tract (GIT)

The GIT (digestive system) has various functions such as food ingestion, propulsion, digestion and absorption of nutrients, water and vitamins (Corwin, 2008). Due to the different compartments of the GIT with specialized conditions, such as enzyme concentration and corresponding pH, it is possible to degrade non absorbable large molecules to molecules which can be absorbed. Thereby location of absorption is dependent on factors such as the molecular properties, the different conditions in the compartments and by the anatomical structure of the compartment such as surface organization. Thus, physiological factors of the gastrointestinal compartments such as anatomy, pH, bacterial microflora, residence time of food and presence of transport proteins impact molecule absorption (El-Kattan and Varma, 2012).

2.4.1 The human GIT

2.4.1.1 Anatomy and function

The human GIT extends from the oral cavity to the anus. It is divided into the following different specialized compartments: oral cavity, esophagus, stomach, small intestine and large intestine. The outcomes of this compartmentalization are different physiological and chemical conditions along the GIT (Rehner and Daniel, 2002). Figure 2-4 gives a detailed schematic overview of the GIT structure.

2.4.1.2 Upper GIT

Once food has been ingested into the oral cavity its particle size will be reduced through the process of chewing and afterwards reaches the esophagus by swallowing. Here the bolus is transported via peristaltic contractions into the stomach. Hereby salivary secretion in the oral cavity facilitates the lubrication and stirs food with two digestive enzymes: *α*-amylase (cleaves internal *α*-1,4-glycoside bonds present in starch) and lingual lipase (hydrolysis of dietary lipid) (Johnson, 2007; Weisbrodt, 2007).

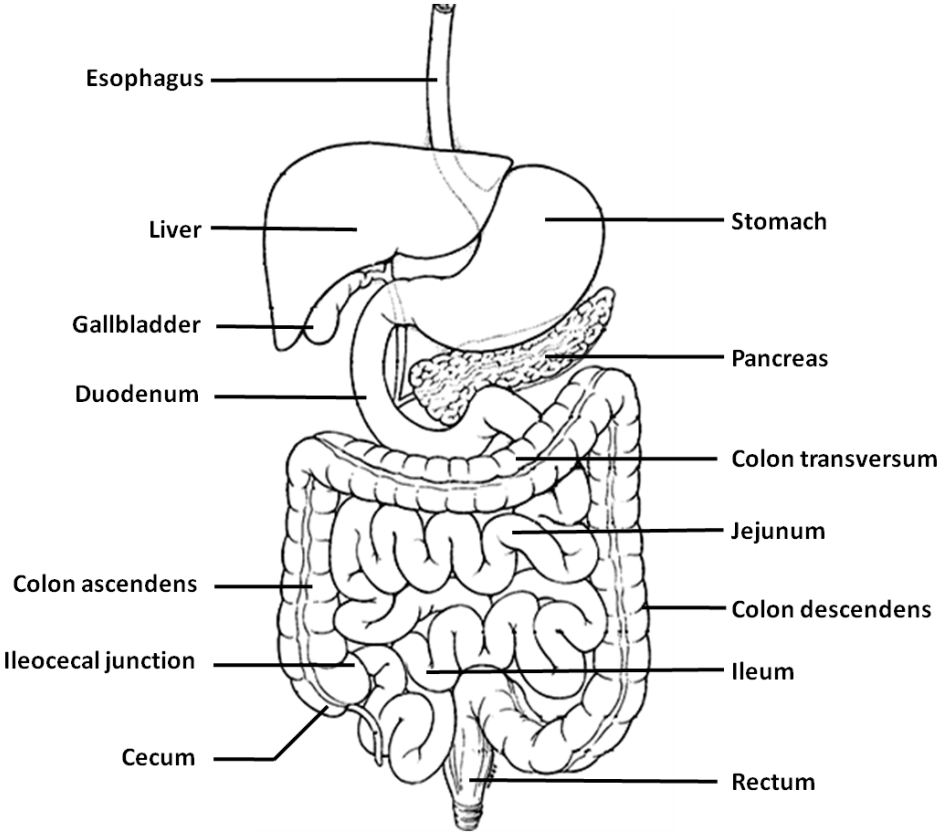


Figure 2-4: Schematic detailed overview of the human GIT (copied from (Corwin, 2008).

2.4.1.2.1 Stomach

In the stomach the bolus is stirred with gastric juice and particle size is decreased again. The major constituents of gastric juice are pepsin, mucus, hydrogen ions and the intrinsic factor. Despite the activity of saliva enzymes and the proteolytic activity of pepsin in the stomach, gastric digestion plays only a minor role in the degradation of macromolecules (Rehner and Daniel, 2002). In a fasted state, the pH of gastric fluid ranges from 1 to 3.5 and can rise in fed state up to 4.5 and higher (Fallingborg, 1999; Rehner and Daniel, 2002) (see Figure 2-5).

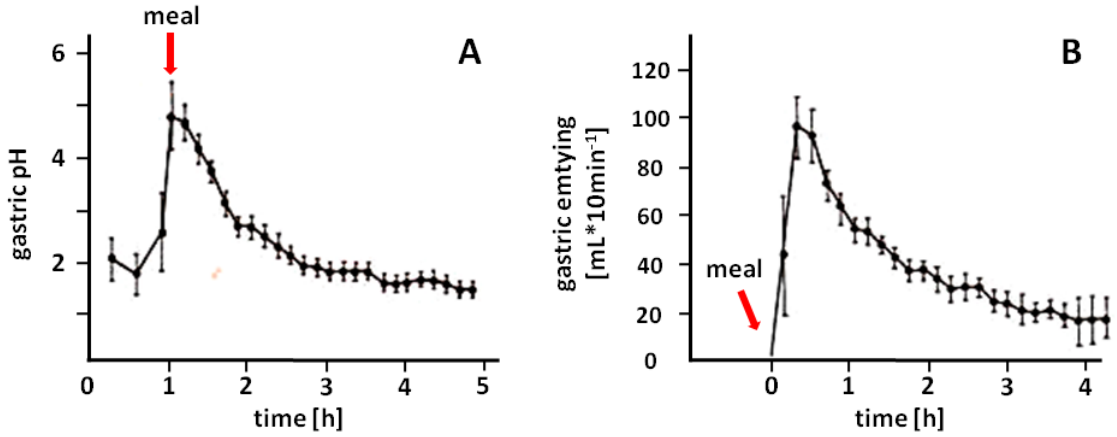


Figure 2-5: Changing of gastric pH (A) and gastric emptying rate (B), after consumption of a meal (400 mL, half viscous, pH 6, 40% carbohydrates, 40% fat, 20% protein). Modified according to (Malagelada et al., 1976).

Gastric emptying is accomplished by coordinated contractile activity of the stomach, pylorus and proximal small intestine (Weisbrodt, 2007). Solid food passes the stomach only after a reduction of its particle size which requires an adequate resistance time of such foods in the stomach. Only particles with 1 mm^3 or less are easily emptied by gastric motility, whereas liquids passes the stomach immediately (Weisbrodt, 2007) (see Figure 2-5).

Furthermore, the chemical composition of foods affects the rate of gastric emptying. For instance, foods that are high in lipids, H^+ or that differ considerably from isotonicity pass the stomach at a slower rate than observed for near-isotonic saline solutions (Rehner and Daniel, 2002; Weisbrodt, 2007). Receptors in the upper small intestine regulate the gastric emptying. These receptors respond to physical properties (osmotic pressure) and chemical composition (H^+, lipids) of the bolus (Weisbrodt, 2007). In addition to the type of meal, other factors such as body position, emotional state, and activity (exercise) can affect the rate of gastric emptying (Chereson, 1996).

Thereupon the motility is influencing the amount of bolus reaching the small intestine. More specifically, in a study which observed the gastric emptying time after consumption of different breakfasts, the following emptying times were reported: high carbohydrate 120 ± 75 min; high fat and protein content 580 ± 375 min; and 150 mL water 45 ± 110 min (measured by a radio capsule) (Zimmermann and Leitold, 1992).

2.4.1.2.2 Small intestine

Anatomically the small intestine extends from the gastroduodenal junction (pylorus) to the duodenum, followed by the jejunum to the ileum. It ends at the ileocecal junction (Figure 2-4). In the duodenum, pancreas and gallbladder effluent are excreted to the chyme. As a consequence of the hydrogen carbonate content of pancreas secretion and duodenal gland efflux, the pH is raised in the aboral direction of the small intestine (pH in fed state: duodenum 5.4, jejunum 5.2 – 6.0, ileum 7.5) (Hörter and Dressman, 2001; Rehner and Daniel, 2002).

Furthermore, pancreas effluent contains important enzymes for digestion, especially for carbohydrate digestion, proteolysis and lipolysis. The major function of the gallbladder is to emulsify the fat in the chyme by bile acids. These acids are produced by hepatocytes in the liver and are stored within the gallbladder until excretion. So the liver and gallbladder are also important for digestion because bile acids increase the absorption of fats and consequently high lipophilic drugs (El-Kattan and Varma, 2012). Additionally, once molecules are absorbed in the small intestine they can be transformed in the liver and excreted via the gallbladder into small intestine (enterohepatic circulation). More specifically, the liver plays a major role in detoxification and excretion of xenobiotics. Absorbed molecules undergo a biotransformation and can be excreted in the liver as well as in the small intestine itself which can lead to a limited oral bioavailability (see chapter 2.6.2.4) (Dietrich et al., 2003; El-Kattan and Varma, 2012).

The motility of the small intestine homogenizes the chyme with digestive fluids and enzymes, facilitating contact with the intestinal mucosa of the entire chyme and propulsion in an aboral direction (Weisbrodt, 2007). Thus, the small intestine is the part of the GIT where most digestion and absorption take place. For this, the small intestine has a specialized mucosa in order to maximize the intestinal absorptive surface area (Rehner and Daniel, 2002) with circular folds, villi and microvilli at the enterocyte. These structures create an absorptive surface area of 300 to 400 m^2 in the small intestine. In Figure 2-6 the anatomy and different tissue layers of the small intestine are shown. The villi and the tunica mucosa are situated at the luminal side of the small intestine, followed by the thin muscle layer of muscularis mucosa. The tela submucosa, the longitudinal and circular muscle layers of lamina muscularis follow which regulate the motility of the small intestine. The tunica serosa (a tissue nerved with blood vessels) completes the small intestine wall at the serosal side.

Besides the enterocytes, other specialized cells are occurring in the small intestine such as paneth and microfold cells.

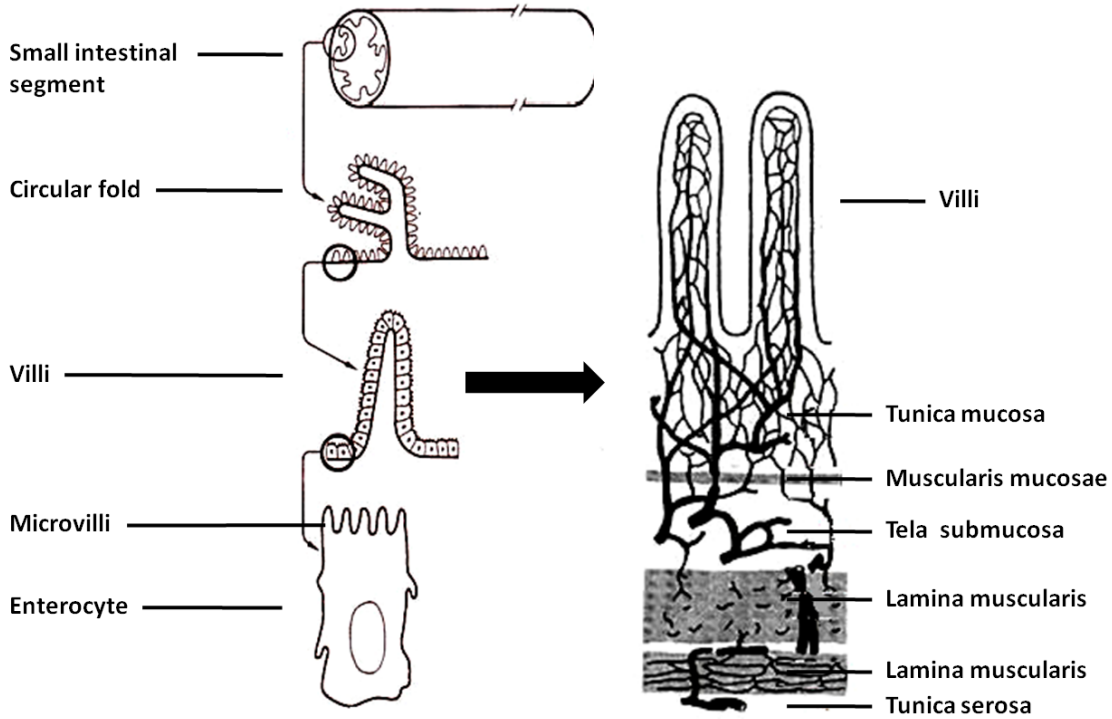


Figure 2-6: Anatomy and surface enlarging factors of the small intestine on the left and architecture of the different tissue layers with vasculature on the right. According to (Rehner and Daniel, 2002).

Furthermore, the small intestinal microbiota is also involved in the process of digestion, as shown by Knaup et al. investigating the degradation of several quercetin glycosides in the ileal fluid (Knaup et al., 2007). Colonization of microbiota in the small intestine varies within the different parts and increases from the duodenum with 10^3 cfu*mL^{-1} to the jejunum and ileum with 10^4 – 10^8 cfu*mL^{-1} (Blaut and Clavel, 2007) .

The total transit time of chyme in the small intestine varies from 2.8 up to 6.3 hours (Ibekwe et al., 2006). Hereby the flow from the small intestine to the large intestine is partly regulated by the ileocecal junction at the end of the small intestine (Weisbrodt, 2007).

2.4.1.3 Large intestine

The large intestine can anatomically be divided into the cecum, ascending-, transverse-, and descending colon and the rectum (Figure 2-4). The major function of the large intestine is to absorb water and electrolytes. In total the absorptive surface area of the large intestine is not important. Typical surface anatomy present in the small intestine such as villi and microvilli cannot be observed (Rehner and Daniel, 2002).

Because of the high amount of microbiota in the colon (10^9 – 10^{12} cfu*mL^{-1}(Blaut and Clavel, 2007)) it is a compartment where chyme compounds, which were not absorbed in the upper GIT, are fermented forming short chain fatty acids such as butyrate from non absorbed carbohydrates. The metabolic activity of these bacterial microflora can play a major role in liberation of drugs by hydrolysis, dehydroxylation, deamidation, decarboxylation, and reduction of acid groups (see chapter 2.6.2.1) (El-Kattan and Varma, 2012). The pHs of the colon are: cecum 5.7; ascending colon 5.6; transverse colon 5.7 and descending colon 6.6 (Fallingborg, 1999). Contractions organize the aboral movement of contents via the colon and evacuation of feces (Weisbrodt, 2007). A colonic transit time of 25 up to 30 hours is reported (Silbernagl and Despopoulos, 2003).

2.4.1.4 Unstirred water layer

Adjacent to the intestinal membrane an unstirred water layer is present with a thickness of 25 µm in humans. The effect of this polar unstirred water layer is insignificant on the extent of absorption, even on high lipophilic molecules (Chiou, 1994; El-Kattan and Varma, 2012).

2.4.2 Stoma of the terminal ileum

A stoma of the terminal ileum is a surgical resection of the colon, where a port between the ileum and the abdominal wall is created (Figure 2-7). The reasons for this surgery can be colorectal cancer or other bowl diseases (such as Crohn´s disease and *ulcerative colitis*). Via the artificial stoma the ileal effluent is flushed into a bag (external appliance connected with the terminal ileum).

After the resection the remaining part of the GIT undergoes an adaption since the ileostomy effluent decreases from 1.5 – 2.0 $L*d^{-1}$ within a few weeks after resection to about 0.4 - 0.6 $L*d^{-1}$ (Ladas et al., 1986; Fallingborg, 1999).

Different studies, summarized by (Fallingborg, 1999), demonstrate that a stoma does not affect the pH of the remaining gut, although small changes can be observed after surgery. For instance, the bacterial count in the neoterminal ileum is higher (10^5 up to 10^7 $cfu*g^{-1}$) than in healthy subjects (with normal gut) and the microbiota composition is sometimes different with higher aerobic and fewer anaerobic species present (Fallingborg, 1999). Furthermore, in ileostomists a possible slightly increased small intestinal transit time, when compared to normal subjects, was observed (Ladas et al., 1986; Fallingborg et al., 1990; Fallingborg, 1999).

The ileostomy model became a suitable tool for investigations of colonic availability of various compounds as well as for absorption studies focusing on the upper GIT and small intestine. Nevertheless, the above mentioned deviations have to be taken into account.

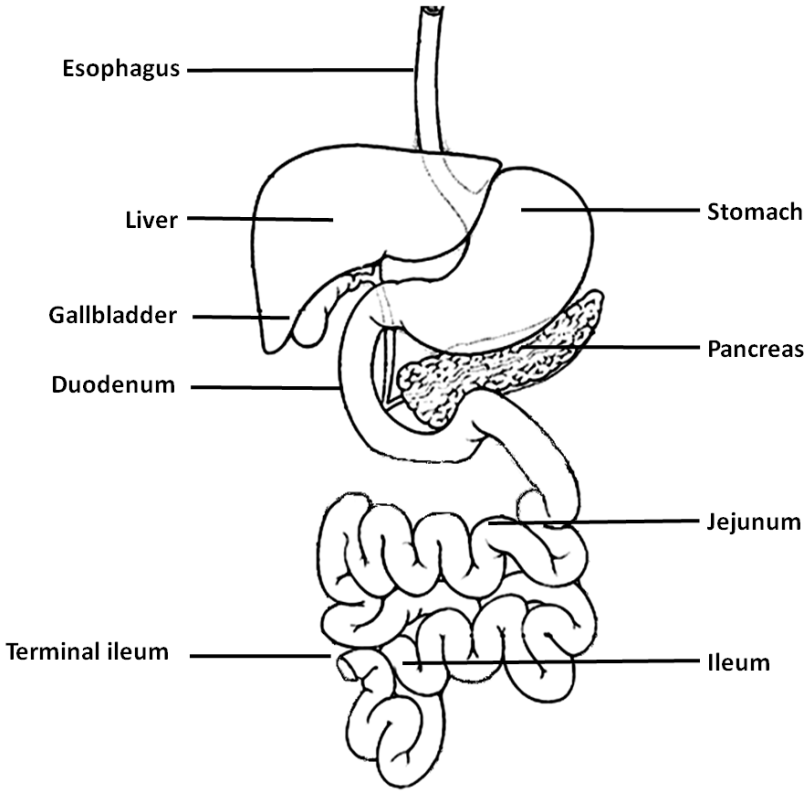


Figure 2-7: Schematic detailed overview of the human GIT with a stoma at the terminal ileum. The large intestine was resected by surgery. Modified according to (Corwin, 2008).

2.4.3 Mechanisms of absorption in the GIT

The selective permeability and surface area of the different GI membranes (located in the stomach, small intestine, and large intestine) regulate the absorption of molecules from the chyme. The characteristic of this barrier function is mostly governed by the properties of the lipid bilayer. According to the physico-chemical properties and the potential affinity to transport proteins of a molecule and the occurrence of specific transport proteins along the GIT, several mechanism of absorption are possible (Figure 2-8) (Rehner and Daniel, 2002; El-Kattan and Varma, 2012): (a) simple diffusion, (b) facilitated diffusion and (c) carrier mediated active transport. Overall, the absorption process through the cell membrane is divided into three partial events: entrance of substrates through the luminal cell membrane, traversion of a metabolic active intracellular space and exit through the serosal membrane into the blood (Daniel, 1986).

(a): Passive simple diffusion describes the molecule spread into an available space (*e.g.* intracellular space) through random motion (Brownian motion). It is driven by a concentration gradient and is leading to a concentration balance in both compartments at ideal conditions (net flux = 0).

So via the transcellular diffusion a spread from the luminal side (high molecule concentration) through the lipid bilayers to the serosal side (low molecule concentration) occurs. Furthermore, because of the lipid bilayer´s hydrophobicity this way of absorption is governed by the physico-chemical properties of the diffusing molecules. For instance, lipophilic molecules (*e.g.* benzene) diffuse very fast, followed by small uncharged polar molecules (*e.g.* H_2O, glycerine) and huge uncharged polar molecules (*e.g.* glucose). In contrast, for inorganic ions and charged organic molecules (charged amino acids) overcoming of the lipid bilayer is limited, because of its polar properties and the hydration shell. In summary, compounds which are absorbed via the transcellular pathway are unionized, with lipophilicity of Log P > 0 and MW > 300 $g*mol^{-1}$(Rehner and Daniel, 2002; El-Kattan and Varma, 2012).

Other than transcellular diffusion, paracellular diffusion can also take place (Figure 2-8). In cell membranes forming an impermeable barrier (such as enterocytes), tight junctions (TJ) connect cells and enclose the paracellular space. Thus the properties of TJ determine the passive diffusion of this transport process. They revealed a selective permeability for cations and became tighter in aboral direction. In general,

molecules that are small (*e.g.* MW < 250 g*mol^{-1}) and hydrophilic in nature (log P < 0) are absorbed via paracellular diffusion (Rehner and Daniel, 2002; El-Kattan and Varma, 2012).

(b): Equally to simple diffusion the facilitated diffusion leads to a concentration balance in both compartments. Due to the uptake of molecules showing adverse diffusion properties, several transport systems of facilitated diffusion are located in the membrane. These transport systems are crossing the lipid bilayer and facilitating permeability based on the reaction mechanisms of catalysis. The specialized membrane proteins are able to decrease the high activation energy which is necessary for passing the bilayer of *e.g.* polar or charged molecules (Figure 2-8).

(c) Whereas diffusion mechanisms are accompanied by an increase of entropy, active transport mechanisms can accumulate a molecule in one compartment. This means a decrease of entropy and therefore energy is required for the transport. So active transport can lead to an increased absorption of a drug (active uptake) or a

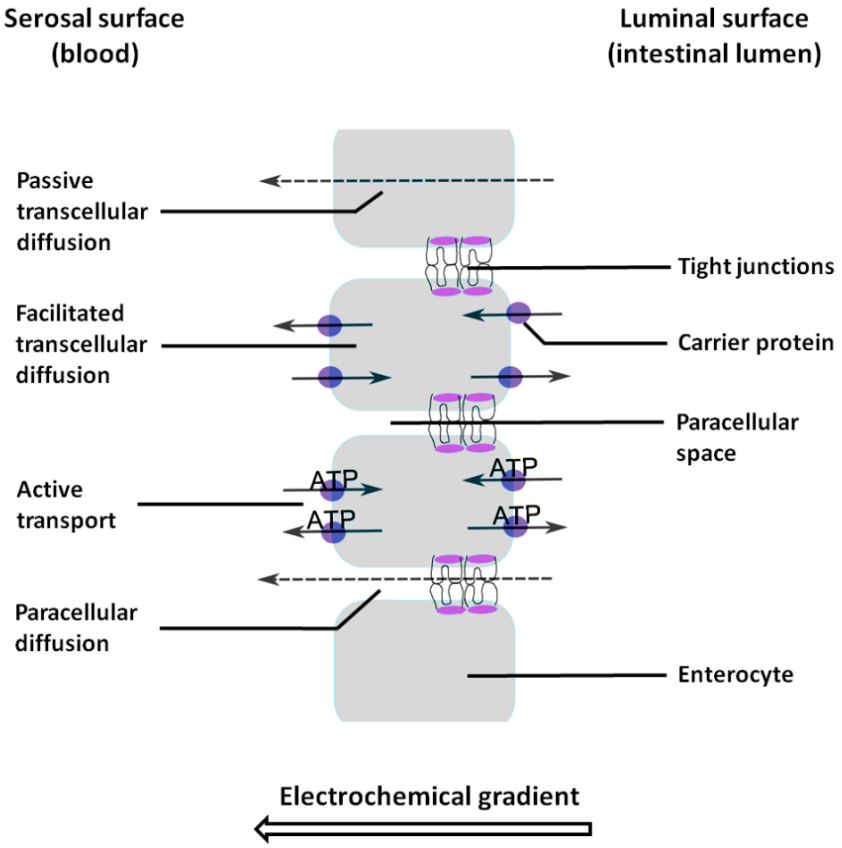


Figure 2-8: Possible mechanism of intestinal absorption from luminal side to the serosal side according to (Deußer, 2010). ATP = Adenosine triphosphate.

reduced bioavailability (active efflux) with or against the direction of an electrochemical gradient (see Figure 2-9). Two different carrier mediated active transport ways are described.

The primary active transport requires energy which is generated by exergonic reactions such as cleavage of adenosine triphosphate (ATP). Primary active transporters have an ATP binding cassette (ABC-transporters) and include a variety of transporters in the GIT. Nevertheless, only a few play a key role in intestinal drug absorption such as *p*-glycoprotein (P-gp), multidrug resistance protein (MRP) and breast cancer proteins (BCRP). These transport proteins can be located at the luminal or serosal membrane of the enterocyte (see Figure 2-8).
P-gp (MDR1) is a transmembrane efflux pump which shows an affinity to a wide range of compounds. Therefore, it is speculated that it plays a major physiological role in the absorption of xenobiotics. Unlike P-gp, the expression of BCRP along the small intestine does not vary significantly. It is highly expressed on the luminal side of enterocytes and it transports substrates back into the lumen. Due to this and the broad substrate specificity, BCRP has been noted as an important efflux transporter which limits drug absorption along the GIT. While P-gp and BCRP are localized on the luminal side of the brush border membrane (luminal efflux), certain MRPs are localized on the luminal and serosal membrane of the enterocyte. MRP 2 and 4 (luminal efflux) are located on the luminal membrane and MRP 1 and 3 (serosal efflux) on the serosal membrane of the enterocyte.

Secondary active transport processes via the solute carrier (SLC) superfamilies require the concentration gradient generated by primary active transport processes as an energy source (H^+, Na^+, Ca^{2+} gradients). Relevant intestinal SLC transporters are sodium dependent glucose transporter (SGLT1, uptake of glucose), monocarboxylic acid transporter (especially MCT1), peptide transporter (PepT1) and organic anion polypeptide transporter (OATP1A2) (Rehner and Daniel, 2002; El-Kattan and Varma, 2012). MCT1 is highly expressed in the intestine and is known as a low affinity, high capacity transporter for unbranched aliphatic monocarboxcylates on the apical membrane of enterocytes (*e.g.* uptake of ferulic acid (Poquet et al., 2008)). The PepT1 transporter is also a low affinity, high capacity transporter localized on the apical side mainly in the duodenum and plays a major role in

intestinal uptake of peptidomimetics that include *β*-lactam antibiotics. In the OATPs solute carrier family OATP2B1 and OATP1A2 play a key role in the intestinal uptake of xenobiotics which are both located on the luminal membrane of the enteroycte. Due to an inhibition of OATP1A2 attributed to grapefruit juice ingestion, the uptake of talinolol was reduced. This illustrates that transporters might be potential sites for diet-drug interactions (El-Kattan and Varma, 2012).

As both active transport ways (primary and secondary) are dependent in a direct or indirect way on the hydrolysis of ATP, their inhibition by a metabolic inhibitor such as NaN_3 is possible (Konishi and Shimizu, 2003; Poquet et al., 2008). Overall, according to the orientation of a transporter protein (efflux or uptake) the uptake of a substrate is reduced or increased (El-Kattan and Varma, 2012).

Furthermore, an absorption in the gastrointestinal tract of molecules via specialized microfold cells (M-cells) is possible. These special cells are located at the peyer´s patches in the intestine. M-cells are lacking of mucus and microvilli but therefore provide broader microfolds. This allows accessibility of particles to the luminal surface so macromolecules or particles can be absorbed (*e.g.* proteins) by endocytosis (Hussain et al., 2001; Rehner and Daniel, 2002).

2.4.4 Transport kinetics

For simple passive diffusion the transport rate (flux) of a molecule is defined by the concentration difference. Thus it cannot be saturated and molecule flux *vs*. molecule concentration have a linear relationship (first order transport kinetics) (Figure 2-9) (Rehner and Daniel, 2002; Silbernagl and Despopoulos, 2003).

Facilitated diffusion or active transport processes are characterized by non linear transport kinetics. Reasons for this are factors limiting the transport. In detail, the facilitated diffusion is influenced by a limited number of binding sites at the transport protein. Consequently, the transport kinetics (molecular flux *vs*. molecule concentration) depends on the luminal drug concentration and shows a hyperbole relation (Figure 2-9) with a limited maximum flux (second order kinetics) (Rehner and Daniel, 2002; El-Kattan and Varma, 2012).

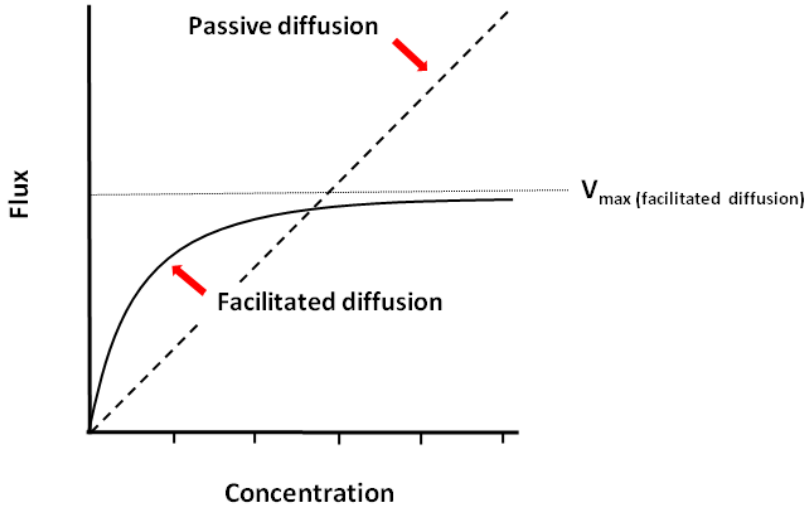


Figure 2-9: Transport kinetics of simple passive diffusion and facilitated diffusion displayed as concentration *vs*. flux. Only facilitated diffusion reaches a flux maximum (V_{max}). According to (Rehner and Daniel, 2002).

2.5 The pig as intestinal absorption model

Men and pigs are omnivorous and show physiological and anatomical similarities of the GIT and the intestinal barrier (Vodicka et al., 2005). Despite the different lengths of the small intestine (pig 15 - 22 meters, men 5 – 6 meters) the lengths per kg*BW are very similar for both species with around 0.1 $m*kg^{-1}*BW^{-1}$(Patterson et al., 2008). Most similarities are observable in the upper part of the GIT (Figure 2-10). In particular, the pig GIT can also be divided into stomach, duodenum, jejunum and ileum. However, comparative considerations of the large intestine of both species are possible concerning its functionality. The large intestine of a pig is found in a spiral conformation (Figure 2-10), but can also be divided into colon ascendens, colon transversum, colon descendens and rectum.

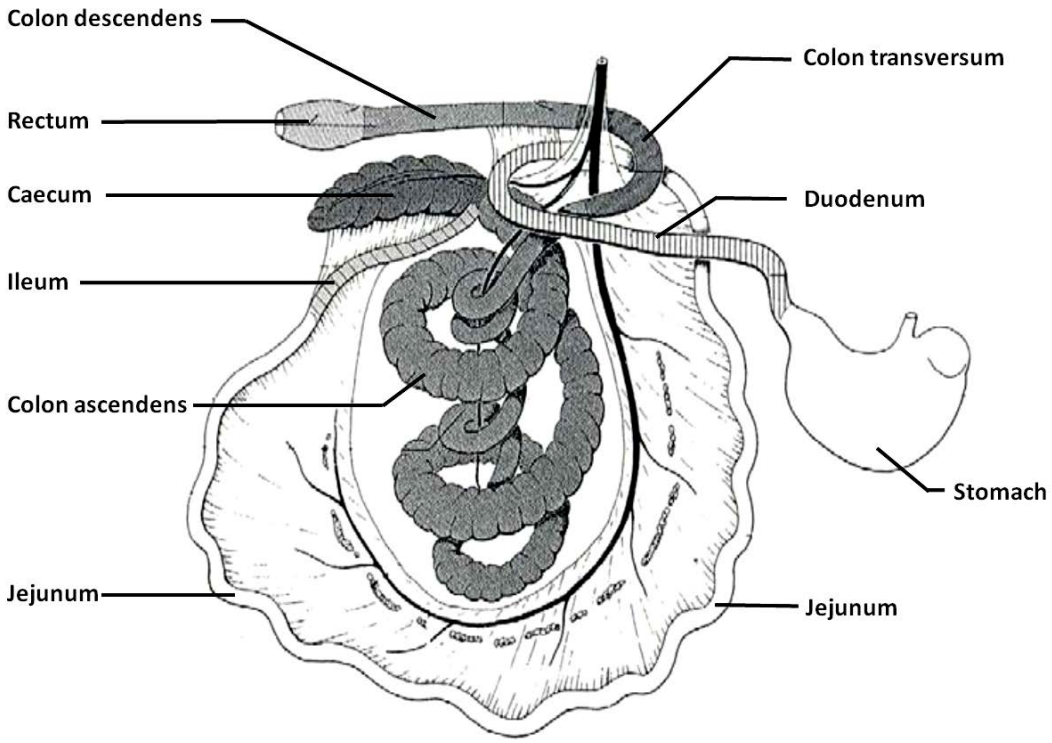


Figure 2-10: Schematic detailed overview of the pig GIT (Charalambakis, 2008). The stomach and small intestine of pig shows strong similarities to the upper GIT of man. Comparative considerations of the large intestine is possible concerning the functionality (Patterson et al., 2008).

Microscopically structures of human and pig intestines are comparable as well (Crump, 1975; Cooper et al., 1997). The morphology of the small intestine is divided into several tissues like the human mucosa: tunica serosa, tunica muscularis, tela submucosa and tunica mucosa (see Figure 2-11). The tunica serosa is a connective tissue and is affiliated to the smooth muscle tissue of tunica muscularis. Next to this, the tela submucosa (a lax connective tissue) is located, along with blood vessels, nerves and adenoid tissue. Following this is the tunica mucosa which consists of three tissue parts: tela submucosa, lamina muscularis mucosae (a thin smooth muscle tissue) and lamina propria mucosae (a lax connective tissue). The wall of the small intestine ends up with the epithelium mucosae at the luminal side (Mosimann and Kohler, 1990).

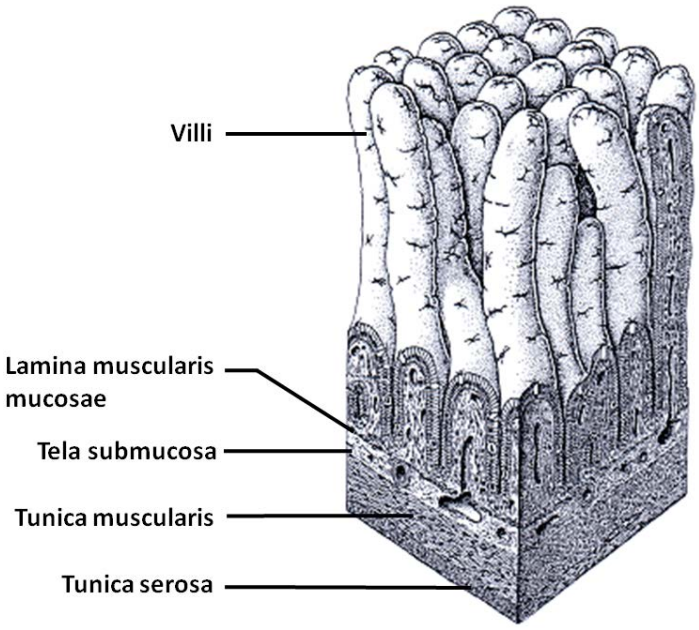


Figure 2-11: Cross section of the pig jejunum (Mosimann and Kohler, 1990).

Beyond anatomical similarities, the associated metabolic processes are similar as well (Patterson et al., 2008). However, literature data on this issue is not coherent. As previous literature reported a limitation of phase II conjugation in pig (missing sulfation) (Williamson et al., 2000), more recent data indicated methylation and glucuronidation of polyphenols as major phase II metabolism besides sulfation (Wu et al., 2005; Espín et al., 2007; Deußer, 2010).

Because of the strong similarities in the physiology of digestion as well as the associated metabolic processes (Crump, 1975; Rowan et al., 1994; Patterson et al., 2008), pig is reported as the best non-primate human-nutrition model (Wu et al., 2005; Patterson et al., 2008).

2.6 Bioavailability

Oral bioavailability describes the absorbed amount of an ingested drug (such as CGA) which becomes available at the site of drug action. However, the drug concentration cannot be readily measured at the direct site of drug action. Based on the premise that the drug concentration at the site of action is in equilibrium with its concentration in blood, the bioavailability can be determined by the concentration of the drug in the systemic circulation or urine (Chereson, 1996).

2.6.1 Factors influencing bioavailability

Various factors are able to influence the bioavailability of a drug. These factors can be widely classified in the dosage form, the proband's physiology and the molecular properties of the ingested drug (see Table 2-7). The molecular properties of the drug (physico-chemical properties) and the dosage form have a major influence on bioavailability. Whereas interindividual differences in proband´s physiology can lead to interindividual differences in bioavailability. Such differences in bioavailability can be attributed to physiological differences such as GI motility, gastric emptying rate or different disease states (see Table 2-7). Especially the gastric emptying rate can be affected by several factors (see chapter 2.4.1.2) and can have a major influence on acid-resistant drugs (Chereson, 1996).

Furthermore, bioavailability of drugs can be decreased when the drug is a substrate of efflux transporters situated along the apical side of the GIT such as MDR 1 or MRP 2 (Dietrich et al., 2003).

Table 2-7: Summary of eminent factors that can influence the bioavailability of a drug (such as CGA) after oral ingestion. These factors can be widely classified in the dosage form, the proband's physiology and the molecule property, according to (Chereson, 1996; Lipinski et al., 1997; Dietrich et al., 2003; Williamson et al., 2011; El-Kattan and Varma, 2012).

Factors related to oral bioavailability

Dosage form	Proband (physiological factors)	Molecule property
Concentration, amount	Variations in absorption power along GI tract	Physico-chemical properties
Size of ingested load	Variations in pH of GI fluids	- Molecule weight
Interactions with other substances	Gastric emptying rate	- Hydrophobicity (log P)
- Food	Intestinal motility	- pKa
- Fluid volume	Perfusion of the GIT	Substrate of efflux transporters?
- Other drugs/xenobiotics	Presystemic and first-pass metabolism	Mechanism of absorption
Formulation: *e.g*. solutions, capsules	Age, sex, weight	
	Disease states	

2.6.2 Bioavailability of CGA

The bioavailability of CGA was elucidated by numerous *in vitro* and *in vivo* experiments showing its complexity, with partially contradictory outcomes. Therefore, the individual steps of CGA bioavailability must be taken into account, such as liberation, absorption, distribution, metabolization and elimination (LADME) with consideration of the methodical differences used in the individual studies. Specifically metabolization of CGA was determined with different analytical methodologies in the past and therefore needs detailed consideration. In Figure 2-12 a schematic overview of 5-CQA (**1d**) LADME profile is given.

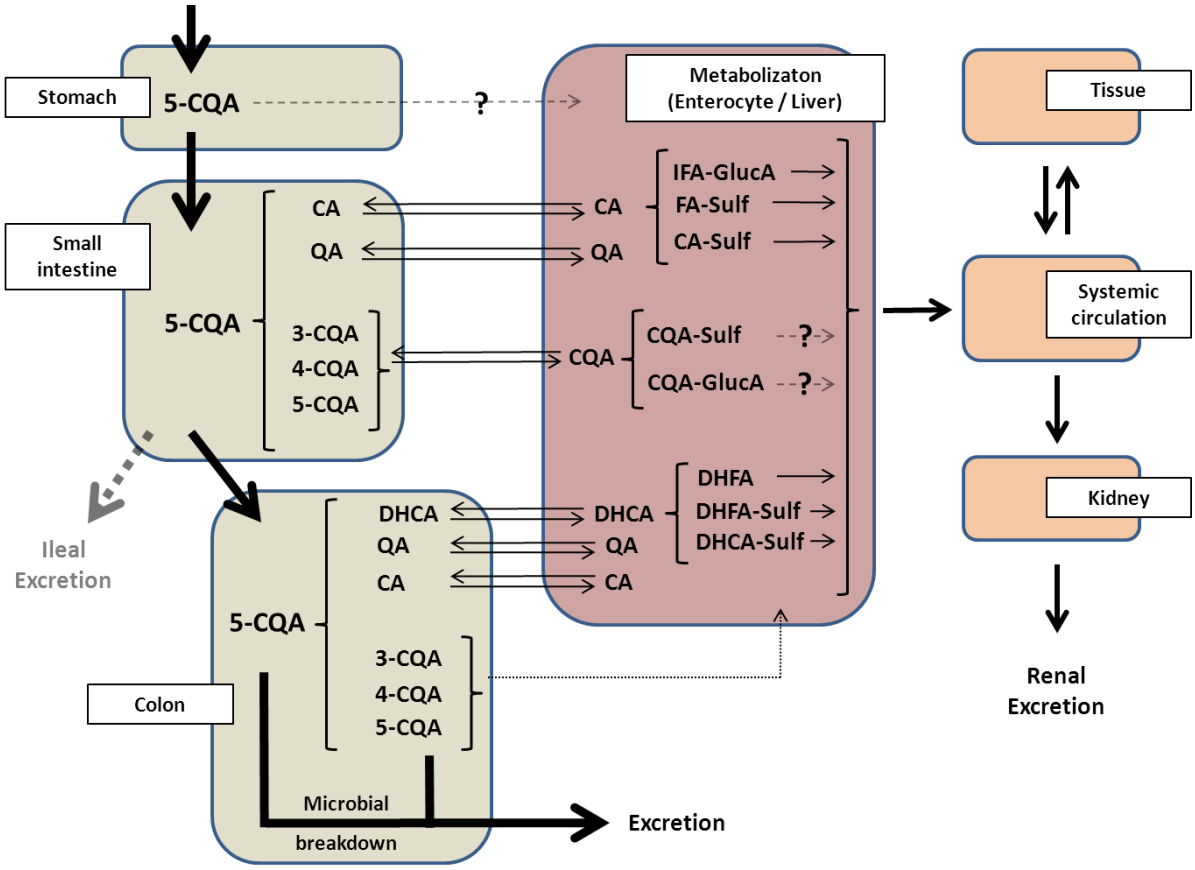


Figure 2-12: Ingestion, liberation, absorption, metabolization, distribution and excretion pathways of 5-CQA (LADME) in a healthy human (with colon) and ileostomists (without colon, ileal excretion), modified according to (Scalbert and Williamson, 2000; Stalmach et al., 2009; Stalmach et al., 2010; Williamson et al., 2011). Bold arrows indicate major pathways. CQA = caffeoylquinic acid, CA = caffeic acid, QA = quinic acid, DHCA = dihydrocaffeic acid, DHFA = dihydroferulic acid; FA = ferulic acid, IFA = isoferulic acid, Sulf = sulfate, GlucA = glucuronide.

2.6.2.1 Degradation of CGA and liberation of HCA during GI passage

Observing the way food passes through the GIT (see Figure 2-12), degradation of CGA and liberation of HCA from CGA increases from stomach to colon.

The liberation of free phenolic acids and QA (**10a**) from CGA plays only a minor role in the upper GIT. More specifically, Rechner et al. showed that the acidic environment of the gastric lumen did not affect coffee CGA composition (Rechner et al., 2001). Additionally, a recent study investigated a high stability of CA and 5-CQA in simulated gastric juice and saliva (Kahle et al., 2011). Only a reduced liberation of CA (< 1%) was detected *in vivo* in a ligated rat stomach for 5-CQA (Lafay et al., 2006) as well as a limited liberation of HCA from coffee CGA *in vitro* in a gastric cell layer study via an esterase most likely located on the apical side (Farrell et al., 2011).

In vitro a degradation of 5-CQA was observed in a simulated duodenal juice. In this assay (pH 7.2) caffeic acid methylester, CA (**5a**), QA (**10a**), FA (**7a**), DHCA (**6a**) and 3- and 4-CQA (**1b-c**) were formed within 24 hours of incubation (Kahle et al., 2011). As the conditions of this study are not physiological (extended incubation time and increased pH, *cf*. chapter 2.4.1.2) the data cannot be transmitted to the *in vivo* situation. In another incubation with human duodenal fluid (*in vitro,* 4 h of incubation) (Olthof et al., 2001; Lafay et al., 2006) or ileal effluent (*ex vivo,* 2 h of incubation) 5-CQA (**1d**) was not degraded (Olthof et al., 2001; Lafay et al., 2006). In literature a cinnamoyl esterase is described as being located along the human small intestine (Andreasen et al., 2001) so a moderate liberation of CGA into QA (**10a**) and HCA is conceivable as already detected in human intervention studies with ileostomists (Kahle et al., 2007; Stalmach et al., 2010).

It is well known that considerable amounts of ingested CGA reach the colon (Olthof et al., 2001; Kahle et al., 2007; Stalmach et al., 2010; Hagl et al., 2011). In this compartment the microbial metabolism leads to the liberation of QA and HCA and further numerous degradation products, respectively (see Figure 2-13) (Olthof et al., 2003). In particular the microorganisms *Escherichia coli*, *Bifidobacterium lactis*, and *Lactobacillus gasseri* are reported to be able to express cinnamoyl esterase activity (Rechner et al., 2004) and therefore are partly responsible for the observed degradation.

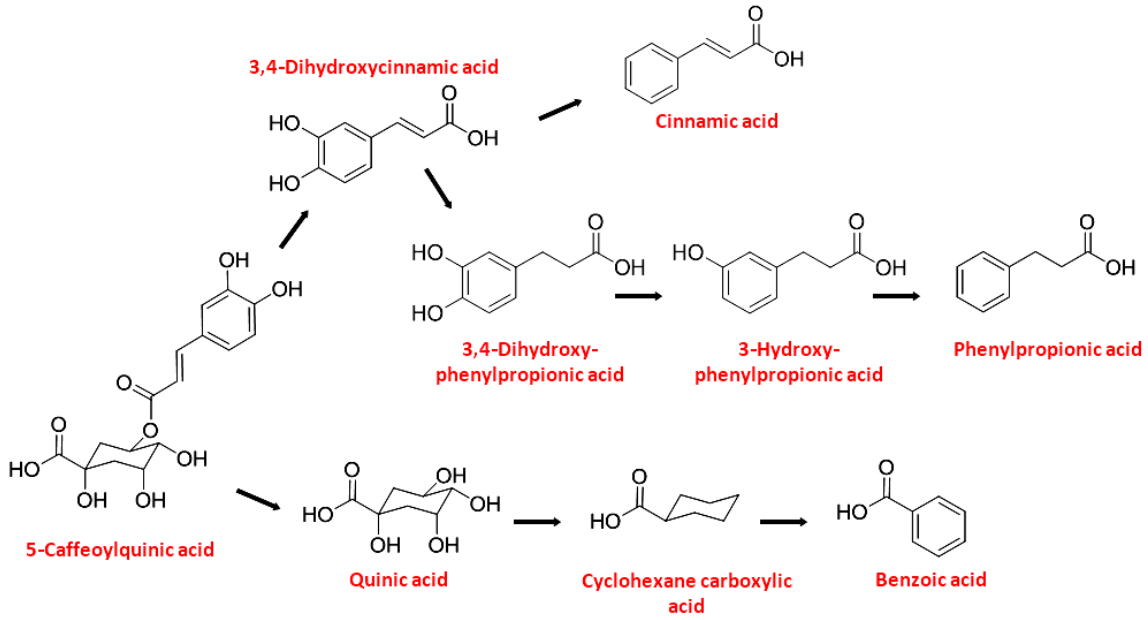


Figure 2-13: Proposed cleavage of 5-caffeoylquinic acid to 3,4-dihydroxycinnamic acid (CA) and QA and subsequent microbial degradation in the colon, according to (Olthof et al., 2003; Rechner et al., 2004).

CGA are rapidly cleaved into QA (**10a**) and HCA (*e.g*. 3,4-dihydroxycinnamic acid, (CA)) with a subsequent degradation via reduction at the aliphatic double bond, dehydroxylation and aromatization (Olthof et al., 2003; Rechner et al., 2004). Hereby benzoic- and 3-phenylhydroxypropionic acid are the major metabolites of human colonic microbiota (Gonthier et al., 2006). Until now it is not known if different subgroups of CGA (CQA (**1a-d**), FQA (**3a-c**), CQL (**2a-b**), diCQA (**4a-c**)) show differences in kinetics and affinity to a cinnamoyl esterase (liberation of CQA, HCA and QA (**10a**) and further degradation) during the GI – passage.

2.6.2.2 Absorption

In vivo investigations of individual coffee CGA absorption rates are limited because CGA undergo large interesterification- and biotransformation reactions in the human body. Thus, different research groups have performed *in vitro* and *ex vivo* studies to achieve reliable individual absorption data, summarized in Table 2-8.

Small amounts of CGA and HCA were absorbed in the experiments performed (Table 2-8). The absorption of the HCA seems by trend to be higher in comparison to the esterified ones (CGA) *in vitro* and *ex vivo* (Table 2-8). In most *in vivo* studies higher

plasma levels of free HCA and only low plasma levels of intact CGA (FQA (**3a-c**), CQA (**1b-d**), CQL (**2a-b**)) after consumption of CGA were reported, affirming these observations (Nardini et al., 2002; Wittemer et al., 2005; Azzini et al., 2007; Matsui et al., 2007; Bergmann et al., 2009; Stalmach et al., 2009).

Farrell et al. investigated the permeability of coffee CGA in cell monolayer experiments (gastric cell line, Caco-2) and therefore used coffee with its natural CGA composition and varying CGA concentrations. In the Caco-2 cell line they figured out that the major coffee CGA were all permeable, with the exception of 3,5-diCQA (**4b**). Furthermore, their work revealed a strong hint of decreasing permeability of 5-acyl CGA compounds within the CGA subgroup (5-FQA (**3c**), 5-CQA (**1d**)). The gastric permeability of diCQA (**4a-c**) was counting a multiple compared to other coffee polyphenols, especially 3,5-diCQA (**4b**) (Farrell et al., 2011). These observations may partially explain the data of one group which showed high amounts of CQA (**1b-d**) and even diCQA (**4a-c**) in plasma after coffee CGA consumption (Monteiro et al., 2007; Farah et al., 2008).

Individual absorption experiments with single compounds and physiological concentrations were performed in T84 colon cell line model (Bergmann et al., 2009). The structure-absorption relation showed a high absorption of the lipophilic cinnamic acid. In contrast, the more polar hydroxycinnamic acids showed a strongly decreased absorption in the following order: IFA (**7g**), FA (**7a**), CA (**5a**), p-CouA. These observations indicate a correlation of the physico-chemical properties of a compound and its permeability in the used T84 monolayer. Furthermore, different absorption rates of single CGA and HCA *in vitro* indicate limitations of comparing *in vivo* bioavailability studies with different CGA and HCA compositions. Thus, the ileal excretion of CGA after coffee consumption (Stalmach et al., 2010) or after apple juice consumption (Kahle et al., 2007) showed a limited comparability because coffee and apple juice showed a different CGA profile.

Since the physico-chemical properties of a molecule can be an important factor in influencing its bioavailability, especially for a passive absorbed compound (see chapter 2.4.3), a structure-absorption relation (SAR) for the main coffee CGA is of interest. Concluding a SAR with the existing literature data on coffee CGA absorption is constricted because of the different concentrations used in these experiments and the limited number of investigated compounds (CGA and HCA) in the past. More

specifically, no individual experiments have been performed with single FQA (**3a-c**) and diCQA (**4a-c**) up to now.

Table 2-8: Summary of *in vitro* (cell line) and *ex vivo* absorption studies with single chlorogenic acids, hydroxycinnamic acids and QA, modified according to (Deußer, 2010). c = incubation concentration; t = incubation time.

Compound	c (µM)	t (min)	Experimental design	Absorbed amount (%)	Reference
Hydroxycinnamic acids					
CA (5a)	20	240	pig ileal mucosa*	3.7 ± 0.1	(Deußer, 2010)
	20	240	T 84	n.d.	(Bergmann et al., 2009)
	5,000	40	Caco-2	0.2 ± 0.0	(Konishi and Kobayashi, 2004)
FA (7a)	20	240	pig ileal mucosa*	1.2 ± 1.1	(Deußer, 2010)
	20	240	T 84	2.3 ± 0.2	(Bergmann et al., 2009)
	5,000	40	Caco-2	3.4 ± 0.2	(Konishi and Shimizu, 2003)
	30	60	Caco-2/HT29-MTX	9.4°	(Poquet et al., 2008)
p-CoA	20	240	pig ileal mucosa*	3.3 ± 1.8	(Deußer, 2010)
	20	240	T 84	n.d.	(Bergmann et al., 2009)
	1,000	40	Caco-2	1.6 ± 0.2	(Konishi et al., 2003)
Caffeoylquinic acids					
1-CQA (1a)	10	240	pig ileal mucosa*	n.d.	(Deußer et al., 2013)
	10	240	T 84	n.d.	(Bergmann et al., 2009)
3-CQA (1b)	50	240	pig ileal mucosa*	1.5 ± 0.2	(Deußer et al., 2013)
	50	240	T 84	n.d.	(Deußer, 2010)
4-CQA (1c)	10	240	pig ileal mucosa*	n.d.	(Deußer et al., 2013)
	10	240	T 84	n.d.	(Deußer, 2010)

Compound	c (µM)	t (min)	Experimental design	Absorbed amount (%)	Reference
5-CQA (1d)	50	240	pig ileal mucosa*	1.9 ± 1.0	(Deußer et al., 2013)
	50	240	T 84	n.d.	(Bergmann et al., 2009)
	5,000	40	Caco-2	0.1 ± 0.0	(Konishi and Kobayashi, 2004)
	5,000	60	Caco-2	0.1 ± 0.0	(Dupas et al., 2006)
p-Coumaroylquinic acids					
4-p-CoQA	10	240	pig ileal mucosa*	n.d.	(Deußer et al., 2013)
	10	240	T 84	n.d.	(Deußer, 2010)
5-p-CoQA	10	240	pig ileal mucosa*	n.d.	(Deußer et al., 2013)
	10	240	T 84	n.d.	(Deußer, 2010)
D-(-)-Quinic acid (10a)	80	240	pig ileal mucosa*	0.5 ± 0.3	(Deußer et al., 2013)
	80	240	T 84	0.5 ± 0.1	(Deußer, 2010)

**ex vivo* Ussing chamber experiments; ° calculated on the basis of permeation rate

n.d. = not detected

2.6.2.2.1 Site of CGA and HCA absorption

The site of absorption is discussed controversially in literature. Several groups assume the colon as a major part of CGA absorption, but there is recent evidence for absorption taking place in the upper parts of the GIT such as the stomach and small intestine.

