Influence of breakfast consumption on the chlorogenic acid metabolism in humans

Vom Fachbereich Chemie der Technischen Universität Kaiserslautern
zur Verleihung des akademischen Grades
„Doktor der Naturwissenschaften“
genehmigte Dissertation.

D386

Datum der wissenschaftlichen Aussprache: 19.10.2017

vorgelegt von Diplom-Lebensmittelchemikerin
Denise Scherbl

Kaiserslautern 2017

für meine Familie

Auch aus Steinen,

die einem in den Weg gelegt werden,

kann man Schönes bauen.

Johann Wolfgang von Goethe

Der experimentelle Teil der Arbeit entstand im Zeitraum von April 2011 bis Dezember 2013 in der Arbeitsgruppe von Frau Prof. Dr. rer. nat. Elke Richling im Fachbereich Chemie, Fachrichtung Lebensmittelchemie und Toxikologie der Technischen Universität Kaiserslautern.

Tag der wissenschaftlichen Aussprache: 19.10.2017

Prüfungskommission:

Vorsitzender:	Prof. Dr. rer. nat. Helmut Sitzmann
1. Berichterstatter:	Prof. Dr. rer. nat. Elke Richling
2. Berichterstatter:	Prof. Dr. Dr. med. Dieter Schrenk

Ich danke Frau Prof. Dr. Elke Richling für die Überlassung des interessanten Themas und die stete Diskussionsbereitschaft und Unterstützung während und nach meiner Promotionszeit.

Table of contents

Publications

Journal publications

Watzek, N., Boehm, N., Feld, J., **Scherbl, D.**, Berger, F., Merz, K.H., Lampen, A., Reemtsma, T., Tannenbaum, S., Skipper, P. L., Baum, M., Richling, E., Eisenbrand, G. (2012), *N*7-Glycidamide-guanine DNA adduct formation by orally ingested acrylamide in rats: a dose response study encompassing human diet-related exposure levels. *Chemical Research in Toxicology*, 25(2): 381-390.

Watzek, N., **Scherbl, D.**, Feld, J., Berger, F., Doroshyenko, O., Fuhr, U., Tomalik-Scharte, D., Baum, M., Eisenbrand, G., Richling, E. (2012), Profiling of mercapturic acids of acrolein and acrylamide in human urine after consumption of potato crisps. *Molecular Nutrition and Food Reasearch*, 56(12): 1825-1837.

Scherbl, D., Watzek, N., Kleesen, K., Hilsch, K., Eisenbrand, G., Richling, E. (2012), *In vitro* reaction kinetics of acrolein with DNA to generate N^2-Hydroxypropano-2´-deoxyguanosine DNA adducts.

Scherbl, D., Renouf, M., Marmet, C., Poquet, L., Cristiani, I., Dahbane, S., Emady-Azar, S., Sauser, J., Galan, J., Richling, E. (2016), Breakfast consumption induces retarded release of chlorogenic acid metabolites in humans. *European Food Research and Technology*, 243(5): 791-806.

Oral presentations

Scherbl, D., Watzek, N., Baum, M., Berger, F., Eisenbrand, G., Feld, J., Hengstler, J. G., Schug, M., Richling, E. (2011), Acrylamid wird in primären Rattenleberzellen effizient durch Bindung an Glutathion inaktiviert. 40. Deutscher Lebensmittelchemikertag, Halle (Saale), Germany

Scherbl, D., Watzek, N., Feld, J., Berger, F., Doroshyenko, O., Fuhr, U., Tomalik-Scharte, D., Baum, M., Eisenbrand, G., Richling, E. (2012), Acrolein exposure from heat-treated foods may be higher than that to acrylamide: Monitoring of urinary mercapturic acids in humans, Association of Applied Biologists, Munich, Freising, Germany

Scherbl, D., Erk, T., Barron, D., Dionisi, F., Hauser, J., Muentnich, S., Renouf, M., Steiling, H., Williamson, G. Richling, E. (2013), Gastrointestinal structure- and dose-response relationships for absorption of coffee polyphenols, Second International Conference on Cocoa, Coffee and Tea, Neapel, Italy

Poster presentations

Scherbl, D., Watzek, N., Baum, M., Berger, F., Doroshyenko, O., Feld, J., Fuhr, U., Tomalik-Scharte, D., Eisenbrand, G., Richling, E. (2012), Mercaptursäuren im Humanurin als Marker für die Exposition von Acrolein und Acrylamid. Lebensmittelchemische Gesellschaft; Fachgruppe in der Gesellschaft Deutscher Chemiker, Regionalverbände Süd-West, Kaiserslautern, Germany

Watzek N., Boehm N., Feld J., **Scherbl D.**, Berger F., Merz K.H., Lampen A., Reemtsma T., Tannenbaum S., Skipper P. L., Baum M., Richling E., Eisenbrand G. (2012), Low dose oral application of acrylamide to rats: mercapturic acid excretion and *N*7-GA-Gua DNA adduct formation, Gesellschaft für Umwelt - Mutationsforschung e.V. (GUM), 26. GUM-Tagung, Mainz, Germany

Watzek N., **Scherbl D.**, Berger F., Feld J., Eisenbrand G., Richling E. (2012), Acrolein and acrylamide: excretion of mercapturic acids after consumption of potato chips, 78. Jahrestagung der Deutschen Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie e.V. (DGPT), Dresden, Germany

Eisenbrand G., **Scherbl D.**, Watzek N., Fuhr, U., Tomalik-Scharte, D., Richling, E. (2012), Mercapturic acid excretion in human urine indicates higher exposure to acrolein than acrylamide after ingestion of potato chips, Society of Toxicology, 51st Annual Meeting, San Francisco (California), USA

Watzek N., Boehm N., Feld J., **Scherbl D.**, Berger F., Merz K.H., Lampen A., Reemtsma T., Tannenbaum S., Skipper P. L., Baum M., Richling E., Eisenbrand G. (2012), Mercapturic acid excretion and *N*7-GA-Gua DNA adduct formation after low dose oral application of acrylamide to rats, American Association for Cancer Research, 103nd Annual Meeting, Chicago (Illinois), USA

Scherbl, D., Watzek, N., Eisenbrand, G., Richling, E. (2012), Acrolein und Acrylamid: Renale Mercaptursäureausscheidung nach Verzehr von Kartoffelchips, 41. Deutscher Lebensmittelchemikertag, Muenster, Germany

Eisenbrand, G., Watzek, N., **Scherbl, D.**, Baum, M., Berger, F., Fuhr, U., Doroshyenko, O., Tomalik-Scharte, D., Richling, E. (2013), Acrylamide and Acrolein: heat induced contaminants in food, Society of Toxicology, 52st Annual Meeting, San Antonio (Texas), USA

Müntnich, S., **Scherbl, D.**, Richling, E. (2013), *In-vitro* Studien zur Absorption von Kaffeeinhaltsstoffen mit Hilfe des USSING-Kammer-Modells, Lebensmittelchemische Gesellschaft; Fachgruppe in der Gesellschaft Deutscher Chemiker, Regionalverbände Süd-West, Karlsruhe, Germany

Scherbl, D., Erk, T., Barron, D., Dionisi, F., Hauser, J., Muentnich, S., Renouf, M., Steiling, H., Williamson, G., Richling, E. (2013), Coffee polyphenols: Investigation of structure- and dose-dependent absorption in the gastrointestinal tract. 42. Deutscher Lebensmittelchemikertag, Braunschweig, Germany

List of tables

List of figures

Abbrevations

3-PPA	3-phenylpropionic acid
3,4-MPPA	3-(4-methoxyphenyl)propionic acid
4-MCA	4-methoxycinnamic acid
ax	axial
bidest	double distilled
BMI	body mass index [kg/m^2]
bw	body weight
°C	degree celsius
c	centi (10^{-2})
CA	caffeic acid
CAG	caffeic acid glucuronide
CAS	caffeic acid sulfate
CFQA	caffeoylferuloylquinic acid
CGA	chlorogenic acid
CinA	cinnamic acid
CoA	coenzyme A
COMT	catechol-*O*-methyltransferase
CQA	caffeoylquinic acid
CQA15L	caffeoylquinic acid-1,5-lactone
CQAS	caffeoylquinic acid sulfate
CQL	caffeoylquinic acid lactone, caffeoylquinide
DAD	diode array detection
DC	decarboxylation
DH	dehydrogenase
DHCA	dihydrocaffeic acid
DHCAG	dihydrocaffeic acid glucuronide
DHCAS	dihydrocaffeic acid sulfate
DHFA	dihydroferulic acid
DHFAG	dihydroferulic acid glucuronide
DHFAS	dihydroferulic acid sulfate
DHiFA	dihydroisoferulic acid
DHiFAG	dihydroisoferulic acid glucuronide
DHiFAS	dihydroisoferulic acid sulfate
diCQA	dicaffeoylquinic acid

Abbrevations

diMeDHCA	dimethoxydihydrocaffeic acid
DiMeCA	dimethoxycaffeic acid
DMeOX	demethoxyesterase
DMSO	dimethylsulfoxide
e.g.	for example, *exempli gratia*
eq	equatorial
ESI	electrospray ionization
EST	esterase
et al.	*et alii*
EtOH	ethanol
f	femto (10^{-15})
FA	ferulic acid
FAG	ferulic acid glucuronide
FAS	ferulic acid sulfate
FIA	flow injection analysis
FQA	feruloylquinic acid
FQA15L	feruloylquinic acid-1,5-lactone
FQAG	feruloylquinic acid glucuronide
FQAS	feruloylquinic acid sulfate
g	gram
GIT	gastrointestinal tract
h	hour
HCl	hydrochloric acid
HCOOH	formic acid
HPLC	high performance (pressure) liquid chromatography
HPLC-ESI-MS/MS	high performance-electrospray ionization-tandem mass spectrometry
iFA	isoferulic acid
iFAG	isoferulic acid glucuronide
iFAS	isoferulic acid sulfate
J	coupling constant
Keap1	Kelch Like ECH Associated Protein 1
kg	kilogram
l	liter
m	meter
m	milli (10^{-3})
M	molar (mol/L)

*m*CoA	*meta*-coumaric acid
*m*CoAG	*meta*-coumaric acid glucuronide
*m*CoAS	*meta*-coumaric acid sulfate
*m*DHCoA	*meta*-dihydrocoumaric acid
*m*DHCoAG	*meta*-dihydrocoumaric acid glucuronide
*m*DHCoAS	*meta*-dihydrocoumaric acid sulfate
MeCN	acetonitrile
MeDHFA	methyldehydroferulic acid
MeFA	methylferulic acid
MHz	megahertz
min	minute
mol	SI unit for an amount of substance
MRM	multiple reaction monitoring
MS	mass spectrometer, mass spectrometry
MS/MS	tandem mass spectrometry
µ	micro (10^{-6})
n	variable quantity
n	nano (10^{-9})
Nrf2	NF-E2 p45 subunit-related factor 2
NMR	nuclear magnetic resonance
NRC	Nestlé Research Center
nm	nanometer
*o*CoA	*ortho*-coumaric acid
*o*DHCoA	*ortho*-dihydrocoumaric acid
p	pico (10^{-12})
p.a.	pro analysis
*p*CoA	*para*-coumaric acid
*p*CoAG	*para*-coumaric acid glucuronide
*p*CoAS	*para*-coumaric acid sulfate
*p*CoQA	*para*-coumaroylquinic acid
*p*CoQA15L	*para*-coumaroylquinic acid-1,5-lactone
*p*DHCoA	*para*-dihydrocoumaric acid
*p*DHCoAG	*para*-dihydrocoumaric acid glucuronide
*p*DHCoAS	*para*-dihydrocoumaric acid sulfate
pH	negative decimal logarithm of the hydrogen ion activity in a solution (potentia hydrogenii)

pK_S	logarithm of the acid dissociation constant
Q	quadrupole
QA	D-(-)-quinic acid
RA	reductase
RP	reversed phase
rpm	rotations per minute
RT	room temperature
s	second
SD	standard deviation
SIDA	stable isotope dilution analysis
SPE	solide phase extraction
ST	sulfuryl-*O*-transferase
T	temperature
UDP-GT	uridindiphosphate glucuronosyl transferase
UV	ultra-violet
Vis	visible

Summary

Chlorogenic acids (CGA) are phenolic compounds that form during the esterification of certain *trans*-cinnamic acids with (-)-quinic acid. According to several human intervention studies, they may have potential health benefits. Coffee is the main source of CGA in human nutrition, and is consumed either alone or in combination with a variety of foods. For this reason, the presented study aimed to clarify whether the simultaneous consumption of food, for example, a breakfast rich in carbohydrates, with instant coffee affects the absorption and bioavailability of CGA. The research specifically focused on how various food matrices, which are consumed at the same time as a coffee beverage, will influence kinetic parameters such as area under the curve (AUC), maximum plasma concentration (c_{max}), and time needed to reach maximum plasma concentration (t_{max}).

In a randomized crossover study, fourteen healthy participants consumed either pure instant coffee or coffee with a carbohydrate- or fat-rich meal. All of the subjects consumed the same quantity of CGA (3.1 mg CGA/kg body weight). Blood samples, collected at various time points up to 15 h after instant coffee consumption, were quantitatively analysed. Additionally, three urine collection intervals were chosen over a time period of 24h. High performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) was used to determine the CGA present, along with the concentrations of respective metabolites.

During a blind data review meeting, 20 of the 56 analysed plasma metabolites were chosen for further statistical analysis. A total of 36 metabolites were monitored in the urine samples. Similar as in the plasma samples, between-treatment differences, measured through AUC, C_{max}, and t_{max}, of various CGA derived metabolites were to estimate. Each treatment was also analysed in terms of the correlation between the plasma AUC and urinary excretion of seven metabolites.

It is already known that inter-individual variations in CGA absorption depends on gut microbial degradation and affects the efficacy of these compounds. Microorganisms present in the gastrointestinal tract metabolise CGA to form dihydroferulic acid (DHFA) and dihydrocaffeic acid (DHCA) derivatives, which precede the subsequent formation of a wide range of metabolites. Therefore stool samples were collected from the participants within 12 h before the second study day. Subsequent an *ex-vivo* incubation of faecal samples with 5-O-caffeoylquinic acid (5-CQA), the main chlorogenic acid found in coffee was performed. An HPLC system connected to a CoulArray® detector was used to measure the concentrations of 5-CQA and its metabolites. Reduced concentrations of 5-CQA as well as the appearance of DHCA and caffeic acid (CA) in the gut microbiota medium, were

monitored to calculate the inter-individual kinetics for each compound. In addition, these samples were analysed for microbiota content by an external laboratory (L&S, Bad Bocklet, Germany). These results were used to distinguish whether the decreased or increased content of a specific microorganism was related to an individual's decreased or increased metabolic efficiency. Finally, we used to aforementioned results to evaluate if any correlation could be drawn between the plasma appearance, urinary excretion and ability of microorganisms to degrade 5-CQA.

Strong inter-individual variation was observed for AUC, C_{max} and t_{max}. The AUC measured the quantity of CGA in plasma samples. We noted that pure instant coffee consumption resulted in slightly higher CGA bioavailability than instant coffee with the additional consumption of a meal. However, these differences were not statistically significant. Additionally, the metabolites were divided into groups, according to similarity and chemical properties. They were further classified into three groups according to their physical structure and predicated from the area of appearance: directly from coffee (*quinics*), after first degradation and metabolism (*phenolics*, all *trans*-cinammic acids and their sulfates and glucuronides) as well as colonic degradation and metabolism (*colonics*, all dihydro compounds). These respective metabolic classes showed significant differences in the AUC values of certain classes yet no significant between-treatment differences. Our results corroborated earlier studies in that the three caffeoylquinic acid (CQA) isomers were absorbed to a lower extent whereas all feruloylquinic acids (FQA) were detected in comparably high amounts in the plasma samples of the volunteers. However, the amount of these quinic acid conjugates in the plasma samples accounted for only 0,5% of the total amount of identified. In contrast, at least 8.7% of the investigated compounds were identified to be *phenolics*. Dihydro compounds, the so known *colonics*, were identified as the most common metabolites (90.8%). Additionally, dihydroferulic acid (DHFA), *meta*-dihydrocoumaric acid (*m*DHCoA), dihydrocaffeic acid-3-sulfate (DHCA3S) and dihydroisoferulic acid (DHiFA) were identified to account for 78% of the studied metabolites, and thus represent the most abundant compounds circulating in the plasma after coffee consumption.

Irrespective of treatment, the t_{max} value for early metabolites (quinic and phenolic compounds) was observed between 0 and 2 h after the ingestion of coffee and t_{max} value for late metabolites (colonic metabolites) was observed between 7 and 10 h. The amount of colonic metabolites had not returned to the baseline level 15 h after the ingestion of coffee. The co-ingestion of breakfast and coffee, when compared to the ingestion of coffee alone, significantly increased the C_{max} values for all quinic and phenolic compounds, as well as two colonic metabolites (DHCA and DHiFA). These differences also revealed that the three treatments differed in terms of the kinetics of release. Thus, future studies should use an

extended plasma collection time with shorter intervals (e.g. 2 h) to provide a full pharmacokinetic profile.

There were no statistically significant between-treatment differences in the urine samples collected 24 h after coffee ingestion. However, urine samples collected within six hours of the consumption of coffee alone or in combination with a fat-rich meal showed significantly higher CGA quantities than samples collected at the same time point for coffee ingested with a carbohydrate-rich. Strong inter-individual variability and the fact that only 14 healthy subjects participated in the study hindered the identification of any clear trend between the plasma concentrations of metabolites and their excretion in urine.

Four hours after the *ex vivo* incubation of 5-CQA with individual faecal samples the sum of 5-CQA, CA, and DHCA varied strongly between participants. These findings could result from binding effects of the phenolic compounds with faecal constituents, further degradation or metabolism, and/or the release of bound phenolic substances before the experiment started. We hypothesized that for participants with high plasma AUCs of dihydro compounds, their incubation samples show also high concentrations of CA and DHCA in the incubation medium after four hours. No significant correlation could be found.

This study and all of the outcomes were exploratory. Due to the limited number of participants, we could only investigate tendencies for how the co-ingestion of food affects the bioavailability of CGAs and their respective metabolites following coffee consumption. Therefore, the achieved results are only indicative. Despite this limitation, the data highlight that even though all three treatments had strong similarities in the total bioavailability of CGAs and metabolites from instant coffee, there were between-treatment differences in the kinetics of release. The co-ingestion of breakfast and coffee favoured a slow and continuous release of colonic metabolites while non-metabolized coffee components were observed in plasma within the first hour when coffee was ingested alone.

In conclusion, both a shift in gastrointestinal transit time and the plasma metabolite composition were observed when the ingestion of coffee alone or in combination with breakfast were compared. These results showed that breakfast consumption induces the retarded release of chlorogenic acid metabolites in humans. The data from our human intervention study suggest that the bioavailability of chlorogenic acids from coffee and their derivatives does not only depend on chemical structure, molecular size and active or passive transport ability, but is also influenced by inter-individual differences. Therefore, we strongly recommend that future studies include metabolism experiments that focus on microbiota genotypes and/or the genotyping of individual subjects. This type of research could be pivotal to elucidating whether, and how, genotype affects the metabolic profile after chlorogenic acid intake.

Zusammenfassung

Chlorogensäuren (CGA) sind Ester der trans-Zimtsäure und (-)-Chinasäure. Die Ergebnisse humaner Interventionsstudien weisen auf einen potentiellen Nutzen dieser Substanzklasse für die Gesundheit hin. Kaffee stellt eine der Hauptquellen an CGA in der Ernährung dar. Kaffee wird alleine oder in Kombination mit einer Vielzahl an Lebensmitteln konsumiert. Es war bisher nicht bekannt, ob der gleichzeitige Konsum mit anderen Lebensmitteln die Resorption von CGA aus Kaffee beeinflussen kann. Aus diesem Grund war es das primäre Ziel dieser Studie aufzuklären, ob die Aufnahme eines Lebensmittels, beispielsweise eines kohlenhydratreichen Frühstücks, gemeinsam mit Kaffee, einen Effekt auf die Absorption und Bioverfügbarkeit der CGA zeigt. Der Fokus dieser Forschungsarbeit lag darin, die kinetischen Parameter wie die Fläche unter der Kurve (area under the curve (AUC)), die maximale Plasmakonzentration (C_{max}) und die Zeit, die benötigt wird, um die maximale Plasmakonzentration zu erreichen (t_{max}), zu erfassen.

In einer randomisierten ’crossover‘-Studie konsumierten vierzehn gesunde Probanden entweder Instantkaffee allein oder Instantkaffee zusammen mit einer kohlenhydrat- oder fettreichen Mahlzeit. Jeder Proband konsumierte die gleiche Menge CGA bezogen auf sein Körpergewicht (3,1 mg CGA / kg Körpergewicht). Blutproben, die zu verschiedenen Zeitpunkten bis 15 h nach Kaffeekonsum gesammelt wurden, wurden im Anschluss analysiert. Zusätzlich wurden drei Urinsammelzeiträume über 24 h gewählt. Hochleistungsflüssigkeitschromatographie-Elektrospray-Massenspektrometrie (HPLC-ESI-MS/MS) wurde genutzt, um die Konzentrationen der CGA und deren entsprechende Metaboliten zu bestimmen.
Während einem ’blind data review‘ wurden 20 der 56 analysierten Plasmametaboliten für weitere statistische Analysen ausgewählt. In den Urinproben wurden insgesamt 36 Metaboliten erfasst und ebenso wie im Plasma die Unterschiede der jeweiligen Darreichungen, gemessen an der AUC, C_{max}, und t_{max} verschiedener CGA und ihrer Metaboliten, ermittelt. Für jede Darreichung wurden sieben Metaboliten zusätzlich auf ihre Korrelation von AUC im Plasma und auf die renale Ausscheidung hin untersucht.
Es ist bekannt, dass interindividuelle Unterschiede der CGA-Absorption und die Wirksamkeit dieser Verbindungen von einem mikrobiellen Abbau abhängen. Mikroorganismen, die im Gastrointestinaltrakt lokalisiert sind, sind dazu in der Lage, CGA zu Dihydroferulasäure (DHFA) und Dihydrokaffeesäure (DHCA) zu metabolisieren. Diese Derivate gehen einer großen Anzahl im Organismus gebildeter Metaboliten voraus. 12 h vor dem zweiten Studientag wurden Stuhlproben von jedem Probanden gesammelt und im Anschluss wurde

eine ex vivo-Inkubation mit Faecesproben und 5-O-Caffeoylchinasäure (5-CQA) durchgeführt. Ein HPLC-System mit CoulArray®-Detektor wurde verwendet, um die Konzentrationen von 5-CQA und seinen Metaboliten zu bestimmen. Die ermittelten Konzentrationen von 5-CQA, DHCA und Kaffeesäure (CA) im Darm-Mikrobiota-Medium (gut microbiota medium (GMM)) wurde genutzt, um die interindividuellen Kinetiken jeder Substanz zu bestimmen. Zusätzlich wurden die einzelnen Proben auf ihre mikrobielle Zusammensetzung hin in einem externen Labor (L&S, Bad Bocklet, Germany) untersucht. Diese Ergebnisse wurden genutzt, um erniedrigte oder erhöhte Gehalte an spezifischen Mikroorganismen in individuellen Proben mit den Ergebnissen der ex vivo-Inkubation zu vergleichen. Es war von Interesse, ob erhöhte Gehalte an spezifischen Mikroorganismen zu einem erhöhten Abbau von 5-CQA und somit zu einem vermehrten Auftreten von DHCA und CA führten. Schließlich wurden diese Ergebnisse genutzt, um mögliche Korrelationen zwischen dem Auftreten der CGA Metaboliten im Plasma, deren renale Ausscheidung und der Fähigkeit der Mikroorganismen 5-CQA abzubauen, zu evaluieren.

Starke interindividuelle Unterschiede wurden für AUC, C_{max} und t_{max} beobachtet. Es war zu beobachten, dass der Konsum von Instantkaffee allein in einer gering erhöhten AUC, im Vergleich zum Verzehr des Kaffees in Kombination mit einer Mahlzeit, resultierte. Diese Ergebnisse zeigten allerdings keine statistische Signifikanz. Die Metaboliten wurden in verschiedene Klassen eingeteilt. Die Einteilung erfolgte nach physikalischer Struktur und chemischen Eigenschaften, sowie der Erscheinung und dem Ort der Modifikation: *quinics* schlossen die Substanzen ein, die schon im Kaffee detektiert werden konnten, *phenolics* (trans-Zimtsäuren und deren Sulfate und Glucuronide) beinhalteten alle Substanzen, die einem ersten Abbau und Metabolismus unterlagen und *colonics* (alle Dihydro-Verbindungen) stellten die Klasse an Substanzen dar, die einem weiteren Abbau und Metabolismus im Kolon unterlagen. Für die entsprechenden Metabolitklassen, konnten signifikant unterschiedliche AUC-Werte für bestimmte Klassen, aber nicht zwischen den Darreichungen ermittelt werden. Unsere Ergebnisse bestätigen ältere Untersuchungen, bei denen die drei Caffeoylchinasäureisomere (CQA) zu einem geringeren Anteil absorbiert wurden als die entsprechenden Feruoylchinasäureisomere (FQA). Die Gehalte der *quinics* in den Plasmaproben wurden jedoch nur als 0,5% der Gesamtmenge erfasst. Im Gegensatz dazu waren 8,7% aller untersuchten Substanzen *phenolics*. *Colonics* wurden als meist vorkommende Metaboliten mit 90,8% identifiziert. Zusätzlich wurden Dihydroferulasäure (DHFA), meta-Dihydrocumarsäure (mDHCoA), Dihydrokaffeesäure-3-Sulfat (DHCA3S) und Dihydroisoferulasäure (DHiFA) mit 78% aller untersuchten Metaboliten, als die Hauptmetaboliten, die nach Kaffeekonsum im Plasma zirkulieren, identifiziert.

Ungeachtet der Darreichung war t_{max} für die frühen Metaboliten (*quinics* und *phenolics*) zwischen 0 und 2 h und t_{max} für die späten Metaboliten (*colonics*) zwischen 7 und 10 h nach der Kaffeeaufnahme. Die Gehalte der *colonics*, waren innerhalb von 15 h nach Kaffeekonsum nicht zum Basislinienlevel zurückgekehrt. Die kombinierte Aufnahme von Kaffee mit Frühstück im Vergleich zu Kaffee ohne Frühstück führte zu einer signifikanten Abnahme von C_{max} für alle *quinics* und *phenolics* sowie zwei der im Kolon gebildeten Metaboliten (DHCA and DHiFA). Es zeigte sich, dass sich die drei Darreichungen in der Kinetik der Freisetzung unterschieden. Daher sollte bei zukünftigen Studien verlängerte Plasmaprobennahmen in kürzeren Intervallen (z. B. 2 h) erfolgen, um ein vollständiges pharmakokinetisches Profil zu erhalten.
Für die unterschiedlichen Darreichungen konnten keine statistisch signifikanten Unterschiede zwischen den 24 h-Sammelurinproben festgestellt werden. Jedoch wiesen Urinproben, die innerhalb der ersten sechs Stunden nach Kaffeekonsum allein oder in Kombination mit einer fettreichen Mahlzeit gesammelt wurden, signifikant höhere CGA-Gehalte, als die Proben, die im gleichen Zeitraum nach Kaffeekonsum in Kombination mit einer kohlenhydratreichen Mahlzeit gesammelt wurden, auf. Starke interindividuelle Schwankungen und die Tatsache, dass nur 14 Probanden an der Studie teilgenommen haben, erschwerten die Ermittlung eines klaren Trends zwischen den Plasmakonzentrationen der Metaboliten und deren renaler Ausscheidung.
Die Ergebnisse der Inkubationen zeigten nach einer Inkubationszeit von vier Stunden (5-CQA mit den Stuhlproben) stark variierend Gehalte von 5-CQA, CA und DHCA zwischen den einzelnen Proben. Diese Beobachtung könnte durch Bindungseffekte der phenolischen Substanzen an fäkalen Bestandteilen, weiterem Abbau oder Metabolismus und / oder der Freisetzung von bereits vor dem Experiment gebundenen phenolischen Substanzen erklärt werden. Zur Untersuchung möglicher weiterer Korrelationen wurden die Einzel-AUC-Gehalte der Dihydroverbindungen im Plasma der Probanden in hohe und niedrige Gehalte unterteilt. Im Anschluss wurde untersucht, ob entsprechende Inkubationsproben gleicher Probanden hohe bzw. niedrige Konzentration von CA und DHCA im Inkubationsmedium aufwiesen. Es konnte keine Korrelation nachgewiesen werden.

Diese Studie und alle erhaltenen Ergebnisse sind als Pilotstudie zu sehen. Aufgrund der eingeschränkten Anzahl an Teilnehmern konnten nur Tendenzen für den Effekt eines Lebensmittels auf die Bioverfügbarkeit von CGAs und deren Metaboliten aus Kaffee beobachtet werden. Deshalb sind die gezeigten Ergebnisse nur indikativ. Trotz dieser Einschränkung ist hervorzuheben, dass die gezeigten Daten keinen Einfluss des Frühstücks auf die Bioverfügbarkeit der CGA und ihrer Metaboliten aus Kaffee aufwies, aber ein bedeutender Unterscheid in deren Kinetik der Resorption vorlag. Die kombinierte Aufnahme

eines Frühstücks mit Kaffee führte zu einer verlangsamten und kontinuierlichen Bildung der Kolonmetaboliten. Die nicht-metabolisierten Chlorogensäuren aus Kaffee wurden hingegen innerhalb der ersten Stunde nach Kaffeeverzehr im Plasma nachgewiesen.

Zusammenfassend konnten, beim Vergleich der Ergebnisse der verschiedenen Darreichungen, sowohl Verschiebungen der gastrointestinalen Transitzeit als auch der Zusammensetzung der Plasmametaboliten festgestellt werden. Der Frühstückverzehr führte zu einer verzögerten Resorption der Chlorogensäuren im Menschen. Die Daten unserer Humanstudie zeigten, dass die Bioverfügbarkeit von CGA aus Kaffee nicht nur von der Struktur, der Molekülgröße und aktiver oder passiver Transportfähigkeit des Gewebes des Organismus abhängt, sondern oder auch von individuellen Unterschieden des Probanden. Deshalb empfehlen wir, dass zukünftige Metabolismusexperimente die Typisierung der Mikrobiota und / oder die Genotypisierung eines Individuums, beinhalten. Diese Forschung könnte entscheidend sei, um zu klären, ob und wie, die Zusammensetzung der Mikrobiota als auch der Genotyp eines Individuums den Metabolismus und die Bioverfügbarkeit von Chlorogensäuren beeinflussen können.

1 Introduction

Classically, chlorogenic acids (CGA) are a family of esters formed between certain *trans* cinnamic acids and (-)-quinic acid [*Clifford, 1999, Clifford, 2000, IUPAC, 1969*]. The cinnamic acid conjugates are known to occur widely in dicotyledenous plants [*Mølgaard&Ravn, 1988*], especially in coffee. Previous studies have investigated, and linked, physiological effects such as free-radical scavenging, metal chelation, modulation of signal transduction, gene expression, modulation of enzymatic activity, and activation of transcription factors to the CGAs [*Nardini et al.,* 2002]. Also Coffee, one of the most popular beverages worldwide, has been widely studied in many scientific reports for its potential effect on health [*Natella et al., 2002*]. Recent epidemiological studies indicate that coffee consumption reduces the risk of degenerative diseases such as type 2 diabetes [*Nyambe-Silavwe and Williamson, 2016*], cardiovascular disease, or cancer [*Zhang et al., 2009*]. To date, the specific coffee ingredients that are associated with these effects have not been adequately established.
Coffee is rich in several different compounds including the main coffee antioxidants, the chlorogenic acids (CGAs) [*Stalmach et al., 2006*]. Caffeic, ferulic, and *p*-coumaric acids react with quinic acid to form a range of conjugates respectively caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs), and *p*-coumaroylquinic acids (*p*-CoQAs) [*Clifford et al., 2000*]. Although coffee alkaloids, like trigonelline, roasting products such as melanoidins, and the *N*-methylpyridinium ion in particular, are considered antioxidants [*Lemanska et al., 2001*].Nevertheless, physiological effects such as free-radical scavenging, metal chelation, modulation of signal transduction, gene expression, modulation of enzymatic activity, and activation of transcription factors, have been studied and linked to the CGAs [*Nardini et al., 2002*].

Bioavailability is extremely important for assessing the preventive efficacy effects of coffee consumption. Studies have focused on the bioavailability of hydroxycinnamic acids and their derivatives in rodents and mammals. For instance, Azuma *et al*. found glucuronide and sulphate conjugates of caffeic and ferulic acid in rat plasma after consumption of caffeic acid, whereas ingested 5-CQA was found almost exclusively in the small intestine [*Azuma et al., 2000*]. To date, only a limited number of studies in humans have investigated the systemic circulation after absorption of coffee CGAs [*Erk et al., 2013, Renouf et al., 2014, Stalmach et al., 2009*]. In a study by Olthof *et al*., 5-CQA is poorly absorbed in the human small intestine [*Olthof et al., 2003*]; the prevailing microbiota degrade it prior to absorption [*Plumb et al., 1999*], metabolizing it into hippuric acid [*Olthof et al., 2003*]. In addition, Rechner and coworkers observed glucuronide as well as sulfate conjugates of caffeic acid (CA) in plasma

samples of five volunteers after coffee consumption (in sum 449.1 mg, quantified relative to 5-CQA). In addition, free acids as well as glucuronides and sulfates of ferulic (FA), iso-ferulic (iFA), methylferulic (MeFA) and hippuric acids were detected in urinary samples of these five probands and were concluded as specific biomarkers for the bioavailability and metabolism of dietary caffeic acid (CA) esters [*Rechner et al., 2001*]. The colonic degradation and metabolism is well investigated by Ludwig and coworkers. Up to 6 h, they investigated the identity and quantity of breakdown products generated during incubation of espresso coffee with human fecal samples. They detected 11 catabolites with dihydrocaffeic, dihydroferulic, and 3-(3´-hydroxyphenyl)propionic acids as the major end products, comprising 25 – 75% of the total catabolites [*Ludwig et al., 2013*]. In another study, increases in free and conjugated caffeic acid, but no CQAs, were reported in human plasma [*Nardini et al., 2002*]. Monteiro *et al*. detected inter-individual variations of CGA concentrations in plasma after coffee consumption [*Monteiro et al., 2008*]. Farah *et al*. detected 30% hydroxycinnamic acids in plasma after consumption of encapsulated green coffee [*Farah et al., 2008*]. In volunteers, 29% of the CGAs consumed in coffee were detected as metabolites, like dihydroferulic acid, dihydrocaffeic acid-3-sulphate, and -4-sulphate in urine [*Stalmach et al., 2009*]. These results were confirmed by a study with ileostomy subjects investigating CGA absorption and metabolism in a dose-response manner [*Erk et al., 2012*]. In summary, the absorption or non-absorption of the CGA in the small intestine is mainly depending on the chemical structure and any attached chemical groups. The absorbed substrates may appear in plasma as methylated, sulphated and glucuronidated derivatives or as unchanged molecules. When the CGA were not absorbed and reach the colon, they are substrates for extensive catabolism by colonic microbiota. Subsequent absorption in the colon results in similar derivatives in plasma like found already after absorption in the small instetine or as glycine derivatives [*Williamson and Clifford, 2017*].

A majority of the German population daily consumes coffee or its products during breakfast. However, at present, data regarding the influence of food consumption on CGA absorption and availability is limited. For example, one study found that milk did not affect bioavailability parameter of CGAs from coffee; instead non-dairy creamer showed a delayed t_{max} of CGAs [*Renouf et al., 2010*]. Additionally, there is currently no data available regarding the effects of consumed food composition on absorption rates and excretion of CGAs after coffee consumption. As coffee is one of the most popular beverages worldwide and oftentimes consumed in combination with food, we performed a human intervention study with healthy volunteers who consumed only coffee (T1), coffee with a carbohydrate-rich breakfast (T2), and coffee with a fat-rich breakfast (T3). Blood and urine samples were collected and analyzed for information on absorption, body distribution, and excretion. We have observed,

for the first time, the absorption and excretion of coffee ingredients (CGAs and their derivatives) as affected by food matrix simultaneously consumed with coffee.

2 State of knowledge

2.1 Phenolcarbonic acids - Classification, occurence, role

Phenolcarbonic acid is a general term for phenolic compounds such like hydroxycinnamic acids and hydroxybenzoic acids as well as their derivatives (see Figure 1).

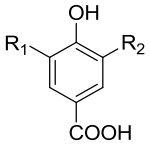
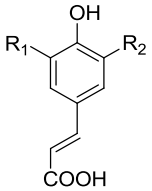
hydroxybenzoic acids		R_1	R_2	hydroxycinnamic acids		R_1	R_2
	**GA**	OH	OH		***p*CoA**	H	H
	PA	OH	H		**FA**	H	OCH_3
	SyA	OCH_3	OCH_3		**SA**	OCH_3	OCH_3
hydroxybenzoic acid	**VA**	OCH_3	H	hydroxycinnamic acid	**CA**	H	OH

Figure 1 Chemical structures of hydroxycinnamic (*p*CoA: *p*-coumaric acid, FA: ferulic acid, SA: sinapinic acid, CA: caffeic acid) and hydroxybenzoic acids (GA: gallic acid, PA: protocatechuic acid, SyA: syringic acid, VA: vanillic acid) [after *Watzl, 2001*]

Caffeic acid (CA, 3,4-dihydroxycinnamic acid), *p*-coumaric acid (*p*CoA, *trans*-4-hydroxycinnamic acid) and ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) are representative for hydroxycinnamic acids, and gallic acid (GA, 3,4,5-trihydroxybenzoic acid) and vanillic acid (VA, 4-hydroyx-3-methoxybenzoic acid) for hydroxybenzoic acids. Their main occurrence is in fruits of *rosaceae*, potatoes, red wine and coffee as free compounds or as esters of acids, sugars and alcohols [*Mattila&Hellström, 2007, Römpp, 1996*]. Mostly, hydroxycinnamic acids are esterified with quinic acid (QA) what give rise to a new substance class, for example the quinic acid esters with *trans*-CA called caffeoylquinic acids (CQA) (see Figure 2) [*Clifford, 1999*].

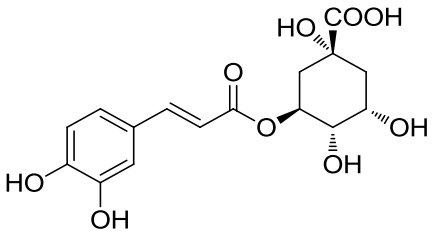


Figure 2 Chemical structure of 5-*O*-caffeoylquinic acid (5-CQA)

Additionally, esters from *p*CoA with QA (*p*-coumaroylquinic acid, *p*CoQA) and FA with QA (feruloylquinic acid, FQA) exist in plants. These three QA ester groups belong to the so

called family of chlorogenic acids (CGA). In addition to the monoesters, there is also the presence of diesters in plants. Diesters comprise either two molecules of the same hydroxycinnamic acid or an alloy. For example two molecules of CA esterified with QA make dicaffeoylquinic acid (diCQA) and an alloy of CA and FA, generates a caffeoylferuloylquinic acid (CFQA) [*Clifford, 1999*]. Possible positions for conjugations are: 1,3- 1,4-, 1,5-, 3,4- and 3,5-diCQA or the correlated CFQA [*ChemSpider*].

2.1.1 Biosynthesis of chlorogenic acids

Chlorogenic acids are formed via the shikimate pathway, which is also important for the biosynthesis of specific amino acids. At this pathway, phosphoenol pyruvate (PEP) and erythrose-4-phosphate (E4P) are precipitated and after seven metabolic steps converted into chorismate, the precursor of many aromatic secondary metabolites is formed only in microorganisms and plants, never in animals (see Figure 3): After condensation of PEP and E4P the shikimic acid pathway intermediate 3-dehydroquinate occurs. It is transformed into quinic acid (QA) or after a number of two steps into shikimic acid. Via shikimate-5-phosphate chorismate is formed [*Stryer, 1995*], where the pathway bifurcates into the formation of tyrosine and phenylalanine, respectively. Thereby the different hydroxycinnamic acids are formed. This final biosynthesis steps occurs via the phenylpropanoid pathway. After deamination of phenylalanine by ammonium lyase, *trans*-cinnamic acid is formed. *p*CoA is hydroxylated at position C-4 and by hydroxylation at position C-3 CA is formed. Catechol-*O*-methyl transferase (COMT) methylates CA to FA, which is finally transformed into sinapic acid [*Dixon et al., 2002*]. Last step is the formation of different mono esters, like 5-CQA, 5-FQA or 5-*p*CoQA: After coupling of *trans*-cinnamic acid to coenzyme A (CoA) by CoA lyase, followed the transfer to QA by hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase [*Farah et al., 2006, Lepelley et al., 2007*]. The subsistence of the 3- and 4-isomers may be based on acyl migration [*Farah&Donangelo, 2006*].

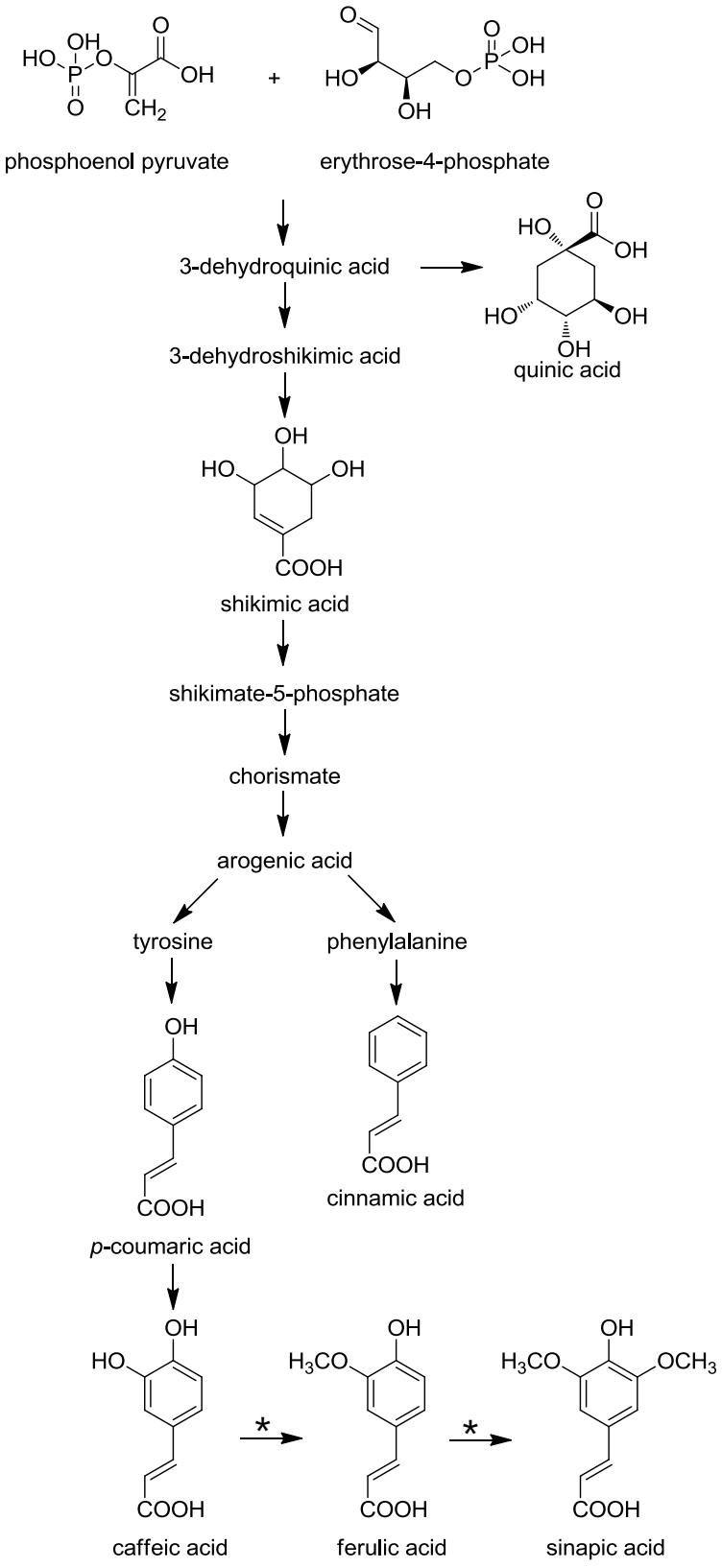


Figure 3 Biosynthesis of CGA in plants via shikimat and phenylpropanoid pathway (*via catechol-*O*-methyl transferase (COMT)) [modified after [*Farah&Donangelo, 2006*].

2.1.2 Occurrence and intake

In comestible goods, hydroxycinnamic acids are present in higher amounts in a large number of fruits and vegetables. Fruits like apples, grapes and blueberries, vegetables like lettuce, spinach and broccoli and cereal brans are rich in hydroxycinnamic acids (see Table 1) [*Clifford, 1999, Phenolexplorer*].

Table 1 Exemplary amounts of phenolic acids and derivatives in selected foodstuffs; FW: fresh weight. DW: dried weight.

Comestible goods	amount	references
apple	3 – 57 mg/100 g FW	*Dragovic-Uzelac et al., 2005, Mattila&Kumpulainen, 2002, Möller&Herrmann, 1983, van der Sluis et al., 2001, van der Sluis et al., 2002, Vrhovsek et al., 2004*
aronia berry	103 mg/100 g FW	*Mattila et al., 2006*
artichoke heads	202 mg/100 g FW	Romani *et al., 2006*
peanuts	106 mg/100 g FW	*Mattila&Hellstrom, 2007*
bilberry	3 – 85 mg/100 g FW	*Ehala et al., 2005, Mattila et al., 2006*
Arabica coffee beverage	89 mg/100 mL	*Moreira et al., 2005*
Robusta coffee beverage	205 mg/100 mL	*Moreira et al., 2005*
Instant coffee Nestlé Green Blend®	10.26 g/100 g DW	*Witt, 2015*
potato peel	32-636 mg/100 g FW	*Weidel, 2014*
potato flesh	7-306 mg/100 g FW	*Weidel, 2014*
white wine	2 – 10 mg/100 ml	*Frankel et al., 1995, Minussi et al., 2003, Rodríguez-Delgado et al., 2001, Teissedre&Landrault, 2000, Tinttunen&Lehtonen, 2001*
salad (lollo rosso)	52 mg/100 g FW	*Mattila&Hellstrom, 2007*

Comestible goods	amount	references
common wheat, refined flour	0 – 10 mg/100 g FW	*Adom et al., 2005, Selinheimo et al., 2006*
hard wheat, refined flour	14 mg/100 g FW	*Lempereur et al., 1997*
spearmint, dried	613 – 1491 mg/100 g FW	*Kivilompolo&Hyötyläinen, 2007, Wang et al., 2004*

Coffee and potatoes represent the major sources of phenolic acids. The available epidemiological data allows no exact calculation of the daily intake for CGA, but it is well known that coffee and coffee products play an important role due to the large CGA content in coffee. According to the different human diets, the daily CQA intake ranges between 100 mg and up to 1 g [*Clifford&Knight, 2004*].

Radtke and coworkers calculated a mean intake of phenolic acids of 222 mg/d in a Bavarian human subgroup (63 woman and 56 men, age 19 – 49 years), though there was a large range from 9 – 989 mg/d [*Radtke et al., 1998*]. Likewise, they reported a higher intake of hydroxycinnamic acids (211 mg/d thereof 205 mg CA/d) compared to hydroxybenzoic acids (11 mg/d). Coffee consumption of woman exceeds that of men, so that the gender dimorphism for CA intake is a factor of 1.3 higher for woman. The intake of the other phenolic acids such as vanillic or sinapinic acid is higher for men than for woman. A reason could be the different diet of the two genders. Cereal bran, which is rich in FA, is oftentimes not considered for the overall intake. Therfore, it is possible that the amount of real intake is far higher [*Radtke et al., 1998*]. Further findings of a Finnish study reported in a daily intake of 641 ± 363 mg phenolic acids, and a total intake of 863 ± 415 mg/d as sum of all polyphenols. Here again, CA was the main phenolic acid with 417 ± 325 mg/d. In addition, FA from cereal bran was considered and calculated with 120 ± 60 mg/d, as the second most consumed hydroxycinnamic acid after CA [*Ovaskainen et al., 2008*].

Wide ranges of phenolic acid intake amounts results from different diets and diverse pattern in food. Cultivar, season, cultivation and ripeness of the fruits and vegetables have an influence on quantities. Secondly, the processing changed the distribution pattern and content of CGA, whereat raw products usually have higher contents [*Radtke et al., 1998*].

2.1.2.1 Coffee and the influence of roasting and brewing on chlorogenic acid contents

Coffee is a beverage prepared by brewing from the roasted and grinded seeds of the coffee plant. Also, the row or roasted seeds of several species of a small evergreen bush of the

family *Rubiacea* of the biological genus *Coffea*, are called coffee. Coffee is among the most widely consumed beverages in the world [*Belitz et al., 2009*]. In Germany, 162 L per capita were consumed in 2014 [*deutscher Kaffeverband*].
Coffee is native to Africa, more precisely the contemporary Ethiopia. By the 16th century, it had reached the rest of the Middle East, then Constantinople and Venice. By the middle of the 17th century, coffee was widely consumed across all over Europe. Today, averages of 140 million bags coffee containing 60 kg are collected per year [*deutscher Kaffeeverband*]. Almost the half of the worldwide produced coffee comes from Brazil and Vietnam.
Preferentially, the coffee plant grows in high tropical altitude as evergreen shrubs or small trees, which are 3 – 12 m tall when unpruned. Such, used for harvesting, are pruned to keep them 2 – 2.5 m height. At the beginning, jasmine-like fragrant flowers prosper. These flowers turn into the stone fruits, the so called coffee berries. The fruit of berry has a green outer skin, which with increasing maturation colored red-violet till deep red. The ripe coffee berries were hand-picking harvested one or two times a year. Different varieties of the 70 *Coffea* species exist. The two most commonly grown are the *Coffea arabica* with 75% of worldwide production, and the *Coffea canephora* (the so called Robusta) with 25% [*Belitz et al., 2009*].

Pre preparation of coffee beverage, the processing methods of coffee berries occur in one of two processes: dry/natural or wet. At the *dry process* the whole fruit is spread out on sun-drying terraces, whereat the flesh of the berries peels off and also the parchment and the silver skin. After that, dehulled and cleaned coffee beans are classified, packed and shipped away. The *wet process* is more sophisticated and used for premium-priced coffee beans. Freshly harvest beans are brought to a pulper, which detaches the skin and the pulp without damaging the seed. During this process, the silver skin is still preserved. Beans are carried into water stream fermentation tanks, the water is drained off and the beans are left to ferment. After collection, the beans are dried, processed by dehulling machines, packed and shipped [*Belitz et al., 2009*].
After arrival of dried, green coffee beans in the coffee roasting facility, a heat treated process called roasting is used, which forms the delightful aroma and taste. Roasting process consists of four major phases: drying, development, decomposition and full roasting. Finally, these phases result in denaturation of proteins, evaporation of water, changes in structure and color and an increase in volume of the beans. Heat is transferred either by ‘contact roasting’ or by ‘contact convection’. ‘Contact roasting’ uses direct contact of the beans with the walls of the roasting equipment. ‘Contact convection’ means, that hot air pass through the beans. The roasting process is characterized by degradation of existent and formation of new compounds. This varies greatly, depending on coffee variety and extent of roasting, and also on species of coffee beans. The extent of roasting depends on the wished roast profile

to achieve flavor characteristics. The roast temperature can vary between 196°C and 245°C [*Coffee Glossary*]. There is a shift of the amino acid composition caused by degradation processes via acidolysis, which results in a decrease of about 30% of solids. The fraction of carbohydrates, mainly polysaccharides, fragmented into soluble compounds, however the lipid fraction survives the roasting process with only minor changes. The content of components like chlorogenic acids (CGA) decreases by roasting, while melanoidins are formed via Maillard reaction. Different contents of coffee ingredients for non-roasted and roasted coffee of species *Coffea arabica* and *canephora* (Robusta) are shown in Table 2 [*Belitz et al., 2009*].

Table 2 Composition of green and roasted (medium degree of roasting) *Coffea arabica* and *canephora* (Robusta)[a,b] [*Belitz et al., 2009*].

components [values in % of solids]	**green coffee**		**roasted coffee**	
	Arabica	**Robusta**	**Arabica**	**Robusta**
carbohydrates	55 – 65.5	40 – 55.5	38.0	41.5
proteins	8.5 – 12.0		10.0	
chlorogenic acids[c]	6.7 – 9.2	7.1 – 12.1	2.6	3.1
lipids	16.5	10.0	17.0	11.0
trigonelline	0.6 – 1.2		0.1 – 0.7	
caffeine	0.8 – 1.4	1.7 – 4.0	1.3	2.4
minerals	3.0 – 5.4		4.6	
melanoidins			23.0	
aroma substances			0.1	

[a] Values in % of solids.