The early occurrence of CGA and its metabolites after coffee consumption in the systemic circulation (C_{max} and T_{max}) (Stalmach et al., 2009; Renouf et al., 2010a) as well as *in vitro* (gastric cell line) (Farrell et al., 2011), *ex vivo* (pig ileal mucosa) (Deußer, 2010) and an animal study (ligated rat stomach) (Lafay et al., 2006) indicate a considerable contribution of the small intestine and stomach on CGA absorption. However, on the basis of several studies using the ileostomy model it is known that the absorption of CGA in the upper parts of the GIT varies (see Table 2-9). Following the absorption thesis of Olthof and coworkers in ileostomists (Olthof et al., 2001)

($CGA_{CONSUMED} – CGA_{ILEAL\text{-}EXCRETION} = CGA_{ABSORBED}$) means an absorption in the upper GIT of 95% for caffeic acid (**5a**) and 22 - 74% for CQA (**1b-d**). Thus, high amounts can reach the colon (colonic availability) (see Table 2-9). Recent comparison of CGA absorption after coffee consumption in healthy volunteers (with colon) and ileostomy volunteers (without colon) revealed the importance of the colon with a three times higher absorption of CGA in the healthy volunteers (Rio et al., 2010). The degradation products of CGA formed in the colon such as DHCA (**6a**), DHFA (**8c**) (Renouf et al., 2010a) or a potential liberation of CQA (**1b-d**) and CA (**5a**) from diCQA (**4a-c**) contribute to a higher total absorption in healthy volunteers.

However, comparative considerations of the described ileostomy studies in Table 2-9 are limited as some bioavailability factors that might influence the absorption are varying. Especially the different dosage forms such as apple beverage (Kahle et al., 2007; Hagl et al., 2011), coffee (Stalmach et al., 2010) and pure compounds dissolved in hot water (Olthof et al., 2001), as well as different doses, consumed volumes from 200 mL to 1,000 mL have to be considered. Furthermore, the polyphenol composition of apple beverages is different to that of coffee with dihydrochalcone derivatives, flavan-3-ols and flavonols (Kahle et al., 2007; Hagl et al., 2011). Due to the lack of data on T_{max} (ileal excretion), it is not possible to observe an influence of the GI transit time on absorption of CGA. Altogether, the high deviation in CQA ileal excretion might be caused by the different matrices (dosage forms), the different polyphenol compositions, the different consumed CGA doses and the different analytical methods used for CGA determination such as HPLC-DAD or LC-MS/MS measurement (with or without metabolite detection). Since several bioavailability factors are varying in these publications (see Table 2-9), the influence of one parameter such as dose cannot be determined. Therefore further studies are needed to answer the influence of individual parameters on bioavailability such as dose-response studies.

Table 2-9: Administered amount of chlorogenic acids (CGA) and corresponding ileal excretion in % (equivalent to colonic availability) observed in the ileostomy model. CQA = caffeoylquinic acid (1a-d); CA = caffeic acid (5a).

Administered amount (µmol)	Dosage form Matrix		Age (a)	BMI ($kg*m^{-2}$)	Subjects	T_{max} (h)	**Ileal excretion (%)**	Reference
2,800 5-CQA	water (200mL)	with light breakfast	63	27	4 F 3 M	n.d.	**67***	(Olthof et al., 2001)
2,800 CA	water (200mL)	with light breakfast	63	27	4 F 3 M	n.d.	**5***	(Olthof et al., 2001)
358 CQA	cloudy apple juice (1,000 mL)	fasted (light meal after 4h)	40.8	n.d.	8 F 3 M	≈ 2	**26***	(Kahle et al., 2007)
335 CQA	apple smoothie (700 mL)	not specified	52.9	25	5 F 5 M	n.d.	**78***	(Hagl et al., 2011)
385 CGA	coffee (200mL)	fasted (light meal after 3h)	41-54	26	2 F 3 M	n.d.	**71**	(Stalmach et al., 2010)

*no metabolites were observed in these studies; t_{max} = time of maximum ileal excretion; n.d. not determined; F = female; M = male

2.6.2.2.2 Mechanisms of CGA and HCA absorption

The way of CGA and HCA absorption is principally discussed either with a passive diffusion or an active transport process. As the active transport process can be limited by concentration (see Figure 2-9) the mechanism of absorption could be a decisive factor influencing the bioavailability. Due to the lack of *in vivo* dose-response studies a limitation of bioavailability by dose is not clarified up to now. However, several *in vitro* studies provide some hints for the absorption mechanism as well as a potential efflux transport which can also limit bioavailability.

For instance several CGA, namely FA (**7a**), CA (**5a**) and 5-CQA (**1d**), were transported via the SLC transporter MCT through a monolayer (Caco-2) as a poor substrate (Konishi and Kobayashi, 2004). Contrary to this, the same group investigated non saturable transport of 5-CQA (**1d**) in a Caco-2 monolayer indicative for diffusion (Konishi and Kobayashi, 2004). This was confirmed by a hypothesized transcellular diffusion of FA (**7a**) through a co-cultured Caco-2 / HT29-MTX cell monolayer (Poquet et al., 2008) and a dose independent absorption of FA (Deußer, 2010).

The recent work of Farrell and coworkers (Farrell et al., 2011) indicated a much more complex situation and lead to the conclusion that absorption mechanisms have to be discussed for individual coffee CGA, respectively. In detail, for coffee solution mainly passive diffusion in a gastric cell layer was observed, but a potential carrier mediated transport for certain 4-acyl CGA subgroup (CQA (**1c**); FQA (**3b**)) was hypothesized. Beyond this a potential active efflux process (secretion) for diCQA (**4a-c**), decreasing its total absorption rate was discussed. Such efflux transport was observed via selective inhibition of MRP- and P-gp transporters in the secretion of ferulic acid glucuronide and dihydroferulic acid (Poquet et al., 2008).

2.6.2.3 Distribution in the human body

As the concentration of an ingested drug (such as CGA) cannot be detected readily at the direct site of drug action an equilibrium between its concentration in blood and concentration at the site of action is assumed (Chereson, 1996).

Therefore, no data of CGA distribution in the human body in different tissues exist despite quantitative data of plasma concentration summarized by (Williamson et al., 2011) or urine concentration (see chapter 2.6.2.5).

Pharmacokinetic data are only available from healthy volunteers after the ingestion of a single coffee dose (see Table 2-10). Comparing the plasma maximum CGA concentration (C_{max}) per 50 mg CGA dose, the differences in the systemic availability of CGA are apparent, particularly for CA (**5a**) and CQA (**1b-d**).

However, recent data showed that plasma AUC and C_{max} of coffee metabolites appear proportionally to the ingested coffee dose in healthy volunteers (Renouf et al., 2013). Furthermore, the studies summarized in Table 2-10 used all the same matrix (coffee) for CGA administration, other parameters than food matrix are influencing the bioavailability of CGA. The coffee consumed in the described studies (see Table 2-10) had a volume from 190 to 400 mL and was normally consumed in a fasted state. Further factors might influence C_{max} such as age, BMI, T_{max} and different analytical measurements.

Table 2-10: Pharmacokinetic data of chlorogenic acids after the consumption of coffee beverage by healthy volunteers (dose calculated from total CGA content derived directly from the references indicated). Summarized by (Williamson et al., 2011).

Compound	Dose (mg)	Dosage form (mL)		Age (a)	BMI ($kg*m^{-2}$)	Subjects	C_{max} (nM)	T_{max} (h)	AUC ($\mu mol*h^{-1}*L^{-1}$)	C_{max}*50 mg^{-1} dose	Reference
CA (**5a**)	96	200	fasted	n.d.	n.d.	10 M	426	~1	n.d.	**222**	(Nardini et al., 2002)
	1,236	190	fasted (light meal after 2 h)	22-55	n.d.	4 F 2 M	1,560	1.4	2.8	**63**	(Monteiro et al., 2007)
	145	200	fasted (lunch after 3 h)	19-35	24	3 F 8 M	92	1	0.3	**32**	(Stalmach et al., 2009)
	335	400	not specified	34	24	5 F 4 M	81	1.6	n.d.	**12**	(Renouf et al., 2010a; Renouf et al., 2010b)
FA (**7a**)	145	200	fasted (lunch after 3 h)	19-35	24	3 F 8 M	76	0.6	0.5	**26**	(Stalmach et al., 2009)
	335	400	not specified	34	24	5 F 4 M	139	0.6 and 6.0°	n.d.	**21**	(Renouf et al., 2010a; Renouf et al., 2010b)
IFA (**7g**)	335	400	not specified	34	24	5 F 4 M	98	1.8	n.d.	**15**	(Renouf et al., 2010a; Renouf et al., 2010b)
DHCA (**6a**)	145	200	fasted (lunch after 3 h)	19-35	24	3 F 8 M	346	5	2.8	**119**	(Stalmach et al., 2009)
	335	400	not specified	34	24	5 F 4 M	200	10	n.d.	**30**	(Renouf et al., 2010a)

Compound	Dose (mg)	Dosage form (mL)		Age (a)	BMI ($kg*m^{-2}$)	Subjects	C_{max} (nM)	T_{max} (h)	AUC ($\mu mol*h^{-1}*L^{-1}$)	C_{max}*50 mg^{-1} dose	Reference
CQA (**1b-d**)	96	200	fasted	n.d.	n.d.	10 M	0	n.d.	n.d.	**0**	(Nardini et al., 2002)
	1,236	190	fasted (light meal after 2 h)	22-55	n.d.	4 F 2 M	4,890	2.3	11.5	**198**	(Monteiro et al., 2007)
	145	200	fasted (lunch after 3 h)	19-35	24	3 F 8 M	2	0.6 to 1	0.04	**0.8**	(Stalmach et al., 2009)

°Two peaks of FA were observed. n.d. = not determined; F = female; M = male

2.6.2.4 Metabolization

As the presystemic metabolism or first-pass metabolism in the small intestine and liver is able to reduce the systemic availability of an oral ingested drug, metabolization is a decisive bioavailability factor (Chereson and Banakar, 1996).

The routes by which a drug (xenobiotic, such as CGA) might be metabolized are numerous and depend on the drug itself. Metabolization includes reactions such as oxidation, reduction, hydrolysis, hydration, conjugation and condensation reactions. Normally, xenobiotics metabolism is divided into two phases: phase I (functionalization reactions) and phase II (conjugative reactions). An overview of chemical reactions associated with phase I or II are given in Table 2-11. Phase I enzymes are thought to act as a preparation of a xenobiotic by formation of a chemically reactive functional group on which the phase II reactions can occur. Phase I enzymes are found in the liver, GIT, lung and kidney. They primarily consist of the cytochrome P 450 superfamily of microsomal enzymes (Gibson and Skett, 2001).

Table 2-11: Reactions of xenobiotic metabolism classed as phase I or phase II metabolism according to (Gibson and Skett, 2001).

Phase I	Phase II
Oxidation	Glucuronidation / glucosidation
Reduction	Sulfation
Hydrolysis	Methylation
Hydration	Acetylation
Dethioacetylation	Amino acid conjugation
Isomerisation	Glutathione conjugation
	Fatty acid conjugation
	Condensation

Conjugative reactions of phase II enzymes generally increase hydrophilicity and thereby enhance excretion in the bile or urine (Gibson and Skett, 2001). The phase II enzymes consist of many superfamilies such as sulfotransferase, glutathione S-transferases and UDP-glucuronosyltransferases and can be found in several tissues such as liver, kidney and GIT (Xu et al., 2005). In Table 2-12 an overview of the

phase II enzymes is given with the corresponding conjugation reaction and corresponding functional group of xenobiotics.

Table 2-12: Phase II conjugation reactions, corresponding enzyme and corresponding functional groups of xenobiotics, according to (Gibson and Skett, 2001)

Phase II reaction	Enzyme	Functional group
Glucuronidation	UDP-Glucuronosyltransferase	-OH; -COOH; -NH_2; -SH
Glycosidation	UDP-Glycosyltransferase	-OH; -COOH; -SH
Sulfation	Sulfotransferase	-NH_2; -SO_2NH_2
Methylation	Methyltransferase	-OH; -NH_2
Acetylation	Acetyltransferase	-NH_2; -SO_2NH_2; -OH
Amino acid conjugation		-COOH
Glutathione conjugation	Glutathione-*S*-transferase	Epoxide; Organic halide
Fatty acid conjugation		-OH
Condensation		Various

Once CGA are absorbed, an extensive biotransformation in the human body occurs (see Figure 2-14). Therefore, the bioavailability of CGA in its unconjugated form is limited due to metabolization. Phase II conjugation reactions are the major biotransformation steps in humans (Holst and Williamson, 2008). Furthermore, as CGA showed free hydroxyl groups (see Figure 2-1), the function of phase I reactions plays only a minor role in its metabolization (Wong et al., 2010).

As reported by Rio et al. the typical metabolic actions observed for CGA *in vivo* are methylation, sulfation and glucuronidation (Rio et al., 2010). Additionally *in vitro* a glutathione conjugate formation by rat hepatocyte microsomes was observed for CA, DHCA and CGA (postulated for 5-CQA) (Moridani et al., 2001). Biotransformation probably already occurs in the stomach with hydrolyzation of CGA and subsequent methylation of HCA, observed in a gastric cell monolayer model (Farrell et al., 2011). Kahle et al. reported 5-CQA and CA to be stable whilst incubated with simulated gastric juice (Kahle et al., 2011), so the gastric cell monolayer itself reveals the metabolic activity.

Esterases located in upper GIT are able to liberate HCA from CGA. Consequently, HCA are liberated and subsequently undergo further metabolic reactions: *e.g.* CA (**5a**) to its 3- and 4-*O*-sulfates (**5e-d**), FA (**7a**) to its 4-*O*-sulfate (**7c**), CA (**5a**) to form IFA (**7g**), its 3-O-suflate (**7f**) and glucuronide (**7e**) as observed *in vivo* (Rio et al.,

2010) and *in vitro* using human intestinal and liver S9 homogenates (Wong et al., 2010). The main HCA conjugates in urine are sulfates, with the exception of IFA which is mainly glucuronidated (Wong et al., 2010). Caffeoylquinde-, feruloylquinde-sulfates, and feruloylquinde- glucuronides are the only CGA conjugates which have been identified up to now (Stalmach et al., 2009; Redeuil et al., 2011). Furthermore, influences of different ingested CGA doses on the routes by which CGA and liberated HCA are metabolized have not been clarified up to now.

The colon is the major action site for liberation of HCA from CGA and conversion of it to dihydro compounds (as already described in chapter 2.6.2.1) (Rio et al., 2010). More specifically, once FA (**7a**) or benzoic acid (a microbial catabolite of CA (**5a**) or QA (**10a**)) are absorbed they will be conjugated to FA-glycine (**7d**) or to hippuric acid (Olthof et al., 2003; Rio et al., 2010). Furthermore a minor contribution of the hepatic NADPH-cytochrome P450 phase I metabolism in the formation of dihydrocinnamic acids (*e.g*. DHCA (**6a**), DHFA (**8c**)) was discussed by Stalmach 2009 and coworkers (Stalmach et al., 2009).

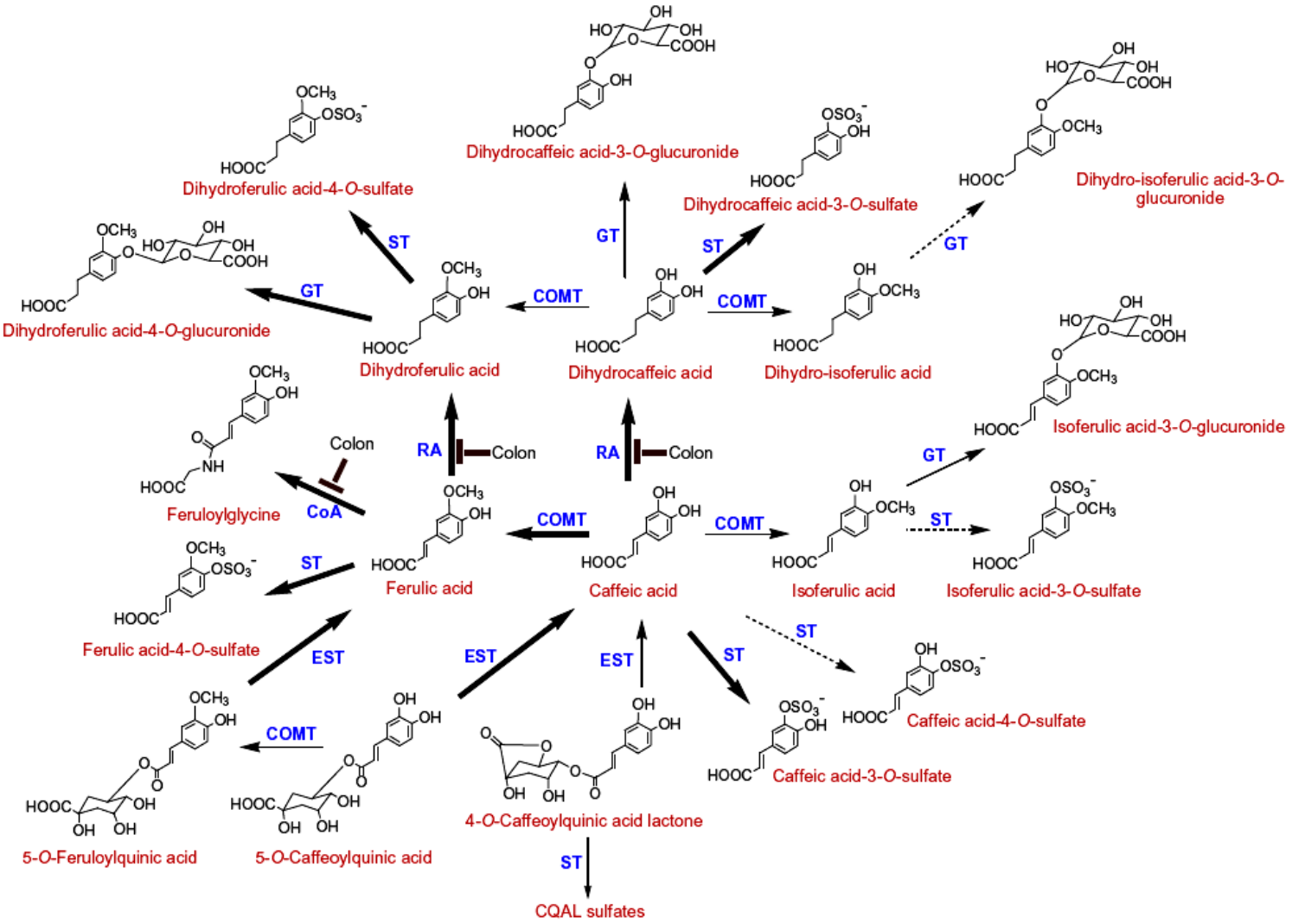


Figure 2-14: Metabolism pathway of CGA after acute ingestion of coffee in human volunteers: COMT = catechol methyltransferase; ST = sulfotransferase; EST = esterase; RA = reductase; GT = UDP-glucuronyltransferase; CoA = co-enzyme; Bold arrows indicate major pathways. According to (Stalmach et al., 2010).

2.6.2.5 Excretion

Up to now, excretion of CGA after coffee consumption was observed by monitoring renal excretion, although an excretion via the enterohepatic circulation is theoretically possible as well.

The overall renal excretion of an oral ingested drug such as CGA can be presumed to give evidence of its bioavailability as the concentration of a drug at the site of drug action cannot be directly measured readily (Chereson, 1996). A comparative consideration of data on renal excretion after CGA or HCA consumption is hindered, because factors which may influence the bioavailability differ in the considered studies (see Table 2-13). Different doses and dosage forms with partially different CGA and HCA compositions were administered and additionally different analytical methods were used for detection of compounds in urine.

More specifically, Olthof and coworkers observed a different renal excretion after the consumption of CA or 5-CQA as pure compounds dissolved in hot water by ileostomists (Olthof et al., 2001). This finding provides evidence for a different absorption and excretion behavior according to the CGA or HCA composition of the consumed food. The consumed artichoke leaf extract (containing CGA and flavanoids) (Wittemer et al., 2005), the polyphenol rich extract (Ito et al., 2005) and the high bran cereal breakfast (Kern et al., 2003a) showed completely different polyphenol profiles to that of coffee, so the comparability of the described studies is less. Studies which determined the renal excretion of CGA after coffee consumption are different in administered dose and dosage forms, too. Farah and coworkers administered an encapsulated green coffee extract, which had to be disintegrated in the GIT before absorption (Farah et al., 2008), Rechner administered 6 cups of coffee within 8 hours (Rechner et al., 2001) and Stalmach administered a single dose of coffee to healthy (with colon) and ileostomists (without colon) (Stalmach et al., 2009; Stalmach et al., 2010) see Table 2-13.

Thus, it is not possible to draw a conclusion based on available literature data, whether the different doses and dosage forms are affecting the bioavailability. However, Wittemer et al. reported a renal excretion of about 4% of the ingested CGA after the consumption of two artichoke leaf extracts with two different CA equivalent amounts indicating a dose independent excretion (Wittemer et al., 2005).

Additionally, the analytical methodology influences the outcome, as shown by the group of Stalmach (Stalmach et al., 2009; Stalmach et al., 2010). They used an analytical method without enzyme hydrolysis of CGA metabolites and detected a multiple level of renal excretion compared to others (see Table 2-13). The metabolites were measured directly by Stalmach et al. with a high selective HPLC-MS system in the multiple ion mode. Other groups reported significantly lower amounts excreted renally by using enzymatic hydrolysis of metabolites. These groups measured the aglyca by HPLC-DAD (Olthof et al., 2001; Rechner et al., 2001; Kern et al., 2003a; Farah et al., 2008), LC-MS/MS (Ito et al., 2005), or HPLC coulometric array detection (Wittemer et al., 2005).

On the one hand an intervention study with different CGA doses in a single food matrix is required to investigate the influence of different doses on CGA bioavailability. On the other hand an individual dose and different matrices are required to answer the influence of different food matrices on bioavailability. Additionally, those studies have to be performed with a CGA source with a defined CGA and HCA composition and an analytical methodology for the excreted compounds without enzymatic hydrolysis.

Table 2-13: Summary of renal excretion after consumption of chlorogenic or hydroxycinnamic acids (within food matrix) in probands with colon (healthy volunteers). Renal excretion is given in % of ingested dose based on total cinnamates. Comparative consideration of renal excretion between the single studies is limited because of the different consumed doses, different dosage forms and different analytical methods used for measurement (with or without metabolite detection). FA = ferulic acid (7a).

Dose	Dosage form			Age	BMI	Subjects	Renal excretion	Reference
(mg)	Matrix		Consumption	(a)	($kg*m^{-2}$)		% of dose*	
Healthy subjects (with colon)								
107°°	artichoke leaf extract	with hydroxypropyl-methylcellulose gel	not specified	25	22	7 F 7 M	**4.7** ǂ	(Wittemer et al., 2005)
148	coffee	200 mL	fasted (lunch after 3 h)	19 - 35	24	3 F 8 M	**29**	(Stalmach et al., 2009)
154°°	artichoke leaf extract	with hydroxypropyl-methylcellulose gel	not specified	25	22	7 F 7 M	**4.0** ǂ	(Wittemer et al., 2005)
259	polyphenol rich beverage	4 g instant coffee; 0.3 g green tea extract; 10 g cocoa powder; 18 g grape skin extract; 200 mL	not specified	25	22	5 F 4 M	**3.8** ǂ	(Ito et al., 2005)
259	FA in high bran cereal	100 g commercial breakfast (containing 85% wheat bran)	together with breakfast	32	26	3 F 3 M	**3.1** ǂ	(Kern et al., 2003a)
170	green coffee extract	encapsulated, 0.4 g	fasted (light meal after 1 h)	22 - 55	n.d.	5 F 5 M	**5.5** ǂ	(Farah et al., 2008)
898	coffee	6 cups within 8 hours	not specified	31	25	5 M	**≈ 5.9** ǂ	(Rechner et al., 2001)

*as total cinnamates; °° CA equivalents; ǂ after enzymatic hydrolysis (without metabolite identification); n.d. = not determined; F = female; M = male

Table 2-14: Summary of renal excretion after consumption of chlorogenic or hydroxycinnamic acids (as pure compound or within food matrix) in probands without a colon (ileostomy volunteers). Renal excretion is given in % of ingested dose based on total cinnamates. Comparative consideration of renal excretion between the single studies is limited because of the different consumed doses, different dosage forms and different analytical methodology used for measurement (with or without metabolite detection). CA = caffeic acid (5a), 5-CQA = 5-caffeoylquinic acid (1d).

Dose (mg)	Dosage form: Matrix	Dosage form	Consumption	Age (a)	BMI ($kg*m^{-2}$)	Subjects	Renal excretion % of dose*	Reference
Subjects without colon (ileostomists)								
139	coffee	200 mL	fasted (light meal after 3 h)	41 – 54	26	2 F 3 M	**8**	(Stalmach et al., 2010)
500	CA	200 mL hot water	with light breakfast	63	27	4 F 3 M	**10.7** ǂ	(Olthof et al., 2001)
1,000	5-CQA	200 mL hot water	with light breakfast	63	27	4 F 3 M	**0.6** ǂ	(Olthof et al., 2001)

* as total cinnamates; °° CA equivalents; ǂ after enzymatic hydrolysis (without metabolite identification); n.d. = not determined; F = female; M = male

3 Aims

Several previous studies on CGA oral bioavailability revealed contradictory data (Olthof et al., 2001; Kahle et al., 2007; Stalmach et al., 2010; Hagl et al., 2011; Williamson et al., 2011). Different factors can have an influence on the oral bioavailability of CGA, such as the dosage form, dose, proband's physiology or the molecular properties of the drug (Chereson, 1996; Lipinski et al., 1997; Dietrich et al., 2003; Williamson et al., 2011; El-Kattan and Varma, 2012). As different studies with healthy (see Table 2-10, State of knowledge) or ileostomy volunteers (see Table 2-9 and Table 2-14, State of knowledge) showed methodological differences in their analytical strategies (sample preparation with or without enzymatic cleavage of CGA conjugates), dosage form (such as different CGA composition from different food matrix) or dose, the influence of a single parameter on bioavailability could not be observed.

As coffee is the richest source of CGA in the western diet and a common coffee beverage (200 mL) serves between 70 and 350 mg CGA (Clifford, 1999; Farah and Donangelo, 2006) the main objective of this thesis was to monitor the effects of CGA and QA dose from coffee on their oral bioavailability. Especially an influence of ingested dose on the site where absorption occurs and metabolization was here in a closer focus. Therefore, ileostomy volunteers consumed three different doses of CGA and QA by coffee brew and the effect of dose was determined on: ileal excretion, plasma concentration, and renal excretion of QA, CGA and its metabolites. This dose-response study was performed in cooperation with the Department of Medicine II, Gastroenterology, University of Wuerzburg, Germany.

Furthermore, the CGA composition of coffee beverages or other food matrices containing CGA vary (Farah and Donangelo, 2006; Neveu et al., 2010). As varying CGA compositions show different physico-chemical properties this could also have an influence on CGA bioavailability. However, this has not been sufficiently investigated for coffee CGA up until now. Especially, the effect of the physico-chemical properties of CGA from coffee on their individual absorption rates has not been investigated. Here, the objective was to determine effects of the physico-chemical properties on absorption of CGA and QA. This was performed *ex*

vivo using the Ussing chamber model, with pig jejunum and individual compounds in physiological concentrations.

4 Results

In this work we investigated the bioavailability of coffee CGA and QA in the upper parts of the GIT. More specifically, we observed an influence of the consumption of different coffee doses on CGA and QA bioavailability in ileostomists and furthermore the influence of the chemical structure of CGA and QA on the bioavailability of those molecules in the pig jejunal mucosa model.

Therefore, we established on the one hand an *ex vivo* absorption experiment with pig jejunal mucosa in the Ussing chamber using CGA and QA present in coffee to observe effects of the chemical structure on their bioavailability. For this, these molecules were applied in physiological concentrations (20 µM) and their total absorbed amounts were determined (luminal to serosal).
Moreover, the mechanism of absorption was elucidated for 5-CQA, which is the dominating CGA in coffee. In order to differentiate between diffusion and active transport the absorption rates (flux) were measured at concentrations from 20 µM up to 3,500 µM (luminal to serosal). Secretion of CGA (serosal to luminal direction) were measured with 5-CQA at 20 µM with and without co-incubation of the metabolic inhibitor NaN_3 (10mM). Furthermore, active efflux transporters were detected in the pig jejunal mucosa by Western blot analysis.

In order to investigate the influence of different CGA doses on CGA bioavailability we performed a randomized, double-blinded and dose-response intervention study. On three separate days ileostomists consumed decaffeinated coffee with three different doses of CGA (HIGH 4,525 µmol; MEDIUM 2,219 µmol; LOW 1,053 µmol) and QA (HIGH 2,546 µmol, MEDIUM 1,373 µmol, LOW 695 µmol). The influence of the different consumed doses on total absorbed amounts (renal excretion), colonic availability (ileal excretion), metabolization and plasma concentrations was observed. Further, the stability of the mentioned coffee molecules in ileal fluid was investigated to observe any possible degradation during sample collection.

4.1 Stability of CGA in the small intestine (ileal fluid incubation)

In order to simulate the ileal passage of CGA from coffee and to observe any possible CGA degradation and interesterification during sample collection in the dose-response intervention study (see chapter 4.3) we performed an *ex vivo* incubation experiment.

This experiment was performed by incubating instant coffee with ileal fluid in an anaerobic chamber at physiological conditions (anaerobic, + 37°C) and concentrations (0.5 g instant coffee per L ileal fluid which corresponds to 132 µM CGA). Additionally, in order to determine the influence of ileal fluid on coffee CGA stability (*e.g.* ileal matrix, ileal micro flora), a further stability study was performed analog using only the incubation buffer (blank = without ileal fluid).

The recovered amount of the CGA was lower than the applied amount. In the 0 h samples 22.8% of CGA were recovered with ileal fluids whereas 58.4% of CGA were found in the incubation experiment with buffer samples. Because of this the initial concentration detected at time point 0 h was set at 100% and the stability and degradation of CGA during incubation was calculated in % of initial concentration (see Figure 4-1).

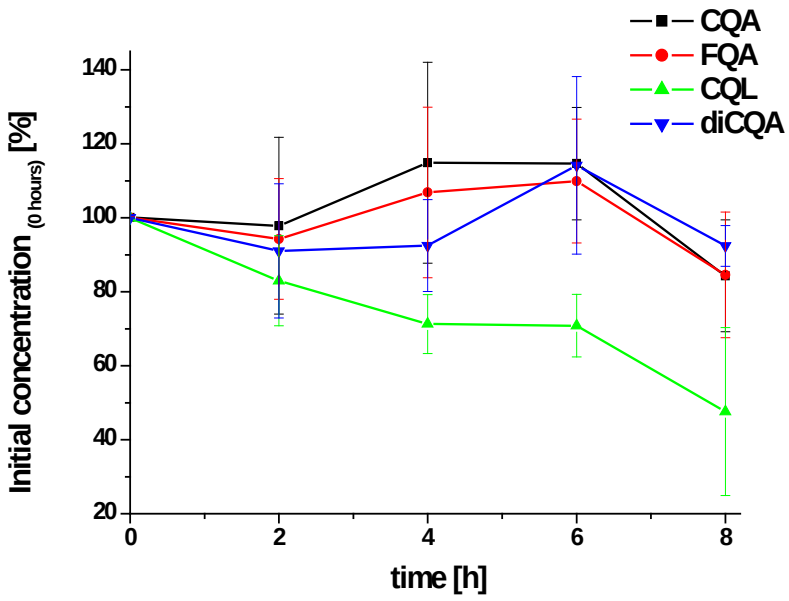


Figure 4-1: Stability of coffee CGA subgroups during *ex vivo* incubations with ileal fluid simulating the passage of the small intestine (n = 5). Values are shown in % of the initial concentration at time point 0 h (incubation conditions see chapter 7.3.4). CQA = caffeoylquinic acid (1a-d); FQA = feruloylquinic acid (3a-c); CQL = caffeoylquinide (2a-b), diCQA = dicaffeoylquinic acid (4a-c).

It was shown that the CGA from coffee under study were stable during the incubation time of eight hours with ileal fluid, despite caffeoylquinides (**2a-b**) (Figure 4-1).
The concentration of CQA (**1a-d**) and FQA (**3a-c**) slightly increased within the first 4 hours of incubations and for diCQA (**4a-c**) within the first 6 hours of incubation. This effect was also observed in incubations without ileal fluid (buffer), indicating a non microbial influence. Approximately 85% of the initial detected concentration of CQA (**1a-d**), FQA (**3a-c**) and diCQA (**4a-c**) remained stable in ileal fluid until the end of incubation (8 h), whereas the stabilities in the buffer (without ileal fluid) were lower especially for diCQA (**4a-c**) (Figure 4-2) (see Table 10-2, Appendix).

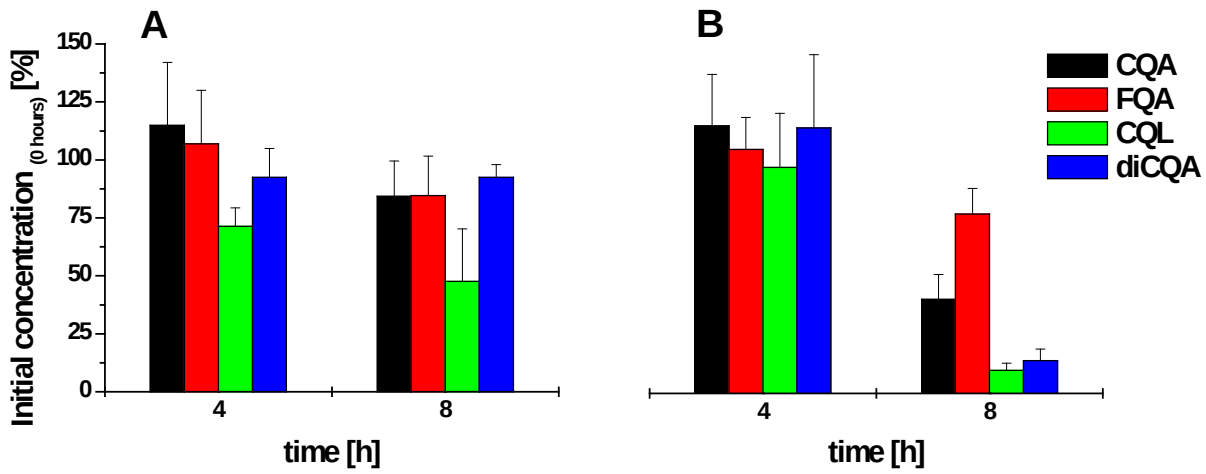


Figure 4-2: Stability of CGA from coffee in: A = ileal fluid (inoculum) (n = 5) and B = buffer solutions (n = 2) at representative time points of 4 and 8 hours (given in % of initial amount). Incubation conditions see chapter 7.3.4. CQA = caffeoylquinic acid (1a-d); FQA = feruloylquinic acid (3a-c); CQL = caffeoylquinide (2a-b), diCQA = dicaffeoylquinic acid (4a-c).

The incubation experiment using ileal fluid revealed that the CQL (**2a-b**) concentrations continuously decreased during the entire incubation time of 8 h and finally reached 48 ± 23% (mean value of 3- and 4-CQL) of the initial detected concentration at time point 0 h (see Figure 4-2 and Table 10-2, Appendix). Experiments using buffer media (without ileal fluid) showed that CQL remained more stable for the first 4 hours of incubation; followed by a strong decrease within the next 4 hours. Overall, the investigated compounds in the incubation buffer solutions showed reduced stability in comparison to the observed stabilities in the incubation

experiment with ileal fluid (see Figure 4-2). Nevertheless, free CA (**5a**) or FA (**7a**) as proposed hydrolysis products were detected only in inoculum incubations. Here, the degradation of coffee CGA in inoculum was apparently linked to the formation of free CA (**5a**) and FA (**7a**) (see Figure 4-3).

Additionally, we observed some interesterifications within the CGA subgroups, because the amount recovered at the end of incubations (8 hours) within a subgroup varied. For instance, about 94 ± 17% of the initial measured 3-CQA (**1b**) and 107 ± 25% of the initial measured 3-FQA (**3a**) concentration was found when incubated with ileal fluid. Contrary to this, about 74 ± 18% of the initial measured 5-CQA (**1d**) and 67 ± 14% of the initial measured 5-FQA (**3c**) concentration was found. This indicates a moderate interesterification within the CGA subgroups (see Table 10-2, Appendix).

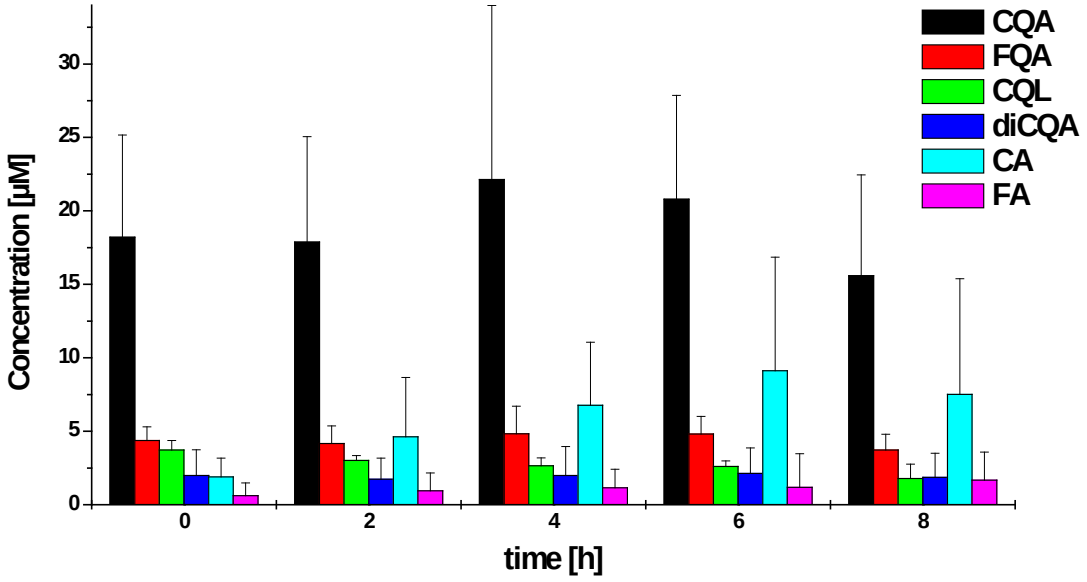


Figure 4-3: Degradation of CGA from coffee and formation of free caffeic acid (CA) (5a) and ferulic acid (FA) (7a) during incubations with ileal fluids (n = 5). Experimental conditions see chapter 7.3.4 (used concentration: 0.5 g instant coffee per L ileal fluid which corresponds to 104.7 µM CQA; 12.0 µM FQA; 6.3 µM CQL; 8.4 µM diCQA; 0.9 µM CA). CQA = caffeoylquinic acid (1a-d); FQA = feruloylquinic acid (3a-c); CQL = caffeoylquinide (2a-b), diCQA = dicaffeoylquinic acid (4a-c).

4.2 Enzyme activities of ileal fluid

Additionally to the *ex vivo* ileal fluid stability experiment with CGA from coffee, we determined in a semi-quantitative method the enzyme activities of ileal fluids. Different enzyme activities of ileal fluids from single volunteers could provide explanations for different CGA profiles and amounts excreted via ileal fluid in the dose-response study (see chapter 4.3).

The enzyme activities were checked with an Api®-ZYM test kit (see Figure 4-4) which was incubated with ileal fluids. After an incubation time of 4 hours enzymatic activities were detected by color reactions in individual micro wells. In all ileal fluids under study we observed esterase activity (test kit No. 3 and 4 in Table 10-3, Appendix). Despite the ileal effluent of ileo 5, a *β*-glucuronidase activity in the ileal fluids of ileo 1 to 4 was detected (micro well no. 15 in Table 10-3, Appendix).

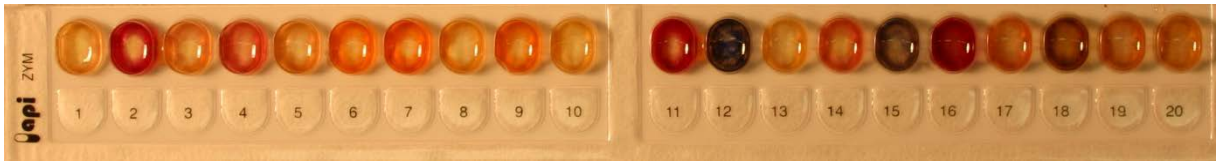


Figure 4-4: Representative Api®-ZYM test results after incubation with ileal fluid of ileostomist no. 3. The dark colored micro wells (1 to 20) indicate enzyme activities of ileal fluids, such as a *β*-glucuronidase activity in micro well no. 15. Coding of well no. (1 to 20) is given in Table 10-3, Appendix. Experimental conditions see chapter 7.3.4.6.

4.3 Human dose dependent intervention study with instant coffee

Until now the question of how the ingested doses of CGA and QA from coffee influence their absorption and metabolism remains unresolved. To assess absorption in the small intestine, we performed a dose-response study with a randomized, double-blinded, crossover design with five ileostomist subjects. After a two day polyphenol-free diet, in three separate occasions, the volunteers consumed, coffee with different total CGA contents (HIGH 4,525 µmol; MEDIUM 2,219 µmol; LOW 1,053 µmol) and free QA contents (HIGH 2,546 µmol; MEDIUM 1,373 µmol; LOW 695 µmol). CGA, QA and metabolite concentrations in plasma, ileal effluent and urine were subsequently identified and quantified by HPLC-DAD, -ESI-MS and -ESI-MS/MS.

4.3.1 CGA and QA contents of instant coffee brews used for the dose-response study

The main CGA found in the coffee brews under study were CQA (**1a-d**) with 1-CQA (**1a**) as a minor component, and 3-CQA (**1b**), 4-CQA (**1c**) and 5-CQA (**1d**) as major ones. The CGA contents of the three different coffee brews (soluble coffee dissolved in hot water) are summarized in Table 10-5, Appendix. Free caffeic acid (**5a**) was only detectable in low amounts. Other CGA, such as FQA (**3a-c**), diCQA (**4a-c**) and CQL (**2a-b**), were also detected in lower amounts than the CQA (see Figure 4-5).

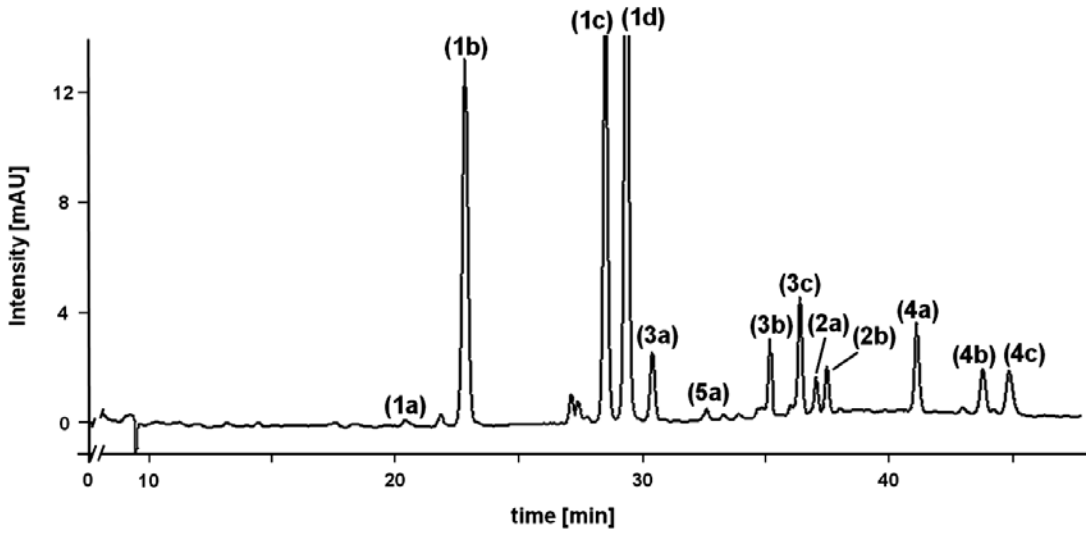


Figure 4-5: Representative HPLC-DAD chromatogram (at 320 nm) of an instant coffee brew (used in the intervention study for trial LOW = 6.5 g instant coffee powder reconstituted in 650 mL water). (1a): 1-CQA, (1b): 3-CQA, (1c): 4-CQA, (1d): 5-CQA, (3a): 3-FQA, (5a): CA, (3b): 4-FQA, (3c): 5-FQA, (2a): 3-CQL, (2b): 4-CQL, (4a): 3,4-diCQA, (4b): 3,5-diCQA, (4c): 4,5-diCQA. Unmarked peaks were not identified, (HPLC conditions see chapter 7.2.1: HPLC system I conditions 1a).

The total CGA contents in coffee brews consumed were 4,525 µmol (1,642 mg), 2,218 µmol (805 mg) and 1,053 µmol (382 mg) for the HIGH-, MEDIUM- and LOW- doses, respectively. This corresponds to an ingestion of 59.7 ± 2.0, 29.1 ± 1.5 and 13.8 ± 0.5 µmol*kg^{-1}*BW^{-1}. The average coffee volume was 381 ± 31 mL. The free D-(-)-quinic acid contents were 2,547, 1,373 and 695 µmol (HIGH; MEDIUM; LOW), respectively. This corresponds to an ingestion of 33.6 ± 3.6, 18.0 ± 1.7 and 9.1 ± 0.8 µmol*kg^{-1}*BW^{-1}. Individual CGA and QA amounts consumed under study are given in Figure 4-6.

The different CGA and QA concentrations in the consumed dosages also affected the pH of coffee brews served: 5.4 (HIGH); 6.0 (MEDIUM); 6.8 (LOW).

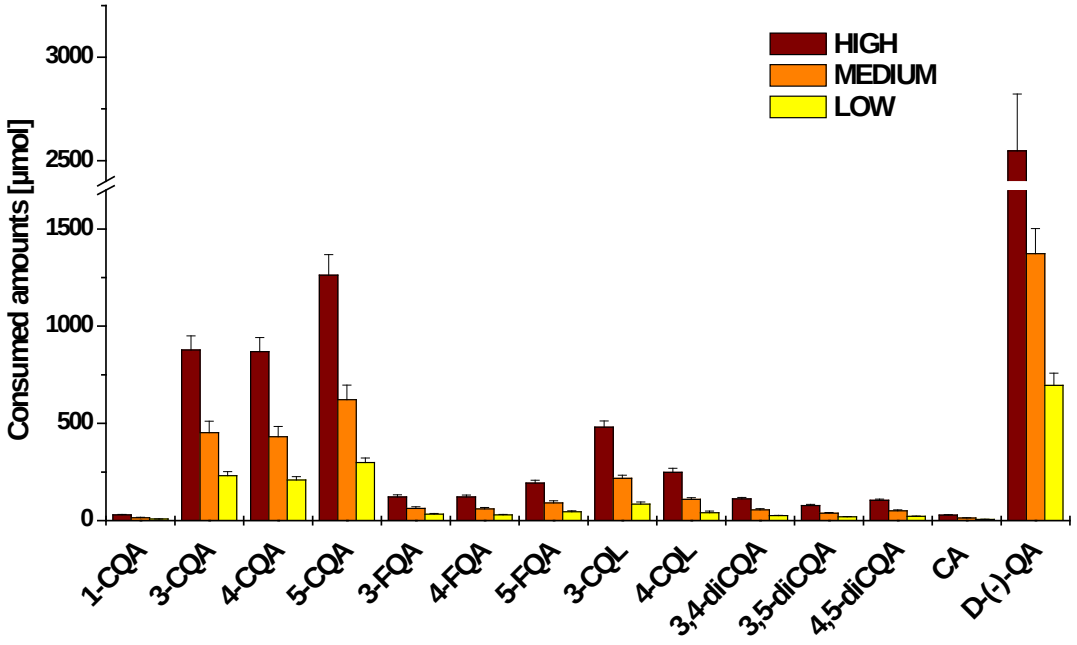


Figure 4-6: Amounts of CGA and QA (in µmol) in the three different coffee brews (HIGH, MEDIUM, LOW) in the double blinded randomized trials (n = 5). CQA = caffeoylquinic acid (1a-d); FQA = feruloylquinic acid (3a-c); CQL = caffeoylquinide (2a-b), diCQA = dicaffeoylquinic acid (4a-c), CA = caffeic acid (5a), QA = quinic acid (10a).

4.3.2 Ileal elimination of CGA, QA and metabolites after coffee consumption

Ileostomy fluids were collected - 12 to 0, 0, 0.5, 1, 2, 3, 4, 6, 8 hours *post* coffee consumption, frozen and subsequently freeze dried. CGA and QA were extracted by solid-liquid extraction and identified and quantified by HPLC-DAD, -ESI-MS and -ESI-MS/MS.

4.3.2.1 Identification of CGA, QA and metabolites in ileal fluids

Identification of CGA, QA and corresponding metabolites in ileal fluids was achieved by co-chromatography and matching MS^2 spectra with available reference compounds (see chapter 7.1.2). CGA compounds without available references were

assigned using literature data (Clifford et al., 2003; Stalmach et al., 2009; Marmet et al., 2014) and similar MS^2 spectra of corresponding precursor compounds. Glucuronides were additionally confirmed by neutral loss scan experiments (176 amu). A total of 37 CGA and corresponding metabolites were identified in ileal fluids after coffee consumption. An overview is given in Table 4-1.

Sulfates of CQA were detected with a precursor ion of *m/z* 433 and their characteristic product ions, especially the *m/z* 353 ion which indicates a loss of 80 amu. The fragmentation pattern of 3-*O*-CQA-sulfate (**1g**) shows the *m/z* 135 and *m/z* 191 product ion ratio similar to 3-*O*-CQA (**1b**). The 4-*O*-CQA-sulfate (**1h**) gave a characteristic *m/z* 173 product ion similar to 4-O-CQA (**1c**) and 5-*O*-CQA-sulfate (**1i**) showed the dominating *m/z* 191 ion similar to 5-O-CQA (**1d**). Furthermore, we were able to identify all CQA-sulfates conjugated at position 3´ of the caffeic acid moiety by co-chromatography using reference compounds (provided by Denis Barron from NRC). Two CQA-glucuronides were detected in the product ion scan mode at *m/z* 529 via the product ion *m/z* 353 indicating the loss of a glucuronide unit (176 amu). Identification was achieved according to similar MS^2 spectra of corresponding precursor compounds. 3-*O*-CQA-glucuronide (**1e**) was identified by the product ion ratio *m/z* 135 and *m/z* 191 and 4-*O*-CQA-glucuronide (**1f**) by the characteristic *m/z* 173 product ion. However, it was not possible to determine the conjugation position at the caffeic acid moiety due to of lack of references.

CQL-sulfates (**2e-f**) showed a $[M-H]^-$ of *m/z* 415. The product ion spectrum is dominated by *m/z* 161, typically for CQL, and *m/z* 335 is generated by a specific loss of 80 amu, according to the work of Stalmach and coworkers (Stalmach et al., 2009). CQL-glucuronides (**2c-d**) revealed a $[M-H]^-$ of *m/z* 511. The product ion spectrum was also dominated by *m/z* 161 and *m/z* 335 generated by a specific loss of 176 amu. Due to lack of references it was not possible to identify the conjugation position of the caffeic acid moiety.

Sulfates of FQA were detected by the precursor ion of *m/z* 447 and characteristic product ions of the FQA, especially the *m/z* 367 which indicates a loss of 80 amu. 3-*O*-FQA-4´-*O*-sulfate (**3g**) has similar product ions (*m/z* 193 and *m/z* 134) to 3-*O*-FQA (**3a**). 4-*O*-FQA-sulfate (**3h**) has the characteristic *m/z* 173 product ion similar to 4-O-FQA (**3b**). Confirmation was also achieved by co-chromatography and comparison of MS^2 fragmentation patterns with a reference mix of FQA-sulfates

(Menozzi-Smarrito et al., 2011). Three FQA-glucuronides were detected with a $[M-H]^-$ of *m/z* 529. The neutral loss scan mode and the product ion *m/z* 367 indicate the loss of a glucuronide unit (176 amu). 3-*O*-FQA-4´-*O*-glucuronide (**3d**) was identified by the product ion *m/z* 193 similar to 3-FQA (**3a**), 4-*O*-FQA-4´-*O*-glucuronide (**3e**) by the characteristic product ion *m/z* 173 typical for 4-FQA (**3b**) and 5-*O*-FQA-4´-*O*-glucuronide (**3f**) by the dominating *m/z* 191 product ion typical for 5-FQA (**3c**). Confirmation of feruloylquinic acid glucuronide was also performed by co-chromatography and comparison of MS^2 fragmentation patterns with a mix of feruloylquinic acid glucuronide references synthesized by Menozzi-Smarrito and coworkers (Menozzi-Smarrito et al., 2011).

The identification of isoferuloylquinic acid-glucuronide (IFQA-GlucA) was assumed by the data obtained from FQA-GlucA. So we assumed the same product ion characteristics as feruloylquinic acid glucuronides. We identified 3-*O*-IFQA-3´-*O*-glucuronide (**3i**) and 4-*O*-IFQA-3´-*O*-glucuronide (**3j**) corresponding to the MS^2 fragmentation pattern. Feruloylglycine was identified by confirming the MS^2 fragmentation pattern reported by Stalmach and coworkers. (Stalmach et al., 2009).

All CGA consumed via coffee brews by the volunteers were identified in the ileal fluids. We have observed an extensive metabolization in the upper parts of the GIT of ileostomists with 24 metabolites being formed (see Table 4-1). Among the group of CGA the dominating mechanisms were sulfation, glucuronidation and hydrolyzation.