[b] Water content of raw coffee: 7 – 13%

[c] Main components: 5-*O*-caffeoylquinic acid (chlorogenic acid: Arabica 3.0 – 5.6%; Robusta 4.4 – 6.6%).

Influence of roasting and brewing on chlorogenic acid contents

Coffee is considered as major source for CGA in the human diet. Green coffee contains the highest amounts (see Table 2) and is jointly responsible for bitterness, acidness and adstringency. The contents range from about 0.8 to 11.9% of dry mass [*Campa et al., 2005, Farah&Donangelo, 2006*]. Approximately 80% of this CGA is represented by caffeoylquinic acids (CQA). From the three available CQA-isomers, 3-*O*-caffeoylquinic acid (3-CQA), 4-*O*-caffeoylquinic acid (4-CQA) and 5-*O*-caffeoylquinic acid (5-CQA), 5-CQA is the major isomer. The three di-caffeoylquinic acids (diCQA) isomers are the next most-abundant CGA with up to 15%. With up to 6.2% represented the feruloylquinic acid (FQA) isomers a minor group [*Farah et al., 2005*]. During processing, the CGA composition changes, which take part in the generation of color, flavor and aroma [*Montavon et al., 2003, Trugo&Macrae, 1984*]. Total CGA contents in commercial roasted coffee ranged from about 0.5 to 7%, according to processing, roasting degree, blend and analytical conditions. The content of quinides (caffeoylquinic acid lactones) ranges from about 0.4 to 0.5%. Every 1% loss of dry matter, leads to 8 to 10% loss of CGA [*Clifford, 1999, Clifford, 2000, Clifford et al., 2003, Farah&Donangelo, 2006*]. CGA are thermally instable, what effect a breakage of the carbon-carbon bonds and gives completely degraded phenolic derivatives. Table 3 presents the contents of CGA in *C. arabica Cv.* Bourbon (Brazil) in relation to the roasting time. As described above, 5-CQA was the major CGA in green coffee with a content of 3126.1 ± 29.7 mg/100 g. When coffee was roasted for 6 min, isomerization processes occurred, followed by a decrease of 5-CQA to 1995.8 ± 19.9 mg/100 g and an increase of 3-CQA and 4-CQA to 816.1 ± 13.1 mg 3-CQA/100 g (483.3 ± 6.1 mg 3-CQA/100 g in green coffee) and 999.6 ± 45.1 mg 4-CQA/100 g (543.5 ± 9.3 mg 4-CQA/100 g in green coffee), respectively. Though, 9 min roasting reduced all three isomers to 244.3 ± 17.9 mg 5-CQA/100 g, 155.8 ± 7.6 mg 4-CQA/100 g and 122.8 ± 3.0 mg 3-CQA/100 g. In this dark roasting process, CQA were hydrolyzed and transferred into low molecular weight compounds like for example caffeic acid (CA) [*Clifford, 2000, Farah et al., 2005, Trugo&Macrae, 1984*]. Similar results were found for the FQA isomers. 5-FQA was the major compound with a content of 210 ± 1.8 mg/100 g in green coffee, whereas 4-FQA was determined with a content of 40.1 ± 1.8 mg/100 g and 3-CQA of 28.3 ± 0.9 mg/100 g. After 6 min roasting, 5-FQA was decreased to 109.1 ± 8.2 mg/100 g and 4-FQA and 3-FQA were increased to 68.0 ± 3.8 mg/100 g and 47.8 ± 7.9 mg/100 g, respectively. Intensive roasting over 9 min revealed decreased values of 22.1 ± 0.8 mg 5-FQA/100 g, 19.9 ± 1.0 mg 4-FQA/100 g and 9.7 ± 0.2 mg 3-FQA/100 g. The three diCQA isomers were not isomerized, but only degraded from approxametely 250 mg/100 g in green coffee to less than 10 mg/100 g in dark roasted coffee. Additionally, the roasting process supports the

Table 3 Total CGA and CGL amounts for *C. arabica Cv.* Bourbon (Brazil) and coffee beans roasted at different conditions (green, light (6 min of roasting), dark (9 min of roasting); T = 230 °C); Results are shown as the means of roasting in duplicates and extractions in triplicates ± standard deviation, expressed in mg/100 g of coffee, dry weight, nd: not detected [*Farah et al., 2005*].

	green	light	dark
3-CQA	483.3 ± 6.1	816.1 ± 13.1	122.8 ± 3.0
4-CQA	543.5 ± 9.3	999.6 ± 45.1	155.8 ± 7.6
5-CQA	3126.1 ± 29.7	1995.8 ± 19.9	244.3 ± 17.9
3-FQA	28.3 ± 0.9	47.8 ± 7.9	9.7 ± 0.2
4-FQA	40.1 ± 1.8	68.0 ± 3.8	19.9 ± 1.0
5-FQA	210.7 ± 1.8	109.1 ± 8.2	22.1 ± 0.8
3,4-DiCQA	236.2 ± 4.0	119.7 ± 2.3	7.4 ± 1.2
3,5-DiCQA	254.9 ± 4.7	88.6 ± 3.8	5.1 ± 2.7
4,5-DiCQA	278.9 ± 12.5	127.8 ± 4.0	9.2 ± 1.8
3-CQA15L	nd	160.2 ± 2.3	95.7 ± 4.7
4-CQA15L	nd	92.1 ± 2.9	53.1 ± 3.5
3-FQA15L	nd	14.1 ± 1.2	21.2 ± 1.9
4-FQA15L	nd	7.0 ± 0.4	7.8 ± 0.3
3,4-DiCQA15L	nd	4.1 ± 0.4	1.0 ± 0.2
3-*p*-CoQA15L	nd	4.6 ± 0.4	4.4 ± 0.2
4-*p*-CoQA15L	nd	5.3 ± 0.2	4.0 ± 1.6
total	5202 ± 70.8	4658.9 ± 115.9	783.5 ± 48.6

transformation of the CGA, diCQA and CoGA into quinides due to dehydration and formation of an intramolecular bond [*Clifford, 2000, Farah et al., 2005, Trugo&Macrae, 1984*]. Converted CGA from the green coffee beans formed the corresponding lactones such as 3-*O*-caffeoyl-1,5-qunide (3-CQA15L, 160.2 ± 2.3 mg/100 g in light roasted coffee) and 4-*O*-caffeoyl-1,5-qunide (4-CQA15L, 92.1 ± 2.9 mg/100 g in light roasted coffee), whereat the major compound was 3-CQA15L. Both isomers are shown in Figure 4 Formation of 3- and 4-*O*-caffeoylquinide (3-CQA15L and 4-CQA15L) by thermal rearrangement of 4-CQA [after *Farah et al., 2005*].. Additionally, after roasting for 6 min 3-FQA15L (14.1 ± 1.2 mg/100 g), 4-FQA15L (7.0 ± 0.4 mg/100 g), 3,4-DiCQA15L (4.1 ± 0.4 mg/100 g), 3-*p*-CoQA15L

(4.6 ± 0.4 mg/100 g) and 4-*p*-CoQA15L (5.3 ± 0.2 mg/100 g) were determined, but also these compounds were degraded when coffee was roasted for 9 min [*Farah et al., 2005*].

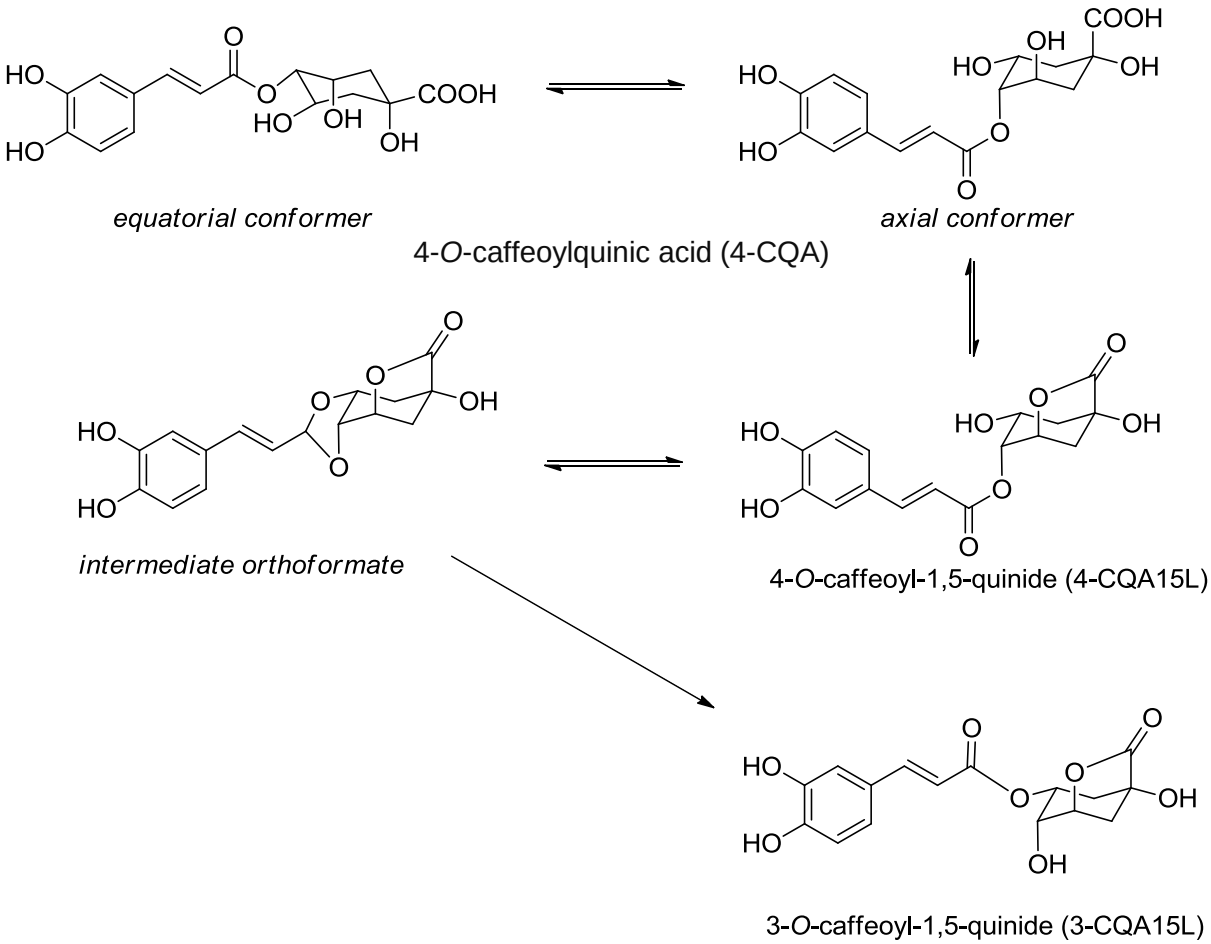


Figure 4 Formation of 3- and 4-*O*-caffeoylquinide (3-CQA15L and 4-CQA15L) by thermal rearrangement of 4-CQA [after *Farah et al., 2005*].

Table 4 presents more data of CGA contents in commercially more sold Ethiopian green coffee beans and coffee beans roasted at different conditions. The conditions are much stronger than in the previous experiment, but similar results were observed. 5-CQA is with a content of 5070 ± 161 mg/100 g the major CGA in green coffee. During the light roasting process (12 min, 230 °C) occurred isomerization brought an increase of 3-CQA and 4-CQA, in parallel to decrease of 5-CQA. Stronger roasting conditions caused a decrease of all three isomers up to less than 20 mg/100 g in the coffee beans. In contrast to the *C. arabica Cv.* Bourbon (Brazil), 4-FQA is the major compound with a content of 358 ± 8 mg/100 g in green coffee, whereas 5-FQA showed a content of 29 ± 0 mg/100 g. During the roasting process, 4-FQA was decreased and not any longer detectable under city (17 min, 250 °C) and French roasting (21 min, 250 °C) conditions. 5-FQA contents increased to 52 ± 2 mg/100 g under

light roasting conditions and decreased under stronger roasting conditions. All diCQA isomers were degraded during the roasting process and not any longer detectable under city and French roasting conditions. All in all, the total CGA amount was 6909 ± 236 mg/100 g in green coffee decreasing to 34 ± 3 mg/100 g in French roasted coffee [*Moon et al., 2009*].

Table 4 Total CGA amounts in commercial Ethiopian green coffee beans and coffee beans roasted at different conditions (green, light roasted (12 min, 230 °C), medium roasted (14 min, 240 °C), city roasted (17 min, 250 °C), French roasted (21 min, 250 °C)). Results are shown as the means in triplicates ± standard deviation, expressed in mg/100 g of coffee, dry matter basis; nd: not detected; * trace or not detected [*Moon et al., 2009*].

	green	light roast	medium roast	city roast	French roast
3-CQA	208 ± 5	545 ± 10	337 ± 7	38 ± 1	7 ± 1
5-CQA	5070 ± 161	1511 ± 35	927 ± 18	79 ± 1	16 ± 1
4-CQA	428 ± 13	756 ± 23	487 ± 10	59 ± 2	11 ± 1
4-FQA	358 ± 8	128 ± 3	89 ± 2	*	nd
5-FQA	29 ± 0	56 ± 2	42 ± 1	nd	nd
3,4-diCQA	101 ± 5	65 ± 1	29 ± 1	nd	nd
3,5-diCQA	575 ± 30	56 ± 0	21 ± 3	nd	nd
4,5-diCQA	133 ± 14	92 ± 1	40 ± 1	nd	nd
total	6909 ± 236	3209 ± 75	1972 ± 43	176 ± 4	34 ± 3

In Table 5 the total CGA amounts in brewed *Coffea arabica* and *canephora* (Robusta) beverages are shown. The total amount depends on the type of coffee and is 87.7 mg/100 mL for coffee Arabica and 204.6 mg/100 mL for coffee Robusta [*Moreira et al., 2005*]. Finally, it is of interest which amount of the respective CGA in powder is transferred in the brewed beverage, which is described to be more than 90% when a water volume/coffee powder ratio of >16 is used [*Lang et al., 2013b*].

Table 5 Total CGA amounts [mg/100 mL] for *Coffea arabica* and *canephora* (Robusta) beverages [*Moreira et al., 2005*]

	Arabica [mg/100 mL]	Robusta [mg/100 mL]
3-CQA	N/A	32.3
4-CQA	19.0	36.5
5-CQA	43.1	75.8
4-FQA	13.3	30.1
5-FQA	4.6	16.6
3,4-DiCQA	3.5	6.0
3,5-DiCQA	2.7	4.4
4,5-DiCQA	1.5	3.1
sum	87.7	204.6

Next to roasting, the beans undergo different important processing steps until the finished coffee beverage is available. The mechanical removement of the flesh from the cherry which yields the green coffee bean before roasting per se could have an influence. After roasting, the beans are either packed or grounded into a powder and the powder is used to brew the coffee beverage with hot water. At each of these processing steps it must be considered that the chemical content of the coffee beans change [*Matei et al., 2012*]. The influence of the roasting process of the chlorogenic acid content was well investigated in several studies. In contrast, not many data of the influence of brewing of coffee powder with boiling water on the CGA pattern are available. For instance, Matei and coworker reported from novel compounds to be formed from CGA during the brewing process. Hydroxylation of the chlorogenic acid cinnamoyl subsituentd by conjugation of water formed hydroxydihydrocaffeic acid derivatives in the first step. As second step acyl-migration products were detected, including different caffeoylquinic acid regioisomers, which formed finally *cis*-caffeoylquinic acids via *trans-cis* isomerization. The quantity of these novel compounds in the brewed coffee beverage is not determined yet [*Matei et al., 2012*].

Additionally, a number of people are consuming instant coffee or also be denoted as coffee extract. Coffee extract is a solid product offered as powder, grain or flocculation, which contains as minimum 950 g dry matter of coffee extract per kilogram. In modern processes, the roasted and grounded beans are used in 8 – 12 percolates, whereat the coffee grounds are separated and beverage is used for the final product. Separation techniques extract the

flavor compounds via an active cooling step. These flavor compounds are readded to the final concentrated extract, which is obtained in several extraction and flash evaporation steps. The final processing step is spray drying or freeze drying, what agglomerate the coffee extract, which can be packed and shipped [*Römpp, 1996*].

Analysis of CGA contents in commercially available instant coffees revealed large variations. The investigations included 13 samples from the UK and nine samples from Brazil. For the instant coffee samples form the UK, the content was between 3.6 and 10.7% of dry mass, whereat the samples from Brazil showed values between 0.6 and 5.9% of dry mass. Differences in CGA levels were explained by the use of different roasting degrees and different blends. The low content or absence of lactones and diCQAs were explained by a hydrolysis or loss during processing [*Trugo et al., 1984, Nogeuira et al., 2003, Bennat et al., 1994*]. Based on the beneficial biological effects of various coffee ingredients, such as CGA (see Chapter 2.1.5 Physiological effects of hydroxycinnamic acids and their derivatives), the manufacturers are anxious to optimize the roasting process in order to protect physiological active substances like CGA. For this reason, some manufacturers add green beans to the roasted coffee to increas the content of CGA. It is important to consider that the preservation of the typical taste and aroma of coffee plays an important role for the consumer expectations. An example for such an optimized processed coffee is Nescafé Green Blend® – a typical tasting and desirable smelling instant coffee with an elevated amount of CGA.

2.1.3 Absorption, metabolism and bioavailability of hydroxycinnamic acids and their derivatives

An accurate appraisement of the different existent data of the biological effects of hydroxycinnamic acids and their derivatives is not possible as long as it is not completely clarified which influence potential differences in dosing, interactions with the food matrix, and differences in bioavailability may have [*Bohn, 2014*]. “Bioavailability can be defined as the fraction of a nutrition or non-nutrition that is available for the human body for physiological functions and/or storage.” [*Bohn, 2014, p. 6*] For hydroxycinnamic acids and their derivatives this process includes the following digestive steps: For the first step, the release of hydroxycinnamic acids and their derivatives from the food matrix is of interest. The second step investigates the changes in hydroxycinnamic acids and their derivatives during gastric and small-intestinal digestion. The third step includes the cellular uptake of aglycons and some conjugated hydroxycinnamic acids by enterocytes. The fourth step is the microbiological fermentation of non-absorbed hydroxycinnamic acids or their derivatives as

well as those re-excreted via bile or the pancreas to yield additional metabolites. Step five investigates the phase I and II enzyme modifications that occur upon absorption [*Bohn, 2014*]. The main reactions caused by bacteria and phase I (such as cytochrome P450 monooxygenase, alcohol dehydrogenase, aldehyde dehydrogenase, esterases, and others) and II enzymes (such as methyltransferase, sulfotransferase, UDP-glucuronosyltransferase, and others) are deglycosylation, dihydroxylation, demethylation, deconjugation [*Cermak et al., 2006*], epimerization, ring cleavage, hydrolysis, and chain-shortening reactions [*Ferruzzi, 2010*]. Step six includes the transport in the bloodstream and subsequent tissue redistribution. Finally in step seven, the excretion via the kidney or re-excretion into the gut via bile and pancreatic juices is of interest [*Bohn, 2014*].

In general, the percentage of absorbed polyphenols is usually low [*Konishi et al., 2005*]. Basically, the absorption may starts in the oral cavity, but due to the short interaction time between amylase and polyphenols, the impact of enzymatic digestion is assumed to be low [*Laurent et al., 2007*]. Hydroxycinnamic acids compared with their respective esters are basically different in their properties such as molecular size and hydrophilicity, consequently different absorption ways may be assumed. Free hydroxycinnamic acids could easily be absorbed in the small intestine as well as the colon. Esters mostly have to be hydrolyzed by esterases of the small intestine or of the colonic microbiota prior absorption [*Mateos et al., 2006*].

Absorption *in vitro*

In the late 20th and early 21st century, different researchers investigated the absorption of of CGA with the aid of *in vitro* studies with different cells systems. A simple model for the possible route for absorption of ingested 5-CQA and CA was published by Konishi and Kobayashi (see Figure 5). In Caco-2 cells, in the presence of a proton gradient (apical pH, 6.0; basolateral pH, 7.4), they observed a considerable amount of CA to be transported to the basolateral side, although >99% of apically loaded 5-CQA remained on the apical side. Potentially, the transit of CA occurred via paracellular diffusion, by a proton gradient or as substrate of a monocarboxylate transporter (MCT). The intestinal transport mechanism of CQA was nondistinctive [*Konishi&Kobayashi, 2004*]. As a substrate for MCT, a monoanionic carboxyl group and a nonpolar side chain are necessary structural elements [*Rahman et al., 1999*]. CQA seems theoretically to provide the structural criteria to be a MCT substrate, but the ester group may impede the interaction.

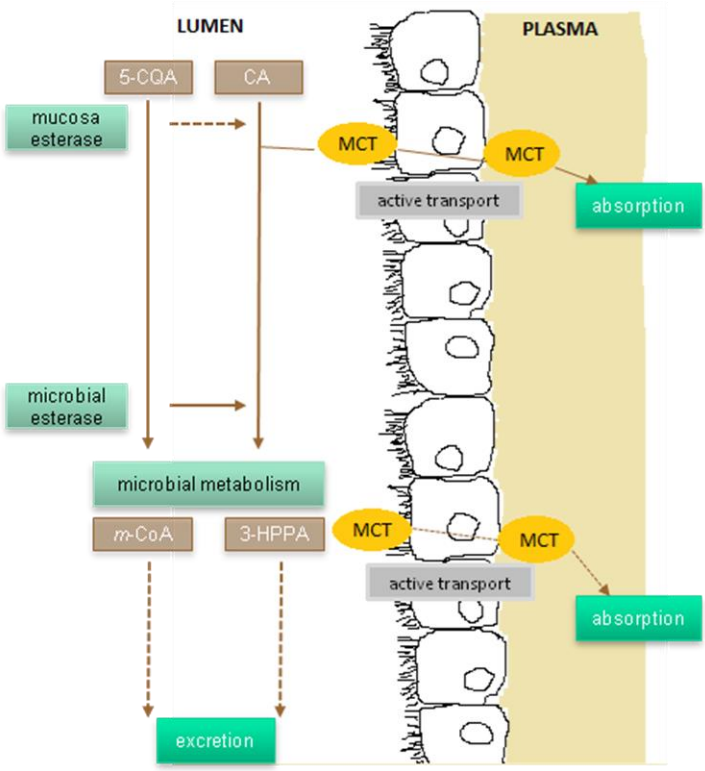


Figure 5 Possible routes (simplified pictured) for absorption in the small intestine of ingested 5-*O*-caffeoylquinic and caffeic acid; 5-CQA: 5-*O*-caffeoylquinic acid, CA: caffeic acid, MCT: monocarboxylate transporter, *m*-CoA: *m*-coumaric acid, 3-HPPA: 3-(*m*-hydroxyphenyl)propionic acid [modified after *Konishi&Kobayashi, 2004*]

Recent studies investigated the possible absorption routes with more modern experiemental setups. Human-differentiated Caco-2 cell monolayers were used to simulate the transformation processes of CGA after reaching the small intestine. The hydrolysis of caffeoylquinic and dimethoxycinnamoyl quinic acid, but not feruloylquinic acids, by enterocyte, mammalian esterases and pancreatic esterases is potentially a contributing mechanism to small intestinal absorption. The hydrolysis is relatively slow and not the entire amount of CGA is hydrolysed [*Encarnacao et al., 2015*]. In Caco-2 cells an absorption rate of 5-CQA of about 0.1% at physiological concentrations equivalent to gut lumen concentrations (0.1 ~ 1 mM) was found. In this experimental setup, the transepithelial transport was demonstrated to be bidirectional permeation with no transport into the basolateral side. As the permeation rate was concentration-dependent and not saturable, Liang and Kitts hypnotized a passive diffusion [*Liang and Kitts, 2015*]. In cultured gastric epithelial monolayers and the Ussing chamber model using ileal pig mucosa, different rates of absorption of several CGA have been reported [*Erk et al., 2013a*]. Hereby, pig mucosa was used as it provides strong similarities with the human gastrointestinal tract (GIT) and in combination with the Ussing chamber, it mimics the functional and morphological

organization of the intestinal epithelium. Across pig mucosa, CGA, metabolites and QA were found to be absorbed in the following order: diCQA (trace) < CQA < CA < FQA < QA. Erk and coworkers determined only low amounts of esters, especially of CQA, being absorbed. The percentage of initially applied CGA that was absorbed through the jejunal pig mucosa was approximately 2% for FQA and approximately 1% for CQA depending on the pH. The crucial factors for absorption have been suggested to be the molecular weight, hydrophilicity and physicochemical properties of the subgroups, as well as the presence of stretchable and elastic bonds, such as in QA [*Erk et al., 2013a*]. All these structure and dose dependency were confirmed in Ussing chamber experiments with the human colon carcinoma cell line Caco-2 [*Müntnich, 2013*]. Non-saturable transport and linear dose-flux relationship were observed with pig mucosa as well as Caco-2 cells for different CGA at various concentrations [*Erk et al., 2013a, Scherbl et al, 2014*].

After absorption to the blood stream, the CGA and free acids arrive in the systemic circulation. For interactions, *in vitro* experiments investigated the binding ability e.g. to bovine serum albumin. The distribution of CA occurred coupled with bovine serum albumin by a binding ability between 61 to 95%. In contrast, the binding capacity of FA and *p*CoA to bovine serum albumin is proved to have a binding ability of less than 10% [*Adzet et al., 1988*]. The strength of binding affinity to human serum albumin follows the order CA >> CQA >>> QA [*Muralidhara&Prakash, 1995*].

Metabolism *in vitro*

For the investigation of compartments and mechanisms, which generate these metabolites, different experiments were performed. *In vitro* studies in Caco-2 cells provided first data of hydroxycinnamic acids and derivatives metabolism, respectively. Extra and intra cellular esterases hydrolyzed esters and in addition, phase I and II enzymes, such as UDP-glucuronosyl transferase (UDP-GT) and sulfotransferase (ST), could simulate parts of biotransformation *in vivo* [*Kern et al., 2005*]. Poquet and coworkers investigated the transport of FA across a cell monolayer of co-cultured Caco-2 and HT29-MTX cells *in vitro* and demonstrated the formation of several conjugates, such as ferulic acid sulfate (FAS), ferulic acid glucuronide (FAG), and dihydroxyferulic acid (DHFA) [*Poquet et al., 2008*].

Absorption, metabolism and bioavailability *in vivo*

Additionally to the various *in vitro* experiments, different *in vivo* studies investigated the amounts and mechanism of absorption as well as (systemic) availability of CGA. Unfortunately, the available data on absorption but especially on metabolism are not sufficient and further investigation of CGA metabolism is of importance. As nowadays known,

metabolism takes mainly place into the intestinal mucosa, the liver and the kidney. The typ of metabolites formed depends on the place of absorption and the kind of enzymes involved [*Stalmach et al., 2009*]. Furthermore, the microbiota, such like *Escherichia coli*, *Bifidobacterium lactis* and *Lactobacillus gasseri,* has an importance when talking about metabolism. This is discussed in detail in chapter *2.1.4 Influence of the human microbiota on the intestinal metabolism of hydroxycinnamic acids and* their derivatives.

Azuma and coworkers demonstrated that in rats only traces of 5-CQA were absorbed from the alimentary tract after orally administration of 700 µmol 5-CQA/kg bw. 5-CQA easily entered blood vessels after i. p. injection and was afterwards partly metabolized [*Azuma et al., 2000*]. A study with ileostomists demonstrated a comparatively low absorption of esterified compounds such as 5-CQA compared to free acids like CA [*Olthof et al., 2001*]. These findings could be approved by a study from Nardini and coworkers, in which ten healthy volunteers consumed a coffee beverage (95.8 ± 4.6 mg 5-CQA/200 ml, consumed within 10 min after brewing). In the collected plasma samples, a significant rise in free and bound plasma CA levels were measured, even if free CA was absent in coffee brews. It was argued that 5-CQA is the source of CA in plasma, but it has to be considered that they failed to detect 5-CQA in plasma samples. In light of this, Nardini and coworkers could not completely excluded that traces of 5-CQA were present in plasma samples below the limit of detection, but concluded that CA is bioavailable to humans, even if present in the diet in bound forms, such as 5-CQA [*Nardini et al., 2002*]. Olthof and coworkerss executed intervention studies with ileostomists to achieve more information on the absorption of CGA, but also CA. They worked with ileostomy subjects, as degradation by the colonic microbiota is almost excluded. Thus, a calculation of absorption can take place by substract the amount excreted in ileostomy effluent from the ingested amount. After consumption of about 1000 mg of total CGA from coffee beverage, 67% of the ingested 5-CQA was excreted into ileostomy effluents within 24 h. This means, a maximum of 33% of the ingested 5-CQA was absorbed in the small intestine of the ileostomists. In contrast, 95% of the ingested CA (500 mg) was absorbed [*Olthof et al., 2001*].

In further investigations, Rechner and coworkers observed glucuronide as well as sulfate conjugates of CA in plasma samples of five volunteers after coffee consumption (in sum 449.1 mg, quantified relative to 5-CQA). In addition, free acids as well as glucuronides and sulfates of ferulic acid (FA), iso-ferulic acid (iFA), methylferulic acid (MeFA) and hippuric acid were detected within 24 h in urinary samples of these five volunteers. All these free acids as well as conjugates were concluded as specific biomarkers for the bioavailability and metabolism of dietary caffeic acid esters [*Rechner et al., 2001a*]. Stalmach and members determined 71 ± 7% CGA and their metabolites in ileal excretions within 24 h after ingestion

of 385 µmol CGA. Thereof, 78% were lead compounds whereat 22% were metabolites preferrably free and sulfated CA and FA, what argued for a first metabolism step through the small intestine. Overall 21 metabolites were determined by Stalmach and coworkers whereof only 17 were detectable in urinary samples. The amounts determined in urinary samples corresponded with 8 ± 1% of the ingested CGA dose. The observed metabolites are summarized and pictured in Figure 6. The main compounds found in urine were FA4S, CA3S, iFA3G and DHCA3S. In contrast to the results achieved in ileostomist (8%), urinary excretion by humans with an intact colon corresponded to 29 ± 4% of CGA intake. This higher amount can be linked by higher levels of DHFA, FAG, DHCAG, DHCAS, DHFAG and DHFAS. These findings highlight the high contribution of colonic metabolism to the overall pattern of metabolites in healthy people. Additionally, in this study, metabolites in plasma were investigated. The major compounds in plasma after 5 h were DHCA, DHCA3S, DHFA and DHFA4S. A secondary compound in plasma was the sulfate of CQA15L [*Stalmach et al., 2009, Stalmach et al., 2010*]. Not sulfated CQA15L was a substrate for intestinal esterases and could be hydrolyzed into CA [*Crozier et al., 2012*]. The transformation into CA3S occured by sulfuryl-*O*-transferase (ST) after hydrolysis of CGA into CA [*Stalmach et al., 2009*]. Additionally, a tissular methylation by COMT to FA and iFA was emphasized [*Gonthier et al., 2003*]. The sulfation of DHCA and DHFA by ST occurred in the liver. In contrast to experiments using the human colon carcinoma cell line Caco-2, *in vivo* the colon mucosa was not able to sulfate phenolic acids [*Poquet et al., 2008*]. The major metabolite of QA is hippuric acid formed by aromatization into benzoic acid and subsequent conjugation with glycine, whereat the stepwise metabolization of CA could also result in the formation of hippuric acid (see Figure 7) [*Gonthier et al., 2003*]. Microbial reduction followed by sulfation in the liver, forms glucuronidation products of primary metabolites by UDP-glucuronyltransferase (UDP-GT) as well as conjugation of FA with glycine [*Gonthier et al., 2003*]. The sulfate/glucuronide-ratio is 5.6 to 1 based on a higher affinity of phenolic acids to ST as to UGT [*Kern et al., 2003, Stalmach et al., 2010*]. Erk and coworkers confirmed these results in ileal effluent, but reported additionally that the ratio depends on the ingested amount of CGAs. They reported a sulfation/glucuronidation-ratio of 8.2:1 for an ingestion of 4525 ± 155 µmol total chlorogenic acids, 10.4:1 for an ingestion of 2219 ± 113 µmol total chlorogenic acids and 13.4:1 for an ingestion of 1053 ± 40 µmol total chlorogenic acids [*Erk et al., 2012*]. Lang and coworkers performed and human intervention study with 13 healthy volunteers who consumed a single cup of coffee beverage (350 mL; 526 mg CGA absolut) after a 10-day washout period.

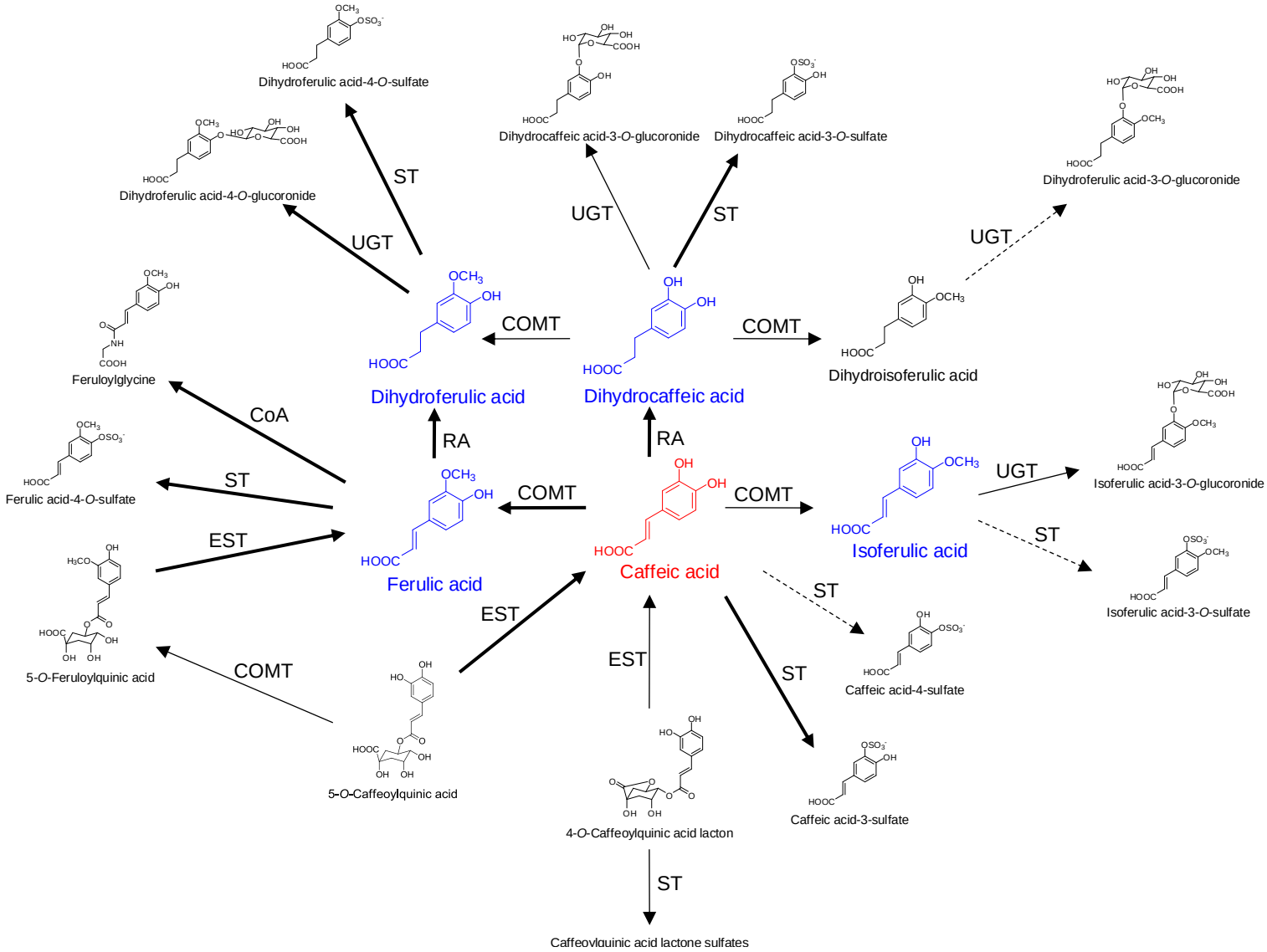


Figure 6 Metabolism of CGA after ingestion via coffee by human volunteers. 5-CQA and 5-FQA are the illustrated structures in this figure but their respective 3- and 4-isomers as well as 4- and 3-CQA15L would be metabolized in a similar manner; COMT: catechol-*O*-methyltransferase, EST: esterase, RA: reductase, UDP-GT: UDP-glucuronyltransferase, ST: sulfuryl-*O*-transferase; CoA: coenzyme A; bold arrows indicate major routes [modified after [*Stalmach et al., 2010*].

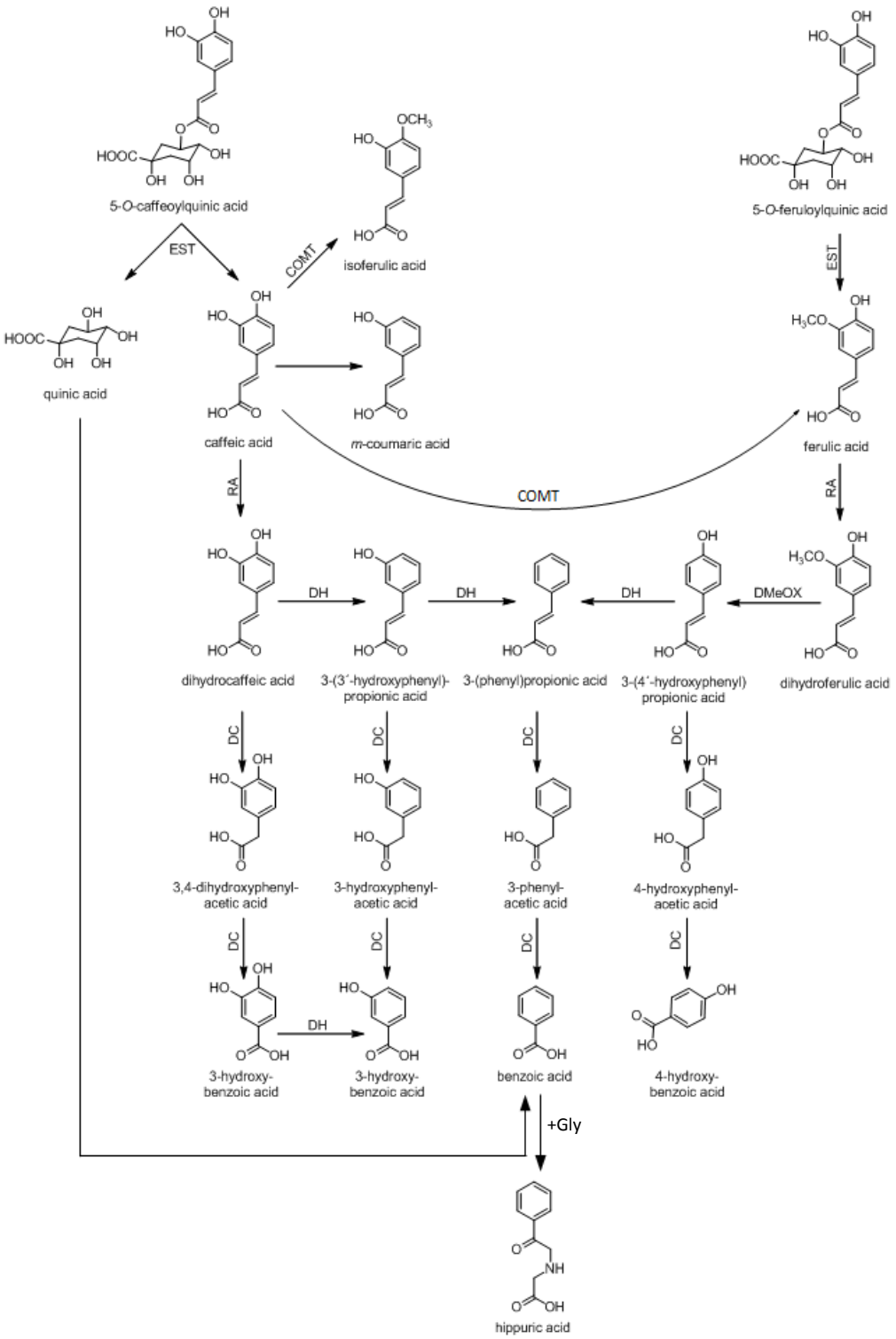


Figure 7 Proposed catabolic pathway of CGA and its microbial degradation in the colon. EST: esterase, RA: reductase, DH: dehydrogenase, DMeOX: demethoxyesterase, DC: decarboxylation, COMT: catechol-*O*-methyltransferase; modified according to [*Gonthier et al., 2003, Ludwig et al., 2013, Stalmach et al., 2009*]

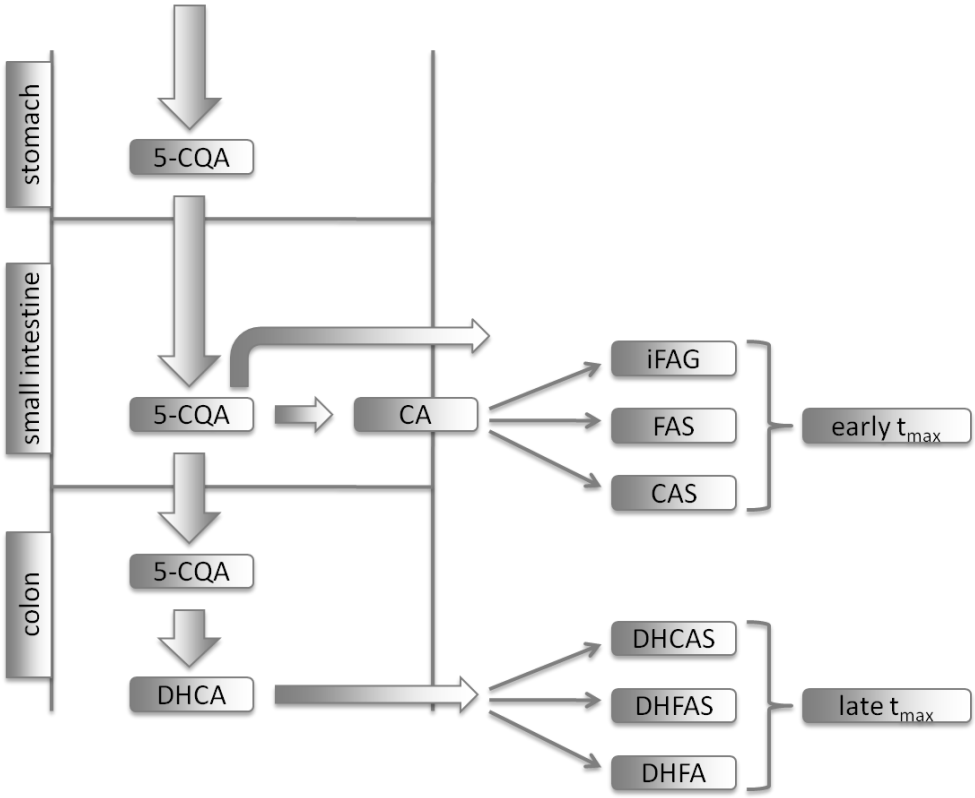


Figure 8 Pathway of absorption and metabolism of CGA using the example of 5-CQA. Note: Some isomerization can occur between different isomers of CQA and of FQA under physiological conditions [*Renouf et al., 2010b, Williamson et al., 2011*].

They observed different pharmacokinetic properties of metabolites that allow a separation of metabolites into two groups. With an early appearance in plasma after approximately 1 h, FA and FAS were detected. Secondly, particularly CGA metabolites formed by the intestinal microbiota, such as DHFA, FAG and DHFAS were determined late and persisted in the plasma for 6 h and longer [*Lang et al., 2013a*]. This is in line with data published by Renouf and coworkers (see Figure 8) reporting CA, FA and iFA equivalents appearing early within the first hour after ingestion and DHCA and DHFA showing the C_{max} 8 to 12 h after ingestion of 900 µmol total CGAs [*Renouf et al., 2010b, Renouf et al., 2010c*]. Additionally, an accumulation was observed by Lang and coworkers due to their extended half-life times during habitual consumption of several cups of coffee per day [*Lang et al., 2013a*]. The possibility, that absorbed and metabolized compounds could be reabsorbed in the colon (enterohepatic circulation), where glucuronides are cleaved by *β*-glucuronidase activity [*Williamson et al., 2000*], is for example in line with data determined for FA which is reabsorbed in the level of the colon what resulted in a second peak of apperance in plasma [*Renouf et al., 2010b*].

As already mentioned before, Erk and coworkers performed a crossover study with five ileostimists which consumed a coffee beverage with three different CGA contents to determine the absorption of CGAs (CQAs and CQLs and their corresponding metabolites quantified as 5-CQA equivalents, FQAs and metabolites quantified as 5-FQA equivalents, FA and feruloylglycine quantified as FA equivalents, FA-glucuronide derivatives quantified as iFA-glucuronide equivalents, sulfated derivatives quantified as FA-sulfate equivalents and CA and DHCA metabolites quantified as 3' conjugated meatbolites) in the small intestine and to investigate additionally the impact of the ingested dose. Plasma samples were collected for 0 – 8 h, urine smples were collected for -24 – 48 h and ileostomy effluents were collected for 8 – 12 h. As already mentioned, in ileal effluent and urinary samples CA and derivatives as well as FA and derivatives were in this study predominantly sulfated. In contrast, iFA determined in the urinary samples were to a greater extent glucuronidated than sulfated. With increasing doses, CGA were preferred conjugated to glucuronic acid. Total ileal excreted amounts were determined between 69 ± 9% for the high dose and 77 ± 4% for the low dose of the CGA metabolites, which is in line with the findings of Stalmach and coworkers [*Erk et al., 2012*]. Inferential, 25 to 30% of the ingested CGA were absorbed in the small intestine comparable to other single-dose studies with ileostomists [*Olthof et al., 2001, Stalmach et al., 2010*]. In general, systemic available metabolites were predominantly renally excreted. In the urine samples, Erk and coworkers determined CGA to 8.0 ± 4.6% for the high dose and 14.6 ± 6.8% for the low dose coffee, with a diversified spectrum of metabolites and a faster transit time for ileostomists after ingestion of the higher coffee dose [*Erk et al., 2012*]. Taken together, in the frame of this dose response study, the consumption of high CGA amounts influenced the gastrointestinal transit time and affected CGA absorption and metabolism, even if Erk and coworkers cannot explain the controversial bioavailability shown [*Erk et al., 2012*].

In contrast to previous studies, a major part of population is consuming coffee or its products every day in combination with food. In light of this, the influence of breakfast consumption on the bioavailability of CGA from coffee should also be of scientific importance. Up to date, only one study focused on the consumption of coffee beverage (332 mg CGA absolute) pure, with 10% whole milk or with sugar and nondairy creamer (as a premixture). 12 h after coffee consumption, blood samples were taken and phenolic acid equivalents were determined. The comparison of the results after pure black coffee consumption with them after the additional consumption of milk did not significantly change the areas under the curve (AUC), maximum plasma concentrations (c_{max}), or the time needed to reach c_{max} (t_{max}). A significant decrease in c_{max} of CA and iFA was observed as well as a significant longer t_{max} of FA and iFA for the treatment with sugar and nondairy creamer [*Renouf et al., 2010c*]. However, the

AUC did not significantly differ. During alnalysis a full enzymatic cleavage of all samples to hydrolyze all CGA and conjugates was performed. So only the overall pattern of plasma appearance was observed. Due to this fact, a change in the distribution pattern of single plasma metabolites cannot be exclude and must be investigated seperately [*Renouf et al., 2013*].

The studies from Erk and coworkers with ileostomists showed possible effects of food matrices on CGA bioavailability. In these studies different sources of CGA (cloudy apple juice, apple smoothie and coffee) were used. The variation in food matrices and phenolic composition had a major influence on intestinal availability and also of interesterification of the investigated subclass of CGA [*Erk et al., 2013b*]. Nevertheless, it is of scientific interest to investigate in more detail the influence of a food matrix on the bioavailability and metabolism of CGA in humans.

2.1.4 Influence of the human microbiota on the intestinal metabolism of hydroxycinnamic acids and their derivatives

In the centuries BC, the origins of anaerobic microbiology emerged, when Hippocrates described the clinical symptoms and high mortality rate associated with what we now known as tetanus [*William, 2012*]. In 1680, Van Leeuwenhoek discovered the anaerobes, as he observed the presence of ‚animalcules' which could exist in the absence of air [*Gest, 2004*].

Nowadays, a definition of ‚anaerobic' in the respective case is necessary. Numerous definitions have been offered for anaerobes or sometimes also obligate anaerobes, such as they create energy and metabolize its substrate without using oxygen and they demonstrates extreme oxygen-sensitivity which means they cannot grow in an aerobic atmosphere. Finally, the best definition of an obligate anaerobe is an organism for which anaerobic cultivation methods give optimum growth and for which oxygen is inhibitory [*Brazier, 1982, Phillips&Willis, 1981, Sisson et al., 1987, Wren, 1977, McKinney, 2004*].

Irrespective of methodology, the state of microbiota depends on the quality of the collected samples. It is essential that samples, in which the anaerobic microbiology will be investigated, have to be protected from oxygen toxicity during the period from collection of species to handling in the laboratory [*Brazier, 1982, Phillips&Willis, 1981, Sisson et al., 1987, Wren, 1977, McKinney, 2004*].

For the handling of respective samples in the laboratory, numerous types of established anaerobic chambers are commercially available. Typically, they are based on the introduction or generation of a gas mixture containing hydrogen into a jar, followed by catalytic conversion of the residual oxygen with hydrogen in the presence of a cold catalyst

to generate water, thus establishing an anaerobic environment [*Brazier, 1982, Phillips&Willis, 1981, Sisson et al., 1987, Wren, 1977*].

Within the human digestive system different microorganisms live in endosymbiotic relationships to fulfill their functions. E.g. during the gastrointestinal tract (GIT) passage bacteria have different tasks such as the fermentation of indigestible carbohydrates. It is well known, that also CGA are metabolized extensively to a wide range of low-molecular catabolites during the pass along the GIT. The dietary phenolic compounds are transformed to a much smaller number of metabolites, but the formation pathways and kinetics are not clearly understood. To understand these mechanisms, the knowledge of the identity and type of responsible microorganisms is important, but not as easy to identify them. Investigations determined special esterases as enzymes for hydrolization of ester bonds of CGA. These esterases are not present in human tissues, but in colonic microbiota, some digestive fluids and also in smaller amounts in small intestine microbiota. In detail, Couteau and members identified *Escherichia coli*, *Bifidobacterium lactis* and *Lactobacillus gasseri* as esterase-producing bacteria with the ability to hydrolyze the ester bonds of CGA amongst others [*Couteau et al., 2001*]. First studies dealed with the identification and quantification of structural changes of phenolic compounds driven by the importance that beneficial biological effects and bioavailability may largely depend on their molecular structure [*Selma et al., 2009, Stalmach et al., 2010, Williamson&Clifford, 2010*]. Ludwig and coworkers investigated the identity and quantity of breakdown products generated during incubation of espresso coffee with human fecal samples. Up to 6 h incubation period, they detected 11 dominating catabolites including dihydrocaffeic acid, dihydroferulic acid, and 3-(3´-hydroxyphenyl)propionic acid as the major end products, comprising 25 – 75% of the total catabolites. Additionally, they observed a clear influence on the rate and extent of degradation in relation to the composition of the gut microbiota of individual volunteers [*Ludwig et al., 2013*].

The detailed proposed catabolic pathways (enzymes, microbiota, etc.) are shown in Figure 7, where CA is the direct product of CQA hydrolysis, whereat FA and IFA were tissulare formed by methylation of CA. The microbiota formed various metabolites of CA, such as *m*CoA and hydroxylated derivatives of phenylpropionic, benzoic and hippuric acids. *m*CoA was formed by dehydroxylation, 3,4-hydroxyphenylpropionic, and 3-hydroxyphenylpropionic acids by hydrogenation and dehydroxylation. Finally benzoic acid was formed by dehydroxylation and subsequent *β*-oxidation in tissue. A conjugation of benzoic acid with glycine led to the formation of hippuric acid [*Booth, 1963, Chesson et al., 1999, Griffiths, 1964, Gumbinger et al., 1993, Perez-Silva, 1966, Quick&Cooper, 1931, Scheline, 1968*]. It has to be kept in mind, that through enterohepatic circulation conjugated compounds are excreted by the liver as bile

constituents into the intestine, and the deconjugated compounds are again metabolized or ‚regenerated' by microbial enzymes before being reabsorbed into the circulating system.