Table 4-1: Product ion scan of chlorogenic acids and metabolites detected in ileal fluid after coffee consumption (HPLC-MS^2 conditions see chapter 7.2.2: HPLC system IV: conditions 1a).

	t_R	CE	(*m/z*)	
Compound	[min]	eV	$[M-H]^-$	product ion
3-*O*-FQA-*O*-GlucA **(3d)**	18.7	- 40	543	367; 193; 113; 175; 149; 85
3-*O*-CQA-3´-*O*-Sulf **(1g)**	19.0	- 40	433	353; 191; 179; 135
3-*O*-CQA-*O*-GlucA **(1e)**	20.0	- 40	529	353; 179; 191; 135
3-*O*-FQA-*O*-Sulf **(3g)**	20.1	- 40	447	367; 193; 191; 134; 173
1-*O*-CQA **(1a)**	20.9	- 50	353	191; 85
3-*O*-CQA **(1b)**	21.8	- 50	353	135; 191; 93
5-*O*-FQA-*O*-GlucA **(3f)**	22.2	- 40	543	367; 191; 113; 85; 173

Compound	t_R [min]	CE eV	(*m/z*) $[M-H]^-$	product ion
4-*O*-FQA-O-GlucA **(3e)**	23.0	- 40	543	367; 173; 193; 113; 85
DHCA-3´-*O*-Sulf **(6d)**	23.6	- 50	261	59; 80; 121; 135; 181
3-*O*-IFQA-*O*-GlucA **(3i)**	24.3	- 40	543	367; 193; 113; 173; 85
4-*O*-CQA-*O*-GlucA **(1f)**	24.5	- 40	529	353; 173; 179.0; 191; 135
4-*O*-CQA-3´-*O*-Sulf **(1h)**	24.5	- 40	433	353; 173; 179; 191; 135
4-*O*-FQA-O-Sulf **(3h)**	25.2	- 40	447	367; 173; 191; 193; 134
5-*O*-CQA-3´-*O*-Sulf **(1i)**	25.3	- 40	433	353; 191
CA-4´-*O*-Sulf **(5d)**	25.7	- 50	259	135; 107; 80; 179; 97
DHFA-4´-*O*-Sulf **(8b)**	25.7	- 40	275	59; 80; 135; 195
CA-3´-*O*-Sulf **(5e)**	27.4	- 50	259	135;107; 80; 97; 179
CA-3´-*O*-GlucA **(5c)**	27.7	- 40	355	135; 179; 85
DHCA **(6a)**	28.5	- 50	181	59; 41; 109; 93; 121; 138
4-*O*-CQA **(1c)**	29.0	- 50	353	135; 191; 93; 173
4-*O*-IFQA-O-GlucA **(3j)**	29.0	- 40	543	367; 173; 113; 193
FA-4´-*O*-Sulf **(7c)**	29.1	- 40	273	134; 178; 149; 193; 121; 97; 80
5-*O*-CQA **(1d)**	29.8	- 50	353	191; 85; 93; 127
3-*O*-FQA **(3a)**	30.0	- 50	367	134; 117; 193
IFA-3´-*O*-Sulf **(7f)**	31.0	- 40	273	134; 178; 193; 137; 149; 80
CQL-*O*-GlucA **(2c-2d)**	33.0	- 40	511	335; 161; 135; 179
CA **(5a)**	33.1	- 50	179	134
CQL-*O*-Sulf **(2e-2f)**	34.0	- 50	415	335; 161; 135; 173; 179
FA-glycine **(7d)**	34.3	- 30	250	206; 191; 177; 149; 163; 134; 100
4-*O*-FQA **(3b)**	34.6	- 50	367	134; 93; 173
5-*O*-FQA **(3c)**	36.0	- 50	367	93; 191; 134
3-*O*-CQL **(2a)**	36.7	- 50	335	161; 133
4-*O*-CQL **(2b)**	37.8	- 50	335	161; 133
FA **(7a)**	41.5	- 30	193	134; 178
3,4-*O*-diCQA **(4a)**	55.3	- 50	515.5	173; 179; 135; 191
3,5-*O*-diCQA **(4b)**	57.5	- 50	515.5	191; 179; 135
4,5-*O*-diCQA **(4c)**	63.2	- 50	515.5	173; 179; 135; 191

4.3.2.2 Quantification of CGA, QA and metabolites in ileal fluid

Determination of CGA in ileal fluid samples was performed by HPLC-DAD (see chapter 7.3.1.6.2.2), QA was quantified by LC-MS/MS using stable isotope dilution analysis (see chapter 7.3.1.6.3). Quantification of metabolites in ileal fluids was performed semi-quantitative by HPLC-MS in the multiple ion mode (see chapter

7.3.1.6.2.3). In Figure 4-7 a typical HPLC-MS chromatogram of caffeoyl quinic acid metabolites in the multiple ion mode (molecular ion $[M-H]^-$) is given.

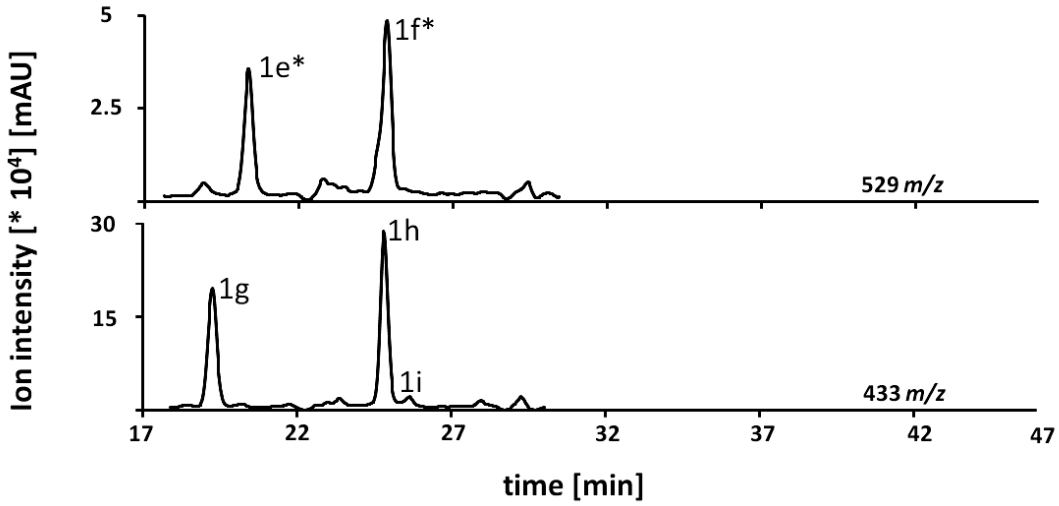


Figure 4-7: Typical HPLC-MS molecular ion $[M-H]^-$ chromatogram of caffeoylquinic acid glucuronides (*m/z* 529) and caffeoylquinic acid sulfates (*m/z* 433) detected in ileal fluid of ileo no. 2 two hours after coffee consumption (HIGH) in the multiple ion mode. Further chromatograms in the multiple ion mode (molecular ion $[M-H]^-$) are given in Figure 10-1, Appendix. Experimental conditions see chapter 7.3.1.6.2.3. 1e* = 3-*O*-CQA-*O*-GlucA; 1f* = 4-*O*-CQA-*O*-GlucA; 1g = 3-*O*-CQA-3´-*O*-Sulf; 1h = 4-*O*-CQA-3´-*O*-Sulf, 1i = 5-*O*-CQA-3´-*O*-Sulf. * = conjugation position was suspected at 3´.

4.3.2.3 Dose-response effects of different ingested CGA and QA doses

To assess dose-response effects on absorption in the upper GIT we determined the ileal excretion of CGA after the consumption of different CGA doses from coffee. All compounds identified in the coffee brews were recovered in the ileal fluids to a certain extent. In total, between 68.8 ± 9.0% (HIGH), 72.4 ± 4.7% (MEDIUM) and 77.4 ± 4.3% (LOW) of the ingested amounts were excreted via the ileal fluids. The total ileal excretions are shown in Table 10-7, Appendix.

Among the different polyphenol subgroups the ileal excretion rates showed minor variations (Table 10-6, Appendix). The ileal excretion rate of caffeoylquinides (**2a-b**) was considerably decreased (Table 10-6, Appendix).

The recovery rates of QA (**10a**) showed high inter-individual variations and were 86.5 ± 14.5% (HIGH); 81.9 ± 8.1% (MEDIUM) to 77.6 ± 17.5% (LOW) for subjects

1 to 4. For subject no. 5, QA ileal recovery was much lower: 38.1 ± 0.9% (HIGH); 23.9 ± 1.0% (MEDIUM); 12.5 ± 0.3% (LOW).

The dose-response effect of coffee CGA and QA consumption and excretion is shown in Figure 4-8. A higher consumption leads to a higher amount excreted ileally (Table 10-7, Appendix).

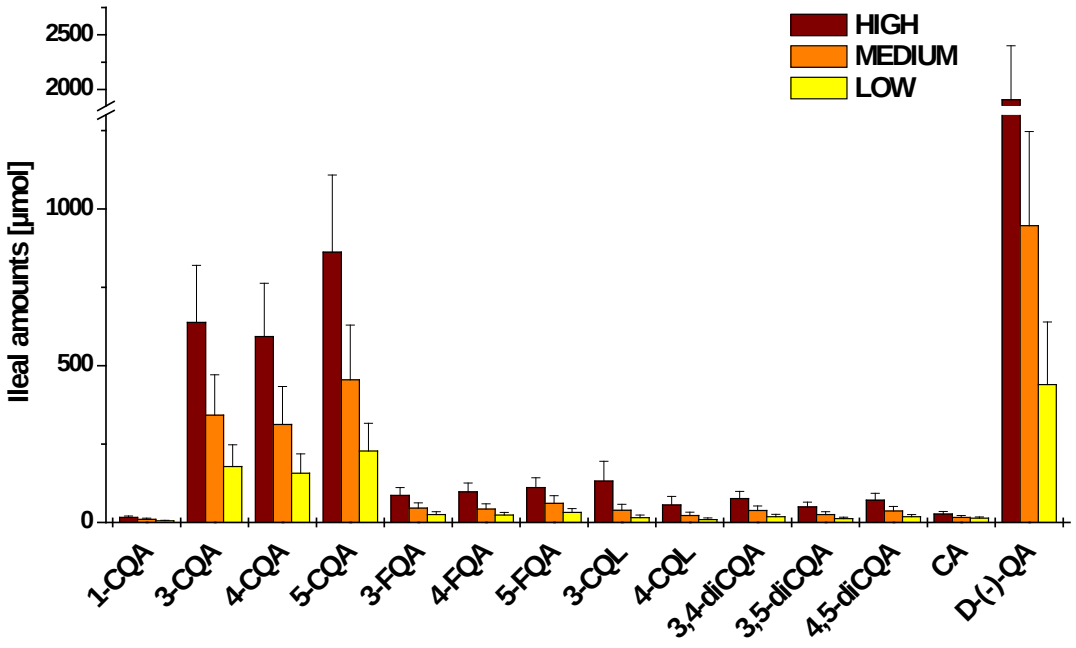


Figure 4-8: Dose-response relationships of CGA and QA (in µmol) excreted into ileal fluids of five probands after consumption of three different CGA doses from coffee (HIGH, MEDIUM, LOW) in double blinded randomized trials (n = 5). CQA = caffeoylquinic acids (1a-d); FQA = feruloylquinic acids (3a-c); CQL = caffeoylquinides (2a-b), diCQA = dicaffeoylquinic acids (4a-c), CA = caffeic acid (5a), QA = quinic acid (10a).

4.3.2.4 Dose-response effects of different ingested CGA and QA doses (by instant coffee brew) with focus on its metabolization in the upper gastrointestinal tract

Additionally to the observed amount of CGA excreted ileally, we determined the metabolization profile of CGA from coffee and the influence of the ingested doses on the metabolization profile.

About 6.7 ± 2.1% (HIGH), 7.7 ± 1.2% (MEDIUM) and 8.9 ± 1.3% (LOW) of the ingested CGA dose were metabolized or hydrolyzed during the passage of the GIT. Sulfation was the dominating conjugation, with a sulfation : glucuronidation ratio of 8.2 : 1 (HIGH), 10.4 : 1 (MEDIUM) and 13.4 : 1 (LOW).

Especially, CGA with a CA moiety were sulfated (Figure 4-9). Hereby the hydroxyl group at position 3´ of the CA molecule was important for conjugation reactions (Table 10-7, Appendix, Figure 2-1). Due to a lack of references, this could not be confirmed for CQA-GlucA. Further conjugation reactions observed were: methylation, verified by detected IFA compounds **(7f, 3i, 3j)**, glycine conjugation monitored by feruloylglycine **(7d)** (Figure 4-10) and dehydrogenase reactions monitored by dihydro compounds **(6a, 6d, 8b)**. Dimethylations **(9a + 9b)** were not observed in the ileal effluents.

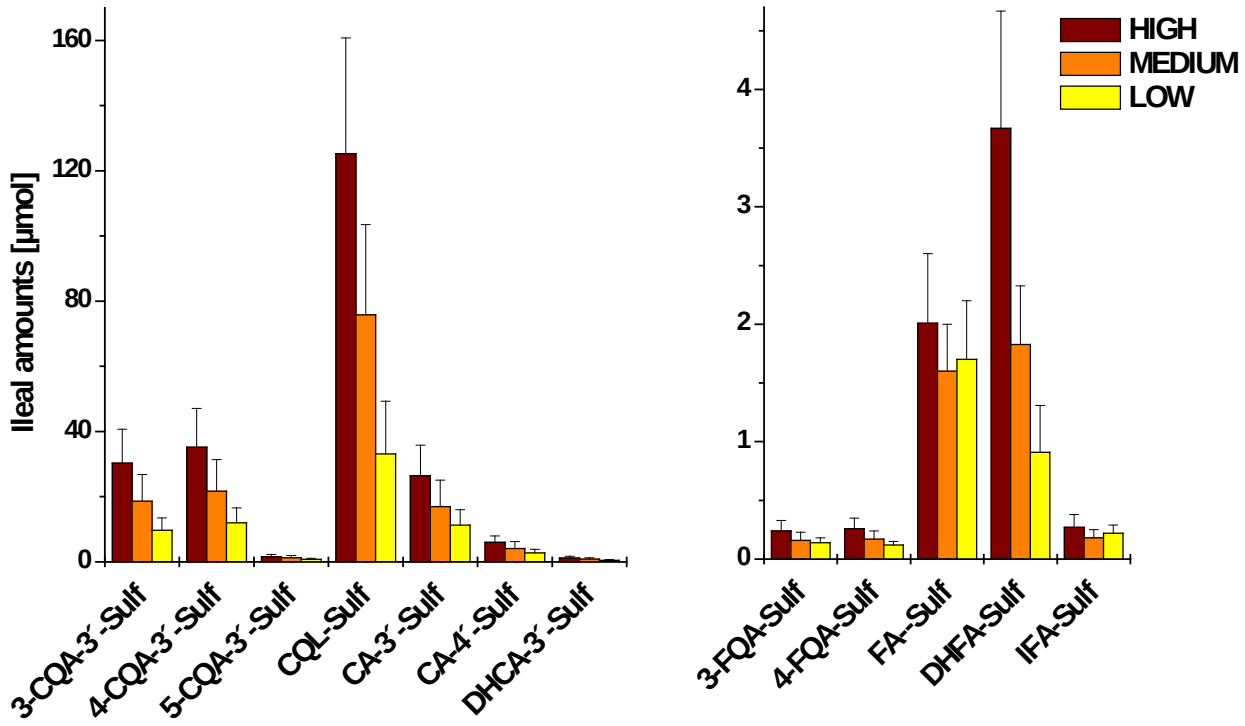


Figure 4-9: Total amounts of excreted sulfated CGA metabolites (in µmol) in ileal fluids of five probands after consumption of three different CGA doses via coffee brew (HIGH, MEDIUM, LOW) in double blinded randomized trials (n = 5). CQA = caffeoylquinic acid; FQA = feruloylquinic acid; CQL = caffeoylquinide, CA = caffeic acid; DHCA = dihydrocaffeic acid; FA = ferulic acid; IFA = isoferulic acid; DHFA = dihydroferulic acid; Sulf = sulfate.

Molecules esterified with QA (**10a**) at position 5 (5-CQA (**1d**), 5-FQA (**3c**)) dominate the polyphenol profile from coffee brews. Nevertheless, metabolization for these compounds was less (Figure 4-9 and Figure 4-10) as only small amounts of 5-CQA-3´-Sulf **(1i)** were detectable.

CA conjugates showed a relationship between the consumed CGA dose and the ileally excreted amount of CA conjugates. This characteristic was not as strong for the consumed FA (**7a**) and FA linked metabolites, especially FA-Sulf (**7c**) and IFA-Sulf (7f) (see Figure 4-9).

Furthermore, we detected a moderate hydrolytic activity, indicated by the high content of free CA (**5a**) and the occurrence of DHCA (**6a**) and FA (**7a**) in the ileal fluids (Figure 4-9 and Figure 4-10).

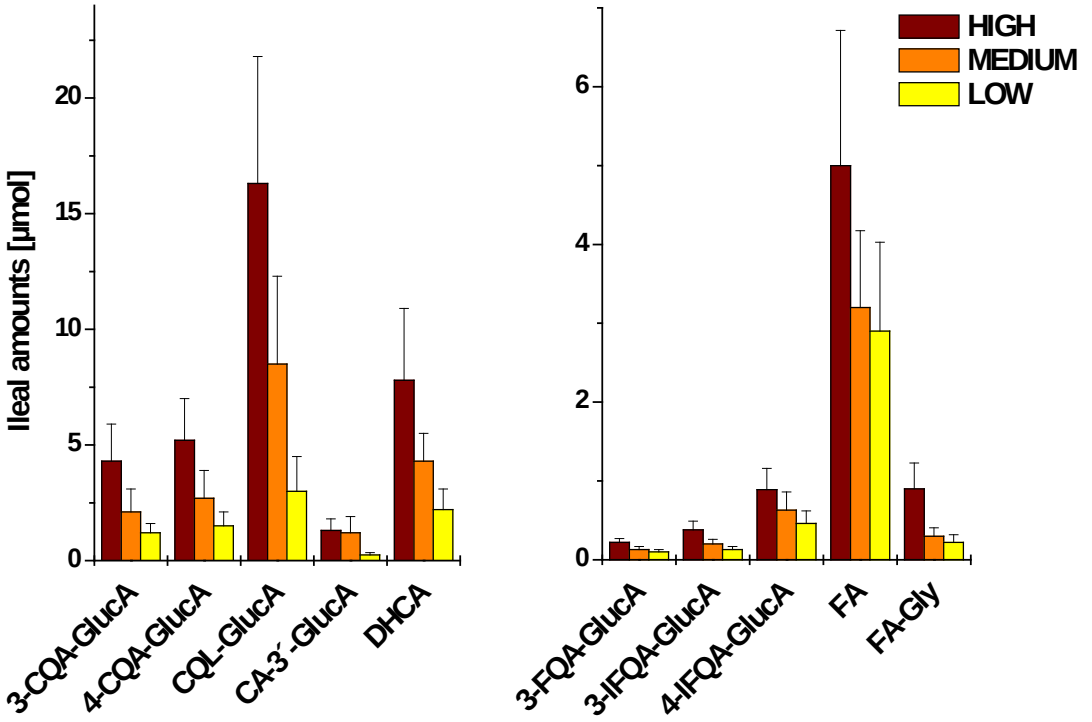


Figure 4-10: Total amounts of excreted glucuronidated, hydrolyzed and glycine conjugated CGA metabolites (in µmol) in ileal fluids of five probands after consumption of three different CGA doses via coffee brew (HIGH, MEDIUM, LOW) in double blinded randomized trials (n = 5). CQA = caffeoylquinic acid; FQA = feruloylquinic acid; IFQA = isoferuloylquinic acid; CQL = caffeoylquinide, CA = caffeic acid; DHCA = dihydrocaffeic acid; FA = ferulic acid; GlucA = glucuronide; Gly = glycine.

4.3.2.5 Physiological concentrations of CGA and QA in ileal fluids after coffee consumption

By determining the weight of ileal fluid and the total amounts of CGA being excreted into the ileostomy effluents it was possible to calculate the physiological CGA concentrations. The calculations revealed that the concentrations of the intact esters (CQA (**1a-d**), FQA (**3a-c**), and diCQA (**4a-c**)) in the excreted ileal fluids were, at their maximum, at least twice as high as in the coffee consumed and that the concentrations of CA and QA in the ileal excretion reached a multiple of their respective concentrations in the coffee brews. But no dose-response effects were observable between the consumption of different coffee concentrations and the maximum of physiological CGA concentrations (Table 10-9, Appendix).

4.3.2.6 Gastrointestinal transit time (GI-TT) of CGA, QA and metabolites after coffee consumption

As the gastrointestinal transit time (GI – TT) of a drug can have a major influence on its bioavailability we examined the ileal excretion T_{max} of 5-CQA (**1d**), the major coffee CGA component as a parameter of GI - TT. The passage time of consumed coffee through the upper gastrointestinal tract varied between the five subjects. In particular, the GI - TT of one subject (no 5) differed significantly (Figure 4-11, $p < 0.03$) in comparison to other volunteers. Independently of the coffee dose, subject 5 revealed a shift in passage time of several hours by $T_{max\ (5\text{-}CQA)}$ as shown in Figure 4-11. This finding was confirmed by the separate GI - TT determinations using an anthocyanin-rich (colored) beverage. In this study all volunteers showed a GI - TT of 45 min, despite of ileo 5 with a GI - TT of up to 5 hours.

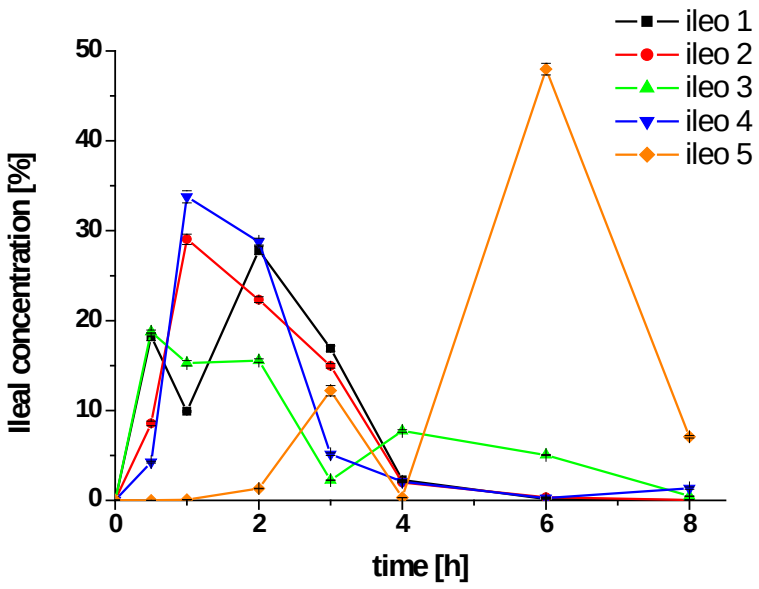


Figure 4-11: Representative kinetics of CGA and corresponding metabolites excreted via ileal fluid (% of ingested dose) (n = 5) after consumption of a MEDIUM coffee.

The mean $T_{max(5\text{-}CQA)}$ of ileo 1 to 4 accelerated with increasing consumed coffee dose: 0.5 ± 0.0 h (HIGH); 0.8 ± 0.3 h (MEDIUM), 1.6 ± 1.0 h (LOW): This effect was not determined for $T_{max(5\text{-}CQA)}$ of ileo 5: 4.0 h (HIGH); 6.0 h (MEDIUM) and 4.0 h (LOW). T_{max} of compounds originating in coffee were all in accordance to $T_{max(5\text{-}CQA)}$. Whereas the T_{max} of metabolites were delayed for approximately 1 hour (see Table 10-8, Appendix).

Only small amounts of CGA and metabolites were detectable in the ileal fluids (despite ileo no.5) eight hours after coffee consumption, indicating a completed GI - passage of compounds (see Figure 4-11).

4.3.3 Renal excretion of CGA, QA and metabolites

Urine samples were collected - 24 to 0, 0, 0.5, 1, 2, 3, 4, 6, 8, 8 to 24 and 24 to 48 hours *pre* and *post* coffee consumption, rapidly acidified and frozen at - 80 C°. QA was measured in urine by stable isotope dilution analysis without additional sample preparation. CGA and metabolites were isolated by solid-phase extraction (SPE). Identification and quantification were performed by HPLC-ESI-MS and -ESI-MS/MS.

4.3.3.1 Identification and quantification of CGA, QA and metabolites in urine samples

Identification of CGA, QA and corresponding metabolites in urine samples was performed by co-chromatography and matching MS^2 spectra with available reference compounds (see chapter 7.1.2) as already described in chapter 4.3.2.1. Some compounds without available references were assigned using literature data (Clifford et al., 2003; Stalmach et al., 2009; Marmet et al., 2014) and similar MS^2 spectra of corresponding precursor compounds. Glucuronides were confirmed additionally by neutral loss experiments (176 amu), as already described in chapter 4.3.2.1.

We identified up to 38 CGA (metabolized or unmetabolized) in the urine samples of the ileostomists after coffee consumption (Table 4-2). Furthermore, neither 1-CQA (**1a**), diCQA (**4a-c**) nor dimethylated **(9a + 9b)** compounds were detectable in the urine samples under study.

Quantification of CGA and corresponding metabolites in urine was performed as described in chapter 4.3.2.2 by HPLC-ESI-MS in the multiple ion mode (see chapter 7.3.1.5.2.2 and Figure 10-1, Appendix); for QA by HPLC-ESI-MS/MS in the multiple reaction monitoring mode (see chapter 7.3.1.5.3). In order to determine the concentration of CGA, QA, and corresponding metabolites in urine independent of the excreted urine volume we additionally measured the creatinine amounts in all urine samples (see chapter 7.3.1.5.4).

Table 4-2: Product ion scan (HPLC-MS^2) to identify chlorogenic acids and metabolites in urine samples after coffee consumption (HPLC-MS^2 conditions see chapter 7.2.2: HPLC system IV: conditions 1a). CQA = caffeoylquinic acid; FQA = feruloylquinic acid; IFQA = isoferuloylquinic acid, CQL = caffeoylquinide, diCQA = dicaffeoylquinic acid; CA = caffeic acid; DHCA = dihydrocaffeic acid; FA = ferulic acid; IFA = isoferulic acid; DHFA = dihydroferulic acid; GlucA = glucuronide; Sulf = sulfate.

	t_R	CE	(*m/z*)	
Compound	[min]	eV	$[M-H]^-$	product ion
3-*O*-FQA-*O*-GlucA **(3d)**	18.7	- 40	543	367; 193; 113; 175; 149; 85
3-*O*-CQA-3´-*O*-Sulf **(1g)**	19.0	- 40	433	353; 191; 179; 135
3-*O*-CQA-*O*-GlucA **(1e)**	20.0	- 40	529	353; 179; 191; 135
3-*O*-FQA-*O*-Sulf **(3g)**	20.1	- 40	447	367; 193; 191; 134; 173
3-*O*-CQA **(1b)**	21.8	- 50	353	135; 191; 93
5-*O*-FQA-*O*-GlucA **(3f)**	22.2	- 40	543	367; 191; 113; 85; 173
DHCA-4´-*O*-Sulf **(6c)**	22.9	- 50	261	59; 88
4-*O*-FQA-O-GlucA **(3e)**	23.0	- 40	543	367; 173; 193; 113; 85
DHCA-3´-*O*-Sulf **(6d)**	23.6	- 50	261	59; 80; 121; 135; 181
CA-4´-*O*-GlucA **(5b)**	24.0	- 40	355	135; 179
3-*O*-IFQA-*O*-GlucA **(3i)**	24.3	- 40	543	367; 193; 113; 173; 85
4-*O*-CQA-*O*-GlucA **(1f)**	24.5	- 40	529	353; 173; 179.0; 191; 135
4-*O*-CQA-3´-*O*-Sulf **(1h)**	24.5	- 40	433	353; 173; 179; 191; 135
4-*O*-FQA-O-Sulf **(3h)**	25.2	- 40	447	367; 173; 191; 193; 134
CA-4´-*O*-Sulf **(5d)**	25.7	- 50	259	135; 107; 80; 179; 97
DHFA-4´-*O*-Sulf **(8b)**	25.7	- 40	275	59; 80; 135; 195
DHCA-3´-*O*-GlucA **(6b)**	26.0	- 40	357	181; 85; 113; 59; 138
CA-3´-*O*-Sulf **(5e)**	27.4	- 50	259	135;107; 80; 97; 179
DHFA-4´-*O*-GlucA **(8a)**	27.4	- 40	371	85; 59; 195
CA-3´-*O*-GlucA **(5c)**	27.7	- 40	355	135; 179; 85
FA-4´-*O*-GlucA **(7b)**	28.0	- 40	369	134; 178; 193; 85; 59; 149
DHCA **(6a)**	28.5	- 50	181	59; 41; 109; 93; 121; 138
4-*O*-CQA **(1c)**	29.0	- 50	353	135; 191; 93; 173
4-*O*-IFQA-O-GlucA **(3j)**	29.0	- 40	543	367; 173; 113; 193
FA-4´-*O*-Sulf **(7c)**	29.1	- 40	273	134; 178; 149; 193; 121; 97; 80
5-*O*-CQA **(1d)**	29.8	- 50	353	191; 85; 93; 127
3-*O*-FQA **(3a)**	30.0	- 50	367	134; 117; 193
IFA-3´-*O*-Sulf **(7f)**	31.0	- 40	273	134; 178; 193; 137; 149; 80
IFA-3´-*O*-GlucA **(7e)**	32.0	- 40	369	178; 193; 134; 85; 59

Compound	t_R [min]	CE eV	(*m/z*) $[M-H]^-$	(*m/z*) product ion
CQL-*O*-GlucA **(2c-2d)**	33.0	- 40	511	335; 161; 135; 179
CA **(5a)**	33.1	- 50	179	134
CQL-*O*-Sulf **(2e-2f)**	34.0	- 50	415	335; 161; 135; 173; 179
FA-glycine **(7d)**	34.3	- 30	250	206; 191; 177; 149; 163; 134; 100
4-*O*-FQA **(3b)**	34.6	- 50	367	134; 93; 173
5-*O*-FQA **(3c)**	36.0	- 50	367	93; 191; 134
3-*O*-CQL **(2a)**	36.7	- 50	335	161; 133
4-*O*-CQL **(2b)**	37.8	- 50	335	161; 133
FA **(7a)**	41.5	- 30	193	134; 178

4.3.3.2 Dose-response effects of different ingested CGA and QA doses according to their total renal excretion and renally excreted metabolites

In order to observe dose-response effects on CGA bioavailability we measured the amount of CGA and conjugates in urine samples after the ingestion of increasing CGA doses from coffee. The total renal excretion of CGA differed considerably among the ileostomists; in particular, subject no. 5 showed a longer and significantly higher renal excretion compared to the other ileostomists ($p < 0.01$; Figure 4-15).

Between 8.0 ± 4.9% (HIGH), 12.1 ± 6.7% (MEDIUM) and 14.6 ± 6.8% (LOW) of the ingested CGA amounts and metabolites were detected in urine (Table 10-10, Appendix). Thus renal excretions of CGA and metabolites increased with decreasing coffee doses. We found 16.4 ± 6.0% (HIGH), 14.8 ± 1.5% (MEDIUM) and 13.7 ± 2.7% (LOW) of the ingested QA in urine after coffee consumption.

In contrast to the dominating 5-acyl compounds in the consumed coffees (5-CQA (**1d**), 5-FQA (**3c**), see Figure 4-6), in urine the unmetabolized 3-acyl compounds like 3-FQA (**3a**) and 3-CQA (**1b**) were dominating independent of the administered doses (Figure 4-12).

The maximum concentrations of CGA and metabolites in urine were calculated in nmol*g^{-1} creatinine (C_{max}) after ingestion of the three different CGA doses from coffee. C_{max} of CGA in urine was in relationship to the ingested dose for all CGA and QA (**10a**) (Table 10-12, Appendix).

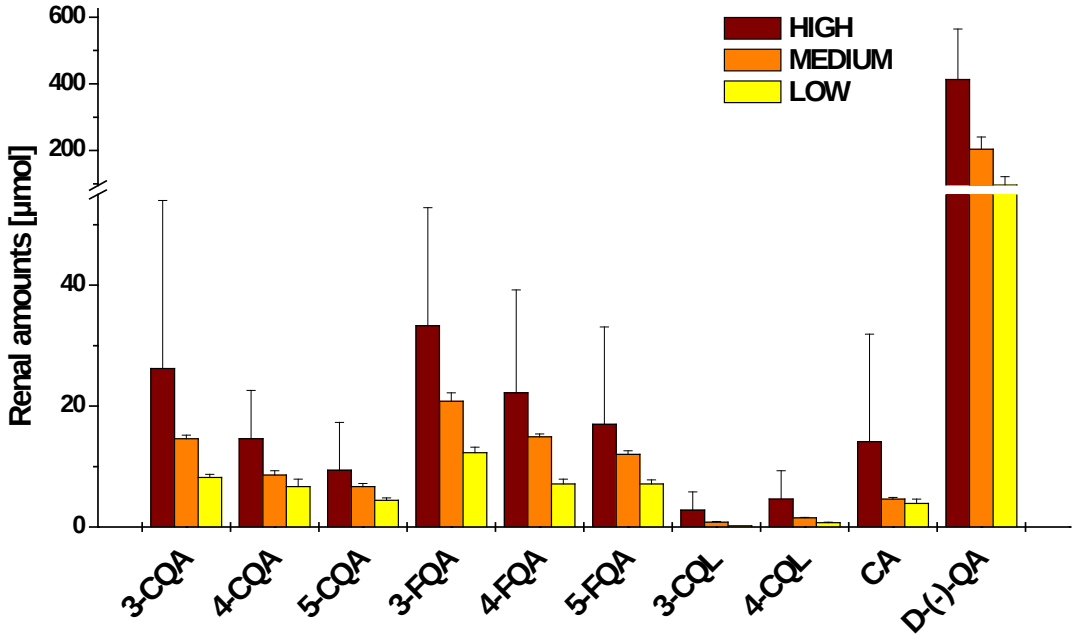


Figure 4-12: Amounts of CGA and D-(-)-quinic acid (QA (10a)) (in µmol) renally excreted in five probands after consumption of three different CGA doses via coffee brew: (HIGH, MEDIUM, LOW) in double blinded, randomized trials (n = 5). CQA = caffeoylquinic acid; FQA = feruloylquinic acid; CQL = caffeoylquinide; CA = caffeic acid.

In order to asses dose-response effects on the routes by which CGA are metabolized, we detected the renally excreted CGA conjugates. The rate of metabolized or hydrolyzed CGA was 67.0 ± 6.6% (HIGH), 69.2 ± 6.5% (MEDIUM) and 69.8 ± 3.4% (LOW) with a sulfation : glucuronidation ratio of 0.7 : 1 (HIGH), 1 : 1 (MEDIUM) and 1.3 : 1 (LOW) (for details see, Table 10-10, Appendix). Similar to the metabolization profile in the ileal fluids, no metabolites of the 5-CQA (**1d**) or 5-FQA (**3c**) were detected in urine samples. Further, the 3´position of the caffeic acid moiety was preferred for sulfation.

The influence of increasing CGA doses consumed with coffee on metabolization is shown in Figure 4-13 and Figure 4-14.

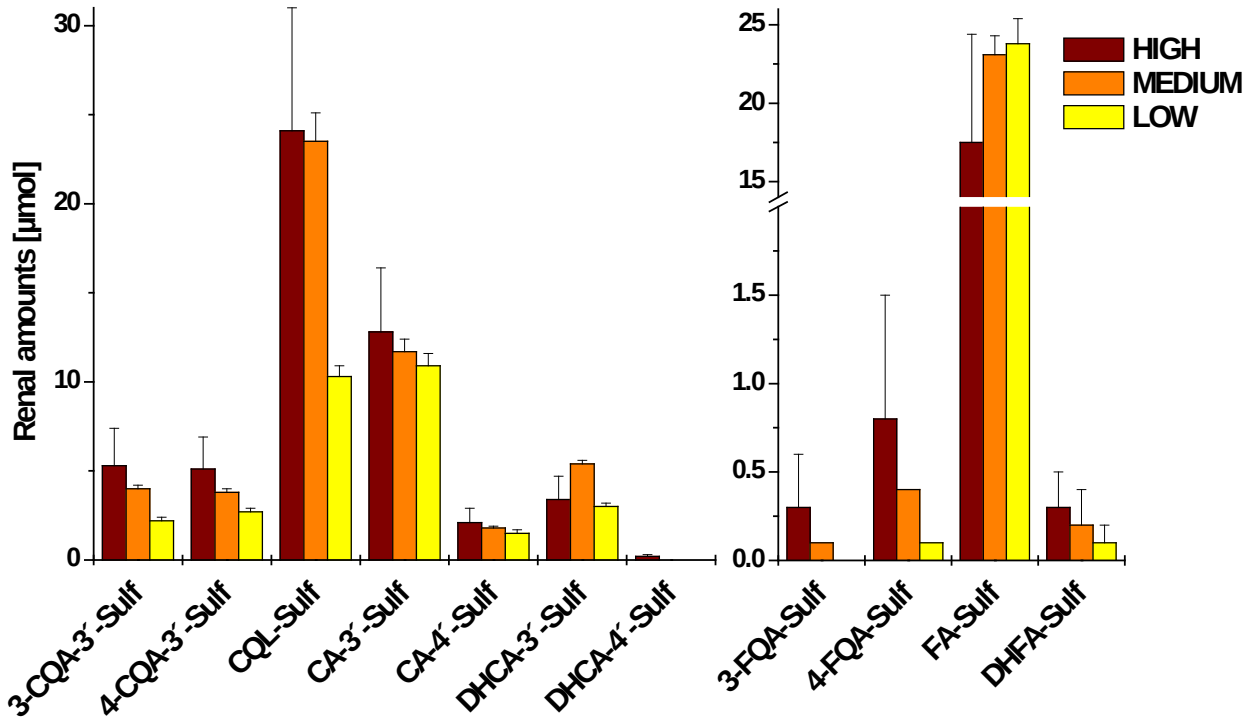


Figure 4-13: Summary of sulfated CGA metabolites (in µmol) renally excreted in five probands after consumption of three different CGA doses via coffee brews: (HIGH, MEDIUM, LOW) in double blinded, randomized trials (n = 5). CQA = caffeoylquinic acid; FQA = feruloylquinic acid; CQL = caffeoylquinide; CA = caffeic acid; DHCA = dihydrocaffeic acid; FA = ferulic acid; DHFA = dihydroferulic acid; Sulf = sulfate.

After the consumption of the highest coffee dose (HIGH) no increase of the sulfated compounds: CQL-Sulf **(2e + 2f)**, CA-3´-Sulf **(5e)**, DHCA-3´-Sulf **(6d)**, FA-Sulf **(7c)** and FA-Gly **(7d)** and DHFA-GlucA **(8a)** was observed.

In addition, no sulfation of the hydroxycinnamic acid IFA (**7g**) took place during all three trials - only glucuronidation was observed (Figure 4-14).

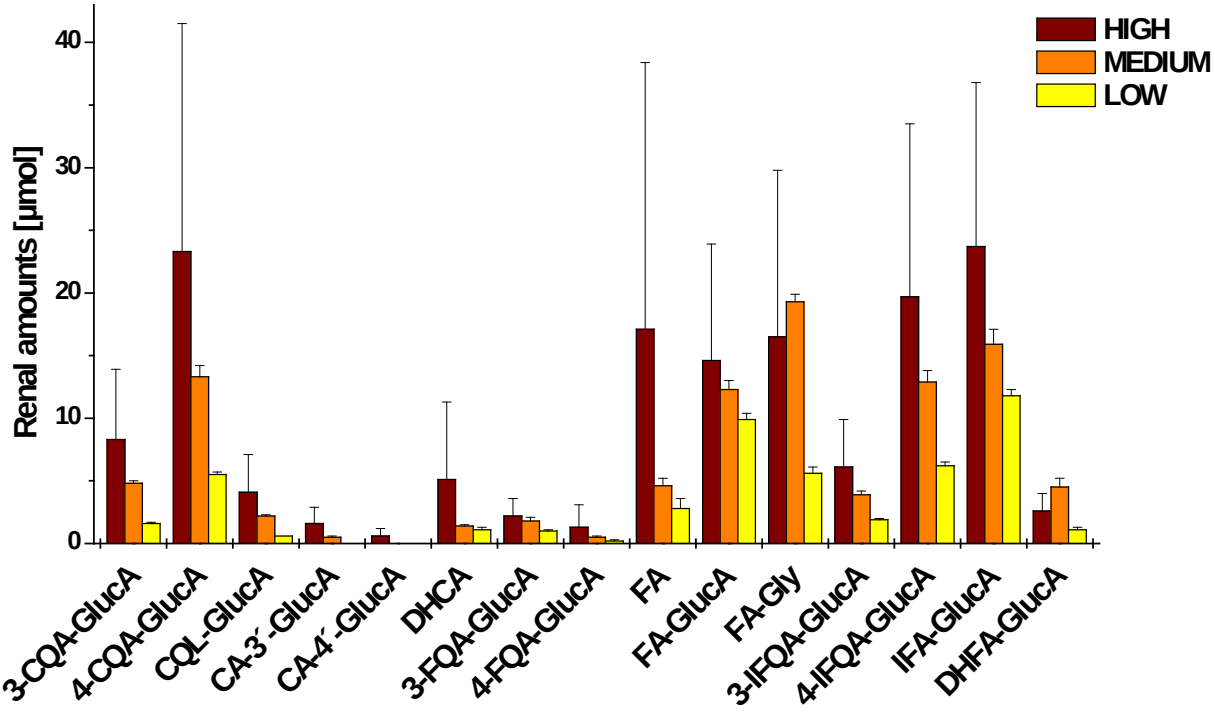


Figure 4-14: Total amounts of excreted glucuronidated, hydrolyzed and glycine conjugated CGA metabolites renally excreted (in µmol) in five probands after consumption of three different CGA doses via coffee brews: (HIGH, MEDIUM, LOW) in double blinded, randomized trials (n = 5). CQA = caffeoylquinic acid; FQA = feruloylquinic acid; IFQA = Isoferuloylquinic acid; CQL = caffeoylquinide; CA = caffeic acid; DHCA = dihydrocaffeic acid; FA = ferulic acid; IFA = isoferulic acid; DHFA = dihydroferulic acid; GlucA = glucuronide; Gly = glycine.

4.3.3.3 Kinetics of CGA, QA and corresponding metabolite being renally excreted after coffee consumption

Kinetics of maximum concentration of CGA, QA and corresponding metabolites in urine samples after consumption of the different coffee doses were similar in the samples of all volunteers (Table 10-11, Appendix). The maximum concentrations of 5-CQA (**1d,** $T_{max(5\text{-}CQA)}$) in urine were observed at: 2.0 ± 0.0 h (HIGH); 2.0 ± 0.0 h (MEDIUM); 2.6 ± 1.2 h (LOW). Ileostomist 5 showed a prolonged renal excretion time (Figure 4-15) and thus, a significantly higher amount excreted (**$p < 0.01$). The time points of maximum renal QA (**10a**) excretions were also similar for all of the

volunteers and showed an extended $T_{max(QA)}$: 6.2 ± 2.5 h (HIGH), 5.6 ± 1.2 h (MEDIUM) and 7.4 ± 1.2 h (LOW).

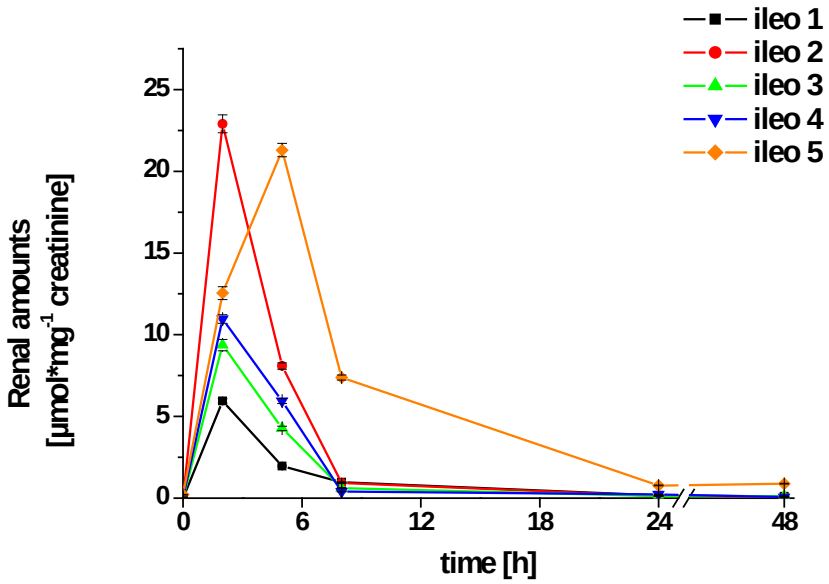


Figure 4-15: Representative kinetics of the sum of chlorogenic acids and corresponding metabolites (in µmol*mg^{-1} creatinine) detected in urine samples of ileostomists (n = 5) after coffee consumption of a MEDIUM coffee dose.

4.3.4 CGA and QA amounts in plasma

Blood samples were collected at the following time points: 0, 0.5, 1, 2, 4, 6 and 8 hours *post* coffee consumption, immediately centrifuged to generate plasma, subsequently stabilized and frozen at - 80°C (for details see chapter 7.3.1.7.1). At some study trials (LOW, MEDIUM, and HIGH dose consumption) data of one ileostomist was missing therefore, all plasma data was shown as the mean of 4 ileostomists (n = 4) instead of five (for details see chapter 7.3.1.7.1 and 7.3.1.7.2.2).

Due to the lack of references for quantification, the concentrations of CGA in plasma were measured after enzymatic hydrolysis of the sulfates and glucuronides by HPLC-MS/MS analysis in the selected reaction monitoring (SRM) mode (see chapter 7.3.1.7.2.1). The quantification of QA was performed by stable isotope dilution analysis by HPLC-MS/MS in the MRM mode as described in chapter 7.3.1.7.3.

4.3.4.1 Dose-response effects of different ingested CGA and QA doses with focus on its plasma concentration

We determined increasing CGA concentrations in plasma correlating with an increased consumption of CGA dose from the coffee beverages. The overall AUC of all CGA were 4,412 ± 751 nM*h_{0-8}^{-1} (HIGH), 2,394 ± 637 nM*h_{0-8}^{-1} (MEDIUM) 1,782 ± 731 nM*h_{0-8}^{-1} (LOW). AUC for plasma QA were 24.4 ± 5.6 µM*h_{0-8}^{-1}(HIGH), 14.8 ± 5.0 µM*h_{0-8}^{-1} (MEDIUM) and 8.0 ± 0.5 µM*h_{0-8}^{-1} (LOW), respectively.

No diCQA (**4a-c**) were detectable in plasma after consumption of the different coffee doses. All compounds, except for IFA (**7g**) and DiMeDHCA (**9b**), showed a dose-dependent relationship in plasma AUC or C_{max} (Figure 4-16; Table 10-13, Appendix).

In contrast to the CGA profile in the consumed coffee, (5-acyl compounds are dominating, see Figure 4-6) the 4-acyl compounds of CQA (**1c**) and FQA (**3b**) were dominating in plasma (Table 10-13, Appendix) indicating a possible acyl migration.

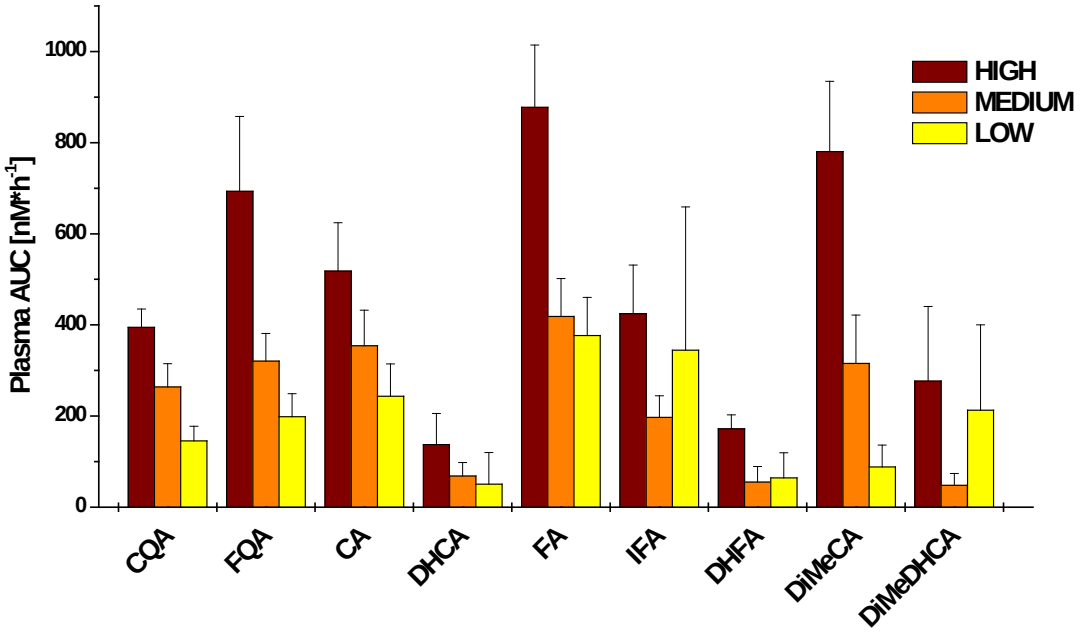


Figure 4-16: Summary of area under the curves (AUC) of plasma samples (in nM*h_{0-8}^{-1}) of four probands after the consumption of HIGH, MEDIUM and LOW CGA doses by coffee (data expressed as mean values ± SD; n = 4). Plasma samples were enzymatically treated (glucuronidase and sulfatase), for details see chapter 7.3.1.7.1. CQA = caffeoylquinic acid; FQA = feruloylquinic acid; CA = caffeic acid; DHCA = dihydrocaffeic acid; FA = ferulic acid; IFA = isoferulic acid; DHFA = dihydroferulic acid; DiMeCA = dimethoxycaffeic acid; DiMeDHCA = dimethoxydihydrocaffeic acid.

4.3.4.2 Kinetics of plasma CGA, QA and corresponding metabolite concentrations after coffee consumption

After one hour nearly all CGA were almost at their maximal plasma concentration in each trial (see Table 10-13, Appendix). Volunteer 5 showed a slower elimination of CGA in comparison to the other volunteers (Figure 4-17) and thus, AUC was significantly increased ($p < 0.05$).

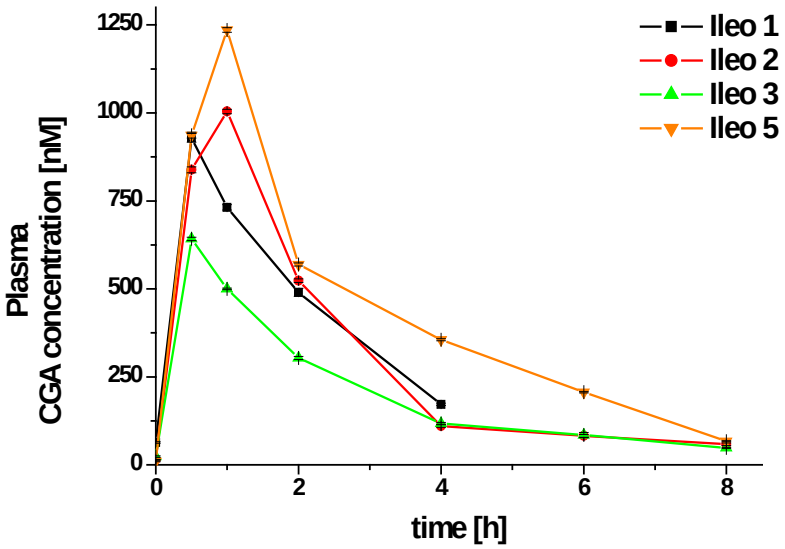


Figure 4-17: Representative kinetics of CGA and corresponding metabolites plasma concentrations (after enzyme hydrolysis) after consumption of a MEDIUM coffee dose by four ileostomist (ileo), (n = 4). Ileo 4 not shown (see chapter 7.3.1.7.1).

In all volunteers the plasma samples of the QA (**10a**) concentrations reached their maxima within about 4.5 hours after coffee consumption - much later than the max of CGA (see Figure 4-18 and Table 10-13, Appendix). Only a small decrease of QA was observed eight hours after coffee consumption (see Figure 4-18). Thus, elimination of QA from plasma was not complete eight hours after coffee consumption.

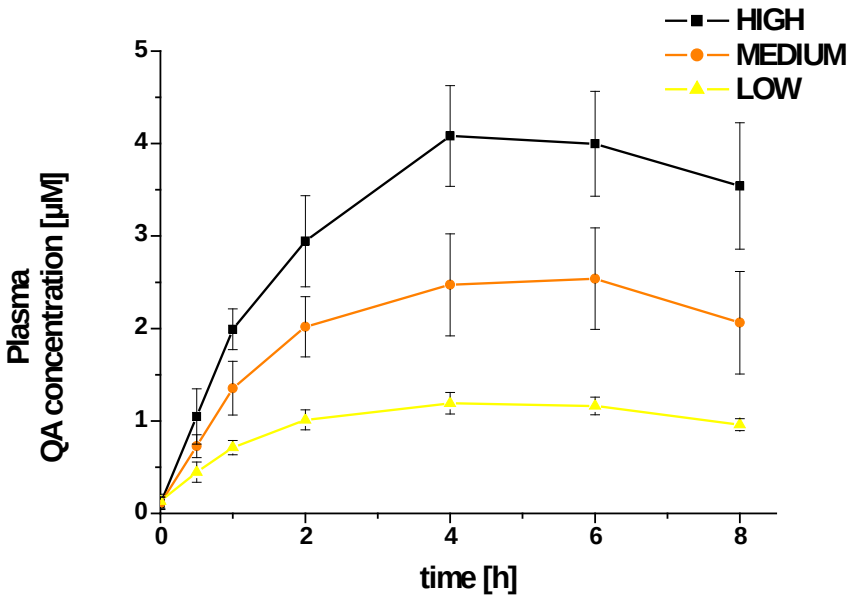


Figure 4-18: Plasma concentrations of free D-(-)-quinic acid (10a), after the consumption of three different CGA and QA doses from coffee (HIGH, MEDIUM and LOW) by four ileostomists (data expressed as mean values ± SD; n = 4).

4.4 Individual coffee CGA and QA absorption (*ex vivo* in the pig jejunum Ussing model)

The influences of CGA structural properties on bioavailability are not clearly observable as CGA underlie an extensive biotransformation. Furthermore, *in vivo* several physiological factors such as GI - TT may influence CGA absorption. Therefore, we performed an *ex vivo* transport experiment using pig jejunal mucosa in the Ussing chamber model (with standardized experimental conditions, see chapter 7.3.2) to investigate the influence of the molecular properties / chemical structure (SAR) and doses (DAR) on absorption of CGA and QA in the upper GIT. In order to use relevant physiological concentrations we calculated the maximum concentrations in the upper GIT after coffee consumption (LOW) using data from the intervention study with five ileostomists, see chapter 4.3. It was possible to calculate CGA concentrations by the use of ileal fluid weight and the total amounts of CGA excreted after coffee consumption (LOW) into the ileal effluents (Table 10-9, Appendix).

4.4.1 Absorption (to serosal direction)

Ussing chamber experiments conducted with physiologically relevant concentrations (20 µM, 4 h incubation) of 12 CGA and QA indicated a relationship between absorption and molecular properties (such as molecular structure (see chapter 8) and log D (Table 5-1, Discussion, calculated with MARVIN SKETCH 5.3.1). In increasing order, the amounts of initially applied compounds that passed through the jejunal mucosa from the mucosal (pH 6.0) to the serosal side (pH 7.4) were (approximately) 1% for CQA (**1a-d**), 1.5% for CA (**5a**), 2% for FQA (**3a-c**), and 4% for QA (**10a**) (Table 10-14, Appendix); only trace amounts of diCQA (**4a-c**) were detected on the serosal side. No significant differences were detectable within the CQA (**1a-d**) and FQA (**3a-c**) subgroups (Figure 4-19).

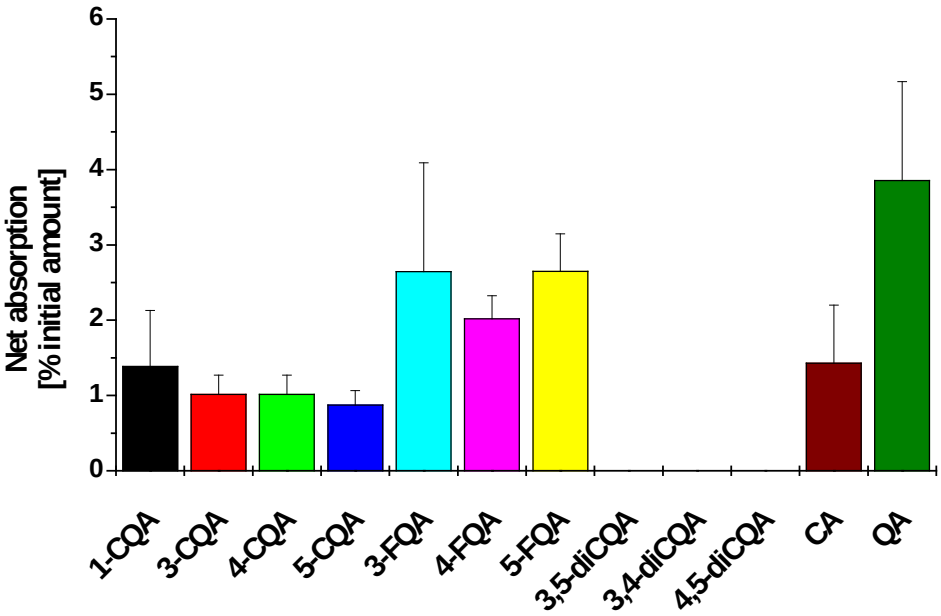


Figure 4-19: Percentages (% of initial amounts) CGA and D-(−)-quinic acid (20 µM) recovered at the serosal side of pig mucosa after 4 h incubation (pH 6 at luminal side, pH 7.4 at serosal side) (mean ± SD; n = 4). Experimental design see chapter 7.3.2.3. CQA = caffeoylquinic acids (1a-d); FQA = feruloylquinic acids (3a-c); diCQA = dicaffeoylquinic acids (4a-c), CA = caffeic acid (5a), QA = quinic acid (10a).

The kinetics of transport to the serosal side revealed an increase in absorption through the jejunal tissue during the entire incubation time. In short, the transport was

directly proportional to the serosal concentration for all investigated compounds (Figure 4-20, Table 10-15, Appendix).

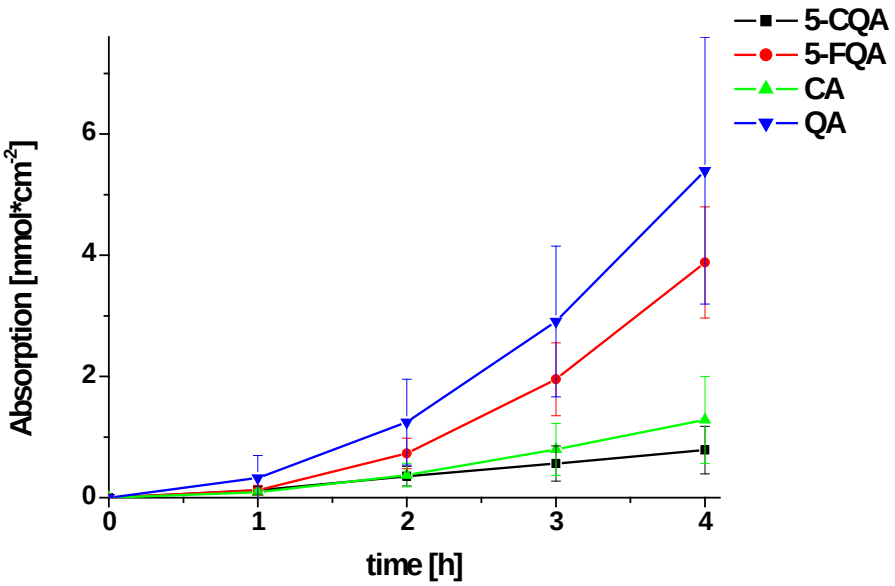


Figure 4-20: Transport kinetics (in nmol*cm^{-2}) to the serosal side for 5-caffeoylquinic acid (5-CQA (1d)), 5-feruloylquinic acid (5-FQA (3c)), quinic acid (QA (10a)) and caffeic acid (CA (5a)) for 4 h at 20 µM. Experimental details see chapter 7.3.2.3.

4.4.2 CGA and QA amounts associated with the pig jejunal mucosa

The analytical method used for CGA extraction (see chapter 7.3.2.6, material and methods section) did not distinguish between cytosolic or tissue associated CGA. Therefore, CGA were detected on or in the tissue samples (tissue associated) in amounts of 1 to 1.5% of the initially applied amount (see Figure 4-21), except for diCQA (**4a-c**) detected in lower amounts. Approx. 0.2% were detected for 3,4-diCQA (**4a**) and 3,5-diCQA (**4b**) and trace amounts for 4,5-diCQA (**4c**).

When 5-CQA (**1d**) (20 µM) was incubated at the serosal compartment 0.5 ± 0.3% and when co-incubated with the metabolic inhibitor NaN_3 0.8 ± 0.3% of the amount applied initially was recovered.

In the DAR experiment with increasing 5-CQA (**1d**) concentrations (0.02 to 3.5 mM) 0.6 ± 0.1% (at 0.5 mM) up to 1.9 ± 0.6% (at 3.5mM) were associated with the tissue (see Table 10-14, Appendix).

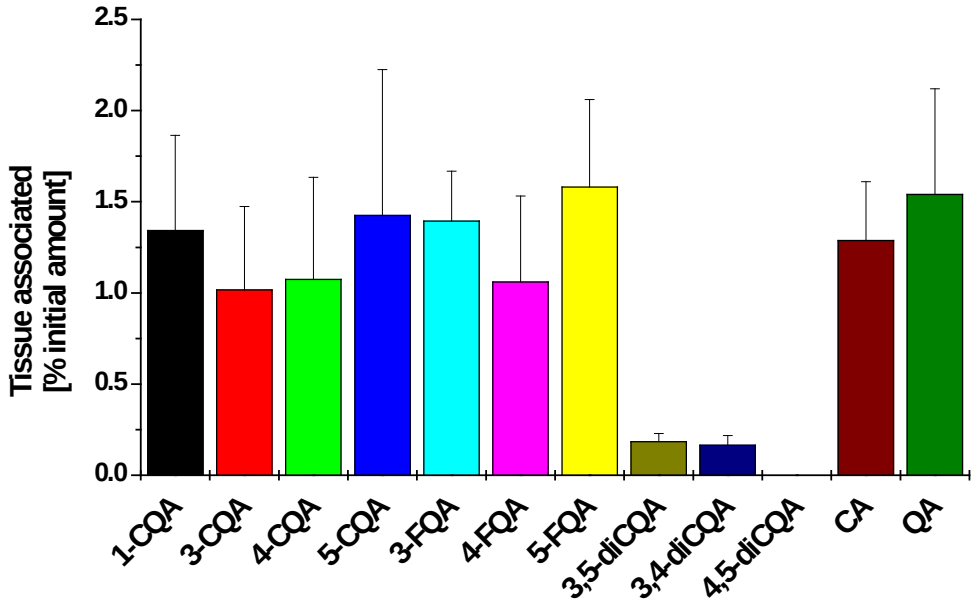


Figure 4-21: Percentages (% of initial amounts) of CGA associated with pig jejunal mucosa after 4 h of incubation at 20 µM (pH 6 at luminal side, pH 7.4 at serosal side, mean ± SD; n = 4). CQA = caffeoylquinic acid; FQA = feruloylquinic acid; CA = caffeic acid; diCQA = dicaffeoylquinic acid; QA = D-(-)-quinic acid. Experimental details see chapter 7.3.2.3.

All data is provided in detail in Table 10-14, Appendix.

4.4.3 Secretion of 5-caffeoylquinic acid (5-CQA) (to luminal direction)

In the CGA secretion experiment (sample applied at serosal side and transfer to luminal direction was monitored) 5-CQA (**1d**) (20 µM) was applied at the serosal side (pH 7.4) and the amount transferred to the mucosal compartment (pH 6.0) was measured. Secretion occurred at a significantly higher rate (2.3 ± 0.6%) than absorption (0.9 ± 0.2%; $p < 0.001$). When the metabolic inhibitor NaN_3 was used a significant decrease in secretion was observed (1.3 ± 0.5%; $p < 0.05$; see Table 10-14, Appendix, Figure 4-22). This provides evidence for an active-transport inhibition.

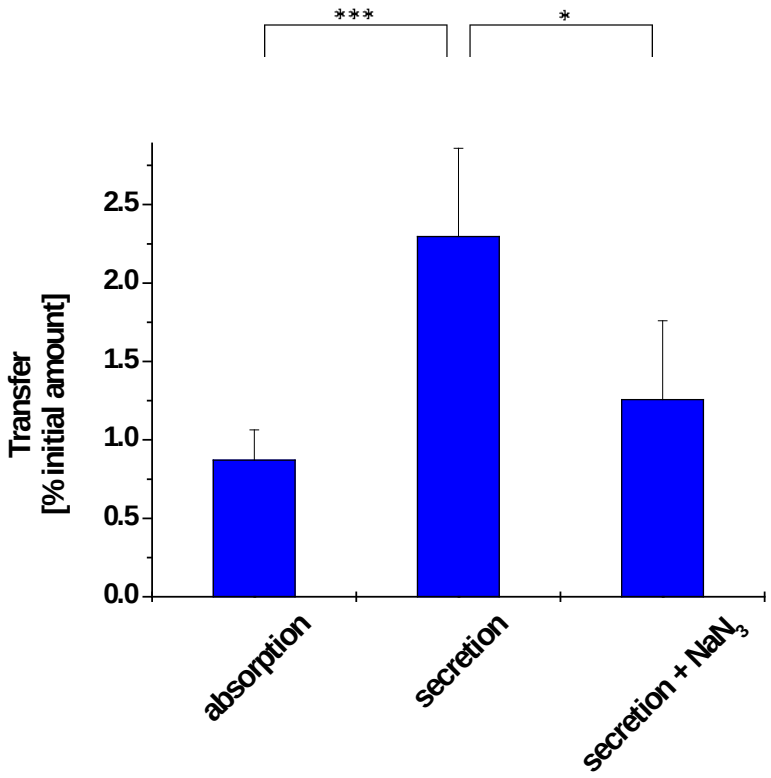


Figure 4-22: Total absorption and secretion of 5–CQA (% of initial amounts) through the pig jejunal mucosa after 4 h incubation at 20 µM (*$p < 0.001$). 5-CQA was applied on either the mucosal side (absorption) or the serosal side (secretion) and coincubation of NaN_3 (10 mM) with 5-CQA on the serosal side (*$p < 0.05$), (pH 6 at luminal side, pH 7.4 at serosal side) (charted data are mean ± SD; n = 4.)**

4.4.4 Identification of ABC-transporters in the pig jejunal mucosa

As the experiments with the metabolic inhibitor NaN_3 (see chapter 4.4.3) indicated an active CGA secretion, we performed Western blot experiments with pig jejunal mucosa to identify active efflux transporters. The ABC efflux transporters MRP 2 and the P-gp transporter MDR 1 were identified in pig jejunal mucosa for the first time (see Figure 4-23) with the support of Johanna Hauser (diploma thesis), (see chapter 7.3.3 for experimental conditions).

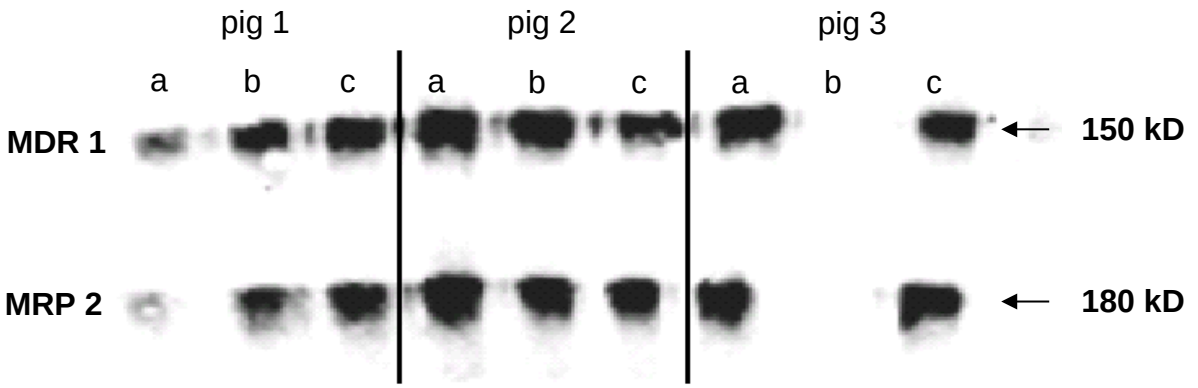


Figure 4-23: Western blot analysis of the ABC-transporters MDR 1 (MW = 150 kD) and MRP 2 (MW = 180 kD) in the pig jejunal mucosa. Analytical conditions see chapter 7.3.3 (Western blot analysis was performed by Johanna Hauser).

4.4.5 Dose-dependent transfer rates of 5-CQA (to serosal direction)

In the dose-absorption experiment using pig mucosa and 5-CQA (applied at doses of 0.02 to 3.5 mM), the total amount transported to the serosal side was approximately 1% independent of the applied dose (Table 10-14, Appendix). Saturation of absorption was not observed up to the highest concentration of 3.5 mM.

However, after 2 h of incubation the flux (dose–transport rate in $nmol*h^{-1}*cm^{-2}$) relationship was linear (Figure 4-24) indicating a passive diffusion of 5-CQA (**1d**) (*cf*. Figure 2-9, see State of knowledge).

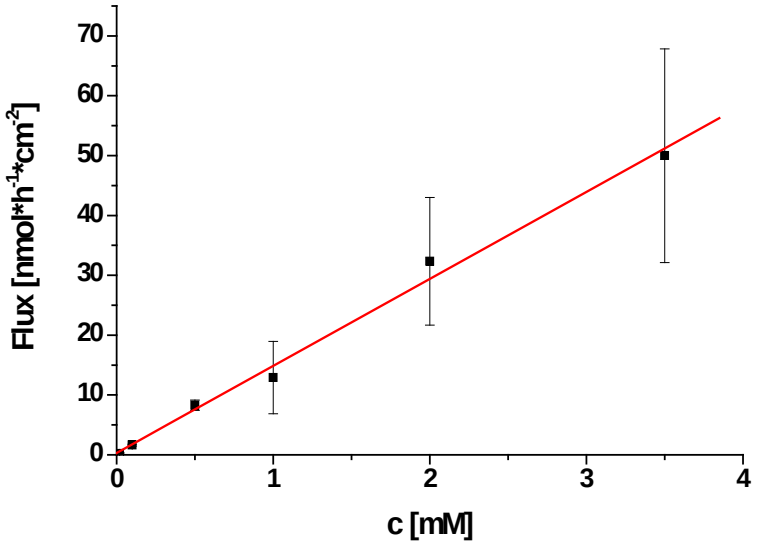


Figure 4-24: Flux rates for 5-CQA [nmol*h^{-1}*cm^{-2}] (concentration: 0.02 to 3.5 mM) from mucosal to serosal side at 2 h of incubation with pig jejunal mucosa in the Ussing chamber (mean ± SD; n = 4). R^2 = 0.99.

4.4.6 Mass balance of individual coffee compounds in the Ussing chamber experiments

Some interesterifications of CGA were observed in the mucosal compartment. CQA (**1a-d**) and FQA (**3a-c**) interesterified into their corresponding isomers, with QA esters linked at position 4 being the most unstable (see Table 4-3). The measured CGA composition in the mucosal compartment after 4 h incubation with 5-CQA (**1d**) is shown in Figure 4-25 with 3- (**1b**) and 4-CQA (**1c**) as interesterification products and CA (**5a**) as a product of hydrolysis.

On the serosal side, only the FQA subgroup formed interesterification artefacts (see Table 4-3). The interesterification rates of 5-CQA (**1d**) did not change with increasing concentrations (up to 3.5 mM). In the secretion experiments (serosal application) with 5-CQA at pH 7.4, interesterification in the serosal compartment occurred at a higher rate (17.8% on initial amount) than in all other absorption experiments (see Table 4-3).

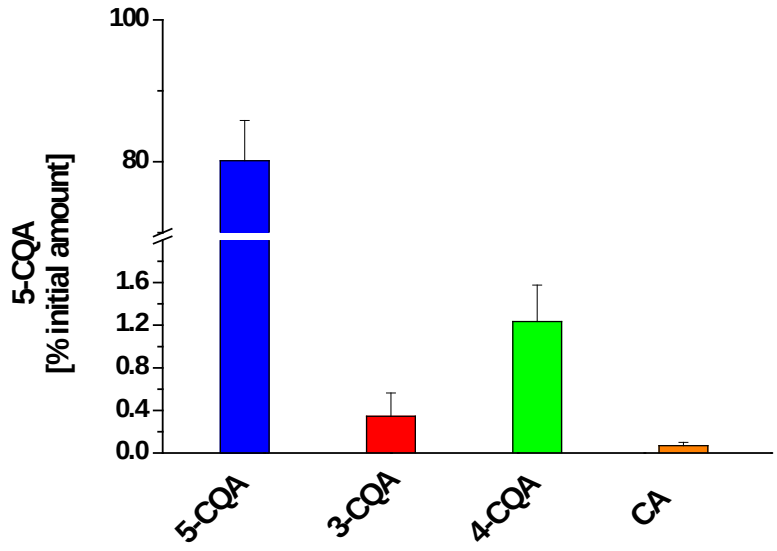


Figure 4-25: Composition of mucosal compartment (% of initial amount) after 4 h incubation in Hank's balanced salt solution (HBSS) (pH 6) at + 37°C in the Ussing chamber with 5-CQA (20 µM) (pH 7.4 at serosal side, mean ± SD; n = 6).

Interesterification of diCQA (**4a-c**) was only observed for 3,5-diCQA (**4b**) in low amounts. Some diCQA (**4a-c**) showed less stability with a liberation of free CA (**5a**) up to 6.2% of the initial concentration. The hydrolytic reaction products, CA of CQA, were observed only on the mucosal side (see Table 4-3).
As an indication of some hydrolytic reactions the free hydroxycinnamic acids, namely CA (**5a**) and FA (**7a**), were detectable after incubations with CQA (**1a-d**) (from 0.1 up to 0.4% CA), and 5-FQA (**3c**) (0.8% FA), respectively (see Table 4-3).

The stability of a compound (mass balance) after 4 h of incubation was calculated as the sum of recovered amount of incubated molecule, its interesterification and hydrolytic products in the individual Ussing chamber compartments (serosal, mucosal, tissue). We recovered mass balances after 4 hours from 69.8 up to 121.1% of the initial amounts applied (shown in Table 4-3).

Table 4-3: Summary of data for interesterification, hydrolysis and mass balance (sum of mucosal, serosal and tissue located amounts) 4 h after incubation of CGA (20 µM) in the Ussing chamber model using pig jejunal tissue. Details see chapter 7.3.2.3 (n.d. = not detected; < LOQ = below LOQ), (mean ± SD as a percentage of the initial concentration; n = 4).

	Interesterification		Hydrolysis	Mass balance
Application (20 µM)	Mucosal	Serosal	Mucosal	Entire distribution
Mucosal (pH 6.0)				
1-CQA (**1a**)	< LOQ	n.d.	0.4 ± 0.2	100.3 ± 10.0
3-CQA (**1b**)	3.7 ± 0.4	n.d.	0.4 ± 0.1	104.9 ± 7.6
4-CQA (**1c**)	6.1 ± 1.4	n.d.	0.4 ± 0.2	103.6 ± 12.1
5-CQA (**1d**)	1.6 ± 0.6	n.d.	0.1 ± 0.0	99.3 ± 8.4
3-FQA (**3a**)	1.1 ± 0.6	0.6 ± 0.2	n.d.	106.6 ± 17.6
4-FQA (**3b**)	15.7 ± 3.2	0.8 ± 0.2	n.d.	103.4 ± 8.1
5-FQA (**3c**)	2.0 ± 0.2	0.3 ± 0.1	0.8 ± 0.1	106.0 ± 4.2
3,5-diCQA (**4b**)	1.7 ± 0.4	n.d.	6.2 ± 1.1	69.8 ± 21.5
3,4-diCQA (**4a**)	n.d.	n.d.	n.d.	84.6 ± 4.2
4,5-diCQA (**4c**)	n.d.	n.d.	4.0 ± 1.1	99.2 ± 11.1
CA (**5a**)				100.4 ± 7.8
D-(-)-QA (**10a**)				108.4 ± 13.4
Serosal (pH 7.4)				
5-CQA (**1d**)	0.2 ± 0.1	17.8 ± 1.8	n.d.	121.5 ± 16.8

4.4.7 Metabolic actions of pig jejunal tissue on 5-CQA

At the highest concentration of 5-CQA (3.5 mM) applied, a metabolite was identified in the serosal compartment of the Ussing chamber. A CQA-glucuronide was identified in the product-ion scan mode at *m*/*z* 529 via its product ions at *m*/*z* 191 (QA (**10a**)) and *m*/*z* 162 (CA (**5a**) minus H_2O), indicating the loss of a glucuronic acid unit (176 amu). The loss of 176 amu was confirmed by a HPLC-MS/MS neutral-loss experiment. Furthermore, the product ion at *m/z* 191 gives a hint for 5-CQA (**1d**) fragmentation.

5 Discussion

5.1 Liberation of HCA and degradation of CGA from coffee in ileal fluid (*ex vivo* stability experiments)

By performing *ex vivo* experiments we have simulated the ileal passage of CGA from coffee to monitor any possible CGA degradation during ileal transit *in vivo* and, or sample collection (ileal bags) in our dose-response intervention study (see chapter 4.3). For this we have performed an *ex vivo* incubation experiment with CGA and ileal effluent at physiological ileal conditions (see chapter 4.1).

The instant coffee concentrations in the inoculum (0.5 $g*L^{-1}$) were significantly smaller than the instant coffee concentrations consumed during the LOW dose treatment (10 $g*L^{-1}$) in the coffee intervention study (see chapter 4.3.1). As a recovery rate of 70% of the consumed CGA in ileal effluent is known (see Figure 5-1) an incubation concentration of 0.5 g instant coffee per L ileal effluent corresponds to a physiological concentration that can be easily reached after the consumption of coffee.

However, the overall recovery of CGA used in the study was low which was presumably the effect of the high organic solvent concentration in the used extraction solution (99% EtOH). Other extraction methods achieved higher recovery rates (Olthof et al., 2001; Kahle et al., 2011) using considerably lower organic solvent concentrations as well as used for ileal fluids extraction after coffee consumption (*in vivo* study) in this work (see chapter 7.3.1.6.1).

Because of the low recovery of CGA, the initial measured concentration at time point 0 h was set at 100%. Therefore, stability and degradation of CGA compounds were shown in % of the initial measured concentration and the achieved results can be only considered as a hint of the actual behaviour of CGA during ileal passage. Nevertheless, in literature comparable investigations were performed for 5-CQA (**1d**) and CA (**5a**) with ileal fluid confirming our observations (Olthof et al., 2001; Kahle et al., 2011) of a negligible influence of ileal conditions on coffee CGA degradation, except for CQL (**2a-b**) degradation.

The observed changes in CGA concentration during the *ex vivo* simulations using ileal fluids are shown in Figure 4-1, Results. Specifically concentrations of CQL (**2a-b**) were continuously decreasing during the eight hours of the experiment

whereas concentrations of CQA (**1a-d**), FQA (**3a-c**) and diCQA (**4a-c**) were less affected. Moreover, CQA (**1a-d**), FQA (**3a-c**) and diCQA (**4a-c**) concentrations increased during the incubation time before its decrease during the last two hours of the experiment as shown in Figure 4-1, Results. Here, we presume a release of these compounds from polymeric compounds such as melanoidins, where CGA can be bond covalently or non covalently during roasting (Nunes and Coimbra, 2010). Simultaneously, a degradation of all other CGA was observed during incubations of instant coffee with ileal fluids following the formation of the free hydroxycinnamic acids (CA (**5a**), FA (**7a**)) as shown in Figure 4-3, Results.

Phenolic compounds from coffee with an intramolecular ester bridge such as the lactones (CQL (**2a-b**)) showed the highest degradation within 8 hours of incubation (48% of the initial measured concentration was recovered), whereas the QA esters (CQA, FQA, diCQA) remained much more stable (at least 84% of the initial measured concentration was recovered) after 8 hours (Table 10-2, Appendix). The recovery rates of CGA in ileal effluent after coffee consumption revealed a similar outcome with a decreased recovery of CQL in comparison to CQA, FQA or diCQA (Table 10-6, Appendix).

Several mechanisms such as chemical, microbial or enzymatic reactions could affect the outcomes of our observations. Therefore, additional experiments were performed without ileal fluid, using only buffer solutions (Figure 4-2, Result section) indicating that ileal conditions without ileal fluid and ileal microbiota such as chemical processes might cause the observed effects.

Hence, the enzymatic esterase in the ileal effluents detected via Api®-ZYM test kit (see Figure 4-4, Results) was not cleaving the ester bond between HCA and QA. Furthermore, the microbiota content deviations of ileal effluents (Table 10-4, Appendix) (Fallingborg, 1999) might not have influenced the stability of CGA and HCA. Here, we observed *ex vivo* moderate interesterifications within the CQA (**1b-d**) subgroup and significant interesterifications for the FQA (**3a-c**) (*$p < 0.05$) subgroup. The 5-acyl compounds decreased (**1d, 3c**) and the 3-acyl increased (**1b, 3a**) (Table 10-2, Appendix), respectively. This means that after 8 hours of incubation the CQA and FQA profiles were closer to the equilibrium than the CQA and FQA profiles of the incubated instant coffee. Previously, these findings were observed for CQA in ileal effluents after consumption of apple beverages (Kahle et al., 2007; Hagl et al., 2011) or coffee (Stalmach et al., 2010).

In the diCQA (**4a-c**) and the CQL (**2a-b**) subgroup interesterification seemed to be sterically hindered. Nevertheless, in the diCQA (**4a-c**) subgroup, 3,5-diCQA (**4b**) showed a decreased stability during the *ex vivo* incubations with ileal fluid (Table 10-2, Appendix). These findings are in agreement with the recovery of 3,5-diCQA in the ileal effluent after consumption of different coffee doses (Table 10-6, Appendix) or by (Stalmach et al., 2010). These changes upon the coffee CGA profile at ileal conditions were corroborated by the Ussing chamber experiments with individual CGA (see chapter 4.4.6). Due to these individual incubations we know now that interesterification from 5-acyl into 3-acyl occurs via the 4-acyl compound as an intermediate product (Figure 5-7).

In conclusion, the CGA under study were stable within the first six hours of ileal fluid incubations. Thus, CGA consumed from coffee were stable during the ileal passage and sampling in the ileal bags in the dose-response intervention study performed (see chapter 4.3). This confirms that the ileostomy model is a suitable tool in CGA bioavailability research. However, the CQL showed a decreased stability and might be partially degraded during ileal passage or sampling in the ileal bags. This has to be taken into account by interpreting data from human intervention studies collecting ileostomy bags after coffee consumption.

5.2 Human dose-response intervention study with coffee polyphenols and QA

As existing bioavailability data of chlorogenic acids in literature after single dose consumption used different study designs such as different doses or different dosage forms with different polyphenol compositions and different analytical strategies (with or without metabolite detection), a comparative consideration of the somewhat controversial bioavailability data was not possible. Thus, the influence of the above mentioned study factors on bioavailability cannot be observed.

Due to this, we performed a dose-response study with different CGA doses (HIGH, MEDIUM, LOW), coffee as dosage form and one analytical strategy. Here, the effect of the dose on the bioavailability in the upper GIT and on the sites within the GIT where absorption occurs (*e.g.* colonic availability) was observed as this was the only factor differing during the study trials. Therefore, ileostomists consumed different coffee doses in $mg*kg^{-1}*BW^{-1}$, in a randomized, double blinded, crossover trial and

the observed effects on coffee polyphenol and free QA (**10a**) absorption, ileal excretion and metabolism were documented.

5.2.1 CGA and QA amount in instant coffee

The main CGA subclass found in the administered instant decaffeinated coffees under study was CQA (**1a-d**) with 5-CQA (**1d**) as major ones (Table 10-5, Appendix). Other CGA, such as FQA (**3a-c**), diCQA (**4a-c**) and CQL (**2a-b**) were also detected in lower amounts than the CQA (see Figure 4-5, Results)**.** CGA profile in g % dry matter basis of the instant coffee powder under study was: 7.0% CQA, 1.0% FQA, 1.0% diCQA, and 1.4% CQL. This profile was confirmed by previous investigations on the coffee CGA profile in g % dry matter basis (*cf*. Table 2-6, State of knowledge (Clifford, 1999; Farah and Donangelo, 2006)). Moreover, the 5-acyl compounds (5-CQA (**1d**) and 5-FQA (**3c**)) were dominating their subgroup and the major quinide was the 3-CQL (**2a**) (Farah et al., 2005b). Free acids such as CA (**5a**) and QA (**10a**) were observed as reported previously (Hucke and Maier, 1985; Badoud and Pratz, 1986; Farah and Donangelo, 2006; Farah, 2012).

The total CGA amount in g % dry matter basis of instant coffee powder was 10% CGA which is close to the instant coffee used by the group of (Renouf et al., 2013)(*cf*. Table 2-6, State of knowledge) (Farah and Donangelo, 2006), and a CGA amount which is comparable to CGA amounts of green unroasted coffee beans (*cf*. Table 2-5, State of knowledge) (Farah and Donangelo, 2006).

A common coffee serves ~ 400 µmol CGA per 200 mL cup (Clifford, 1999). The total CGA contents consumed via coffee brew in our intervention study were 4,525 µmol (1,642 mg), 2,218 µmol (805 mg) and 1,053 µmol (382 mg) for the HIGH-, MEDIUM- and LOW- doses, respectively (Table 10-5, Appendix). This means a 2.5 to 10 times higher dose than normally ingested (Clifford, 1999). This enabled us to observe possible dose-response effects on bioavailability in the upper GIT by acute ingestion.

5.2.2 Ileal elimination of CGA, QA and metabolites after coffee consumption

Because of the colon resection in ileostomists it is possible to detect the colonic availability of orally ingested nutritional compounds such as CGA from coffee by analyzing the ileal effluents. Furthermore, bioavailability and metabolization in the

upper GIT could be observed without the intensive colonic microbial degradation of nutritional compounds as occurring in healthy volunteers with a colon.

5.2.2.1 Effects on ileal excretion by an increasing consumption of CGA and QA from coffee

After coffee consumption by ileostomy volunteers, we recovered about ⅔ of the CGA metabolites in ileal fluids (see Table 10-7, Appendix), indicating that about ⅓ of them were absorbed in the small intestine, comparable to a previous single-dose study on a different group of ileostomist volunteers (Stalmach et al., 2010). Other studies using single doses detected a similar colonic availability of CGA (Olthof et al., 2001; Hagl et al., 2011) in spite of the group of Kahle et al. (Kahle et al., 2007) that found a considerable smaller colonic availability (see Figure 5-1). Comparative considerations to our data are limited due to the missing metabolite detections in these studies (see Table 2-9, State of knowledge) and different consumed CGA compositions. Nevertheless, the CGA recoveries in the bioavailability studies (Olthof et al., 2001; Hagl et al., 2011) (without metabolite detection) are assumed to be close to our observations (see Figure 5-1), because less than 10% of consumed CGA were metabolized or hydrolyzed when passing the upper GIT, as observed in our dose-response study after coffee consumption (see chapter 4.3.2.4, Results).

For the determination of CGA and potential metabolites the groups of (Olthof et al., 2001; Hagl et al., 2011) and (Kahle et al., 2007) used an HPLC-DAD system. In contrast, the group of Stalmach and our current study used an HPLC-MS system in the multiple ion mode (see chapter 7.3.1.6.2). The HPLC-MS as well as the HPLC-DAD analytical system showed a similar sensitivity for CGA (LOD $_{\text{5-CQA absolute}}$: HPLC-DAD = 0.2 ng; HPLC-MS (multiple ion mode) = 0.5 ng). The high number of CGA and CGA metabolites in ileal effluent and a similar absorption wavelength (λ_{max}) of CGA compounds complicated a chromatographic separation of all compounds. Such a chromatographic separation is necessary for HPLC-DAD detection but not for HPLC-MS detection. The HPLC-MS system in the multiple ion mode showed a high selectivity by detection of the mass to charge ratio. Thus, we conclude CGA metabolites detection by HPLC-DAD might be incomplete due to low selectivity. Hence, the technical limitations of the HPLC-DAD method are not suitable for CGA metabolite detection in contrast to the HPLC-MS system.

Therefore, identification of CGA, QA and corresponding metabolites was conducted with the HPLC-MS system (see chapter 7.3.1.6.2.1). More specific, Co-chromatography, matching MS^2 spectra of available reference compounds (see chapter 7.1.2) and literature data (Clifford et al., 2003; Stalmach et al., 2009) were used for identification. CGA compounds without available references such as FA-Gly (**7d**) were identified using literature data (Clifford et al., 2003; Stalmach et al., 2009) and similar MS^2 spectra of corresponding precursor compounds.

As no reference compounds of isoferuloylquinic acid-glucuronide (IFQA-GlucA (**3i, 3j**)) or its precursor compound isoferuloylquinic acid (IFQA) were available identification was assumed by the data obtained from FQA-GlucA. Comparative consideration of recently published IFQA product ion characterization was limited as the reference FQA compounds were already showing different retention times and product ions presented in that reference, in contrast to our observations (Kuhnert et al., 2010). Nevertheless, a later retention time of IFQA-GlucA was confirmed (Kuhnert et al., 2010).

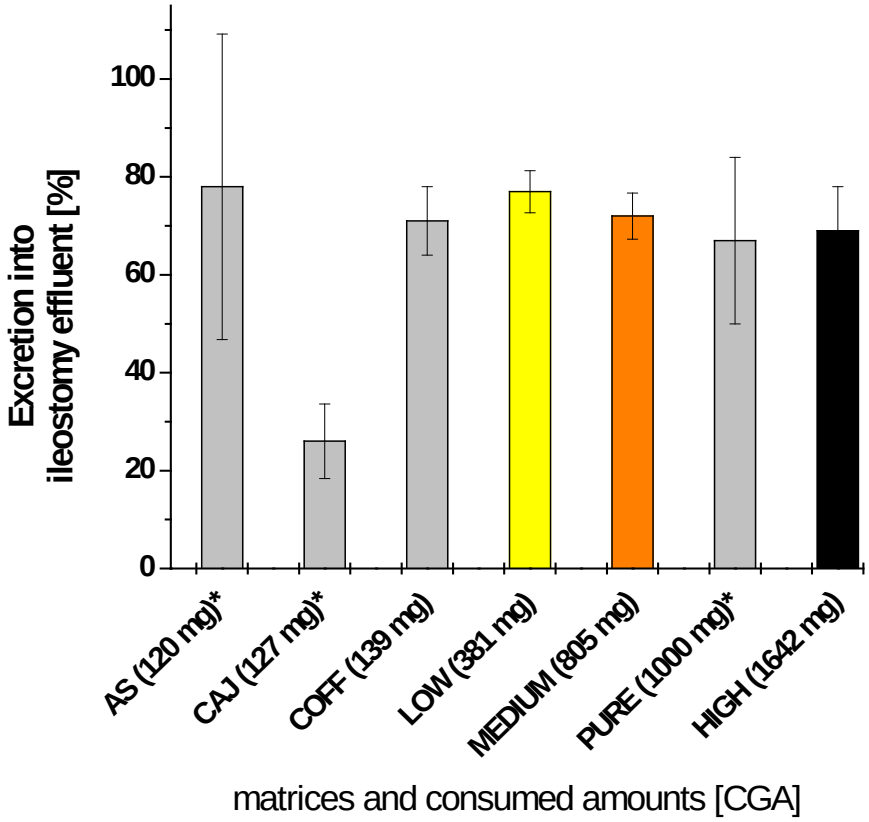


Figure 5-1: Amounts (%) of chlorogenic acids (CGA) excreted into ileostomy effluents and corresponding amounts of CGA consumed, published in literature (grey bars) compared to our findings (LOW, MEDIUM and HIGH). Different CGA matrices and amounts were used in single dose studies published, such as AS = apple smoothie with 120 mg CGA (Hagl et al., 2011); CAJ = cloudy apple juice with 127 mg CGA (Kahle et al., 2007); COFF = coffee with 139 mg CGA (Stalmach et al., 2010) and PURE = 1,000 mg 5-CQA in water (Olthof et al., 2001). In our dose-response study three different coffees LOW, MEDIUM, HIGH with 382, 805 and 1,642 mg CGA were consumed. * = without identification of CGA metabolites.

The consumption of different doses of CGA via coffee did not greatly affect absorption in the small intestine, see Figure 5-1.

Thus, the existing contradictory data in literature on ileal excretion of CGA (Kahle et al., 2007) must have been influenced by other bioavailability factors such as food matrix or dosage forms.

Furthermore, we determined no relationship of consumed coffee dose versus the maximal physiological CGA concentrations (C_{max}) in the ileal effluents, $C_{max\ 5\text{-}CQA}$:

4.1 (HIGH), 6.0 (MEDIUM), 2.3 (LOW) µmol per g ileal fluid, (see Table 10-9, Appendix). Water consumption was allowed *ad libitum* during the remaining study trial once the ileostomist had drunk the coffee. We assume that potentially different amounts of water being consumed within the three trials affected the C_{max} values more than different amounts of coffee CGA doses consumed. Moreover, maximal physiological CGA concentrations in the excreted ileal fluids were higher as in the consumed coffees. As the upper GIT of ileostomists is adapted to water absorption (see chapter 2.4.2, State of knowledge) (Ladas et al., 1986; Fallingborg, 1999), a concentration process of CGA in the upper GIT due to water absorption is conceivable.

The observed interesterification of CGA in the *ex vivo* ileal fluid experiments (see chapter 4.1 and Table 10-2, Appendix) could also be observed *in vivo* in the dose-response study with ileostomists independent of the doses after coffee consumption (see Table 10-6, Appendix). More specifically, recovery rates of 3-acyl compounds of CQA (3-CQA, **1b**) and FQA (3-FQA, **3a**) were higher than their corresponding 5-acyl compounds. Furthermore, 3,5-diCQA (**4b**) showed the lowest recovery rate of the diCQA (**4a-c**) subgroup (Table 10-6, Appendix). This effect is much more distinctive in the *ex vivo* ileal fluid incubation experiments compared to the *in vivo* dose-response experiment with ileostomists because of the much longer CGA residence time used in the *ex vivo* experiments (8 hours) in comparison to the residence time of consumed coffee CGA in the ileum (see Figure 4-11, Results).
These observations indicate a small interesterification as discussed in detail in chapter 5.3.4 and are in agreement with a previous coffee intervention study with ileostomists (Stalmach et al., 2010).
We calculated a considerably lower recovery rate for CQL (**2a-b**) in relation to the other CGA from coffee investigated; see Table 10-2 and Table 10-6, Appendix. We conclude that the lower ileal recovery of consumed CQL (**2a-b**) in the *in vivo* dose-response study resulted in a higher absorption rate and/or in a partial degradation during passage of the ileum. This thesis is corroborated by the high content of CQL metabolites detected either in ileal fluid or urine in the current *in vivo* study or by others (Stalmach et al., 2010) and an observed degradation of CQL *ex vivo* at ileal conditions (see chapter 4.1).

The high inter-individual differences in $T_{max\ 5\text{-}CQA}$ did not relate to the total amounts of CGA reaching the ileal effluents. Especially ileo 5 showed a significant decelerated excretion compared to ileo 1-4 ($p < 0.03$) (see Figure 4-11, Results) but a similar total amount of ileal excreted CGA.

However, the excreted QA content showed significant inter-individual differences, especially in subject no. 5 who had a very long transit time (ileo 5 compared to ileo 1-4, $p < 0.01$). On the one hand these inter-individual differences were confirmed by an additional study observing the GI – TT after consumption of an anthocyanin rich test beverage, and on the other hand high inter-individual differences have already been observed in transit time by others (6.2 – 12.8 hours) (Fallingborg et al., 1990).

Additionally, we could show that ingestion of a higher coffee dose lead to a faster transit time ($T_{max\ 5\text{-}CQA\ (ileo\ 1\text{-}4)}$ in hours: HIGH 0:5 ± 0:0; MEDIUM 0.8 ± 0.3; LOW 1.6 ± 1.0) ($p < 0.09$). Considering that these factors might influence the gastric emptying time, playing a key role in upper GI - TT (chapter 2.4.1.2), a relationship between the different coffee doses / concentrations seems probable.

5.2.2.2 Effects on CGA metabolite amounts by increasing coffee doses consumed

Once absorbed into the enterocyte CGA undergo phase II reactions such as sulfation, glucuronidation, methylation or hydrolysis (Kern et al., 2003b; Wong et al., 2010). Subsequently, the formed metabolites can reach the circulatory system or they secrete back into the gut lumen and could be excreted via ileal fluid. The bioavailability of CGA from coffee is limited by metabolization. Up until now it is not clear if consumption of different doses influences the metabolic pathways of CGA from coffee.

In our dose-response study the amounts being metabolized or hydrolyzed after the ingestion of different CGA doses indicate an extensive metabolic activation occurring during the passage of the upper GIT. Moreover, the determined metabolites showed a clear delay in ileal excretion in comparison to the unmetabolized CQA (*e.g.*: T_{max} at the HIGH trial: CQA (**1a-d**) ≈ 1.2 *vs*. CQA-Sulf (**1g-i**) ≈ 2.2 h or CQA-GlucA (**1e-f**) ≈ 2.2 h; Table 10-8, Appendix).

A proportionally lower amount (in %) of CGA was metabolized or hydrolyzed with consumption of increasing CGA doses which could conceivably be due to enzyme saturation, limitation of substrates, limited transport capacities at the enterocyte

(influx and efflux) or an influence of the GI - TT. In an similar study ileostomy probands consuming one dose of coffee, Stalmach et al. (Stalmach et al., 2010) observed that 22% was metabolized or hydrolyzed with a low dose of CGA ingestion (2.7 times smaller than our LOW dose) with a sulfation : glucuronidation ratio of 10.5 : 1. In agreement with Stalmach et al., we also identified sulfation as the major phase II conjugation reaction in the upper GIT, but we additionally observed an increase in proportion of glucuronidation with increasing CGA dose.

We detected no inter-individual differences in the sulfation : glucuronidation ratios of ileal effluents after coffee consumption. This indicates that the inter-individual differences of *ß*-glucuronidase activity measured in the ileal effluents (ileo 1-4) (see Table 10-3, Appendix) had no detectable influence on the glucuronide amounts in ileal effluents.

The selectivity preference for sulfation and glucuronidation on the 3´-hydroxyl of CA (**5a**) and DHCA (**6a**) as also observed by others (Wong et al., 2010) was consistent over the three applied doses in both the ileal effluents as well as in the urine samples. Moreover phase II conjugations of 5-acyl compounds such as 5-FQA (**3c**) or 5-CQA (**1d**) were sterically hindered. We detected only small amounts of 5-CQA-3´-Sulf (see Figure 4-9, Results) similar to a previous study on one dose (Stalmach et al., 2010).

The group of Wong (Wong et al., 2010) and Stalmach (Stalmach et al., 2009; Stalmach et al., 2010) showed an extensive metabolism of CGA during the passage through the body. We confirm an extensive metabolism of CGA (Stalmach et al., 2009; Stalmach et al., 2010; Wong et al., 2010) and additionally novel metabolites in urine. Furthermore, we observed that FA-Gly, DHCA and DHFA can be formed in probands without a colon after coffee consumption.

Small amounts of the free HCA (CA (**5a**), FA (**7a**)) as well as free DHCA (**6a**) indicate some esterase activity in the upper GIT (Table 10-7, Appendix). Despite the potential esterase activity along the upper GIT (Andreasen et al., 2001; Lafay et al., 2006; Farrell et al., 2011) the chemical hydrolytic activity in the ileal fluids as already discussed in chapter 5.1 may also contribute to the free acid content.

Passive diffusion into the enterocyte should be limited for molecules which are high in molecular weight and having an adverse log D. The latter (log D) describes the

distribution of an ionizable molecule (such as CGA) between an octanol and water phase at a certain pH and is therefore a suitable descriptor for the lipophilicity of a molecule.

We postulate an active efflux mechanism at the enterocyte for sulfated or glucuronidated metabolites, especially for the intact esters high in molecular weight with an adverse log D (*e.g.*: CQA-Sulf (**1g-i**) or -GlucA (**1e-f**), FQA-Sulf (**3g-h**) or –GlucA (**3d-f**)) as previously reported with FA (**7a**) metabolites *in vitro* (Poquet et al., 2008).

5.2.3 Renal excretion of CGA and QA after coffee consumption

Based on the premise of a concentration equilibrium of CGA and QA between the systemic circulation and urine (Chereson, 1996) we determined the influence of increasing CGA and QA doses on renal excretion. Due to a lack of references (such as CGA sulfates and glucuronides) we achieved semi-quantitative data (see chapter 7.3.1.5.2.1).

5.2.3.1 Effect of an increasing amount of CGA and QA consumed by coffee on renal excretion

Renal excretions correlated negatively with increasing CGA doses ingested and high inter-individual variations (Table 10-10; Appendix). We presume a relationship between GI - TT and CGA bioavailability. More specifically, one volunteer showed a much longer GI - TT (Ileo 5) and therefore the renal excretion was twice as high as for the other subjects. The relationship between GI – TT and renal excretion of each volunteer shown in Figure 5-2 gives a hint that a decelerated passage of CGA in the gastrointestinal tract leads to a higher absorption rate.

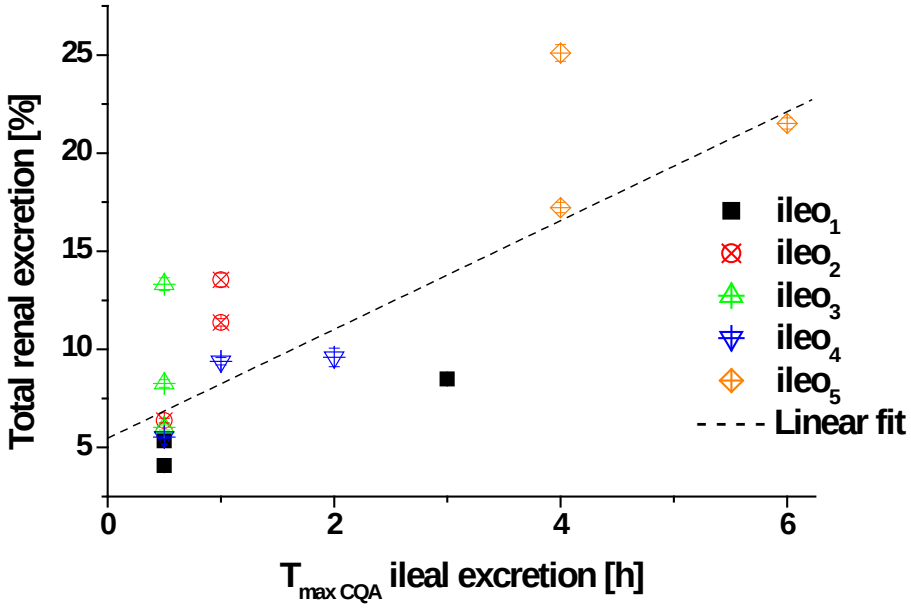


Figure 5-2: Relationship of total renal excretion of consumed CGA in %, with maximum of ileal excretion ($T_{max\ CQA}$) in hours of each ileostomist given for all doses (HIGH; MEDIUM; LOW). Linear fit: $R^2 = 0.64$; y = 2.8x + 6.2.

Due to the extensive biotransformation that CGA undergo in the human body it is not possible to calculate recovery rates of individual CGA. Therefore, total renal excretion of CGA is reported here. It was determined between 8.0 ± 4.9% for the high dose and 14.6 ± 6.8% for the low dose. Other research groups detected similar renal amounts being renally excreted in ileostomists of 8% after ingestion of 385 µmol CGA from coffee (with a comparable semi-quantitative analytical method as used in our study) (Stalmach et al., 2010) and 11% after administration of 2,800 µmol pure CA (**5a**) (Olthof et al., 2001) (enzyme treatment was used as analytical method) (see Table 2-14, State of knowledge). In healthy volunteers the total renal recovery of CGA after coffee consumption was reported to be about 3 times higher in contrast to ileostomists. This was reported by Stalmach et al., 2009 and Rio et al., 2010 who used a comparable analytical method to our study (Stalmach et al., 2009; Rio et al., 2010).

In previous bioavailability studies healthy probands consumed CGA and considerable lower total excretions of CGA were observed (see Table 2-13, State of knowledge). However, these groups used different analytical methods (mostly with enzymatic cleavage of CGA metabolites), different CGA dosage forms such as encapsulated

green coffee and different CGA compositions in ingested food such as artichoke leaf extract (see Table 2-13, State of knowledge). Due to this, the reported total renal CGA excretions determined in these studies (Rechner et al., 2001; Ito et al., 2005; Wittemer et al., 2005; Farah et al., 2008) are not further discussed.

As previously reported, we found unmetabolized CGA in urine (Olthof et al., 2001; Stalmach et al., 2009; Stalmach et al., 2010; Kahle et al., 2011). Hereby, especially 3-FQA (**3a**) and 3-CQA (**1b**) of these two CGA subgroups were predominant in urine, independent of the administered dose (Table 10-10, Appendix, Figure 5-3), whereas in plasma the 4-acyl compounds were predominant after enzyme hydrolysis (Figure 5-3, Table 10-13, Appendix). The 5-acyl compounds (5-FQA (**3c**), 5-CQA (**1d**)) were detected in both compartments only in minor amounts. The group of Renouf et al. determined after coffee consumption and enzymatic hydrolysis of plasma in terms of apparent plasma appearance the following order: 3-FQA > 5-FQA > 4-CQA > 4-FQA > 5-CQA > 3-CQA (Renouf et al., 2013). The differences to our observations (see Figure 5-3) might be caused due to the fact that healthy volunteers (with colon) consumed the coffee in this study.

Interesterification reactions at the physiological plasma pH could be a reason for this. Such interesterification reactions were also observed in the *ex vivo* incubation experiments with CGA and ileal effluents, see Table 10-2, Appendix. Especially, the 3-acyl compounds (3-FQA (**3a**), 3-CQA (**1b**)) showed high recoveries in these *ex vivo* experiments as well as in urine and plasma after coffee consumption (see Figure 5-3).

Furthermore, the elimination of CGA and metabolites via urine could be affected by the various phase II conjugation reactions. The SULT enzyme family seems to prefer the 3- and 4-acyl CQA and FQA, similar to the UGT enzyme family, which additionally showed a high affinity for 4-CQA and 4-IFQA (see Figure 5-3, Table 10-10, Appendix). Both enzyme families showed an inhibition for 5-CQA (**1d**) or 5-FQA (**3c**) conjugation.

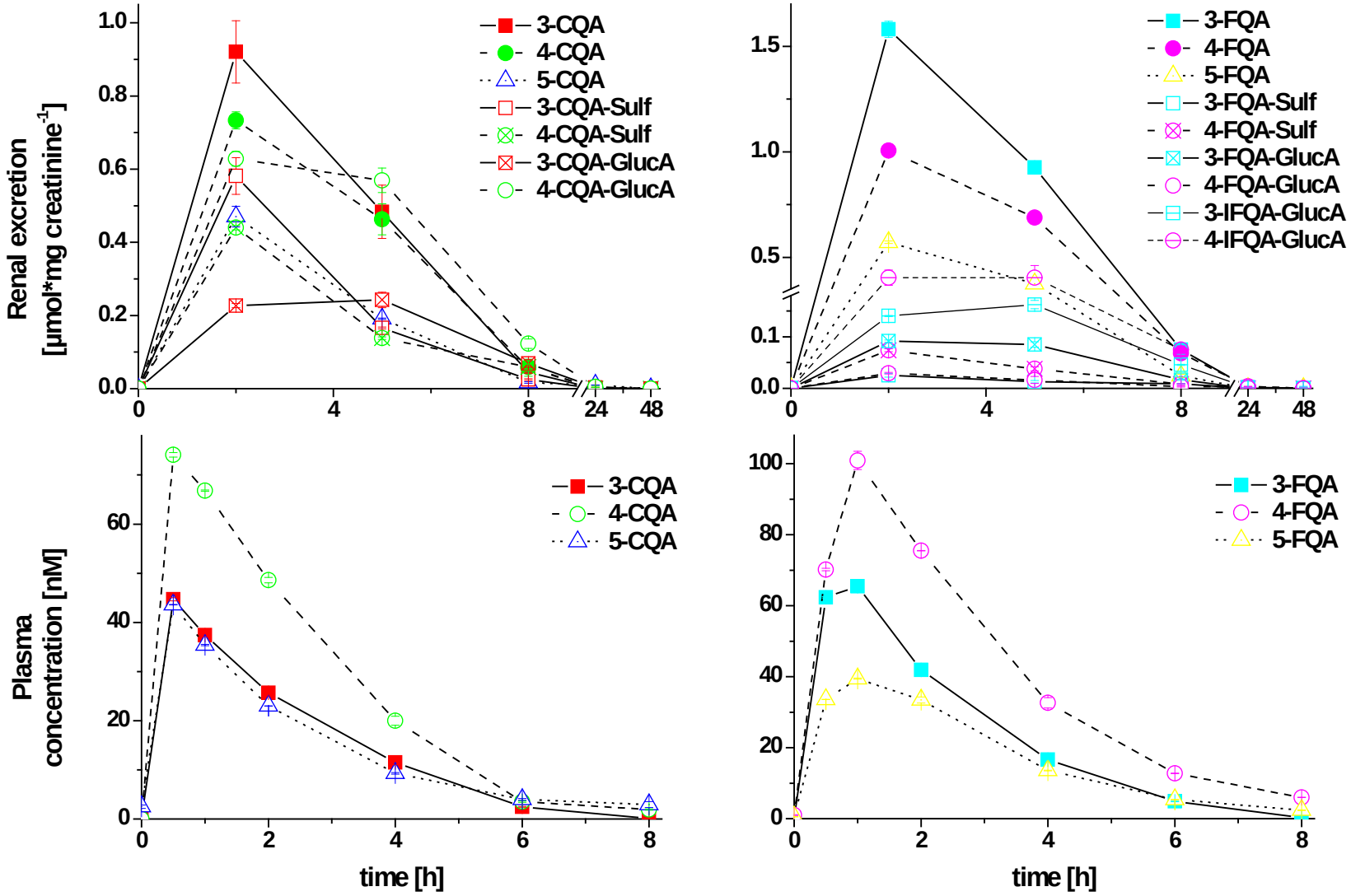


Figure 5-3: Representive kinetics of caffeoylquinic acids (CQA), feruloylquinic acids (FQA), isoferuloylquinic acids (IFQA) and metabolites in urine (µmol*mg^{-1} creatinine) and plasma (after enzyme hydrolysis, in nM) of ileostomist no. 2 after the consumption of coffee (HIGH CGA dose, 4,525 µmol). Sulf = sulfate, GlucA = glucuronide.

Despite unmetabolized CGA, we were not able to identify any diCQA (**4a-c**) in plasma or in urine. Caffeoylquinides (CQL) (**2a-b**) were highly absorbed; particularly its sulfated metabolites (CQL-Sulf) were detectable in urine (Table 10-10, Appendix). In comparison to CGA the QA (**10a**) moiety of CQL (**2a-b**) have an intra-molecular ester bridge which decreases the polarity. Specifically, the calculated log $D_{(pH6.0)}$ for CQL was + 0.4 which is more suitable for passive absorption than the log $D_{(pH\ 6.0)}$ for CQA of – 2.9 (calculated with MARVIN SKETCH 5.3.1).