2.1.5 Physiological effects of hydroxycinnamic acids and their derivatives

Polyphenol rich diets correlated with different health effects such as free-radical scavenging, metal chelation, modulation of enzymatic activity, alteration of signal transduction pathways and exhibit *in vitro* many biological activities such as an antimutagenic, anticarcinogenic, anti-inflammatory and antioxidant potential [*Budryn et al., 2014, Dziki et al., 2014, Liang and Kitts, 2015, Stocker, 1999*]. The importance of these compounds was also shown in epidemiological studies. A decrease of diseases such likes cancer, coronary heart diseases, osteoporosis and dementia were observed when these kind of compounds were ingested [*Sato et al., 2011, Steinmetz&Potter, 1996*]. Also antioxidant, antidiabetic and anticarcinogen effects are demonstrated as properties of CGA [*Karthikeyan et al., 2011, Natella et al., 2002, Thom, 2007b*].

Antioxidative effects

In the organism, as a toxic but natural byproduct of aerobic metabolism, reactive oxygen species (ROS) are formed [*Eisenbrand, 2005*]. Normal cells maintain their capability to neutralize reducing or oxidizing compounds by the storage of other reducing or oxidizing compounds. An imbalance of this pool of compounds, which help the cells with their repair and detoxification functions, can lead to a damage of cellular and extracellular macromolecules. An exceeding ROS level above the physiological normal level refers to oxidative stress [*Heber et al., 2006, Schmidt, 2007*]. Oxidative modifications at cell structures such like DNA, RNA, proteins and lipids by ROS are associated with the pathogeneses of chronicle disease and the natural aging process [*Balaban et al., 2005*]. In combination with many endogenous defense mechanisms such as enzymes (superoxiddismutase, katalase, glutathion peroxidase, etc.), also compounds with a low molecular size are able to inhibit the oxidation of for example DNA, and consequently DNA damages. These compounds are named antioxidants, such as vitamin C, A and E, and have the capability to intercept free radicals and minimize oxidative chain reactions, subsequently. The reason is an decrease in oxidative stress [*Eisenbrand, 2005*].

Already in the last century, the antioxidative potential of CGA was hypotized. Radical scavenging activity and thus the *in vitro* antioxidant effects of CGA are stronger than the known antioxidant impacts of ascorbic acid and α-tocopherole [*Ohnishi et al., 1994, Sawa et*

al., 1998, Vinson et al., 1995]. The –C=C-COOH group linked to the phenyl ring as in cinnamic acids ensures greater proton-donating ability and plays consequently a role in stabilizing the radical by resonance. FA is expected to be more effective than *p*CoA because the electron-donating methoxy group allows increased stabilization of the resulting aryloxyl radical trough electron delocalization after hydrogen donation by the hydroxyl group [*Rice-Evans, 1996*]. The radical may react with a second radical ($R^{\cdot}$), acquiring a stable quinone structure (see Figure 9) [*Pietta, 2000*].

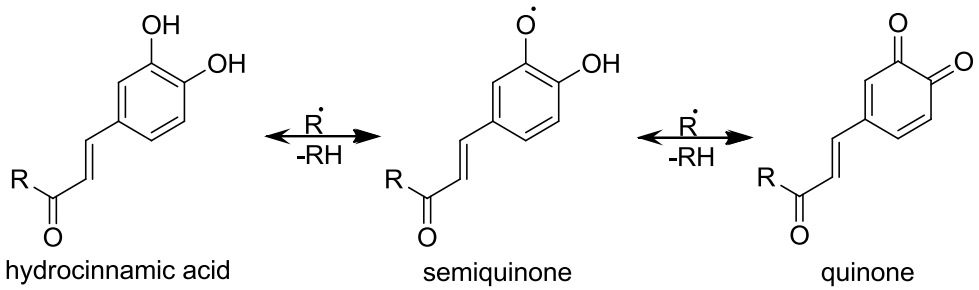


Figure 9 Scavenging of ROS ($R^{\cdot}$) by hydroycinnamic acid among building of quinone [modified according to [*Pietta, 2000*].

In vitro as well as *in vivo* studies proved this antioxidant potential of 5-CQA and CA. In an *in vitro* ischemia/reperfusion model, Sato and members showed the antioxidant potential of 5-CQA as well as CA. Via 2-methyl-6-p-methoxyphenylethynylimidazopyrazynone (CMPEC) assay, an assay for measuring superoxide anion-scavening activities of various compounds, an IC_{50} of 10.1 ± 9.3 µM for 5-CQA and of 41.0 ± 12.2 µM for CA was determined. This test showed a stronger antioxidative activity for CA than for 5-CQA. In addition, the total antioxidant performance (TAP) showed concentration dependence for both substances [*Sato et al., 2011*].

Sirota and coworker hypotized that CA might act as an electrophile in addition to its nucleophilic properties, and is capable of inducing the Nrf2/Keap1 (NF-E2 p45 subunit-related factor 2/Kelch Like ECH Associated Protein 1) pathway in cancer cells. Nrf2 is one example of genes involved in the cellular antioxidant defense. They finally found that CA induced Nrf2 translocation into the nucleus and consequently its transcription what is predominantely induced *via* the double bound in CA (Michael acceptor). The mechanism of induction of Nrf2 pathway and phase II enzymes by CA occurred on one hand by the electrophilic moiety in CA. It is essential for the oxidation of the Keap1 protein. The nucleophilic moiety (the catechol/quinone moiety) can provide scavenging ability, but it

cannot contribute directly to Nrf2 induction. In this process also generated hydrogen peroxide (produced by the catechol group) was involved or may also induce the process [*Sirota et al., 2015*].

Two similar *in* vivo studies were performed in which the volunteers consumed coffee rich in CGA. Both groups observed a decreas of total DNA strand breaks in lymphozytes [*Hoelzl et al., 2010*] and blood samples [*15*] as well as an increased expression of Nrf2.

Antiinflammatory effects

Inflammation is the activation of the immune system as protective response of body tissues to germs, toxicants, pathogens, damaged cells, or irritants. Substances or treatments with the property to reduce the inflammation are known to have an anti-inflammatory effect [*Ferrero-Miliani et al., 2007*].

In an animal model of chronic neuroinflammation performed by Wenk and coworkersr, a lipopolysaccharide was infused into the 4th ventricle of 3-month-old F-344 rats for 14 days. This treatment is known to reproduce a variety of behavioral, neurochemical and neuropathological changes associated with Alzheimer's disease. Knowing the potential beneficial effect of ferulic acid (FA), 3 or 30 mg FA/kg/day were administered subcutaneously. A dose-dependent reduction in microglia activation within the temporal lobe was observed, when rats were treated with FA. In addition, they suggested a relation of the antioxidant properties of FA and a significant attenuation of the pathological process within the brain associated with Alzheimer's diseas. However, this age-associated process can only be decreased, if the treatment takes place in the early stage [*Wenk et al., 2004*]. Another study evaluated the anti-inflammatory, antinociceptive and antipyretic activities of 5-CQA in male Wistar rats. 5-CQA doses of 10, 50 and 100 mg/kg administered orally inhibited carrageenin-induced paw edema compared to the control. An additional inhibition of the numbers of flinches in the late phase of formalin-induced pain test was observed. Dos Santos and coworkers suggested an inhibitory action of 5-CQA in the peripheral synthesis / release of inflammatory mediators involved in these responses [*dos Santos et al., 2006*].

Antidiabetic effects

Diabetes mellitus is a group of metabolic diseases caused by the lack of insulin or of insulin resistance of cells. There are different types of diabetes mellitus but the two main types are type I and type II. Referring to the classification after ICD-10-WHO, type I diabetes is a primary insulin-dependent diabetes mellitus, whereat type II diabetes is a primary non-insulin-dependent diabetes mellitus and the most commonly with 90 to 95% of all diabetes diseases [*ICD*-10-WHO Version 2016, *Mutschler, 1975*]. Supernutrition, overweight (obesity)

and somatic anergia play an important role in the occurrence of diabetes mellitus typ II [*Mutschler, 1975*].

Founded by a great interest in diabetes mellitus, already 30 years ago different groups investigated the antidiabetic effects of CGA. It was shown that CA and CQA had an inhibition effect on the α-amylase activity. Additionally, investigations had shown an inhibition effect of glucose absorption from intestine as well as an inhibition effect on the α-glucosidase activity by CA, FA and CQA [*Adisakwattana et al., 2009, Funk et al, 2005, Ishikawa et al., 2007, Iwai et al., 2006, Narita et al., 2009, Welsch et al., 1989*]. Welsch and coworkers investigated in an *in vitro* experiment the interaction of 5-CGA, FA and CA, respectively and the sodium-dependent glucose transporter in brush border membrane vesicles isolated from rat small intestine. Welsch and coworkers reported a decrease of 80% of the Na^+-dependent glucose transport capacity via, when the vesicles were treated with 1 mM 5-CQA. The treatment with 1 mM FA and CA, respectively gave still a reduction of 35 – 40% [*Welsch et al., 1989*].

In an *in vivo* study with male albino Wistar rats, a 3.5 mg CA/kg bw solution was given to rats (gavage) and after 10 min a solution of glucose (200 mg/kg bw) in combination with CA (3.5 mg CA/kg bw) was fed. In a time period of 0 to 90 min, blood samples were collected and glucose was determined. In untreated rats as well as in treated rats, glycaemia started to rise 5 min after ingestion of glucose and peak values were reached at 10 to 15 min. Thereat, the glycemic peak after CA-treatment during the oral glucose tolerance test was significantly ($p < 0.05$) reduced by ~22% at 10 min and ~17% at 15 min [*Bassoli et al., 2008*]. In forty male Sprague-Dawley rats, a high dose of CGA (90 mg/kg) inhibited a high-fat diet-induced elevation in SGLT1 expression. High-dose CGA controlled the levels of blood glucose and insulin to maintain glucose homeostasis [*Peng et al., 2015*].

A similar result showed van Dijk and coworkers in a human oral glucose tolerance test (oGTT) where 15 overweight men consumed either 12 g decaffeinated coffee, 1 g 5-CQA or a placebo (1 g mannitol) in a randomized order. The glucose and insulin concentrations were determined. In blood samples, early glucose and insulin concentrations tended to be lower than those of the collective treated with the placebo during an oGTT [*van Dijk et al., 2009*].

Due to this, Thom and coworkers performed two human intervention studys in parallel. The first study should carry out the differences in blood glucose concentrations (glucose absorption) after ingestion of three different test beverages. Therefor, three human collectives volunteered: Group one ingested only a solution containing 25 g sucrose (control group), the second group consumed normal instant coffee plus 25 g sucrose and the third group were treated with a CQA-enriched coffee plus 25 g sucrose. After consumption of the CGA-enriched coffee, the AUC of blood glucose was significantly reduced as compared with

the control group, but not with the group which ingested normal instant coffee plus 25 g sucrose.

As a controlled body weight conduces as prevention for diabetes mellitus type II, in the second 12-weeks study, the body mass of 30 moderately overweight people was monitored, when 50% of the group consumed 5-CQA enriched instant coffee and the other half only the instant coffee. A weight reduction for CQA-enriched coffee drinkers of 5.4 ± 0.6 kg compared with 1.7 ± 0.9 kg for the instant coffee drinkers was observed. The difference in weight loss between the two groups was significant ($p < 0.05$). Thom and coworkers concluded that consumption of an with 5-CQA-enriched coffee may have an significant effect on the absorption and utilization of glucose from the diet [*Thom, 2007a*]. A similar effect was reported by Bakuradze and coworkers in a human intervention study comprising 33 healthy volunteers. A daily ingestion of 750 mL of freshly brewed coffee rich in green coffee constituents and roast products for four weeks showed a body weight ($p<0.01$) and body fat reduction ($p<0.05$) [*Bakuradze et al.*, 2011]. Bakuradze and coworkers performed a second similar human intervention study with 84 volunteers. Against former findings, no changes in body weight were reported [*Bakuradze et al., 2015*].

Anticarcinogenic effects

Phenolic acids have the capability to impede following mechanism: Inhibition of phase I enzymes, interactions with the activated carcinogen, interactions with the DNA, induction of phase II enzymes and antioxidative activity. Accordingly, phenolic acids may also inhibit both the initiation and promotion phase of the cancerogenesis [*Watzl, 2001*].

As one of these studies, Kampa and members performed an *in vitro* experiment using T47D human breast cancer cells. They investigated the antiproliferative action of six phenolic acids (CA, FA, syringic acid, sinapinic acid, protocatechuic acid and 3,4-dihydroxyphenylacetic acid). The tested compounds (10^{-7} M) showed an inhibitory antiproliferative effect on T47D cells, with the maximum effect on the 5th day of incubation. The most potent inhibitor of cell growth by 80% was CA, followed by FA, protocatechuic acid and 3,4-dihydroxyphenylacetic acid, which showed a similar inhibition on cell growth by 40%. For the six compounds under study, the IC_{50} values varying from the nanomolar to the picomolar range (CA: $2.2 \cdot 10^{-9}$ M, FA: $2.3 \cdot 10^{-9}$ M, syringic acid: $< 10^{-12}$ M, sinapinic acid: $7 \cdot 10^{-11}$ M, protocatechuic acid: $2 \cdot 10^{-11}$ M and 3,4-dihydroxyphenylacetic acid: $< 10^{-12}$ M) [*Kampa et al., 2004*]. As shown for quercetin and kaempferol before [*Ciolino et al., 1998, Ciolino et al., 1999*], also CA regulated CYP1A1 gene expression through binding to the aryl hydrocarbon receptor (AhR). This means that the activation of transcription factors could be blocked, if CA is binding to AhR instead of the possible formation of an AhR-TCDD (2,3,7,8-tetrachlorodibenzodioxin)

complex, which induces CYP1A1, resulting in an increase in DNA binding activity of NF-κB and apoliprotein 1 [*Kampa et al., 2004*].

In the human colon cancer cell line HT29 an inhibition (IC_{50} = 133 ± 18 µg/mL) of growth after treatment with a polyphenol-rich extract of an apple juice was shown *in vitro*. This concentration is clearly below the concentration of determined polyphenols in consumer-relevant apple juice (about 500 µg/mL). Testing of single compounds, CA gave the strongest growth inhibitory properties (IC_{50} = 132 ± 19 µM), whereas 5-CQA showed only marginal growth inhibition (IC_{50} = 205 ± 53 µM) [*Kern et al., 2005*]. In addition, Karthikeyan and coworkers investigated the potential of FA on radiosensitidation in two cervical cancer cell lines (HeLa and ME-180). In this *in vitro* experiment, the percentage of growth inhibition (MTT assay), the colony survival, the levels of lipid peroxidation (TBARS, CD and LHP), the antioxidant status (SOD, CAT, GPx and GSH), the oxidative DNA damage (% tail DNA, tail length, tail moment and Olive tail moment), the apoptotic morphological changes (AO/EtBr staining) and the intracellular ROS levels (DCFH-DA) were estimated. In both cell lines, FA (10 µg/mL) enhanced radiation effects by increasing lipid peroxidative markers as well as by significant enhancement of ROS levels. In contrast, FA treatment without radiation increased intracellular ROS levels what indicated the prooxidant nature. Also the observed enhanced oxidative DNA damage and apoptotic morphological changes when cervical cancer cells were treated with FA plus radiation, suggested the radiation sensitizing property of FA [*Karthikeyan et al., 2011*].

To examine whether CQA showed anticarcinogenic effects *in vivo*, the inhibition effect on 8-hydroxydeoxyguanosine formation in animal organs was investigated. Therefore an oxygen radical-forming carcinogen, 4-nitroquinoline-1-oxide (25 ppm) was administered in the drinking water to all rats. Additionally, a selected group of these rats were fed a diet containing 250 ppm CA. The 8-hydroxydeoxyguanosine level in the DNA of the rat tongue, the target tissue, was significantly reduced in the CA-treated group (250 ppm). This may suggest an indication for mechanisms of cancer chemoprevention by CQA [*Kasai et al., 2000*]. Also (spontaneous) DNA strand breaks play a role in carcinogenesis. Bakuradze and coworks showed in two different studies a significant decrease of spontaneous DNA strand breaks of the coffee-consuming volunteers, as compared to the control group (only water) [*Bakuradze et al., 2015, Bakuradze et al., 2016*].

Toxicological effects

The acute toxicity of CQA and CA was examined in a multitude of toxicological studies and is considered to be low [*Tice, 1998*].

Schafer and coworkers determined the acute oral toxicity and found LD_{50} values for CQA and CA in redwing blackbirds of more than 100 mg/kg bw (sex and strain not provided) [Schafer et al., 1983]. In mice, dosages in the range of 3.5 – 381 mg CQA/kg bw showed no adverse effects [*Hach&Heim, 1971*]. Intraperitoneal administration of 4000 mg CQA/kg bw induced death in 4 of 6 treated rats, while doses lower than 2497 mg/kg bw revealed no obvious toxicity [*Chaube&Swinyard, 1976*]. In contrast the LD_{50} for CA was in a range of 1500 mg/kg bw after i.p. administration (death of 5/8 rats). Dosages below 1250 mg/kg bw were nontoxic [*Chaube&Swinyard, 1976*]. However, the relevant exposure route for humans is the oral administration. Dosages between 50 – 500 mg CA/kg bw given to rats by gavage revealed no clinical symptoms of toxicity. The only substance related effect was reduced glutathione-S-transferase (GST) activity in the liver of these rats [*Ploemen et al., 1993*].

After administration of up to 0.2% CQA or CA respectively via the isocaloric and isonitrogenous diet of mice no clinical symptoms of toxicity were detectable. Substance related effects were limited to effects in modulating hepatic xenobiotic activating-detoxification enzymes [*Kitts&Wijewickreme, 1994*]. There are different short-term and sub-chronic studies in rats known, using a dose range of 10,000 – 20,000 ppm in the diet. Adverse effects observed caused by substance uptake were reduced kidney and adrenal weights as well as hyperplasia and increased cell proliferation of the forestomach [*Eklund, 1975, Hirose et al., 1987, Ito et al., 1993, Kagawa et al., 1993*]. Additionally, daily i.p. injections on Wistar rats for 8 days with dosages up to 500 mg CQA/kg bw and 187.5 mg CA/kg bw respectively were not lethal [*Chaube&Swinyard, 1976*].

There is one prenatal developmental toxicity study with CA in rats available, where i.p. administrations of 40 – 187.5 mg/kg bw day on days 5 through 12 of gestation induced rib defects in 7.4% of the 21-day-old fetuses [*Chaube&Swinyard, 1976*]. At no dosage, fetal central nervous system defects or maternal and fetal mortality were detectable.

Hirose and coworkers showed a carcinogenic potential in a long term study in rats (n = 20). For two years, rats were fed with CA-enriched feed (2%). Histological examination revealed that CA induced forestomach squamous cell carcinoma in 57% of the animals ($p < 0.001$) [Hirose, 1990]. An extrapolation for humans occurs in 140 g CA/d which is 140 fold higher than maximal daily intake of CGA. Additionally, carcinogenicity was determined in mice, where administrations of CQA given via the diet (CQA-enriched feed, 2%) over 96 weeks induced squamous-cell papillomas and carcinomas of forestomach, alveolar type II-cell tumors of the lung andrenal-cell adenomas [*Hagiwara et al., 1991, IARC, 1993*]. In rats, administrations of CA-enriched feed (2%) over 51 to 104 weeks showed a significantly increased in papillomas of the forestomach [*Hagiwara et al., 1991, IARC, 1993*] while shorter exposure periods with the same diet not resulted in such effects [*Hirose et al., 1992, Kagawa et al., 1993*]. The dosage of CA-enriched feed (2%) for two years induced carcinomas of the

forestomach, increased the incidence of renal adenomas [*Hagiwara et al., 1991, IARC, 1993*] and did produce significant hyperplasia [Hirose et al., 1993], whereat no hepatocellular adenomas or carcinomas, glandular stomach adenomas or carcinomas [Hagiwara et al., 1996], or kidney adenomas were observed [*Hirose et al., 1993*]. In contrast, the administration of 250 ppm (0.025% enriched feed) CQA respectively 10,000 ppm CA (1% enriched feed) over 24 respectively 20 weeks to hamsters did not induce any tumors.

Epidemiological data on the toxicological effects of pure CQA and CA are limited. Initially, Freedman and coworkers, observed an allergic response of industrial workers on green coffee [*Kaye&Freedman, 1961*]. Hence, studies were performed investigating the allergic response of CQA and CA. If CQA and CA were administered subcutaneously only CQA was able to show an allergic response. In the passive transfer method, CQA was analyzed against serum from an individual allergic to green coffee, where doses as low as 20 µM induced a positive reaction. However, based on different other studies, no reactions were observed so that CQA seems not to be one of the allergens responsible for atopic allergy to green coffee [*Karr et al., 1978, Layton et al., 1965a, Layton et al., 1968, Layton et al., 1965b*].
In human intervention studies, the plasma total homocysteine concentrations was increased after 5-CQA. For seven days, healthy volunteers ingested 2 g 5-CQA, subsequent plasma was collected 4 to 5 h and 20 h after intake and total homocysteine was determined. Compared to the placebo group, concentrations were 12 respectively 4% higher after 5-CQA intake. It is known that higher homocysteine concentrations in plasma come along with an increased incidence of cardiovascular diseases. Extrapolated, 2 g 5-CQA corresponds with 1.5 L of a strong coffee, which is above the mean intake of 2 – 3 cups of coffee (approximately 172 – 259 mg 5-CQA, Arabica coffee beverage) [*Moreira et al., 2005*]. Moridiani and members shown a formation of mono- and bi-glutathione conjugates when glutathione was present during the peroxidase/H_2O_2-catalyzed oxidation of CA, DHCA or 5-CQA. Finally, glutathione depletion is possible what caused an absence of defense mechanism and following susceptibility to oxidative stress [*Moridani et al., 2001*].

Finally, only topical CQA and CA treatment induced clinical symptoms of toxicity, tumors or death. In epidemiological studies in industry workers who were processing green coffee only allergic effects were detectable whereat CQA seems not to be one of the allergens responsible for atopic allergy to green coffee. Additionally higher homocysteine concentrations and glutathione conjugation were observed in humans. Nevertheless, the toxicological effects are considered to be low [*Tice, 1998*].

2.2 Methodologies

2.2.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) compares to the classical column chromatography with enhancements. The efficiency increased based on reduction of particle size of sorbents material as well as instrumentalization. The fain-grained sorbent material is capable of providing better separation, shorter analytical times, and a more sensitive limit of determination [*Kromidas, 2017*].

The schematic setup of an HPLC unit is shown in Figure 10. After degassing (2), pumps (3) move the mobile phases (1) to an injection valve (autosampler) (4). Following sample injection, the mobile phase moves through the guard (5) and analytical column (6). Afterwards, the sample components to be separate are determined by one of different possible detectors (7). All HPLC detectors are present to monitor physical parameters of the mobile phase and change the signal if a substance elutes from the column. A plot of the detector response as a function of time is called a chromatogram. The data are transmitted (8) and converted at the connected computer (9) [*Cammann, 2000*].

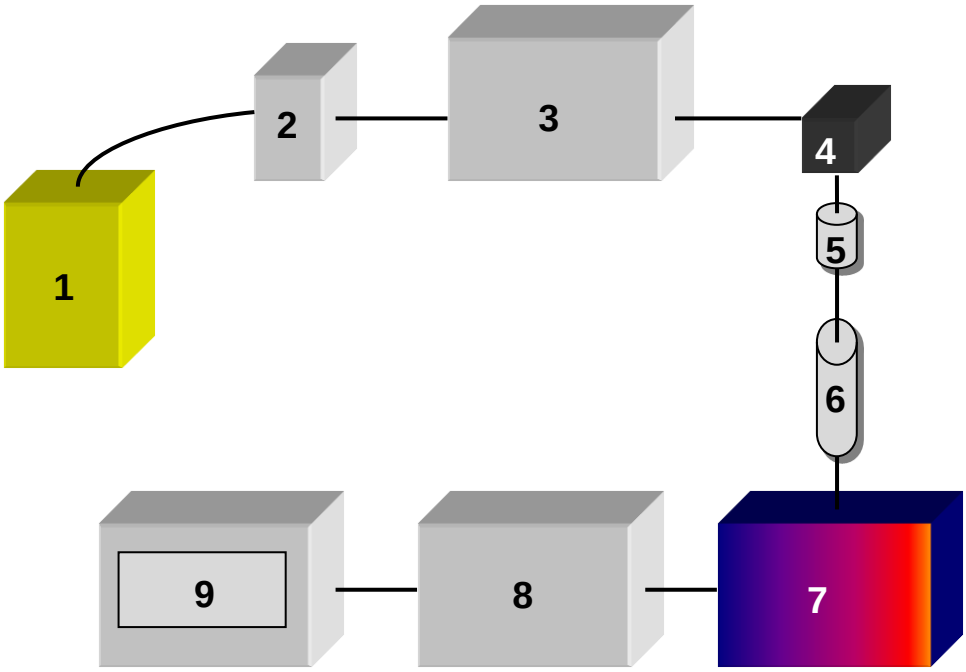


Figure 10 Schematic figure of a high performance liquid chromatography (HPLC) unit: (1) solvent reservoirs, (2) degasser, (3) high pressure pumps, (4) autosampler/sample injection loop, (5) guard column, (6) analytical column, (7) detector, (8) data acquisition, (9) computer [modified after Camman, 2006]

2.2.2 Detectors – CoulArray® detector coupled with high performance liquid chromatography (HPLC)

The CoulArray® detector is a detector coupled with high liquid chromatography (HPLC) system, which offers the ability to detect and quantitate trace levels of electroactive/redoxactive substances in complex matrices. The observed signal might be due to the compound of interest or due to other compounds in the sample, which have the same electroactivity / redoxactivity as the compound of interest. For this problem, the CoulArray® detector offers the possibility to use a multi-electrode detector system in which a series of electrochemical cells (up to 16) are set at different potentials (for each electrode independently controlled from -1000 mV to 2000 mV) to oxidize or reduce the substances that elute from the column. Due to that it is unlikely that two substances have a similar retention time as well as the same redoxactivity, the collection of a number of chromatograms allows the identification (and quantification) of the substance interested in [*RADANAL*].

2.2.3 Detectors – Mass spectrometry (MS) coupled with high performance liquid chromatography (HPLC)

Mass spectrometry is a physical analytical technique that determines the mass to charge ratio (*m/z*) of in vacuum stable ions. MS analysis are performed in different fields (physics, astrophysics, gas phase chemistry, organic chemistry, biology, medicine, etc.) and used to analyse pure samples as well as complex mictures. It is used for determining mass to charge ratios of particles, which could be precursor-ions or product-ions after fragmentation, but also for elucidating the chemical structure. Ionization depends on used method and is caused by to determine analytes. Organic compounds are quantified at concentrations of pico to 10 atto mol, generally. Mass spectrometry works by generation of positive or negative ions and radical ion, respectively, whereas the electrical high voltage allows acceleration of these produced ions. The reached velocity of ions is due to the kinetic energy and the acceleration in the electric field. The rate of ion yield is the determining factor of limit of detection for a mass spectrometer [*Cammann, 2000, Lottspeich, 2008*].

Please Note: Some technical terms referd to the mass spectrometry are provider-specific and based on the instruments and techniques offered by AB Sciex GmbH.

One special technique to ionize molecules is electrospray ionization (ESI). ESI is a viable, mild ionization method used as ion source to couple HPLC with MS. Precursors are not or marginal fragmented and desired precursor-ions or quasimolecular ions such as $[M+H]^+$ are

generated, generally. Under atmospheric pressure the molecules are desolvated, which means formed ions are transferred from solvent into gas phase. That process is classed in four steps: First, solvent containing the analytes is dispersed by electrospray into a fine aerosol and loaded droplets are formed. Extensive solvent evaporation result in decrease of the initial droplet size and in increase of density of charge on the surface. The droplet becomes unstable reaching its Rayleigh limit. At this point, the charge to surface ratio of the droplet increase, the droplets deforms and emits charged jets in a process known as Coulomb fission. After completed desolvation, precursor-ions formed at atmospheric pressure are accelerated first into the prevakuum area and afterwards into the high vacuum region of the mass spectrometer. Curtain gas, what is in general nitrogen, furthers desolvation and collides with neutral particles to prevent the entrance of contaminants through the interface plate. Also, arrangements of lenses and skimmer cones or additional quadrupols are used to desolvate and focus ions into the first quadrupole (Q1). Analysis occurs by a continuous flow, which is injected by a capillary tube, called probe. The probe with impressed high voltage serves as backing electrode to the interface plate, which implicate an electric field. Nebulizer gas around the probe enhances the formation of droplets [*Cammann, 2000, Lottspeich, 2008*]. The schematic representation of electrospray ionization is illustrated in Figure 11.

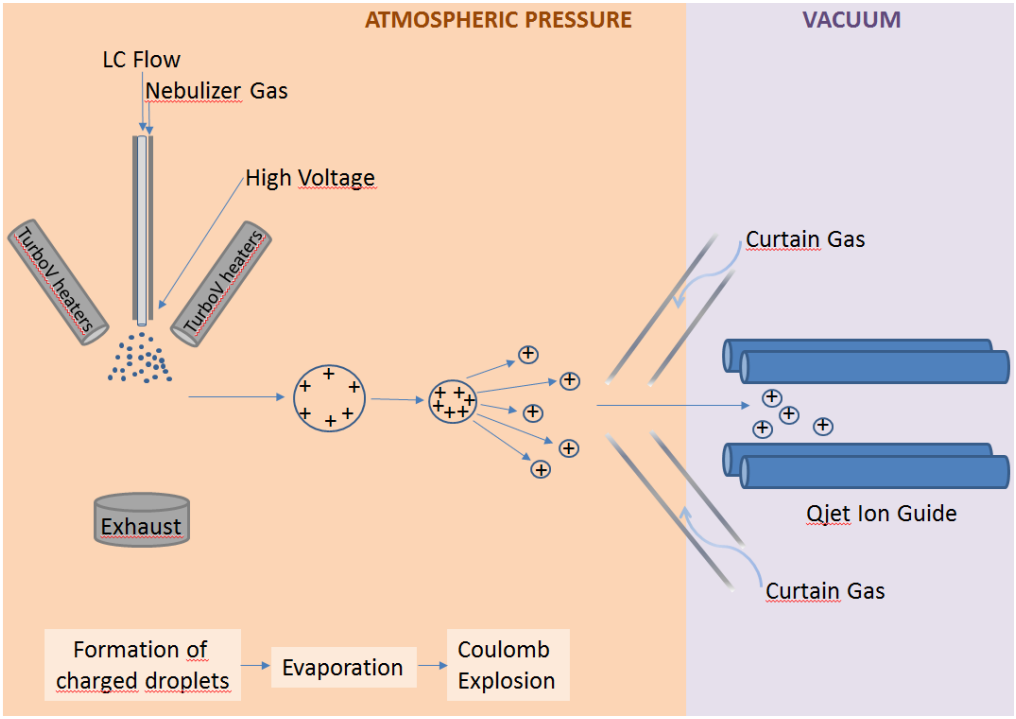


Figure 11 Schematic representation of electrospray ionization [modified after *ABSciex, 2008*]

Following separation, an analysis of compound ions is generated in high vacuum. That avoids scattering and impacts of ions with gas molecules. Separation of *m/z* ratios could occur after six principles: magnetic and sector field separation, quadrupol mass filter, ion trap, ion cyclotron resonance cell and time of flight spectrometer [*Cammann, 2000, Lottspeich, 2008*].

Quadrupol mass filter are commonly used. Here four concentric parallel rods with opposite direct voltage and switching polarity of the electrodes result in an oscillating electrical field. Ions are accelerated in the opposite direction and only ions with a certain *m/z* ratio are confident between the electrodes. Changes to the potentials on the rods allow a wide range of *m/z* values. Triple quadrupole mass spectrometer has four consecutive quadrupole stages, Q0 to Q3, which allow the determination of the chemical structure of compounds, supplementary. Such systems are called tandem mass spectrometer, as well. Q0 is only for motion of ions in an oscillating electric field. First Q1 act as mass filter, where ions are scanned at their *m/z* values. Q2 is no quadrupol as defined, rather a collision cell. Collision gas and impressed voltage fragment precursor-ions in the corresponding product-ions. *m/z* values of formed ions are determined at Q3, the second quadrupol [*Cammann, 2000, Lottspeich, 2008*].

Multiple reaction monitoring (MRM)

Scanning a certain *m/*z value at Q1, fragmentation at Q2 and transmitted an identified product-ion by Q3 to the detector gives the possibility for quantification and is known as multiple reaction monitoring (MRM) mode (see Figure 12 Schematic representation of the multiple reaction monitoring (MRM) mode [modified after *ABSciex, 2008*]) [*Cammann, 2000, Lottspeich, 2008*].

Essential advantage of MRM is the fade-out of interfering signals of complex matrices, such like in biological material. The high sensitivity involves a good signal to noise ratio, with a low limit of detection.

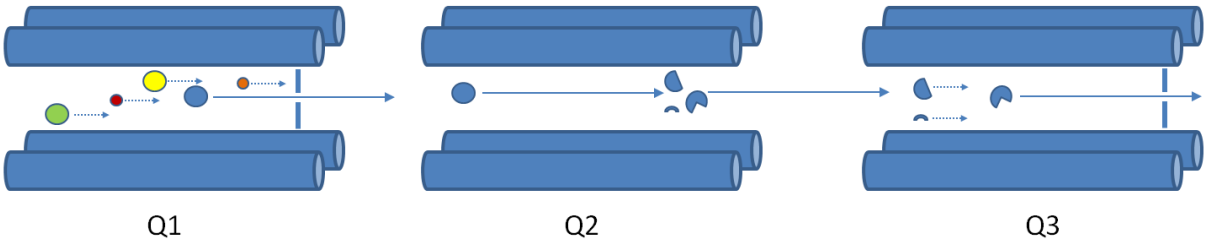


Figure 12 Schematic representation of the multiple reaction monitoring (MRM) mode [modified after *ABSciex, 2008*]

Product-ion scans

Product-ion scans are a commonly employed MS/MS method for sequence analysis or identification and/or structure determination of single components in alloys or after synthesis of standards. A precursor-ion is isolated by setting Q1 to transfer only the *m/z* ratio of that ion into Q2. In the collision cell (Q2), the ion is fragmented and the generated product-ions are mass analyzed in Q3 (see Figure 13 Schematic representation of the product-ion scan [modified after *ABSciex, 2008*]) [*Cammann, 2000*].

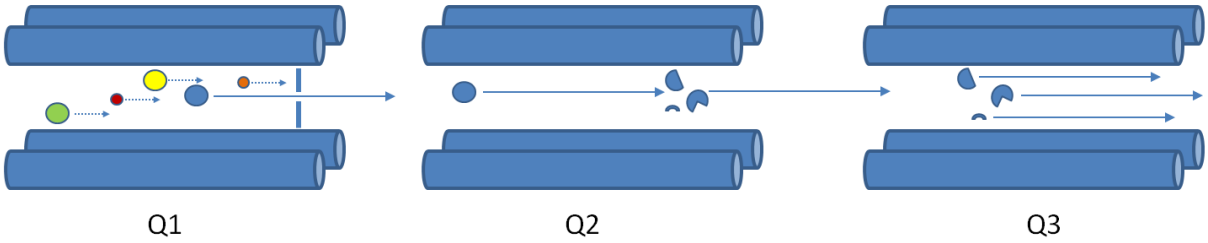


Figure 13 Schematic representation of the product-ion scan [modified after *ABSciex, 2008*]

2.2.4 Calibration and tuning of a AB Sciex Triple Quad or QTrap® mass spectrometer

Calibration is the process of calibrating the mass scale using polypropylene glycol (PPG), called calibration standard, which has peaks with accurately known masses. They provide a benchmark for comparison when calibrating the instrument. Using this calibration standard ensures the mass peaks are assigned to the correct *m/z* values, thereby ensuring the highest possible mass accuracy of instrument. To assure the mass peaks are assigned to the correct *m/z* values, the mass shift have to be equal to or less than 0.1 Da. Tuning the instrument is the process of optimizing the resolution and instrument parameters to ensure the best sensitivity and performance of the mass spectrometer. Optimizing the resolution means adjusting the peak width and peak shape. A maximal sensitivity is resulted in a peak width between 0.6 and 0.8 Da at 50% of the maximal peak high of a given *m/z* ratio. The sensitivity

is disproportioned to the resolution; the higher the resolution, the lower the sensitivity. For optimized analysis, compound-dependent and source-dependent parameters have to be tuned in. Compound-dependent parameters are optimized by infusion via syringe pump for each substance. Optimization occurs by Analyst Software 1.4.2 and 1.6 (AB Sciex GmbH Germany, Darmstadt, Germany) and a flow of 10 µL/min. Source-dependent parameters are determined by flow injection analysis (FIA) via HPLC and injections of a standard solution via the autosampler. That means without column and the accordant conditions (flow and mixture of mobile phase) for one of all analytes. Also for this process steps Analyst Software 1.4.2 and 1.6 (AB Sciex GmbH Germany, Darmstadt, Germany) was used. Compound-dependent and source-dependent parameters and their functions are described in Table 6 and Table 7.

Table 6 Compound-dependent MS parameters and their functions.

parameter	function
declustering potential (DP)	voltage between skimmer and orifice plate, minimization of analyt-eluent-clusters
entrance potential (EP)	focusing and transport of ions in Q0
collision energy (CE)	voltage between Q0 and Q2, control of collision energy
collision cell entrance potential (CEP)	voltage between Q0 and entrance of Q2, focusing of ions in Q2
collision cell exit potential (CXP)	voltage between Q2 and Q3, transport of ions from Q2 to Q3

Table 7 Source-dependent MS parameters and there functions.

parameter	function
curtain gas (CUR)	pressure of gas between curtain plate and orifice plate, prevention against contamination
collision activated dissociation (CAD)	pressure of collision gas, fragmentation of analytes on Q2
ionspray voltage (IS)	voltage of ESI-probe, ionization of analytes
temperatur (TEM)	temperature, increase of volatility of eluate and formation of precursor-ions in gas phase
gas 1 (GS1)	nebulizer gas, formation of small eluate droplet and influence of stability of electrospray
gas 2 (GS2)	turbo gas, evaporation of eluate in eluate droplet

2.2.5 Stable isotope dilution analysis (SIDA)

For the quantification of chemical compounds with a mass spectrometer the stable isotope dilution analysis (SIDA) is recommended. Using SIDA, a defined amount of isotopically labeled standard is added to the sample. After equilibration, the ratio of the isotopologues is stable because of their nearly identical chemical and physical properties. During sample preparation, losses of the analyte are completely compensated by identical losses of the isotopologue. Final mass spectrometry allows differentiation based on the *m/z* ratios. With the known amount of the labeled standard, the content of the analyte can be calculated via the ratios of the peak areas vs. ratios of the concentrations (see Figure 14 Schematic representation of stabile isotop dilution analysis (SIDA) [modified after *Rychlik&Asam, 2008*]) [*Cammann, 2000, Rychlik&Asam, 2008*].

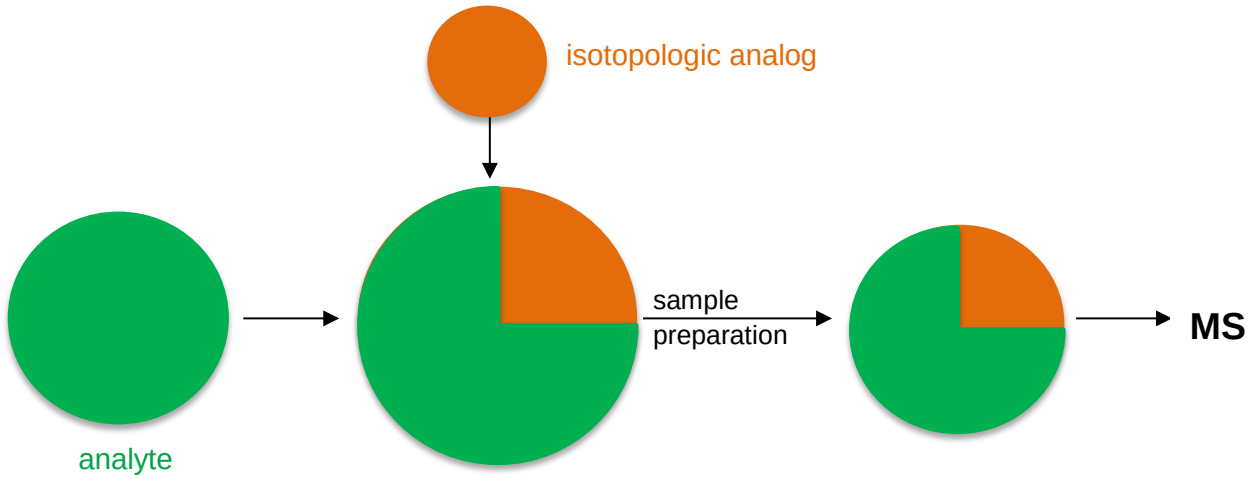


Figure 14 Schematic representation of stabile isotop dilution analysis (SIDA) [modified after *Rychlik&Asam, 2008*]

3 Aim

Chlorogenic acids (CGA) are a family of esters of certain *trans*-cinnamic acids and (-)-quinic acid and well known to provide beneficial effects on human health. Apart from almost every vegetable comestible goods, coffee is reported to be the main source of CGA in human nutrition. A major part of population consumes coffee or its products every day in combination with a meal such as a breakfast. A central point to assign the beneficial effect of CGA from coffee is the influence of a food matrix on the bioavailability and the metabolism of CGA. Only limited data are available on how the bioavailability as well as absorption and excretion of these coffee ingredients and their derivatives are influenced depending on the kind of food consumed at the same time.

In our human intervention study, the consumed coffee beverage (instant study coffee) used to investigate the above mentioned bioavailability, absorption and excretion. Fourteen healthy participants consumed either the coffee beverage only (COFFEE), the coffee beverage with a breakfast rich in carbohydrates (CARB) or the coffee beverage with a breakfast containing high levels of fat (FAT), in a random order, to investigate the influence of a food matrix on the bioavailability of CGA from coffee.

One main point of interest was to determine to what extent the CGA present in the coffee beverage were circulating and excreted. Therefore, existing CGA and their respective metabolite concentrations were determined in the collected plasma and urine samples using high performance liquid chromatography electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS/MS). Thus, the primary goal of the study was to estimate differences in absorption and bioavailability between the different metabolites in plasma and urine after one of the three treatments in terms of: area under the curve (AUC), maximum plasma concentration (C_{max}), and time needed to reach maximum plasma concentration (t_{max}).

Additionally, it was already known that the microbiota is responsible for metabolism of CGA to DHFA and DHCA derivatives, with subsequent formation of a wide range of metabolites. Therefore, as second aim, *ex vivo* incubations of the volunteers' faecal samples with 5-*O*-caffeoylquinic acid as the main cholorgenic acid in coffee were performed to achieve data for metabolic efficiency and interindividual variabilities. In addition, these samples were analysed for their microbiota content by an external laboratory (L&S, Bad Bocklet, Germany). These results were used to correlate a change in special microorganism related to a decreased or increased metabolic efficiency of the individual.

Finally, statistical evaluation was performed between plasma appearances, urinary excretion, and the ability of the microorganisms to degraded 5-*O*-caffeoylquinic acid as coffee constituent to figure out any potential correlations. These results should help to come to the conclusion if and which influence a breakfast consumption have on the chlorogenic acid metabolism in humans.

4 Results and discussion

4.1 Study design and subjects

The study was subdivided in five study days, shown in Figure 15. At the beginning of the study, a general medical screening was carried out to exclude any known or unknown disorders or disease.

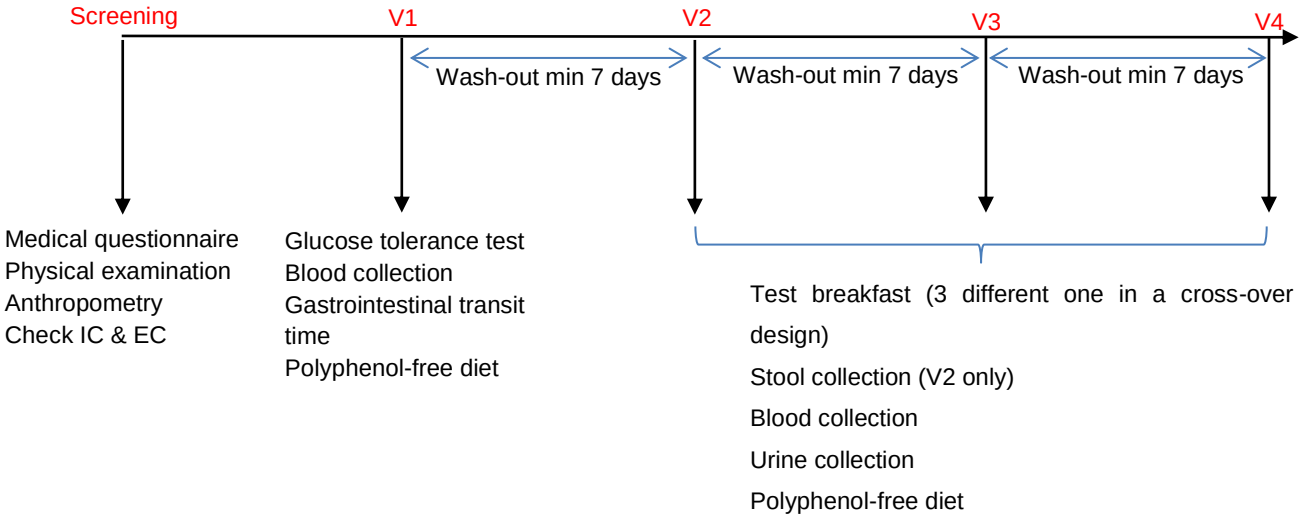


Figure 15 Design oft the performed human intervention study.

Bioimpedance measurements and demographic data

The body composition determined by bioimpedance measurement of each proband can have an impact on the absorption and metabolisation of coffee polyphenols.

Appendixes, Table 51 to Table 53 show the physiological data of the 14 probands. The average of BMI of all probands was 23.0 ± 1.6 kg/m^2, the average of the percental fat free mass was 76.2 ± 7.5%, the average of percental total body water was 55.8 ± 5.5% and the average of percental fat mass was 17.4 ± 6.0 h.
For detailed basline demographic data see chapter 6.4.1 Study design and subjects, page 145.

Oral glucose tolerance test (oGTT)

Oral glucose tolerance test (oGTT) was performed by a single intake of 75 g glucose and measurement of blood glucose concentrations in the peripheral blood (fingertip) were performed after 0, 30, 60, 120, 180, 240 and 300 minutes were performed.

Appendix, Table 55 shows the blood glucose concentrations of each proband (01 – 14) until 300 min after intake of glucose. The fasting plasma glucose of all probands was less than 126 mg/dL and the 2 h-plasma glucose lay between 140 mg/dL and 200 mg/dL. Only the 2 h-plasma glucose of proband 14 was slightly increased in an acceptable magnitude.

Probands

Finally, our subject collective consisted of seven health men and seven healthy women, 29 ± 8 years old. GIT transit time was 17.4 ± 6.0 h and all other inclusion/exclusion criteria were also achieved.

The result-providing study days took place on three separate days (V2 to V4). In each case, test subjects consumed 200 mL of a coffee beverage prepared from instant coffee with 3.1 mg chlorogenic acids/kg bw. As coffee is oftentimes consumed as a breakfast beverage, three different breakfasts were consumed in a cross-over design in a randomized order. As test breakfast, there were the coffee beverage only (COFFEE), the coffee beverage with a breakfast rich in carbohydrates (two bread rolls and honey, CARB) or the coffee beverage with a breakfast containing high levels of fat (one bread roll and peanut butter, FAT). These two breakfasts were equicaloric (~626 kcal calculated with nutrition facts on the product).

Diagnostics of microbiota

The microbiota species and amounts in fecal samples can have an important impact on the metabolization of coffee polyphenols. In order to determine the microbiota contents we transferred aliquots of fresh faeces (time points: between -24 and 0 h) at +4 °C to an external laboratory (L&S laboratories, Bad Bocklet, Germany) (Appendix, Table 54).

For most samples, the microbiota content was in a normal range as defined by L&S laboratories, Bad Bocklet, Germany, and characterised as 95% of values from a normal population. Some probands had an increased or decreased level of some bacteria or fungi. Importantly, the quantity of bacteria or fungi was out of the normal range what means not automatically, that this is caused by illness. In connection with the incubation studies with faeces samples the data were compared.

4.2 Amounts of chlorogenic acids quantities in the coffee samples

The coffee used for the study was dissolved in boiled water and after cooling samples were centrifuged. Thereafter, the supernatant was filtered, diluted and 20 µL was injected to the

HPLC-DAD system. Parameters and solvent gradient are illustrated in Table 39 (Materials and methods) [*Witt, 2012*].

Figure 16 shows a representative HPLC-DAD chromatogram at 325 and 270 nm of an instant study coffee sample, used for the intervention study displaying the respective chlorogenic acids.

Table 8 and Figure 17 show the amounts of chlorogenic acids [%] in the instant coffee sample used for the intervention study. The contents corresponded well with the denoted information on the original product label. There was 9.2 g of CGA per 100 g of instant coffee (compared to 8.7 g of CGA per 100 g of instant coffee as denoted information on the original product label). The different quantities were caused by the additional quantification of 3-FQA and 3,5-DiCQA in the coffee compared to the information on the original product label. Caffeoylquinic acid was the most abundant CGA subclass in the coffee we studied, followed by feruloylquinic acid and di-caffeoylquinic acid.

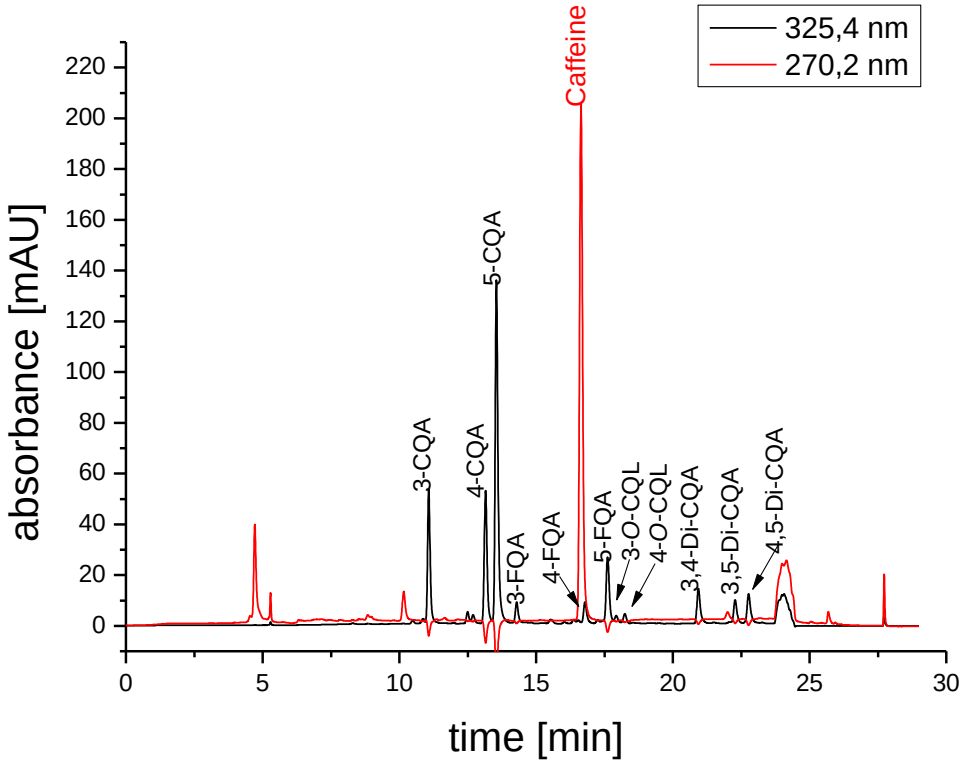


Figure 16 HPLC-Chromatogram of a coffee sample (0.5g/50 ml) at 325.4 and 270.2 nm [*Witt, 2012*].

Table 8 Amounts of chlorogenic acids [%] determined in the instant coffee used in the human intervention study (n = 3).

	amount [%]	SD [%]
3-CQA	1.32*	0.05*
4-CQA	1.41*	0.06*
5-CQA	3.86*	0.13*
3-FQA	0.23*	0.03*
4-FQA	0.23*	0.02*
5-FQA	0.73*	0.03*
3,4-DiCQA	0.58*	0.08*
3,5-DiCQA	0.35*	0.03*
4,5-DiCQA	0.46*	0.06*
QA	1.12	0.04

* [*Witt, 2012*]

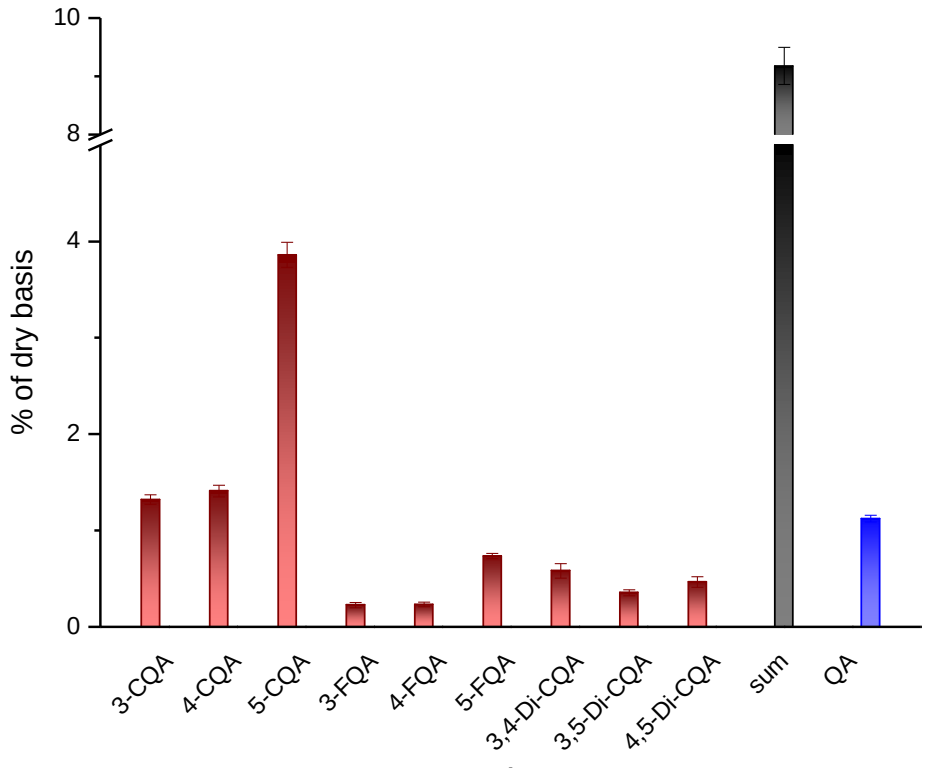
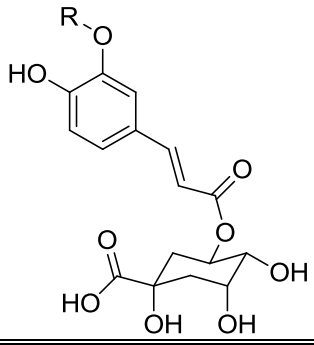


Figure 17 Amounts of chlorogenic acids (sum: sum of all chlorogenic acids) and quinic acid [%] in the instant study coffee used in the human intervention study (n = 3); modified according to [*Witt, 2012*]

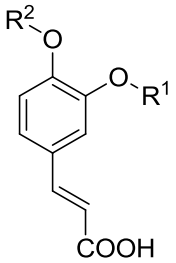
4.3 Systemic availability of instant coffee clorogenic acids and metabolites in plasma

In general, we observed large interindividual variation in AUC, C_{max}, and t_{max} of all determined 56 metabolites in plasma (data not shown). For the statistical calculations, 20 of these 56 plasma metabolites were selected. These data modifications were agreed during a blind data review meeting by the project manager (Mathieu Renouf), biostatisticians (Julien Sauser and Sharam Émady-Azar) and involved parties (Isabelle Cristiani, Samir Dahbane, Laure Poquet, Elke Richling and Denise Scherbl). Table 9 shows the structures of the selected metabolites except 3- and 4-FQA (isomers of 5-FQA, which are esterified on position 3 or 4 with quinic acid). Additionally to the statistical analysis for single metabolites, the metabolites were divided into groups, according to similarity and chemical properties, which were then compared to observe any possible differences (see Table 10). Additionally metabolites were further classified into three groups according to their physical structure and predicated from the area of appearance: directly from coffee (*quinics* - 5-CQA, 3-FQA, 4-FQA and 5-FQA), after first degradation and metabolism (*phenolics* - CA3S, CA4S, FA4G, FA4S, iFA, iFA3G, iFA3S, MeFA) as well as colonic degradation and metabolism (*colonics* - DHCA, DHCA3S, DHFA, DHFA4G, DHFA4S, DHiFA, DHiFA3G, mDHCoA, mDHCoAS).

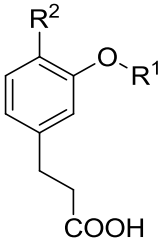
Table 9 Structures of the selected 20 metabolites in plasma for the statistical calculations.



	R
5-CQA	H
5-FQA	CH_3



	R^1	R^2
CA3S	SO_2H	H
CA4S	H	SO_2H
FA4G	CH_3	Gluc*
FA4S	CH_3	SO_2H
iFA	H	CH_3
iFA3G	Gluc*	CH_3
iFA3S	SO_2H	CH_3
MeFA	CH_3	CH_3



	R^1	R^2
DHCA	H	OH
DHFA	OCH_3	OH
DHCA3S	SO_2H	OH
DHFA4G	CH	O-Gluc*
DHFA4S	CH_3	OSO_2H
DHiFA	H	OCH_3
DHiFA3G	Gluc*	OCH_3
mDHCoA	OH	H
mDHCoAS	OSO_3^-	H

*Gluc:

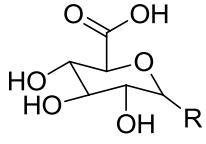


Table 10 Plasma sample grouping for the different derivatives according to similarity and chemical properties.

Chlorogenics	Phenolics	Colonics	Ferulics	Caffeics
5CQA	CA3S	DHCA	5FQA	5CQA
5FQA	CA4S	DHCA3S	3FQA	CA3S
3FQA	FA4G	DHFA	4FQA	CA4S
4FQA	FA4S	DHFA4G	FA4G	iFA
	iFA	DHFA4S	FA4S	iFA3G
	iFA3G	DHiFA	DHFA	iFA3S
	iFA3S	DHiFA3G	DHFA4G	DHiFA
	MeFA	mDHCoA	DHFA4S	DHiFA3G
		mDHCoAS	MeFA	

Coumarics	Sulfates	Glucuronides	Aglycons	Methylated
mDHCoA	CA3S	FA4G	5CQA	iFA
mDHCoAS	CA4S	iFA3G	5FQA	MeFA
	FA4S	DHFA4G	3FQA	DHiFA
	iFA3S	DHiFA3G	4FQA	DHFA
	DHCA3S		DHFA	
	DHFA4S		DHCA	
	mDHCoAS			

Figure 18 to Figure 21 show the time-dependent peak detections (in samples taken after 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12 and 15 h after coffee consumption) for selected subjects (S02 and S05) and treatments (T1: COFFEE and T3: FAT). Data were obtained with MultiQuant™ Software (version 3.0.2, Sciex, Darmstadt, Germany) using the MQ4 integration algorithm. For integration details see 6 Material and methods.

The figures show clear inter-individual differences, but also an influence on t_{max} and C_{max} of a fat-rich meal consumed at the same time with the coffee beverage (Figure 19 and Figure 21) is visible compared to the consumption of pure coffee bavarage (Figure 18 and Figure 20).

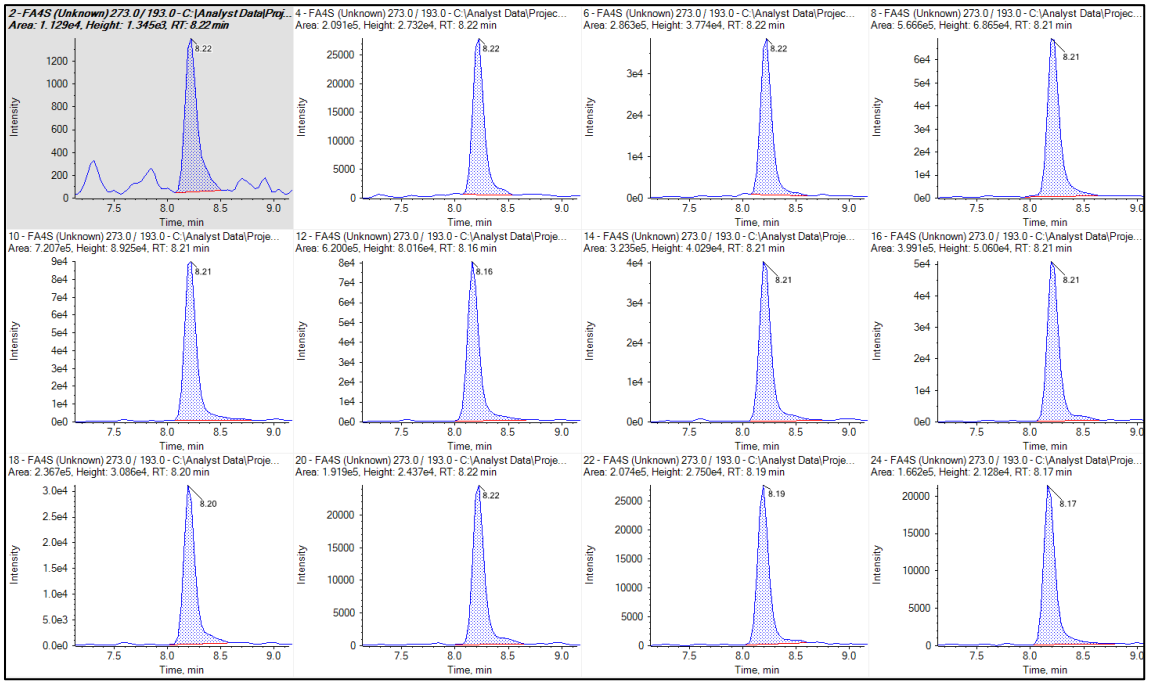


Figure 18 Time-dependent peak detection of FA4S in serum of subject 02 after consumption of pure instant coffee (T1: COFFEE).

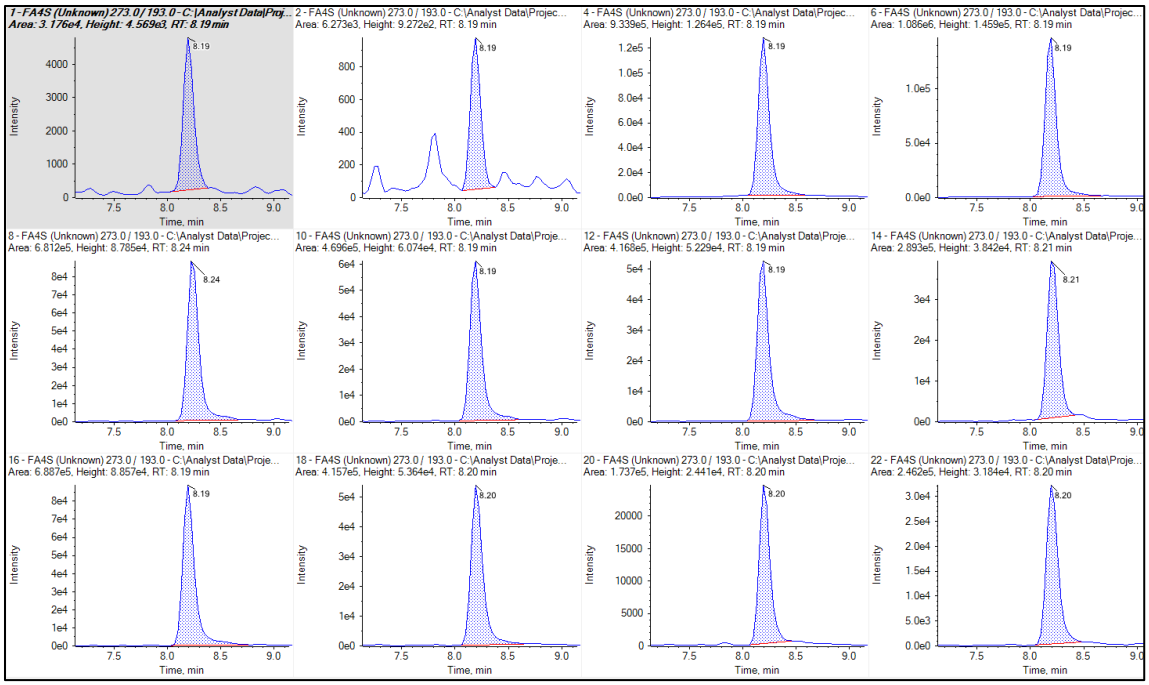


Figure 19 Time-dependent peak detection of FA4S in serum of subject 02 after consumption of coffee with a fat-rich meal (T3: FAT).

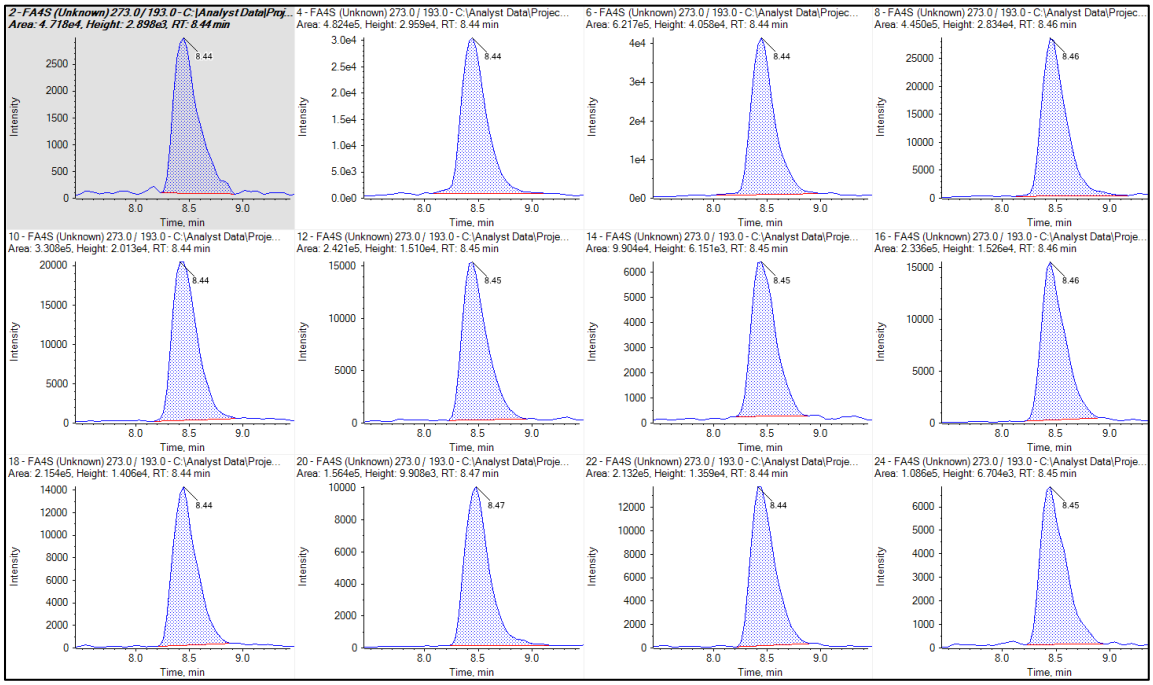


Figure 20 Time-dependent peak detection of FA4S in serum of subject 05 after consumption of pure instant coffee (T1: COFFEE).

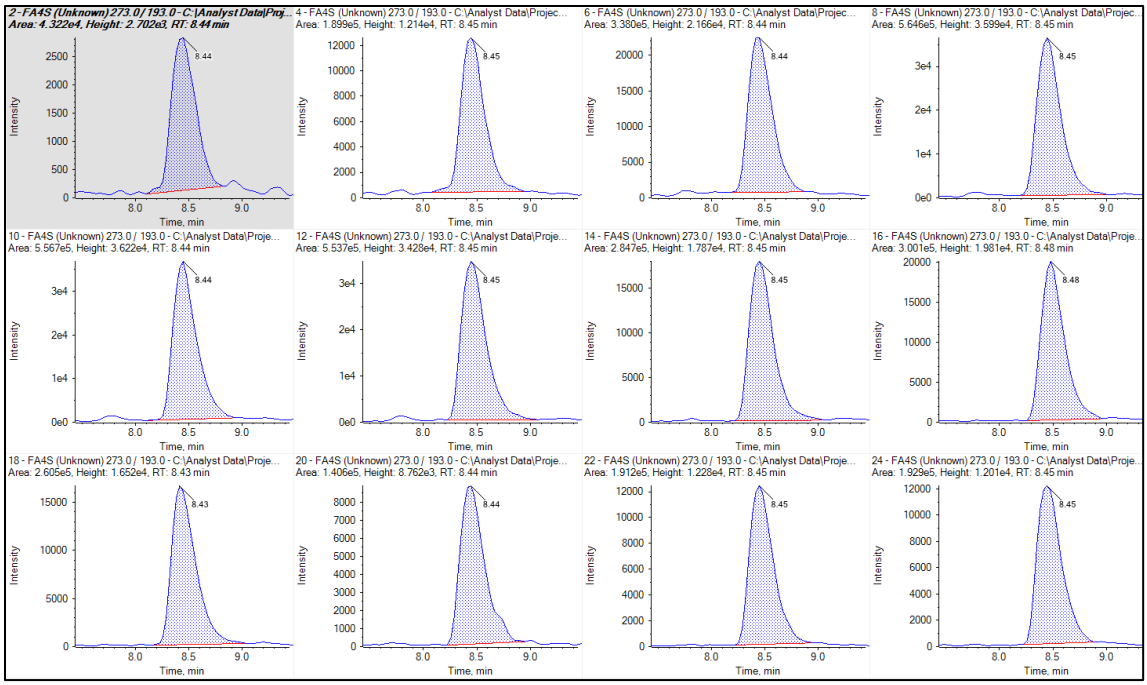


Figure 21 Time-dependent peak detection of FA4S in serum of subject 05 after consumption of coffee with a fat-rich meal (T3: FAT).

Figure 22 to Figure 41 show the plasma kinetics of the selected 20 metabolites as mean data of the values of single subjects. Error bars are for the sake of clarity not shown in these figures.

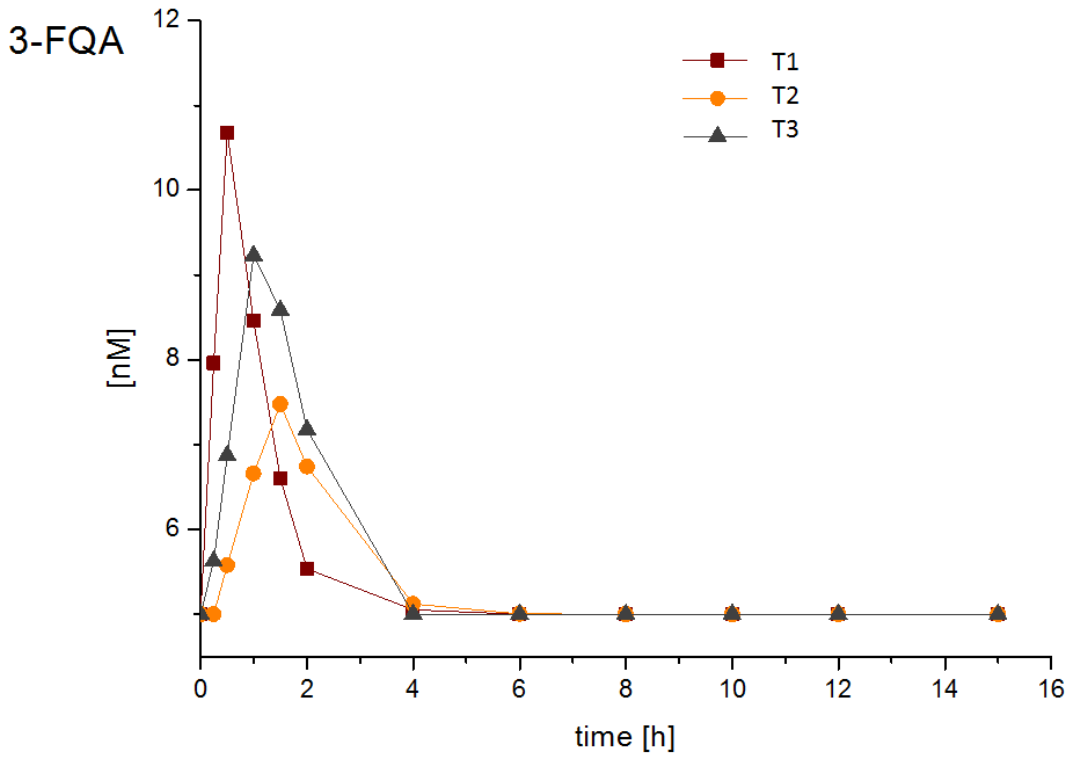


Figure 22 Time kinetics of 3-FQA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

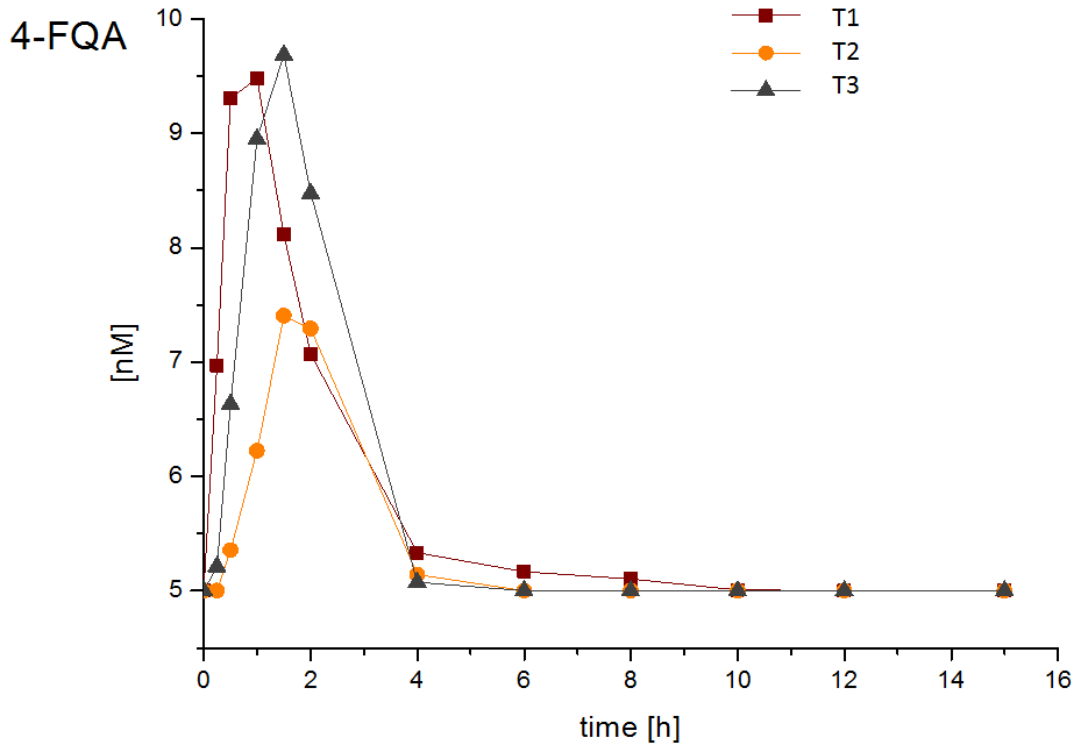


Figure 23 Time kinetics of 4-FQA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

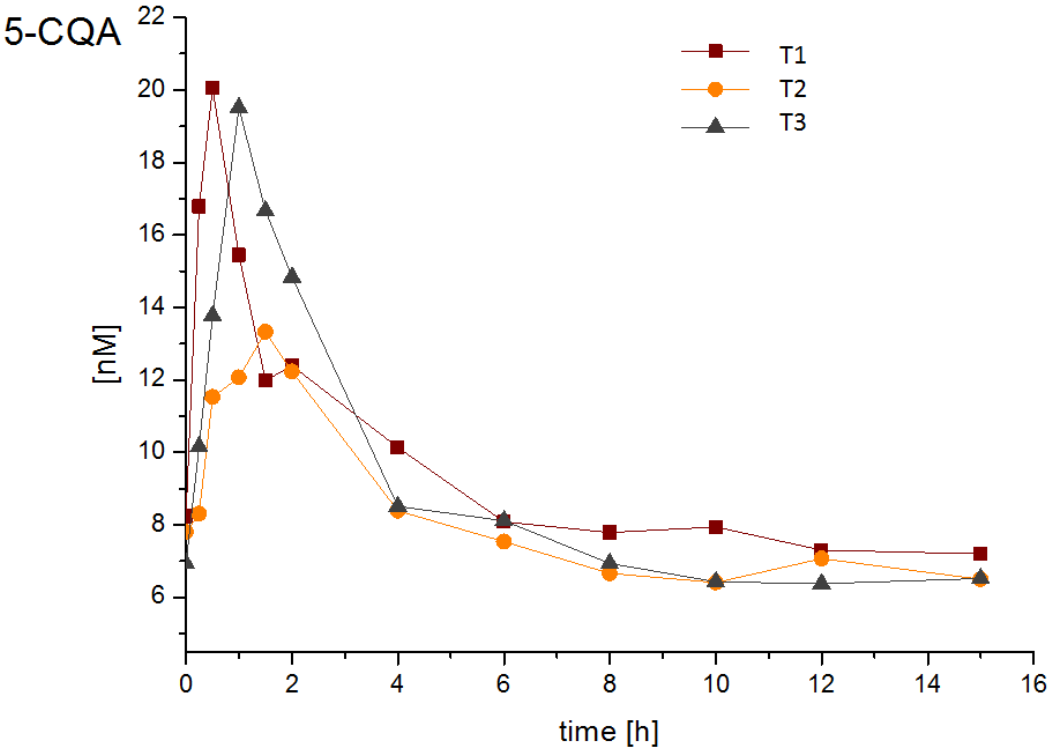


Figure 24 Time kinetics of 5-CQA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

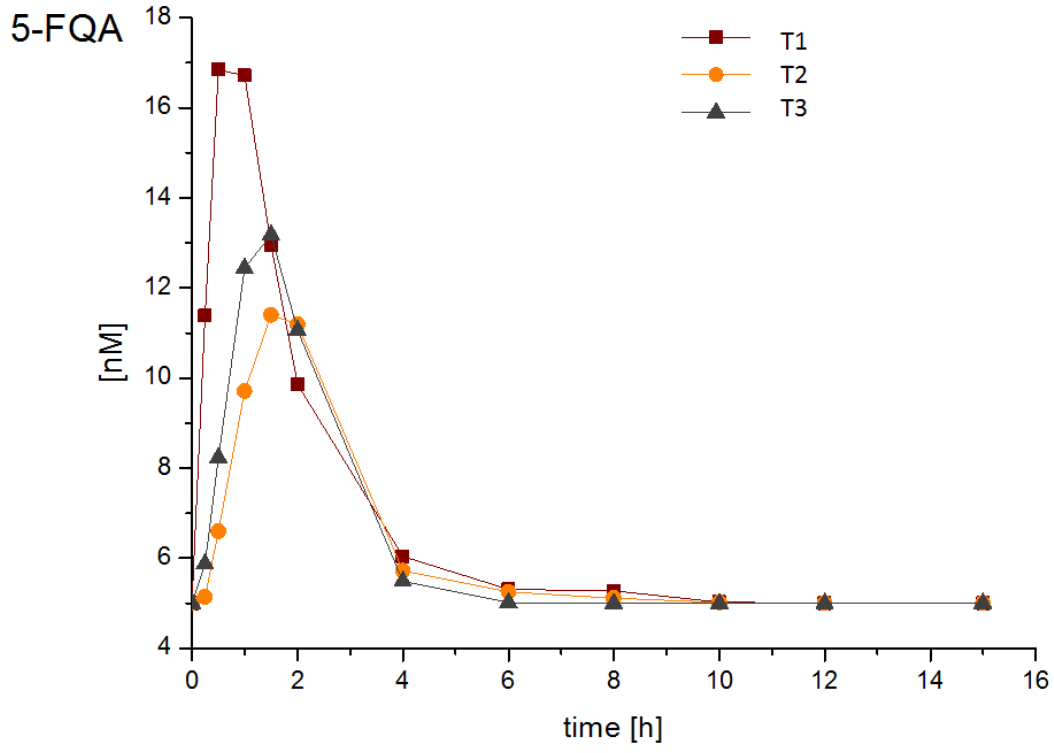


Figure 25 Time kinetics of 5-FQA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

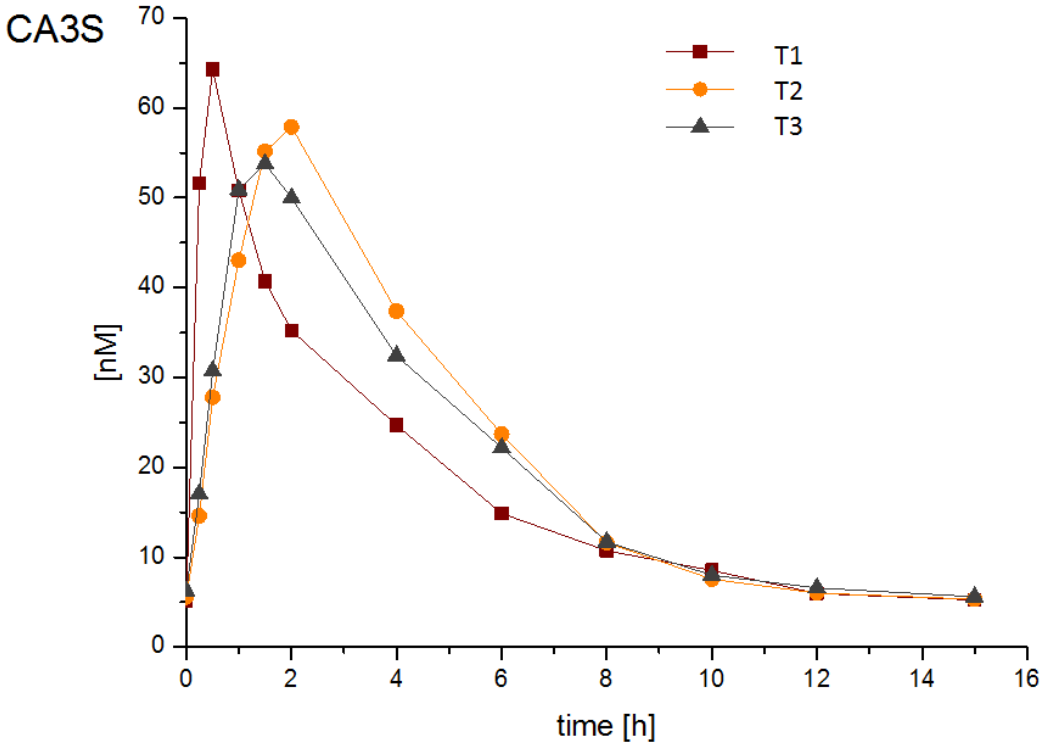


Figure 26 Time kinetics of CA3S from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

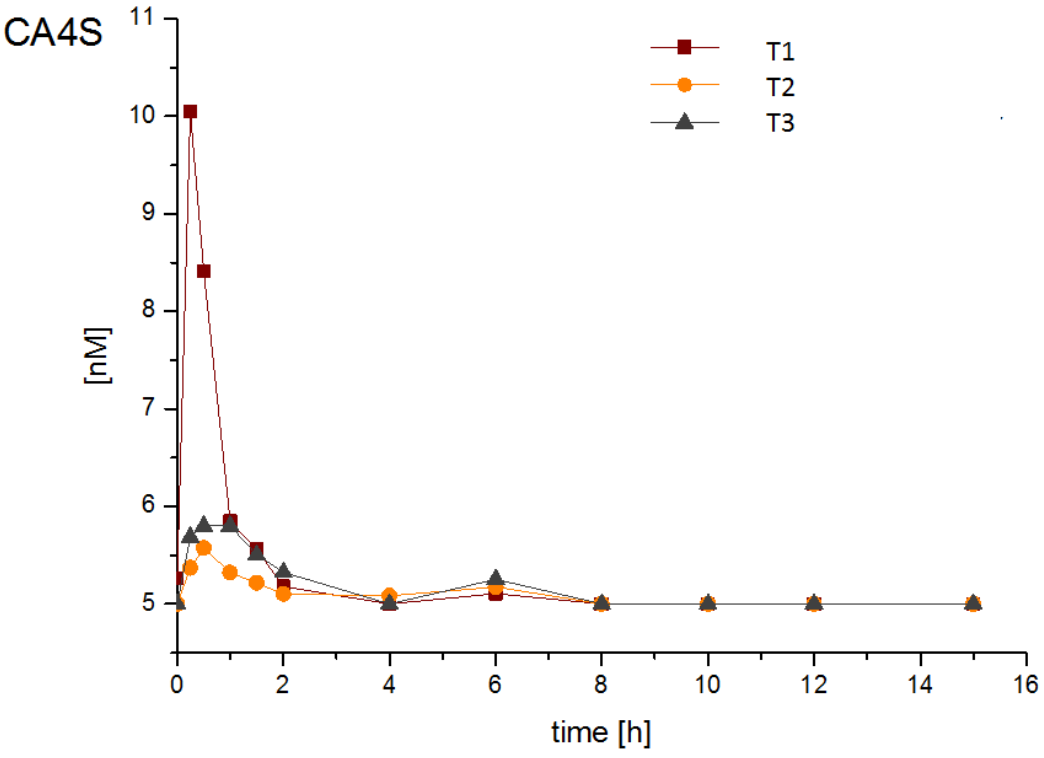


Figure 27 Time kinetics of CA4S from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

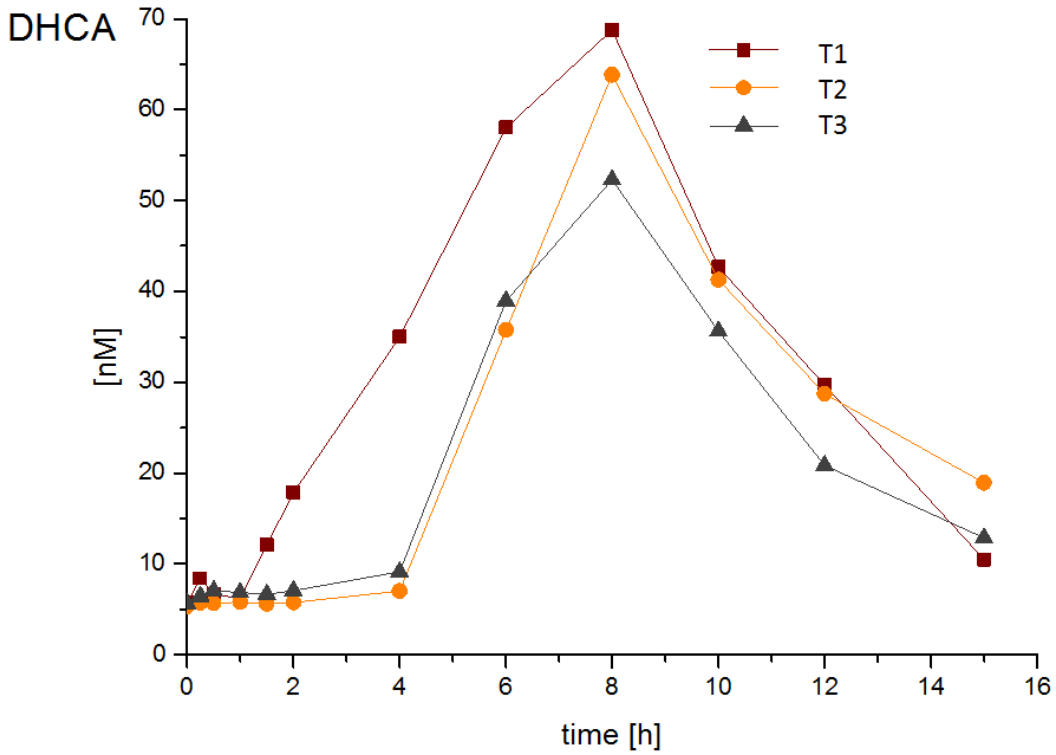


Figure 28 Time kinetics of DHCA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

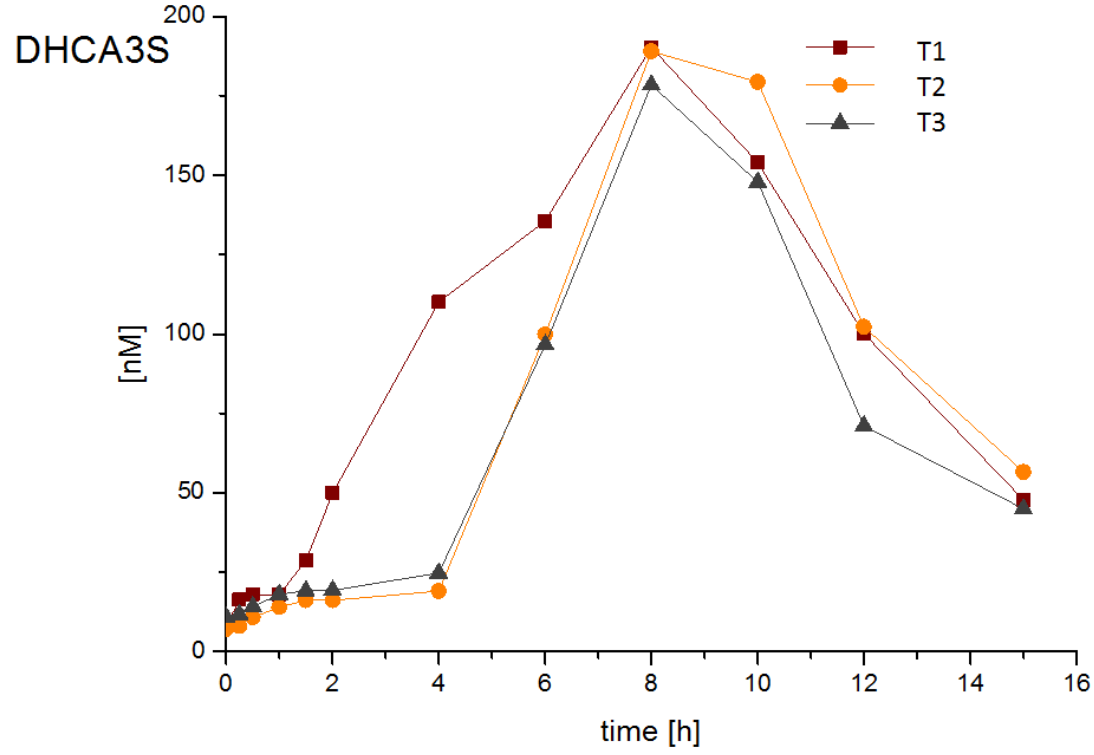


Figure 29 Time kinetics of DHCA3S from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

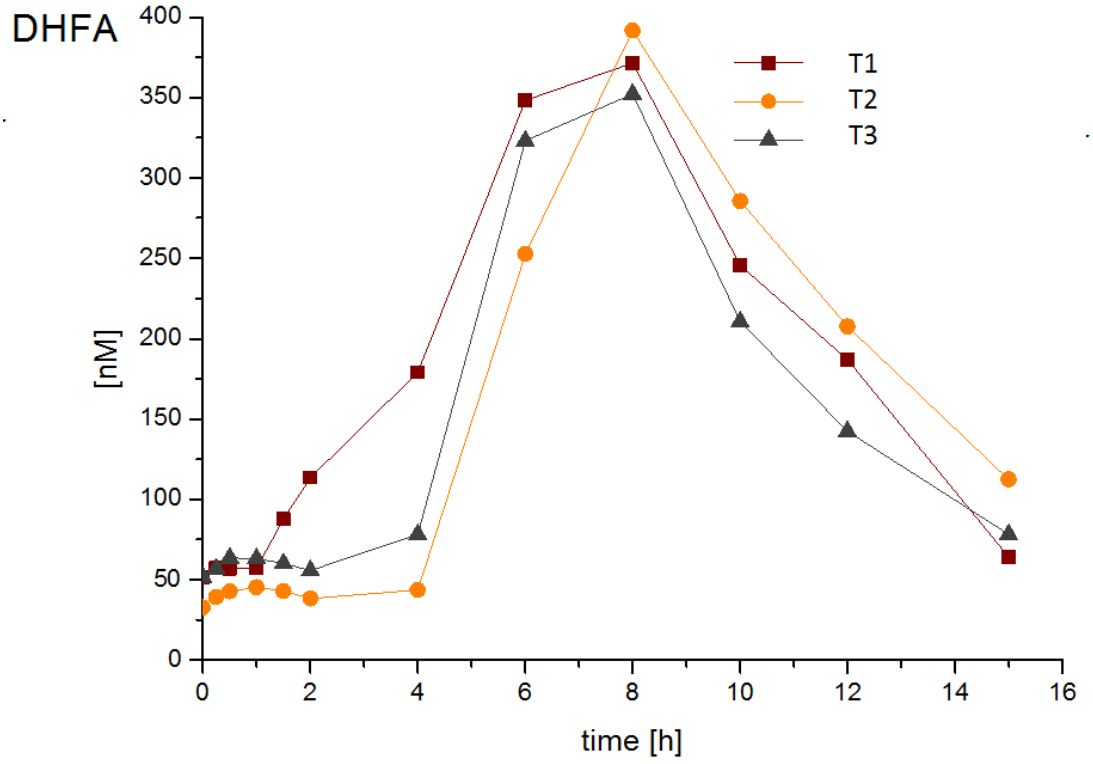


Figure 30 Time kinetics of DHFA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

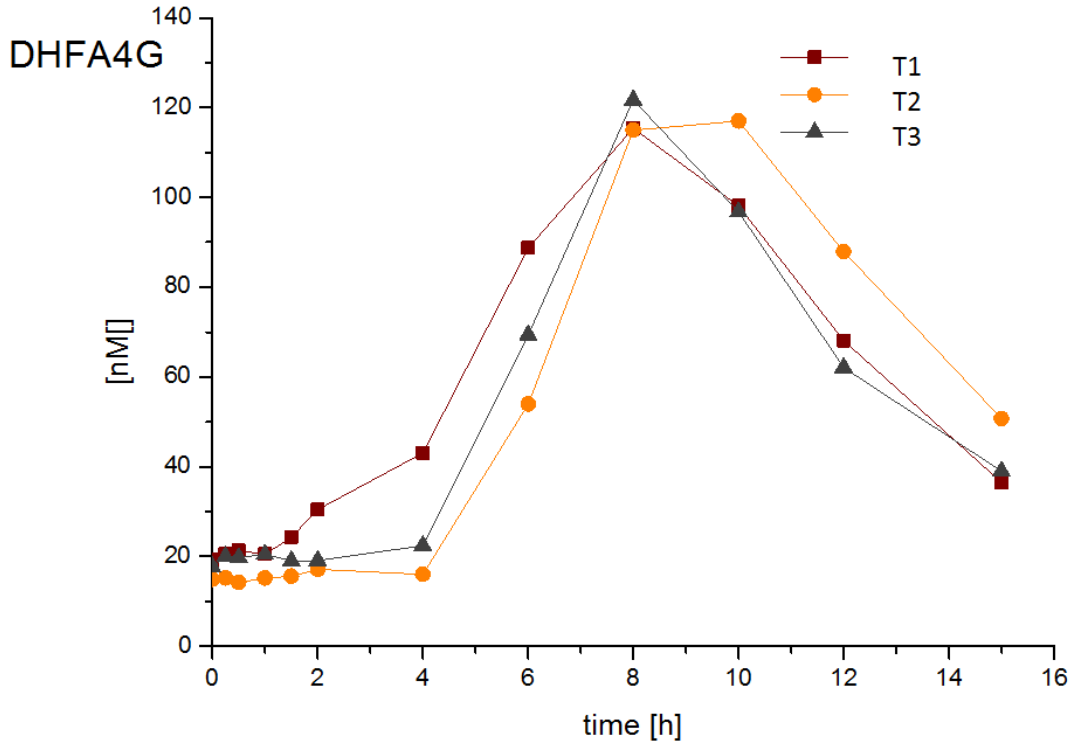


Figure 31 Time kinetics of DHFA4G from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

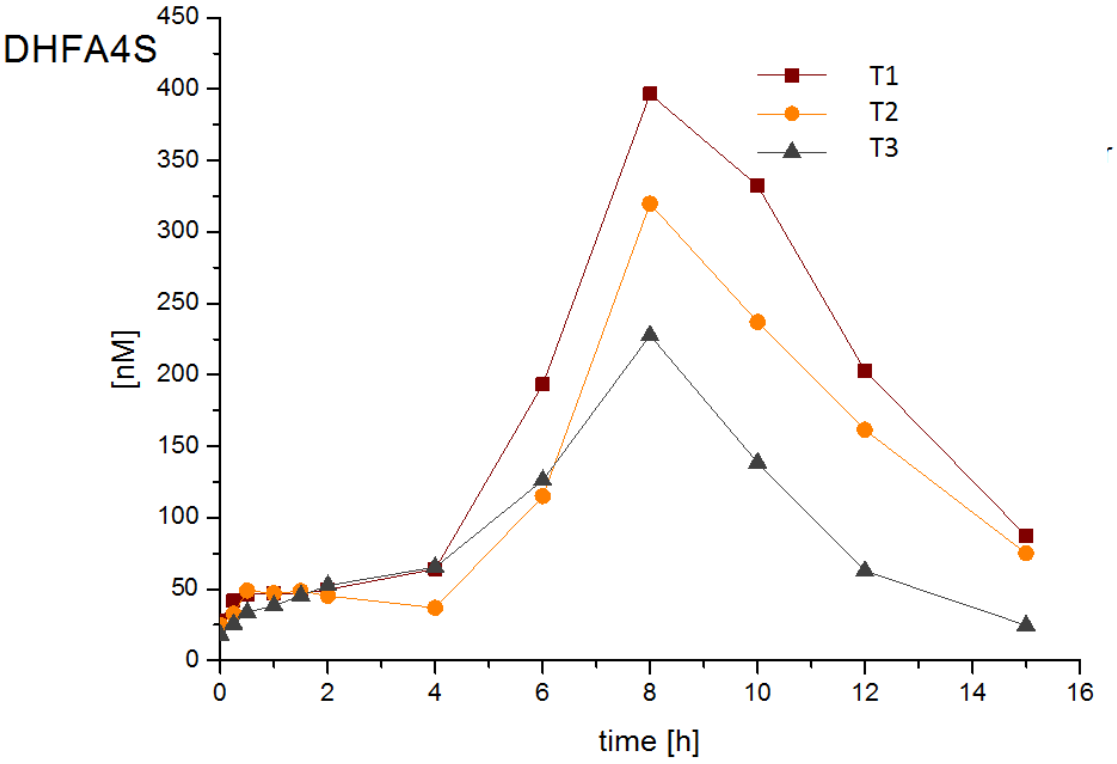


Figure 32 Time kinetics of DHFA4S from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

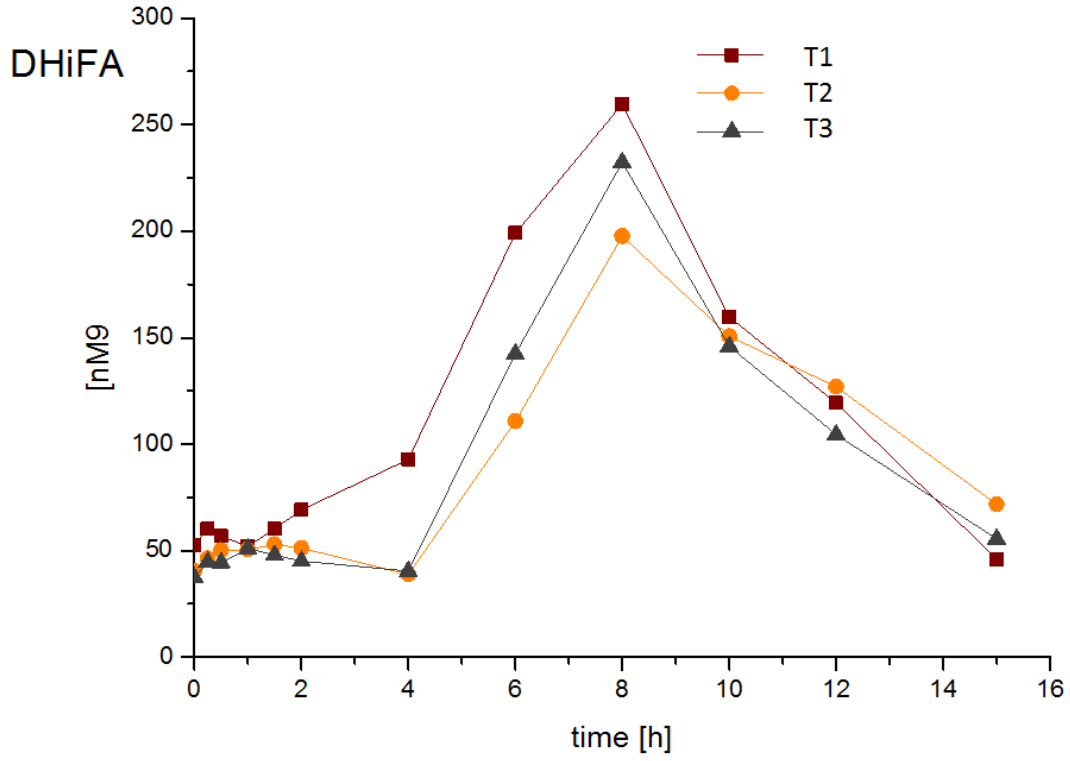


Figure 33 Time kinetics of DHiFA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

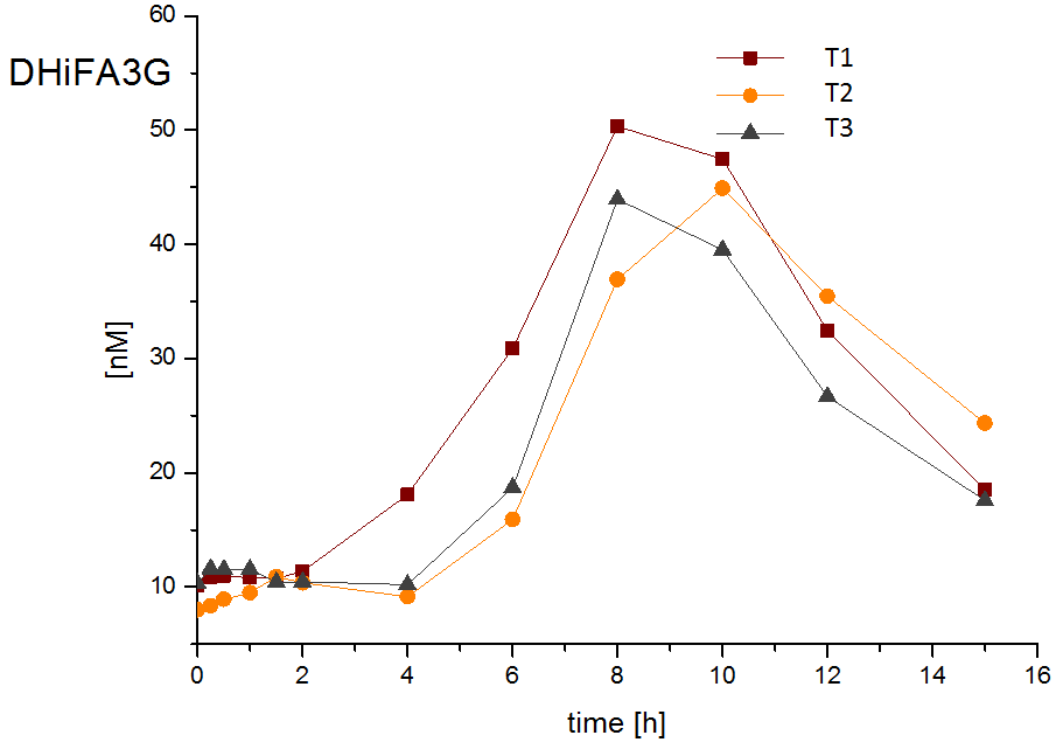


Figure 34 Time kinetics of DHiFA3G from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

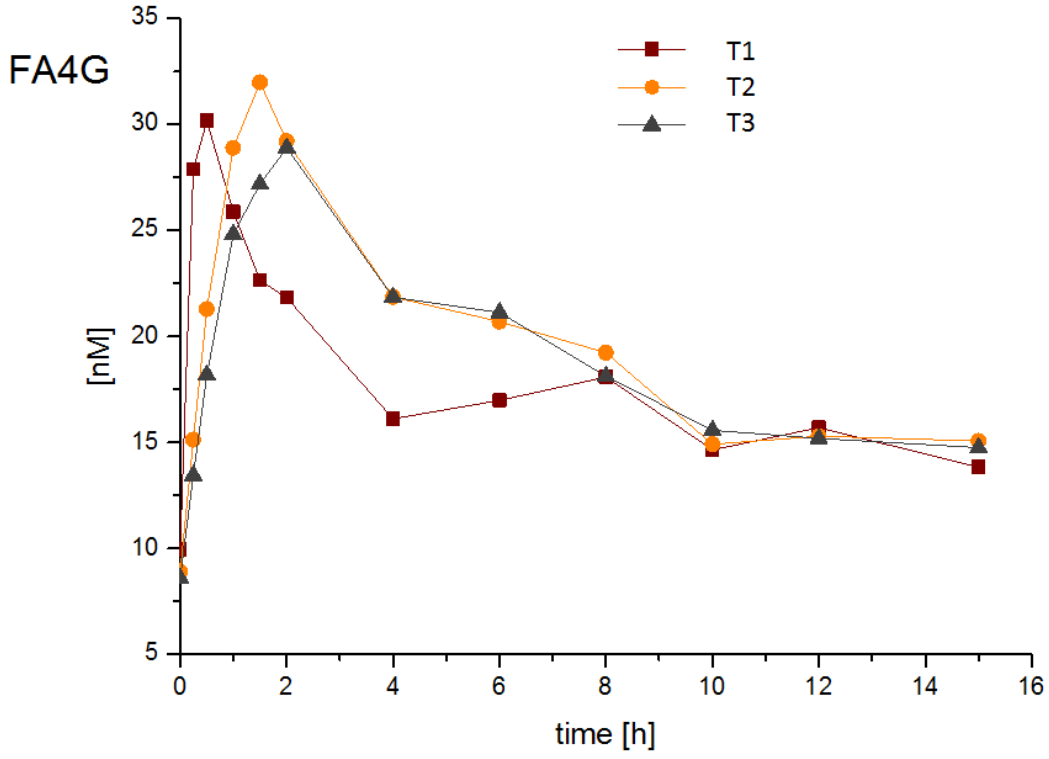


Figure 35 Time kinetics of FA4G from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