As early as 1964 one study reported QA (**10a**) in urine (Halpern, 1964), whereas previous bioavailability studies described its colonic metabolites in urine at its most, *e.g*. hippuric acid (Rechner et al., 2001; Olthof et al., 2003; Kahle, 2008).
In 1970 Adamson and coworkers orally administered 12 g [^{14}C] QA to Rhesus monkeys. This group detected QA being aromatized by the gut flora to hippuric acid and found 32% of the radioactivity as hippuric acid in urine (Adamson et al., 1970). But, in 2011 Pero and Lund observed millimolar concentrations of QA in urine after consumption of a nutritional supplement (AIO + AC-11®) (Pero and Lund, 2011).
However, the renal dose dependent recovery of QA was determined here for the first time. Recovery rates were nearly similar in all trials with about 15%. Hence, the recovery of QA in urine was not affected by the consumed dose or GI-TT. But a potential hydrolysis of CGA into QA and HCA was indicated by measurable free HCA in our studies; and in previous work (Kahle et al., 2007). Taking the potential hydrolysis of CGA into account the actual recovery rates of free QA consumed with the coffee brews under study was estimated to be less than 15%.

5.2.3.2 Effect of increasing CGA dose consumption on CGA metabolite formation

The total percentage of CGA metabolites (conjugates or hydrolytic products) in urine were not affected by the doses consumed, whereas the ratios of sulfation : glucuronidation (sulf : glucA) in urine showed a dose-dependency similar to the formation of metabolites excreted via ileal effluents. The sulf : glucA ratio changed from 0.7 : 1 (HIGH), 1 : 1 (MEDIUM), up to 1.3 : 1 (LOW). In a previous study with ileostomy volunteers (Stalmach et al., 2010), the urinary sulf : glucA ratio was 5.6 : 1. In this study CGA consumption via coffee compared to our lowest coffee dose (LOW) was 2.7 times lower. This could explain the higher amount of sulfation in

this study (Stalmach et al., 2010). We conclude that sulfation is limited by increasing dose conceivably due to enzymatic saturation, limitation of substrates, limited transport capacities of the enterocyte (influx and efflux) or an influence of the GI - TT.
Our findings of a regioselectivity preferences of conjugating enzymes at the hydroxyl position 3´ (Figure 2-1, Sate of knowledge) confirmed the findings of recently published studies (Stalmach et al., 2010; Wong et al., 2010).
Thereupon, the conjugation enzymes being involved in renal and ileal excretions are regulated by the substitutes at the aromatic ring of the hydroxycinnamic acid moieties, dose unaffected. For instance, we determined a sulfation preference for CA (**5a**) and DHCA (**6a**) both molecules have two hydroxyl groups at position 3´ and 4´ (see Figure 4-13, Results). Furthermore, a strong glucuronidation affinity for IFA (**7g**) and IFQA (**3i, 3j**), both molecules with a methoxy group at position 4´ was observed (Figure 4-14, Results).

Moreover, for the first time we described metabolites of the intact CGA and corresponding metabolic profile in urine (such as CQA-Sulf and CQA-GlucA), in spite of sulfated CQL (**2e-f**) which were already reported by (Stalmach et al., 2009; Stalmach et al., 2010). We were able to show that conjugating enzymes involved in renal excretion are also regulated by the hydroxycinnamic acid moiety. For instance, whereas the CQL (**2a-b**), a CA with quinide moiety, showed a strong affinity for sulfation (Figure 4-13, Results), the CQA (**1a-d**) a CA with quinic acid moiety seemed to hinder the preferred sulfation with the consequence of higher affinity to the UGT enzyme family (Figure 4-14, Results).

5.2.4 Influence of coffee dose on plasma CGA and QA occurrence

In order to determine the effect of different consumed CGA and QA doses on oral bioavailability in ileostomists we measured their plasma concentrations and metabolites after consumption of increasing doses of coffee. Since a lack of references for quantification, the concentrations of CGA in plasma were measured after enzymatic hydrolysis of the sulfates and glucuronides (see chapter 7.3.1.7.2.1).
Furthermore, plasma data is available for four probands per study trial only as two volunteers withdrew their permission for blood collection (Ileo 3 at the HIGH trial and Ileo 4 at the MEDIUM trial) and the individual calibration curve of ileo 5 at the study trial LOW could not be used for quantification. Additionally, the needle of Ileo 1 was

blocked after five hours at the MEDIUM trial. Here data for time points six and eight are missing (see chapter 7.3.1.7).

Within the first 30 min after consumption plasma concentrations of CGA showed a strong ascent and T_{max} was reached dose independently within the first hour of consumption (Table 10-13, Appendix, Figure 4-17, Results) for all CGA, as confirmed by other published data (Nardini et al., 2002; Renouf et al., 2010a; Renouf et al., 2010b; Renouf et al., 2013). Hereby, methylation was shown to be a fast phase II conjugation reaction, indicated by the early T_{max} and the high AUC level of methylated hydroxycinnamic acids, *e.g.* for IFA (**7g**) (see Table 10-13, Appendix, Figure 4-16, Results). Considering a possible gastric residence time from 0.2 up to 3.8 hours (Fallingborg et al., 1990) a CGA uptake and methylation in the stomach would be conceivable. An animal study with rats published by (Lafay et al., 2006) and the recently performed work on permeation of CGA through cultured gastric cells (Farrell et al., 2011) confirmed this thesis. Furthermore, the low gastric pH influences the log D of CGA (see Figure 2-2, State of knowledge) so these molecules are more lipophilic which might facilitate diffusion into the blood stream. Plasma appearance was linearly correlated to most compounds within the different ingested doses (see Figure 4-16, Results, Table 10-13, Appendix). A non linear plasma appearance of some compounds might be result from inter-individual difference in the probands physiology.

It is known from ileal excretions of CGA after coffee consumption that about ⅓ of consumed CGA are potentially absorbed dose unaffected (Figure 5-1). Nevertheless, most bioavailability studies on CGA from coffee show an early T_{max} and C_{max} (Table 2-10, State of knowledge) indicating a major influence of the upper GIT on absorption. Thus, the contribution of the large intestine (in healthy volunteers) to plasma T_{max} and C_{max} of CGA and their metabolites is low, except for metabolites formed by colonic microflora. Due to this, T_{max} and C_{max} from CGA bioavailability studies with ileostomy volunteers (without colon) are comparable with data from studies with healthy volunteers (with a colon). Up until now there is no literature data for CGA plasma concentrations in ileostomists after coffee consumption available. We calculated the maximum plasma concentration (C_{max} level) at a theoretically consumed dose of 50 mg CGA from coffee and compared the data from studies with

healthy volunteers with data of the current study shown in Figure 5-4 (according to Table 2-10, State of knowledge (Williamson et al., 2011)).

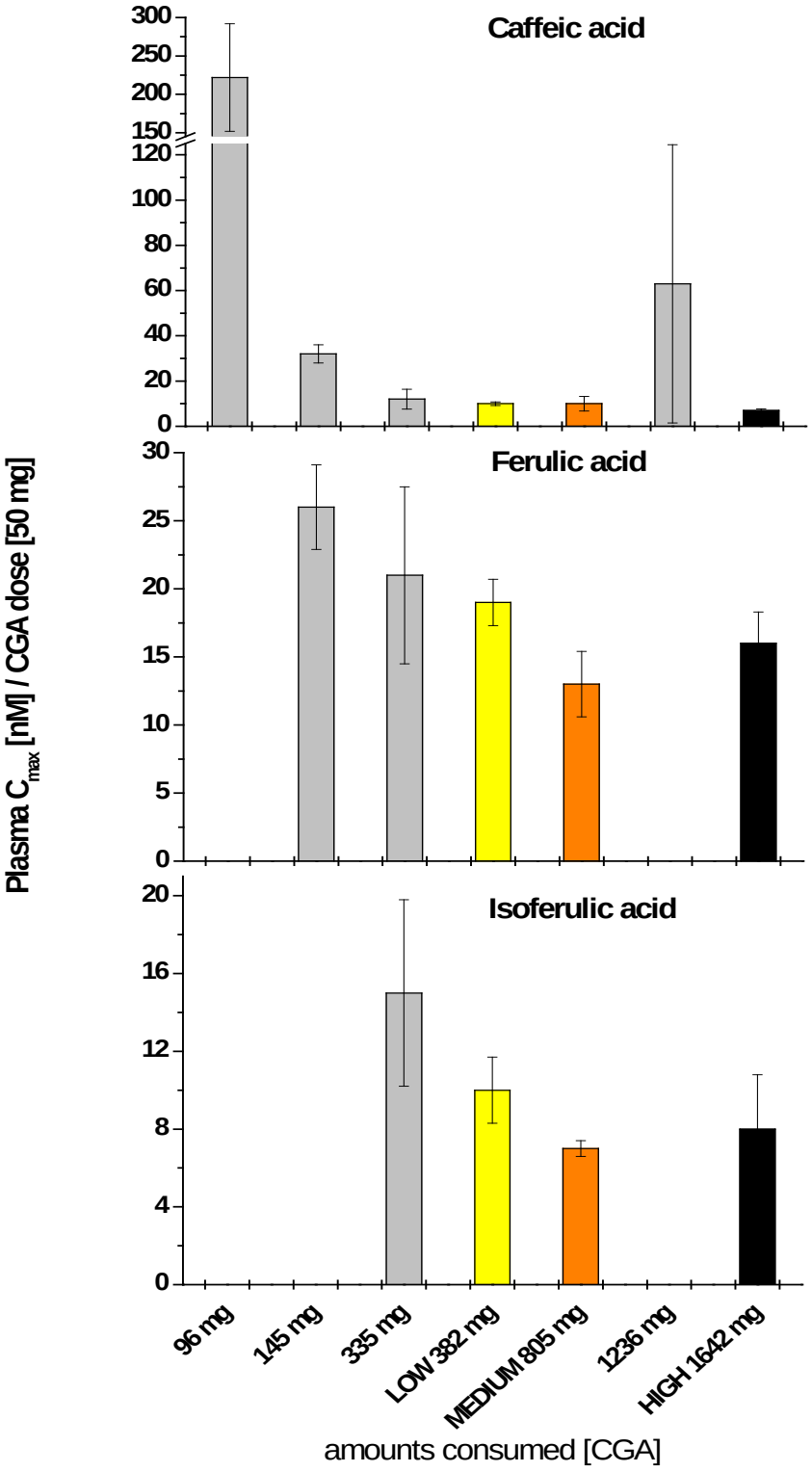


Figure 5-4: Maximum plasma concentrations (C_{max}) (in nM) per 50 mg consumed CGA dose by coffee reported in literature (healthy, with colon) (grey bars) compared to our findings after consumption of different coffee doses (LOW, MEDIUM, HIGH) by four ileostomists (without colon). Different CGA amounts via coffee were consumed in single dose studies published, with 96 mg = (Nardini et al., 2002); 145 mg = (Stalmach et al., 2009); 335 mg = (Renouf et al., 2010a; Renouf et al., 2010b); 1,236 mg = (Monteiro et al., 2007), see Table 2-10, State of knowledge.

CA (**5a**) concentrations in plasma after coffee consumption have been investigated for more than ten years. Previous studies reported a high C_{max} of CA (Nardini et al., 2002; Monteiro et al., 2007) compared to our findings. We observed a dose independent plasma concentration of CA. This was confirmed by the observations of the group of Renouf et al. who performed a dose-response study with healthy volunteers consuming coffee in a dose range from 176 to 704 mg CGA (Renouf et al., 2013). The detected plasma CA concentrations are in agreement with recently published data (Stalmach et al., 2009; Renouf et al., 2010a; Renouf et al., 2010b); see Figure 5-4. The latter research groups have also determined a similar FA (**7a**) and the COMT metabolite IFA (**7g**) C_{max} per 50 mg CGA coffee dose (see Figure 5-4). In the above mentioned dose-response study with healthy volunteers similar C_{max} per 50 mg CGA coffee dose were detected for CA (**5a**), FA (**7a**) and IFA (**7g**) (Renouf et al., 2013)[2]. This indicates the key position of the upper GIT after CGA consumption in absorption and the maximum plasma levels.

In 2002 for the first time Nardini and coworkers reported a very high C_{max} level of CA in humans with a pioneering methodology, so the reported values have to be considered in an qualitative point of view and not quantitatively (Nardini et al., 2002; Williamson et al., 2011). The relevance of the reported data on CA of Monteiro et al. 2007 is also doubtful because of the high standard deviation (see Figure 5-4).

In our study the maximum plasma concentration of the dimethylated compound DiMeCA was quantified for the first time. Moreover, a plasma ratio of FA (**7a**) : DiMeCA (**9a**) of about 1 : 2 60 min after coffee consumption was described recently (Nagy et al., 2011) contrary to our data (FA : DiMeCA ≈ 2 : 1). However, the administered coffee dose in this study remains unknown and plasma was treated with chlorogenic acid esterase prior to analysis so compounds such as FQA (**3a-c**) were cleaved (Nagy et al., 2011).

In contrast to others, for the first time (Nardini et al., 2002; Stalmach et al., 2009; Redeuil et al., 2011) both, CQA (**1a-d**) and FQA (**3a-c**) were detected in plasma as intact compounds in significant amounts (Figure 4-16, Results). C_{max} and AUC in plasma correlated with the different ingested doses (see Table 10-13, Appendix,

[2] Plasma C_{max} is shown only in figures in that publication. Therefore an exact calculation of C_{max}*50 mg $dose^{-1}$ was not possible.

Figure 4-16, Results). AUC as well as the renal excretions seemed to be affected by the different GI – TT, see chapter 5.2.3.1.

The CQA and FQA plasma profiles were not similar to the ones determined in the original beverage (*cf*. Figure 5-3 and Figure 4-6, Results). The 5-acyl compounds 5-CQA (**1d**) and 5-FQA (**3c**) were dominating the CGA profile in the beverage. However, only small amounts of these compounds are present in plasma after coffee consumption. This difference was also observed by others (Stalmach et al., 2009; Renouf et al., 2013).

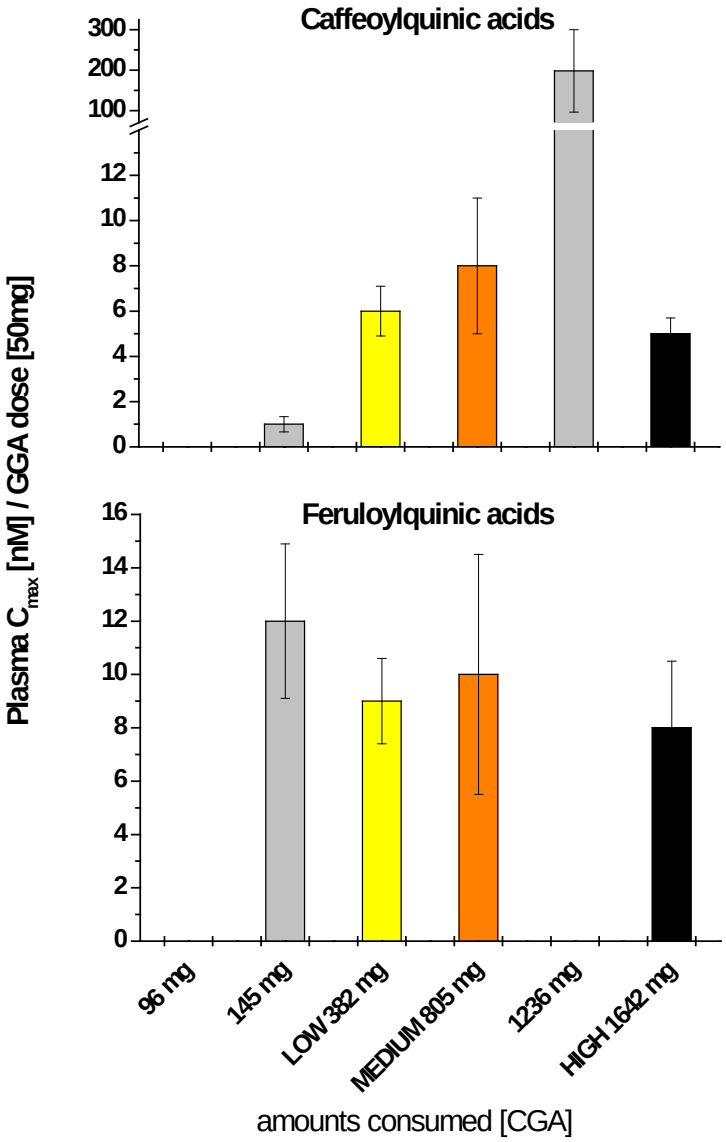


Figure 5-5: Maximum plasma concentrations (C_{max}) of caffeoylquinic and feruloylquinic acids (in nM) per 50 mg consumed CGA dose by coffee reported in literature (healthy, with colon) (grey bars) compared to our findings after consumption of different CGA doses from coffee (LOW, MEDIUM, HIGH) by four ileostomists (without colon). Different CGA amounts were consumed in reported single dose studies, with 96 mg = (Nardini et al., 2002); 145 mg = (Stalmach et al., 2009); 1,236 mg = (Monteiro et al., 2007), see Table 2-10, State of knowledge.

The dominance of the 4-acyl compounds in plasma could be explained by a possible facilitated transport for 4-CQA (**1c**) and 4-FQA (**3b**) as observed by (Farrell et al., 2011) or potential isomerization within the CGA subgroups. Other research groups (Monteiro et al., 2007; Farah et al., 2008) detected the 5-CQA as the major plasma CQA and moreover high amounts of diCQA in plasma after coffee consumption.

Our data and the findings of other groups (Renouf et al., 2013), (summarized by Williamson et al., 2011) were not in agreement with this high amounts of diCQA in plasma after coffee consumption. A possible explanation for this difference might be the different pharmacokinetic data. The time point of maximum plasma concentration (T_{max}) for CQA in two studies (Monteiro et al., 2007; Farah et al., 2008) were considerably late (3.3 h for 5-CQA (**1d**) (Farah et al., 2008) and maximum plasma concentration (C_{max}) for CQA showed multiple levels (Farah et al., 2008) in comparison to our study or the data of the group of (Stalmach et al., 2009; Renouf et al., 2013) (Figure 5-5). Furthermore, the dosage form was completely different in the study reported by Farah since volunteers consumed 451 µmol encapsulated CGA from a green coffee extract. Nevertheless, we detected a dose independent bioavailability of the CQA subgroup after consumption of different CGA doses from coffee by ileostomists (see Figure 5-5).

The subgroup of FQA (**3a-c**) was quantified in plasma of healthy volunteers after coffee consumption by the groups of (Stalmach et al., 2009) and (Renouf et al., 2013). The data agrees (in C_{max}*50 mg dose^{-1}) with our data and corroborates a dose independent bioavailability (see Figure 5-5). These findings are not consistent with other data published (Nardini et al., 2002; Monteiro et al., 2007). In 2002, Nardini et al. did not determine any FQA in human plasma with a pioneering methodology and in 2007 Monteiro et al. noted that FQA were also absent in plasma, despite a very high plasma CQA amount after coffee consumption (see Figure 5-5) (Williamson et al., 2011).

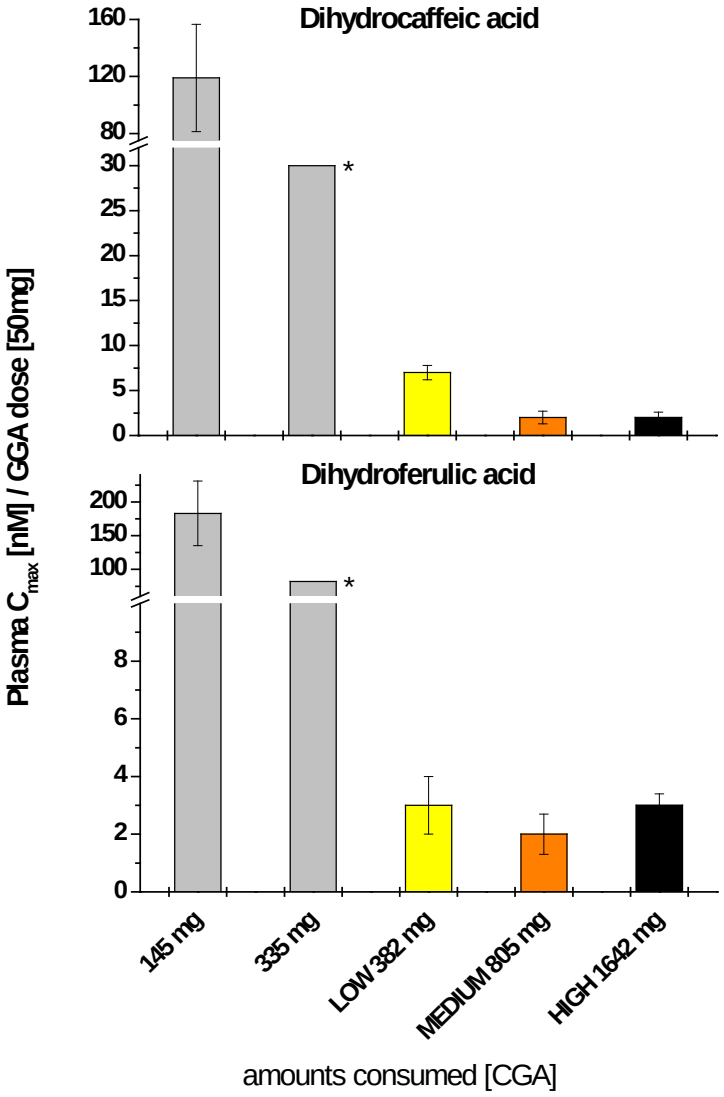


Figure 5-6: Maximum plasma concentrations (C_{max}) (in nM) per 50 mg consumed CGA dose by coffee reported in literature (healthy, with colon) (grey bars) compared to our findings after consumption of different doses (LOW, MEDIUM, HIGH) by four ileostomists (without colon). Different coffee CGA amounts were consumed in published single dose studies, with 145 mg = (Stalmach et al., 2009); 335 mg = (Renouf et al., 2010a; Renouf et al., 2010b), see Table 2-10, State of knowledge. * = SD was not determined in that publication.

The colonic microflora is thought to be the key metabolic site for reduction of hydroxycinnamic acids into dihydro compounds (DHCA (**6a**), DHFA (**8c**)) (Stalmach et al., 2009; Renouf et al., 2010a; Renouf et al., 2013). Other studies with ileostomists confirmed these findings (Stalmach et al., 2010). In our study the plasma concentrations and T_{max} (≤ 0.9 h) of DHFA (**8c**), DHCA (**6a**) and DiMeDHCA (**9b**) indicated also a small contribution of reduction processes (*e.g.* by hepatic

NADPH-cytochrome P450 phase I metabolism or in the enterocyte) in the upper parts of the GIT, see Figure 5-6 (Table 10-13, Appendix).

In general, the plasma C_{max} data in our dose-response study correlates well with recently published data achieved with healthy volunteers (with colon) consuming coffee (Stalmach et al., 2009; Renouf et al., 2010a; Renouf et al., 2010b; Renouf et al., 2013), indicating a major influence of the upper GIT on maximum CGA plasma concentrations (*e.g.* CA (**5a**), FA (**7a**), IFA (**7g**), CQA (**1a-d**), FQA (**3a-c**)). Nevertheless, comparative considerations between ileostomists and healthy volunteers are not possible without restrictions, since overall ileal bacterial count, ileal microbiota composition and GI – TT are different in ileostomy volunteers (Fallingborg, 1999).

A comparative consideration of the coffee CGA uptake into the circulatory system (plasma AUC) with existing literature (with healthy volunteers) was not possible. ⅔ of ingested CGA were recovered in the ileostomy bags and would be available in the colon of healthy persons. For instance, a study using a ≈ 2.7 times smaller dose than our lowest dose (LOW) reported a higher plasma AUC level which was probably due to the esterase activity of the colonic microflora. The AUC was ten times higher for the liberation products CA (**5a**) and FA (**7a**) (Stalmach et al., 2009) and compounds which do not undergo microbial liberation such as FQA (**3a-c**) were found to have an AUC nearly three times higher in this study (Stalmach et al., 2009). So it can be concluded that amounts of CGA that reach the colon were absorbed in its intact form (such as FQA) and after microbial biotransformation (such as CA and FA).
The sum of plasma AUC for CGA and metabolites showed no linear dose-response to the ingested dose (AUC: 4,412 ± 751 $nM*h_{0-8}^{-1}$ (HIGH), 2,394 ± 637 $nM*h_{0-8}^{-1}$ (MEDIUM) and 1,782 ± 731 $nM*h_{0-8}^{-1}$ (LOW)). This was most likely due to the high interindividual variability in absorption which was already observed in total renal excretion and the low number of 4 volunteers. The group of Renouf et al. observed also a strong interindividual variability in plasma appearance but a linear dose-response between the sum of AUC and the ingested CGA dose in ten healthy volunteers (Renouf et al., 2013).

Previous research on QA (**10a**) bioavailability predominantly focused measuring urine levels of QA and its colonic metabolite hippuric acid. Nevertheless, more than 50 years ago QA was once observed in serum of humans (Halpern, 1964). Moreover, recent data revealed free QA in plasma after oral ingestion of 6 g QA (Pero et al., 2009).

In our study the plasma concentrations of QA (**10a**) showed a dose independent absorption. However, the elimination of QA from the circulatory system (Figure 4-18, Results) was not complete ($T_{max} \approx 5$ h) even eight hours after coffee consumption. Pero and coworkers observed a T_{max} of 10.5 h for QA in the plasma of a single healthy man (with colon) and discussed a possible accumulation of QA here (Pero et al., 2009). A possible hydrolysis of absorbed coffee CGA in the circulatory system and a slow elimination could impact this. Maximum plasma concentrations of QA were linear dose-dependent. Because of this, it is imaginable that a habitual coffee drinker (3 to 5 cups during the day) might accumulate QA in the circulating system.

5.2.5 Conclusive considerations

Due to the somewhat controversial and inconsistent bioavailability data for chlorogenic acids in literature, we performed a dose-response study with different CGA doses. Therefore, ileostomists consumed increasing coffee doses in a randomized, double blinded, crossover trial and the dose effects on coffee polyphenol and free D-(-)-quinic acid absorption in the upper GIT, ileal excretion (equivalent to colonic availability in healthy volunteers) and metabolism were observed.

Our results show, that after coffee consumption with different CGA and QA doses the oral bioavailability of CGA in ileostomists was slightly decreased by increasing doses (total renal excretion in %: 14.6 ± 6.8 (LOW), 12.1 ± 6.7 (MEDIUM) and 8.0 ± 4.9 (HIGH)).

As both CGA bioavailability and GI - TT of CGA slightly decreased by consumption of increasing doses, we conclude a dose independent absorption of CGA from coffee. Therefore, the dose-response study revealed that the absorption in the upper GIT after CGA consumption is not influenced by different doses. Thus, other factors such as the dosage form (*e.g.* food matrices), different polyphenol compositions of

ingested foods (*e.g.* CGA composition in artichoke), differences in analytical methodology (*e.g.* with or without enzymatic cleavage of CGA conjugates) or different GI - TT have to be taken into account and investigated further. More specifically, considering the fact that different CGA doses from coffee may influence the GIT - TT, it is conceivable that previous studies on CGA bioavailability with different administered dosage forms and food matrices revealed different bioavailability data (Olthof et al., 2001; Kahle et al., 2007; Stalmach et al., 2010; Hagl et al., 2011).
The inter-individual differences in GI - TT in our study were in agreement with the inter-individual differences of CGA and metabolites plasma curves, ileal and renal excretions, especially for volunteer 5. For instance, the decelerated GI - TT of this volunteer (no. 5), resulted in delayed plasma elimination and prolonged renal excretion (*cf*. Figure 4-11, Figure 4-15, Figure 4-17, Results).
A relationship of coffee dose versus the maximum physiological CGA concentrations in the ileal fluids was not observed probably due to the different amounts of water being consumed during the study by the volunteers (see Table 10-9, Appendix). Nevertheless, the maximal physiological CGA concentrations in the ileostomy effluents were at least twice as high as in the coffee being consumed. The reasons for these relatively high CGA concentrations in ileal fluids may result from a concentration process in the upper GIT – tract due to the absorption of water over time.

Approximately 15% of the QA were available for systemic circulation and about 70% of CGA would be available in the colon independently of the consumed doses. Furthermore, due to the high amounts of free QA in coffee, its high plasma C_{max} and the small amounts being renally eliminated, QA is surely an underestimated compound in coffee.

We observed an extensive metabolism for CGA and HCA via passage through the body in ileostomists with new routes of metabolization determined by HPLC-MS in the multiple ion mode, in contrast to the literature (Kahle et al., 2007; Stalmach et al., 2010). The bioavailability of CGA and intensive metabolization was independent of the ingested dose (rates of renally excreted metabolites in % of total renal excretion: $67.0 \pm 6.6\%$ (HIGH), $69.2 \pm 6.5\%$ (MEDIUM) and $69.8 \pm 3.4\%$ (LOW)). However, the

routes of metabolization shifted for CGA and HCA from sulfation to glucuronidation with increasing CGA doses.

Furthermore, we did not identify any diCQA in urine nor in plasma. The higher molecular weight of diCQA (516.5 g*mol^{-1}) might hinder passage across the GI - barrier. Nevertheless, a lack of diCQA in ileal recovery was observed and may be due to hydrolysis in the GIT liberating CQA (**1a-1d**), CA (**5a**) and QA (**10a**) as already discussed in chapter 5.1.

The plasma appearance of CGA and metabolites after coffee consumption showed that plasma C_{max} of ileostomists is comparable to plasma C_{max} of healthy volunteers, except of colonic metabolites (Stalmach et al., 2009; Renouf et al., 2013). This indicates the key position of the upper GIT in CGA absorption. Dimethoxy derivatives of CA (**5a**) and DHCA (**6a**) (DiMeDHCA (**9b**) and DiMeCA (**9a**)) were observed in plasma by others, too (Nagy et al., 2011; Redeuil et al., 2011). Elimination profiles of dimethoxy derivatives in plasma were similar to all other compounds observed, although we did not identify any of these molecules in urine nor in ileal fluids. Thus, we conclude that the body might not be able to eliminate the high lipophilic molecules via renal or bilary excretion (enterohepatic circulation). We presume a demethylation followed by a subsequent conjugation reaction by SULT or UGT enzymes.

5.3 Absorption of individual CGA and QA from coffee in the Ussing chamber with pig jejunal mucosa (*ex vivo*)

Owing to the dearth of data on CGA absorption in the jejunum, we chose pig jejunal mucosa to be used in the Ussing chamber model as it is the best non-primate human-nutrition model (Patterson et al., 2008). Furthermore, for CGA and QA we investigated the influence of physico-chemical properties (structure-absorption relation, (SAR)) and dose (dose-absorption relation, (DAR)) on their absorption in the jejunum.

On the basis of our data provided by the coffee dose-response study with ileostomists (see chapter 4.3.2), we applied the physiological concentrations of CGA in ileal effluents after coffee consumption in the Ussing chamber model (Table 10-9, Appendix). Thus, we used a concentration of at least 20 µM which should be easily reached in the upper GIT after the consumption of a single cup of coffee or other food containing CGA (Neveu et al., 2010), see Table 2-4, State of knowledge.

5.3.1 Individual structure-absorption relation (SAR) of CGA and QA from coffee in pig jejunal mucosa (to serosal direction)

Our results showed that the investigated CGA crossed the pig *jejunal epithelium* (see Figure 4-19, Results, Table 10-14, Appendix) toward a proton gradient (pH 6.0 $_{mucosal}$ / pH 7.4 $_{serosal}$). Only trace amounts of diCQA passed the mucosa and were observable on the serosal side. These findings are in agreement with our observations in the dose-response study with ileostomists (see chapter 4.3). Here diCQA were absent in urine and plasma after coffee consumption. Mostly all other data, available from bioavailability studies (Williamson et al., 2011), confirmed this as well; except for the observation of Farrell et al., 2011 observing a moderate diCQA uptake in gastric and Caco-2 monolayers (Farrell et al., 2011). In this study the low pH (pH 3) of the gastric monolayer might have influenced the log D of CGA (see Figure 2-2, State of knowledge) and particularly the use of a monolayer in contrast to a “poly-layer” (*jejuna epithelium*) used in our study could explain these different observations.

For the subgroups of CGA the following qualitative SAR were determined: CQA (≈ 1%) < CA (≈ 1.5%) < FQA (≈ 2%) < QA (≈ 4%). This is in accordance with the existing literature for CQA and CA absorption in other parts of the GIT (Konishi

and Kobayashi, 2004; Deußer et al., 2013). In the pig ileal mucosa Deußer and co-workers observed CQA (**1a-d**) absorption only for 3-CQA (1.5 ± 0.2%) and 5-CQA (1.9 ± 1.0%) in contrast to 1-CQA and 4-CQA. This is presumably the consequence of the different concentrations used in their study: 50 µM for 3- and 5-CQA in contrast to 10 µM for 1- and 4-CQA. The above mentioned order of the CGA subgroups can be explained in part by the different physico-chemical properties of the subgroups (see Table 5-1).

Table 5-1: Absorption rates (to serosal direction) of individual coffee polyphenols in % (at 20 µM each) in the Ussing chamber with its corresponding physico-chemical properties: molecular weight and distribution coefficient in octanol/water (log D, calculated with MARVIN SKETCH 5.3.1).

Compound	Absorption (%)	MW (g*mol^{-1})	log D (pH 6)
1-*O*-Caffeoylquinic acid **(1a)**	1.4 ± 0.7	354.3	- 2.99
3-*O*-Caffeoylquinic acid **(1b)**	1.0 ± 0.3	354.3	- 2.88
4-*O*-Caffeoylquinic acid **(1c)**	1.0 ± 0.3	354.3	- 2.83
5-*O*-Caffeoylquinic acid **(1d)**	0.9 ± 0.2	354.3	- 2.88
Caffeic acid **(5a)**	1.4 ± 0.8	180.2	- 0.8
3-*O*-Feruloylquinic acid **(3a)**	2.6 ± 1.4	368.3	- 2.74
4-*O*-Feruloylquinic acid **(3b)**	2.0 ± 0.3	368.3	- 2.68
5-*O*-Feruloylquinic acid **(3c)**	2.6 ± 0.5	368.3	- 2.74
3,4-O-diCaffeoylquinic acid **(4a)**	< LOQ	516.5	- 0.47
3,5-O-diCaffeoylquinic acid **(4b)**	< LOQ	516.5	- 0.5
4,5-O-diCaffeoylquinic acid **(4c)**	< LOD	516.5	- 0.47
D-(-)-Quinic acid **(10a)**	3.9 ± 1.3	192.2	- 5.19

Firstly, the molecule size seems to affect the absorption from the mucosal to the serosal side. This is clearly the case when comparing data for diCQA (MW = 516.5 g mol^{-1}) (see Table 5-1) with CA and QA (180.2 and 192.2 g mol^{-1}). In both the Ussing chamber SAR experiment and in the human intervention study (dose-response coffee consumption) an absorption of diCQA was not detected, whereas CA (**5a**) and QA (**10a**) were systemically available in the jejunal SAR and

human dose-response study (see chapter 4.3.4). A lower molecule size seems to benefit the absorption, whereas the stretchable and elastic atom bonds of QA might also matter. Here QA showed the highest absorption rate (3.9%), despite its hydrophilic log D (see Table 5-1 and Figure 4-19, Results). Deußer reported about 0.5% of QA passing the ileal pig mucosa and a T84 colon carcinoma cell monolayer (Deußer et al., 2013). Both experimental designs simulated different parts of the GIT in contrast to our study. Furthermore, the concentrations used for the incubations were four times higher than the ones used in our study.

In our investigations the FQA were absorbed to a higher extent than the CQA. The differences in chemical structures between CQA and FQA are one methoxy group at the hydroxycinnamic acid moiety. Although this additional group increases the molecular size of FQA its lipophilicity is increasing as well. Thus, we conclude that the influence of log D on absorption is higher than that of the molecule size for *mono*CGA (one QA moiety). The influence of molecule lipophilicity (log D) was already reported recently on HCA permeation through a T84 cell monolayer (Bergmann et al., 2009).

In our dose-response human intervention study both CGA subgroups, CQA and FQA, were also detected in plasma after coffee consumption (see chapter 4.3). Because of the extensive metabolism CGA undergo during the passage of the upper GIT, a comparison of the Ussing chamber data discussed here with data from other human bioavailability studies is not possible (Stalmach et al., 2010). Nevertheless, we are able to conclude that absorption in the pig jejunum observed in our study or in a gastric epithelial monolayer (Farrell et al., 2011) contributes to the early appearance of CGA in plasma after coffee consumption.

Another factor that contributes to absorption, apart from the physico-chemical properties, could be active transport. For instance, Konishi and Kobayashi 2004 identified an active transport for certain hydroxycinnamic acids (Konishi and Kobayashi, 2004). FA (**5a**) and CA (**7a**) may in part be actively transported by a monocarboxylic acid transporter (MCT) across the monolayer (Konishi and Kobayashi, 2004; Poquet et al., 2008). Moreover, an active transport is imaginable for QA (**10a**) which shows strong structural similarities to D-glucose. An active transport of this compound similar to that of glucose uptake via SGLT is conceivable but not supported by our data, especially since high concentrations of QA have been

detected in the human plasma and urine after coffee consumption in our dose-response study (see Table 5-1 and chapter 4.3).

The differences in absorption within the CQA, FQA, and diCQA subgroups were rather small. The position of esterification at the QA moiety did not affect the absorption in the pig jejunum at the concentration of 20 µM. Previous intestinal absorption models have considered other parts of the GIT and used different CGA concentrations (Deußer, 2010; Farrell et al., 2011). Nevertheless, the results of Farrell and co-workers gave a hint for a higher absorption of 3,5-diCQA (**4b**) in contrast to other diCQA (Farrell *et al*., 2011).

Moreover, the Ussing chamber experiments revealed that the incubation time used and the serosal concentrations were in relation. This implies that a longer residence time of coffee CGA at the luminal side corresponds to a higher absorbed CGA amount (see Figure 4-20, Results).

5.3.2 Amounts of CGA and QA located at the jejunal pig mucosa

Except for the diCQA, 1 to 1.5% of the CGA and the QA were associated (inside or membrane bound) with the tissue (Figure 4-21, Results). The low levels of diCQA associated with tissue are in accordance with their limited absorption rates. In an Ussing chamber study using T84 cell monolayers, CA (**5a**) and 5-CQA (**1d**) were identified mainly in the cytosol of the cells (Deußer, 2010). This in agreement with the previously hypothesized transcellular diffusion of hydroxycinnamic acids through a co-cultured Caco-2 / HT29-MTX cell monolayer (Poquet et al., 2008).

The significant differences (*$p < 0.05$) in tissue associated 5-CQA (**1d**) after a mucosal application (1.4 ± 0.8%) *vs*. serosal application (0.5 ± 0.3%) (see Table 10-14, Appendix), could be explained by the unstirred water layer at the mucosal side, where 5-CQA might be associated as well.

5.3.3 Secretion of 5-CQA (to mucosal direction)

The amount of 5-CQA (**1d**) secreted from the serosal to mucosal side was significantly higher than the amount absorbed ($p < 0.001$). Coincubations with the metabolic inhibitor NaN_3 ($p < 0.05$) reduced the secretion (Figure 4-22, Results), indicating the involvement of an active efflux process. Farrell and co-workers have

already discussed the effects of increased CGA secretion in contrast to absorption; the presence of an active efflux transport was postulated (Farrell *et al*., 2011). Selective inhibition showed an influence on MRP- and P-gp transporters in the secretion of the CGA, FA glucuronide (**7b**) and DHFA (**8c**) reported by Poquet (Poquet et al., 2008). In our study, we were able to identify MRP 2 and the P-gp transporter MDR 1 in pig jejunal mucosa by Western blot analysis (see Figure 4-23, Results). Up until now no such transporters have been reported for pig jejunum. In the human jejunum, these two ABC efflux transporters are located on the mucosal side (Taipalensuu et al., 2001; Seithel et al., 2006). A potentially similar organization of transporters in the pig jejunum might contribute to the significantly higher secretion than absorption and hence reduction of 5-CQA bioavailability.

5.3.4 Stability and interesterification of individual coffee compounds in the *ex vivo* experiment

Interesterification reactions and hydrolytic activities were observed in the mucosal compartment (see Table 4-3, Results); being in agreement with other studies (Kahle et al., 2007; Hagl et al., 2011) and with the incubation experiments with CGA and ileal effluents as already discussed in chapter 5.1. Furthermore, the observed moderate hydrolytic activity was in agreement with other *in vivo* studies (Stalmach et al., 2010). In Figure 4-25, Results, levels of the identified interesterification (CQA) and hydrolytic (caffeic acid, **5a**) products of 5-CQA (**1d**) at pH 6 on the mucosal side are given.

The highest rates of interesterification were observed for 4-acyl CQA (**1c**) and FQA (**3b**) because this *para*-position is sterically benefited (see Figure 5-7). 3- and especially 5-acyl CQA (**1b, 1d**) and FQA (**3a, 3c**) exhibited lower rates of interesterification and 1-CQA (**1a**) exhibited none (see Table 4-3, Results). Due to the steric preference of position 4, the 3- and 5-acyl mono CGA migrated nearly exclusively to this position. Additionally, 4-acyl mono CGA migrated dominantly into the 3-acyl one (see Figure 5-7). The migration strongly depends on the pH in the medium, as shown when 5-CQA (**1d**) was applied at the serosal side with the higher pH of 7.4 (interesterification of approximately 18%) (see Table 4-3, Results) and by previous results (Farah et al., 2006). Whereas previously no interesterification of 5-CQA (**1d**) was reported after an incubation with T84 cells in HBSS medium (pH 7.4;

4 hours) in the Ussing chamber (Bergmann et al., 2009). However, Bergman and coworkers did not increase the buffer capacity of the HBSS medium with $NaHCO_3$ which is necessary to stabilize pH at 7.4 when gassed with CO_2. We observed a decrease of pH in HBSS without additional $NaHCO_3$ from 7.4 to 6.9, because of the formation of H_2CO_3 in the HBSS buffer when gassed with CO_2. Hence, pH might have changed during the experiment of Bergman et al. and therefore, no interesterification, of 5-CQA (**1d**) was observed.

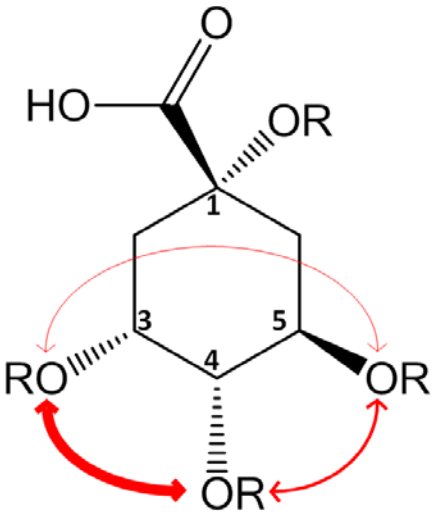


Figure 5-7: Structure of D-(-)-quinic acid (10a). Schematic acyl migration (interesterification) pathways of *mono*CGA (FQA (3a-c), CQA (1a-d)) during incubation of individual *mono*CGA in the Ussing chamber experiments (pH 6). Experimental conditions see chapter 7.3.2. R = H, CA (5a) or FA (7a). Bold arrows are major acyl migration pathways. Acyl migration at position 1 (*e.g.* 1-CQA) is sterically inhibited and at the *para* position 4 sterically benefited.

Lower interesterification levels were observed for the diCQA (**4a-c**), but simultaneously exhibited an increased hydrolysis. Due to the steric benefit of position 3 and especially the *para* position 4 at the QA molecule (Figure 5-7), 3,4-diCQA (**4a**) showed neither hydrolysis nor acyl migration (see Table 4-3, Results). The molecule 4,5-diCQA (**4c**) was less stable and showed some hydrolysis (4%) but no acyl migration as the benefited position 4 is already substituted. However, we observed some hydrolysis (6%) and interesterification (1.7%) of 3,5-diCQA (**4b**) into the more stable 3,4-diCQA (**4a**).

These findings are in agreement with our observations of 3,5-diCQA (**4b**) being less stable in contrast to 3,4-diCQA (**4a**) in the incubation experiments with CGA in ileal fluids (see chapter 5.1 and Table 10-2, Appendix). Additionally, in the dose-response study (chapter 4.3 and Table 10-6, Appendix)) 3,5-diCQA showed the lowest recovery rates of diCQA subgroup in ileal effluents after coffee consumption being in agreement to another coffee consumption study with one single coffee dose (Stalmach et al., 2010).

5.3.5 Metabolic actions of pig jejunal tissue on 5-CQA

As a consequence of the low dose of CGA used in the SAR experiments (20 µM) metabolites were not detectable. At higher concentrations (≥ 100 µM), applied to the pig ileal mucosa, conjugation reactions of FA (**7a**) have been reported previously (Deußer, 2010). In our dose-response study (see chapter 4.3) and in a recently published study a significant proportion of CGA metabolism occurred in the human upper GIT, except for 5-CQA (Stalmach et al., 2010), metabolites including free and sulfated caffeic and ferulic acids and others occurring. In those studies no glucuronidation of CQA was observed in the upper GIT. Here, *ex vivo* trace amounts of a 5-CQA glucuronide were detected on the serosal side of the pig mucosa when 5-CQA was applied at high concentration (3.5 mM). Glucuronidation as the major phase II metabolism in pigs was reported before (Wu et al., 2005; Espín et al., 2007).

5.3.6 Pig jejunal absorption mechanism of the main polyphenol (5-CQA) from coffee

The DAR experiment was performed with the most abundant CGA, 5-CQA (**1d**). In our *in vivo* dose-response study with ileostomists (see chapter 4.3) we discovered an influence of the GI – TT on CGA bioavailability. Such effects could be excluded in the *ex vivo* Ussing chamber absorption experiments. The concentrations used here were 0.02 up to 3.5 mM, covering the physiological range in the GIT after the consumption of a cup of the coffee (LOW) (see Table 10-9, Appendix). Dose-response studies indicated a non-saturable, dose-independent absorption of 5-CQA in pig jejunum as the flux ($nmol*h^{-1}*cm^{-2}$) was linear over the concentration range (Figure 4-24, Results). Thus, we conclude a passive diffusion being the major absorption

mechanism in the pig jejunal mucosa (*cf*. Figure 2-9, State of knowledge). Furthermore, the dependency of CGA absorption on its molecular weight and lipophilicity (log D) as shown in chapter 5.3.1 gives evidence for diffusion processes as well.

This is in agreement with previous studies using gastric and Caco-2 cell monolayers (Konishi and Kobayashi, 2004; Farrell et al., 2011). In these studies, a linear relationship and a non saturable absorption were reported indicating a diffusion process. In the dose-response study with ileostomists we discovered evidence of a dose-independent absorption of CGA in the upper GIT (see chapter 4.3). Nevertheless, 5-CQA has been shown to be a poor substrate for MCT in a Caco-2 cell model (Konishi and Kobayashi, 2004) and a non-linear transport has been observed in a gastric cell model for some CGA by others (Farrell et al., 2011).

5.3.7 Conclusive Considerations

As the absorption of CGA *in vivo* is influenced by various bioavailability factors such as extensive biotransformation or differences in proband's physiology (*e.g*. GI – TT) investigations on individual CGA absorption are limited (see chapter 4.3.2) (Stalmach et al., 2010). Therefore, the influence of the physico-chemical properties on absorption of individual CGA (SAR) and the mechanism of CGA absorption (DAR) were investigated using the Ussing chamber model (with its standardized experimental conditions). Furthermore, owing to the dearth of data on CGA absorption in the jejunum, we chose pig jejunal mucosa for the Ussing chamber model.

It was shown that an absorption in the upper GIT contributes to the early plasma appearance of CGA after coffee consumption (Table 10-13, Appendix) (Renouf et al., 2010a). Additionally, the absorption of CGA from coffee in the jejunum strongly depends on their physico-chemical properties (absorption rate: CQA (≈ 1%) < CA (≈ 1.5%) < FQA (≈ 2%) < QA (≈ 4%)) and the absorption rate of the main CGA from coffee (5-CQA (**1d**)) was not saturable by increasing doses, indicating a passive diffusion as the absorption mechanism.

Furthermore, we conclude the following relationship: the longer the incubation time of CGA with pig jejunal mucosa in the Ussing chamber, the higher the absorbed CGA amounts. Therefore, incubation time and absorbed CGA amount were in a direct

proportion (Figure 4-20, Results). This outcome confirmed our thesis of a relation between the total GI – TT of CGA after coffee consumption and the total absorbed CGA amount in humans. More specifically, *in vivo* an increased total renal CGA excretion is associated with an increased GI – TT of CGA (see Figure 5-2). Thus, the GI – TT becomes a decisive parameter for CGA uptake.

In spite of this, the detected active efflux transport of 5-CQA (**1d**) is limiting its bioavailability. Nevertheless, it can be enhanced *ex vivo* by the metabolic inhibitor NaN_3. This observation confirms our postulated thesis of an active efflux transport of CGA and especially CGA-metabolites at the enterocyte.

Enhancing bioavailability of CGA might also be possible via competitive inhibition of efflux transporters by other polyphenols as already discussed by others (Williamson et al., 2007; Walter et al., 2010). Such interactions have not been sufficiently investigated, but the flavanol quercetin seems to show hereupon an influence. Its metabolites have shown an interaction with MRP2 and its aglyca led to an increased absorption of the polyphenol hesperitin in a Caco-2 cell model (Williamson et al., 2007; Brand et al., 2010). Thus, quercetin glycosides which also occur in apple juice besides CGA (Kahle et al., 2005) can be released by ileal microbiota (Knaup et al., 2007) or by lactase-phlorizin hydrolase (Day et al., 2003) and may increase the absorption of other polyphenols. Such possible synergistic effects make the bioavailability of polyphenols more sophisticated and may partially explain the differences in ileal recovery of CGA after apple juice consumption (Kahle et al., 2007) in comparison to coffee consumption (see Figure 5-1).

6 General discussion

The lifelong daily exposure of CGA and their potentially beneficial effects on human health generates a growing interest in them. A precise knowledge of CGA bioavailability is important for the understanding of health effects related to CGA consumption since bioavailability describes the amount of CGA and metabolites becoming available at the site of action.

However, several factors have an influence on the oral bioavailability of a drug such as CGA. The dosage form (*e.g.* matrix), dose, proband's physiology or the molecular properties of the drug can affect its bioavailability (Chereson, 1996; Lipinski et al., 1997; Dietrich et al., 2003; Williamson et al., 2011; El-Kattan and Varma, 2012). In the diet, daily intake of CGA (dose) varies from 25 up to 1000 mg a day (Clifford, 1999). As CGA show a wide distribution among edible plants (Neveu et al., 2010) the dosages (matrix) varies, as well. Furthermore, the CGA composition in food (*e.g*. apple juice, artichoke leaf extract, coffee) can be different (Farah and Donangelo, 2006; Neveu et al., 2010) resulting in different physico-chemical properties of the CGA compositions.

Up to now studies on oral bioavailability of CGA with different study designs (*e.g*. dose or dosage form) might be the reason for the inconsistent and contradictory data (Olthof et al., 2001; Nardini et al., 2002; Kahle et al., 2007; Monteiro et al., 2007; Stalmach et al., 2009; Renouf et al., 2010a; Renouf et al., 2010b; Stalmach et al., 2010; Hagl et al., 2011; Williamson et al., 2011; Renouf et al., 2013). Hence, we addressed the question: can different administered doses of CGA (dose-absorption relation) or different CGA compositions (structure-absorption relation) influence their bioavailability in the upper GIT?

As coffee is one of the richest dietary sources of CGA (Clifford, 1999; Farah and Donangelo, 2006) we used decaffeinated instant coffee in our study to observe dose-response effects on oral bioavailability in the upper GIT, on metabolization and on the sites where absorption occurs within the GIT. Therefore, ileostomy volunteers consumed three different doses (HIGH, MEDIUM, LOW) of CGA and QA by instant coffee on separate occasions. The consumed doses were 2.5 to 10 times higher than amounts normally ingested by a single cup of coffee. A possible effect of dose on bioavailability by acute ingestion was determined by ileal effluents (equivalent to colonic

availability in healthy subjects), plasma, and renal concentrations of QA, CGA and metabolites.

Furthermore, the influence of the physico-chemical properties of CGA and QA from coffee on its bioavailability was monitored since structure-absorption relation (SAR) bioavailability data of the individual coffee CGA and QA was inconsistent. This was performed *ex vivo* with the so called Ussing chamber model using pig jejunum mucosa applying individual polyphenol compounds from coffee (1-, 3-, 4-, 5-CQA (**1a-d**), 3-, 4-, 5-FQA (**3a-c**), CA (**5a**), 3,4-, 3,5-, 4,5-diCQA (**4a-c**), QA (**10a**)) in physiological concentrations. Additionally, we performed a dose-response experiment in the Ussing chamber to clarify the mechanism of CGA absorption as the data of CGA absorption mechanism is controversial as well (Konishi and Kobayashi, 2004; Deußer, 2010; Farrell et al., 2011).

Our results show that after coffee consumption of different doses of CGA and QA by ileostomy subjects, bioavailability was affected by the GI – TT. An accelerated ileal excretion reduced the systemic bioavailability of nearly all CGA when increasing doses of CGA from soluble coffee were ingested (total renal excretion in %: 14.6 ± 6.8 (LOW), 12.1 ± 6.7 (MEDIUM) 8.0 ± 4.9 (HIGH)). As both CGA bioavailability and GI - TT slightly decreased by consumption of increasing doses, we conclude a dose independent absorption. The total bioavailability of QA is observed independent of dose as well.

These observations were confirmed by Ussing chamber experiments with pig jejunum mucosa where on one hand a dependency of total absorption and residence time was observed and on the other hand a dose independent absorption of 5-CQA (**1d**).

Hence, the GI – TT is a decisive bioavailability parameter for CGA uptake in the upper GIT. Consequently, considering differing gastric residence times after consumption of different meals (Zimmermann and Leitold, 1992) this means an influence of GI – TT by the chemical composition of ingested food (see chapter 2.4.1.2, State of knowledge) might influence the CGA absorption in a much more complex way than different CGA amounts itself. Due to this, the GI – TT has to be taken into account in bioavailability studies in the future.

For the first time, a high availability of QA in the upper GIT (approx. 15% renally excreted) and the dose independent colonic availability of CGA (about 70%) was

observed *in vivo*. So the sites where CGA absorption occurs within the GIT was regardless of the dose.

We detected a much more extensive metabolism during the passage of CGA through the body in ileostomists than expected from literature (Stalmach et al., 2010). Nevertheless, for the first time significant amounts of unmetabolized CGA (3-, 4-, 5-CQA (**1b-d**), 3-, 4-, 5-FQA (**3a-c**), 3-, 4-CQL (**2a-b**)) from coffee were detected in urine supporting their systemic availability. Whereas in the coffee beverage the 5-acyl compounds 5-CQA (**1d**) and 5-FQA (**3c**) were dominant within a CGA subgroup, the 4-acyl compounds, 4-CQA (**1c**) and 4-FQA, (**3b**) were predominant at the site of action. This has to be taken into account for future *in vitro* studies. The routes of CGA metabolization were influenced by the dose since a shift from sulfation to glucuronidation occurred with increasing doses.