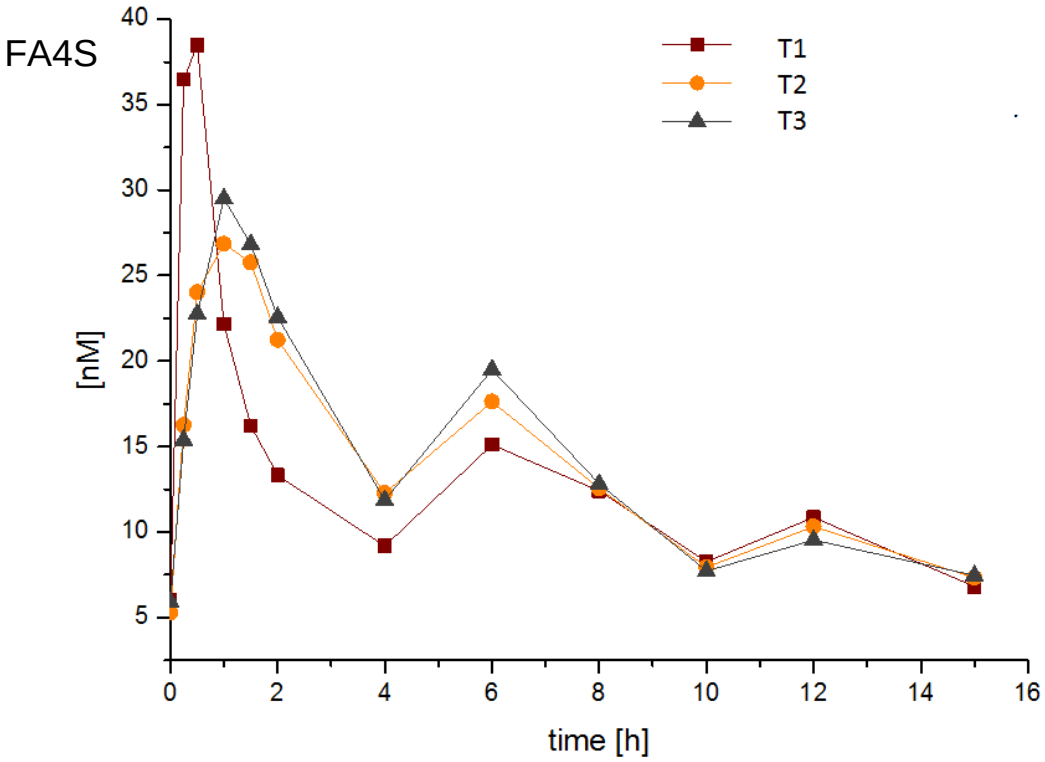


Figure 36 Time kinetics of FA4S from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

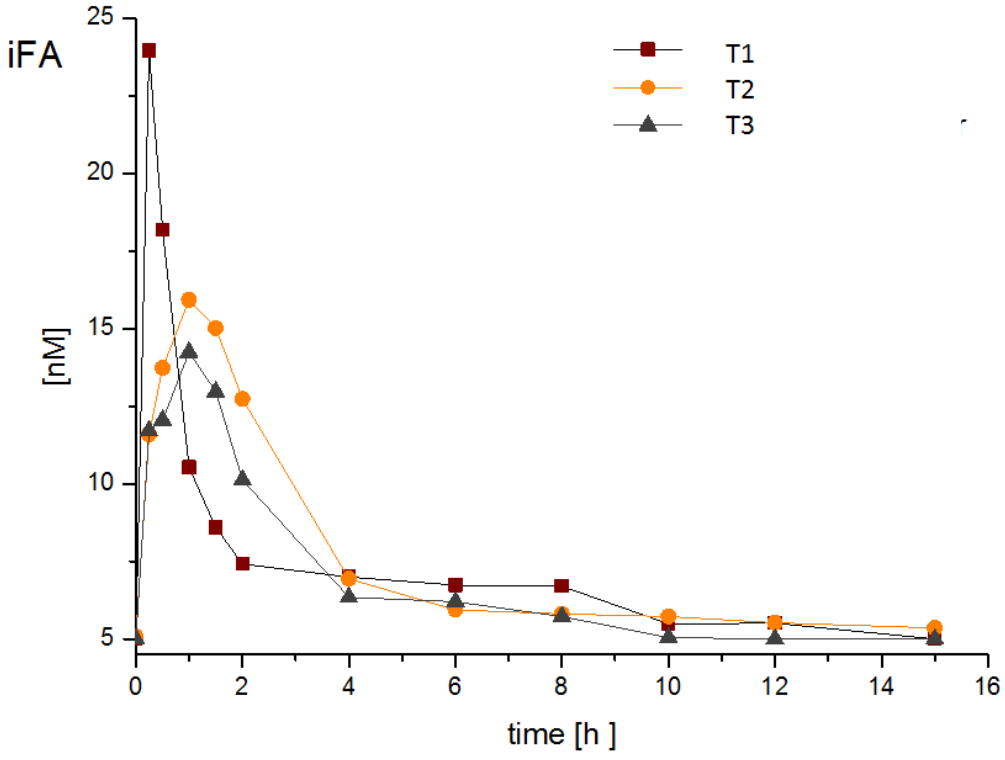


Figure 37 Time kinetics of iFA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

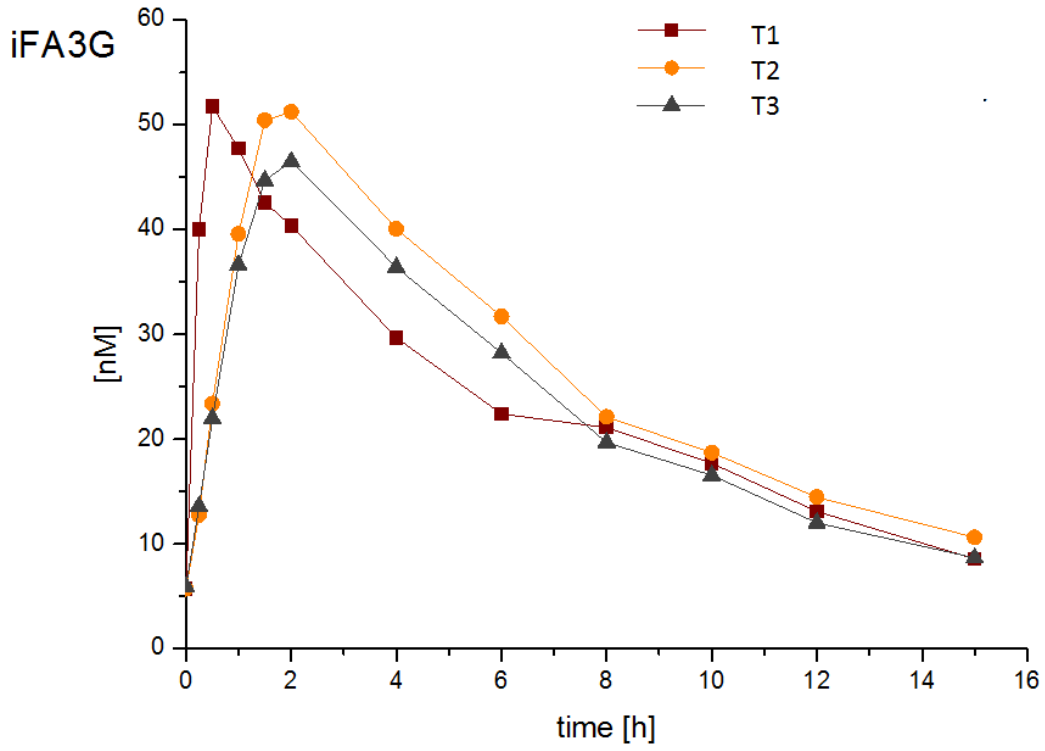


Figure 38 Time kinetics of iFA3G from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

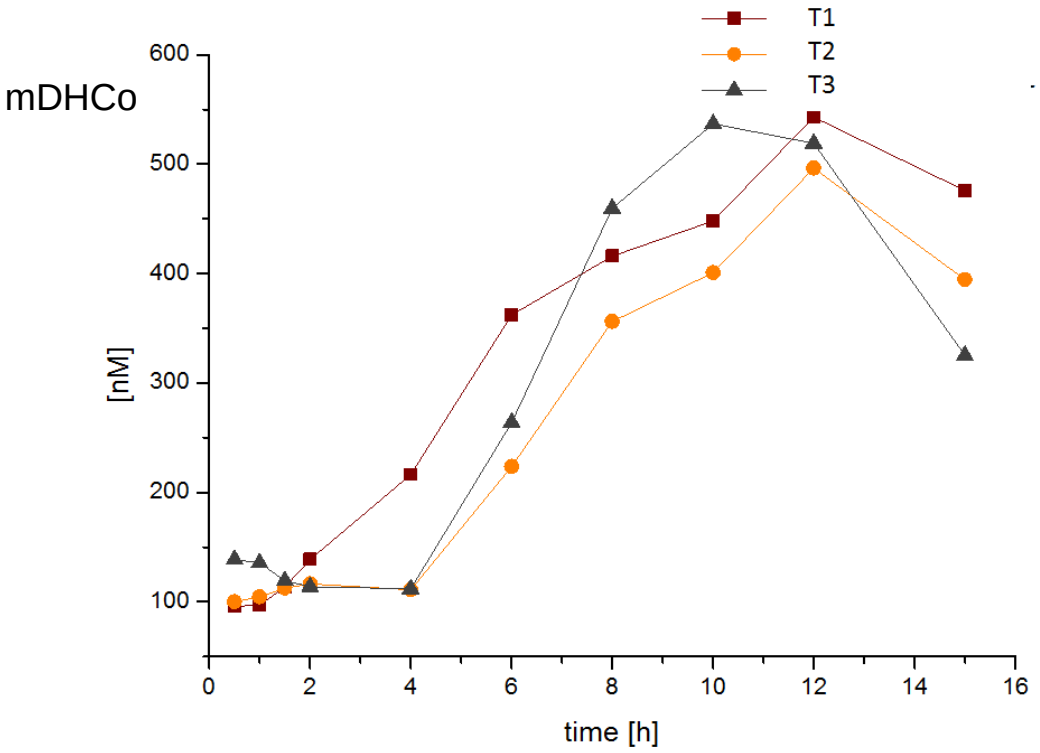


Figure 39 Time kinetics of mDHCoA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

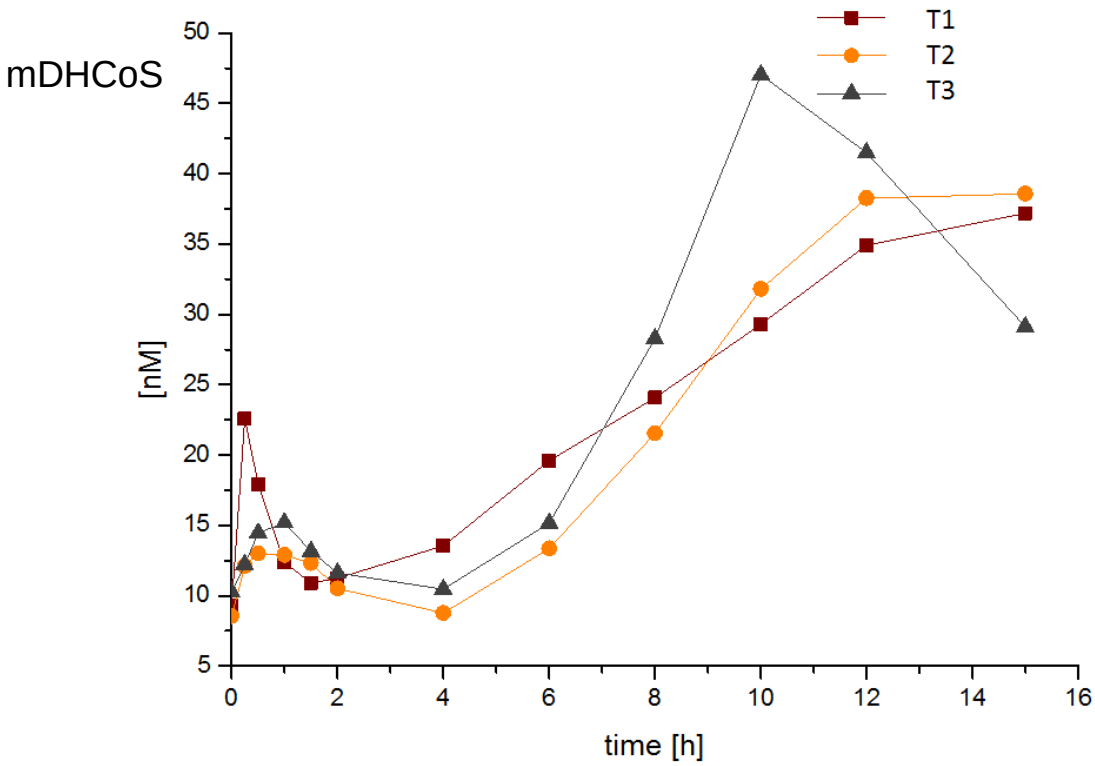


Figure 40 Time kinetics of mDHCoAS from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

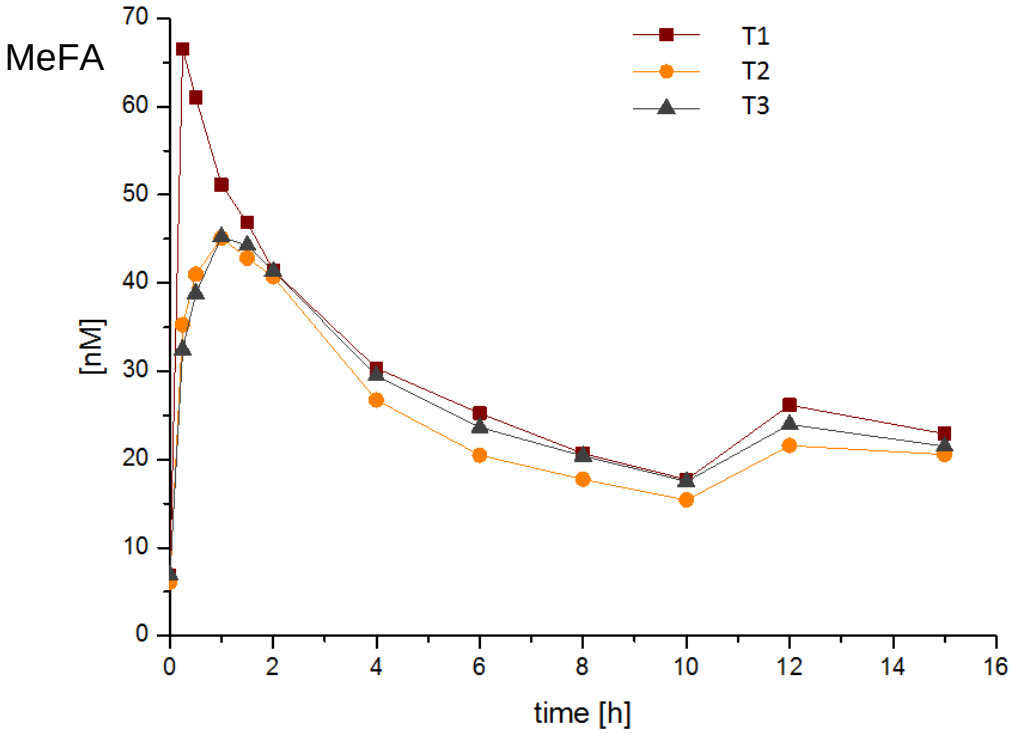


Figure 41 Time kinetics of MeFA from 0 up to 15 h. Data are means [nM] of the plasma values of single volunteers. T1: COFFEE, T2: CARB, T3: FAT

Table 11 shows the number of detected metabolites in plasma samples by subject and treatment. In addition, Table 12shows the number of subjects with a specific detected metabolite in plasma depending on the treatment. The data modifications were agreed during the above-mentioned blind data review meeting and included following: For iFA3S, almost all data were very close to the LOQ (see Appendix, Table 60), hence this metabolite was removed from all the analysis. Subject 07 had for all colonic metabolites inconsistent and incoherent kinetics for the treatment with pure coffee (COFFEE) and was removed for all metabolites. Subject 08 was removed from FA4G analysis, based on inconsistent kinetics between treatments. For 5-CQA, subject 02 had strong outliers, which were excluded and replaced by the LOQ (see Appendix, Table 60) value. For mDHCoA and mDHCoAS, the kinetics were incomplete for the most of the data. Accordingly, Cmax and tmax were not relevant and were not computed.

Table 11 Number of detected metabolites in plasma; samples of each subject and depending on treatments (total n = 20). Data modifications deviant from the norm were agreed during the blind data review meeting (see page 64). NE: not evaluated.

subject	COFFEE	CARB	FAT
01	20	18	19
02	20	19	19
03	20	20	20
04	20	20	19
05	19	18	17
06	20	19	20
07	NE	18	20
08	19	17	18
09	20	20	19
10	20	20	20
11	20	20	20
12	19	18	18
13	20	20	20
14	20	18	19

Details of the different 20 structures see Table 9.

Table 12 Number of subjects with specific detected metabolites in plasma samples depending on treatments (total n = 14). Data modifications deviant from the norm were agreed during the blind data review meeting (see page 64).

metabolite	COFFEE	CARB	FAT
3-FQA	13	13	14
4-FQA	11	9	12
5-CQA	13	14	14
5-FQA	13	14	14
CA3S	13	12	13
CA4S	13	9	9
DHCA	13	14	13
DHCA3S	13	13	14
DHFA	13	14	14
DHFA4G	13	14	14
DHFA4S	13	14	14
DHiFA	13	14	14
DHiFA3G	13	14	14
FA4G	12	13	13
FA4S	13	14	14
iFA	13	14	14
iFA3G	13	14	14
mDHCoA	13	14	13
mDHCoAS	13	14	13
MeFA	13	14	14

Area under the curve (AUC)

AUCs were calculated as described in chapter 6.4.5 Area under the curve (AUC).

The individual sum of AUC for plasma metabolites by treatment showed strong varying values (see Table 13), both interindividual and intraindividual. For instance, the lowest value was 315 µM*min for subject 14 (FAT) and the highest value 1,706 µM*min for subject 05 (COFFEE). Examples for strong intraindividual differences were subject 05 and subject 09. For subject 05 sum of AUC for plasma metabolites was 606 µM*min (FAT) compared to 1,706 µM*min (COFFEE). For subject 09 it was 353 µM*min (FAT) compared to 953 µM*min (COFFEE).

Table 13 Individual sum of AUC for plasma metabolites by subject and by treatment [nM*min]. Data modifications deviant from the norm were agreed during the blind data review meeting (see page 64). NE: not evaluated.

subject	COFFEE	CARB	FAT
01	974,862	700,417	852,199
02	1,046,453	788,510	720,931
03	673,906	749,112	821,840
04	622,874	820,865	606,084
05	1,705,997	950,539	605,720
06	794,508	388,212	662,454
07	NE	856,833	727,578
08	447,583	466,914	542,497
09	952,887	717,541	353,312
10	887,310	772,052	772,187
11	1,688,207	1,142,593	1,047,529
12	357,267	299,743	520,925
13	503,516	829,744	626,276
14	367,366	455,847	314,538

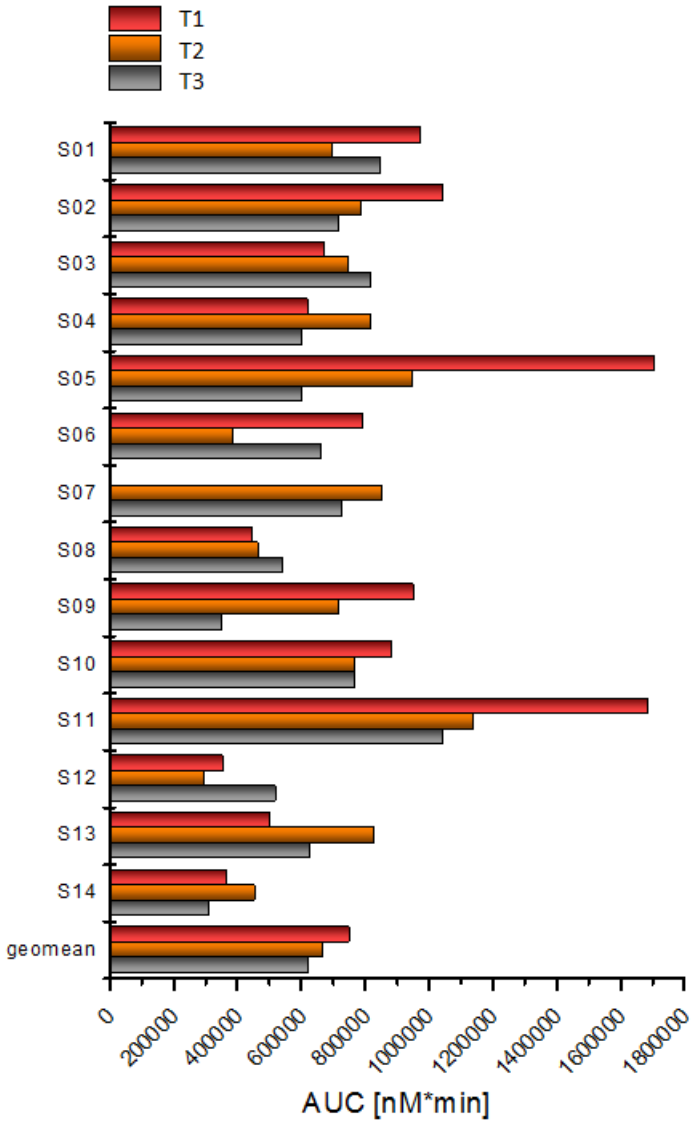


Figure 42 Sum of AUCs by treatment shown for single subjects as geometric mean. T1: COFFEE, T2: CARB, T3: FAT (n = 2; lower and upper bounds are hidden for a better overview)

Table 14 shows the summary statistics on sum of AUCs with lower and upper bounds by treatments and Figure 42 the barplot related to the AUCs of single subjects as well as summary statistics. The geomeans of summary statistics in sum of AUCs were approximately 752 µM*min for COFFEE treatment, 669 µM*min for CARB treatment and 626 µM*min for FAT treatment. The three treatments did not show any statistically significant differences among themselves, when comparing the sum of AUCs of all metabolites determined (see Table 15). Indeed, the ratio between the mean of CARB with respect to COFFEE was 0.876 with 95% CI equals to [0.713, 1.076] with an associated p-value of 0.0207. Comparing FAT to COFFEE showed a mean ratio of 0.825 and a 95% CI of [0.672, 1.014] with a p-value of 0.067. In tendency, intake of coffee in combination with a meal may

cause a decreased sum of plasma AUC. Whereas, the comparison of FAT treatment to CARB led to a mean ratio of 0.943 with a 95% CI of [0.770, 1.154] and a p-value of 0.566. The two treatments did not show differences even if the composition of ingredients varied.

Breakfast had no significant effect on CGA absorption when calculated as summary statistics on sum of plasma metabolite AUCs. A detailed observation of the metabolic profiles of the 14 study subjects demonstrated that there is no consistent influence of a food matrix on the bioavailability of CGAs from coffee. Only for a few metabolites exceptions were observed.

Table 14 Summary statistics on sum of plasma AUCs with lower and upper bounds [nM*min]. Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52).

	n	geomean	lower	upper
COFFEE	13	751,791	451,021	1,253,133
CARB	14	669,009	459,058	974,983
FAT	14	626,070	451,913	867,343

Table 15 Model-based treatments effect on sum of plasma AUCs with 95% confidence intervals.

	estimate	lower	upper	p-value
CARB / COFFEE	0.876	0.713	1.076	0.207
FAT / COFFEE	0.825	0.672	1.014	0.067
FAT / CARB	0.943	0.770	1.154	0.566

Overall, for the single metabolites and COFFEE treatment, the highest AUCs were observed for mDHCoA (142,728 nM*min; correspond to 25.8% of sum of all determined metabolites), DHFA (117,846 nM*min; correspond to 21.3%), DHCA3S (74,525 nM*min; correspond to 13.5%) and DHiFA (61,695 nM*min; correspond to 11.2%) (see Table 16). Thus, 71.8% of the sum of the plasma AUCs for all metabolites identified comprised of these four metabolites.

In addition, our initial analysis of metabolites by group showed that for all three treatments, plasma contained low levels of *quinics* and high levels of *colonics*, based on their AUC values. For example, we observed higher quantities of *phenolics* (48,253 nM*min, 8.7%: CA3S, CA4S, FA4G, FA4S, iFA, iFA3G, iFA3S, MeFA) and *colonics* (501,840 nM*min, 90.8%: DHCA, DHCA3S, DHFA, DHFA4G, DHFA4S, DHiFA, DHiFA3G, mDHCoA, mDHCoAS) than for *chlorogenics* (2,842 nM*min, 0.5%: 5-CQA, 3-FQA, 4-FQA, 5-FQA) (all COFFEE data).

Table 16 Summary statistics on plasma AUC for each metabolite and treatment with lower and upper bounds [nM*min]. Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52 and Table 12).

metabolite	n	geomean	lower	upper
3-FQA				
COFFEE	13	290.8	136.8	617.9
CARB	14	63.8	3.2	1280.1
FAT	14	316.6	130.9	765.9
4-FQA				
COFFEE	13	81.7	1.3	5171.3
CARB	14	9.0	0.1	1772.6
FAT	14	93.4	1.7	5106.2
5-CQA				
COFFEE	13	1232.5	570.1	2664.6
CARB	14	720.1	276.9	1872.6
FAT	14	1515.2	746.7	3074.8
5-FQA				
COFFEE	13	1236.6	629.1	2430.7
CARB	14	817.7	452.1	1479.0
FAT	14	932.2	529.4	1641.6
CA3S				
COFFEE	13	6927.3	1550.2	30955.5
CARB	14	2053.8	11.4	369739.5
FAT	14	4440.1	99.9	197291.3
CA4S				
COFFEE	13	97.7	21.9	437.0
CARB	14	2.3	0.0	175.9
FAT	14	3.3	0.0	342.1
DHCA				
COFFEE	13	24010.1	13824.3	41700.9
CARB	14	15994.3	8205.6	31176.3
FAT	14	5466.5	116.2	257065.3
DHCA3S				
COFFEE	13	74524.8	37649.7	147516.1
CARB	14	19917.4	285.1	1391615.0
FAT	14	48627.4	22000.4	107481.3
DHFA				
COFFEE	13	117846.3	59374.9	233899.4
CARB	14	115717.9	62402.1	214586.3
FAT	14	101955.4	64952.5	160038.6
DHFA4G				
COFFEE	13	33193.9	13982.0	78803.4
CARB	14	33671.2	15091.6	75124.5
FAT	14	30455.1	15472.4	59946.2

metabolite	n	geomean	lower	upper
DHFA4S				
COFFEE	13	26796.5	4298.7	167041.1
CARB	14	29088.4	6340.0	133458.8
FAT	14	22702.7	5605.5	91947.6
DHiFA				
COFFEE	13	61695.0	30292.9	125648.8
CARB	14	54884.2	35671.6	84444.8
FAT	14	56745.8	31619.8	101837.4
DHiFA3G				
COFFEE	13	12239.0	5192.9	28845.8
CARB	14	11544.0	4861.3	27413.3
FAT	14	9900.5	4912.3	19954.0
FA4G				
COFFEE	12	5939.4	3247.8	10861.7
CARB	13	7806.9	3715.2	16404.9
FAT	13	7273.5	2815.0	18793.0
FA4S				
COFFEE	13	3963.7	1244.1	12628.7
CARB	14	5297.8	1812.2	15487.2
FAT	14	5215.7	1826.2	14895.8
iFA				
COFFEE	13	1324.5	516.8	3395.0
CARB	14	1673.7	830.4	3373.5
FAT	14	1048.6	336.9	3264.4
iFA3G				
COFFEE	13	14955.4	9623.1	23242.2
CARB	14	17164.0	11021.4	26730.0
FAT	14	14782.6	9667.8	22603.5
mDHCoA				
COFFEE	13	142728.1	37515.5	543010.1
CARB	14	121833.4	44070.5	336809.8
FAT	13	166986.3	78657.7	354503.6
mDHCoAS				
COFFEE	13	33193.9	13982.0	78803.4
CARB	14	33671.2	15091.6	75124.5
FAT	13	30455.1	15472.4	59946.2
MeFA				
COFFEE	13	26796.5	4298.7	167041.1
CARB	14	29088.4	6340.0	133458.8
FAT	14	22702.7	5605.5	91947.6

Isomers (CA3S and CA4S, DHCA3S and DHCA4S, DHFA snd DHiFA, DHFA3G and DHFA4G) showed preferential regions for conjugation, if comparing the formation rates. For example, both metabolites, CA3S (6927.3 nM*min, COFFEE) and DHCA3S

(74524.8 nM*min, COFFEE), were preferred structures compared to the corresponding 4-isomers (CA4S: 97.7 nM*min, COFFEE, DHCA4S: N/A). We observed a similar structural preference also for methylation, as we found greater AUCs for DHFA (117846.3 nM*min, COFFEE) and DHFA4G (33193.9 nM*min, COFFEE), as for DHiFA (61695.0 nM*min, COFFEE) and DHiFA3G (12239.0 nM*min, COFFEE), respectively. If glucuronidation would be the favored conjugation, DHiFA3G would be preferred. In addition, methylation seemed also favored. In our search for methylated metabolites, we found that the quantities of DHiFA and DHFA were three- and ten-times higher than the quantity of DHCA (24010.1 nM*min, COFFEE), respectively. Also high quantities of iFA (1324.5 nM*min, COFFEE) and MeFA (26796.5 nM*min, COFFEE), and not pure CA and FA (as aglycons), were detected. Furthermore, caffeic and coumaric acid derivatives seemed to be preferred sulfated, whereas FA and DHFA derivatives appeared to be preferred glucuronidated. For example, FA4G (5939.4 nM*min, COFFEE) was more abundant than FA4S (3963.7 nM*min, COFFEE) and DHFA4G (33193.9 nM*min, COFFEE) was more abundant than DHFA4S (26796.5 nM*min, COFFEE). These effects were independent from treatment.

Compounds are metabolized as soon as they enter the body. Liorach and coworkers, found glucuronidation take place faster and glucuronides appear ealier in urine of humans than sulfates [*Liorach et al., 2010*]. Erk and coworkers were able to specify this finding in more detail as results given from an ileostomists study, showed that CA and derivatives were predominantly sulfated, and FA and derivatives were to a greater extent glucuronidated and renally excreted [*Erk et al., 2012, Guy et al., 2009*]. This is in line with our data indicating that for ferulic acid and derivatives glucuronidation was preferred. Nevertheless, as caffeic and coumaric acid and derivatives seemed to be preferred sulfated, also the pH-value, the stabilization by a π-electron system, and the steric influence of the methyl group of FA, may play a role which final conjugation step is occurring. In addition, Wen and Walle investigated the hepatic metabolic stability and intestinal absorption of either methylated or non-methylated polyphenols *in vitro.* For the non-methylated polyphenols a rapidly elimination was observed when a human liver S9 fraction including the appropriate cofactors for conjugation and oxidation was used. In contrast, methylation indicated to give a higher resistance to hepatic metabolism, and due to an increased stability. Additionally, a 5 – 8 times larger uptake of methylated flavones than of the nonmethylated ones was observed [*Wen and Walle, 2006*].
All these findings may explain the higher amounts of methylated compounds in humans, as well.

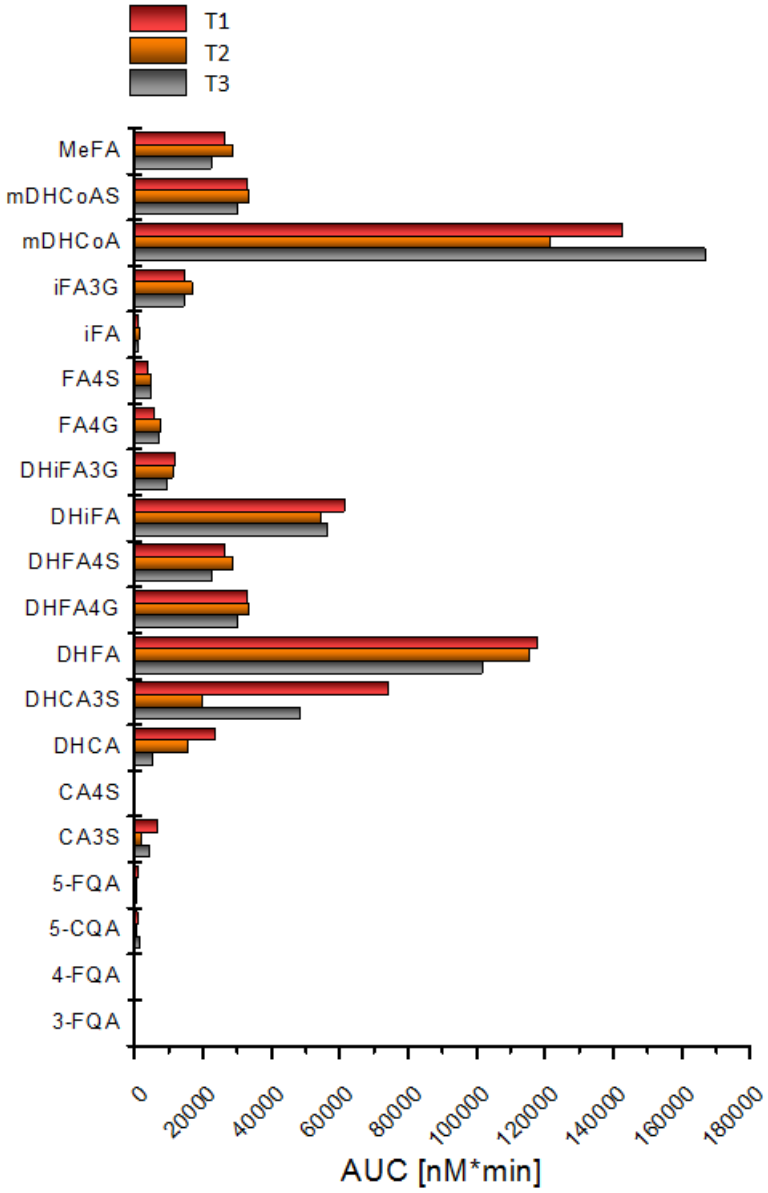


Figure 43 Plasma bioavailability as AUC by treatment and metabolites ploted as geometric means. T1: COFFEE, T2: CARB, T3: FAT. (n = 13 or 14 (see Table 16)) Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52). Lower and upper bounds are hidden for a better overview.

Table 17 and Figure 43 show the correlation of AUCs dependent on treatment (CARB / COFFEE, FAT / COFFEE and FAT / CARB) with a detectable treatment effect on AUC with 95% confidence interval for selected metabolites. All the quinic acid esters showed no significance between FAT and COFFEE treatment, except 5-FQA. However, they showed a strong significance between CARB and COFFEE as well as FAT and CARB treatment, except 5-FQA which showed no significance between FAT and CARB. The only *phenolics* giving significance were CA4S for CARB and COFFEE as well as FAT and COFFEE (see definition of *quinics*, *phenolics*, *colonics*, *caffeics*, *ferulics*, *sulfates*, *glucuronides*, *aglycons*

and *methylated in* Table 10), iFA for FAT and CARB, iFA3G for CARB and COFFEE and MeFA for CARB and COFFEE. None of the *colonics* gave significance.

In summary, only for the early metabolites (compare t_{max} values in Table 25) a treatment effect on AUC could be observed, even if the effects are not consistent between the treatments. To investigate, if this effect was compound dependent, a higher number of data would be useful to get statistical significances.

Table 17 Model-based treatments effect on plasma AUCs for each metabolite with 95% confidence intervals.

metabolite	estimate	lower	upper	p-value
3-FQA				
CARB / COFFEE	0.269	0.088	0.818	0.021
FAT / COFFEE	1.332	0.437	4.059	0.614
FAT / CARB	4.961	1.672	14.716	0.004
4-FQA				
CARB / COFFEE	0.137	0.026	0.706	0.018
FAT / COFFEE	1.424	0.276	7.350	0.673
FAT / CARB	10.411	2.107	51.449	0.004
5-CQA				
CARB / COFFEE	0.590	0.358	0.971	0.038
FAT / COFFEE	1.220	0.738	2.018	0.437
FAT / CARB	2.069	1.270	3.370	0.003
5-FQA				
CARB / COFFEE	0.645	0.494	0.843	0.001
FAT / COFFEE	0.736	0.563	0.961	0.024
FAT / CARB	1.140	0.879	1.479	0.324
CA3S				
CARB / COFFEE	0.260	0.040	1.705	0.160
FAT / COFFEE	0.551	0.082	3.715	0.540
FAT / CARB	2.119	0.339	13.239	0.422
CA4S				
CARB / COFFEE	0.018	0.002	0.156	0.000
FAT / COFFEE	0.026	0.003	0.224	0.001
FAT / CARB	1.435	0.183	11.279	0.731
DHCA				
CARB / COFFEE	0.709	0.131	3.831	0.690
FAT / COFFEE	0.222	0.041	1.193	0.079
FAT / CARB	0.313	0.060	1.640	0.169
DHCA3S				
CARB / COFFEE	0.288	0.042	1.953	0.202
FAT / COFFEE	0.556	0.081	3.816	0.551
FAT / CARB	1.932	0.287	13.008	0.499

metabolite	estimate	lower	upper	p-value
DHFA				
CARB / COFFEE	0.984	0.737	1.313	0.912
FAT / COFFEE	0.859	0.646	1.142	0.295
FAT / CARB	0.873	0.659	1.156	0.342
DHFA4G				
CARB / COFFEE	0.999	0.751	1.327	0.992
FAT / COFFEE	0.906	0.682	1.204	0.496
FAT / CARB	0.907	0.688	1.197	0.491
DHFA4S				
CARB / COFFEE	1.116	0.796	1.564	0.525
FAT / COFFEE	0.862	0.618	1.202	0.381
FAT / CARB	0.772	0.558	1.069	0.119
DHiFA				
CARB / COFFEE	0.868	0.589	1.279	0.474
FAT / COFFEE	0.897	0.609	1.321	0.581
FAT / CARB	1.033	0.707	1.509	0.867
DHiFA3G				
CARB / COFFEE	0.911	0.621	1.336	0.632
FAT / COFFEE	0.781	0.532	1.145	0.204
FAT / CARB	0.857	0.593	1.238	0.411
FA4G				
CARB / COFFEE	1.267	0.793	2.024	0.323
FAT / COFFEE	1.180	0.738	1.886	0.490
FAT / CARB	0.931	0.595	1.457	0.756
FA4S				
CARB / COFFEE	1.192	0.902	1.575	0.216
FAT / COFFEE	1.214	0.922	1.598	0.167
FAT / CARB	1.018	0.778	1.333	0.895
iFA				
CARB / COFFEE	1.172	0.796	1.724	0.421
FAT / COFFEE	0.765	0.526	1.115	0.163
FAT / CARB	0.653	0.449	0.951	0.026
iFA3G				
CARB / COFFEE	1.182	1.012	1.380	0.035
FAT / COFFEE	1.019	0.872	1.190	0.816
FAT / CARB	0.862	0.741	1.002	0.054
mDHCoA				
CARB / COFFEE	0.832	0.477	1.453	0.518
FAT / COFFEE	1.176	0.659	2.100	0.583
FAT / CARB	1.413	0.805	2.482	0.229
mDHCoAS				
CARB / COFFEE	0.861	0.156	4.756	0.864
FAT / COFFEE	0.288	0.051	1.607	0.156
FAT / CARB	0.334	0.062	1.807	0.203
MeFA				
CARB / COFFEE	0.749	0.582	0962	0.024
FAT / COFFEE	0.860	0.669	1105	0.238
FAT / CARB	1.148	0.900	1466	0.266

The above-mentioned findings are summarized in Table 18 as statistical significant differences for AUC for each metabolite and each treatment.

Table 18 Summary of statistical significant differences observed for individual metabolite plasma AUC. “>1” means the numerator is higher. “<1” means the denominator is higher.

	CARB / COFFEE	FAT / COFFEE	FAT / CARB
3-FQA	<1		>1
4-FQA	<1		>1
5-CQA	<1		>1
5-FQA	<1	<1	
CA4S	<1	<1	
iFA			<1
iFA3G	>1		
MeFA	>1		

Additionally to the statistical analysis for single metabolites, the metabolites were divided into groups, according to similaries and chemical properties, which were then compared to determine any possible differences (see Table 10).

Table 19 shows the summary statistics on sum of AUCs for *quinics*, *phenolics*, *colonics*, *caffeics*, *ferulics*, *sulfates*, *glucuronides*, *aglycons* and *methylated* with lower and upper bounds by treatments (see also Figure 44). For all three treatments, our initial analysis of the metabolites by main groups (*quinics*, *phenolics* and *colonics*) showed very low sums of AUCs from plasma after only coffee consumption for the *quinics*, and high sums for the *colonics*.

Table 19 Summary statistics on sum of plasma AUCs for *quinics*, *phenolics*, *colonics*, *ferulics*, *sulfates*, *glucuronides*, *aglycons* and *methylated* with lower and upper bounds [nM*min].

	n	geomean	lower	upper
quinics				
COFFEE	13	3,414	1,826	6,383
CARB	14	2,263	1,290	3,968
FAT	14	3,419	1,911	6,117
phenolisc				
COFFEE	13	58,020	40,983	82,138
CARB	14	63,832	43,215	94,285
FAT	14	61,827	43,968	86,939
colonics				
COFFEE	13	678,482	386,128	1,192,192
CARB	14	595,412	392,430	903,384
FAT	14	552,352	378,628	805,786
caffeics				
COFFEE	13	112,489	698,28	181,215
CARB	14	106,115	778,43	144,654
FAT	14	105,239	765,12	144,750
ferulics				
COFFEE	13	259365	110039	611331
CARB	14	250560	119811	523994
FAT	14	224745	132393	381519
sulfates				
COFFEE	13	181427	76256	431647
CARB	14	154480	65667	363410
FAT	14	134077	65284	275359
glucuronides				
COFFEE	13	72318	41530	125931
CARB	14	75231	42765	132343
FAT	14	66874	40415	110655
aglycons				
COFFEE	13	148379	79507	276910
CARB	14	136054	74984	246862
FAT	14	121122	77630	188980
methylated				
COFFEE	13	207110	115228	372258
CARB	14	189808	112683	319721
FAT	14	184298	123553	274908

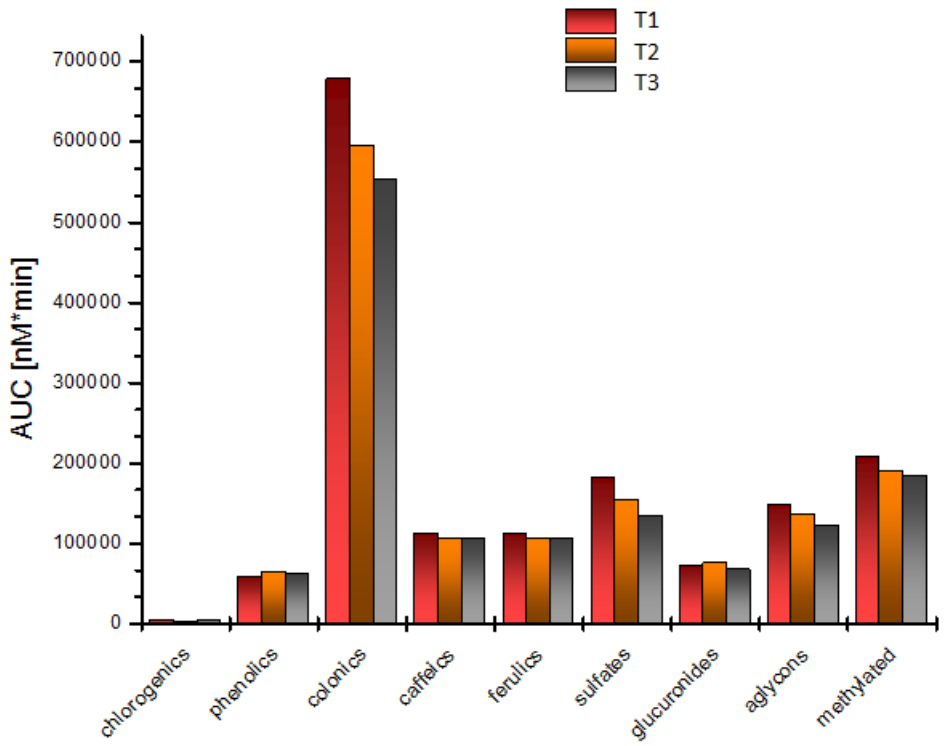


Figure 44 Sum of plasma AUCs for *chlorogenic*, *phenolic*, *colonic*, *caffeic*, *ferulic*, *sulfated*, *glucuronided*, *aglycon* and *methylated* compounds by treatment plotted as geometric means. T1: COFFEE, T2: CARB, T3: FAT. (n = 13 or 14 (see Table 19)); Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52). Lower and upper bounds are hidden for a better overview.

In the plasma samples of the 14 healthy volunteers, we determined approximately 0.5% (3,414 nM*min) as *quinics* of the sum of the AUCs of all metabolites identified, 8.7% (58,020 nM*min) as *phenolics* and the highest quantity (approximately 90.8% (678,482 nM*min)) revealed the ones formed in the colon (*colonics*). The single metabolites which were related to the three groups may be subjected to varying amounts. To point it out, the geomeans were between 9.0 nM*min (4-FQA, CARB) and 1,236.6 nM*min (5-FQA, COFFEE) for the *quinics*, between 2.3 nM*min (CA4S, CARB) and 29,088.4 nM*min (MeFA, CARB) for the *phenolics* and between 5,466.5 nM*min (DHCA, FAT) and 117,846 nM*min (DHFA, COFFEE) for the *colonics*. For all treatments, the four *colonics* with the highest plasma concentrations were mDHCoA (142,728 nM*min; 25.8%)), DHFA (117,846 nM*min; 21.3%), DHCA3S (74,525 nM*min; 13.5%), and DHiFA (61,695 nM*min; 11.2%). Noticeable for these compounds was a slow distribution phase followed by a much slower terminal elimination phase, giving a long time period with relatively high plasma concentrations (cp.

Figure 41). Thus, 71.8% of the sum of the plasma AUCs for all metabolites identified comprised of these four metabolites.

Calculating the amounts as percent of sum of AUC of all determined metabolites (COFFEE treatment) gave 15.2% for the *caffeics*, 35.0% for the *ferulics*, 24.5% for the *sulfates*, 9.8% for the *glucuronides*, 20.1% for the *aglycons* and 28.0% for the *methylated* compounds.

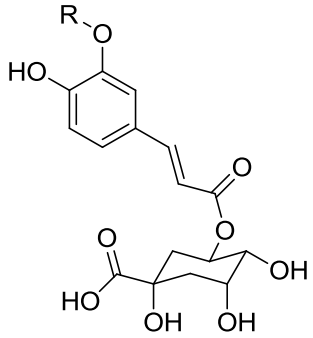
Table 20 shows the effects of treatments on AUC with 95% confidence interval for grouped derivatives. For quinic derivatives, significant statistical differences were observed between CARB and COFFEE as well as between FAT and CARB treatment. For phenolic, colonic, caffeic and ferulic derivatives, the three treatments do not show any statistically significant differences among themselves. For sulfated derivatives, a significant statistical difference was observed between FAT and COFFEE treatment. For remaining derivatives, that is glucuronide, aglycons and methylated derivatives, the three treatments did not show any statistically significant differences among themselves.

Table 20 Model-based effects of treatments on sum of plasma AUCs for *chlorogenics*, *phenoloics*, *colonics*, *ferulics*, *sulfates*, *glucuronides*, *aglycons* and *methylated* with 95% confidence intervals.

	estimate	lower	upper	p-value
chlorogenics				
CARB / COFFEE	0.673	0.503	0.9	0.008
FAT / COFFEE	1.016	0.759	1.36	0.914
FAT / CARB	1.511	1.137	2.007	0.004
phenolics				
CARB / COFFEE	1.08	0.96	1.216	0.202
FAT / COFFEE	1.046	0.929	1.177	0.455
FAT / CARB	0.969	0.863	1.087	0.587
colonics				
CARB / COFFEE	0.868	0.689	1.093	0.228
FAT / COFFEE	0.805	0.639	1.014	0.065
FAT / CARB	0.928	0.741	1.162	0.513
caffeics				
CARB / COFFEE	0.937	0.723	1.215	0.625
FAT / COFFEE	0.93	0.717	1.205	0.581
FAT / CARB	0.992	0.769	1.278	0.949
ferulics				
CARB / COFFEE	0.965	0.769	1.21	0.757
FAT / COFFEE	0.865	0.69	1.086	0.211
FAT / CARB	0.897	0.719	1.118	0.334
sulfates				
CARB / COFFEE	0.834	0.68	1.023	0.082
FAT / COFFEE	0.724	0.591	0.888	0.002
FAT / CARB	0.868	0.712	1.058	0.162
glucuronides				
CARB / COFFEE	1.021	0.856	1.219	0.814
FAT / COFFEE	0.908	0.761	1.084	0.286
FAT / CARB	0.889	0.748	1.056	0.181
aglycons				
CARB / COFFEE	0.913	0.704	1.185	0.494
FAT / COFFEE	0.813	0.627	1.055	0.119
FAT / CARB	0.89	0.691	1.147	0.369
methylated				
CARB / COFFEE	0.906	0.698	1.175	0.456
FAT / COFFEE	0.879	0.678	1.141	0.333
FAT / CARB	0.971	0.753	1.252	0.82

According to previous studies, intact CGA were determined only at low levels in plasma, and 3-, 4-, and 5-CQA could not be detected after coffee consumption at all [*Matsui et al., 2007*, *Nardini et al., 2002, Stalmach et al., 2009*]. Our results confirmed these findings, as only 0.5% of all the metabolites we detected were quinic conjugates. For the *quinics*, we only identified 5-CQA in measureable amounts in plasma, but we were able to detect all FQA isomers in the same plasma samples. This is in contrast to the total amount of CGA in the instant coffee that was consumed, in which the CQA were detected at much higher concentrations as the FQA (5-CQA: 46%, 3-CQA: 12%, 4-CQA: 16%, 5-FQA: 9%, and 4-FQA: 2%). Finally, the mechanisms of absorption of FQA and CQA are not fully investigated. Erk *et al*. also observed that FQA were absorbed *in vivo* to a higher extent than CQA. They speculated that this finding is related to the different chemical structures of the compounds. The increasing molecular size and higher lipophilicity of FQA might lead to a better absorption. Additionally, the increasing permeation of FQA may be explained by absorption mechanisms, presumably active transport as discussed by Erk *et al.* [*Erk et al., 2013*]. Due to high concentrations of CA3S, CQA from coffee may be cleaved into CA, and then further sulfated. It is reported that FQA seem to be more stable against metabolism and/or hydrolysis [*Farrell et al., 2011*] but it is also conceivable, that CQA are methylated to FQA. Moreover, Konishi and coworkers showed that FA has a higher affinity for the monocarboxylic transporter (MCT) than CA [*Konishi et al., 2006*]. If we transfer this knowledge to FQA and CQA, CQA may not be a good MCT substrate, which could explain the lower absorption of CQA compared to FQA in our study. Even if metabolite transport might occur via passive diffusion, less CQA would be absorbed [*Konishi et al., 2005, Murota&Terao, 2003*] because CQA have a lower predicted octanol-water partition coefficient (log*P*) and are thus more polar than FQA [*ChemSpider a&b*]. In detail, the logP value is a partition coefficient which indicates the ratio of the concentration of a chemical in a two-phase system of n-octanol and water. The logP value of 5-CQA is lower than the one of 5-FQA meaning that 5-CQA is more hydrophil than 5-FQA. The different chemical structures, monoisotopic masses and log*P* values of 5-CQA and 5-FQA are shown in Table 21.

Table 21 Molecular formula, monoisotopic masses and log*P* values of 5-CQA and 5-FQA [*ChemSpider a&b*]



	5-CQA (R = H)	5-FQA (R = CH_3)
molecular formula	$C_{16}H_{18}O_9$	$C_{17}H_{20}O_9$
monoisotopic mass	354.095 Da	368.111 Da
log*P*	-0.36	-0.14

As described, in our study, we observed higher amounts of *phenolics* (8.7%: CA3S, CA4S, FA4G, FA4S, iFA, iFA3G, iFA3S, MeFA) and *colonics* (90.8%: DHCA, DHCA3S, DHFA, DHFA4G, DHFA4S, DHiFA, DHiFA3G, mDHCoA, mDHCoAS) than for *chlorogenics* (0.5%: 5-CQA, 3-FQA, 4-FQA, 5-FQA). Notably, these compounds were subject to a slow distribution phase followed by an even slower terminal elimination phase. Thus for an extended time period, the plasma concentrations of these metabolites, i.e. 78% of all the metabolites we investigated, were relatively high. Stalmach and coworkers identified DHFA4S, DHCA3S, FA4S, DHFA, and CA3S as the most abundant metabolites in eleven healthy volunteers after ingestion of a single cup of instant coffee containing 412 µmol of CGA [*Stalmach et al., 2009*].

Also coumaric acid derivatives were determined as abundant metabolites in our study. For example mDHCoAS was detected as metabolite circulating in high concentrations in the plasma samples after coffee consumption. Researchers should investigate whether these compounds are responsible for the beneficial effects of coffee. Literature data suggests that, for example, DHCA has photo-protective effects – it interferes with biological events that initiate after UV exposure in HaCaT cells [*Poquet et al., 2008*]. Another beneficial effect, a higher scavenging effect than that of α-tocopherol and CA, has been reported [*Lekse et al., 2001*, *Silva et al., 2000*]. However, limited data on biological effects of DHFA, mDHCoA, DHCA3S and DHiFA are available, and these metabolites should be investigated in the future.

Higher AUCs for the *quinics* and early metabolites were detectable when only a single dose of coffee was ingested. Fasting conditions led to lower gastric pH-values (pH 1.5 – 3) as during meal intake (pH > 5). That may have a stabilizing effect on the CGA. The meals may trigger impaired absorption, but only for early metabolites. This finding may be explained by a longer resting time in the stomach and upper GIT based on the food matrix. This delay in absorption in the stomach and GIT may cause longer presence in the GIT and therefore an increased degradation by intestinal enzymes and bacteria. This is notably for the *quinics*, except for 5-FQA. In addition, Price and coworkers have observed a fasting-induced suppression of glucuronidation in rats [*Price and Jollow, 1989*]. We cannot confirm this effect with data of our human intervention study, in which no significant differences were detected regarding sum of AUCs of glucuronides between all treatments. However, an additional fasting-induced suppression cannot be ruled out as the results received after treatment of 14 participants gave an overview but no verified data. Comparing the results for the two treatments with either carbohydrate-rich (CARB) or fat-rich (FAT) food, the trend showed higher *quinics* AUCs after FAT treatment than after CARB treatment. The approximate 33 g additional fat ingested by the fat-rich (FAT) food, (compared to high-carb food treatment (CARB)) may have allowed better partitioning, or better access to the membrane or transporter, as mentioned by Jacobsen *et al*. before [*Jacobsen et al., 1999*]. These trends were not detected for 5-FQA, and also iFA showed the opposite effect. Nevertheless, only low fat-induced effects on AUC were observed. The reason may be the hydrophilicity of CGA, meaning that dietary lipids have a limited influence on the absorption and bioavailability on this kind of polyphenols. This finding was confirmed by an *in vitro* digestion model, which showed no higher bioaccessibility of phenolic acids from lipid-rich cacao liquor (45% fat) in comparison to cacao powder (15% fat) [*Ortega et al., 2009*].

Maximum plasma concentration (C_{max})

As secondary outcome the effect of the different treatments on the plasma kinetics other than the AUCs and sum of AUCs were investigated.

Table 22 shows the summary statistics on plasma C_{max} with lower and upper bounds by treatments for each metabolite (see also Figure 45). The geomeans were between 7.6 nM (3-FQA, CARB) and 19.2 nM (5-CQA, COFFEE) for the *quinics*, between 5.8 nM (CA4S, CARB) and 60.7 nM (MeFA, COFFEE) for the *phenolics* and 40.1 nM (DHiFA3G, FAT) and 449.2 nM (DHFA, COFFEE) for the *colonics*.

Table 22 Summary statistics on plasma C_{max} for each metabolite and treatment with lower and upper bounds [nM]. Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52 and Table 12).

metabolite	n	geomean	lower	upper
3-FQA				
COFFEE	13	10.7	8.6	13.5
CARB	14	7.6	5.4	10.6
FAT	14	9.9	7.4	13.2
4-FQA				
COFFEE	13	9.5	6.4	13.9
CARB	14	7.4	5.2	10.4
FAT	14	9.4	6.2	14.2
5-CQA				
COFFEE	13	19.2	12.9	28.5
CARB	14	13.6	8.5	21.6
FAT	14	18.4	11.7	28.9
5-FQA				
COFFEE	13	17.5	13.2	23.0
CARB	14	12.0	9.5	15.3
FAT	14	14.1	10.9	18.1
CA3S				
COFFEE	13	57.1	28.1	116.0
CARB	14	46.2	17.3	123.1
FAT	14	49.9	22.7	109.9
CA4S				
COFFEE	13	9.8	7.0	13.8
CARB	14	5.8	4.5	7.3
FAT	14	6.2	4.9	7.9
DHCA				
COFFEE	13	88.5	56.3	139.4
CARB	14	62.7	35.6	110.6
FAT	14	50.5	20.6	124.3
DHCA3S				
COFFEE	13	240.3	126.4	456.8
CARB	14	149.4	43.2	516.4
FAT	14	169.4	76.9	373.3

metabolite	n	geomean	lower	upper
DHFA				
COFFEE	13	449.2	262.8	767.9
CARB	14	416.4	236.9	732.2
FAT	14	399.0	229.7	693.0
DHFA4G				
COFFEE	13	120.0	62.6	22985.0
CARB	14	117.2	59.1	23267.0
FAT	14	110.5	58.2	21001.0
DHFA4S				
COFFEE	13	111.8	23.1	54154.0
CARB	14	106.5	25.9	43892.0
FAT	14	95.4	26.9	33782.0
DHiFA				
COFFEE	13	279.6	174.4	44830.0
CARB	14	212.3	137.4	32812.0
FAT	14	234.1	139.9	39183.0
DHiFA3G				
COFFEE	13	47.9	24.6	9301.0
CARB	14	41.6	22.4	7738.0
FAT	14	40.1	20.6	7801.0
FA4G				
COFFEE	12	30.5	22.5	4155.0
CARB	13	30.2	19.1	4770.0
FAT	13	28.8	18.3	4540.0
FA4S				
COFFEE	13	37.0	20.8	6583.0
CARB	14	25.8	14.7	4543.0
FAT	14	29.2	17.2	4952.0
iFA				
COFFEE	13	23.0	16.1	3283.0
CARB	14	17.2	12.1	2444.0
FAT	14	16.5	10.6	2545.0
iFA3G				
COFFEE	13	51.3	37.3	7046.0
CARB	14	49.2	33.5	7241.0
FAT	14	47.0	32.6	6787.0
MeFA				
COFFEE	13	60.7	36.7	10030.0
CARB	14	44.1	28.1	6918.0
FAT	14	44.2	27.6	7058.0

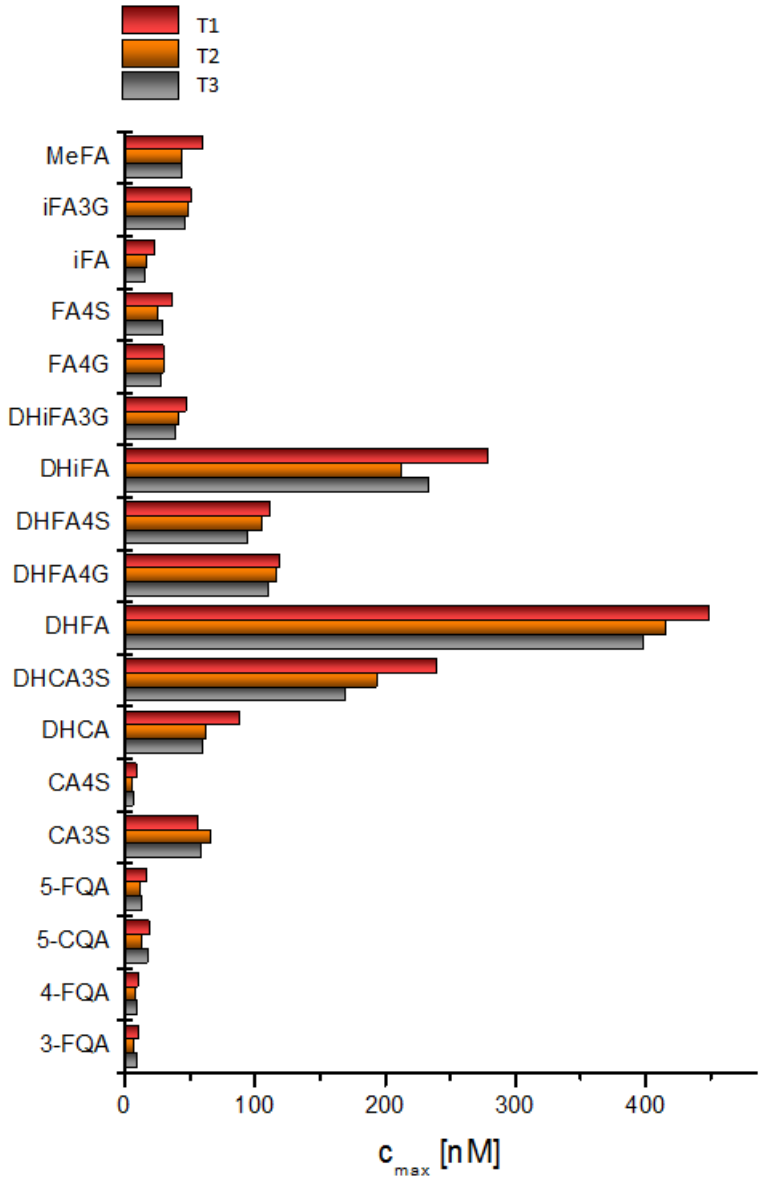


Figure 45 Plasma C_{max} by treatment and metabolites plotted as geometric means. T1: COFFEE, T2: CARB, T3: FAT. (n = 13 or 14 (see Table 22)); Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52 and). Lower and upper bounds are hidden for a better overview.

Table 23 shows the correlation of C_{max} dependent on treatment with a detectable treatment effect on C_{max} with 95% confidence interval for selected metabolites. The C_{max} variable was noticeably affected by breakfast consumption. For all *quinics,* there were significant correlations in C_{max} between CARB and COFFEE as well as FAT and CARB. Five of eight *phenolics* also showed significant correlations between treatments: CA3S for CARB / COFFEE and CA4S, FA4S, iFA and MeFA for CARB / COFFEE as well as FAT / COFFEE. Only two *colonics*, DHCA for FAT and COFFEE and DHiFA for CARB and COFFEE, showed significant correlations between treatments.

Table 23 Model-based treatments effect on C_{max} for each metabolite with 95% confidence intervals.

metabolite	estimate	lower	upper	p-value
3-FQA				
CARB / COFFEE	0.715	0.630	0.811	0.000
FAT / COFFEE	0.936	0.825	1.062	0.303
FAT / CARB	1.309	1.157	1.480	0.000
4-FQA				
CARB / COFFEE	0.794	0.717	0.879	0.000
FAT / COFFEE	1.014	0.916	1.123	0.786
FAT / CARB	1.277	1.157	1.410	0.000
5-CQA				
CARB / COFFEE	0.740	0.633	0.865	0.000
FAT / COFFEE	1.044	0.892	1.222	0.591
FAT / CARB	1.410	1.211	1.643	0.000
5-FQA				
CARB / COFFEE	0.681	0.598	0.775	0.000
FAT / COFFEE	0.796	0.699	0.906	0.001
FAT / CARB	1.168	1.030	1.325	0.016
CA3S				
CARB / COFFEE	0.779	0.630	0.964	0.021
FAT / COFFEE	0.835	0.673	1.036	0.102
FAT / CARB	1.072	0.872	1.317	0.510
CA4S				
CARB / COFFEE	0.604	0.513	0.712	0.000
FAT / COFFEE	0.651	0.553	0.767	0.000
FAT / CARB	1.078	0.923	1.259	0.345
DHCA				
CARB / COFFEE	0.729	0.490	1.084	0.118
FAT / COFFEE	0.571	0.385	0.849	0.006
FAT / CARB	0.784	0.531	1.158	0.222
DHCA3S				
CARB / COFFEE	0.632	0.359	1.110	0.110
FAT / COFFEE	0.631	0.358	1.114	0.112
FAT / CARB	0.999	0.569	1.756	0.998
DHFA				
CARB / COFFEE	0.967	0.731	1.278	0.812
FAT / COFFEE	0.878	0.666	1.158	0.357
FAT / CARB	0.908	0.692	1.193	0.490
DHFA4G				
CARB / COFFEE	0.962	0.760	1.217	0.746
FAT / COFFEE	0.892	0.705	1.129	0.342
FAT / CARB	0.928	0.738	1.167	0.521

metabolite	estimate	lower	upper	p-value
DHFA4S				
CARB / COFFEE	1.037	0.786	1.369	0.795
FAT / COFFEE	0.881	0.670	1.158	0.363
FAT / CARB	0.849	0.650	1.109	0.230
DHiFA				
CARB / COFFEE	0.760	0.577	0.999	0.049
FAT / COFFEE	0.838	0.637	1.103	0.207
FAT / CARB	1.103	0.844	1.442	0.472
DHiFA3G				
CARB / COFFEE	0.865	0.662	1.130	0.286
FAT / COFFEE	0.833	0.638	1.088	0.180
FAT / CARB	0.963	0.745	1.245	0.775
FA4G				
CARB / COFFEE	1.042	0.871	1.246	0.653
FAT / COFFEE	0.996	0.833	1.191	0.965
FAT / CARB	0.956	0.807	1.133	0.603
FA4S				
CARB / COFFEE	0.690	0.604	0.789	0.000
FAT / COFFEE	0.767	0.672	0.876	0.000
FAT / CARB	1.112	0.977	1.266	0.109
iFA				
CARB / COFFEE	0.722	0.647	0.806	0.000
FAT / COFFEE	0.700	0.629	0.779	0.000
FAT / CARB	0.969	0.871	1.079	0.569
iFA3G				
CARB / COFFEE	0.979	0.859	1.116	0.755
FAT / COFFEE	0.933	0.818	1.064	0.298
FAT / CARB	0.952	0.838	1.082	0.451
MeFA				
CARB / COFFEE	0.731	0.673	0.795	0.000
FAT / COFFEE	0.733	0.674	0.797	0.000
FAT / CARB	1.002	0.925	1.087	0.955

The above-named findings are summarized in Table 24 as statistical significant differences for C_{max} for each metabolite and each treatment.

Table 24 Summary of statistical significant differences observed for individual metabolite plasma C_{max}. ">1" means the numerator is higher. "<1" means the denominator is higher.

	CARB / COFFEE	FAT / COFFEE	FAT / CARB
3-FQA	<1		>1
4-FQA	<1		>1
5-CQA	<1		>1
5-FQA	<1	<1	>1
CA3S	<1		
CA4S	<1	<1	
DHCA		<1	
DHiFA	<1		
FA4S	<1	<1	
iFA	<1	<1	
MeFA	<1	<1	

We observed that there were significant differences in C_{max} between treatments for *quinics* and *phenolics* (see Table 23 and Table 24). Except for the two metabolites DHCA and DHFA, there were no significant differences in C_{max} between treatments for the *colonics*. As previously shown for the plasma AUCs of our study, C_{max} values detected for most metabolites measured after consumption of coffee without a meal were significantly higher than for metabolites measured after consumption of coffee with a meal. This may be due to faster metabolite absorption, resulting in a fast distribution phase and followed by an even faster terminal elimination phase (sharper but shorter curves). Additionally, we observed significant correlations of C_{max}, only for the *quinics*, and carbohydrate (CARB) and fat-rich (FAT) treatments. A fat-rich meal led to higher amounts of *quinic* metabolites in plasma than a high-carb meal. This result suggests that there is a substantial influence of treatments on C_{max} for the *quinics*.

Time at which C_{max} is observed (t_{max})

The reported influence of threatments on C_{max} also affected the time at which C_{max} occured. Therefore, we also investigated the of t_{max} in relation to the different treatments.

Table 25 shows the summary statistics on plasma t_{max} with lower and upper bounds by treatments (see also Figure 46). The first appearing metabolite was iFA with a t_{max} of 15.8 min, when only coffee was ingested. However, all metabolites defined in the *quinic* or *phenolic* group ahowed at latest a t_{max} value of 41.7 min (for iFA3G, COFFEE). A fat rich meal led to a delay up to 118.9 min for FA4G. In contrast to this, the defined group of *colonic* metabolites showed an accordingly delayed t_{max} compared to the metabolites of the *quinic* and *phenolic* groups, respectively. The range of t_{max} was between 396.5 min (DHFA; COFFEE) and 612.3 min (DHiFA3G, CARB). For most of the dihydro metabolites, excepted DHFA4S and DHiFA, did not reach a baseline value, even after 15 h (see Figure 28 Figure 31, and Figure 34, Figure 39, Figure 40). It is conceivable that we showed probably only the beginning of the colonic metabolites entering in the bloodstream.

After initial detection at 6 h post-treatment, the metabolites FA4S and MeFA were detected a second time at 12 h (see Figure 36 and Figure 41). Also, after COFFEE there was an early increase in detection of the DH compounds in plasma, when compared to the other treatments (see for examples Figure 28, 30 and 34).

Table 25 Summary statistics on plasma t_{max} for each metabolite ad treatment with lower and upper bounds [min]. Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52 and Table 12).

metabolite	n	geomean	lower	upper
3-FQA				
COFFEE	13	28.4	20.2	40.0
CARB	13	68.6	44.7	105.2
FAT	14	69.4	49.4	97.3
4-FQA				
COFFEE	11	36.2	23.2	56.7
CARB	9	93.5	70.3	124.2
FAT	12	83.3	67.3	103.2
5-CQA				
COFFEE	13	30.0	20.1	44.8
CARB	14	71.4	46.1	110.5
FAT	14	65.5	47.4	90.3
5-FQA				
COFFEE	13	37.1	24.0	57.5
CARB	14	95.3	71.9	126.2
FAT	14	75.6	53.7	106.5

metabolite	n	geomean	lower	upper
CA3S				
COFFEE	13	27.0	16.7	43.5
CARB	12	112.8	73.8	172.2
FAT	13	85.7	49.3	149.0
CA4S				
COFFEE	13	16.7	12.9	21.7
CARB	9	89.4	38.2	209.2
FAT	9	48.3	24.3	95.6
DHCA				
COFFEE	13	398.5	275.0	577.4
CARB	14	483.7	369.7	632.9
FAT	13	483.5	411.3	568.4
DHCA3S				
COFFEE	13	454.7	318.7	648.8
CARB	13	536.9	449.1	641.9
FAT	14	509.3	439.4	590.2
DHFA				
COFFEE	13	396.5	246.3	638.3
CARB	14	493.7	393.7	619.1
FAT	14	473.4	397.7	563.6
DHFA4G				
COFFEE	13	464.9	328.0	658.8
CARB	14	546.7	446.0	670.1
FAT	14	497.4	412.0	600.6
DHFA4S				
COFFEE	13	444.7	310.0	637.9
CARB	14	520.3	419.5	645.3
FAT	14	479.6	393.3	584.9
DHiFA				
COFFEE	13	437.2	307.9	620.7
CARB	14	508.2	396.9	650.8
FAT	14	507.8	430.7	598.6
DHiFA3G				
COFFEE	13	507.4	377.5	682.2
CARB	14	612.3	512.9	730.9
FAT	14	531.1	439.3	642.0
FA4G				
COFFEE	12	26.7	14.0	51.2
CARB	13	89.6	55.5	144.5
FAT	13	118.9	61.7	229.3
FA4S				
COFFEE	13	21.8	15.2	31.2
CARB	14	67.9	36.0	128.3
FAT	14	71.4	35.2	144.7
iFA				
COFFEE	13	15.8	13.1	19.2
CARB	14	56.9	31.3	103.3
FAT	14	52.6	25.7	107.6

metabolite	n	geomean	lower	upper
iFA3G				
COFFEE	13	41.7	19.2	90.7
CARB	14	109.2	73.3	162.6
FAT	14	118.1	77.8	179.3
MeFA				
COFFEE	13	16.7	12.9	21.7
CARB	14	55.3	35.7	85.6
FAT	14	59.3	29.6	118.6

Table 26 shows the correlation of t_{max} dependent on treatment with a detectable treatment effect on t_{max} with 95% confidence interval for selected metabolites. Most metabolites we measured showed significant differences in t_{max} between CARB and COFFEE as well as between FAT and COFFEE treatment. However, four metabolites, namely DHCA3S, DHFA4G, DHFA4S and DHiFA, showed no significant correlation between FAT and COFFEE treatament. Additionally, CA4S and DHiFA3G revealed significance between FAT and CARB (pc. Figure 41).

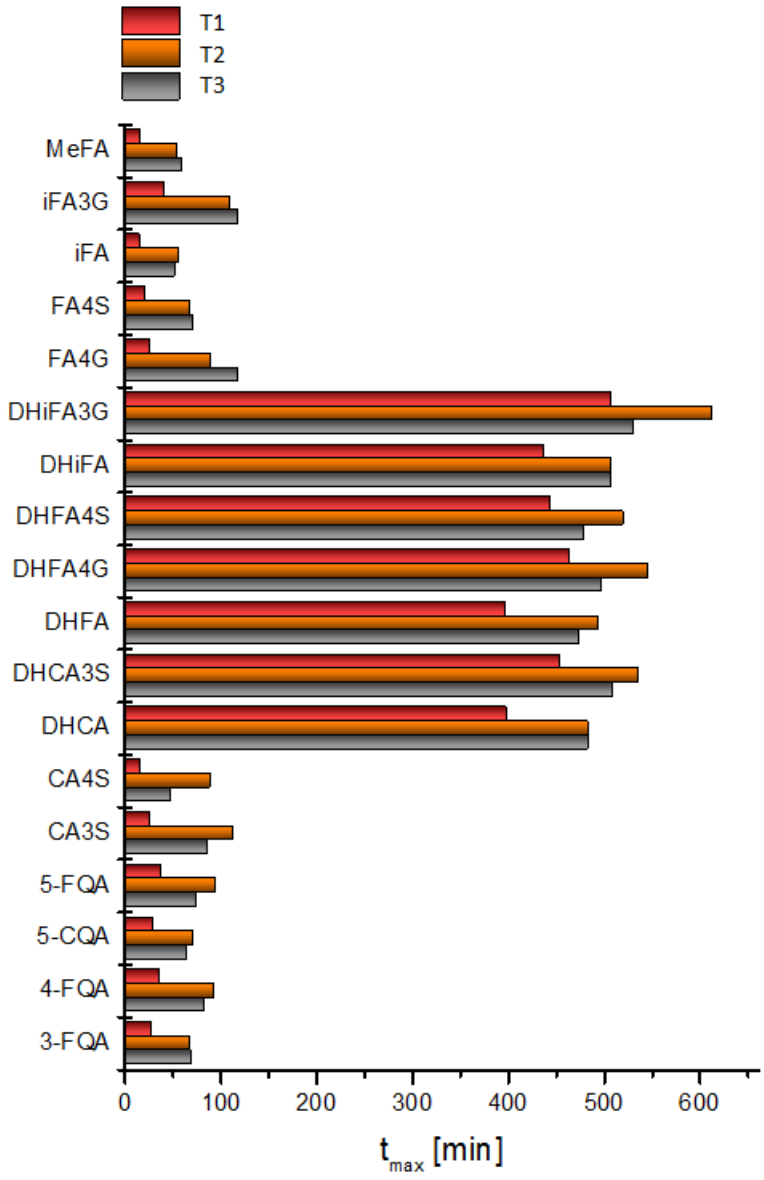


Figure 46 Plasma t_{max} by treatment and metabolites plotted as geometric means. T1: COFFEE, T2: CARB, T3: FAT. (n = 13 or 14 (see Table 25)); Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52). Lower and upper bounds are hidden for a better overview.

Table 26 Model-based treatments effect on plasma t_{max} for each metabolite with 95% confidence intervals.

metabolite	estimate	lower	upper	p-value
3-FQA				
CARB / COFFEE	2.412	1.813	3.207	0.000
FAT / COFFEE	2.438	1.843	3.226	0.000
FAT / CARB	1.011	0.764	1.338	0.939
4-FQA				
CARB / COFFEE	2.574	1.931	3.431	0.000
FAT / COFFEE	2.298	1.760	3.000	0.000
FAT / CARB	0.893	0.673	1.183	0.430
5-CQA				
CARB / COFFEE	2.378	1.765	3.204	0.000
FAT / COFFEE	2.178	1.615	2.938	0.000
FAT / CARB	0.916	0.684	1.227	0.557
5-FQA				
CARB / COFFEE	2.564	1.963	3.350	0.000
FAT / COFFEE	2.036	1.559	2.660	0.000
FAT / CARB	0.794	0.611	1.032	0.085
CA3S				
CARB / COFFEE	4.108	2.916	5.788	0.000
FAT / COFFEE	3.200	2.282	4.488	0.000
FAT / CARB	0.779	0.555	1.093	0.148
CA4S				
CARB / COFFEE	5.549	3.323	9.264	0.000
FAT / COFFEE	3.028	1.814	5.055	0.000
FAT / CARB	0.546	0.317	0.940	0.029
DHCA				
CARB / COFFEE	1.203	1.026	1.412	0.023
FAT / COFFEE	1.196	1.016	1.408	0.032
FAT / CARB	0.994	0.846	1.168	0.941
DHCA3S				
CARB / COFFEE	1.183	1.027	1.363	0.020
FAT / COFFEE	1.111	0.967	1.278	0.137
FAT / CARB	0.940	0.816	1.082	0.388
DHFA				
CARB / COFFEE	1.253	1.056	1.487	0.010
FAT / COFFEE	1.195	1.009	1.415	0.039
FAT / CARB	0.953	0.807	1.126	0.574
DHFA4G				
CARB / COFFEE	1.176	1.038	1.332	0.011
FAT / COFFEE	1.065	0.940	1.207	0.320
FAT / CARB	0.906	0.802	1.023	0.112

metabolite	estimate	lower	upper	p-value
DHFA4S				
CARB / COFFEE	1.174	1.016	1.357	0.029
FAT / COFFEE	1.078	0.933	1.244	0.308
FAT / CARB	0.918	0.797	1.056	0.230
DHiFA				
CARB / COFFEE	1.161	1.013	1.331	0.032
FAT / COFFEE	1.160	1.012	1.330	0.033
FAT / CARB	0.999	0.875	1.141	0.991
DHiFA3G				
CARB / COFFEE	1.212	1.071	1.370	0.002
FAT / COFFEE	1.051	0.929	1.189	0.429
FAT / CARB	0.867	0.770	0.977	0.019
FA4G				
CARB / COFFEE	3.503	2.326	5.274	0.000
FAT / COFFEE	4.658	3.093	7.015	0.000
FAT / CARB	1.330	0.895	1.975	0.158
FA4S				
CARB / COFFEE	2.999	2.018	4.455	0.000
FAT / COFFEE	3.242	2.190	4.798	0.000
FAT / CARB	1.081	0.735	1.590	0.692
iFA				
CARB / COFFEE	3.637	2.495	5.301	0.000
FAT / COFFEE	3.351	2.318	4.843	0.000
FAT / CARB	0.921	0.637	1.332	0.663
iFA3G				
CARB / COFFEE	2.611	1.716	3.971	0.000
FAT / COFFEE	2.810	1.846	4.277	0.000
FAT / CARB	1.076	0.713	1.625	0.726
MeFA				
CARB / COFFEE	3.325	2.331	4.744	0.000
FAT / COFFEE	3.565	2.500	5.086	0.000
FAT / CARB	1.072	0.758	1.517	0.694

The above-mentioned findings are summarized in Table 27 as statistical significant differences for t_{max} for each metabolite and each treatment.

Table 27 Summary of statistical significant differences observed for individual metabolite plasma t_{max}. ">1" means the numerator is higher. "<1" means the denominator is higher.

	CARB / COFFEE	FAT / COFFEE	FAT / CARB
3-FQA	>1	>1	
4-FQA	>1	>1	
5-CQA	>1	>1	
5-FQA	>1	>1	
CA3S	>1	>1	
CA4S	>1	>1	<1
DHCA	>1	>1	
DHCA3S	>1		
DHFA	>1	>1	
DHFA4G	>1		
DHFA4S	>1		
DHiFA	>1	>1	
DHiFA3G	>1		<1
FA4G	>1	>1	
FA4S	>1	>1	
iFA	>1	>1	
iFA3G	>1	>1	
MeFA	>1	>1	

As shown in our study, also other research groups reported that the dihydro compounds, found with the highest plasma concentrations, are typical colonic metabolites, since more extensive hydrolysis occurs in colon and less in the small intestine [*Plumb et al., 1999*]. This means microbiota is responsible for degradation to DHFA and DHCA derivatives [*Williamson et al., 2011*], which circulated later in plasma and had a delayed t_{max} in comparison to the other metabolites from the *quinic* or *phenolic* groups [*Renouf et al., 2010a*, *Renouf et al., 2010b*, *Renouf et al., 2010c*]. Finally, in accordance to published data [*Renouf et al., 2010b*, *Lang et al., 2013a*], the metabolites detected in this study could be separated into two groups: early metabolites, with a t_{max} occurring between 0 - 2 h, and late metabolites, with a t_{max} occurring between 7 - 10 h post-ingestion of the instant coffee we studied. Some metabolites had t_{max} values that occur really early in the plasma after coffee consumption. These results corroborates previous findings for phenolic acids that some compounds could be absorbed or metabolized by the stomach and/or in the upper part of the small intestine [*Poquet et al., 2008*, *Zhao et al., 2004*, *Konishi et al., 2006*]. In a previous study, metabolites that were detected later in plasma such as FA, no return to baseline was observed for most healthy subjects 12 h after coffee consumption [*Renouf et al., 2010b*]. Our study demonstrated that even after 15 h the metabolite concentrations did not reach to baseline and this showed probably only the beginning of the colonic metabolites entering in the

bloodstream. Plasma collection at time points up to 24 h, as performed in other studies [*Guy et al., 2009*], or even up to 48 h, seem to be meaningful. Nevertheless, for such late time points, sample collection should be performed with shorter time intervals, e.g. every two hours. In this line, it might be possible that even a 48 h polyphenol free diet prior coffee intake was not sufficient to reach a full wash out of the colonic CGA metabolites circulating in the blood of the volunteers.

Comparable to the findings described by Farrell and coworkers, we determined methylated compounds like iFA and MeFA, but no CA, directly (~16 min) after coffee consumption. Additionally, we found that only the phase II conjugates FA4S and FA4G appear early post-coffee consumption in plasma. No FA was detected. We confirmed the hypothesis of Farrell and members, that the stomach can be an important site for absorption of pharmacologically active compounds, such as polyphenols from coffee. Furthermore, the ability of the human gastric mucosal cells (apart from the liver) to form methylate conjugates as well as execute biotransformation reactions by SULT and UGT was shown, as also previously observed *in vitro* [*Farrell et al., 2011, Poquet et al., 2008*].

We also identified two t_{max} for MeFA and FA4S in plasma, as described by other authors for other metabolites [*Renouf et al., 2010b*, *Guy et al., 2009*]. This biphasic behavior suggests that absorption occurs both in the stomach/small intestine (first peak) and in the colon (second peak). It may also suggest that plasma appearance is influenced by the enterohepatic circulation.

The t_{max} for all metabolites were strongly affected by treatment. Ingestion of the coffee beverage in combination with the food matrix led to enhanced absorption, probably already in the stomach or in the upper part of the small intestine. For example, FA4G reached t_{max} 27 min after consumption of coffee only, and 119 min after consumption of coffee with a fat-rich meal. Overall, the delay in t_{max} seemed to be more pronounced for the earlier metabolites as for the colonic once. Presumably, CGA may bind to digestive proteins, lipids and carbohydrates. This would reduce the possibility for enzymes such as esterases to cleave the CGA to release the free acids. Additionally, this would increase gastrointestoinal bulk and may also reduce the availability of the coffee CGA, at least in the stomach and small intestine. On the way down to the colon, CGA and the released free acids are absorbed and transformed into their corresponding dihydro derivatives. Thus, the arrival into the colon was delayed, which explains the later t_{max} for the *colonics* in plasma in general. This delay was also caused by the food matrix, but not as much as for the earlier metabolites such as FA4G.

It seemed that a CARB treatment had a stronger effect on t_{max} than FAT treatment. At a very early stage in research of others groups, *in vitro* experiments with isolated brush border

membrane vesicles (150 – 200 µg protein) demonstrated that low levels of selected phenolic acids (CQA, CA, FA at concentrations of 1 mM) can effectively reduce NA^+-dependent glucose uptake from a reaction mixture containing 50 µM D-[^{3}H]-glucose determined by a liquid scintillation spectrometer. At 1 mM, CQA reduced glucose uptake by approximately 81%, while only a slight reduction of 35 and 38% respectively was observed in the presents of CA and FA [*Welsch et al., 1989a&b*]. At the present time, it is assumed that several polyphenols inhibit glucose uptake transporters, such like MCTs, SGLT1 and GLUTs, but also the opposite effect, including reduction of polyphenol uptake by sugar-rich ingredients, may have a meaningful impact [*Peng et al., 2015, Shimizu et al., 2010, Williamson, 2013*]. This interdependence influence may explain the stronger effects on t_{max} between COFFEE and CARB in comparison to COFFEE and FAT as well as the significant differences of CA4S and DHiFA3G when comparing CARB and FAT treatment.

4.2 Availability of instant coffee chlorogenic acids and metabolites in urine

For the statistical calculations, Table 28 shows the number of subjects where specifically detected metabolites in urine where detected after each treatment. The data modifications were agreed during a blind data review meeting and included following: For urinary excretion, absolute values were used instead of expressing them as a percentage ingested dose, except for QA, which was determined as µmol QA/g creatinine. Moreover, when the measurements were below the LOQ for a given time period, the contribution was set to 0.

Table 28 Number of subjects with a specific detected metabolite in urine samples (0 - 24 h) by treatment. NA: not available. Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52).

metabolite	COFFEE	CARB	FAT
3CQA	14	14	14
3FQA	14	14	14
4CQA	14	14	14
4FQA	14	14	14
5CQA	14	14	14
5CQA4S	7	7	7
5FQA	14	14	14
CA	14	14	14
CA3G	1	NA	1
CA3S	14	14	14
CA4G	12	13	13
CA4S	14	14	14
CQA3G	5	1	5
CQA3S	9	9	9
CQL	14	13	14
CQLG	14	13	14
DHCA	14	14	14
DHCA3G	14	14	14
DHCA3S	14	14	14
DHCA4G	13	13	13
DHCA4S	14	14	14
DHFA	13	13	13
DHFA4G	14	14	14
DHFA4S	14	14	14
DHIFA	NA	1	NA
DHIFA3G	14	14	14
DHIFA4S	13	12	13
FA	10	12	11
FA4S	14	13	14
FAG	14	14	14
FQA4G	10	13	12

metabolite	COFFEE	CARB	FAT
FQA4S	14	14	14
IFA	3	4	3
IFA3G	14	14	14
IFA3S	14	14	14
Quinic acid	14	14	14

The total 24 h urine as well as the time spans (0 – 6 h, 6 – 12 h and 12 – 24 h) for urinary excretion summary statistics for the sum of the metabolites concentrations are shown in Table 29 and Figure 47. In total, as geomean of the 14 urinary samples and dependent from treatment between 354.2 – 406.2 nmol/mL of the detected metabolites were excreted. In the time period from 0 to 6 h, we detected 63.2 – 95.4 nmol/mL, which was increasing up to 186.8 – 237.0 nmol/mL in the time period from 6 to 12 h. From 12 to 24 h, between 61.2 – 75.3 nmol/mL of all quantified metabolites were excreted. This is almost in the range of the first time period.

Table 29 Summary statistics on sum of metabolites in urine samples (0 – 24 h, 0 – 6 h, 6 – 12 h and 12 – 24 h) with lower and upper bounds [nmol/mL]. Data modifications deviant from the norm were agreed during the blind data review meeting (see page 52).

	n	geomean	lower	upper
0 - 24 h				
COFFEE	14	406.2	210	785.6
CARB	14	396.8	207.1	760.3
FAT	14	354.2	200.2	626.6
0 - 6 h				
COFFEE	14	95.4	45.0	202.4
CARB	14	63.2	39.1	102.1
FAT	14	87.7	55.1	139.5
6 - 12 h				
COFFEE	14	204.8	97.1	431.9
CARB	14	237.0	105.5	532.2
FAT	14	186.8	87.9	397.0
12 – 24 h				
COFFEE	14	65.0	26.7	157.8
CARB	14	75.3	37.6	150.5
FAT	14	61.2	34.9	107.3

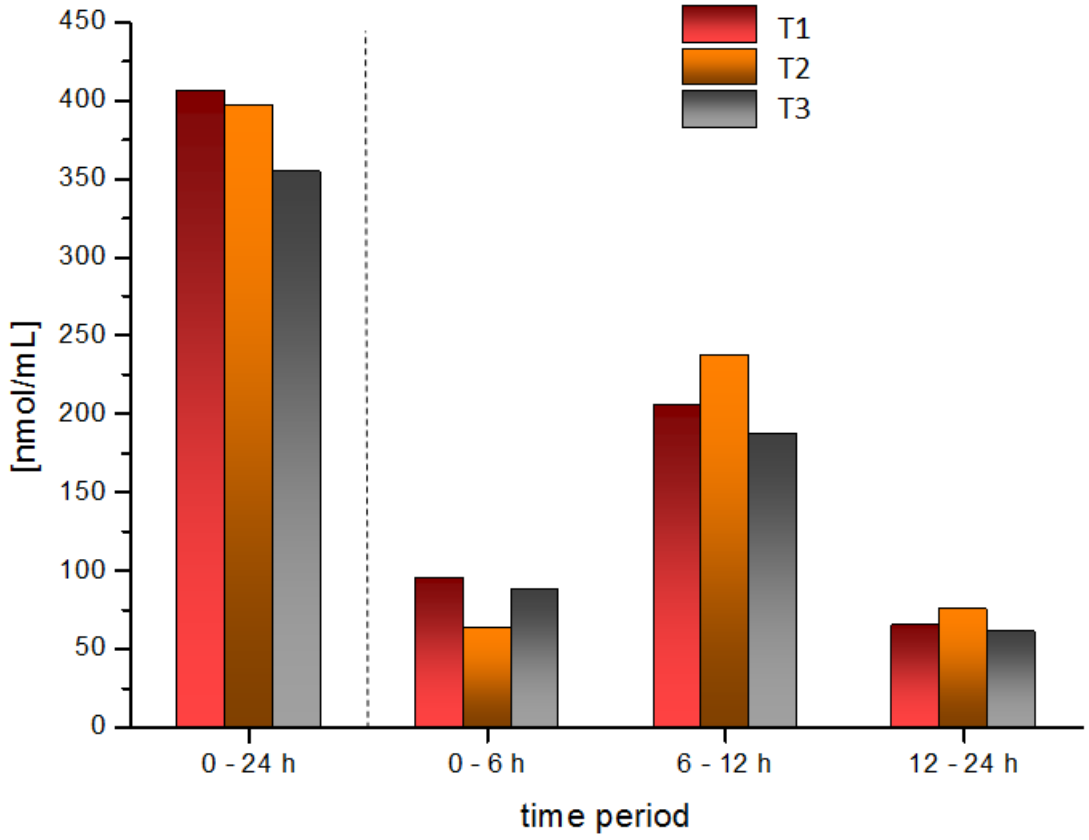


Figure 47 Sum of metabolites in urine samples (0 – 24 h, 0 – 6 h, 6 – 12 h and 12 – 24 h) by treatment plotted as geometric means. T1: COFFEE, T2: CARB, T3: FAT (n = 14; Lower and upper bounds are hidden for a better overview)

Table 30 shows the correlation of sum of metabolites in urine samples dependent on treatment as well as time periods of urine collection. No statistical significant differences were observed in the sum of 24 h urinary excretion for each of the three treatments. For the 0 – 6 h period, statistical significant differences with 95% confidence interval between CARB and COFFEE as well as between FAT and CARB treatment were observed. No other time periods or comparisons showed significant differences between treatments. Summation of the metabolite concentrations after each treatment from time periods 0 – 6 h and 6 – 12 h showed equal values, meaning we observed no differences in total excretion between 0 – 12 h (data not graphically shown).

Table 30 Model-based treatments effect on sum of metabolites in urine samples (0 – 24 h, 0 – 6 h, 6 – 12 h and 12 – 24 h) with 95% confidence intervals.

	estimate	lower	upper	p-value
0 - 24 h				
CARB / COFFEE	0.977	0.771	1.238	0.845
FAT / COFFEE	0.872	0.688	1.105	0.256
FAT / CARB	0.893	0.704	1.131	0.347
0 - 6 h				
CARB / COFFEE	0.662	0.539	0.813	0.000
FAT / COFFEE	0.919	0.748	1.128	0.419
FAT / CARB	1.388	1.13	1.704	0.002
6 - 12 h				
CARB / COFFEE	1.157	0.802	1.67	0.435
FAT / COFFEE	0.912	0.632	1.317	0.624
FAT / CARB	0.788	0.546	1.138	0.204
12 – 24 h				
CARB / COFFEE	1.158	0.787	1.706	0.456
FAT / COFFEE	0.943	0.64	1.388	0.765
FAT / CARB	0.814	0.553	1.198	0.296

There were no significant differences between treatments in the sum of metabolites in urine samples collected 24 h after treatment. It has to be mentioned that the interindividual variance in the urine samples was high, thus a larger number of volunteers would be required to investigate the possible effects of treatments on bioavailability. Additionally, shorter collection intervals (e.g. time periods with two or three hours per period) would provide a more useful pharmacokinetic profile with a stronger validity.

For the 0 – 6 h time period, we observed statistically significant differences between the CARB and COFFEE groups, as well as between the FAT and CARB groups. There were no other significant differences in the sum of metabolites in urine samples between treatments or time periods. A summation of the metabolite concentrations after each treatment over the time periods 0 – 6 h and 6 – 12 h produced equal values

The return of the concentrations in the last periode of time (12 – 24 h) to the level determined in the first time period (-24 – 0 h) indicated that the most metabolites were excreted between the first 24 h.

Figure 47 shows the detailed kinetics, and it seems that the excretion of the summarized amount of metabolites for CARB treatment was delayed in comparison to the other both treatments. This is in line with the results for plasma, which showed also a delayed t_{max} for

every metabolite determined after CARB treatment. Also for the FAT treatment a delayed t_{max} for metabolites in plasma was observed, what we could not confirm for urinary excretion. It should be kept in mind that time periods of six hours revealed no clear full pharmacokinetic profile.

Based on an evaluated and validated HPLC-ESI-MS/MS method using isotopically labeled QA for quantification [*Erk et al., 2009*], QA kinetics in urine samples were investigated in more detail. Figure 48 shows the boxplots related on sum of time spans (-24 – 0 h, 0 – 6 h, 6 – 12 h and 12 – 24 h) of urinary excretion by treatment. In addition, the summary statistics of AUCs are figured. Comparing the AUCs, the three treatments did not show any statistically significant differences among themselves. Indeed, between time period 0 – 6 h and 6 – 12 h for CARB and FAT treatment significant differences on t_{max} with an associated p-value of < 0.001 and < 0.05 respectively were determined.

The data obtained for quinic acid were in line with the overall findings of summarized excreted urine metabolites as described before. QA as well as the polyphenols in the beverage alone passes faster through the GIT as the same amount of QA as well as polyphenols in the beverage in combination with a food matrix, which gave a retarded emptying of stomach and thus a time-delayed metabolization and excretion of the polyphenols under study [*Biesalski and Grimm, 2007*]. Especially for CARB treatment, strong significant differences in urinary excretion were observed between time period 0 – 6 h and 6 – 12 h, whereas the effect for FAT was significant but marginal.

Table 31 shows the amounts of ingested coffee and corresponding QA contents in relation to urinary excreted QA values by subjects and treatments. Additionally, the statistics summarized as means ± SD and corresponding percentage of excreted QA relating to the ingested amounts are specified. Approximately 60% relating to the ingested QA was renal excreted, whereas no statistically significant differences were determined among the three treatments.

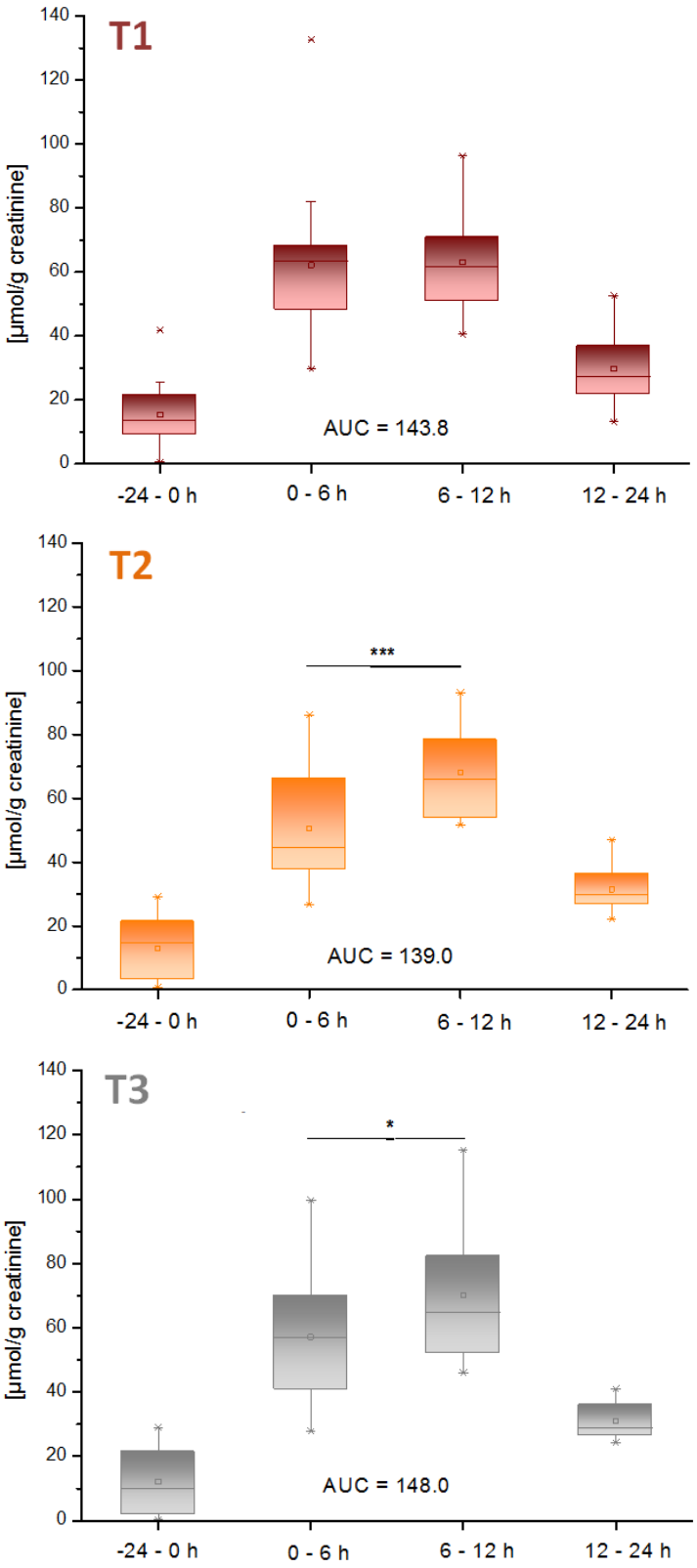


Figure 48 Model-based effects of treatments as boxplots related on sum of time spans (-24 – 0 h, 0 – 6 h, 6 – 12 h and 12 – 24 h) of urinary excretion [µmol/g creatinine] for quinic acid. n = 14; normal distribution according to Anderson and Darling, one-sided, pared t-test; *: < 0.05; *: < 0.001. T1: COFFEE, T2: CARB, T3: FAT**

In a human intervention study reported in literature, Adamson and coworkers detected 20 - 60% of the ingested QA as hippuric acid in urine and the remaining percentage was mostly excreted as unmetabolized QA [*Adamson et al., 1970*]. Unfortunately, we did not detect hippuric acid in the urine samples, but the determined 60% of QA were in line with these data. Nevertheless, we have to keep in mind that the in the body circulating QA was not only free QA from coffee but might be liberated from different QA esters, such as CQA and FQA. High amounts of QA may be released from QA esters after cleavage by esterases during the GIT passage. Hence, it was not possible to define the percentage of excreted QA referred to the free QA from coffee but. It is always the sum of free QA and released QA from QA esters, which is ingested, metabolized and excreted.

Table 31 Amounts of ingested coffee [g] and corresponding QA contents [mg] in addition to urinary excreted QA values [mg] by subjects and treatments. Summary statistics as means ± SD and corresponding percentage of excreted QA relating to the ingested amounts.

subject	COFFEE			CARB			FAT		
	ingested coffee [g]	ingested QA [mg]	excreted QA [mg]	ingested coffee [g]	ingested QA [mg]	excreted QA [mg]	ingested coffee [g]	ingested QA [mg]	excreted QA [mg]
01	2.6	29.4	15.2	2.6	29.6	17.6	2.7	29.8	18.9
02	2.1	23.7	6.9	2.1	23.7	13.2	2.1	23.7	11.5
03	2.9	32.5	21.8	2.9	32.0	20.0	2.9	32.5	18.2
04	2.6	29.3	13.3	2.6	28.9	14.2	2.7	29.8	22.5
05	2.3	25.3	16.2	2.3	25.7	13.6	2.3	26.1	17.6
06	2.2	24.6	15.7	2.1	23.7	10.5	2.1	23.9	14.6
07	2.8	31.7	23.1	2.8	31.7	18.0	2.8	31.7	18.1
08	2.9	32.2	13.5	2.9	32.5	27.0	2.8	31.8	17.4
09	2.0	22.0	12.9	2.0	22.0	25.0	2.0	22.5	12.9
10	2.1	23.9	19.6	2.2	24.5	16.8	2.1	23.7	14.3
11	2.4	26.5	16.8	2.4	26.8	16.5	2.3	26.2	17.8
12	3.1	35.3	25.6	3.2	35.4	18.8	3.1	35.3	21.2
13	2.4	26.8	14.2	2.4	26.4	11.5	2.4	26.5	11.9
14	2.8	31.3	23.1	2.8	30.9	24.3	2.8	30.9	22.2
mean		28.2	17.0		28.1	17.6		28.2	17.1
SD		3.9	4.9		3.9	4.9		3.9	3.5
percentage excreted relating to ingested amount		**60.3%**			**62.7%**			**60.6%**	

4.3 Correlation between plasma AUC and 24 h urinary excretion

After the evaluation of the plasma AUC and 24 h urinary excretion, in Table 32 the correlation between plasma metabolite AUCs and metabolites from 24 h urine samples is described. The inverse correlations for DHFA (only CARB treatment), DHFA4S and FA4S (only COFFEE treatment) were not significant. The positive correlations for CA3S, DHCA, DHCA3S, DHFA (COFFEE and FAT treatment), DHFA4G and FA4S (CARB and FAT treatment) were not significant either. However, apparent data trends suggested that increased plasma metabolite AUCs led to increased metabolite concentrations in the urine samples. Interindividual differences hindered the emergence of any significance in the data from only 14 healthy subjects.

Table 32 Correlation between plasma AUC and 24 h urinary excretion of CA3S, DHCA, DHCA3S, DHFA, DHFA4G, DHFA4S, FA4S depending on the treatment.

	correlation
CA3S	
COFFEE	0.238
CARB	0.339
FAT	0.073
DHCA	
COFFEE	0.570
CARB	0.499
FAT	0.680
DHCA3S	
COFFEE	0.469
CARB	0.533
FAT	0.251
DHFA	
COFFEE	0.218
CARB	−0.044
FAT	0.215
DHFA4G	
COFFEE	0.477
CARB	0.526
FAT	0.445
DHFA4S	
COFFEE	−0.062
CARB	−0.163
FAT	−0.184
FA4S	
COFFEE	−0.261
CARB	0.319
FAT	0.075

The possibility to evaluate the plasma and urinary data was limited due to the low amount of subjects in this study. Caused by this, also the correlation of the plasma AUC and 24 h urinary excretion gave no clear trend. Up to now, just limited data are published. Farah and coworkers found a negative correlation between apparent bioavailability and urinary recovery of CGA ($r = -0.76$; $P = 0.01$). The group tried to explain this behavior by interindividual differences in metabolic rates and/or preferential excretion routes [*Farah et al., 2008*]. Further investigations are needed to produce reliable data which give the possibility to have a clear conclusion on any correlations between plasma AUCs and urinary excretion.