The major mechanism of CGA absorption is governed by their physico-chemical properties (especially log D and molecular weight) in the upper part of the GIT, but differences of CGA subgroups in absorption were less (absorption rate: CQA (≈ 1%) < CA (≈ 1.5%) < FQA (≈ 2%) < QA (≈ 4%)) as we were able to show by our Ussing chamber experiments using ileal pig mucosa.

We conclude that after coffee consumption at high CGA and QA doses the bioavailability of CGA was not limited and that different CGA profiles can have a minor influence on bioavailability in the upper GIT. The variations observed on CGA bioavailability in the current *in vivo* study were based on differences in volunteers physiology (GI - TT) and could not explain the differences in literature data reported (Monteiro et al., 2007; Williamson et al., 2011). According to the contradictory data from literature on CGA bioavailability it is possible that the different consumed dosage forms (matrices) influence the absorption behaviour of CGA. Additionally, differences in analytical methodologies used (*e.g.* with or without enzyme cleavage of CGA conjugates) might have influenced the outcomes as well. On the basis of those considerations a bioavailability study with a consumption of CGA in different matrices with one analytical method is suggested.

7 Materials and methods

7.1 Materials

7.1.1 Register of chemicals

3,4,5-trimethoxyphenylacetic acid	Fluka, Steinheim (Germany)
Acetic acid	Merck, Darmstadt (Germany)
Acetonitrile	J.T.Baker, Deventer (Netherland)
Acrylamide	Roth, Karlsruhe (Germany)
ALIGAL	80% N_2 20% CO_2, Drumm, Kaisersalutern (Germany)
Ammonia	Sigma Aldrich, Steinheim (Germany)
Ammonium persuflate (APS)	Roth, Karlsruhe (Germany)
$CaCl_2$ x 2 H_2O	Sigma Aldrich, Steinheim (Germany)
Carbogen gas	AirLiquide, Düsseldorf (Germany)
$CoCl_2$ x 6 H_2O	Merck, Darmstadt (Germany)
Creatinine	Sigma Aldrich, Steinheim (Germany)
$CuSO_4$ x 5 H_2O	Merck, Darmstadt (Germany)
Cysteinehydrochloride x H_2O	Sigma Aldrich, Steinheim (Germany)
Dimethylsulfoxid	Merck, Darmstadt (Germany)
EDTA	Merck, Darmstadt (Germany)
EtOH *p.a*.	Roth, Karlsruhe (Germany)
$FeSO_4$ x 7 H_2O	Merck, Darmstadt (Germany)
Formic acid	Chemsolute, Renningen (Germany)
Goat anti-mouse IgG-HRP	Santa Cruz Biotechnology, Heidelberg (Germany)
H_2O bidist.	Büchi, Essen (Germany)
H_3PO_4	Sigma Aldrich, Steinheim (Germany)
Hanks´ balanced salt solution	Sigma Aldrich, Steinheim (Germany)
Hydrochloric acid	J.T.Baker, Deventer (Netherland)
K_2CO_3	Sigma Aldrich, Steinheim (Germany)
KCl *p.a.*	Sigma Aldrich, Steinheim (Germany)
L-ascorbic acid	Sigma Aldrich, Steinheim (Germany)
MAb C219 (P-gp)	Abcam, Cambridge (UK)

MAb M2 III-6 (MRP 2)	Abcam, Cambridge (UK)
MeOH	J.T.Baker, Deventer (Netherland)
$MgCl_2$ x 6 H_2O	Sigma Aldrich, Steinheim (Germany)
Milk powder	Roth, Karlsruhe (Germany)
$MnSO_4$ x 1 H_2O	Merck, Darmstadt (Germany)
$Mo_7(NH_4)_6O_{24}$ x 4 H_2O	Merck, Darmstadt (Germany)
Mouse mAb (beta-actin)	Cell Signaling, Frankfurt am Main (Germany)
Na_2SO_4, waterfree	Sigma Aldrich, Steinheim (Germany)
NaCl	Merck, Darmstadt (Germany)
NaH_2PO_4	Sigma Aldrich, Steinheim (Germany)
$NaHCO_3$	Merck, Darmstadt (Germany)
NaOH	Merck, Darmstadt (Germany)
Natriumazid	Sigma Aldrich, Steinheim (Germany)
$NiCl_2$	Sigma Aldrich, Steinheim (Germany)
o-Coumaric acid	Sigma Aldrich, Steinheim (Germany)
Perchloric acid 70-72% GR for analysis	Merck, Darmstadt (Germany)
Picric acid	Sigma Aldrich, Steinheim (Germany)
Resazurin	Sigma Aldrich, Steinheim (Germany)
See Blue Plus 2	Invitrogen, Darmstadt (Germany)
Sodium acetate	Merck, Darmstadt (Germany)
Sodium dodecylsulfate (SDS)	Roth, Karlsruhe (Germany)
Solution I	EtOH / H_2O / formic acid (30/69.9/0.1, *v/v/v*)
Solution II (Homogenize buffer)	Tris (6.057 g) + EDTA (146.12 mg) diluted in 500 mL H_2O and adjusted to pH 7.6
Sulfatase (Cat No S-9754)	Sigma Aldrich, Steinheim (Germany)
Tetramethylethylenediamine	Sigma Aldrich, Steinheim (Germany)
Tris	Roth, Karlsruhe (Germany)
Urea	Merck, Darmstadt (Germany)
$ZnSO_4$ x 7 H_2O	Merck, Darmstadt (Germany)
β-glucuronidase (Cat No G-7396)	Sigma Aldrich, Steinheim (Germany)

7.1.2 Register of used CGA, QA and metabolites

1-*O*-Caffeoylquinic acid	Provided by Hannah Deuser (Bergmann et al., 2009)

5-*O*-Caffeoylquinic acid	Sigma Aldrich, Steinheim (Germany)
3-*O*-Feruloylquinic acid	Provided by Denis Barron (NRC)
4-*O*-Feruloylquinic acid	Provided by Denis Barron (NRC)
5-*O*-Feruloylquinic acid	Provided by Denis Barron (NRC)
Caffeic acid	Sigma Aldrich, Steinheim (Germany)
Ferulic acid	Sigma Aldrich, Steinheim (Germany)
Dihydrocaffeic acid	Fluka, Steinheim (Germany)
D-(-)-Quinic acid	Sigma Aldrich, Steinheim (Germany)
U-^{13}C-D-(-)-Quinic acid	Isolife, Wageningen (Netherland)
Isoferulic acid	Extrasynthèse, Lyon (France)
Dihydroferulic acid	Fluka, Steinheim (Germany)
Dimethoxycaffeic acid	Sigma Aldrich, Steinheim (Germany)
Dimethoxydihydro caffeic acid	Sigma Aldrich, Steinheim (Germany)
d$^{13}C_2$-Caffeic acid	Orphachem S.A., Clermont Ferrand (France)
3-*O*-Caffeoylquinic acid	Synthesis and isolation (see chapter 7.3.5.1)
4-*O*-Caffeoylquinic acid	Synthesis and isolation (see chapter 7.3.5.1)
3-*O*-Caffeoylquinide	Synthesis and isolation (see chapter 7.3.5.2)
4-*O*-Caffeoylquinide	Synthesis and isolation (see chapter 7.3.5.2)
1,3-*O*-diCaffeoylquinic acid	Biopurify Phytochemicals Ltd., Chengdu Sichuan (China)
1,5-*O*-diCaffeoylquinic acid	Biopurify Phytochemicals Ltd., Chengdu Sichuan (China)
3,4-*O*-diCaffeoylquinic acid	Synthesis and isolation by Eric Heinen
3,5-*O*-diCaffeoylquinic acid	Alexis Biochemicals, Lörrach (Germany)
4,5-*O*-diCaffeoylquinic acid	Biopurify Phytochemicals Ltd., Chengdu Sichuan (China)

The following CGA metabolites were all kindly provided by Denis Barron from the Nestle´ research centre in Lausanne (NRC).

Ferulic acid 4´-*O*-glucuronide

Isoferulic acid 3´-*O*-glucuronide

Caffeic acid 4´-*O*-glucuronide

Caffeic acid 3´-*O*-glucuronide

Dihydroferulic acid 4´-*O*-glucuronide

Dihydrocaffeic acid 4´-*O*-glucuronide
Dihydrocaffeic acid 3´-*O*-glucuronide
Dihydro caffeic acid 4´-*O*-sulfate
Dihydro caffeic acid 3´-*O*-sulfate
Dihydro ferulic acid 4´-*O*-sulfate
Caffeic acid 4´-*O*-sulfate
Caffeic acid 3´-*O*-sulfate
Ferulic acid 4´-*O*-sulfate
Isoferulic acid 3´-*O*-sulfate

Mix of FQA-4´-*O*-sulfates, containing:
3-*O*-FQA-4´-*O*-sulfate; 4-*O*-FQA-4´-*O*-sulfate; 5-*O*-FQA-4´-*O*-sulfate
Mix of FQA-4´-*O*-glucuronides, containing:
3-*O*-FQA-4´-*O*-glucuronide; 4-*O*-FQA-4´-*O*-glucuronide; 5-*O*-FQA-4´-*O*-glucuronide
Mix of CQA-3´-*O*-sulfates, containing:
3-*O*-CQA-3´-O-sulfates; 4-*O*-CQA-3´-O-sulfates; 5-*O*-CQA-3´-O-sulfates
Mix of CQA-4´-*O*-sulfates, containing:
3-*O*-CQA-4´-O-sulfates; 4-*O*-CQA-4´-O-sulfates; 5-*O*-CQA-4´-O-sulfates

7.1.3 Consumable material

AnaeroGen™	Oxoid Ltd., Basingstoke, Hamphire (UK)
Api® ZYM	BioMeriéux, Marcy-l`Etoile (France)
Bicinchoninic acid assay, Kit Uptima	KMF Laborchemie, Darmstadt (Germany)
Cryo tubes 1 mL	Greiner Bio-one, Frickenhausen (Germany)
Falcons 15 and 50 mL	Greiner Bio-one, Frickenhausen (Germany)
HPLC-vial	Buddeberg, Mannheim (Germany)
HPLC-vial-inserts	Wagner & Munz, München (Germany)
Incubation vessels 15 mL	Sarstedt, Nümbrecht (Germany)
Monovette tubes 9 mL	Potassium EDTA-gel, Sarstedt, Nümbrecht (Germany)
Pipette tips	Greiner Bio-one, Frickenhausen (Germany)
PVDF-filter	13 mm, 0.45 µm, Pall Corporation, Port, Washington, Michigan (USA)

SPE-column	Bond-elut PH 500 mg, 3 mL, Varian, Agilent Technologies, Böblingen (Germany)
Syringe 1 mL	Terumo, Somerset, New Jersey (USA)
Tubes 1.5 mL and 2 mL	Greiner Bio-one, Frickenhausen (Germany)
Urine collection flask	2.5 L, Sarstedt, Nümbrecht (Germany)

7.2 Equipment

7.2.1 High performance liquid chromatography – diode array-detection (HPLC-DAD)

HPLC system I: HPLC-DAD (Agilent HPLC System)

Degasser	G1379B, Agilent, Waldbronn (Germany)
Binary HPLC-pump	G1312A, Agilent, Waldbronn (Germany)
Autosampler	+ 4°C, G1329A with G1320B, Agilent, Waldbronn (Germany)
Column oven	+ 30°C, G1316A, Agilent, Waldbronn (Germany)
DAD-detector	G1315D, Agilent, Waldbronn (Germany)
Detection wavelengths	254 nm, 270 nm, 320 nm
Software	Chemstation Revision B.02.01. SR2, Agilent, Waldbronn (Germany)

Conditions 1a

Eluate A	H_2O (0.1% formic acid)		
Eluate B	ACN		
Gradient	ascending:	time (min)	ACN (%)
		0	5
		21	17.5
		27	24
		30	28
		60	28
Flow	0.5 mL*min^{-1}		
Injection volume	20 µL		
Column	Synergy polar-RP, 80 Å, 250 x 4.6 mm, 4 µm, Phenomenex, Aschaffenburg (Germany)		

Conditions 1b

Eluate A	H_2O (0.1% formic acid)		
Eluate B	ACN		
Gradient	ascending:	time (min)	ACN (%)
		0	7
		80	27
Flow	1.1 mL*min^{-1}		
Injection volume	20 µL		
Column	Atlantis T3, 3 µm, 4.6 x 150 mm, Waters, Eschborn (Germany)		

Conditions 1c

Eluate A	H_2O (0.1% formic acid)
Eluate B	ACN
Gradient	isocratic, 12% eluate B in 40 min
Flow	0.8 mL*min^{-1}
Injection volume	20 µL
Column	Atlantis T3, 3 µm, 4.6 x 150 mm, Waters, Eschborn (Germany)

HPLC system II: HPLC-DAD (Agilent HPLC System)

Degasser	G1379B, Agilent, Waldbronn (Germany)
Binary HPLC-pump	G1312B, Agilent, Waldbronn (Germany)
Autosampler	+ 4°C, G1367C with G1330B, Agilent, Waldbronn (Germany)
Column oven	+ 40°C, G1316B, Agilent, Waldbronn (Germany)
DAD-detector	G1315C, Agilent, Waldbronn (Germany)
Detection wavelengths	254 nm, 270 nm, 320 nm
Software	Chemstation Revision B.04.01.- SP1 [647], Agilent, Waldbronn (Germany)

Conditions 1a

Eluate A	H_2O (0.1% formic acid)
Eluate B	ACN
Gradient	ascending: time (min) ACN (%) 0 1 10 8 20 18
Flow	0.8 mL*min^{-1}
Injection volume	40 µL
Column	Eclipse plus C18, 2.1 x 50 mm, 1.8 µm, Agilent, Waldbronn (Germany)

Conditions 1b

Eluate A	H_2O (0.1% formic acid)
Eluate B	ACN
Gradient	isocratic, 12% eluate B in 40 min
Flow	0.8 mL*min^{-1}
Injection volume	20 µL
Column	Atlantis T3, 3 µm, 4.6 x 150 mm, Waters, Eschborn (Germany)

HPLC system III: HPLC-DAD (Hewlett Packard 1090 series, compact system)

Degasser	Helium-online-degassing
Binary HPLC-pump	Hewlett Packard 1090 series, photodiode array
Autosampler	Hewlett Packard 1090 series, photodiode array
Column oven	+ 40°C, Hewlett Packard 1090 series, photodiode array
DAD-detector	Hewlett Packard 1090 series, photodiode array
Detection wavelengths	254 nm, 280 nm, 320 nm, 360 nm
Software	HP Chemstation for LC

Conditions 1a

Eluate A	H_2O (0.1% formic acid)
Eluate B	ACN

Gradient	isocratic, 9% eluate B
Flow	1.0 mL*min^{-1}
Injection volume	20 µL
Column	Waters symmetry C18, 4.6 x 250 mm, 5 µm, Waters, Eschborn (Germany)

Conditions 1b

Eluate A	H_2O (0.1% formic acid)
Eluate B	ACN
Gradient	isocratic, 12% eluate B in 40 min
Flow	0.8 mL*min^{-1}
Injection volume	20 µL
Column	Atlantis T3, 3 µm, 4.6 x 150 mm, Waters, Eschborn (Germany)

7.2.2 High performance liquid chromatography – mass-spectrometry (HPLC-MS)

HPLC system IV: HPLC-MS

Degasser	DG-2080-53 3-Line-Degasser, Jasco, Groß-Umstadt (Germany)
System control	LC-Net II/ADC, Jasco, Groß-Umstadt (Germany)
Binäry HPLC-pump	PU-2080 plus intelligent HPLC pump, Jasco, Groß-Umstadt (Germany)
Thermostat controlled autosampler	AS-2057 plus intelligent sampler, + 4°C, Jasco, Groß-Umstadt (Germany)
Mass spectrometer	API 3200 triple quadrupole LC-MS/MS mass spectrometer with valcovalve, Applied Biosystems, Darmstadt (Germany)
Software	Analyst 1.4.2, Jasco, Groß-Umstadt (Germany)

Conditions 1a

Eluate A	H_2O (0.1% formic acid)
Eluate B	ACN

Gradient	ascending:	time (min)	ACN %
		1	5
		21	17.5
		27	24
		30	28
		48	28
Flow	0.5 mL*min^{-1}		
Injection volume	80 µL		
Column	Synergy polar-RP, 80 Å, 250 x 4.6 mm, 4 µm, Phenomenex, Aschaffenburg (Germany)		
Ionization	electrospray ionization (ESI-neg.), - 4.5 kV		
Operating mode	product ion (MS2)		
Resolution Q1:	unit		
Resolution Q3:	unit		
Curtain gas	nitrogen, 30 psi at + 370°C		
Gas 1	70 psi		
Gas 2	40 psi		
CAD	3.0 units		
Electron multiplier voltage	2.0 kV		

7-1: Product ion scan (MS^2) conditions for CGA and metabolites.

Compounds	Q1 *m/z*	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Caffeoylquinic acids	353	- 25.0	- 2.5	- 32.4	- 50.0	- 2.0
Caffeoylquinic acid-*O*-GlucA	529	- 35.0	- 4.5	- 37.2	- 40.0	- 2.0
Caffeoylquinic acid-*O*-Sulf	433	- 25.0	- 2.5	- 34.6	- 40.0	- 2.0
Caffeoylquinides	335	- 25.0	- 2.5	- 31.9	- 50.0	- 2.0
Caffeolyquinide-*O*-GlucA	511	- 40.0	- 4.5	- 36.7	- 40.0	- 2.0
Caffeoylquinide-*O*-Sulf	415	- 25.0	- 2.5	- 34.1	- 50.0	- 2.0
Feruloylquinic acids	367	- 25.0	- 2.5	- 32.8	- 50.0	- 2.0
Iso/-feruloylquinic acid-*O*-GlucA	543	- 30.0	- 9.0	- 37.5	- 40.0	- 2.0
Feruloylquinic acid-O-Sulf	447	- 25.0	- 3.5	- 35.0	- 40.0	- 2.0
Caffeic acid	179	- 25.0	- 2.5	- 27.7	- 50.0	- 2.0
Caffeic acid-*O*-GlucA	355	- 35.0	- 4.5	- 32.5	- 40.0	- 2.0
Caffeic acid-*O*-Sulf	259	- 25.0	- 2.5	- 50.0	- 50.0	- 2.0
Dihydrocaffeic acid	181	- 25.0	- 2.5	- 27.8	- 50.0	- 2.0
Dihydrocaffeic acid-*O*-GlucA	357	- 35.0	- 4.5	- 32.5	- 40.0	- 2.0
Dihydrocaffeic acid-*O*-Sulf	261	- 25.0	- 2.5	- 29.9	- 50.0	- 2.0
Ferulic acid	193	- 35.0	- 3.0	- 28.1	- 30.0	- 2.0
Iso-/ferulic acid-*O*-GlucA	369	- 30.0	- 9.0	- 32.8	- 40.0	- 2.0
Iso-/ferulic acid-*O*-Sulf	273	- 25.0	- 3.5	- 30.3	- 40.0	- 2.0
Feruloylglycine	250	- 35.0	- 3.0	- 29.6	- 30.0	- 2.0
Dihydroferulic acid -4´-*O*-GlucA	371	- 30.0	- 9.0	- 32.9	- 40.0	- 2.0
Dihydroferulic acid-4´-*O*-Sulf	275	- 25.0	- 3.5	- 30.3	- 40.0	- 2.0
diCaffeoylquinic acids	515.5	- 25.0	- 2.5	- 36.8	- 50.0	- 2.0

Conditions Ib

Eluate A	H_2O (0.1 % formic acid)		
Eluate B	ACN		
Gradient	ascending:	time (min)	ACN %
		0	5
		21	17.5
		27	24
		30	28
		48	28
Flow	0.5 mL*min^{-1}		
Injection volume	40 µL		

Column	Synergy polar-RP, 80 Å, 250 x 4.6 mm, 4 µm, Phenomenex, Aschaffenburg (Germany)
Ionization	electrospray ionization (ESI-neg.), - 4.5 kV
Operating mode	Q1 mi (multiple ions)
Curtain gas	nitrogen, 30 psi at + 370°C
Gas 1	70 psi
Gas 2	40 psi
Electron multiplier voltage	2.1 kV
Dwell time	70 msec

7-2: Multiple ion scan conditions for CGA and metabolites.

Compounds	Q1 *m/z*	DP (V)	EP (V)	CEP (V)
Caffeoylquinic acids	353	- 25.0	- 2.5	- 32.4
Caffeoylquinic acid-*O*-GlucA	529	- 30.0	- 3.5	- 37.2
Caffeoylquinic acid-*O*-Sulf	433	- 27.0	- 3.5	- 34.6
Caffeoylquinides	335	- 25.0	- 2.5	- 31.9
Caffeolyquinide-*O*-GlucA	511	- 40.0	- 4.5	- 36.7
Caffeoylquinide-*O*-Sulf	415	- 40.0	- 4.5	- 34.1
Feruloylquinic acids	367	- 25.0	- 2.5	- 32.8
Iso/-feruloylquinic acid-*O*-GlucA	543	- 27.0	- 5.0	- 37.5
Feruloylquinic acid-O-Sulf	447	- 25.0	- 3.0	- 35.0
Caffeic acid	179	- 25.0	- 2.5	- 27.7
Caffeic acid-*O*-GlucA	355	- 35.0	- 4.5	- 22.0
Caffeic acid-*O*-Sulf	259	- 30.0	- 5.0	- 16.0
Dihydrocaffeic acid	181	- 25.0	- 2.5	- 27.8
Dihydrocaffeic acid-*O*-GlucA	357	- 35.0	- 4.5	- 32.5
Dihydrocaffeic acid-*O*-Sulf	261	- 25.0	- 2.5	- 29.9
Ferulic acid	193	- 35.0	- 3.0	- 28.1
Iso-/ferulic acid-*O*-GlucA	369	- 30.0	- 9.0	- 18.0
Iso-/ferulic acid-*O*-Sulf	273	- 25.0	- 3.5	- 16.0
Feruloylglycine	250	- 35.0	- 3.0	- 29.6
Dihydroferulic acid -4´-*O*-GlucA	371	- 30.0	- 9.0	- 32.9
Dihydroferulic acid-4´-*O*-Sulf	275	- 25.0	- 3.5	- 30.3
o-Coumaric acid	163	- 25.0	- 5.0	- 12.0

Conditions Ic

Eluate	H_2O (0.1% formic acid) - ACN; (99/1 ,(*v/v*))
Flow	0.5 mL*min^{-1}
Injection volume	20 µL
Column	Atlantis RP 18 4.6 x 150 mm, 3 µm, Waters, Eschborn (Germany)
Ionization	electrospray ionization (ESI-neg.), - 4.5 kV
Operating mode	MRM (multiple reaction monitoring)
Curtain gas	nitrogen, 25 psi at + 450°C

Gas 1	60 psi
Gas 2	50 psi
Declustering potential	- 55 V
Entrance potential	- 10.5 V
Cell entrances potential	- 28 V
Collison gas	nitrogen, 6 units
Collision energy	- 30 V
Cell exit potential	0 V
Electron multiplier voltage	2.2 kV
Dwell time	2 msec

Conditions Id

Eluate	H_2O (0.1% formic acid) - ACN; (99/1 ,(*v/v*))
Flow	0.5 mL*min^{-1}
Injection volume	70 µL
Column	Atlantis RP 18 4.6 x 150 mm, 3 µm, Waters, Eschborn (Germany)
Ionization	electrospray ionization (ESI-neg.), - 4.5 kV
Operating mode	MRM (multiple reaction monitoring)
Curtain gas	nitrogen, 25 psi at + 370°C
Gas 1	60 psi
Gas 2	50 psi
Declustering potential	- 50 V
Entrance potential	- 11 V
Cell entrances potential	- 30 V
Collison gas	nitrogen, 4 units
Collision energy	- 30 V
Cell exit potential	- 2 V
Electron multiplier voltage	2.3 kV
Dwell time	150 msec

HPLC system V: HPLC-MS

HPLC Transcend system	Thermo Scientific, Dreieich (Germany)

Mass spectrometer	TSQ Vantage Mass Spectrometer, Thermo Scientific, Dreieich (Germany)
PAL System	Thermo Scientific, Dreieich (Germany)

Conditions 1a

Eluate A	H_2O (1% acetic acid)
Eluate B	ACN (1% acetic acid)
Gradient	ascending: time (min) Eluate B%
	0 3
	1 3
	16 20
	26 30
Flow	0.2 mL*min^{-1}
Injection volume	50 µL
Column	UPLC Acquity BEH C18, 1.8 µm, 2.1x150 mm, Waters, Eschborn (Germany) at + 35°C
Ionization	electrospray ionization (ESI-neg.), - 2.5 kV
Operating mode	SRM
Collision Gas Pressure	1.5 mTorr
Cycle time	1 sec
DCV	2 V
Capillary temp.	+ 270°C
Vaporizer temp.	+ 350°C
Sheath gas pressure	40 units
Ion sweep gas pressure	0 units
Aux valve flow	20
Discharge current	- 4.0

Table 7-3: Selective Reaction Monitoring scan (SRM) conditions for CGA.

Compound	Parent mass (*m/z*)	Product 1 (*m/z*)	CE [V]	S-Lens
CA	179.06	135.10	17	56
FA	193.07	134.10	18	57
IFA	193.07	178.10	14	57

Compound	Parent mass (*m/z*)	Product 1 (*m/z*)	CE [V]	S-Lens
DHCA	181.00	137.10	13	60
DHFA	195.08	136.10	17	52
3-CQA	353.09	191.20	21	68
4-CQA	353.10	173.10	17	74
5-CQA	353.10	191.10	18	69
3-FQA	367.11	193.10	19	70
4-FQA	367.11	173.10	15	70
5-FQA	367.11	191.10	22	70
1,3-diCQA	515.12	353.20	19	100
1,5-diCQA	515.12	191.10	31	100
3,4-diCQA	515.12	179.10	29	100
3,5-diCQA	515.12	191.10	31	100
4,5-diCQA	515.12	173.10	29	100
DiMeCA	207.10	103.20	16	62
DiMeDHCA	209.10	150.20	15	75
$d^{13}C_2$-caffeic acid	182.10	137.10	16	52

7.2.3 Ussing Chamber

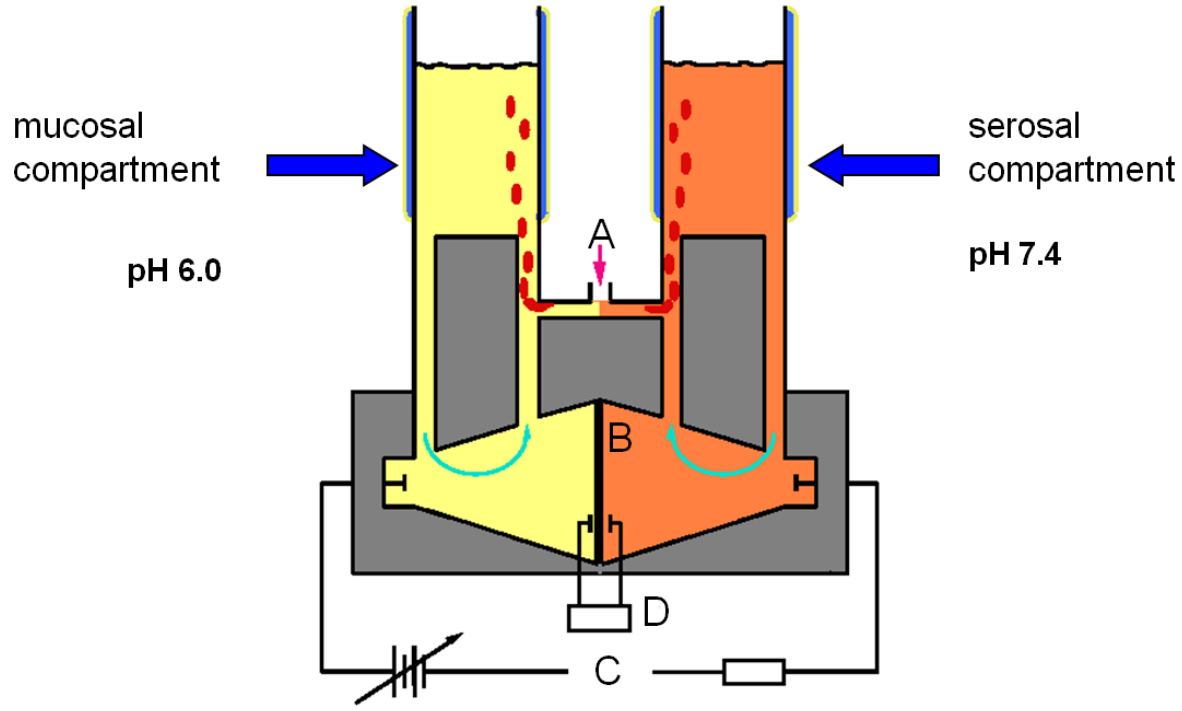


Figure 7-1: Design of the Ussing type chamber: (A) gassing (95% O_2, 5% CO_2); (B) pig jejunal mucosa; (C) tissue conductance (Gt); (D) short-circuit current. Hanks' balanced salt solution at + 37°C was used as medium. Modified according to (Deußer, 2010).

Ussing chamber	K. Mußler, Software Clamp 2.14, 2001 Aachen (Germany)
Area (Figure 7-1, B)	0.785 cm^2
Operating mode:	open circuit
Action replay	1 min, mean value of 2
Pulse duration	1 sec
Pulse distance	1 sec
Number of pulse	1
Gt-parameter:	
Unit	$mS*cm^{-2}$
Amplitude	100 µA
Pulse duration	200 msec (bipolar)

7.2.4 Other equipment

Anaerobic chamber	Arylic glas chamber, self constructed
Biofreezer	Sanyo, München (Germany)
Blot apparatus, Hoefer TE 77	Amersham Biosciences; München (Germany)
Bioimpedance	BF-906, Maltron, Gauting (Germany)
Centrifuge I	5804R, Eppendorf, Hamburg (Germany)
Centrifuge II	5417R, Eppendorf, Hamburg (Germany)
Drying oven	Binder, Tuttlingen (Germany)
Freezer (- 24°C)	Liebherr Premium, Ochsenhausen (Germany)
Lumi-Imager	Roche, Mannheim (Germany)
Lyophilization machine	Christ, Osterode am Harz (Germany)
MS1 Minishaker	IKA, Staufen, (Germany)
NMR, AMX-400	Bruker, Rheinstetten (Germany)
pH meter	Hanna Instruments, Ann Arbor, Michigan (US)
Pipettes	Research, Eppendorf, Hamburg (Germany)
Potter	Schuett, Göttingen (Germany)
UV-VIS Spectrophotometer (UVmini 1240)	SHIMADZU, Duisburg (Germany)
Thermomixer	5436, Eppendorf, Hamburg (Germany)
Ultrasonic bath	Sonorex Super RK 102 P, Bandelin, Berlin (Germany)
Vacuum centrifuge	Unicryo MC2L, UniEqup, Martinsried (Germany)
Vacuum manifold	Supelco, Bellefonte (US)
Vacuum-pump	Rotary vane vacuum pump RZ 5, Vacubrand, Wertheim (Germany)
Vortexer	Genius 3, IKA, Staufen (Germany)
Water bath	Julabo, Seelbach (Germany)
Weighing machine	CP64-0CE, Sartorius, Göttingen (Germany)

7.3 Methods

7.3.1 Human intervention study

This study was performed in the Division of Gastroenterology, Department of Medicine II, University of Wuerzburg.

7.3.1.1 Ethical approval

The study protocol was approved by the Ethics Committee of the Medical Faculty, University of Wuerzburg (No. 124/04 and 32/10).

7.3.1.2 Volunteers

Five female subjects who have undergone colectomy with a terminal stoma of the ileum (but otherwise healthy) have participated in this randomized, double-blinded and 3-period-crossover intervention study. They consumed a single dose of decaffeinated coffee with different amounts of CGA and QA (HIGH, MEDIUM and LOW) on three separate days. All treatments were ingested at one occasion with a minimum on one week washout in between. Ileostomy bags, blood samples and urine samples were collected at selected time points.

7.3.1.2.1 Inclusion and exclusion criteria

Volunteers had to comply with the following inclusion criteria:

- Having undergone colectomy with terminal ileostomy, but otherwise healthy.
- Age > 18 years.

Volunteers which represented one or more of the following criteria were excluded from participation in the study:

- Previous occupational exposure to green coffee beans.
- Having a regular consumption of medication.
- Currently participating or having participated in another clinical trial during the last three weeks prior to the beginning of this study.

7.3.1.2.2 Anthropometry

Anthropometric data of volunteers: average age of 41 ± 3.6 years; BMI of 27.4 ± 2.1 kg/m^2, body fat content of 33.9 ± 2.6% and a basic metabolism rate of 1403.6 ± 58.3 kcal. The reason for colectomy were a M*orbus Crohn`s* disease or *Ulcerative colitis,* volunteers underwent surgery 10.6 ± 6.1 years ago. Anthropometric data of volunteers were measured with Maltron Body Fat-Instrument BF-906 from Juwell medical (for detailed data see Table 10-1, Appendix).

7.3.1.3 Study design

Subjects consumed a single dose of decaffeinated instant coffee with different amounts of CGA and QA (HIGH, MEDIUM and LOW see Table 10-5, Appendix) on three separate days in randomized order. Two days before the start and during study period, volunteers consumed a CGA-free diet and arrived at the study day in a fasted state at 8:00 am. At the beginning of each study day the body weight of each volunteer was measured (see Table 10-1, Appendix) and the total coffee volume to be administrated was calculated (mg*kg^{-1}*BW^{-1}). Instant coffees were reconstituted with boiling water prior to consumption and consumed at a temperature of + 55 – 60°C in less than 10 min. The administered coffee volume was without sugar or milk and served in mg*kg^{-1}*BW^{-1}. A breakfast was served consisting of one white bread and artificial honey (invertase treated sugar product). Volunteers remained fasted for 5 h after breakfast and then consumed a light meal (white bun with ham and cheese) without CGA. Water consumption was allowed *ad libitum*.

Biological fluids were collected at the following time points: Ileostomy fluid - 12 to 0, 0, 0.5, 1, 2, 3, 4, 6, 8 hours, urine - 24 to 0, 0, 0.5, 1, 2, 3, 4, 6, 8, 8 to 24 and 24 to 48 hours, blood samples 0, 0.5, 1, 2, 4, 6 and 8 hours *post* consumption. Sample storage and preparation is described in the following chapters, respectively: ileostomy fluids see chapter 7.3.1.6, urine samples see chapter 7.3.1.5 and blood samples see chapter 7.3.1.7.

After the last blood and ileal fluid sample collection, the volunteers took home a standardized dinner (pasta with cream-cheese sauce and plain yoghurt) and remained on a CGA-free diet until the last urine collection 48 hours after coffee consumption. In Figure 7-2 an overview of the collection intervals of blood, urine and ileostomy bags is given.

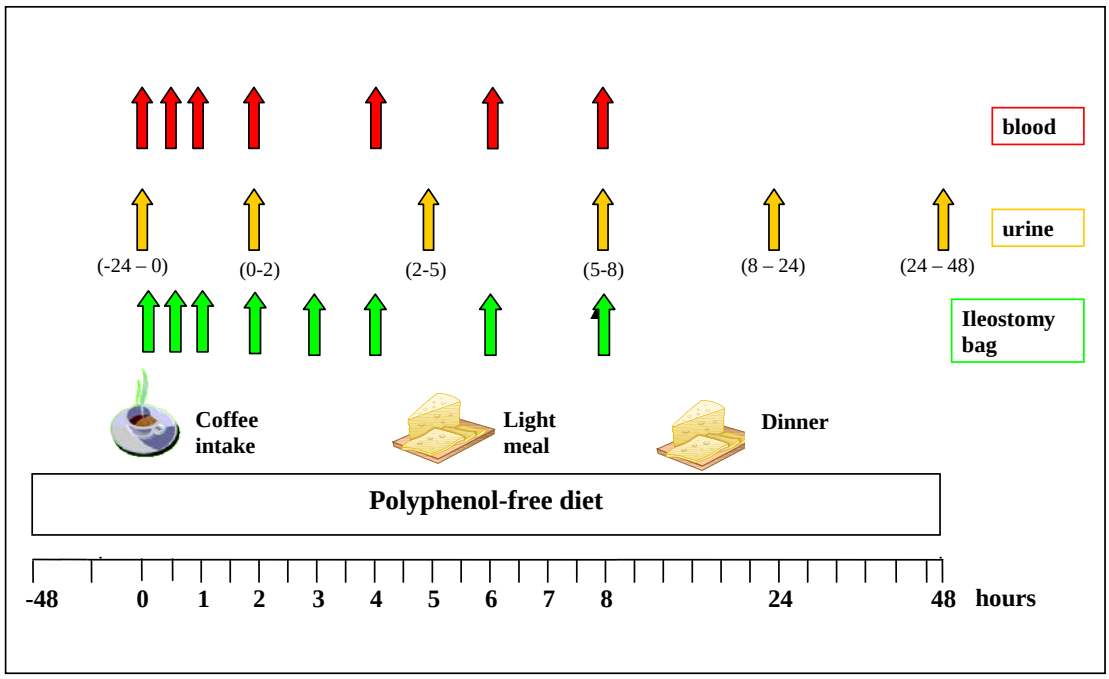


Figure 7-2: Scheme of blood, urine and ileal fluid collection before and after coffee consumption in the ileostomy intervention study. Volunteers consumed a single dose of decaffeinated coffee with different amounts of chlorogenic acid and quinic acid (HIGH, MEDIUM and LOW) on three separate days in randomized order.

Accordingly, on a separate day, volunteers consumed, 2 hours after breakfast, an anthocyanin-rich blueberry beverage to determine the GI - TT by observing the color change of ileal excretion.

7.3.1.4 Instant coffee

On each study day three aliquots of 15 mL of coffee beverage were taken as retain sample and stored at - 80°C. For analysis coffee samples were thawed quickly, centrifuged (5 min, 5,000 x g), membrane filtered (0.45 µm PFDV) and diluted (40 up to 100-fold) with solution I. The internal standard (3,4,5-trimethoxyphenylacetic acid (**ISb**), 30 µL 226.23 mg*L^{-1}) were added to 300 µL of this solution.

7.3.1.4.1 Analysis of coffee CGA

7.3.1.4.1.1 Calibration

CGA were detected at 320 nm and the IS at 270 nm with the HPLC system I. Due to an insufficient chromatographic separation of compounds CQA, FQA and CA were

analyzed with condition 1a (see chapter 7.2.1) whereas CQL, FA and diCQA were analyzed with condition 1b (see chapter 7.2.1).

CGA stock solutions (5-CQA (**1d**), 5-FQA (**3c**), CA (**5a**), FA (**7a**), 3,4-diCQA (**4a**), in solution I) with a maximal concentration range from 333.3 mg*L^{-1} to 10.2 µg*L^{-1} were used. Solutions were analyzed with the HPLC system I (conditions Ia and Ib in chapter 7.2.1) in duplicates. For calibration the observed peak area ratios were plotted versus the concentration ratios with a correlation coefficient of at least R^2 = 0.99. Limit of detection (LOD) and limit of quantification (LOQ) were defined as signal to noise (S/N) ratios: LOD 1:3 and LOQ 1:10, respectively. On this basis, LOQ was calculated from 0.68 to 5.42 ng and LOD from 0.20 to 1.62 ng absolute.

7.3.1.4.1.2 Identification and quantification of CGA

CGA were identified by comparison of retention time, UV spectra and according to the fragmentation patterns with references by HPLC system IV, conditions 1a (Table 4-1, Results). Quantification was based on the achieved calibration curves of references for: FA (**7a**), CA (**5a**). CQA and CQL as 5-CQA (**1d**) equivalents, FQA as 5-FQA (**3c**) equivalents and diCQA as 3,4-diCQA (**4a**) equivalents (for details see HPLC system I, conditions Ia and Ib in chapter 7.2.1).

7.3.1.4.2 Analysis of QA

For the analysis of QA (**10a**) a stable isotope dilution method was established with the isotopically labeled standard U-^{13}C-D-(-)-QA (Isolife, Wageningen (Netherland)) (Erk et al., 2009).

7.3.1.4.2.1 Calibration

Seven reference solutions of QA (**10a**) were prepared in triplicate in solution I with concentrations ranging from 1.2 to 5,000 ng*mL^{-1}. Solutions were analyzed in the MRM mode, and the observed peak area ratios (QA *vs.* U-^{13}C-D-(-)-QA) were plotted *vs* the concentration ratios. LOD and LOQ were defined with signal-to-noise (S/N) ratios of 1:3 and 1:10, respectively. On the basis of the lowest calibration concentrations, an LOQ of 97.7 pg and an LOD of 24.4 pg absolute were calculated.

7.3.1.4.2.2 Identification and quantification of QA

For the quantification of QA (**10a**) a stable isotope dilution analysis with HPLC-MS/MS had been established (Erk et al., 2009). Sample preparation was performed in triplicates.

Coffee solutions were centrifuged (5 min, 5,000 rpm), membrane filtered (0.45 µm PVDF), diluted (1600-fold) with solution I, and 50 µL were spiked with 50 µL of the labeled standard (200 ng*mL^{-1}) and further analyzed by HPLC system IV (conditions Ic, see chapter 7.2.2). For QA quantification the MRM transition from the deprotonated molecular ion [M-H]- m/z 191 to m/*z* 85 was used as the quantifier. Further, the characteristic QA fragmentation ions from [M-H]$^-$ *m/z* 191 to *m/z* 93 and *m/z* 127 were used as qualifier ions to additionally ensure the identity of the detected QA in urine. The MRM transition used for quantification of the isotopically labeled standard U-^{13}C-D-(-)-QA was *m/z* 198 to *m/z* 89 (Figure 7-3).

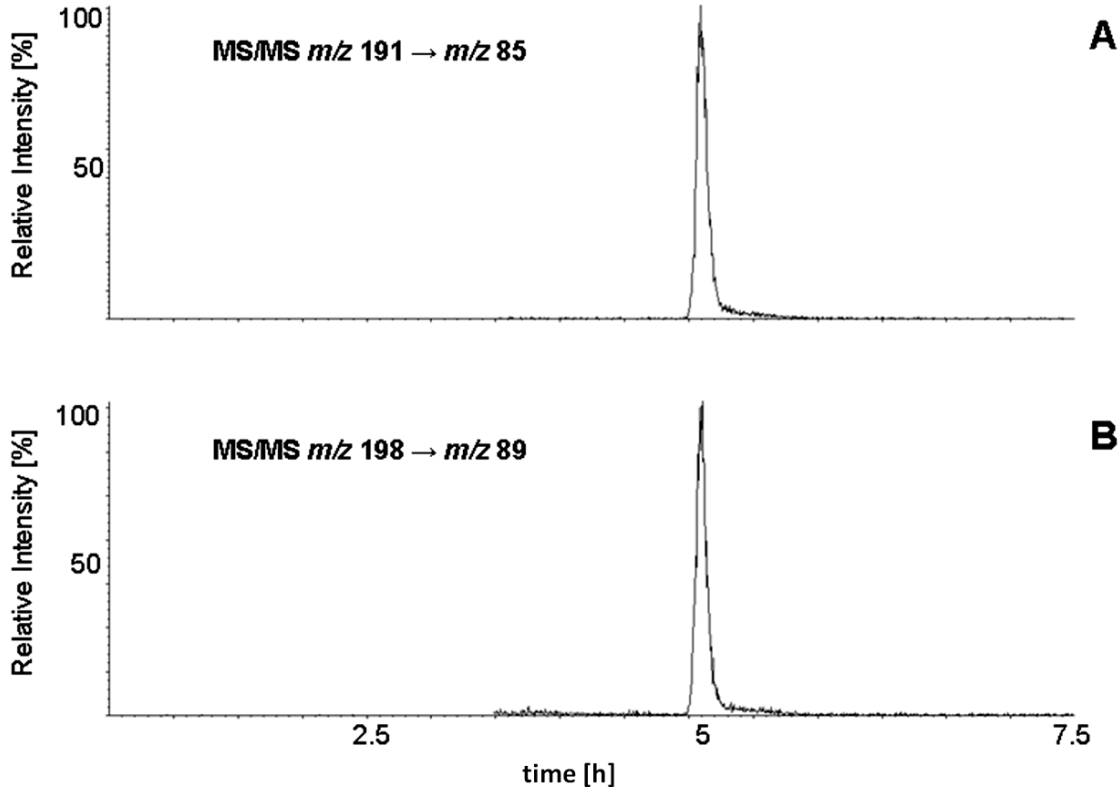


Figure 7-3: HPLC-MS/MS chromatogram monitored in the multiple reaction monitoring (MRM) mode for a model mixture of D-(-)-quinic acid (A) and U-^{13}C-D-(-)-quinic acid (B) at concentrations of 100 ng*mL^{-1} (HPLC system IV, condition Ic see chapter 7.2.2).

7.3.1.5 Urine samples

7.3.1.5.1 Preparation of urine samples

Urine samples were collected before - 24 to 0 and 0, 0.5, 1, 2, 3, 4, 6, 8, 8 to 24 and 24 to 48 hours after coffee consumption. Urine samples were collected in acid washed urine collection flasks containing HCl and ascorbic acid (1 L urine collection flasks containing 10 mL 1 M HCl and 2 mL aqueous ascorbic acid at 100 g*L^{-1}). During the collection intervals, the flasks were kept at + 4 to + 8ºC. At the end of each collection interval the total volume and weight was measured. The pH was adjusted to 3- 4. Five aliquots of 25 mL were filled into a 50 mL falcon and stored at - 80°C.

Unfortunately ileo no. 4 didn't collect the urine sample correctly at the study trial LOW. The sample was collected from 8 to 48 h into one flask.

7.3.1.5.2 Analysis of CGA and metabolites

Aliquots were thoroughly defrosted, warmed to + 37°C, and 3 mL were acidified with H_3PO_4 to a pH of 1.5. In addition, samples were centrifuged (+ 4°C, 5 min, 5,000 x g) and the supernatant (2 mL) was used for solid phase extraction (SPE), (Varian: bond elut PH, 500 mg). All solutions were acidified with HCl (0.01%, *v*/*v*). The SPE - cartridges were preconditioned (2 mL MeOH and 2 mL H_2O) using a vacuum manifold. After sample application (2.0 mL) on the column, the SPE was washed with 1 mL H_2O, dried carefully and afterwards analytes were eluted using 1.8 mL MeOH. The eluate was concentrated in a vacuum centrifuge (60 min), subsequently lyophilized to dryness and stored at – 80°C prior analysis.

Residues were redissolved with 200 µL solution I. To 100 µL of this sample, 10 µL of the IS (*o*-coumaric acid (**ISa**), 61 µM) was added and mixed well. Extraction efficiencies were determined > 90% with test the compounds 5-CQA (**1d**), CA (**5a**), FA (**7a**), DHCA (**6a**), DHFA (**8c**).

7.3.1.5.2.1 Calibration

Calibration curves were generated using a mix of 0 h urine samples (CGA free), spiked with references (see Table 7-4, maximal concentration range 8.2 mg*L^{-1} to 5.5 µg*L^{-1}) and extracted in duplicate as described above. LOQ was from 0.6 to 43.4 ng and LOD was from 0.2 to 13.0 ng (see Table 7-4).

Table 7-4: Limit of detection (LOD) and limit of quantification (LOQ) of the HPLC-MS multiple ion method for CGA and metabolite detection in urine samples (see chapter 7.3.1.5.2.2).

Compound	LOD [ng]	LOQ [ng]
Ferulic acid	4.2	14.1
Ferulic acid-4´-*O*-Sulf	1.3	4.4
Isoferuloylquinic acid-3´-*O*-GlucA	0.7	2.2
Dihydroferulic acid-4´-*O*-Sulf	0.5	1.7
Dihydroferulic acid-4´-*O*-GlucA	0.7	2.3
Caffeic acid	0.2	0.8
Caffeic acid-3´-*O*-Sulf	0.7	2.4
Caffeic acid-3´-*O*-GlucA	2.8	9.4
Dihydrocaffeic acid	13.0	43.4
Dihydrocaffeic acid-3´-*O*-Sulf	0.7	2.4
5-*O*-Caffeoylquinic acid	2.9	9.5
5-*O*-Feruloylquinic acid	0.2	0.6

7.3.1.5.2.2 Identification and quantification of CGA and metabolites

CGA and derivatives identification were achieved on HPLC-system IV, conditions 1a (see chapter 7.2.2). Caffeoylquinic acids and metabolites, Caffeoylquinides and metabolites, Iso- / feruloylquinic acid and metabolites were identified as already reported in chapter 4.3.2.1, Results. Feruloylglycine (**7d**) was identified by comparison of the MS^2 fragmentation pattern reported by (Stalmach et al., 2009). Other hydroxycinnamic acids such as CA (**5a**), FA (**7a**) and derivative compounds were identified by co-chromatography and a matching MS^2 fragmentation pattern with reference compounds provided by NRC.

Quantification of compounds was performed by a multi method (see Figure 10-1, Appendix) with the HPLC system IV, conditions Ib (see chapter 7.2.2). For each analyte the corresponding deprotonated molecular ions $[M-H]^-$ was used as a quantifier in duplicates. Quantification was based on the achieved calibration curves of references (see Table 7-4). Because calibration curves were generated equal to urine samples it was not necessary to use a correction factor for extraction efficacy. Determination was

semi-quantitative, since compounds were quantified based on calibration curves of the available references:

CQA, CQL and their corresponding metabolites, as 5-CQA (**1d**) equivalents; FQA and metabolites as 5-FQA (**3c**) equivalents; FA (**7a**) and FA-Gly (**7d**) as ferulic acid equivalents; FA-GlucA (**7b**) derivatives as IFA-GLucA (**7e**) equivalents; sulfated derivatives as FA-Sulf (**7c**) equivalents. CA- (**5a**) and DHCA (**6a**) metabolites were quantified using the 3´ conjugated metabolites, respectively.

7.3.1.5.3 Quantification of QA

For the quantification of QA (**10a**) in urine the same stable isotope dilution analysis as for coffee was used (see chapter 7.3.1.4.2). Urine sample preparation was performed in duplicates. After unfreezing in a water bath with + 37°C for short, samples were cooled down to room temperature and diluted with bidist. H_2O (20 fold maximum dilution for the high trial). Consequently 40 µL of this dilution were mixed up with 56 µL of the internal standard (U-^{13}C-D-(-)-QA, 200 ng*L^{-1}). To precipitate proteins 16 µL of an acidified methanolic solution (0.25 M HCl in MeOH) were added to this mixture followed by a centrifugation step (+ 4°C, 30 min, 14,000 x g). Samples were quantified with the HPLC system IV, conditions Ic (see chapter 7.2.2) with 20 µL of the received supernatant as injection volume.

7.3.1.5.4 Creatinine determination

Creatinine was measured spectrophotometrically as a colored complex (Knud, 1972). For this, duplicate 400 µL aliquots of diluted urine (normally 1:4) were added to 1,400 µL picric acid solution (9.0 g NaCl, 1.31 g picric acid in 1 L H_2O) and mixed in a cuvette. The reaction was started with the addition of 400 µL sodium hydroxide solution (1 M). Absorption was measured at 510 nm with a UV-VIS spectrophotometer after 10 and 60 sec to obtain:

$$\Delta E = E_{(60\,\mathrm{sec.})} - E_{(10\,\mathrm{sec.})}$$

This difference value and an equally established calibration curve were used to calculate the creatinine concentration. The calibration curve was generated with six standard solutions prepared in duplicate with creatinine concentrations ranging from 34.5 µM to 1105 µM.

7.3.1.6 Ileal fluids

7.3.1.6.1 Preparation of ileal fluid samples

After freeze drying of the ileal bags, the dry weight was determined and samples were carefully homogenized. Aliquots (n = 3) of at least 20 mg were extracted in an Eppendorf tube with 1 mL (solution I). Samples were vortexed shortly at 2,700 min^{-1} and sonicated for 5 min. Subsequently, the samples were centrifuged (5 min., + 4°C, 10,000 x g) and then the supernatant was decanted into a clean Eppendorf tube. The pellet was again re-extracted twice. The received supernatants were combined, filtered through a membrane filter (0.45 µm PVDF). For HPLC-DAD analysis these ileal fluid extract was further diluted (5 up to 20-fold) with solution I. Extraction efficiencies were checked by spiking a CGA-free, re-dissolved ileal extract with a physiological concentration of 5-CQA (**1d**), CA (**5a**) and FA (**7a**) and treated as described above. Recovery rates were determined > 86%.

7.3.1.6.2 Analysis of CGA and metabolites

7.3.1.6.2.1 Identification

Identification of CGA and metabolites was achieved as described in chapter 7.3.1.4.1.2 with HPLC-DAD and chapter 7.3.1.5.2.2 with HPLC-MS/MS.

7.3.1.6.2.2 Quantification of CGA

The obtained extraction solution (see chapter 7.3.1.6.1) was diluted 20 times (solution I) and 30 µL of the IS (3,4,5-trimethoxyphenylacetic acid (**ISb**), 226.23 $mg*L^{-1}$) was added to 300 µL of this solution. CGA determination was performed with the HPLC system I conditions Ia and Ib (see chapter 7.3.1.4.1.1 and 7.2.1).

7.3.1.6.2.3 Quantification of CGA metabolites

####### 7.3.1.6.2.3.1 Sample Preparation

In order to clean up and concentrate ileal samples, a SPE method using a phenylic phase (Varian: bond elut PH. 500 mg) was used for sample preparation. The ethanolic part of the resulting ileal extraction solution (1.0 mL, as described above, see chapter 7.3.1.6.1) was removed in a vacuum centrifuge and subsequently redissolved with

water to 1.0 mL (pH 1.5, acidified with H_3PO_4). This solution was used for SPE extraction as described in chapter 7.3.1.5.2. Residues were redissolved with 200 µL solution I. To 100 µL of this sample, 10 µL of the IS (*o*-coumaric acid (**ISa**), 61 µM) was added and mixed well. Extraction efficiencies were determined > 88% with the test compounds 5-CQA (**1d**) and 5-FQA (**3c**).

7.3.1.6.2.3.2 Calibration

Calibration curves were generated using a CGA free ileal extract, spiked with references (see Table 7-5, maximal concentration range 6.2 mg*L^{-1} to 1.3 µg*L^{-1}). These calibrations were prepared in duplicate as described above and analyzed in duplicate by HPLC system IV conditions 1b (see chapter 7.2.2) in the multiple ion mode. The correlation coefficients were > R^2 = 0.99. LOQ were from 0.7 to 6.2 ng and LOD from 0.2 to 1.9 ng (see Table 7-5).

Table 7-5: Limit of detection (LOD) and limit of quantification (LOQ) of the HPLC-MS multiple ion method used for detection of CGA metabolites in ileal fluid samples (see chapter 7.3.1.6.2.3.3).

Compound	LOD [ng]	LOQ [ng]
Ferulic acid	0.3	1.2
Ferulic acid-4´-*O*-Sulf	0.6	2.0
Isoferuloylquinic acid-3´-*O*-GlucA	0.6	2.0
Dihydroferulic acid-4´-*O*-Sulf	0.4	1.3
Caffeic acid-3´-*O*-Sulf	1.2	4.1
Caffeic acid-3´-*O*-GlucA	0.5	1.5
Dihydrocaffeic acid	1.9	6.2
Dihydrocaffeic acid-3´-*O*-Sulf	1.3	4.4
5-*O*-Caffeoylquinic acid	0.5	1.7
3-*O*-Feruloylquinic acid	0.2	0.7

7.3.1.6.2.3.3 Quantification

Determination was semi-quantitative, since compounds were quantified based on calibration curves of the available references equally as described in chapter 7.3.1.5.2.2.

7.3.1.6.3 Quantification of QA

For the quantification of QA (**10a**) in ileal fluid the extraction solution (see chapter 7.3.1.6.1) was diluted with water (5 up to 400 fold). To 50 µL of this solution 50 µL of the isotopically labeled standard U-^{13}C-D-(-)-QA (200 ng*L^{-1}) was added and mixed up well. For quantification the HPLC system IV, conditions Ic (see chapter 7.2.2) was used.

7.3.1.7 Plasma analysis

7.3.1.7.1 Preparation of blood samples

Blood samples were collected directly before (time point 0 h) and 0.5, 1, 2, 4, 6 and 8 hours after coffee consumption. Blood samples were collected in 9 mL EDTA monovette tubes and were directly centrifuged (3,500 x g, 10 min, + 4°C). To 0.5 mL supernatant (plasma) 20 µL of a fresh 'storage solution' (0.4 M NaH_2PO_4 buffer containing 20% ascorbic acid and 0.1% EDTA) was added and stored at - 80°C. Depending on the blood volume at least three aliquots were obtained.

Unfortunately due to five unsuccessful attempts for placing the needle correctly, two volunteers withdrew their permission for blood collections (Ileo 3 at the HIGH trial and Ileo 4 at the MEDIUM trial). Additionally, the needle of Ileo 1 was blocked after five hours at the MEDIUM trial. Here data for time points six and eight are missing.

7.3.1.7.2 Analysis of CGA and metabolites

7.3.1.7.2.1 Quantification of CGA an metabolites

For measurement of CGA, duplicate samples of 100 µL plasma were spiked with an isotopically labeled IS (5 µL; 1 µM labeled d$^{13}C_2$-caffeic acid) and protein was precipitated (500 µL ethanol) by centrifugation (5 min, + 4°C, 17,500 x g). The residue was further twice re-extracted and the combined supernatants dried under N_2. After evaporation, the residue was incubated at + 37°C for 60 min with *β*-glucuronidase (1,000 units) and sulfatase (60 units) (in pH 5.5, 0.1 M sodium acetate). After adding

10 µL perchloric acid (6 M), CGA was extracted twice (30 min at + 4°C) with ACN. The supernatants were adjusted to pH 7 with potassium carbonate (0.75 M; 40 µL), pooled, dried and re-suspended (water with 1% acetic acid, 5% ACN), and then samples (50 µL) were analyzed by HPLC mass spectrometry.

The selective determination of each target compound was performed by acquisition of characteristic product ions of the precursor ion in the selected reaction monitoring (SRM) mode with HPLC system V, conditions 1a (see chapter 7.2.2).

7.3.1.7.2.2 Calibration

Calibration curves were generated for each volunteer and trial by spiking blank plasma sample (0 hour collection point) with the labeled CA as IS and a stock solution including the compounds described in chapter 7.2.2 with HPLC system V, conditions 1a. The concentration range was 10 to 2,400 nM, except for diCQA, which was 30 to 2,400 nM. The extracted spiked plasma samples at different known concentrations were used to build the calibration curve that corrected at the same time the extraction errors (by ratio to IS), the matrix effects and the extraction recoveries. The LOD was ranged from 1.2 to 13.6 nM, but was set to ~10 nM for all the compounds, except for diCQA where LOQ was set to 30 nM.

Unfortunately the individual calibration curve of ileo 5 at the study trial LOW could not be used for quantification. As blank plasma samples were limited CGA plasma concentrations of ileo 5 after LOW coffee dose consumption could not be determined.

7.3.1.7.3 Analysis of QA

For the measurement of QA (**10a**) a stable isotope dilution analysis was used (see chapter 7.3.1.4.2). A volume of 120 µL plasma was mixed with 30 µL of the IS ^{13}C-QA (5.1 µM; solution I) and deproteinization with 50 µL of acidified EtOH (0.25 M HCl) were added. Samples were vortexed (1 min at 1,600 min^{-1}) and kept at + 4°C for 30 min. After these samples were vortexed briefly and centrifuged (14,000 x g at + 4°C for 30 min). The supernatants were filtered (0.45 µM PVDF), and the extraction was done in duplicates. For Quantification 70 µL of each prepared sample was used as injection volume and measured with HPLC-system IV conditions Id (see chapter 7.2.2). MS was run in the multiple reaction monitoring mode (MRM) and a MRM transition from the deprotonated molecular ion $[M-H]^-$ *m/z* 191 to *m/z* 93 was used as quantifier. Identity of

QA in plasma was confirmed by comparison of the retention time and fragmentation pattern with a commercial reference. MRM transition used for quantification of the isotopically labelled standard U-^{13}C-D-(-)-QA was *m/z* 198 to *m/z* 99.

7.3.1.7.3.1 Calibration

Calibration curve was generated with reference solutions (with concentrations ranging from 0.02 µM to 5.2 µM) prepared in triplicates as described for sample extraction (see chapter 7.3.1.7.3). LOQ was 273 ng and an LOD 91 ng absolute.

7.3.2 Individual *ex vivo* structure-absorption relationship (SAR) of coffee CGA and QA in the pig jejunal Ussing model

7.3.2.1 Study design

The *ex vivo* transport experiments were performed with pig jejunum, provided by a local slaughterhouse (Kuhn & Bolander GmbH, Otterberg, Germany and Haerting GmbH, Kaiserslautern, Germany). German landrace pigs were used; both male and female, aged between 5 and 6 months, of an average body weight of 100 kg. After slaughtering the pigs, the GIT was removed and a 50 cm section of the jejunum was excised 10 cm below the ligament of Treitz. The jejunum was stored in a cooled and carbogen gas–saturated (oxygenated) HBSS buffer and transported immediately to the laboratory.

7.3.2.2 Preparation of the tissue (jejunal *epithelium* and *lamina propria*)

A 10 cm section was then excised and a longitudinal incision made along the mesenteric fixation. A cleaning step with HBSS followed in order to remove chymus residues. The jejunum was fixed in place with needles and the jejunal *epithelium* and *lamina propria* were separated carefully from the *lamina muscularis mucosae* and *tela submucosa* by slide stripping. The separation was monitored with histological examinations. Subsequently, the tissue (*jejunal epithelium* with *lamina propria*) was secured inside the Ussing chamber (K. Mußler, Aachen, Germany; software, Clamp 2.14; area, 0.78 cm^2) and the two chambers were filled with the HBSS buffer (5.0 mL). The tissue was used for incubation experiment if reaching a tissue conductance form 9 to 22 ms*cm^{-2}.

7.3.2.3 Incubation transfer experiments

Physiological conditions were simulated throughout the incubation. The HBSS buffer contained glucose (1.0 g*L^{-1}), a physiological salt concentration, and was aerated with carbogen gas (95% O_2 / 5% CO_2) at a temperature of + 37°C. Moistening of carbogen gas was achieved by a washing flask. On the mucosal side the HBSS buffer was adjusted to the physiological pH of 6.0 with 1 M HCl (Fallingborg, 1999), whereas on the serosal side a pH of 7.4 was used (stabilized with 60 mg $NaHCO_3$). The incubation experiments were started after a 15 min equilibration period; by adding a DMSO stock solution of CGA (2.5 µL, 40 mM stock solution for a final concentration of 20 µM) at the mucosal side for absorption experiments and at the serosal side for secretion experiments. The final DMSO concentration in the incubation buffer was 0.05%. CGA were added to a final concentration of 20 µM on the corresponding side. Approx. 200 µL aliquots (HBSS) were taken from both sides each hour during the entire experiment (4 hours). Aliquots were immediately stabilized with 1 M HCl and stored at - 80°C. Each incubation experiment with a single CGA or different concentrations were performed at least in four Ussing chambers, with the jejunum of two different animals.

7.3.2.3.1 Dose-response incubation experiments

Dose-response experiments were conducted on 5-CQA (**1d**) in a final concentration range of 0.02 to 3.5 µM in the compartments. The transepithelial flux (nmol*h^{-1}*cm^{-2}) was calculated on the basis of the serosal 5-CQA concentration 2 h after the experiment had started. Aliquots and tissues were taken and stored as described above. Linearity of the transepithelial flux (nmol*h^{-1}*cm^{-2}) plotted against concentration (mM) was checked by means of the coefficient of determination (R^2) (see Figure 4-24, Results).

7.3.2.3.2 Inhibition of transfer with NaN_3

Inhibition of a potential active transport of CGAs via ABC-transporters was investigated with NaN_3. The influence of NaN_3 on absorption or secretion was determined by co-incubating 10 mM NaN_3 in both chambers with 20 µM 5-CQA. Aliquots and tissues were taken and stored as described above.

7.3.2.4 CGA analysis of incubation buffer

The incubation-buffer samples (200 µL) were thawed quickly, membrane filtered ([PVDF, 0.45 µm), and used for HPLC–DAD analysis. For HPLC analysis, 15 µL of the internal standard (IS), 3,4,5-trimethoxyphenylacetic acid (226.2 mg L^{-1}), was added to 150 µL of the received solution. The mixture was analyzed in duplicate with HPLC-DAD at 320 nm.

CA (**5a**), 5-CQA (**1d**), 5-FQA (**3c**) and 4,5-diCQA (**4c**) were analyzed with HPLC system I condition 1a (see chapter 7.3.1.4.1 and 7.2.1).

The compounds 4-FQA (**3b**), 3-FQA (**3a**), 3,4-diCQA (**4a**) and 3,5-diCQA (**4b**) were analyzed with HPLC system II, condition 1a (see chapter 7.2.1). The absolute LOQ was calculated in the range 0.61 to 0.72 ng and an LOD in the range 0.15 to 0.18 ng. Quantification was based on the calibration curves of references. For FQA, the 3-feruloylquinic acid and for diCQA, the 3,5-diCQA were used for quantification and the results are presented as 3-FQA and 3,5-diCQA equivalents.

7.3.2.5 QA analysis in incubation buffer

Determination of QA (**10a**) was done with 50 µL filtered buffer mixed up with 50 µL of isotopically labeled standard U-^{13}C-D-(-)-QA equal to chapter 7.3.1.4.2.

7.3.2.6 Extraction and analysis of tissue (jejunal *epithelium* and *lamina propria*)

After the incubation experiment, the remaining tissue was exhaustively washed and covered with 1.5 mL buffer solution (0.4 M Na_2HPO_4, pH 4). Samples were briefly vortexed and homogenized with a Potter. To precipitate proteins, 1.0 mL of the generated suspension was mixed with 200 µL of an acidified ethanol solution (EtOH/formic acid, 96.5% / 3.5%, *v*/*v*). Samples were centrifuged (10 min, 16,500 x g, + 4°C) and the supernatants were filtered (PVDF membrane, 0.45 µM) and used for HPLC-DAD analysis. CGA analysis was performed as described in chapter 7.3.2.4. and QA analysis was performed as described in chapter 7.3.2.5.

7.3.2.7 Identification of CGA metabolites

Identification of metabolites was performed as described in chapter 7.3.1.5.2.2.

7.3.3 Identification of the transport proteins MDR 1 and MPR 2 in the pig jejunum

For tissue analysis, samples were thawed in 1.5 mL homogenization buffer (6.057 g Tris and 146.12 mg EDTA in 500 mL bidist. H_2O, pH 7.6) and 25 µL protease inhibitor cocktail. Samples were homogenized, centrifuged (15 min at + 4°C and 6,500 x g) and the supernatant were centrifuged (30 min at + 4°C and 100,000 x g). The received pellets were re-suspended as per the manufacturer's instructions and the protein content was determined with the bicinchoninic acid assay (Kit Uptima, KMF Laborchemie, Darmstadt, Germany). The suspensions were diluted with loading buffer [50 mL of 0.5 M Tris/HCl (pH 6.8), 1.24 g SDS, 40 mL glycerin, 0.16 g bromophenolblue, and 5 mL mercaptoethanol in 100 mL bidist. H_2O], resulting in a final protein content of 14 to 40 µg per 15 µL sample. After 15 min at room temperature (RT), lysis was performed (+ 95°C, 5 min) and samples were centrifuged (15 min at RT and 13,000 rpm). The samples (15 µL) were loaded in the gel wells [stacking gel: 2.54 mL H_2O, 1.23 mL Tris (1.5 M, pH 8.8), 1.15 mL acrylamide (30%), 49.2 µL SDS (10%), 24.6 µL APS (10%), and 2.46 µL TEMED; separation gel: 1.1 mL H_2O, 0.5 mL Tris (0.5 M, pH 6.8), 0.25 mL acrylamide (30%), 20 µL SDS (10%), 20 µL APS (10%), and 2 µL TEMED] and molecular weight marker (10 µL) was loaded in the first lane of every gel. Proteins were separated by gel electrophoresis [100 V, 1 to 2 h] and then transferred to a polyvinylidene fluoride membrane. This was performed in a semi-dry blot apparatus at 45 mA for 90 min (Hoefer TE 77, Amersham Biosciences, Muenchen, Germany). Membranes were blocked by storing for 1 h in blocking buffer (100 mL TBS-T, 5% milk powder) and then incubated overnight at + 4°C with the primary antibodies, mAb C219, mAb M2 III-6, and mouse mAb (each diluted 200-fold with TBS-T). After washing twice with TBS-T, the membranes were incubated for 1 h with the secondary antibody, goat anti-mouse IgG-HRP (diluted 5,000-fold with TBS-T). Bands were visualized by stirring in a detection solution [10 mL A (50 mg luminol in 200 mL 0.1 M Tris/HCl), 1 mL B (11 mg *p*-coumaric acid in 10 mL DMSO, and 100 µL C (50 µL H_2O_2 in 1 mL Tris/HCl)] and using a Lumi-Imager (Lumi-Analyst 3.1, Roche, Mannheim, Germany).

7.3.4 Stability measurements of CGA in ileal fluid

7.3.4.1 Study design and volunteers

In order to determine the stability of CGA in the ileal fluids we performed an *ex vivo* incubation model. Therefore, we sampled ileal effluents and incubated it with instant coffee (same as in the intervention study) in the anaerobic chamber. The experiment lasted 8 hours and was performed under physiological conditions with a coffee concentration (132.2 µM CGA) that could easily be reached after coffee consumption.
CGA free ileal effluents were provided by five ileostomist (7.3.1.2); which were remaining in CGA-free diet 48 hours before sampling. The ileal fluids were stored by volunteers in gas proof boxes containing an anaerobic atmosphere created by Anaero Gen™ under cooled conditions and were in this kind transported to the laboratory.

7.3.4.2 Incubation buffer

Ex vivo simulation of CGA stability in ileal effluent was performed with an carbonate-phosphate buffer according to Lebet et al. (Lebet et al., 1998). Following the instructions in Table 7-6 the trace element solution was prepared first with bidist. water and 10 mL of it was added to the carbonate-posphate buffer (Table 7-6) in 1 L. The pH of buffer was adjusted to 6.3 (1 M HCl) and oxygen was removed in a first step by heating in a water bath (30 min at + 80°C).

Table 7-6: Composition of the anaerobic incubation buffer (carbonate-phosphate buffer) in bidist. water according to (Lebet et al., 1998).

Carbonate-phosphate buffer		Trace element solution	
Compound	Unit	Compound	Unit
$NaHCO_3$	9.24 g*L^{-1}	$FeSO_4$ x 7 H_2O	368.0 mg*100 mL^{-1}
Na_2HPO_4	2.83 g*L^{-1}	$MnSO_4$ x 1 H_2O	115.9 mg*100 mL^{-1}
NaCl	0.47 g*L^{-1}	$ZnSO_4$ x 7 H_2O	44.0 mg*100 mL^{-1}
KCl	0.45 g*L^{-1}	$CoCl_2$ x 6 H_2O	12.0 mg*100 mL^{-1}
Urea	0.40 g*L^{-1}	$NiCl_2$	10.0 mg*100 mL^{-1}
$CaCl_2$ x 2 H_2O	0.07 g*L^{-1}	$CuSO_4$ x 5 H_2O	9.8 mg*100 mL^{-1}
Na_2SO_4, waterfree	0.10 g*L^{-1}	$Mo_7(NH_4)_6O_{24}$ x 4 H_2O	1.7 mg*100 mL^{-1}
$MgCl_2$ x 6 H_2O	0.10 g*L^{-1}		
Trace element solution	10 mL*L^{-1}		
Resazurin (1 mg*mL^{-1})	1 mL*L^{-1}		

After cooling to room temperature 5 mL of cysteine hydrochloride (0.1 g*mL^{-1}) was added and remaining oxygen was removed in a second step by gassing with helium until a color change from blue to a weak pink was observed (approx. 3 hours).

7.3.4.3 Incubation of CGA

The following steps were performed in an anaerobic chamber (Figure 7-4) continuously flushed with N_2 : CO_2 gas mixture (80/20, *v/v*) according to the work of Knaup et al. 2007 (Knaup et al., 2007).

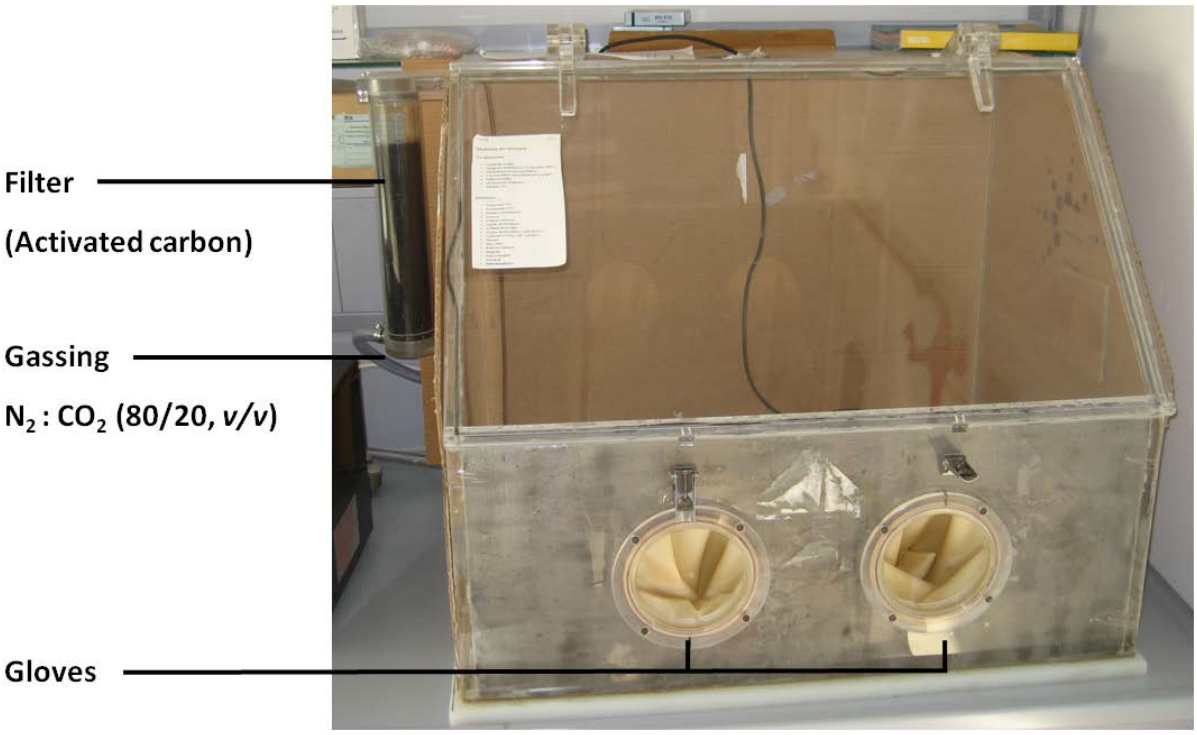


Figure 7-4: Anaerobic chamber for *ex vivo* incubation experiments.

The incubation experiments were performed in duplicates for each volunteer. The pH values of the ileal fluids were measured and the ileal fluids were mixed with the same volume of the pre-gassed anaerobic incubation buffer. Coarse particles of this mixture were removed by filtration using a riddle. The filtrate was used as inoculum. For the incubation aliquots of 1.950 µL inoculum were added to the incubation vessels containing a 50 µL aliquot of the coffee DMSO stock solution (800 mg of instant coffee powder in 20 mL DMSO and 20 mL bidist. water, which corresponds to 104.7 µM CQA; 12.0 µM FQA; 6.3 µM CQL; 8.4 µM diCQA; 0.9 µM CA). Vessels were mixed up well, closed and incubated at + 37°C for 4 min, 2, 4, 6 and 8 hours in a drying oven. The

incubation was stopped by adding 10 µL 1 M HCl, vortexed and was subsequently placed in liquid nitrogen. In order to determine the influence of ileal fluid on coffee CGA stability, a further stability study was performed according to the procedure described above using only the anaerobic carbonate-phosphate buffer (blank = without ileal fluid). As control, a blank value with 50 µL DMSO/bidist. water (1/1, v/v, without coffee CGA) and inoculums of each volunteer were prepared and subjected to the procedure described above.

7.3.4.4 Sample extraction and analysis

After freeze drying the inoculum and blank samples were extracted. For this 1.2 mL EtOH (with 1% formic acid) was added to the incubation vessels, vortexed for short (2,700 min^{-1}) and sonicated for 5 min. Subsequently, samples were centrifuged (5 min, + 4°C, 5,000 x rpm) and then the supernatants were decanted into a clean Eppendorf tube. The pellets were re-extracted three times. The received supernatants were combined, concentrated in a vacuum centrifuge, subsequently lyophilized to dryness and stored at – 80°C prior to analysis. Recovery rate was determined with 93% for the test compound 5-CQA (**5a**) in a spiked CGA-free ileal fluid. Nevertheless, the overall recovery of CGA at time point 0 h in the incubation experiment was 22.8% when incubated with inoculum and 58.4% when incubated with buffer solution.

7.3.4.5 Quantification of CGA

The dried extract was redissolved in 1 mL solution I, filtered through a membrane filter (0.45 µm PVDF) and analyzed as described in 7.3.1.4.1.

7.3.4.6 Enzyme activity of ileal fluid

To determine enzymatic activities of ileostomy fluids, the semi-quantitative micro-method Api® ZYM (BioMeriéux, Marcy-l`Etoile (France)) was performed in triplicates for each volunteer. Therefore, volunteers (who were still remaining in CGA-free diet) additionally collected CGA free ileostomy fluid 48 hours after coffee consumption.

The ileal fluids were stored by volunteers in gas tight boxes containing an anaerobic atmosphere created by AnaeroGen™ under cooled conditions and in this way

transported to the laboratory. All following steps were performed in an anaerobic chamber aerated with ALIGAL gas (CO_2/ N_2, 80/20, *v/v*). Preparation of the inoculums were performed by opening the ileal bags and mixing 3.0 mL of ileal fluid with 6.0 mL sterile water. After vortexing for short and centrifuging (10 min, 3,500 x g), the supernatants were used as inoculum. The micro-wells of the enzymatic test stripes were inoculated with 65 µL inoculum or 65 µL sterile water as control. Subsequently, the samples were proofed and incubated for 4 hours at + 37°C. At the end of incubation one drop of ZYM A reagent and one drop of ZYM B reagent were added to each micro-well and after five minutes test stripes were put under a powerful light source (500 W) for about 20 sec with the bulb placed approximately 10 cm above it. Metabolic products formed during incubation time were detected through colored reactions compared to control incubations.

7.3.4.7 Determination of microflora in ileal fluids

The microflora content was determined with the Enterosan-test® technique performed with ileal fluid through the L&S AG (Bad Bocklet, Germany).

Therefore, ileal fluids were sampled on study day two immediately before coffee consumption (sampling point 0 hours) and were stored under cooled conditions (+ 4°C) prior to analysis at the L&S laboratory.

7.3.5 Synthesis and isolation of single CGA

7.3.5.1 Preparation of 3- and 4-caffeoylquinic acid

The preparation of 3-CQA (**1b**) and 4-CQA (**1c**) was achieved by interesterification of 5-CQA (**1d**) using the method of Trugo and Macrae (Trugo and Macrae, 1984a). A 10 $g*L^{-1}$ solution of 5-CQA in water was compounded (200 mg 5-CQA in 20 mL H_2O) and the pH was adjusted to 8 with 4 M NH_3. This solution was heated for 30 min to + 100°C and subsequently cooled down to room temperature. Interesterification was stopped by adding 4 M HCl in order to obtain a pH of 3. The formed CQA mixture (3-CQA, 4-CQA, 5-CQA) was separated using HPLC system III condition 1a (see chapter 7.2.1). Due to obtain pure solid compounds eluates with an individual CQA were combined and lyophilisized. The Purity of the received 3-CQA (99%) and 4-CQA (95%) were examined with HPLC system III condition 1a. Identification was performed with HPLC system IV conditions 1a (see chapter 7.2.2).

7.3.5.2 Preparation of 3- and 4-caffeoylquinide

A freeze dried mixture of 3-CQA, 4-CQA and 5-CQA was generated (Trugo and Macrae, 1984a). This mixture was used to achieve quinides according to the published protocol of (Rúveda et al., 1964).

The CQA mixture (200 mg) was dissolved in 150 mL acetic acid (water free) and were constantly stirred at a temperature of + 100°C for 6 hours. Acetic acid was almost completely removed and 7 mL of solution I was added. Isolation was carried out by HPLC system I condition 1c (see chapter 7.2.1). Identification of the obtained pure compounds (purity > 95%) was performed by comparison of the fragmentation pattern with HPLC system IV conditions 1a (see chapter 7.2.2) and literature data. Further NMR spectroscopy data was used for differentiating 3-CQL (**2a**) and 4-CQL (**2b**). For this, compounds were dissolved in deuterated methanol (CD_3OD) and NMR spectra were obtained. In Figure 7-5 the main NMR spectra characteristics of 3-CQL and 4-CQL are shown. A Bruker AMX – 600 apparatus with WIN – NMR v6.0 software was used.

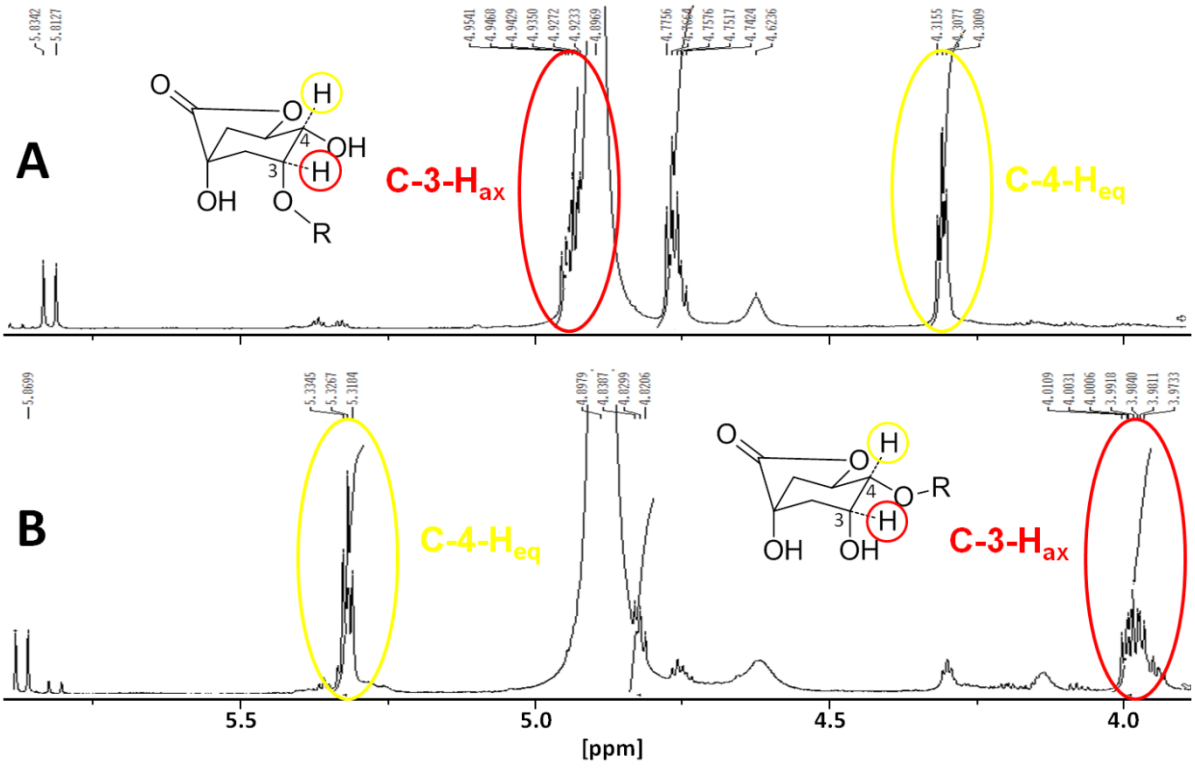


Figure 7-5: Detailed view of the main characteristics of a ^{1}H-NMR spectra of 3-caffeoylquinide (A) and 4-caffeoylquinide (B) in deuterated methanol (CD_3OD). Chemical structure = quinide, R = caffeic acid moiety. For details see Table 7-7.

The achieved ^{1}H-NMR data for 3-CQL (2a) and 4-CQL (2b) (Table 7-7) were in accordance with literature data (Bennat et al., 1994).

Table 7-7: ^{1}H-NMR data of 3-caffeoylquinide (3-CQL) and 4-caffeoylquinde (4-CQL) in deuterated methanol (CD_3OD) at 600 MHz. The reference signals for ^{1}H were δ = 3.35 ppm for CHD_2OD and δ = 4.8 ppm for HDO.

Position*	3-CQL	4-CQL
C-2-H ax	2.12 (dd)	2.00 (dd)
C-2-H eq	2.19 (m)	2.19 (m)
C-3-H ax	4.95 (ddd)	4.01 (ddd)
C-4-H eq	4.31 (dd)	5.33 (dd)
C-5-H ax	4.77 (dd)	4.83 (dd)
C-6-H ax	2.59 (d)	2.43 (m)
C-6-H eq	2.32 (ddd)	2.43 (m)
C-2'-H	7.11 (d)	7.10 (d)
C-5'-H	6.84 (d)	6.82 (d)
C-6'-H	6.99 (dd)	7.01 (dd)
C-7'-H	7.65 (d)	7.67 (d)
C-8'-H	6.34 (d)	6.39 (d)

*see Figure 7-5.

7.3.6 Statistics

Each experiment was repeated multiple times and results are expressed as means ± SD. The reported n values represented the number of independent repeated experiments. Excel 2007 (Microsoft, Unterschleißheim, Germany) was used for statistical evaluations (two-sample T-test of significance) and differences were considered significantly with *$p \leq 0.05$, **$p \leq 0.01$ or ***$p \leq 0.001$. Data achieved in the Ussing chamber experiment was additionally analysed with Nalimov test for outlier detection.

Deviation was calculated on the basis of the following formula:

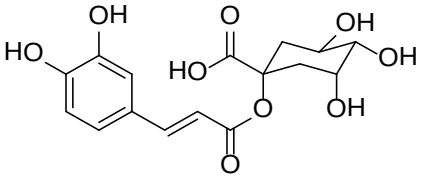
$$Stabw = \sqrt{\frac{\sum(x - \bar{x})^2}{n}}$$

Where x is the sample mean average and n is the sample size.

8 Overview of chemical structures

Table 8-1: Naming of chemical structures and numbering of chlorogenic acids (CGA), its metabolites and D-(-)-QA.

No	Substance	No	Substance
	Caffeoylquinic acids		***diCaffeoylquinic acids***
1a	1-*O*-Caffeoylquinic acid	4a	3,4-*O*-diCaffeoylquinic acid
1b	3-*O*-Caffeoylquinic acid	4b	3,5-*O*-diCaffeoylquinic acid
1c	4-*O*-Caffeoylquinic acid	4c	4,5-*O*-diCaffeoylquinic acid
1d	5-*O*-Caffeoylquinic acid		
1e	3-*O*-Caffeoylquinic acid-3´-*O*-GlucA		***Hydroxycinnamic acids***
1f	4-*O*-Caffeoylquinic acid-3´-*O*-GlucA	5a	Caffeic acid
1g	3-*O*-Caffeoylquinic acid-3´-*O*-Sulf	5b	Caffeic acid-4´-*O*-GlucA
1h	4-*O*-Caffeoylquinic acid-3´-*O*-Sulf	5c	Caffeic acid-3´-*O*-GlucA
1i	5-*O*-Caffeoylquinic acid-3´-*O*-Sulf	5d	Caffeic acid-4´-*O*-Sulf
		5e	Caffeic acid-3´-*O*-Sulf
	Caffeoylquinides	6a	Dihydrocaffeic acid
2a	3-*O*-Caffeolyquinide	6b	Dihydrocaffeic acid-3´-*O*-GlucA
2b	4-*O*-Caffeoylquinide	6c	Dihydrocaffeic acid-4´-*O*-Sulf
2c	3-*O*-Caffeolyquinide-3´-*O*-GlucA	6d	Dihydrocaffeic acid-3´-*O*-Sulf
2d	4-*O*-Caffeolyquinide-3´-*O*-GlucA	7a	Ferulic acid
2e	3-*O*-Caffeoylquinide-3´-*O*-Sulf	7b	Ferulic acid-4´-*O*-GlucA
2f	4-*O*-Caffeoylquinide-3´-*O*-Sulf	7c	Ferulic acid-4´-*O*-Sulf
		7d	Feruloylglycine
	Iso-/ Feruloylquinic acids	7e	Isoferulic acid-3´-*O*-GlucA
3a	3-*O*-Feruloylquinic acid	7f	Isoferulic acid-3´-*O*-Sulf
3b	4-*O*-Feruloylquinic acid	7g	Isoferulic acid
3c	5-*O*-Feruloylquinic acid	8a	Dihydroferulic acid -4´-*O*-GlucA
3d	3-*O*-Feruloylquinic acid-4´-*O*-GlucA	8b	Dihydroferulic acid-4´-*O*-Sulf
3e	4-*O*-Feruloylquinic acid-4´-O-GlucA	8c	Dihydroferulic acid
3f	5-*O*-Feruloylquinic acid-4´-*O*-GlucA	9a	Dimethoxycaffeic acid
3g	3-*O*-Feruloylquinic acid-4´-*O*-Sulf	9b	Dimethoxydihydrocaffeic acid
3h	4-*O*-Feruloylquinic acid-4´-O-Sulf	10a	D-(-)-Quinic acid
3i	3-*O*-Isoferuloylquinic acid-3´-*O*-GlucA		
3j	4-*O*-Isoferuloylquinic acid-3´-O-GlucA		***Standards***
		ISa	*o*-Coumaric acid
		ISb	3,4,5-*trans*-Trimethoxyphenylacetic acid



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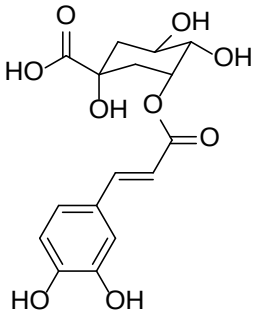

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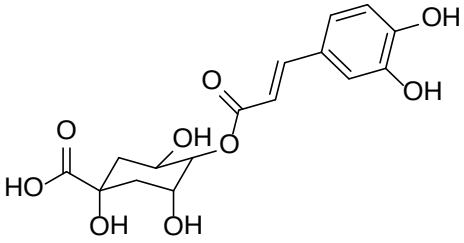

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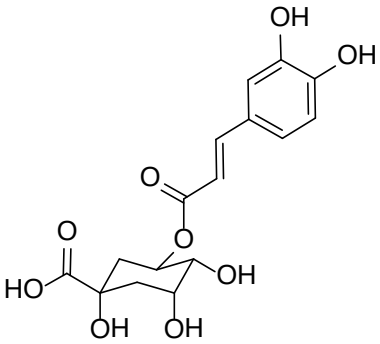

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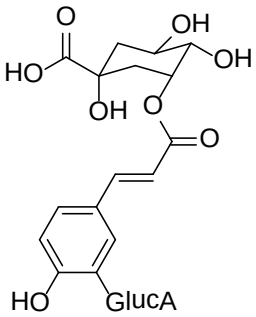

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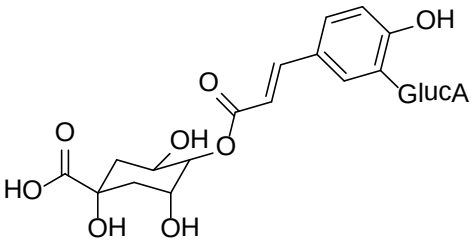

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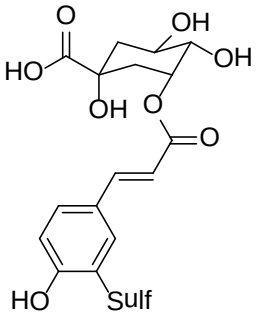

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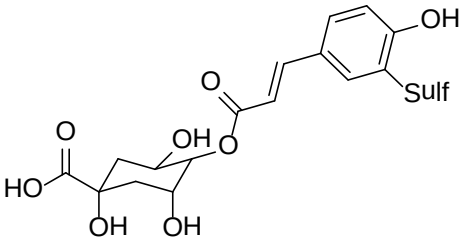

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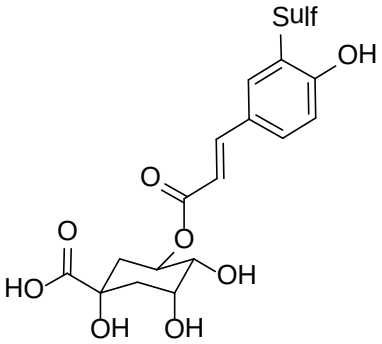

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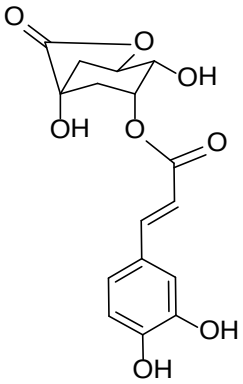

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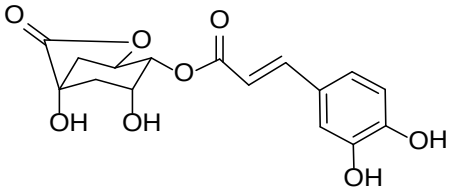

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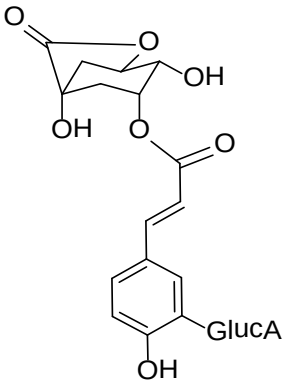

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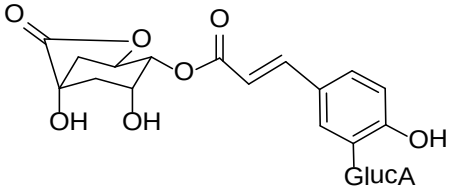

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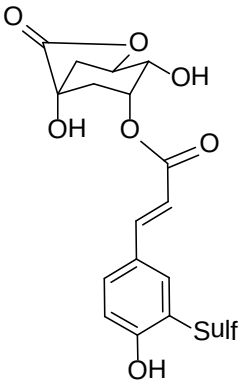

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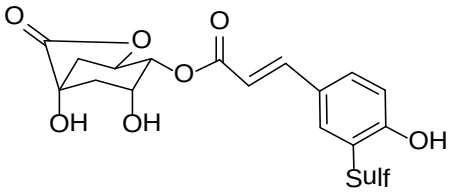

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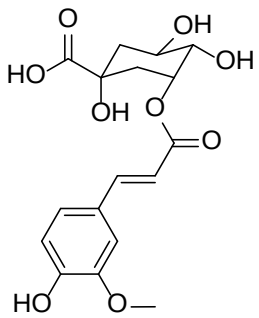

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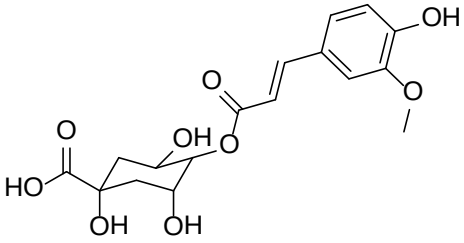

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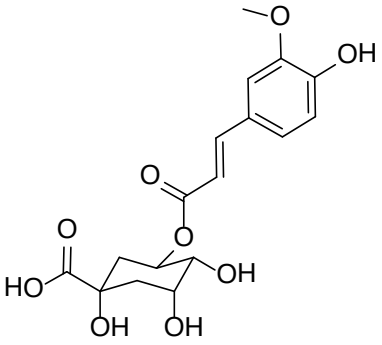

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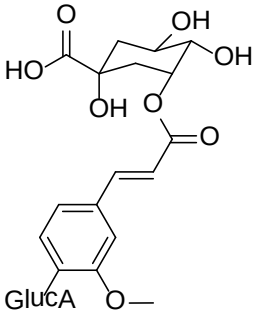

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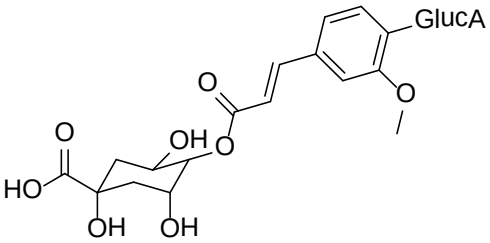

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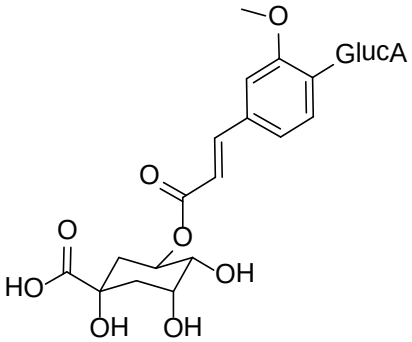

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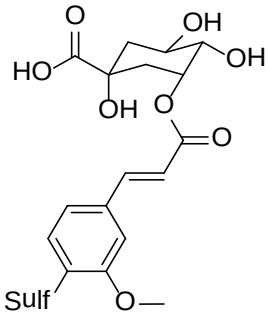

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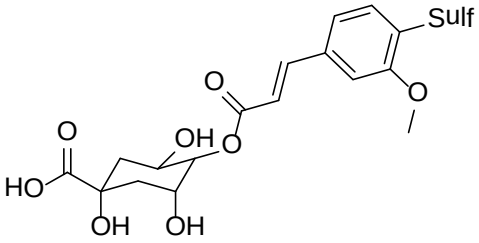

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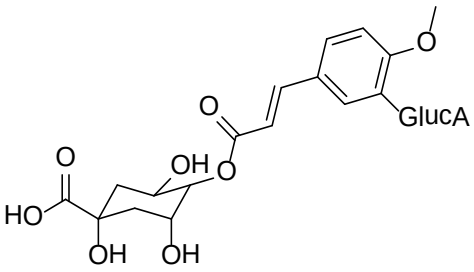

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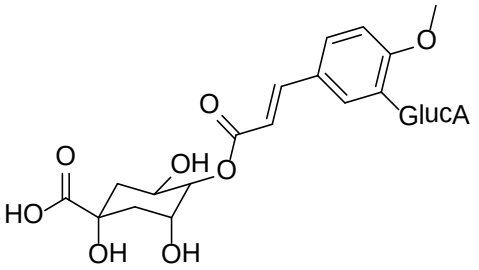

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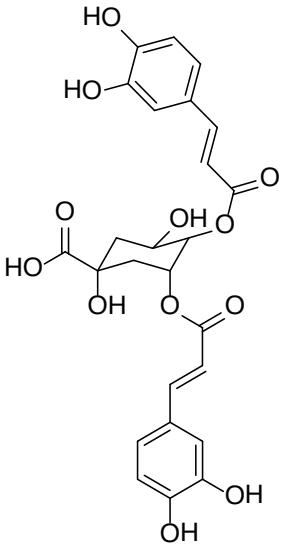

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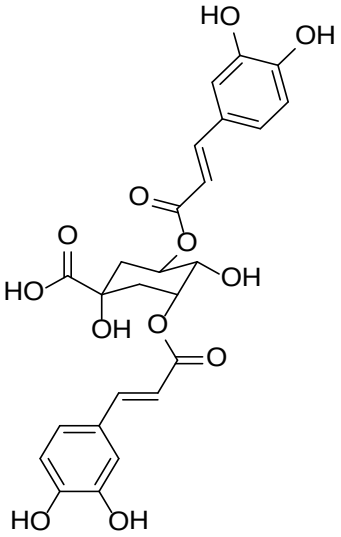

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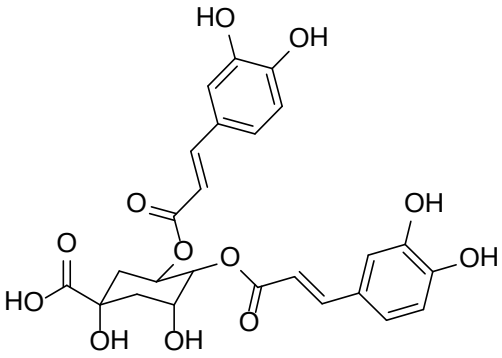

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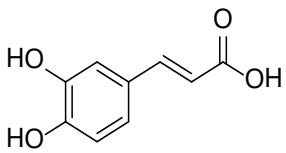

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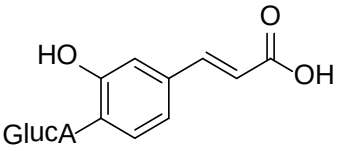

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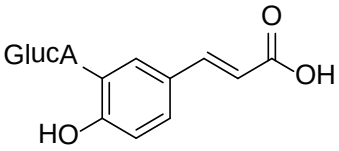

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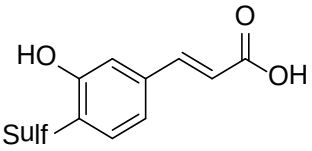

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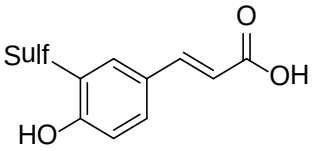

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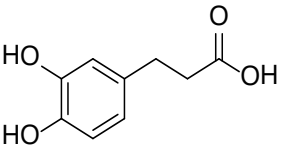

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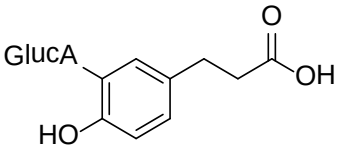

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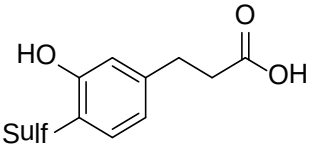

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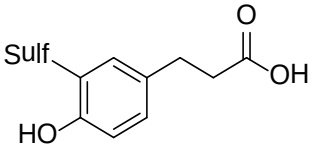

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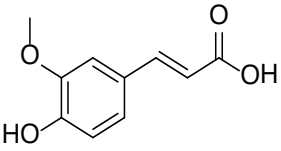

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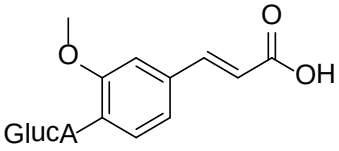

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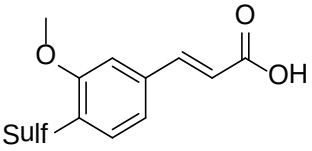

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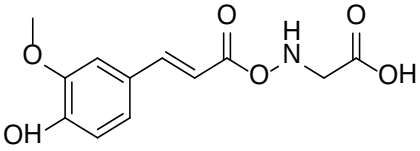

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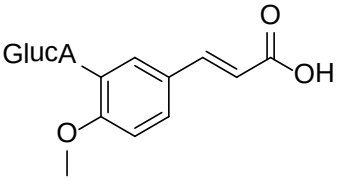

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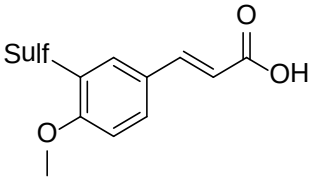

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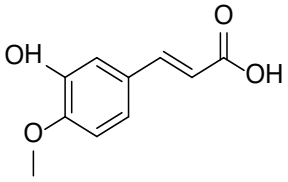

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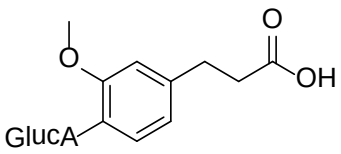

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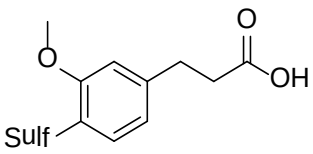

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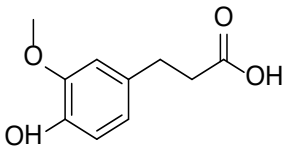

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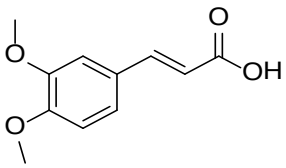

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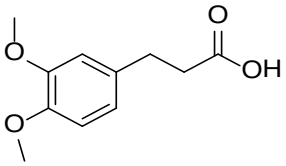

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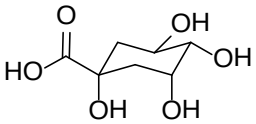

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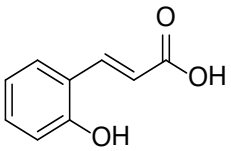

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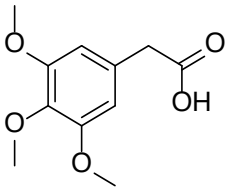

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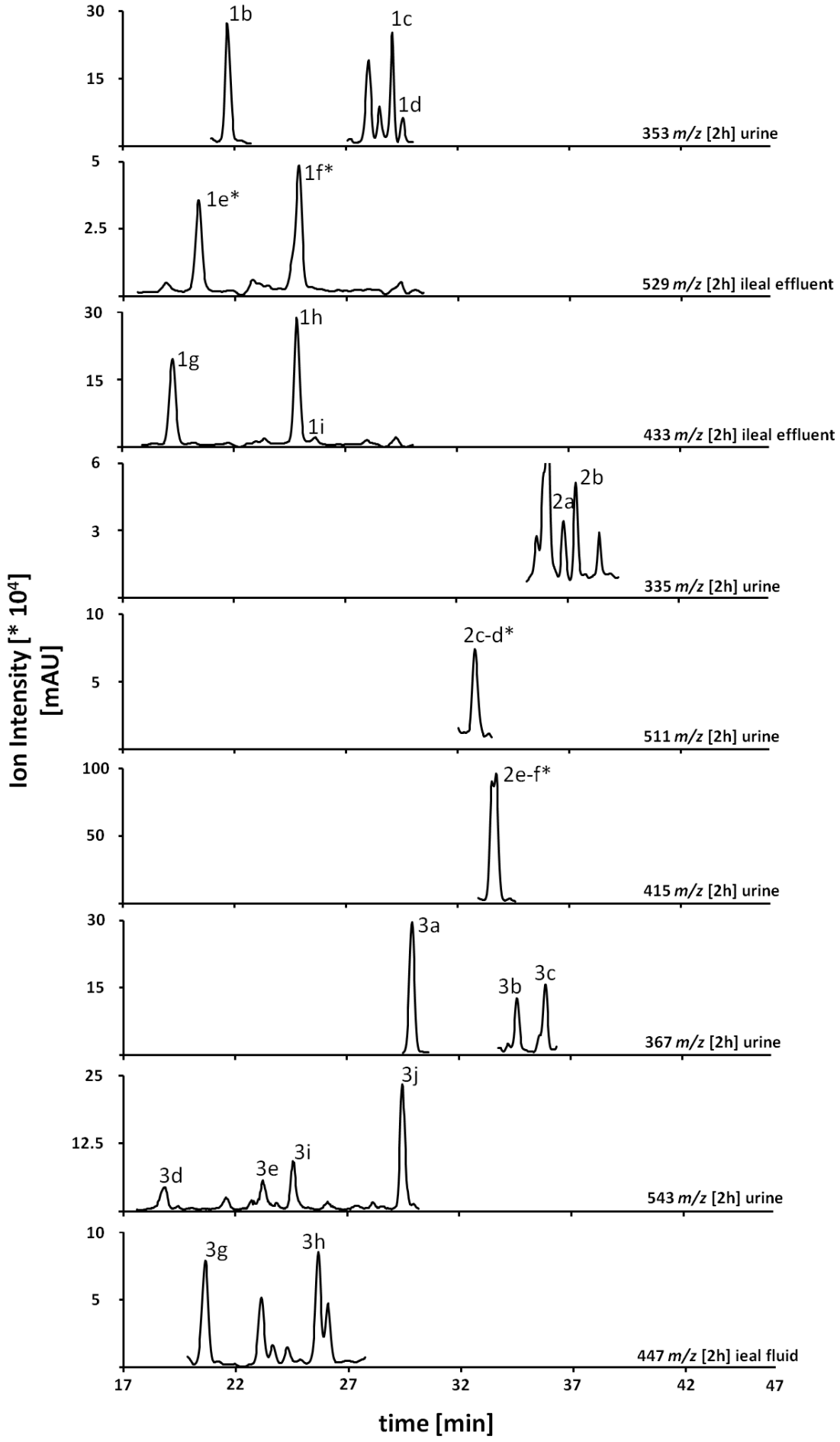
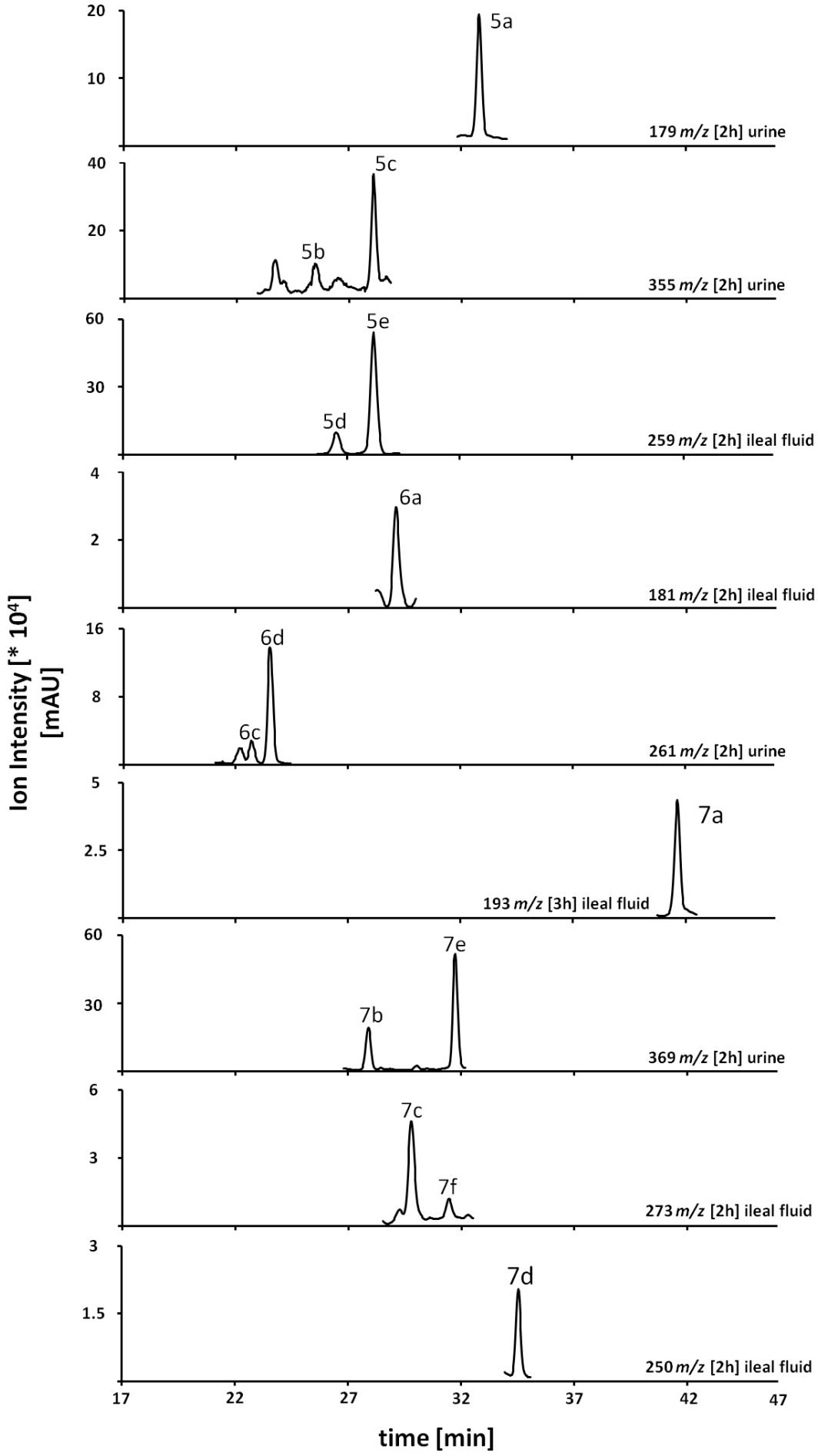
10 Appendix

Table 10-1: Anthropometric data achieved from each individual ileostomist (Ileo 1 to Ileo 5) participating in the human coffee intervention studies.