4.4 Investigation of the colonic metabolism of food intrinsic *in vitro*

Interindividual variability in absorbability and metabolism also depends on individual gut microbiota and therefore microbal degradation. Finally this degradation affects the efficacy of CGAs. Further investigations might accelerate progress in understanding the role of human gut bacteria in the bioavailability of CGAs. In our study, after a 48 h polyphenol-free diet we collected fecal samples of each subject. Incubations of commercially available 5-CQA with these fecal samples of individual volunteers were performed under anaerobic conditions. Afterwards 5-CQA and metabolites were determined and quantified by HPLC CoulArray™ to investigate the degradation and metabolism efficancy of the gut bacteria. The achieved results are shown in Table 33 as well as Figure 49 to Figure 62 and were corrected for endogenous levels of 5-CQA, CA and DHCA which were detected in very low levels in the control fermentations of the fecal samples. We were able to observe that the degradation of 5-CQA was depending on the fecal samples of the subjects following a very interindividual kinetic. Both extremes, full degradation of 5-CQA (see results for subject 04 (Figure 52 and Table 33)) and very limited degradation (see results for subject 13 (Figure 61 and Table 33)) were observable. The results of all other incubations of the other subjects showed varying degradation efficiency in between. As example for the strong interindividual differences, the degradation of 5-CQA and the appearance of the degradants CA, and DHCA for subject 4 and 13 observed for 4 h are shown in Figure 52 and Figure 61. As noticeable for subject 4, a fast degradation of 5-CQA was determined accompanied with the appearance of CA until 2 h. Already after 30 minutes, DHCA was detectable with a clear increase up to 4 h. The 5-CQA incubated with fecal sample of subject 13, showed no distinct cleavage. After 1 h, a decreasing shift was observed in combination with a small increase of CA up to 2 µM until 4 h. Over the whole incubation time, no DHCA was detectable in the incubation from subject 13. As an overview of the 14 subjects, 10.0 ± 2.3 µM determined as sum of 5-CQA, CA and DHCA after 4 h incubation were detectable. Regarding the individual values as sum of 5-CQA, CA and DHCA after 4 h incubation, the lowest value is 6.0 µM (subject 7) and the highest value 13.3 µM (subject 13). The subject were divided into two groups regarding the seven subjects with the lowest DHCA concentration after 4 h and the seven subjects with the highest DHCA concentration after 4 h. Subjects 5, 7, 9, 10, 11, 12 and 13 degraded 5-CQA into the DHCA to a low extend, whereas subjects 1, 2, 3, 4, 6, 8 and 14 degraded 5-CQA into DHCA to a high extend. In Table 34 both groups are listed showing the individual amounts of summarized plasma AUCs [µmol*min] of all dihydro compounds (DHCA, DHCA3S, DHFA, DHFA4S, DHiFA, DHiFA3G, DHiFA3S, MeDHFA) determined. The mean of dihydro compounds for the group with low DHCA formation was 952 µM*min, for the group with high DHCA formation 636 µM*min.

Table 33 Amounts of 5-CQA, CA, and DHCA after 4h anaerobic incubations of 5-CQA (10 µM) with fecal samples of 14 volunteers (S01 – S14). The concentrations were determined as mean ± SD of three separate incubations.

	5-CQA [µM ± SD]	CA [µM ± SD]	DHCA [µM ± SD]		5-CQA [µM ± SD]	CA [µM ± SD]	DHCA [µM ± SD]
S01				**S08**			
0 h	4.29 ± 0.48	1.83 ± 0.27	0.55*	0 h	5.06 ± 0.64	2.33 ± 0.14	0.04 ± 0.00
0.5 h	3.47 ± 0.30	4.15 ± 0.15	0.66 ± 0.02	0.5 h	1.43 ± 0.06	5.71 ± 1.05	0.59 ± 0.16
1 h	1.75 ± 0.62	4.85 ± 1.49	0.76 ± 0.08	1 h	0.01	7.00 ± 0.47	1.15 ± 0.01
2 h	0.59 ± 0.05	7.20 ± 1.59	1.12± 0.19	2 h	n. d.	7.37 ± 0.28	2.23 ± 0.09
4 h	0.50 ± 0.04	7.76 ± 1.11	2.03 ± 0.15	4 h	n.d.	5.25 ± 0.63	3.38 ± 0.06
S02				**S09**			
0 h	6.55 ± 0.31	n. d.	n. d.	0 h	6.05 ± 0.34	0.48 ± 0.06	n. d.
0.5 h	7.47 ± 0.35	0.22*	n. d.	0.5 h	4.27 ± 0.41	1.52 ± 0.10	0.32*
1 h	6.44 ± 0.19	1.12 ± 0.40	n. d.	1 h	3.38 ± 0.16	3.00 ± 0.56	0.44*
2 h	5.58 ± 1.91	3.31 ± 0.98	0.04*	2 h	2.09 ± 0.28	5.49 ± 0.71	0.65 ± 0.44
4 h	1.32 ± 0.70	5.43 ± 0.99	2.22 ± 0.48	4 h	0.4*	5.79 ± 1.27	1.34 ± 0.65
S03				**S10**			
0 h	5.81 ± 0.29	1.26 ± 0.10	0.89 ± 0.03	0 h	7.60 ± 0.63	0.41 ± 0.11	0.14 ± 0.06
0.5 h	5.64 ± 0.69	2.56 ± 0.22	1.19 ± 0.02	0.5 h	8.23 ± 0.51	1.22 ± 0.06	0.21 ± 0.00
1 h	3.69 ± 0.02	3.03 ± 0.20	1.45 ± 0.09	1 h	7.99 ± 0.18	2.10 ± 0.07	0.26 ± 0.00
2 h	2.58 ± 0.28	5.89 ± 0.21	2.44 ± 0.05	2 h	7.18 ± 0.35	3.90 ± 0.36	0.36 ± 0.03
4 h	0.77 ± 0.03	6.95 ± 1.37	3.91 ± 0.42	4 h	4.48 ± 0.63	6.45 ± 0.24	0.66 ± 0.03
S04				**S11**			
0 h	7.06 ± 0.59	0.28 ± 0.04	n. d.	0 h	4.64 ± 0.24	n. d.	n. d.
0.5 h	4.55 ± 0.28	1.44 ± 0.24	0.94 ± 0.14	0.5 h	4.78 ± 0.21	n. d.	n. d.
1 h	2.86 ± 0.15	2.79 ± 0.40	2.45 ± 0.24	1 h	5.13 ± 0.67	0.86 ± 0.20	0.47 ± 0.00
2 h	0.36 ± 0.11	3.63 ± 0.14	5.08 ± 0.18	2 h	4.57 ± 0.11	4.04 ± 1.19	0.62 ± 0.04
4 h	n. d.	n. d.	8.47 ± 0.63	4 h	2.46 ± 1.17	9.89 ± 3.15	0.90 ± 0.16
S05				**S12**			
0 h	9.42 ± 0.73	n. d.	n. d.	0 h	7.74 ± 1.59	n. d.	n. d.
0.5 h	12.0 ± 2.80	0.32 ± 0.08	n. d.	0.5 h	7.85 ± 0.47	1.17 ± 0.14	0.26*
1 h	14.4 ± 3.43	0.94 ± 0.30	n. d.	1 h	6.65 ± 0.18	2.64 ± 0.02	0.29 ± 0.00
2 h	14.1 ± 3.43	2.04 ± 0.82	n. d.	2 h	4.43 ± 0.40	4.95 ± 0.62	0.34 ± 0.02
4 h	9.17 ± 1.81	2.69 ± 0.47	n. d.	4 h	1.62 ± 0.18	7.01 ± 1.35	0.46 ± 0.03
S06				**S13**			
0 h	7.15 ± 0.11	1.60 ± 0.23	0.87 ± 0.41	0 h	14.75 ± 0.33	n. d.	n. d.
0.5 h	6.37 ± 0.26	2.63 ± 0.51	0.98 ± 0.01	0.5 h	14.73 ± 1.59	n. d.	n. d.
1 h	5.28 ± 0.32	3.64 ± 0.97	1.08 ± 0.06	1 h	14.69 ± 0.67	0.02*	n. d.
2 h	4.04 ± 0.65	5.31 ± 0.79	1.62 ± 0.17	2 h	13.45 ± 0.88	0.82 ± 0.36	n. d.
4 h	1.28 ± 0.04	5.99 ± 0.61	4.50 ± 0.55	4 h	11.21 ± 0.38	2.13 ± 0.14	n. d.
S07				**S14**			
0 h	6.51 ± 2.46	n. d.	n. d.	0 h	6.01 ± 1.07	2.45 ± 0.78	0.04*
0.5 h	7.94 ± 0.74	n. d.	n. d.	0.5 h	2.15 ± 0.60	7.33 ± 2.09	0.46 ± 0.20
1 h	8.46 ± 1.89	0.54 ± 0.40	n. d.	1 h	0.44*	6.85 ± 0.78	0.80 ± 0.17
2 h	5.86 ± 1.38	1.13 ± 0.35	n. d.	2 h	n. d.	6.45 ± 1.31	1.56 ± 0.33
4 h	3.88 ± 0.67	1.80 ± 0.31	0.31 ± 0.16	4 h	n. d.	5.02 ± 0.80	3.11 ± 0.53

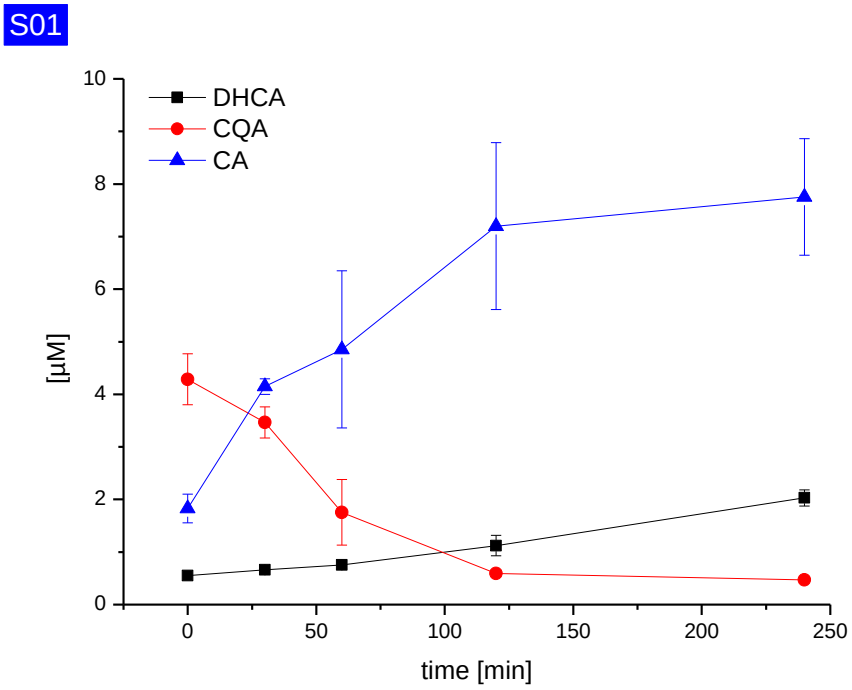


Figure 49 Incubations of 5-CQA (10 µM) with fecal samples of subjects S01. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

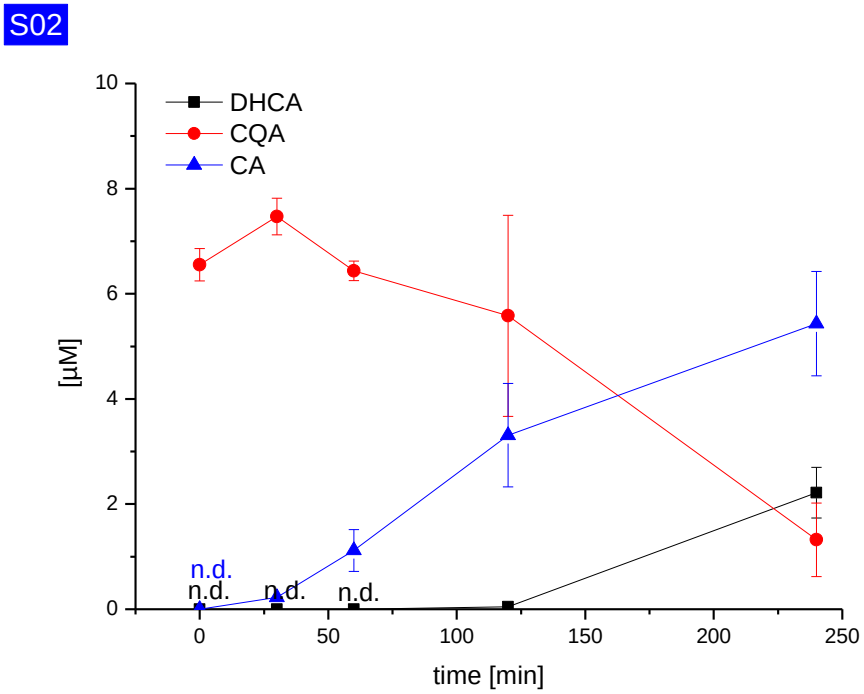


Figure 50 Incubations of 5-CQA (10 µM) with fecal samples of subjects S02. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

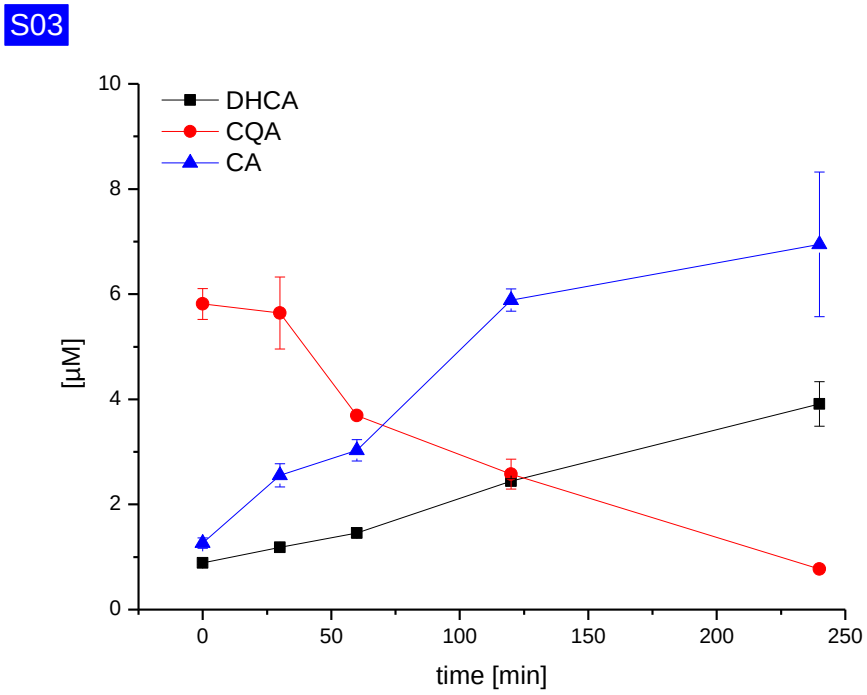


Figure 51 Incubations of 5-CQA (10 µM) with fecal samples of subjects S03. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

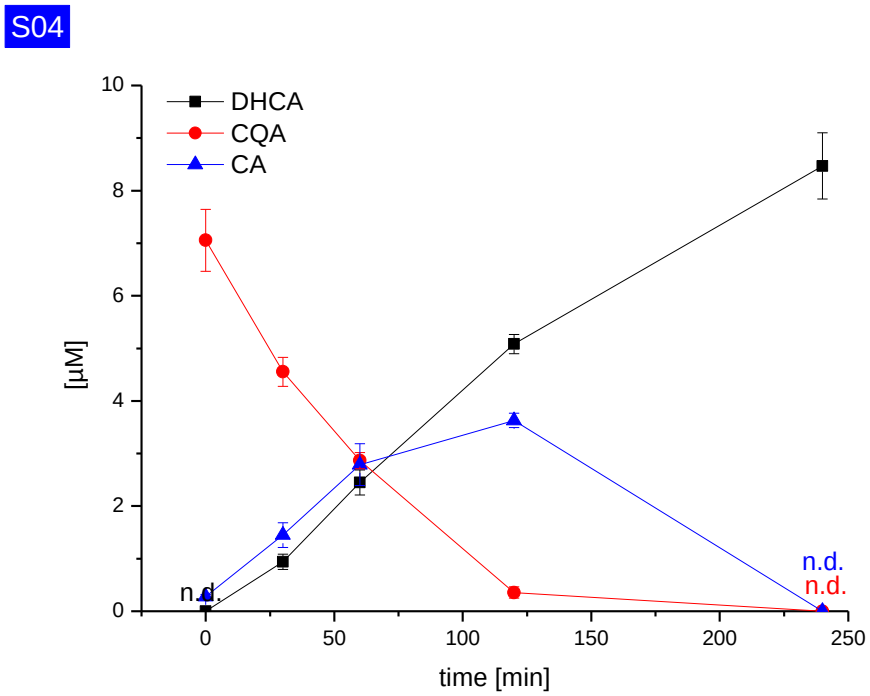


Figure 52 Incubations of 5-CQA (10 µM) with fecal samples of subjects S04. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

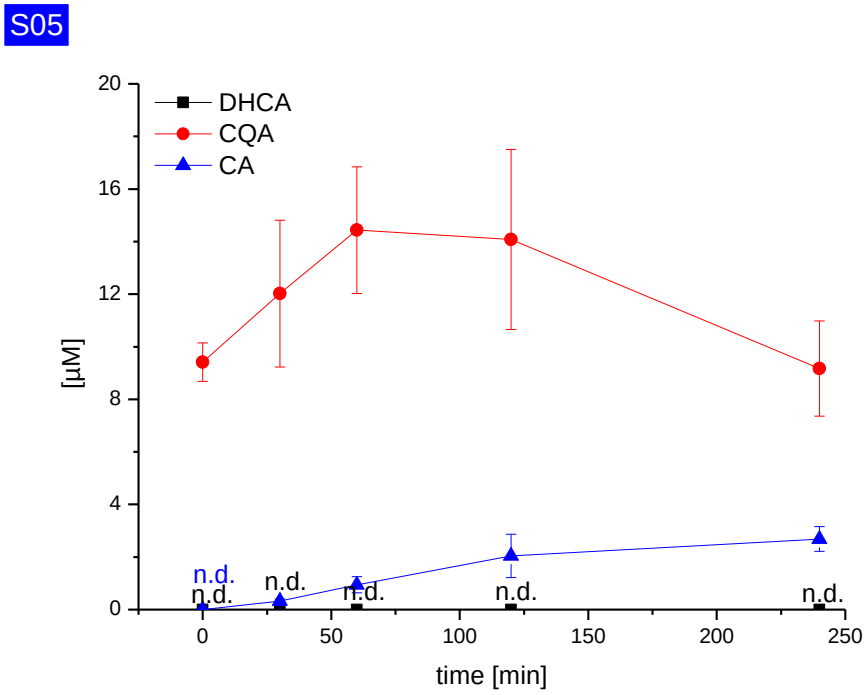


Figure 53 Incubations of 5-CQA (10 µM) with fecal samples of subjects S05. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

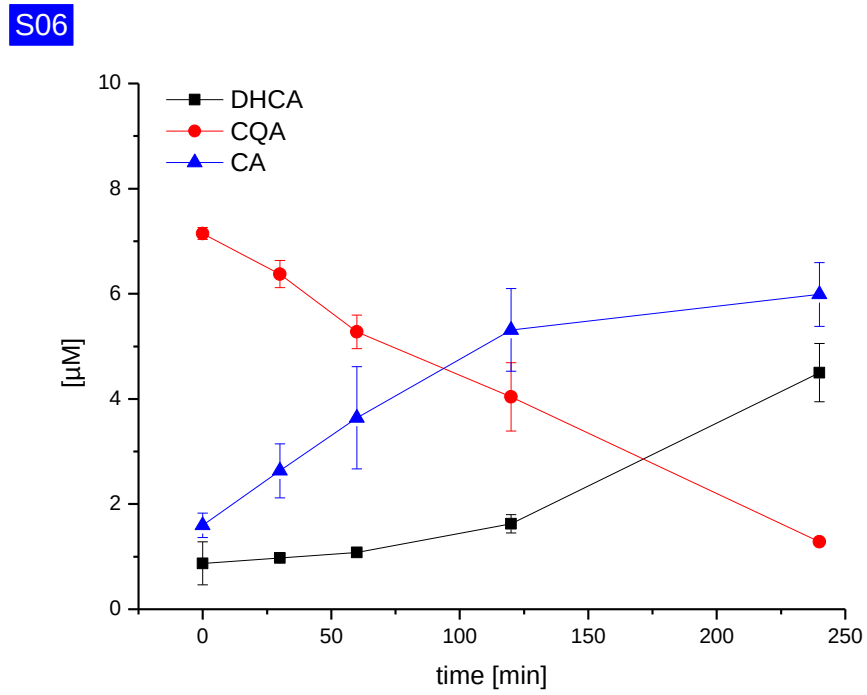


Figure 54 Incubations of 5-CQA (10 µM) with fecal samples of subjects S06. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

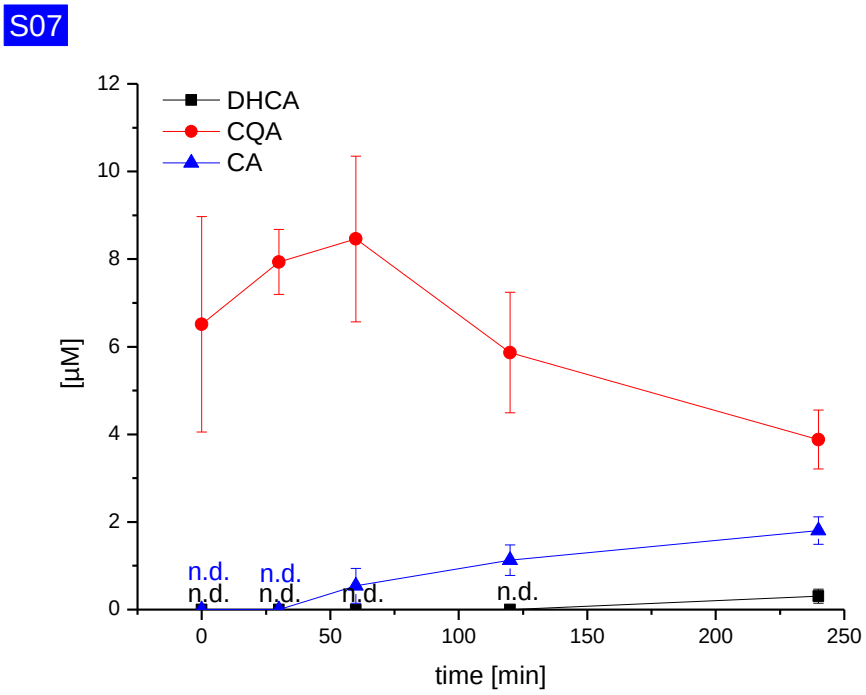


Figure 55 Incubations of 5-CQA (10 µM) with fecal samples of subjects S07. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

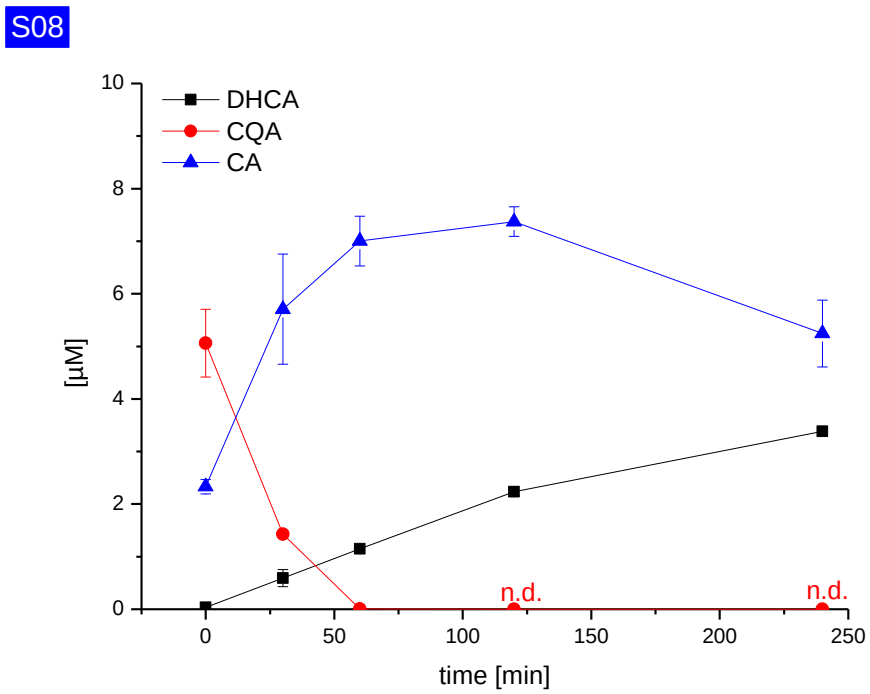


Figure 56 Incubations of 5-CQA (10 µM) with fecal samples of subjects S08. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

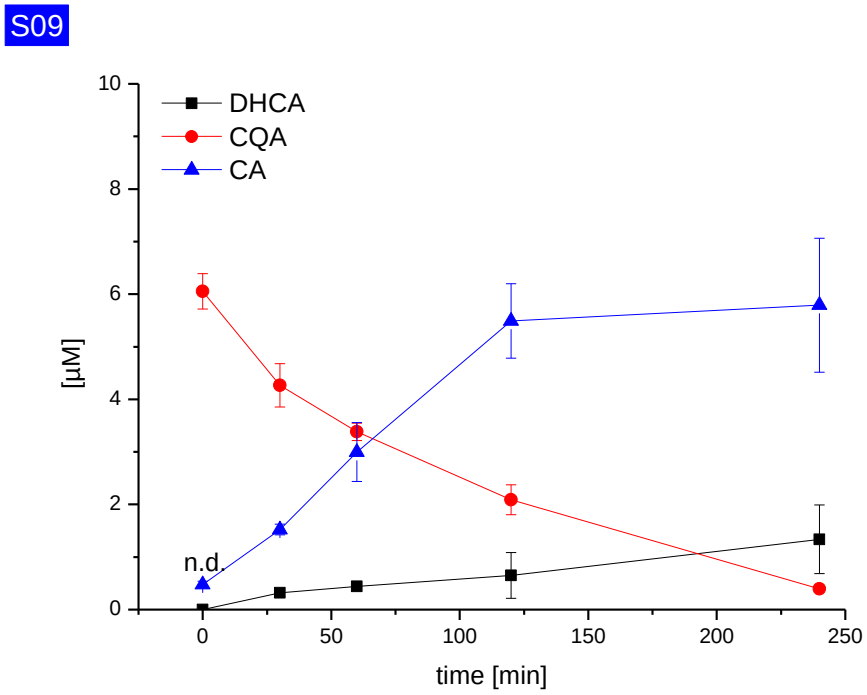


Figure 57 Incubations of 5-CQA (10 µM) with fecal samples of subjects S09. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

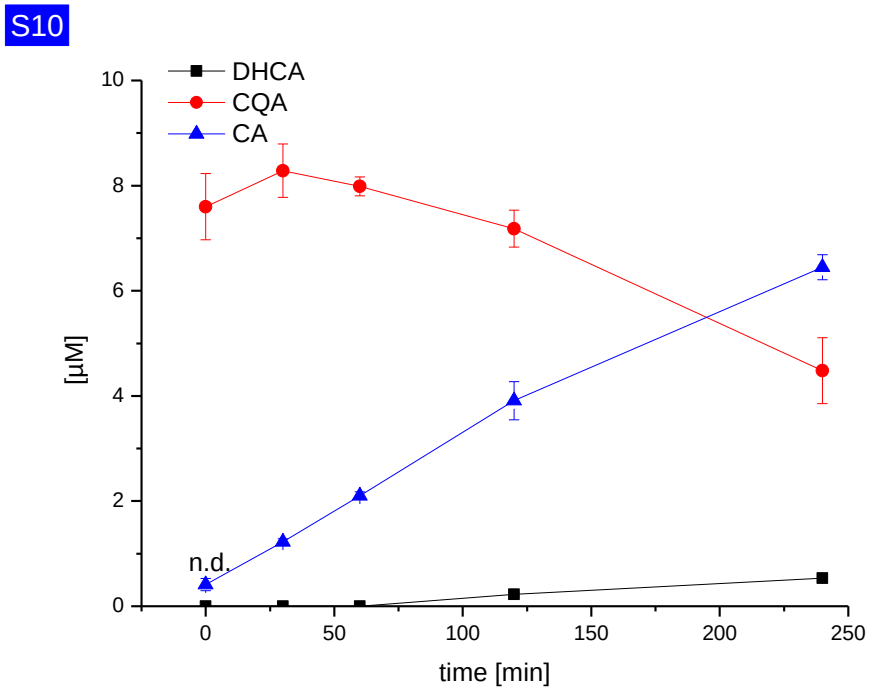


Figure 58 Incubations of 5-CQA (10 µM) with fecal samples of subjects S10. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

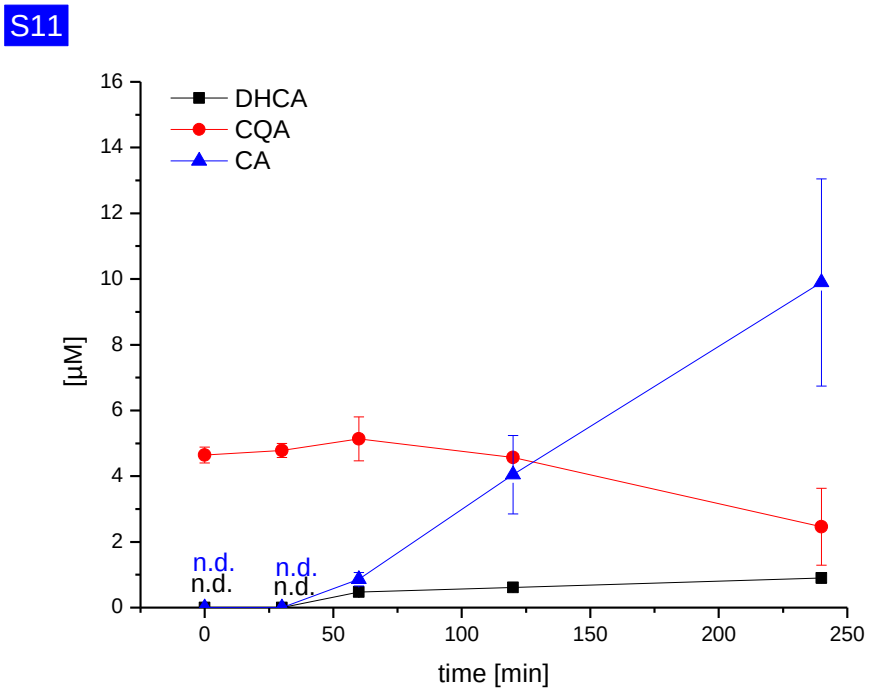


Figure 59 Incubations of 5-CQA (10 µM) with fecal samples of subjects S11. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

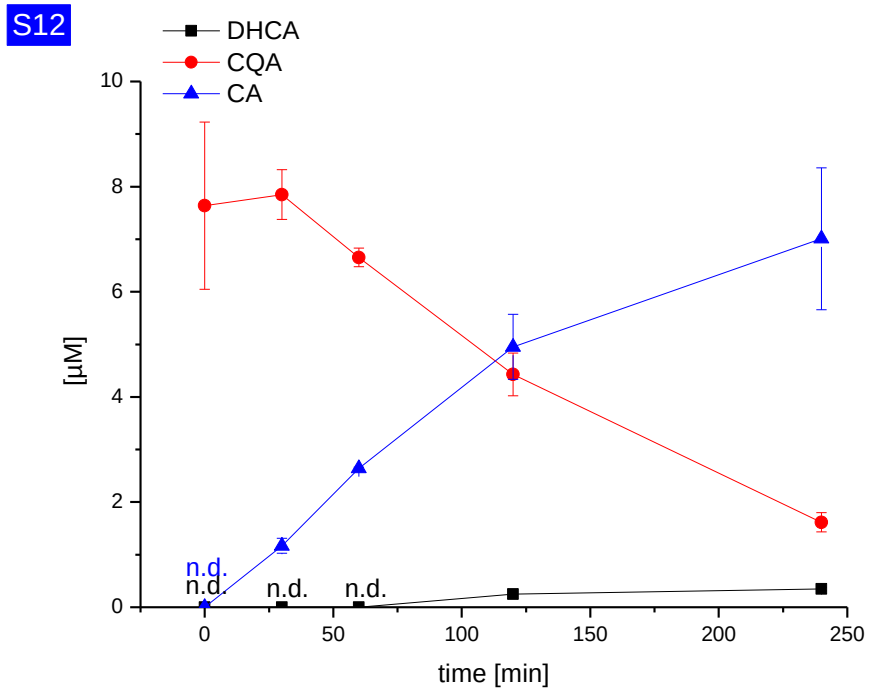


Figure 60 Incubations of 5-CQA (10 µM) with fecal samples of subjects S12. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

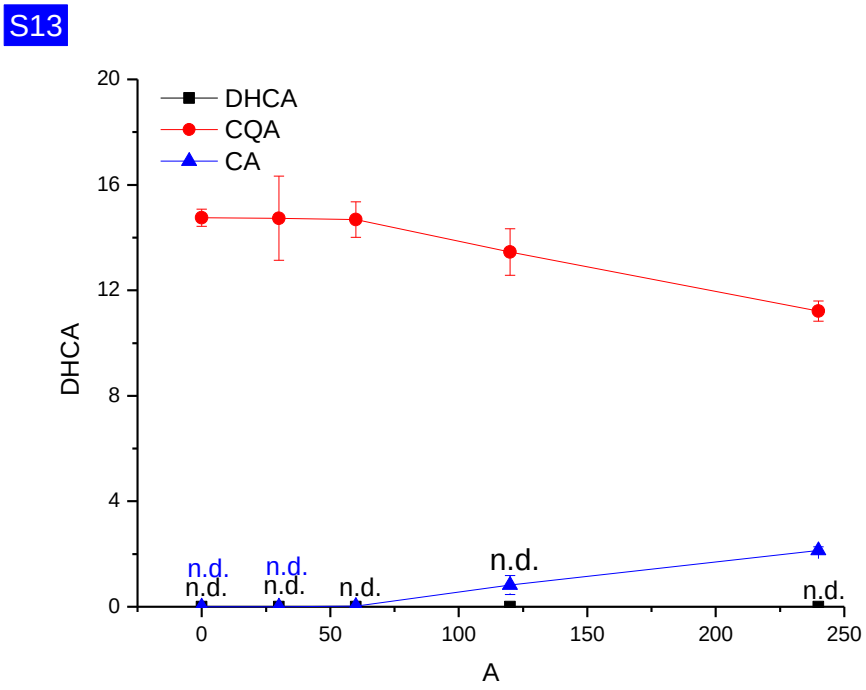


Figure 61 Incubations of 5-CQA (10 µM) with fecal samples of subjects S13. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

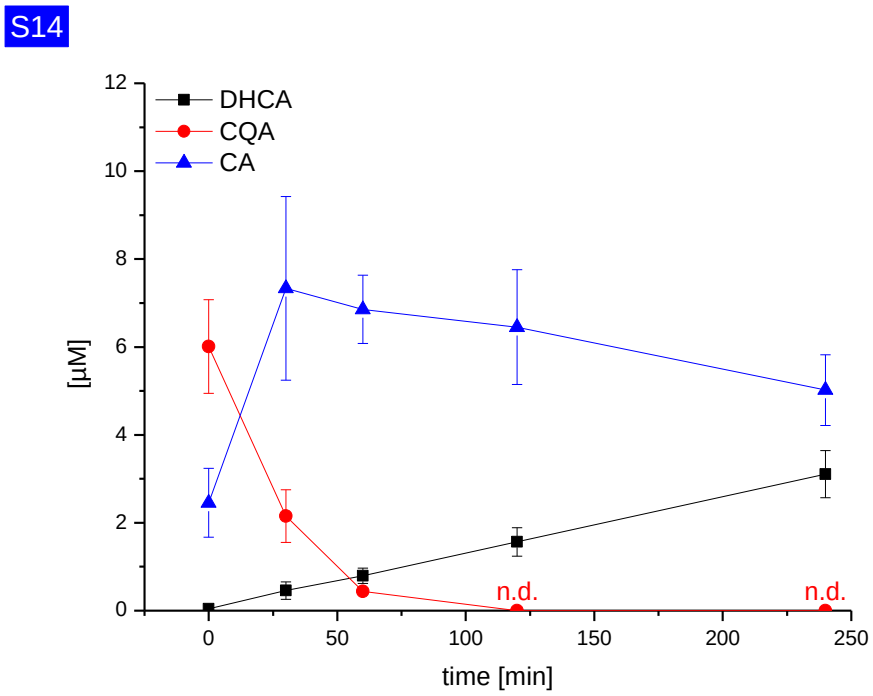


Figure 62 Incubations of 5-CQA (10 µM) with fecal samples of subjects S14. Degradation and appearance (of 5-CQA, CA and DHCA) within 4 h (0, 30, 60, 120, and 240 min). The results are means of three separate incubations with SD in all cases ≤1.59 µM.

Table 34 Plasma AUC [µM*min] of dihydro compounds determined (DHCA, DHCA3S, DHFA, DHFA4G, DHFA4S, DHiFA, DHiFA3G, DHiFA3S, MeDHFA) for each individual subject after ingestion of one cup of coffee (3.1 mg CGA/kg bw). Subjects were separated into two groups split by the final DHCA concentration being above or below 2 µM in incubated fecal sample medium after 4 h.

	low DHCA formation			high DHCA formation		
	subject	respective DHCA amounts [µM]	plasma concentrations [µM*min]	subject	respective DHCA amounts [µM]	Plasma concentrations [µM*min]
	S05	n.d.	1603	S01	2.03 ± 0.15	890
	S07	0.31 ± 0.16	N/A	S02	2.22 ± 0.48	953
	S09	1.34 ± 0.65	910	S03	3.91 ± 0.42	601
	S10	0.66 ± 0.03	849	S04	8.47 ± 0.63	585
	S11	0.90 ± 0.16	1630	S06	4.50 ±0.55	734
	S12	0.46 ± 0.03	284	S08	3.38 ± 0.06	404
	S13	n.d.	439	S14	3.11 ± 0.53	291
average [µM*min]			**953 ± 630**			**637 ± 242**

Overall, metabolism of different food ingredients can take place in all the different tissues of an organism with variable occurrences of the possibilities of metabolism according to the availability of special enzymes or bacteria. Different studies tried to investigate which part of an organism is responsible for which metabolic steps. Investigations of metabolism in the stomach in addition to the small intestine can be performed using ileostomy subjects (for example as investigated by [*Erk et al., 2012, Erk et al., 2013b*]). Investigations on the metabolism of the large intestine are rather difficult due to the impossibility to use an isolated part of the organism such as the large intestine. Hence, the comparison of results received by studies with ileostomy subjects and healthy subjects delivered first knowledge information of the metabolism occurring in the colonic part as shown by Stalmach and coworkers [*Stalmach et al., 2009, Stalmach et al., 2010*]. For detailed information on the metabolising ability of typical human microbiota, only *in vitro* investigations are mostly possible and should be interpreted with caution. In the past, it was showed that the microbiota is responsible for metabolism of CGA to DHFA and DHCA derivatives [*Williamson et al., 2011*], with subsequent formation of a wide range of metabolites including 3-hydroxyphenylpropionic acid, benzoic acid and hippuric acid [*Gonthier et al., 2006, Gonthier et al., 2003, Ludwig et al., 2013*]. Due to a confined experiment, we had only the possibility to determine DHCA formation by fecal microbiota as final metabolite. Our results confirmed the findings of the

other working groups detecting DHCA as one of the metabolites formed by the fecal bacteria from CQA.

In our study, the individual summation of 5-CQA, CA, and DHCA values after 4 h from the incubation of 5-CQA with fecal samples of single probands were in a range between 6.0 and 13.3 µM. Smaller amounts as the expected total of 10 µM indicated on one hand binding effects of phenolic acids to fecal components. On the other hand, a much more detailed catabolic pathway as proposed by Ludwig and coworkers [*Ludwig et al., 2013*], could also explain the lower values determined. In this case, CA is the direct product of CQA hydrolysis, whereat FA and IFA might be tissulare formed in gastric mucosal cells as well as the liver by COMT methylation of CA [*Farrell et al., 2011, Poquet et al., 2008*]. The microbiota and tissues formed broader metabolites of CA, such as *m*CoA and hydroxylated derivatives of phenylpropionic, benzoic, and hippuric acids. To this, *m*CoA can be formed by dehydroxylation, and 3,4-hydroxyphenylpropionic and 3-hydroxyphenylpropionic acids by hydrogenation and dehydroxylation. Finally benzoic acid is formed by dehydroxylation and subsequent *β*-oxidation in tissue. A conjugation of benzoic acid with glycine leads to the formation of hippuric acid [*Booth, 1963, Chesson et al., 1999, Griffiths, 1964, Gumbinger et al., 1993, Perez-Silva, 1966, Quick&Cooper, 1931, Scheline, 1968*]. In our *in vitro* experiment, only the non-tissual metabolism steps were possible due to the experimental setting. This limitation could explain the lower amounts determined as the expected 10 µM, because we did not determin other metabolites except CA and DHCA. Findings of more than 10 µM may be explained by the release of already on fecal compounds bounded 5-CQA, CA or DHCA. No studies on binding effects of CGA and fecal compounds are available, but interactions of phenolics with other moleculs of human origin were investigated. For example, Adzet and coworkers showed the interactions of phenolics with bovin serum albumin in an *in vitro* experiment by determining the binding ratio. They observed CA coupled with bovine serum albumin by a binding ratio between 61 to 95%. In contrast, the binding capacity of FA and *p*-CoA to bovine serum albumin was shown to have a binding ratio of less than 10% [*Adzet et al., 1988*]. The strength of binding affinity to human serum albumin follows the order CA >> CQA >>> QA [*Muralidhara&Prakash, 1995*]. These results confirm the possibility of interactions from CGA and fecal components.

The devision of subjects into two groups split by the final DHCA concentration after 4 h being above or below 2 µM showed no correlation between low plasma AUCs of dihydro compounds and a limited degradation of 5-CQA and appearance of CA and DHCA. Concluding, the bioavailability of coffee chlorogenic acids and its derivatives depend not only on the structure, molecular size and active or passive transport ability of the tissular composition of the organism, rather or also on individual differences, which are difficult to

assess and to forecast. We strongly recommend that future investigations include conduct metabolism experiments that focus on microbiota genotyping of individual subjects.

5 Final discussion

The influence of a food matrix on the bioavailability of coffee chlorogenic acids (CGA) in humans was investigated in an exploratory human intervention study. Taking the limited number of subjects into account, the effects of food matrix on the bioavailability of CGA from coffee and further metabolism only allowed us to observe tendencies. Nevertheless, no clear conclusion can be drawn, as the study with 14 participants revealed no reliable statistical data.

Strong interindividual variations could be observed for area under the curve (AUC), maximum plasma concentration (c_{max}), and time needed to reach maximum plasma concentration (t_{max}). Based on these interindividual variations and given the limited number of subjects no significant effects on the sum of plasma metabolite AUCs were detectable. A detailed observation of the metabolic profiles of the 14 study subjects definitely demonstrated that there is no consistent influence of the food matrix consumed in this study on the bioavailability of CGA from coffee. However, a few metabolites were found to contradict this observation.

Our results confirmed findings of previous studies, in which intact CGA were measured only at low plasma levels [*Matsui et al., 2007*, *Nardini et al., 2002, Stalmach et al., 2009*]. We were able to quantify 5-CQA, but no 3- and 4-CQA in measurable amounts in plasma. In contrast to the total sum of CGA in the instant study coffee, in which the CQA were detected at much higher concentrations as compared to FQA, we found all FQA isomers in our study in comparatively high amounts. It is already known that FQA may be absorbed in a higher quantity as compared to CQA, but the underlying mechanism of absorption is not yet fully investigated [*Erk et al., 2013*]. It is assumed, that these findings are based on the different chemical structures, the increased molecular size and lipophilicity of FQA. A lower predicted log octanol-water partition coefficient (log*P*) of CQA might explain a lower absorption of CQA, even if transport of the metabolites would occur via passive diffusion, as described for *in vitro* monolayer experiments [*Konishi et al., 2005, Murota&Terao, 2003, ChemSpider a&b*]. In addition, FQA are known to be relatively chemically stable, whereas CQA from coffee may be cleaved into CA, and then further metabolized [*Farrell et al., 2011*]. In addition, it is conceivable, that CQA is methylated to FQA. It is known that FA has a higher affinity for the monocarboxylic transporter (MCT) than CA [*Konishi et al., 2006*]. If this hold also true for FQA and CQA, CQA may not be a good MCT substrate.

The main metabolites found in our intervention study were colon-metabolized and conjugated compounds, such as DHCA, DHCA3S, DHFA, DHFA4G, DHFA4S, DHiFA, DHiFA3G, mDHCoA, and mDHCoAS (*colonics*), with approximately 90.8% of all statistically interpreted metabolites. Phenolic compounds accounted for 8.7% in form of CA3S, CA4S, FA4G, FA4S, iFA, iFA3G, iFA3S and MeFA (*phenolics*), but only for 0.5% as quinic compounds (5-CQA, 3-FQA, 4-FQA and 5-FQA (*quinics*)) representing the direct instant coffee constituents without any transformation. In the 14 healthy participants we identified DHFA, mDHCoA, DHCA3S and DHiFA as the most abundant metabolites, with 78% of all metabolites investigated. Interestingly, these compounds were subject to a slow distribution phase followed by an even slower terminal elimination phase. Thus, the plasma concentrations of these metabolites were relatively high for an extended period of time, what may explain the comparably higher quantities than for all other compounds detected. Since coumaric acid derivatives have not been much investigated in previous studies yet, these results suggest, that there should be a further examination, whether these compounds are also capable to be responsible for the beneficial effects of coffee. The same applies to the other three abundant metabolites after study coffee consumption – DHFA, DHCA3S and DHiFA – of which only limited data for the biological effects in humans are available.

The ingested meals may trigger impaired absorption relating to single metabolite AUCs, but only for the early metabolites. A possible reason for the impaired bioavailability may be a delay in absorption resulting in an increased degradation by intestinal enzymes and bacteria.

We observed for *quinics* and *phenolics,* and additionally for DHCA and DHFA, significant differences in C_{max} between treatments. Detected C_{max} values determined after pure coffee consumption were significantly higher than for metabolites measured after ingestion of coffee with a meal. After coffee consumption alone, a faster CGA and metabolite absorption, resulting in a fast distribution phase and followed by an even faster terminal elimination phase. This assumption is reflected by sharper, but compressed curves as compared to consumption of coffee with a meal. According to this finding, we did not observe differences in the sum of the AUC between treatments, reflecting the constant exposure of coffee polyphenols, even though there is a substantial influence of treatment on C_{max}.

Depending on the region where the metabolites are formed and/or absorbed, they can be divided into two groups: The early metabolites, such as *quinics* or *phenolics*, with a t_{max} between 0 and 2 h post-ingestion of the coffee and the late ones, the *colonics*, with a t_{max} between 7 and 10. Some of the early metabolites occurred really early (already 15 min post-dose) in the plasma what may indicate an absorption or metabolization by the stomach and/or in the upper part of the small intestine as already published before [*Poquet et al.,*

2008, *Zhao et al., 2004*, *Konishi et al., 2006*]. The appearance of FA4S, FA4G, iFA and MeFA directly after coffee consumption confirmed previous hypothesis, that the stomach can be an important site for absorption of pharmacologically active compounds [*Farrell et al., 2011*]. Furthermore, these data provided an argument for the ability of human gastric mucosal cells (apart from the liver) to perform methylation as well as biotransformation reactions by SULT and UGT [*Farrell et al., 2011, Poquet et al., 2008*]. The late metabolites, which were found with the highest concentrations in plasma, are typically dihydro compounds formed by the microbiota via hydrolysis in the colon. Additionally, after 15 h no return to baseline was observed for most of the latest metabolites. Therefore, plasma collection at time points up to 24 h, or even up to 48 h, and within shorter intervals, e.g. every two hours, seemed to be meaningful, providing a full pharmacokinetic profile. Nevertheless, this showed probably only the beginning of the colonic metabolites entering in the bloodstream. In this line, it might be possible that even a 48 h polyphenol free diet prior coffee intake was not sufficient to reach a full wash out of the colonic CGA metabolites circulating in the blood of the volunteers.
For two compounds, MeFA and FA4S, we identified two C_{max} in plasma (6 and 12 h). This biphasic behavior suggests that absorption occurs both in the stomach/small intestine (first peak) and in the colon (second peak). It may also suggest that plasma kinetic is influenced by the enterohepatic circulation which causes reabsorption in the small intestine.

The t_{max} values for all metabolites were strongly affected by treatment, as observed for FA4G for example, which had a delayed t_{max} of about 1.5 h when coffee was consumed in combination with uptake of a fat-rich meal. Ingestion of the coffee beverage in combination with the food matrix led to an enhanced absorption, probably in the upper part of the small intestine. Esterases in the small intestine are capable to cleave the CGA releasing the free acids and QA, respectively. On the way up to the colon, the acids are absorbed and transformed into their corresponding dihydro derivatives, the *colonics*. However, the time of arrival to the colon is delayed, which explains the later t_{max} for the *colonics*.

The metabolites were further classified into groups according to published knowledge about their physical structure and/or on whether the metabolites originated directly from the coffee, or whether they sustained initial or colonic degradation and metabolism: *Quinics, phenolics, colonics, caffeics, ferulics, glucuronides, sulfates, aglycons* and *methylated* compounds. No significant differences in the sums of AUCs were detected for all groups, except for the *quinics* and *sulfates.* In contrast to the *quinics*, for which the sum of AUCs of metabolites collected after coffee consumption with a CARB meal was significantly lower than other metabolites collected after pure coffee consumption, the *sulfates* showed a significantly lower

sum of metabolite AUCs collected after treatment with coffee with a FAT meal. These findings may be explained by a longer transition period of metabolites in the gut, caused by the presence of food matrix. This longer transition time potentially increased metabolite degradation or reduced sulfation. The data we collected cannot explain the reduced sulfation. However, it may have been caused by the direct inhibition of SULT conjugation, or by increased degradation due to longer storage in the gut.

There were no significant between-treatment differences in the metabolite concentrations of urine samples collected 24 h after coffee consumption. However, there was high inter-individual variance in the urine samples, and thus, a larger group of volunteers is required to investigate how the different treatments affect bioavailability. Additionally, shorter collection intervals could have provided a more useful pharmacokinetic profile. Nevertheless, there were differences in the urine samples collected during the 0 – 6 h period. The concentrations of metabolites were significantly higher in urine samples of individuals who had only consumed coffee than in the urine samples of indivudals who had consumed coffee with a carbohydrate-rich meal. Furthermore, the treatment that included coffee consumption in combination with a fat-rich meal resulted in significantly higher concentrations of metabolites in urine samples than the treatment that included coffee consumption with a carbohydrate-rich meal. There were no differences in the amounts of urinary metabolites between samples collected from 6 – 12 h and from 12 – 24 h. In addition, we determined quinic acid (QA) in urine samples and correlated the quantity to the amount ingested via the study coffee. We determined 60% QA of the given dosage, but we have to keep in mind, that circulating QA in the body was not only free QA directly from coffee. Moreover, QA may also be released from different QA esters, such as CQA and FQA, after cleavage by esterases during the colonic passage. On the basis of these data, a clear statement concerning the excreted amount of QA related to the ingested amount is not possible. We have to consider the sum of free QA and released QA from QA esters always as a result of ingestion, metabolism, as well as excretion.

The comparison of plasma metabolite concentrations to urinary metabolite concentrations showed no significant differences. The reason for this may be explained by strong interindividual differences and the low number of volunteers, which hindered a clear significance in the data. However, apparent data suggest a trend that higher plasma AUCs led to higher concentrations of urinary metabolites.