	Ileo 1	Ileo 2	Ileo 3	Ileo 4	Ileo 5
Age	40	47	40	36	42
Size (cm)	163	158	166	175	170
Bodyweight (kg)	65.0	73.5	82.7	79.5	79.0
Bodyfat (%) *	29.1	35.6	36.4	33.1	35.2
Bodyfat (kg) *	18.9	26.2	30.1	26.3	27.8
Body Mass Index (BMI)	24.5	29.4	30.0	26.0	27.3
Fat free body mass (kg) *	46.1	47.3	52.6	53.2	51.2
Fat free body mass (%) *	70.9	64.4	63.6	66.9	64.8
Water content (L) *	33.7	34.6	38.5	38.9	37.5
Water content (%) *	51.8	47.1	46.6	48.9	47.5
Basic metabolism (kcal) *	1351	1324	1440	1483	1420
Primary disease	*Morbus Crohn*	*Morbus Crohn*	*Morbus Crohn*	*Morbus Crohn*	*Ulcerative Colitis*
Time point of colectomy	2002	1986	2001	2002	1996

*measured with bioimpedance instrument (Maltron BodyFat-Instrument BF-906, Gauting (Germany))


1b
1c
1d
353 m/z [2h] urine
1e*
1f*
529 m/z [2h] ileal effluent
1g
1h
1i
433 m/z [2h] ileal effluent
2a
2b
335 m/z [2h] urine
2c-d*
511 m/z [2h] urine
2e-f*
415 m/z [2h] urine
3a
3b
3c
367 m/z [2h] urine
3d
3e
3i
3j
543 m/z [2h] urine
3g
3h
447 m/z [2h] ieal fluid
Ion Intensity [* 10⁴] [mAU]
time [min]


Ion Intensity [* 10⁴] [mAU]
20
10
5a
179 m/z [2h] urine
40
20
5c
5b
355 m/z [2h] urine
60
30
5e
5d
259 m/z [2h] ileal fluid
4
2
6a
181 m/z [2h] ileal fluid
16
8
6d
6c
261 m/z [2h] urine
5
2.5
7a
193 m/z [3h] ileal fluid
60
30
7e
7b
369 m/z [2h] urine
6
3
7c
7f
273 m/z [2h] ileal fluid
3
1.5
7d
250 m/z [2h] ileal fluid
17
22
27
32
37
42
47
time [min]

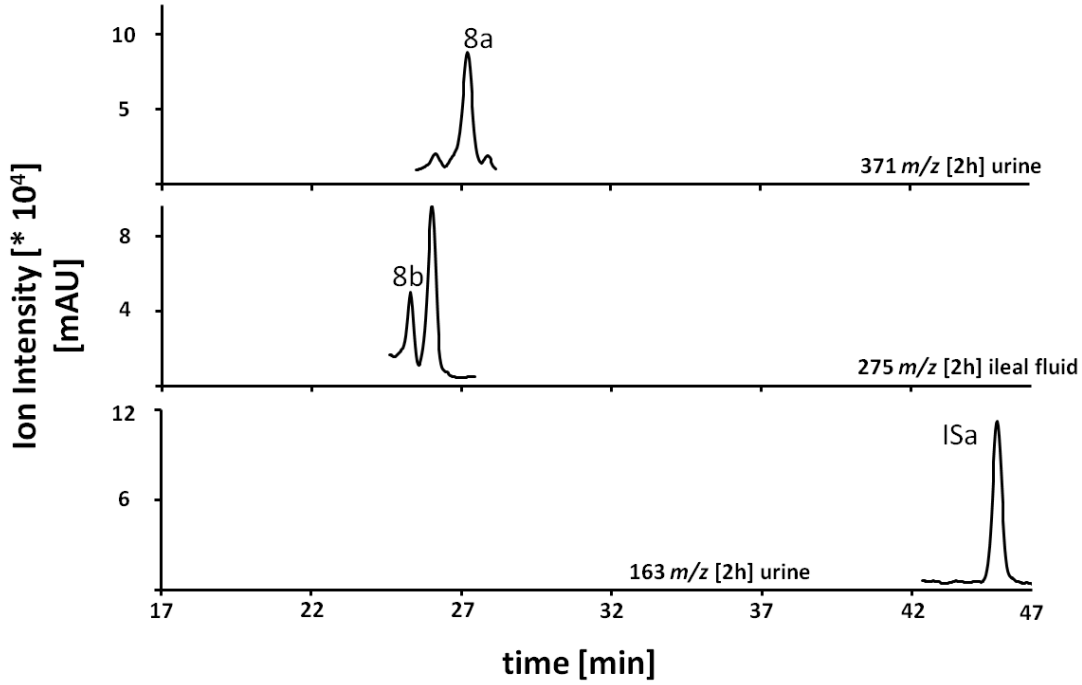


Figure 10-1: HPLC-MS molecular ion $[M-H]^-$ chromatograms of chlorogenic acids and corresponding metabolites detected in urine and ileal fluid of an ileostomist 2 h after coffee consumption. Internal standard a (ISa) – *o*-coumaric acid. HPLC system IV, conditions 1b (see chapter 7.2.2). For identification of peaks and peak numbers see Table 8-1, Overview of Chemical Structures, (* conjugation position was suspected at 3´).

Table 10-2: Recovery rates (in % of the initial measured concentration at time point 0 h) of CGA from instant coffee 8 hours after *ex vivo* incubation with inoculum (ileal fluid/buffer, 1/1, v/v, n = 5) or buffer (n = 2) with the anaerobic chamber indicating interesterification and degradation during incubation (data expressed as mean values ± SD). Details see chapter 7.3.4.

Compound	Inoculum	Buffer
1-*O*-Caffeoylquinic acid	48 ± 31	43 ± 11
3-*O*-Caffeoylquinic acid	93 ± 17	47 ± 16
4-*O*-Caffeoylquinic acid	89 ± 11	41 ± 11
5-*O*-Caffeoylquinic acid	74 ± 18	35 ± 10
3-*O*-Caffeolyquinide	44 ± 20	13 ± 7
4-*O*-Caffeoylquinide	56 ± 30	8 ± 6
3-*O*-Feruloylquinic acid	107 ± 25	107 ± 11
4-*O*-Feruloylquinic acid	97 ± 27	76 ± 58
5-*O*-Feruloylquinic acid	68 ± 14	11 ± 11
3,4-O-diCaffeoylquinic acid	97 ± 10	17 ± 12
3,5-O-diCaffeoylquinic acid	86 ± 11	19 ± 12
4,5-O-diCaffeoylquinic acid	94 ± 10	16 ± 7

Table 10-3: Enzyme activity of ileal fluid of each ileostomist (Ileo 1 to Ileo 5) determined *versus* bidist. water (blank) using Api®-ZYM test (BioMeriéux); n = 3.

No.	Enzyme assayed for:	Ileo 1	Ileo 2	Ileo 3	Ileo 4	Ileo 5	Blank
1	Control	-	-	-	-	-	-
2	Alkaline phosphatase	+	+	+	+	+	-
3	Esterase (C4)	-	(+)	-	+	-	-
4	Esterase Lipase (C8)	+	+	+	+	(+)	-
5	Lipase (C14)	-	-	-	+	-	-
6	Leucine arylamidase	+	+	+	+	+	-
7	Valine arylamidase	+	+	+	+	+	-
8	Cystine arylamidase	-	-	-	-	-	-
9	Trypsin	+	+	+	+	+	-
10	*α*-chymotrypsin	-	-	-	-	-	-
11	Acid phosphatase	+	+	+	+	+	-
12	Naphtol-AS-BI-phosphohydrolase	+	+	+	+	+	(+)
13	*α*-galactosidase	-	-	-	-	-	-
14	*β*-galactosidase	-	+	(+)	(+)	+	-
15	*β*-glucuronidase	(+)	(+)	+	+	-	-
16	*α*-glucosidase	+	+	+	+	+	-
17	*β*-glucosidase	-	-	-	-	-	-
18	N-acetyl-*β*-glucosaminidase	+	+	+	+	+	-
19	*α*-mannosidase	-	-	-	-	-	-
20	*α*-fucosidase	-	-	-	-	-	-

Legend**:** + = positive; (+) = weak positive; - = negative

Table 10-4: Amounts of microflora determined in ileostomy effluents of each volunteer as colony forming unit per g [CfU/g].

Microflora	Ileo 1	Ileo 2	Ileo 3	Ileo 4	Ileo 5
Aerobic					
E. coli	$1x10^8$	$1x10^8$	$8x10^7$	$1x10^8$	$1x10^5$
E. coli-variant	---	$< 10^4$	$< 10^4$	$< 10^4$	---
E. coli-variant lact.-neg.	$1x10^6$	---	---	---	---
E. coli-variant haem.	---	---	---	---	$6x10^3$
Klebsiella sp.	---	$8x10^7$	---	---	---
Proteus sp.	---	$7x10^3$	---	---	---
Enterobacteriaceae	$< 10^4$	---	$< 10^4$	$< 10^4$	$< 10^4$
Enterococcus sp.	$1x10^8$	$1x10^6$	$7x10^7$	$6x10^5$	$5x10^5$
Other aerobics	$< 10^4$	$< 10^4$	$< 10^4$	$< 10^4$	$< 10^4$
Anaerobic					
Bacteroides sp.	$< 10^8$	$< 10^8$	$1x10^3$	$5x10^3$	$1x10^3$
Clostridium sp.	$< 10^6$	$< 10^6$	$7x10^7$	$< 10^6$	$1x10^8$
Bifidobacterium sp.	$1x10^8$	$1x10^6$	$1x10^7$	$1x10^6$	$3x10^7$
Lactobacillus sp.	$1x10^6$	$6x10^5$	$1x10^6$	$1x10^6$	$1x10^6$
Other anaerobis	$< 10^6$	$< 10^6$	$< 10^6$	$< 10^6$	$< 10^6$
Fungi					
Candida sp.	$< 10^2$	$1x10^2$	$5x10^2$	$1x10^3$	$4x10^3$
Geotrichum sp.	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$
Other fungi	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$

Analysis was performed by L+S AG, Bad Bocklet (Germany).

Table 10-5: Amounts of coffee CGA and QA consumed in each trial in µmol (data expressed as mean values ± SD; n = 5)

Compound	HIGH	MEDIUM	LOW
1-*O*-Caffeoylquinic acid	29.2 ± 2.7	14.3 ± 2.2	8.0 ± 0.6
3-*O*-Caffeoylquinic acid	877.5 ± 73.2	451.8 ± 59.2	230.9 ± 21.0
4-*O*-Caffeoylquinic acid	868.8 ± 72.5	431.3 ± 53.1	208.7 ± 17.3
5-*O*-Caffeoylquinic acid	1262.0 ± 106.2	621.5 ± 75.3	298.4 ± 24.1
Total Caffeoylquinic acids	3037.3 ± 148.0	1519.1 ± 109.6	746.0 ± 36.4
3-*O*-Caffeolyquinide	480.6 ± 31.6	218.2 ± 15.7	84.3 ± 12.0
4-*O*-Caffeoylquinide	248.8 ± 20.9	109.7 ± 8.7	40.8 ± 8.1
Total Caffeoylquinides	729.4 ± 37.8	327.9 ± 18.0	125.1 ± 14.6
3-*O*-Feruloylquinic acid	121.6 ± 10.8	63.3 ± 8.3	33.5 ± 3.8
4-*O*-Feruloylquinic acid	121.6 ± 9.5	60.1 ± 7.8	29.5 ± 2.8
5-*O*-Feruloylquinic acid	192.8 ± 15.5	90.5 ± 11.3	45.7 ± 4.3
Total Feruloylquinic acids	436.0 ± 21.1	213.9 ± 16.1	108.7 ± 6.4
3,4-*O*-diCaffeoylquinic acid	112.2 ± 6.8	55.8 ± 5.2	25.7 ± 1.7
3,5-*O*-diCaffeoylquinic acid	77.1 ± 6.4	38.3 ± 3.9	19.4 ± 1.4
4,5-*O*-diCaffeoylquinic acid	105.0 ± 5.7	50.5 ± 4.3	21.6 ± 1.5
Total diCaffeoylquinic acids	294.3 ± 10.9	144.6 ± 7.8	66.8 ± 2.7
Caffeic acid	28.1 ± 2.4	13.3 ± 1.2	6.5 ± 0.5
Total chlorogenic acids	4525.0 ± 154.6	2218.8 ± 112.5	1053.1 ± 39.8
D-(-)-Quinic acid	2546.5 ± 275.9	1372.9 ± 128.9	695.3 ± 62.6

Table 10-6: Ileal amounts of CGA and corresponding metabolites excreted after ingestion of a HIGH- , MEDIUM- and LOW- coffee dose (in % of initial coffee concentration) indicating interesterification during the passage of the gastrointestinal tract, ileostomist n = 5 (data expressed as mean values ± SD; n = 5).

Compound	HIGH	MEDIUM	LOW
1-*O*-Caffeoylquinic acid	55.1 ± 8.8	67.9 ± 5.8	63.7 ± 6.9
3-*O*-Caffeoylquinic acid*	76.5 ± 8.6	80.0 ± 4.3	81.9 ± 7.4
4-*O*-Caffeoylquinic acid*	72.8 ± 8.2	77.8 ± 4.8	81.4 ± 8.9
5-*O*-Caffeoylquinic acid*	68.4 ± 8.3	73.4 ± 5.0	76.5 ± 8.7
Caffeolyquinides*	26.7 ± 9.9	19.5 ± 8.4	47.2 ± 17.2
3-*O*-Feruloylquinic acid*	71.4 ± 10.5	72.5 ± 3.9	74.9 ± 8.5
4-*O*-Feruloylquinic acid*	80.4 ± 9.2	71.2 ± 7.5	79.7 ± 8.7
5-*O*-Feruloylquinic acid*	57.5 ± 8.1	67.6 ± 5.4	69.8 ± 8.3
3,4-*O*-diCaffeoylquinic acid	68.1 ± 7.2	68.1 ± 6.1	72.2 ± 9.0
3,5-*O*-diCaffeoylquinic acid	64.6 ± 7.3	65.1 ± 5.3	63.0 ± 7.6
4,5-*O*-diCaffeoylquinic acid	67.7 ± 8.3	72.4 ± 7.2	83.1 ± 8.4
D-(-)-Quinic acid	76.3 ± 22.9	70.3 ± 24.3	64.6 ± 30.4

* including corresponding metabolites (sulfates and glucuronides).

Table 10-7: Ileal amounts of CGA and metabolites excreted after ingestion of a HIGH- , MEDIUM- and LOW- coffee (in µmol), ileostomist n = 5. (data expressed as mean values ± SD; n = 5)

Compound	HIGH	MEDIUM	LOW
1-*O*-Caffeoylquinic acid	16.1 ± 4.8	9.8 ± 3.8	5.1 ± 2.1
3-*O*-Caffeoylquinic acid	637.7 ± 182.6	341.9 ± 128.8	178.6 ± 68.9
4-*O*-Caffeoylquinic acid	592.8 ± 169.7	312.3 ± 121.4	157.1 ± 61.6
5-*O*-Caffeoylquinic acid	861.6 ± 246.1	455.0 ± 174.6	227.6 ± 88.6
3-*O*-Caffeoylquinic acid-*O*-GlucA	4.3 ± 1.6	2.1 ± 1.0	1.2 ± 0.4
4-*O*-Caffeoylquinic acid-*O*-GlucA	5.2 ± 1.8	2.7 ± 1.2	1.5 ± 0.6
3-*O*-Caffeoylquinic acid-*O*-Sulf	30.3 ± 10.4	18.6 ± 8.2	9.7 ± 3.8
4-*O*-Caffeoylquinic acid-*O*-Sulf	35.2 ± 11.9	21.7 ± 9.7	12.0 ± 4.6
5-*O*-Caffeoylquinic acid-*O*-Sulf	1.6 ± 0.7	1.3 ± 0.7	0.8 ± 0.3
3-*O*-Caffeolyquinide	132.0 ± 63.1	38.5 ± 19.5	15.5 ± 8.0
4-*O*-Caffeoylquinide	55.8 ± 27.6	22.1 ± 10.9	9.3 ± 4.8
3-/4-*O*-Caffeolyquinide-*O*-GlucA	16.3 ± 5.5	8.5 ± 3.8	3.0 ± 1.5
3-/4-*O*-Caffeoylquinide-*O*-Sulf	125.3 ± 35.5	75.8 ± 27.7	33.1 ± 16.2
3-*O*-Feruloylquinic acid	86.4 ± 24.9	45.6 ± 17.1	25.0 ± 9.5
4-*O*-Feruloylquinic acid	97.5 ± 28.5	43.0 ± 16.8	23.4 ± 8.7
5-*O*-Feruloylquinic acid	110.9 ± 31.6	61.1 ± 23.9	31.9 ± 12.4
3-*O*-Feruloylquinic acid-*O*-Sulf	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
4-*O*-Feruloylquinic acid-O-Sulf	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
3-*O*-Feruloylquinic acid-*O*-GlucA	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0
4-*O*-Feruloylquinic acid-O-GlucA	< LOQ	< LOQ	< LOQ
3-*O*-Isoferuloylquinic acid-*O*-GlucA	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
4-*O*-Isoferuloylquinic acid-O-GlucA	0.9 ± 0.3	0.6 ± 0.2	0.5 ± 0.2
Caffeic acid	27.0 ± 8.0	16.2 ± 5.6	13.4 ± 4.4
Caffeic acid-3´-*O*-GlucA	1.3 ± 0.5	1.2 ± 0.7	0.2 ± 0.1
Caffeic acid-3´-*O*-Sulf	26.4 ± 9.4	17.0 ± 8.1	11.3 ± 4.7
Caffeic acid-4´-*O*-Sulf	6.0 ± 2.0	4.1 ± 2.1	2.8 ± 1.1
Dihydrocaffeic acid	7.8 ± 3.1	4.3 ± 1.2	2.2 ± 0.9
Dihydrocaffeic acid-3´-*O*-Sulf	1.2 ± 0.5	0.9 ± 0.4	0.5 ± 0.2

Compound	HIGH	MEDIUM	LOW
Ferulic acid	5.0 ± 1.7	3.2 ± 1.0	2.9 ± 1.1
Ferulic acid-4´-*O*-Sulf	2.0 ± 0.6	1.6 ± 0.4	1.7 ± 0.5
Feruloylglycine	0.9 ± 0.3	0.3 ± 0.1	0.2 ± 0.1
Isoferulic acid-3´-*O*-Sulf	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Dihydroferulic acid-4´-*O*-Sulf	3.7 ± 1.0	1.8 ± 0.5	0.9 ± 0.4
3,4-*O*-diCaffeoylquinic acid	76.4 ± 22.6	38.0 ± 14.5	18.6 ± 7.3
3,5-*O*-diCaffeoylquinic acid	49.9 ± 14.7	24.9 ± 9.3	12.3 ± 4.8
4,5-*O*-diCaffeoylquinic acid	71.1 ± 21.5	36.5 ± 14.2	18.0 ± 6.9
∑	**3093.7 ± 317.0**	**1598.4 ± 110.7**	**816.6 ± 88.9**
% of ingested amount	**68.8 ± 9.0**	**72.4 ± 4.7**	**77.4 ± 4.3**

Table 10-8: Ileal excretion of CGA and metabolites given in T_{max} (hours) after ingestion of a HIGH- , MEDIUM- and LOW- coffee, ileostomist n = 5. (data expressed as mean values ± SD; n = 5).

Compound	HIGH	MEDIUM	LOW
1-*O*-Caffeoylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3
3-*O*-Caffeoylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3
4-*O*-Caffeoylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3
5-*O*-Caffeoylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3
3-*O*-Caffeoylquinic acid-*O*-GlucA	2.2 ± 1.0	3.0 ± 1.5	2.6 ± 1.0
4-*O*-Caffeoylquinic acid-*O*-GlucA	2.4 ± 1.0	2.8 ± 1.6	2.6 ± 1.0
3-*O*-Caffeoylquinic acid-*O*-Sulf	2.1 ± 1.3	3.0 ± 1.7	2.4 ± 1.0
4-*O*-Caffeoylquinic acid-*O*-Sulf	2.2 ± 1.2	3.0 ± 1.8	2.4 ± 1.0
5-*O*-Caffeoylquinic acid-*O*-Sulf	3.3 ± 1.9	4.5 ± 1.5	3.4 ± 1.5
3-*O*-Caffeolyquinide	1.2 ± 1.4	1.2 ± 0.9	1.3 ± 1.3
4-*O*-Caffeoylquinide	1.2 ± 1.4	1.2 ± 0.9	1.3 ± 1.4
3-/4-*O*-Caffeolyquinide-*O*-GlucA	2.0 ± 1.1	2.2 ± 1.9	2.6 ± 1.0
3-/4-*O*-Caffeoylquinide-*O*-Sulf	1.5 ± 1.3	2.1 ± 2.0	2.6 ± 1.0
3-*O*-Feruloylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3
4-*O*-Feruloylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3
5-*O*-Feruloylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3
3-*O*-Feruloylquinic acid-*O*-Sulf	3.0 ± 0.9	3.6 ± 1.4	3.0 ± 0.6
4-*O*-Feruloylquinic acid-O-Sulf	3.0 ± 1.5	3.2 ± 1.5	2.8 ± 0.8
3-*O*-Feruloylquinic acid-*O*-GlucA	3.0 ± 1.7	3.6 ± 1.4	2.5 ± 0.5
4-*O*-Feruloylquinic acid-O-GlucA	2.3 ± 0.9	4.0 ± 1.4	3.0 ± 0.0
3-*O*-Isoferuloylquinic acid-*O*-GlucA	3.2 ± 0.7	3.6 ± 1.4	2.8 ± 0.7
4-*O*-Isoferuloylquinic acid-O-GlucA	2.8 ± 1.2	3.0 ± 0.6	2.8 ± 0.7
Caffeic acid	1.2 ± 1.4	1.2 ± 0.9	1.2 ± 1.4
Caffeic acid-3´-*O*-GlucA	1.6 ± 1.3	1.6 ± 1.0	1.8 ± 1.4
Caffeic acid-3´-*O*-Sulf	2.2 ± 1.2	3.0 ± 1.9	2.4 ± 1.0
Caffeic acid-4´-*O*-Sulf	2.2 ± 1.2	2.3 ± 2.0	2.4 ± 1.0
Dihydrocaffeic acid	1.2 ± 1.4	1.8 ± 2.1	2.2 ± 1.4
Dihydrocaffeic acid-3´-*O*-Sulf	3.6 ± 2.4	2.7 ± 2.0	2.4 ± 1.0

Compound	HIGH	MEDIUM	LOW
Ferulic acid	1.2 ± 1.4	2.7 ± 1.9	2.8 ± 0.7
Ferulic acid-4´-*O*-Sulf	3.2 ± 1.2	4.0 ± 1.7	3.1 ± 1.9
Feruloylglycine	2.6 ± 0.8	3.2 ± 1.5	3.4 ± 1.4
Isoferulic acid-3´-*O*-Sulf	4.6 ± 2.2	3.8 ± 1.9	3.0 ± 0.6
Dihydroferulic acid-4´-*O*-Sulf	1.3 ± 1.4	2.9 ± 2.2	2.5 ± 1.1
3,4-O-diCaffeoylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3
3,5-O-diCaffeoylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3
4,5-O-diCaffeoylquinic acid	1.2 ± 1.4	1.8 ± 2.1	2.1 ± 1.3

Table 10-9: Ileal excretion of CGA and metabolites given in C_{max} (in nmol*g^{-1}ileal fluid) after ingestion of a HIGH- , MEDIUM- and LOW- coffee, ileostomist n = 5, (data expressed as mean values ± SD; n = 5).

Compound	HIGH	MEDIUM	LOW
1-*O*-Caffeoylquinic acid	72 ± 38	123 ± 118	48 ± 17
3-*O*-Caffeoylquinic acid	3011 ± 1668	4601 ± 4682	1771 ± 713
4-*O*-Caffeoylquinic acid	2801 ± 1516	4180 ± 4163	1583 ± 625
5-*O*-Caffeoylquinic acid	4070 ± 2235	6014 ± 6029	2278 ± 902
3-*O*-Caffeoylquinic acid-*O*-GlucA	30 ± 15	21 ± 10	11 ± 7
4-*O*-Caffeoylquinic acid-*O*-GlucA	39 ± 21	26 ± 12	12 ± 9
3-*O*-Caffeoylquinic acid-*O*-Sulf	206 ± 95	187 ± 121	100 ± 57
4-*O*-Caffeoylquinic acid-*O*-Sulf	215 ± 98	276 ± 245	125 ± 63
5-*O*-Caffeoylquinic acid-*O*-Sulf	18 ± 19	16 ± 9	8 ± 6
3-*O*-Caffeolyquinide	756 ± 392	677 ± 631	159 ± 73
4-*O*-Caffeoylquinide	350 ± 243	451 ± 498	91 ± 42
3-/4-*O*-Caffeolyquinide-*O*-GlucA	118 ± 84	76 ± 48	29 ± 23
3-/4-*O*-Caffeoylquinide-*O*-Sulf	509 ± 332	812 ± 644	305 ± 148
3-*O*-Feruloylquinic acid	403 ± 225	610 ± 621	241 ± 97
4-*O*-Feruloylquinic acid	468 ± 239	569 ± 594	239 ± 114
5-*O*-Feruloylquinic acid	523 ± 296	794 ± 799	316 ± 127
3-*O*-Feruloylquinic acid-*O*-Sulf	2 ± 1	2 ± 1	1 ± 1
4-*O*-Feruloylquinic acid-O-Sulf	2 ± 1	1 ± 1	1 ± 1
3-*O*-Feruloylquinic acid-*O*-GlucA	1 ± 1	1 ± 1	1 ± 0
4-*O*-Feruloylquinic acid-O-GlucA	< LOQ	< LOQ	< LOQ
3-*O*-Isoferuloylquinic acid-*O*-GlucA	3 ± 1	2 ± 1	1 ± 1
4-*O*-Isoferuloylquinic acid-O-GlucA	5 ± 3	5 ± 3	3 ± 2
Caffeic acid	134 ± 93	301 ± 419	134 ± 87
Caffeic acid-3´-*O*-GlucA	5 ± 2	7 ± 8	2 ± 1
Caffeic acid-3´-*O*-Sulf	166 ± 90	208 ± 177	124 ± 85
Caffeic acid-4´-*O*-Sulf	42 ± 31	54 ± 58	28 ± 18
Dihydrocaffeic acid	33 ± 25	39 ± 41	18 ± 12
Dihydrocaffeic acid-3´-*O*-Sulf	7 ± 4	9 ± 7	5 ± 4

Compound	HIGH	MEDIUM	LOW
Ferulic acid	24 ± 13	36 ± 33	22 ± 14
Ferulic acid-4´-*O*-Sulf	12 ± 7	20 ± 27	10 ± 7
Feruloylglycine	6 ± 4	2 ± 2	2 ± 1
Isoferulic acid-3´-*O*-Sulf	2 ± 1	2 ± 2	1 ± 1
Dihydroferulic acid-4´-*O*-Sulf	16 ± 8	23 ± 24	8 ± 4
3,4-O-diCaffeoylquinic acid	371 ± 186	502 ± 506	179 ± 68
3,5-O-diCaffeoylquinic acid	242 ± 120	320 ± 315	117 ± 42
4,5-O-diCaffeoylquinic acid	344 ± 163	461 ± 441	174 ± 72

Table 10-10: Renal excretion of chlorogenic acids and metabolites (in µmol) after ingestion of a HIGH-, MEDIUM- and LOW- coffee, ileostomists n = 5 (data expressed as mean values ± SD; n = 5).

Compound	HIGH	MEDIUM	LOW
3-*O*-Caffeoylquinic acid	26.2 ± 27.8	14.6 ± 0.6	8.2 ± 0.5
4-*O*-Caffeoylquinic acid	14.6 ± 8.0	8.6 ± 0.7	6.7 ± 1.2
5-*O*-Caffeoylquinic acid	9.4 ± 7.9	6.7 ± 0.5	4.4 ± 0.4
3-*O*-Caffeoylquinic acid-*O*-GlucA	8.3 ± 5.6	4.8 ± 0.2	1.6 ± 0.1
4-*O*-Caffeoylquinic acid-*O*-GlucA	23.3 ± 18.2	13.3 ± 0.9	5.5 ± 0.2
3-*O*-Caffeoylquinic acid-*O*-Sulf	5.3 ± 2.1	4.0 ± 0.2	2.2 ± 0.2
4-*O*-Caffeoylquinic acid-*O*-Sulf	5.1 ± 1.8	3.8 ± 0.2	2.7 ± 0.2
3-*O*-Caffeolyquinide	2.8 ± 3.0	0.8 ± 0.1	0.2 ± 0.0
4-*O*-Caffeoylquinide	4.6 ± 4.7	1.5 ± 0.1	0.7 ± 0.1
Caffeoylquinide-*O*-GlucA	4.1 ± 3.0	2.2 ± 0.1	0.6 ± 0.0
Caffeoylquinide-*O*-Sulf	24.1 ± 6.9	23.5 ± 1.6	10.3 ± 0.6
3-*O*-Feruloylquinic acid	33.3 ± 19.5	20.8 ± 1.4	12.3 ± 0.9
4-*O*-Feruloylquinic acid	22.2 ± 17.0	14.9 ± 0.5	7.1 ± 0.8
5-*O*-Feruloylquinic acid	17.0 ± 16.1	12.0 ± 0.6	7.1 ± 0.7
3-*O*-Feruloylquinic acid-*O*-Sulf	0.3 ± 0.3	0.1 ± 0.0	< LOQ
4-*O*-Feruloylquinic acid-O-Sulf	0.8 ± 0.7	0.4 ± 0.0	0.1 ± 0.0
3-*O*-Feruloylquinic acid-*O*-GlucA	2.2 ± 1.4	1.8 ± 0.3	1.0 ± 0.1
4-*O*-Feruloylquinic acid-O-GlucA	1.3 ± 1.8	0.5 ± 0.1	0.2 ± 0.1
3-*O*-Isoferuloylquinic acid-*O*-GlucA	6.1 ± 3.8	3.9 ± 0.3	1.9 ± 0.1
4-*O*-Isoferuloylquinic acid-O-GlucA	19.7 ± 13.8	12.9 ± 0.9	6.2 ± 0.3
Caffeic acid	14.1 ± 17.8	4.6 ± 0.3	3.9 ± 0.7
Caffeic acid-3´-*O*-GlucA	1.6 ± 1.3	0.5 ± 0.1	< LOQ
Caffeic acid-4´-*O*-GlucA	0.6 ± 0.6	n.d.	n.d.
Caffeic acid-3´-*O*-Sulf	12.8 ± 3.6	11.7 ± 0.7	10.9 ± 0.7
Caffeic acid-4´-*O*-Sulf	2.1 ± 0.8	1.8 ± 0.1	1.5 ± 0.2
Dihydrocaffeic acid	5.1 ± 6.2	1.4 ± 0.1	1.1 ± 0.2
Dihydrocaffeic acid-3´-*O*-Sulf	3.4 ± 1.3	5.4 ± 0.2	3.0 ± 0.2
Dihydrocaffeic acid-4´-*O*-Sulf	0.2 ± 0.1	< LOQ	< LOQ

Compound	HIGH	MEDIUM	LOW
Ferulic acid	17.1 ± 21.3	4.6 ± 0.6	2.8 ± 0.8
Ferulic acid-4´-*O*-GlucA	14.6 ± 9.3	12.3 ± 0.7	9.9 ± 0.5
Ferulic acid-4´-*O*-Sulf	17.5 ± 6.9	23.1 ± 1.2	23.8 ± 1.6
Feruloylglycine	16.5 ± 13.3	19.3 ± 0.6	5.6 ± 0.5
Isoferulic acid-3´-*O*-GlucA	23.7 ± 13.1	15.9 ± 1.2	11.8 ± 0.5
Isoferulic acid-3´-*O*-Sulf	< LOQ	n.d.	n.d.
Dihydroferulic acid -4´-*O*-GlucA	2.6 ± 1.4	4.5 ± 0.7	1.1 ± 0.2
Dihydroferulic acid-4´-*O*-Sulf	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
∑	**363.0 ± 223.3**	**256.7 ± 141.8**	**154.5 ± 72.1**
% of ingested amount	**8.0 ± 4.9**	**12.1 ± 6.7**	**14.6 ± 6.8**

n.d. = not detected (< LOD)

Table 10-11: Renal excretion of CGA and metabolites given in T_{max} (hours) after ingestion of a HIGH- , MEDIUM- and LOW- coffee, ileostomist n = 5. (data expressed as mean values ± SD; n = 5).

Compound	HIGH	MEDIUM	LOW
3-*O*-Caffeoylquinic acid	2.0 ± 0.0	2.0 ± 0.0	2.6 ± 1.2
4-*O*-Caffeoylquinic acid	2.6 ± 1.2	2.0 ± 0.0	2.6 ± 1.2
5-*O*-Caffeoylquinic acid	2.0 ± 0.0	2.0 ± 0.0	2.6 ± 1.2
3-*O*-Caffeoylquinic acid-*O*-GlucA	3.2 ± 0.3	3.8 ± 1.5	3.2 ± 1.5
4-*O*-Caffeoylquinic acid-*O*-GlucA	2.6 ± 1.2	2.6 ± 1.2	2.6 ± 1.2
3-*O*-Caffeoylquinic acid-*O*-Sulf	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
4-*O*-Caffeoylquinic acid-*O*-Sulf	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
3-*O*-Caffeolyquinide	2.6 ± 1.2	2.0 ± 0.0	2.0 ± 0.0
4-*O*-Caffeoylquinide	3.2 ± 1.5	2.0 ± 0.0	2.0 ± 0.0
3-/4-*O*-Caffeolyquinide-*O*-GlucA	2.0 ± 0.0	2.8 ± 1.3	2.0 ± 0.0
3-/4-*O*-Caffeoylquinide-*O*-Sulf	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
3-*O*-Feruloylquinic acid	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
4-*O*-Feruloylquinic acid	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
5-*O*-Feruloylquinic acid	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
3-*O*-Feruloylquinic acid-*O*-Sulf	3.0 ± 1.4	2.0 ± 0.0	2.0 ± 0.0
4-*O*-Feruloylquinic acid-O-Sulf	2.8 ± 1.3	2.0 ± 0.0	3.0 ± 1.4
3-*O*-Feruloylquinic acid-*O*-GlucA	2.8 ± 1.3	3.2 ± 1.5	3.8 ± 1.5
4-*O*-Feruloylquinic acid-O-GlucA	3.5 ± 1.5	3.2 ± 1.5	3.8 ± 1.5
3-*O*-Isoferuloylquinic acid-*O*-GlucA	3.8 ± 1.5	4.4 ± 1.2	3.2 ± 1.5
4-*O*-Isoferuloylquinic acid-O-GlucA	3.2 ± 1.5	3.8 ± 1.5	3.8 ± 1.5
Caffeic acid	2.6 ± 1.2	3.5 ± 1.5	3.2 ± 1.5
Caffeic acid-3´-*O*-GlucA	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Caffeic acid-4´-*O*-GlucA	3.5 ± 1.5	5.0 ± 0.0	n.d.
Caffeic acid-3´-*O*-sulfate	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Caffeic acid-4´-*O*-Sulf	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Dihydrocaffeic acid	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Dihydrocaffeic acid-3´-*O*-Sulf	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Dihydrocaffeic acid-4´-*O*-Sulf	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0

Compound	HIGH	MEDIUM	LOW
Ferulic acid	2.0 ± 0.0	2.0 ± 0.0	7.5 ± 9.5
Ferulic acid-4´-*O*-GlucA	2.6 ± 1.2	2.0 ± 0.0	2.0 ± 0.0
Ferulic acid-4´-*O*-Sulf	2.0 ± 0.0	2.0 ± 0.0	3.2 ± 2.4
Feruloylglycine	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Isoferulic acid-3´-*O*-GlucA	2.6 ± 1.2	2.0 ± 0.0	2.6 ± 1.2
Isoferulic acid-3´-*O*-Sulf	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Dihydroferulic acid -4´-*O*-GlucA	25.4 ± 19.9	20.0 ± 17.1	32.7 ± 21.7
Dihydroferulic acid-4´-*O*-Sulf	2.8 ± 1.3	2.0 ± 0.0	11.8 ± 18.1

Table 10-12: Concentrations of CGA and metabolites in urine given in nmol*g^{-1}creatinine (C_{max}) after ingestion of a HIGH- , MEDIUM- and LOW- coffee dose, ileostomist n = 5, (data expressed as mean values ± SD; n = 5).

Compound	HIGH	MEDIUM	LOW
3-*O*-Caffeoylquinic acid	1643 ± 1692	961 ± 635	646 ± 404
4-*O*-Caffeoylquinic acid	1088 ± 809	613 ± 433	536 ± 245
5-*O*-Caffeoylquinic acid	592 ± 550	463 ± 330	264 ± 170
3-*O*-Caffeoylquinic acid-*O*-GlucA	406 ± 296	270 ± 210	117 ± 80
4-*O*-Caffeoylquinic acid-*O*-GlucA	1303 ± 1232	680 ± 393	370 ± 176
3-*O*-Caffeoylquinic acid-*O*-Sulf	327 ± 162	296 ± 170	123 ± 33
4-*O*-Caffeoylquinic acid-*O*-Sulf	266 ± 115	239 ± 118	124 ± 29
3-*O*-Caffeolyquinide	222 ± 252	64 ± 51	12 ± 26
4-*O*-Caffeoylquinide	313 ± 324	116 ± 91	59 ± 49
3-/4-*O*-Caffeolyquinide-*O*-GlucA	354 ± 357	141 ± 67	56 ± 37
3-/4-*O*-Caffeoylquinide-*O*-Sulf	2029 ± 723	1969 ± 582	945 ± 326
3-*O*-Feruloylquinic acid	2282 ± 1468	1442 ± 868	1002 ± 477
4-*O*-Feruloylquinic acid	1273 ± 1022	812 ± 584	580 ± 416
5-*O*-Feruloylquinic acid	919 ± 859	731 ± 425	588 ± 364
3-*O*-Feruloylquinic acid-*O*-Sulf	38 ± 31	12 ± 3	< LOQ
4-*O*-Feruloylquinic acid-O-Sulf	86 ± 72	38 ± 37	7 ± 8
3-*O*-Feruloylquinic acid-*O*-GlucA	114 ± 47	100 ± 61	57 ± 37
4-*O*-Feruloylquinic acid-O-GlucA	27 ± 8	24 ± 14	13 ± 7
3-*O*-Isoferuloylquinic acid-*O*-GlucA	241 ± 105	201 ± 146	138 ± 87
4-*O*-Isoferuloylquinic acid-O-GlucA	887 ± 594	697 ± 500	475 ± 267
Caffeic acid	671 ± 775	412 ± 415	390 ± 381
Caffeic acid-3´-*O*-GlucA	158 ± 156	43 ± 19	< LOQ
Caffeic acid-4´-*O*-GlucA	45 ± 32	n.d.	n.d.
Caffeic acid-3´-*O*-Sulf	657 ± 284	629 ± 304	541 ± 195
Caffeic acid-4´-*O*-Sulf	148 ± 71	134 ± 56	75 ± 20
Dihydrocaffeic acid	415 ± 548	122 ± 112	111 ± 112
Dihydrocaffeic acid-3´-*O*-Sulf	202 ± 98	256 ± 139	184 ± 81
Dihydrocaffeic acid-4´-*O*-Sulf	45 ± 51	< LOQ	< LOQ

Compound	HIGH	MEDIUM	LOW
Ferulic acid	981 ± 1145	333 ± 206	240 ± 181
Ferulic acid-4´-*O*-GlucA	596 ± 379	394 ± 183	287 ± 141
Ferulic acid-4´-*O*-Sulf	684 ± 352	630 ± 281	383 ± 190
Feruloylglycine	979 ± 753	694 ± 365	308 ± 169
Isoferulic acid-3´-*O*-GlucA	1315 ± 649	791 ± 397	763 ± 379
Isoferulic acid-3´-*O*-Sulf	< LOQ	n.d.	n.d.
Dihydroferulic acid -4´-*O*-GlucA	35 ± 26	52 ± 56	10 ± 8
Dihydroferulic acid-4´-*O*-Sulf	24 ± 16	15 ± 2	5 ± 5

n.d. not detectable < LOD

Table 10-13: Maximum plasma concentrations (C_{max}), corresponding time point (T_{max}) and AUC of CGA, (after enzyme hydrolysis) and quinic acid for probands after consumption of HIGH-, MEDIUM- and LOW- coffee (data expressed as mean values ± SD; n = 4).

	HIGH			MEDIUM			LOW		
Compound	C_{max} (nM)	$AUC_{(nM*0-8h^{-1})}$	T_{max} (h)	C_{max} (nM)	$AUC_{(nM*0-8h^{-1})}$	T_{max} (h)	C_{max} (nM)	$AUC_{(nM*0-8h^{-1})}$	T_{max} (h)
3-CQA	43 ± 10	129 ± 48	0.6 ± 0.2	33 ± 13	89 ± 27	0.8 ± 0.3	14 ± 6	38 ± 19	1.3± 0.4
4-CQA	73 ± 7	222 ± 61	0.8 ± 0.3	57 ± 24	156 ± 68	0.8 ± 0.3	20 ± 7	64 ± 24	0.9 ± 0.2
5-CQA	44 ± 7	125 ± 38	0.8 ± 0.3	30 ± 12	85 ± 32	0.9 ± 0.2	14 ± 5	43 ± 13	0.6 ± 0.2
3-FQA	96 ± 39	251 ± 85	0.9 ± 0.2	56 ± 26	131 ± 53	0.9 ± 0.2	23 ± 10	61 ± 24	1.0 ± 0.0
4-FQA	117 ± 36	317 ± 85	1.0 ± 0.0	62 ± 24	167 ± 62	0.9 ± 0.2	32 ± 10	94 ± 24	0.9± 0.2
5-FQA	45 ± 8	160 ± 51	1.1 ± 0.5	41 ± 23	119 ± 66	0.9 ± 0.2	16 ± 5	45 ± 6	0.8 ± 0.3
CA	214 ± 22	605 ± 176	0.6 ± 0.2	162 ± 52	445 ± 172	0.9 ± 0.2	77 ± 12	243 ± 71	0.5 ± 0.0
DHCA	63 ± 18	153 ± 65	0.5 ± 0.0	35 ± 11	77 ± 30	0.5 ± 0.0	53 ± 13	51 ± 70	0.8 ± 0.3
FA	518 ± 76	937 ± 157	0.5 ± 0.0	214 ± 39	453 ± 93	0.5 ± 0.0	147 ± 27	377 ± 84	0.5 ± 0.0
IFA	262 ± 91	559 ± 249	0.5 ± 0.0	110 ± 6	190 ± 43	0.5 ± 0.0	76 ± 27	344 ± 315	0.7 ± 0.2
DHFA	85 ± 12	173 ± 27	0.6 ± 0.2	34 ± 12	78 ± 50	0.9 ± 0.2	22 ± 16	64 ± 55	0.5 ± 0.0
DiMeCA	305 ± 63	824 ± 154	0.6 ± 0.2	129 ± 55	357 ± 117	0.8 ± 0.2	58 ± 21	88 ± 48	0.6 ± 0.2
DiMeDHCA	67 ± 55	208 ± 185	0.3 ± 0.2	28 ± 22	42 ± 25	0.8 ± 0.2	59 ± 15	213 ± 186	0.8 ± 0.3
	C_{max} (µM)	$AUC_{(µM*0-8h-1)}$	T_{max} (h)	C_{max} (µM)	$AUC_{(µM*0-8h-1)}$	T_{max} (h)	C_{max} (µM)	$AUC_{(µM*0-8h-1)}$	T_{max} (h)
D-(-)-QA	4.2 ± 0.4	24.4 ± 5.6	5.0 ± 1.0	2.5 ± 0.6	14.8 ± 5.0	4.5 ± 0.9	1.2 ± 0.1	8.0 ± 0.5	5.2 ± 1.0

Table 10-14: Distribution of coffee polyphenols and D-(-)-quinic acid after 4 h incubation with pig mucosa in an Ussing chamber (mean ± SD as a percentage of the initial concentration; n = 4).

	Compartment		
	Mucosal	Serosal	Tissue
Mucosal application (20 µM)			
1-*O*-Caffeoylquinic acid*	97.2 ± 8.6	1.4 ± 0.7	1.3 ± 0.5
3-*O*-Caffeoylquinic acid*	98.8 ± 6.3	1.0 ± 0.3	1.0 ± 0.5
4-*O*-Caffeoylquinic acid*	95.0 ± 7.4	1.0 ± 0.3	1.1 ± 0.6
5-*O*-Caffeoylquinic acid*	95.4 ± 6.8	0.87 ± 0.19	1.4 ± 0.8
Caffeic acid	97.7 ± 6.7	1.4 ± 0.8	1.3 ± 0.3
D-(-)-Quinic acid	103.1 ± 11.5	3.9 ± 1.3	1.5 ± 0.6
3-*O*-Feruloylquinic acid	100.9 ± 15.1	2.6 ± 1.4	1.4 ± 0.3
4-*O*-Feruloylquinic acid	83.9 ± 3.9	2.0 ± 0.3	1.1 ± 0.5
5-*O*-Feruloylquinic acid	98.6 ± 2.9	2.6 ± 0.5	1.6 ± 0.5
3,4-O-diCaffeoylquinic acid	84.4 ± 4.2	< LOQ	0.16 ± 0.05
3,5-O-diCaffeoylquinic acid	61.8 ± 20.0	< LOQ	0.18 ± 0.04
4,5-*O*-diCaffeoylquinic acid	95.2 ± 10.0	< LOD	< LOD
5-*O*-Caffeoylquinic acid (100 µM)*	118.5 ± 13.1	1.6 ± 0.2	1.4 ± 0.7
5-*O*-Caffeoylquinic acid (500 µM)*	130.9 ± 30.5	1.8 ± 0.5	0.6 ± 0.1
5-*O*-Caffeoylquinic acid (1000 µM)*	98.7 ± 6.5	1.1 ± 0.5	1.0 ± 0.8
5-*O*-Caffeoylquinic acid (2000 µM)	106.6 ± 9.9	1.8 ± 1.0	1.3 ± 0.4
5-*O*-Caffeoylquinic acid (3500 µM)	94.7 ± 8.8	1.3 ± 0.5	1.9 ± 0.6
Serosal application (20 µM)			
5-*O*-Caffeoylquinic acid	2.3 ± 0.6	100.7 ± 14.0	0.5 ± 0.3
5-*O*-Caffeoylquinic acid + NaN_3	1.3 ± 0.5	77.1 ± 14.4	0.8 ± 0.3

*data was achieved in cooperation with Johanna Hauser

Table 10-15: Permeation rates of individual CGA and QA through the pig jejunal tissue (in nmol*cm^{-2}) during the incubation experiments in the Ussing chamber at 1, 2, 3 and 4 h. Permeation was determined from mucosal to serosal direction (muc application) or from serosal to mucosal direction (ser application) in concentrations from 20 up to 3500 µM.

Compound	Application	Conc. (µM)	1 h	2 h	3 h	4 h
Caffeoylquinic acids						
1-CQA*	muc	20	0.02 ± 0.04	0.24 ± 0.15	0.46 ± 0.26	0.71 ± 0.43
3-CQA*	muc	20	0.04 ± 0.07	0.24 ± 0.15	0.43 ± 0.20	0.68 ± 0.29
4-CQA*	muc	20	0.11 ± 0.09	0.38 ± 0.22	0.70 ± 0.33	1.06 ± 0.43
5-CQA	ser	20	0.16 ± 0.05	0.66 ± 0.17	1.57 ± 0.67	2.86 ± 1.02
5-CQA + NaN_3	ser	20	0.07 ± 0.06	0.42 ± 0.29	0.88 ± 0.46	1.33 ± 0.81
5-CQA*	muc	20	0.12 ± 0.06	0.35 ± 0.16	0.56 ± 0.29	0.78± 0.39
5-CQA*	muc	100	0.44 ± 0.04	2.07 ± 0.18	4.51 ± 0.24	6.47± 0.66
5-CQA*	muc	500	2.60 ± 0.89	10.91 ± 1.74	22.96 ± 4.4	34.9 ± 8.4
5-CQA*	muc	1000	4.05 ± 2.73	16.95 ± 8.77	34.60 ± 16.60	59.12 ± 28.20
5-CQA	muc	2000	10.47 ± 4.81	42.80 ± 15.48	95.73 ± 20.40	175.50 ± 31.67
5-CQA	muc	3500	9.54 ± 3.98	59.52 ± 21.83	114.08 ± 40.25	218.37 ± 82.52
Feruloylquinic acids						
3-FQA	muc	20	0.10 ± 0.13	0.66 ± 0.27	2.03 ± 1.38	3.65 ± 2.63
4-FQA	muc	20	0.17 ± 0.10	0.67 ± 0.28	1.60 ± 0.58	2.60 ± 0.66
5-FQA	muc	20	0.12 ± 0.08	0.73 ± 0.25	1.95 ± 0.60	3.88± 0.92
Caffeic acid	muc	20	0.09 ± 0.04	0.37 ± 0.20	0.80 ± 0.43	1.28 ± 0.72

Compound	Application	Conc. (µM)	1 h	2 h	3 h	4 h
D-(-)-Quinic acid	muc	20	0.32 ± 0.37	1.25 ± 0.71	2.01 ± 1.24	5.40 ± 2.20

*data was achieved in cooperation with Johanna Hauser

Table 10-16: Jejunal tissue conductance (in % of initial value 0 hours) during the incubation in the Ussing chamber after 1 h, 2 h, 3 h and 4 h with CGA and D-(-)-QA from coffee. Application of compounds was performed on mucosal (muc) or serosal (ser) compartment of the Ussing chamber with concentrations form 20 up to 3500 µM.

Compound	Application	Conc. (µM)	1 h	2 h	3 h	4 h
Caffeoylquinic acids						
1-CQA*	muc	20	125.4 ± 27.8	146.9 ± 30.4	186.0 ± 26.5	234.8 ± 30.7
3-CQA*	muc	20	133.4 ± 36.2	170.5 ± 39.6	218.3 ± 45.9	283.1 ± 44.3
4-CQA*	muc	20	123.9 ± 25.5	138.1 ± 31.8	178.4 ± 43.3	209.7 ± 52.8
5-CQA	ser	20	149.7 ± 18.5	176.7 ± 14.9	210.0 ± 19.7	356.2 ± 62.2
5-CQA + NaN_3	ser	20	140.8 ± 29.8	196.3 ± 54.9	228.7 ± 63.6	274.6 ± 88.8
5-CQA*	muc	20	124.7 ± 27.1	143.6 ± 31.1	176.6 ± 44.8	231.4 ± 49.8
5-CQA*	muc	100	129.8 ± 26.1	138.3 ± 25.5	156.9 ± 28.7	184.5 ± 33.0
5-CQA*	muc	500	151.9 ± 31.0	185.5 ± 35.1	246.5 ± 40.1	423.7 ± 52.5
5-CQA*	muc	1000	137.0 ± 15.8	156.2 ± 17.7	191.0 ± 26.4	282.9 ± 40.4
5-CQA	muc	2000	125.9 ± 34.8	141.4 ± 29.1	156.2 ± 31.2	168.9 ± 17.2
5-CQA	muc	3500	149.9 ± 18.8	165.0 ± 23.8	193.6 ± 35.6	294.4 ± 86.7
Feruloylquinic acids						
3-FQA	muc	20	153.5 ± 25.5	171.1 ± 26.8	189.0 ± 32.0	210.8 ± 37.1
4-FQA	muc	20	139.4 ± 10.1	146.4 ± 14.7	166.1 ± 21.5	185.4 ± 28.2
5-FQA	muc	20	149.0 ± 8.8	184.0 ± 11.6	248.4 ± 21.0	274.7 ± 13.5
Caffeic acid	muc	20	142.1 ± 13.7	172.8 ± 12.6	196.1 ± 12.5	243.0 ± 23.9

Compound	Application	Conc. (µM)	1 h	2 h	3 h	4 h
D-(-)-Quinic acid	muc	20	133.2 ± 21.8	168.9 ± 24.8	212.0 ± 46.3	245.7 ± 56.1
diCaffeoylquinic acids						
3,5-diCQA	muc	20	124.5 ± 28.7	120.5 ± 18.5	168.4 ± 24.4	212.3 ± 21.8
3,4-diCQA	muc	20	157.5 ± 44.7	161.5 ± 38.5	190.6 ± 41.6	232.6 ± 50.4
4,5-diCQA	muc	20	125.8 ± 30.3	151.3 ± 30.6	157.3 ± 28.9	184.6 ± 30.0

*data was achieved in cooperation with Johanna Hauser

Curriculum vitae

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Declaration

Hiermit erkläre ich an Eides statt, dass ich die eingereichte Dissertation selbständig verfasst, die für die Arbeit benutzen Hilfsmittel genannt habe und die Ergebnisse beteiligter Mitarbeiter sowie andere Autoren klar gekennzeichnet habe. Ich habe weder die Dissertation oder Teile der Dissertation als Prüfungsarbeit bei einem anderen Fachbereich eingereicht noch ein Promotionsverfahren bei einer Hochschule beantragt.

Winden, den ……