Overall, metabolism of different food ingredients can take place in all tissues of an organism with varying metabolism depending on the presence of special enzymes or bacteria. The possibility to investigate the metabolic ability of typical human microbiota is and the results

should be interpreted with caution. We performed incubation experiments of 5-CQA with fecal samples of individual volunteers under anaerobic conditions to calculate the inter-individual kinetics for each compound (reduced concentrations of 5-CQA as well as the appearance of CA and DHCA in the gut microbiota medium). As observed before for almost all data (plasma and urine samples), strong interindividual differences were also apparent for the degradation (5-CQA) and formation (CA and DHCA) efficiency of the individual fecal samples. The incubation of 10 µM 5-CQA with individual fecal samples has shown results (summations of 5-CQA, CA, and DHCA after 4 h), which varied in a wide range between 6.0 and 13.3 µM. This suggested that additional degradation and metabolism steps occurred besides the simple degradation from 5-CQA to DHCA. The lower levels as the expected total of 10 µM may be explained by chemical reactions of phenolic acids with fecal components such as proteins, as shown in other *in vitro* experiment, in which *phenolics* were able to interact with bovin serum albumin. Furthermore, the binding capacities of different *phenolics* with bovine serum albumin varied strongly [*Adzet et al., 1988*]. In addition, the more branched catabolic pathway with further formation of different metabolites, such as *m*CoA and hydroxylated derivatives of phenylpropionic, benzoic and hippuric acids, could also explain the low values determined. Higher amounts as the expected may be caused by the initial release of fecal matrix bound 5-CQA, CA or DHCA.
Final correlations between low plasma AUCs of dihydro compounds and a limited degradation of 5-CQA and appearance of CA and DHCA after 4 h in the incubation medium showed no significant difference for the individual fecal samples.

This study and all the outcomes measured within were exploratory. Due to the limited number of subjects, we could only investigate tendencies for effects of food on the bioavailability of CGAs and metabolites from coffee and therefore the revealed results are only indicative. But despite of that limitation it is to highlight that the data show no influence of the breakfast on the total bioavailability of CGAs and metabolites from coffee (AUC), but a significant difference in their kinetics of the release demonstrated by C_{max} and t_{max}. The co-ingestion of breakfast favored a slow and continuous release of colonic metabolites in contrast to the non-metabolized coffee components appearing in the first hour after coffee consumption. This is a relevant result which needs further investigation as it might have important physiological consequence.
In conclusion, the data of the human intervention study suggest that bioavailability of food ingredients depends not only on the chemical structure, molecular size and active or passive transport ability of the organisms, but rather or largely on individual differences. Therefore, we strongly recommend that future investigations should also include the conduction of metabolism experiments that focus on microbiota genotypes in individual subjects. The goal

would be to identify a correlation between genotype and metabolic profiles after chlorogenic acid intake.

6 Material and methods

6.1 General

Chemicals used were of analytical grade. Those not specified were purchased from Fluka, Sigma Aldrich (Schnelldorf, Germany) and Toronto Research Chemicals Inc. (Toronto, Ontario, Canada), in highest purity. Feruloylglycine and 3-*O*-caffeoylquinic acid lactone were synthesized in the working group and were sufficient for sample analysis. For dilutions, solvents and solid phase extractions were operated and produced with fresh bidestilled water ($water_{bidest}$).

6.2 General used consumables, chemicals and equipments

6.2.1 Consumables

reaction tube	2 ml und 1.5 ml, Greiner BIO ONE, Kremsmünster, Österreich
fluted filter	Schleicher & Schuell Bioscience Inc., New Hampshire, United States
Pasteur pipette	disposable pipette og glas circa 150 mm, Brand GmbH & Co KG, Wertheim, Germany
pH paper	pH 9.0-13.0, Honeywell Riedel-de Haën AG, Seelze, Germany
pH strips	Spezialindikator pH 6,5-10,0, Merck,
pipette tip	1000 µl, 100 µl, Greiner BIO ONE, Kremsmünster, Österreich
filter circle	Schleicher & Schuell Bioscience Inc., New Hampshire, United States
centrifuge tube	Centrifugal Filter Devices, 1.6 ml, Amicon, Bioseparation Microcon®, MerckMillipore, Billerica, Massachusetts, United States

6.2.2 Chemicals

compound	abbrevation	
5-*O*-caffeoylquinic acid	5-CQA	Extrasynthèse[1]
genistein	-	Extrasynthèse[1]
sodium chlorid	NaCl	Merck[2]
calcium chlorid	$CaCl_2$	Sigma[3]
sodium hydrogencarbonate	$NaHCO_3$	Sigma[3]
vitamin K_3 (menadione)	-	Sigma[3]
potassium hydrogen phosphate	KH_2PO_4	Merck[2]
iron (II) sulfate heptahydrate	$FeSO_4$ (7 H_2O)	Sigma[3]
Tween 80	-	BD[4]
resazurin	-	Sigma[3]
hemin	-	Sigma[3]
acetic acid (glacial)	CH_3COOH	Merck[2]
propionic acid	-	Fluka[5]
valeric acid	-	Aldrich[6]
isovaleric acid	-	Fluka[5]
tryptycase peptone	-	BD[4]
yeast Extract	-	BD[4]
D-glucose	-	Sigma[3]
L-cysteine	-	Sigma[3]
cellobiose D+	-	Calbiochem[7]
maltose	-	Sigma[3]
fructose	-	Sigma[3]
meat extract	-	Fluka[5]
magnesium sulfate 7 H_2O	$MgSO_4$ (7 H_2O)	Serva[8]
ATCC Vitamin Mix	-	ATCC[9]
ATCC Trace Mineral Mix	-	ATCC[9]
Dubelco phosphate buffer saline	PBS	Sigma[3]
glycerol	-	Merck[2]
sodium hydroxide	NaOH	Merck[2]
methanol Lichrosolv	CH_3OH	Merck[2]
acetonitrile	CH_3CN	J. T. Baker[10]
ortho-phosphoric acid 85%	H_3PO_4	Merck[2]
sodium phosphate monobasic monohydrate	NaH_2PO_4 H_2O	Sigma[3]
dimethyl sulfoxide	DMSO	Sigma[3]
D-(-)quinic acid	QA	Sigma[3]
U-^{13}C-D-(-)-quinic acid	U-^{13}C-QA	IsoLife[11]
p-coumaric acid	pCoA	Sigma-Aldrich Chemie[12]
3-phenylpeopionic acid	3PPA	Sigma-Aldrich Chemie[12]

compound	abbrevation	
Cinnamic acid	CinAc	Sigma-Aldrich Chemie[12]
4-methoxycinnamic acid	4MCA	Sigma-Aldrich Chemie[12]
3-(4-methoxyphenyl)propionic acid	3,4MPPA	Sigma-Aldrich Chemie[12]
methylferulic acid	MeFA	Sigma-Aldrich Chemie[12]
methyldihydroferulic acid	MeDHFA	Sigma-Aldrich Chemie[12]
		Sigma-Aldrich Chemie[12]
caffeic acid	CA	Extrasynthèse[13]
ferulic acid	FA	Extrasynthèse[13]
dihydrocaffeic acid	DHCA	Extrasynthèse[13]
m-coumaric acid	mCoA	Extrasynthèse[13]
o-coumaric acid	oCoA	Extrasynthèse[13]
isoferulic acid	iFA	Extrasynthèse[13]
dihydroferulic acid	DHFA	Fluka[5]
3-caffeoylquinic acid	3-CQA	Chengdu Biopurify Pytochemicals Ltd[14]
4-caffeoylquinic acid	4-CQA	Chengdu Biopurify Pytochemicals Ltd[14]
3-feruloylquinic acid	3-FQA	NRC[15]
4-feruolquinic acid	4-FQA	NRC[15]
5-feruloylquinic acid	5-FQA	NRC[15]
dihydroisoferulic acid	DHiFA	NRC[15]
m-dihydrocoumaric acid	mDHCoA	NRC[15]
o-dihydrocoumaric acid	oDHCoA	NRC[15]
p-dihydrocoumaric acid	pDHCoA	NRC[15]
dihydroferulic-4-*O*-sulfate	DHFA4S	NRC[15]
ferulic-4-*O*-sulfate	FA4S	NRC[15]
caffeic-4-*O*-sulfate	CA4S	NRC[15]
dihydrocaffeic-4-*O*-sulfate	DHCA4S	NRC[15]
dihydrocaffeic-3-*O*-sulfate	DHCA3S	NRC[15]
caffeic-3-*O*-sulfate	CA3S	NRC[15]
isoferulic-3-*O*-sulfate	iFA3S	NRC[15]
dihydroisoferulic-3-*O*-sulfate	DHiFA3S	NRC[15]
5-caffeoylquininc-3-*O*-sulfate	5CQA3S	NRC[15]
5-caffeoylquininc-4-*O*-sulfate	5CQA4S	NRC[15]
5-feruloylquininc-4-*O*-sulfate	5FQA4S	NRC[15]
p-coumaric acid-4-*O*-sulfate	pCoAS	NRC[15]
m-coumaric acid-3-*O*-sulfate	mCoAS	NRC[15]
dihydro-*m*-coumaric acid-3-*O*-sulfate	mDHCoAS	NRC[15]
dihydro-*p*-coumaric acid-4-*O*-sulfate	pDHCoAS	NRC[15]

compound	abbrevation	
caffeic-4-*O*-glucuronide	CA4G	NRC[15]
dihydrocaffeic-4-*O*-glucuronide	DHCA4G	NRC[15]
dihydroferulic-4-*O*-glucuronide	DHFA4G	NRC[15]
caffeic-3-*O*-glucuronide	CA3G	NRC[15]
ferulic-4-*O*-glucuronide	FA4G	NRC[15]
dihydrocaffeic-3-*O*-glucuronide	DHCA3G	NRC[15]
isoferulic-3-*O*-glucuronide	iFA3G	NRC[15]
dihydroisoferulic-3-*O*-glucuronide	DHiFA3G	NRC[15]
5-feruloylquininc-4-*O*-glucuronide	5FQA4G	NRC[15]
m-dihydrocoumaric-3-*O*-glucuronide	mDHCoAG	NRC[15]
p-dihydrocoumaric-4-*O*-glucuronide	pDHCoAG	NRC[15]
p-coumaric-4-*O*-glucuronide	pCoAG	NRC[15]
m-coumaric-3-*O*-glucuronide	mCoAG	NRC[15]
$D^{13}C_2$-caffeic acid	$D^{13}C_2$-CA	Orphachem[16]
Dihydroisoferulic-D_2-3-*O*-glucuronide	D_2-DHiFA3G	TRC[17]
Dihydroisoferulic-D_2-3-*O*-sulfate	D_2-DHiFA3S	TRC[17]

[1]Extrasynthèse, Genay, France

[2]Merck, Darmstadt, Germany

[3]Sigma, a part of Merck, Darmstadt, Germany

[4]BD, Franklin Lakes, New Jersey, United States

[5]Fluka, a part of Merck, Darmstadt, Germany

[6]Aldrich, a part of Merck, Darmstadt, Germany

[7]Calbiochem, a part of Merck, Darmstadt, Germany

[8]Serva, Heidelberg, Germany

[9]ATCC, Teddington, Middlesex, United Kingdom

[10] J. T. Baker, Avantor Performance Materials, Center Valley, Pennsylvania, United States

[11]IsoLife, Wageningen, The Netherlands

[12]Sigma-Aldrich Chemie, Buchs, Switzerland

[13]Extrasynthèse, Lyon, France

[14]Chengdu Biopurify Phytochemicals Ltd, Chengdu, Sichuan, China

[15]NRC, Nestlé Research Center, Vers-chez-les-Blanc, Lausanne, Switzerland

[16]Orphachem S.A., Clermont.Ferrand Cedex, France

[17]TRC, Toronto Research Chemicals, Toronto, Canada

6.2.3 Equipments

thermometer	LHU, LET-100/30, DIN 12778
incubator	CB 210 WTB Binder GmbH, Tuttlingen, Germany
ice machine	Wessamat, Perfect ice! Combi-Line, Kaiserslautern, Germany
Eppendorf pipettes	Eppendorf Group, Hamburg, Germany
hot air gun	Steinel HL 1800E; Herzebrock-Clarholz; Germany
refrigerator	Privileg, Privileg *** (-20 °C) Liebherr Comfort *** (-30 °C) Liebherr Premium (5 °C and -20 °C)
volumetric glassware	Schott Duran®, Mainz, Germany
HPLC-ESI-MS/MS	mass spectrometer API 2000 AB Sciex, Concord, Canada Perkin Elmer Series 2000, Waltham, United States mass spectrometer API 3200 AB Sciex, Concord, Canada HPLC Jasco 2008, Groß-Umstadt, Germany HPLC Agilent 1200 Series, Santa Clara, United States mass spectrometer QTrap 5500 AB Sciex, Concord, Canada HPLC Agilent 1260 Series, Santa Clara, United States
magnetic stirrer	IKA COMBIMAG RCT, Staufen, Germany
Nanodrop	NanoDrop®, ND-1000 Spectrophotometer; Wilmington, United States
pipettes	Pipetten Abimed, Eppendorf, Pipettierhilfe Pipettus® Hirschmann Laborgeräte, Hamburg, Germany
rotary evaporator	Büchi R110 Rotavapor, Essen, Germany
thermo shaker	5436 Eppendorf, Hamburg, Germany
ultrasonic bath	Bandelin Sonorex RK 102 H, Berlin, Germany
analytical balance	Sartorius laboratory, Göttingen, Germany Sartorius CP224S (Feinwaage)

	Sartorius Type 1507 (Grobwaage)
water quench	Köttermann Labortechnik, Uetze, Germany
vacuum exhauster	Vacuubrand Drehschieber Ölpumpe, Wertheim, Germany
vortex	MS1 Minishaker; IKA®, Staufen, Germany
centrifuge	Eppendorf Zentrifuge 5415, Hamburg, Germany
	Eppendorf Zentrifuge 5417R, Hamburg, Germany
	Eppendorf Zentrifuge 5804R, Hamburg, Germany
anaerobic chamber	Don Whitley Anaerobic workstation MG 1000, Shipley, West Yorkshire, United Kingdom
CoulArray Detector	Thermo Fisher, ESA CoulArray Detector model 5600A, Waltham, Massachusetts, United States
Vortex	Genie 2, Scientific Industries, Bohemia, New York, United States
SPE cartridges	Waters Oasis HLB 1 cc/3 mg, Milford, Massachusettts, United States
SPE vacuum manifold	Supelco Visiprep™ 12 or 24, Bellefonte, Pennsylvania, United States
disposable flow control liner	Supelco, Bellefonte, Pennsylvania, United States
pH paper 0 – 14	Merck, Darmstadt, Germany
Milli-Q	Millipore Coperations, a part of Merck, Darmstadt, Germany

6.3 Synthesis and isolation of standards

6.3.1 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) spectra were measured with DPX 400 und Avance 600, instruments of Bruker (Karlsruhe, Germany). For Evaluation, TOPSPIN 1.3 and 1D-WinNMR for graphical formation were used.

The recorded ^{1}H- und ^{13}C-spectra were calibrated again the used solvent (Table 35).

Table 35 Calibration of chemical shift for evaluation of ^{1}H- and ^{13}C-nuclear magnetic resonance spectra.

	DMSO-d_6	D_2O
^{1}H-NMR	2.49	4.7
^{13}C-NMR	39.50	-

Declaration of chemical shift was occurred in δ-scale in ppm. Multiplicities were specified with s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, as well as splitting patterns such like dt = doublet of triplet.

6.3.2 Synthesis of feruloylglycine

Feruloylglycine was synthesized to be used for optimization of compound-related parameters on the mass spectrometer and as a standard for quantification. The synthesis was adapted from Booth *et al*. with slight modifications [*Booth et al., 1957*] as shown in Figure 63. Ferulic acid and glycine ethyl ester hydrochloride were used as basic material, whereas the salt of glycine ethyl ester (glycine ethyl ester hydrochloride) had to be converted into glycine ethyl ester. This step was successfully simulated following a method described by Fischer [*Fischer, 1902*]. A solution of glycine ethyl ester hydrochloride was neutralized with sodium hydroxid solution and afterwards dried with calcium oxide. The filtrate was evaporated and an uncolored liquid occurred. In the first step, the glycinate and dicyclohexyl carbodiimide were dissolved in tetrahydrofurane. Afterwards ferulic acid was dissolved in tetrahydrofurane and added to the before mentioned solution. The reaction mixture was stirred at room temperature for three days and then dicyclohexyl urea was filtered. The filtrate was evaporated and dried, whereat a yellowish solid was achieved. Feruloylglycine ethyl ester was extracted with hot ethanol, filtered and hydrolyzed by being warmed up for about 15 minutes with a slight excess of methanolic potassium hydroxide. Methanol was evaporated under reduced pressure, the residue was taken up in water and acidified to pH 4 with hydrochloric acid. Feruloylglycine was recyrstallized from water, precipitated and dried. A yellowish solid was achieved.

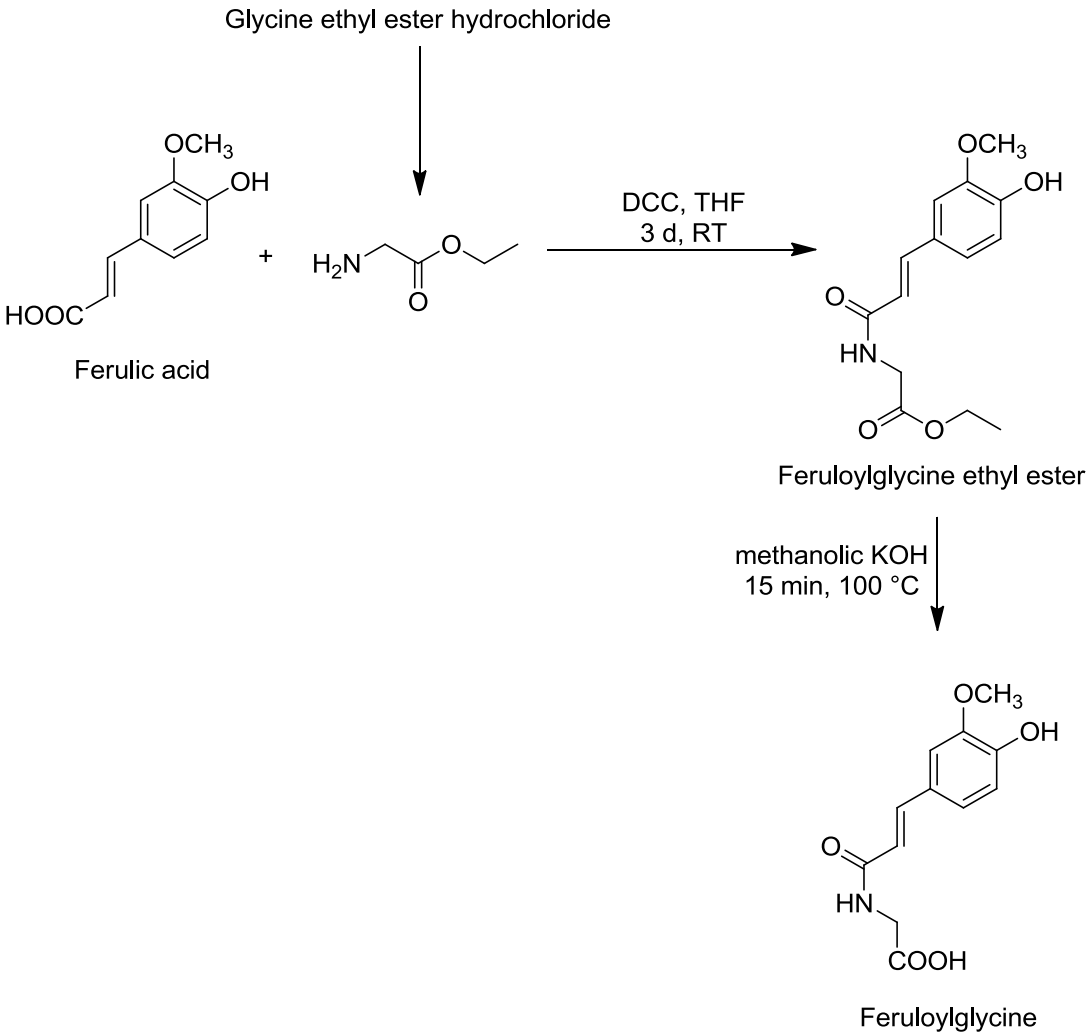


Figure 63 Synthesis scheme for feruloylglycine from ferulic acid and glycine ethyl ester, modified according to [*Booth et al., 1957, Fischer, 1902*]

For the purification of feruloylglycine a preparative RP-HPLC system was used. Separation was performed on an analytical C_{18} HPLC column and detection was achieved at a wavelength of 325 nm (method see Materials and methods, Table 36). The yield was 60% corresponding 1.24 g (4.96 mmol) feruloylglycine.

Table 36 Preparative HPLC parameter for isolation and purification of feruloylglycine.

Column	Reprosil 100		
Material	C18, 5 µm, 250x20 mm		
Mobile phase	A: water (0.1% HCOOH) B: acetonitrile		
wave length	325 nm		
Time	**Flow [ml/min]**	**Solvent A [%]**	**Solvent B [%]**
0.1	5	98	2
20	5	10	90
22	5	10	90
25	5	98	2
30	5	98	2

The chemical characterization and identification of the structure was effected by NMR analysis and the mass to charge ratio (*m/z*) of feruloylglycine was verified by MS analysis.

NMR analysis of feruloylglycine

After dissolving the solid in d_6-DMSO, the ^{1}H-NMR spectroscopy was performed using a DPX 400 spectrometer (Bruker Biospin, Rheinstetten, Germany). The calibration was carried out of the d_6-DMSO with a chemical shift at δ 2.49 ppm.

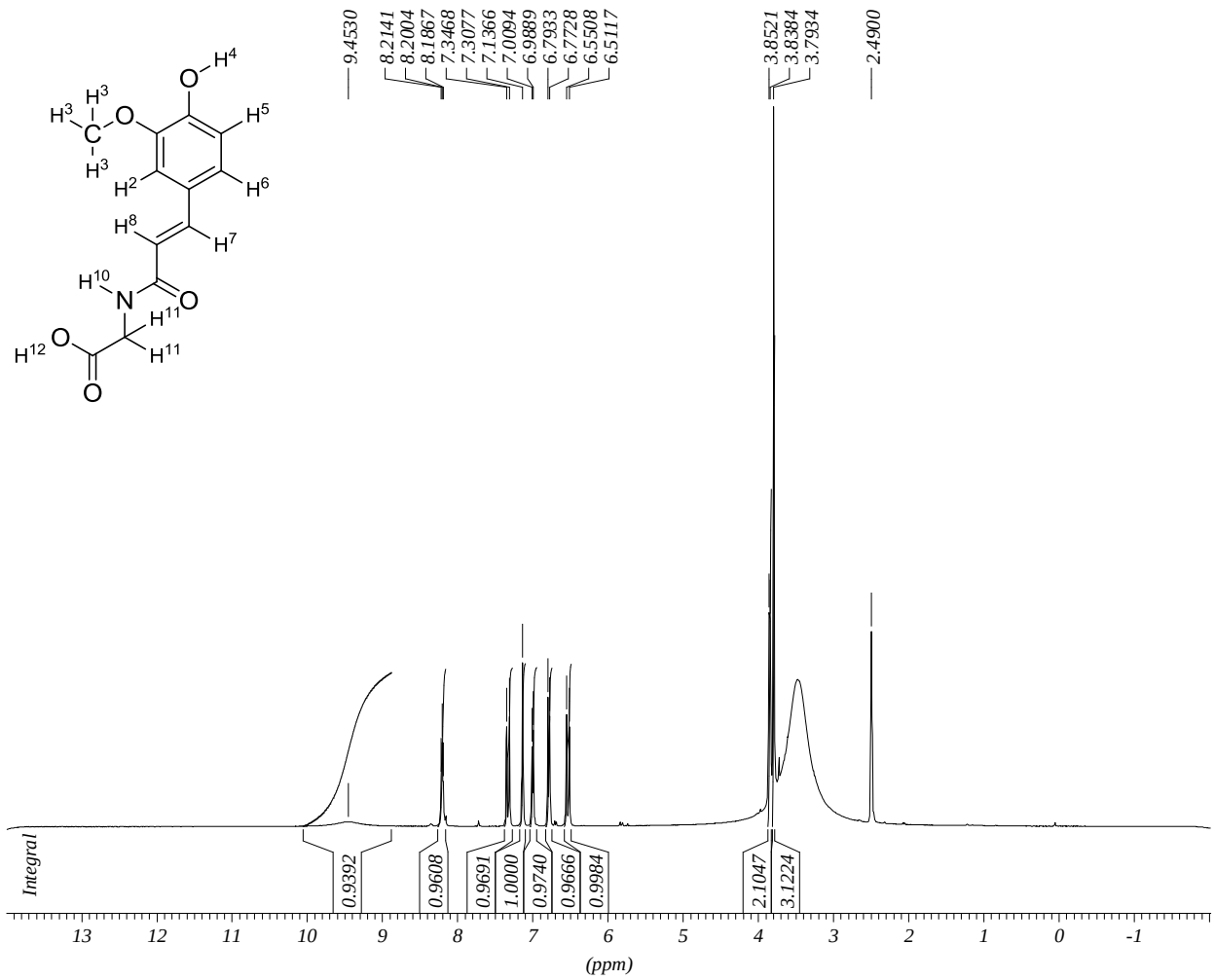


Figure 64 ^{1}H-NMR of feruloylglycine in d_6-DMSO at 293.5 K and 400 MHz.

At the associated ^{1}H-NMR spectrum (Figure 64), in the farthest upfield was the protons at position 3. There was a singlet at δ = 3.79 ppm with an integral of 3. The protons at position 11 arose as doublet at δ = 3.85 ppm with an integral of 2 and a coupling constant of $^{3}J_{HH}$ = 5.48 Hz. At a chemical shift of δ = 6.53 ppm, with an integral of 1 and $^{3}J_{HH}$ = 15,.5 Hz was the proton at position 8 as doublet. In each case, there are doublets for the protons at position 5 and 6 of the aromatic ring, with a chemical shift of δ = 6.78 ppm and δ = 7.00 ppm respectively. Both with an integral of 1 and $^{3}J_{HH}$ = 8.22 Hz. The proton in position 2 was a singlet at δ = 7.14 ppm with an integral of 1. At a chemical shift of δ = 7.33 ppm with an integral of 1 and a coupling constant of $^{3}J_{HH}$ = 15.65 Hz, there is the proton at position 7. At δ = 8.20 ppm, there is a triplet for the proton at position 10 of the NH group with $^{3}J_{HH}$ = 5.69 Hz and an integral of 1. In the farthest downfield, at a chemical shift of δ = 9.45 ppm was the proton at position 12 of the OH group as singlet with an integral of 1.

MS characterization of feruloylglycine

After dissolution of feruloylglycine (100 ng/ml) in ethanol/water/formic acid (30/69.9/0.1; v/v/v) the solution was directly injected via syringe pump into the ion source of a triple quad mass spectrometer (Applied Biosystems, API 3200). The flow rate was set at 10 µl/min and the polarity on the ESI source was negative. The compound was identified by the mass to charge ratio (*m/z)* of 250. After fragmentation in Q2 the compound-dependent parameters of the three fragments with the highest intensity (*m/z* 206, *m/z* 163, *m/z* 134) were optimized (see Figure 65). For the analyses the mass transition *m/z* 250 → 134 was used.

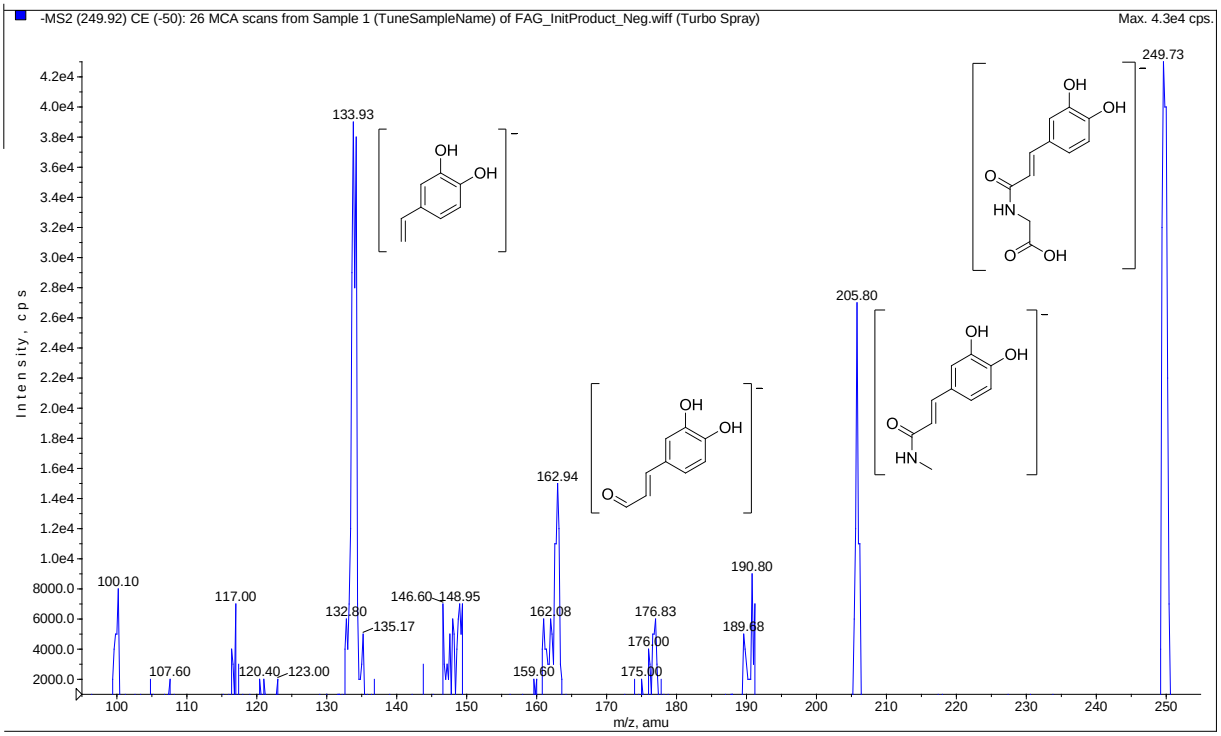


Figure 65 ESI$^-$ product ion scan of feruloylglycine ([M-H]$^-$, *m/z* 250) at Q3 in mass range of *m/z* 90-260.

6.3.3 Synthesis of 3-*O*-caffeoylquinic acid lactone

3- and 4-*O*-caffeoylquinic acid lactones were synthesized to be used for mass spectrometry compound-related parameters optimization and as a standard for quantification. The first step of the synthesis was the isomerization of 5-*O*-caffeoylquinic acid to 3- and 4-*O*-caffeoylquinic acid (3- and 4-CQA) adapted from Trugo and Macrae with slight modifications, briefly [*Trugo&Macrae, 1984*]. The intramolecular esterification to the lactones was performed by the synthesis of Steglich with some modifications [*Neises&Steglich, 1978*].

6.3.3.1 Isomerization of 5-*O*-caffeoylquinic acid to 3- and 4-*O*-caffeoylquinic acid

5-CQA was dissolved in $water_{bidest}$ and pH value was adjusted to 8 with ammonia solution (1 N). For 30 min, the reaction mixture was stirred at 90 °C and the reaction was stopped by acidification with hydrochloric acid solution (adjusted pH-value of 2.5 – 3). The reaction was monitored by HPLC-DAD based on the retention times (3-CQA (4.9 min), 4-CQA (5.9 min), 5-CQA (6.1 min). For the purification of 3-, 4- and 5-CQA a preparative RP-HPLC system was used. The separation was perforemed on an analytical C_{18} HPLC column at a wavelength of 325 nm. In Table 37 of chapter Materials and methods, the analytical parameters are shown. Separation of 3-CQA was successfully performed; 4- and 5-CQA were not separable. Water was evaporated under reduced pressure and 3-CQA was recrystallized from water/ethanol (1/1, v/v), precipitated and dried. An uncolored solid was achieved with a yield of 22% corresponding 111 mg (0.31 mmol). The synthesis scheme is shown in Figure 66.

Table 37 Preparative HPLC parameter for isolation and purification of 3-O-caffeoylquinic acid.

Column	Reprosil 100		
Material	C18, 5 µm, 250x20 mm		
Mobile phase	A: water (0.1% HCOOH) B: acetonitrile		
wave length	325 nm		
Time	**Flow [ml/min]**	**Solvent A [%]**	**Solvent B [%]**
0.1	8	98	2
5	8	88	12
20	8	70	30
21	8	10	90
24	8	10	90
25	8	98	2
29	8	98	2

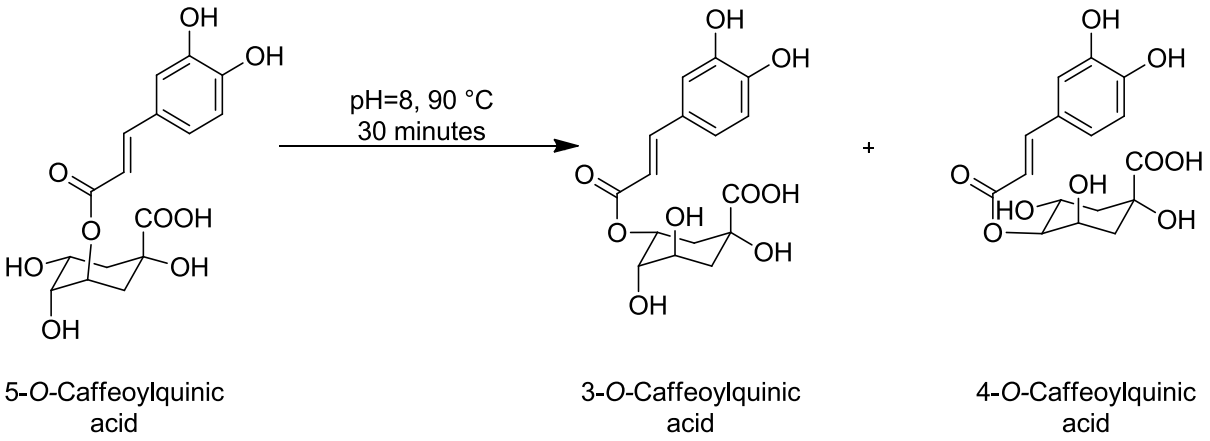


Figure 66 Scheme of isomerization of 5-*O*-caffeoylquinic acid (5-CQA) to 3- and 4-*O*-caffeoylquinic acid (3- and 4-CQA), modified according to [*Trugo&Macrae, 1984*]

The chemical characterization and identification of the structure was effected by NMR analysis and the mass to charge ratio (*m/z*) of 3-CQA was verified by MS analysis.

NMR analysis of 3-*O*-caffeoylquinic acid

After dissolution of the solid in d_6-DMSO, the ^{1}H-NMR spectroscopy was performed using a DPX 600 spectrometer (Bruker Biospin, Rheinstetten, Germany). The calibration was carried out of the d_6-DMSO with a chemical shift by δ 2.49 ppm.

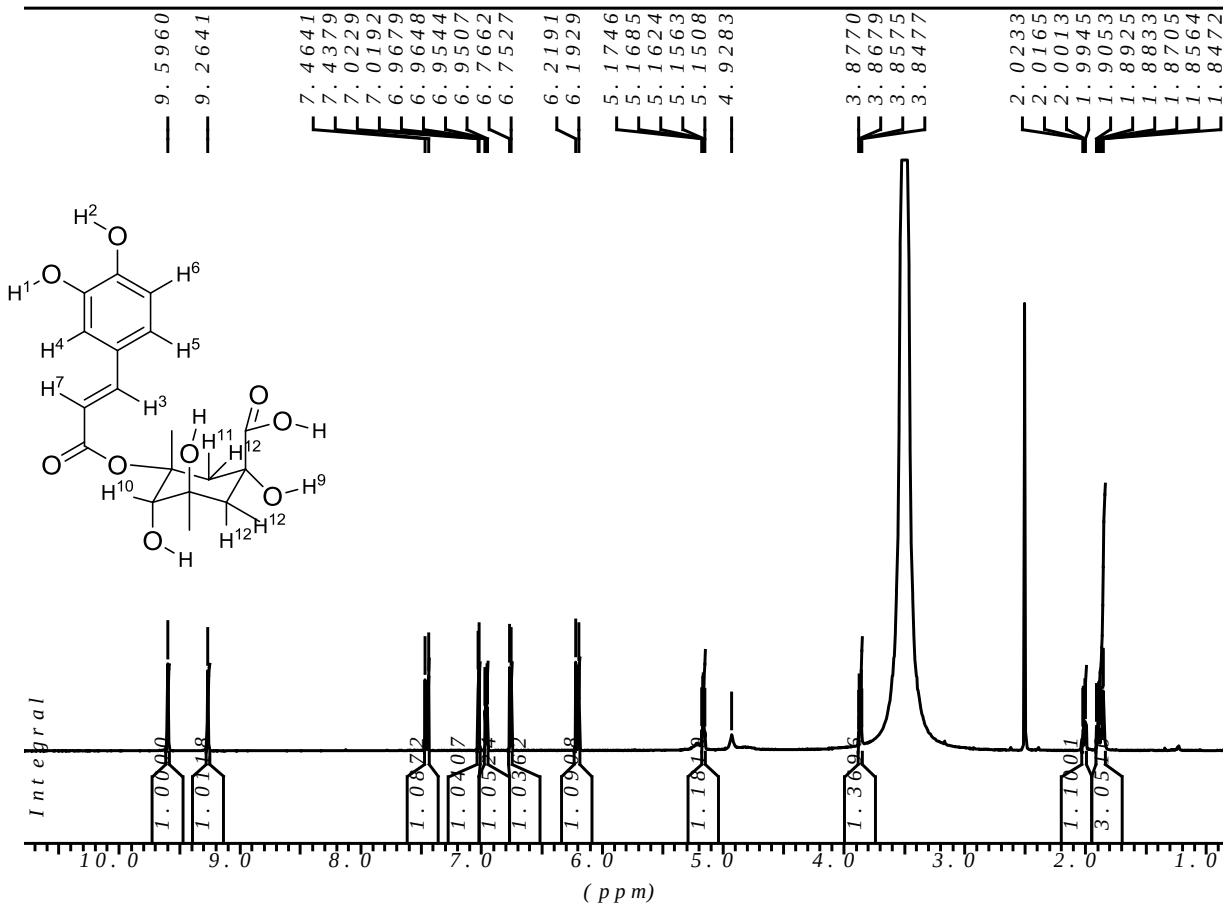


Figure 67 ^{1}H-NMR spectrum of 3-*O*-caffeoylquinic acid (3-CQA) (d_6-DMSO, 600 MHz, 293,5 K)

At the associated ^{1}H-NMR spectrum (Figure 67), in the farthest upfield was the protons at position 12. There is a multiplet at δ = 1.86 ppm with an integral of 3. The protons at position 11 arose as double doublet at δ = 1.99 ppm with an integral of 1 and a coupling constant of $^{3}J_{HHax\text{-}ax}$ = 13.2 Hz and $^{3}J_{HHgeminal}$ = 4.03 Hz. At a chemical shift of δ = 3.84 ppm, with an integral of 1 and $^{3}J_{HHeq\text{-}eq}$ = 8.81 Hz and $^{3}J_{HHeq\text{-}ax}$ = 2.98 Hz was the proton at position 10 as double doublet. There is a singlet for the proton at position 9, the aliphatic hydroxyl group, with a chemical shift of δ = 4.90 ppm. The proton at position 8 was a triplet of a doublet at δ = 5.15 ppm with an integral of 1 and $^{3}J_{HHdiaxial}$ = 7.90 Hz, $^{3}J_{HHax\text{-}eq}$ = 2,78 Hz and $^{3}J_{HHax\text{-}eq}$ = 2.70 Hz. At a chemical shift of δ = 6.19 ppm with an integral of 1 and a coupling constant of $^{3}J_{HH}$ = 15.77 Hz, there is the olefinic proton at position 7. At δ = 6.74 ppm, there is a doublet for the proton at position 6 with $^{3}J_{HH}$ = 8.07 Hz and an integral of 1. There is a double doublet at a chemical shift of δ = 6.94 ppm with an integral of 1 and $^{3}J_{HH}$ = 8.07 Hz and $^{4}J_{HH}$ = 1.84 Hz for the proton at position 5. In each case, there are doublets for the protons at position 4 and 3, with a chemical shift of δ = 7.00 ppm and δ = 7.43 ppm respectively. Both

with an integral of 1 and $^{3}J_{HH}$ = 2.20 Hz and $^{3}J_{HH}$ = 15.78 Hz, respectively. In the farthest downfield, at a chemical shift of δ = 9.24 ppm and δ = 9.58 ppm respectively, there were the protons at position 2 and 1 as singlet with an integral of 1.

MS characterization of 3-*O*-caffeoylquinic acid

After dissolving the sample (100 ng/ml) in ethanol/water/formic acid (30%/69.9%/0.1%; v/v/v) the solution was directly injected via syringe pump into the ion source of a triple quad mass spectrometer (Applied Biosystems, API 3200). The flow rate was set at 10 µl/min and the polarity at the ESI source was negative. The compound was identified by the mass to charge ratio (*m/z)* of 353 and the corresponding fragments (*m/z* 191 quinic acid, *m/z* 179 caffeic acid). After fragmentation in Q2 the compound-dependent parameters of the three fragments with the highest intensity were optimized (see Figure 68). For the analyses the mass transition 353 → 191 was used.

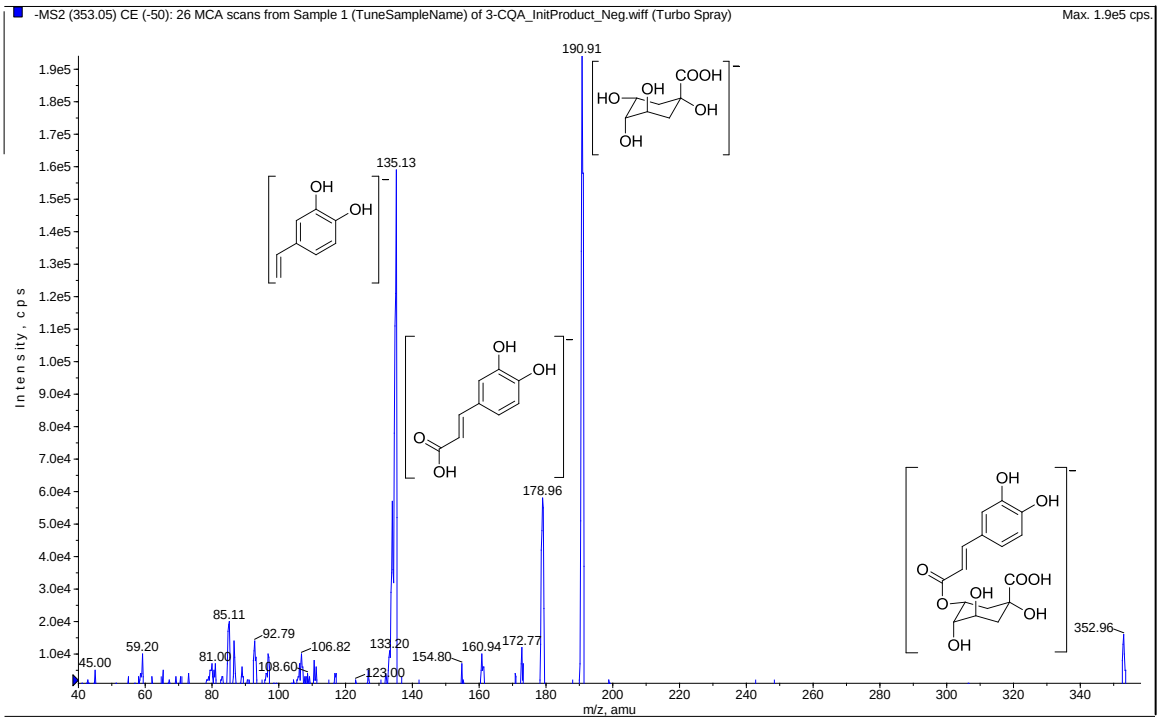


Figure 68 ESI^{-} production scan of 3-*O*-caffeoylquinic acid ([M-H]$^{-}$, *m/z* 353) at Q3 in mass range of *m/z* 40-360, fragmentation: *m/z* 191 quinic acid, *m/z* 179 caffeic acid

On the basis of the correlation of ^{1}H-NMR spectrum by calculation of increment and in comparison with the literature [*He et al., 2010*] and in the comparison to the production ion scan of the precursor *m/z* 353, 3-CQA was identified in positive mode.

6.3.3.2 Synthesis of 3-*O*-caffeoylquinic acid-1,5-lactone (3-CQA15L)

To synthesize 3-O-caffeoylquinic acid lactone (3-CQA15L) 3-CQA was dissolved in tetrahydrofurane (THF) and transformed into the corresponding lactone with dicyclohexyl carbodiimide (DCC). For three hours, the reaction mixture was stired at room temperature. The reaction was monitored by HPLC-DAD based on the corresponding retention time. After evaporation, dicyclohexyl urea was filtered. For the purification of 3-CQA15L a preparative RP-HPLC system was used. The detection was effected on an analytical C18 HPLC column at a wavelength of 325 nm. In Table 38 of chapter Materials and methods, the analytical parameters are shown. Water was evaporated under reduced pressure and 3-CQA15L was precipitated and dried. An uncolored solid was achieved with a yield of 60% corresponding 48 mg (0.14 mmol). The synthesis scheme is shown in Figure 69 [*Neises&Steglich, 1978*].

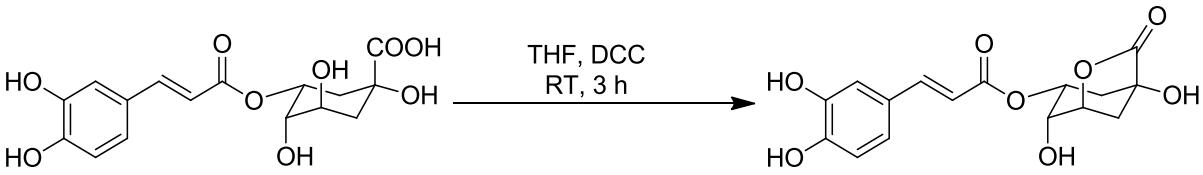


Figure 69 Scheme for the synthesis of 3-*O*-caffeoylquinic acid-1,5-lactone (3-CQA15L)) from 3-*O*-caffeoylquinic acid (3-CQA) modified according to [*Neises&Steglich, 1978*]; THF: tetrahydrofuran, DCC: N,N-dicyclohexylcarbodiimide

Table 38 Preparative HPLC parameter for isolation and purification of 3-*O*-caffeoylquinic acid lactone.

Column	Reprosil 100		
Material	C18, 5 µm, 250x20 mm		
Mobile phase	A: water (0.1% HCOOH) B: acetonitrile		
wave length	325 nm		
Time	**Flow [ml/min]**	**Solvent A [%]**	**Solvent B [%]**
0.1	10	98	2
3	10	84	16
20	10	60	40
21	10	10	90
24	10	10	90
25	10	98	2
29	10	98	2

The chemical characterization and identification of the structure was effected by NMR analysis and the mass to charge ratio (*m/z*) of 3-CQA15L was verified by MS analysis.

NMR analysis of 3-*O*-caffeoylquinic acid-1,5-lactone

After dissolution of the solid in d_6-DMSO, the ^{1}H-NMR spectroscopy was performed using a DPX 400 spectrometer (Bruker Biospin, Rheinstetten, Germany). The calibration was carried out of the d_6-DMSO with a chemical shift by δ 2.49 ppm.

At the associated ^{1}H-NMR spectrum (Figure 70), in the farthest upfield was the protons at position 8. There is a multiplet at δ = 1.93 ppm with an integral of 2. The protons at position 9 arose as double doublet at δ = 2.21 ppm with an integral of 1 and a coupling constant of $^3J_{HH}$ = 13.2 Hz and $^2J_{HH}$ = 4.03 Hz. At a chemical shift of δ = 2.36 ppm, with an integral of 1 and $^3J_{HH}$ = 11.49 Hz was the proton at position 10 as doublet. There is a triplet for the proton at position 7 with a chemical shift of δ = 4.13 ppm, an integral of 1 and a coupling constant of $^3J_{HH}$ = 4,43 Hz. The proton at position 6 was a multiplet at δ = 4.69 ppm with an integral of 2. At a chemical shift of δ = 6.25 ppm with an integral of 1 and a coupling constant of $^3J_{HH}$ = 15.910 Hz, there is the olefinic proton at position 4. At δ = 6.75 ppm, there is a doublet for the proton at position 5 with $^3J_{HH}$ = 8.07 Hz and an integral of 1. There is a double doublet at a chemical shift of δ = 6.99 ppm with an integral of 1 and $^3J_{HH}$ = 8.31 Hz and $^4J_{HH}$ = 1.84 Hz for the proton at position 3. There is a singlet for the proton at position 2 with a chemical shift of δ = 7.04 ppm and an integral of 1. In the farthest downfield, at a chemical shift of δ = 7.52 ppm, there is the proton at position 1 as a doublet with an integral of 1 and a coupling constant of $^3J_{HH}$ = 15.78 Hz.

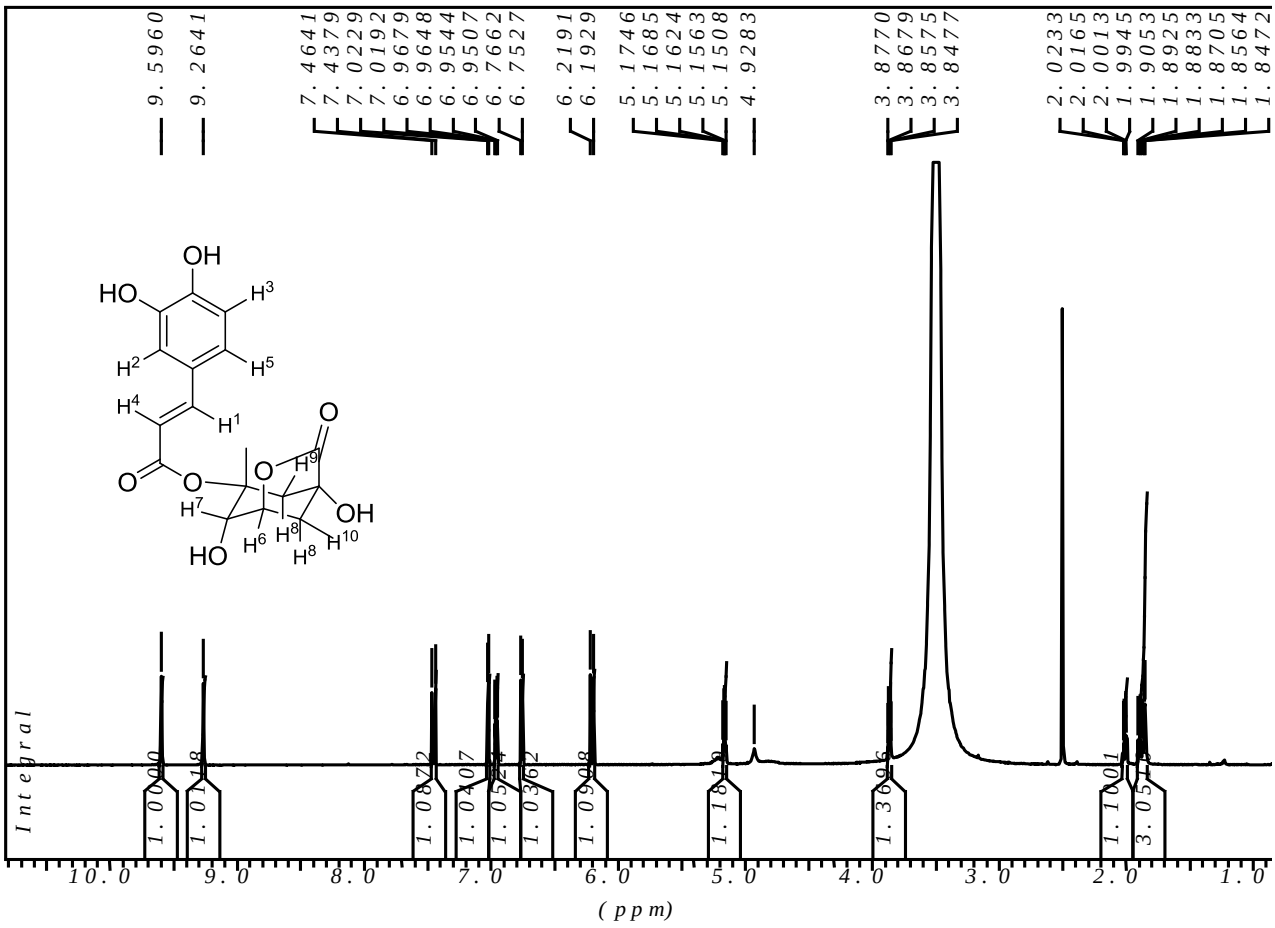


Figure 70 ^{1}H-NMR spectrum of 3-*O*-caffeoylquinic acid-1,5-lactone (d_6-DMSO, 400 MHz, 293,5 K)

MS characerizeation of 3-*O*-caffeoylquinic acid-1,5-lactone

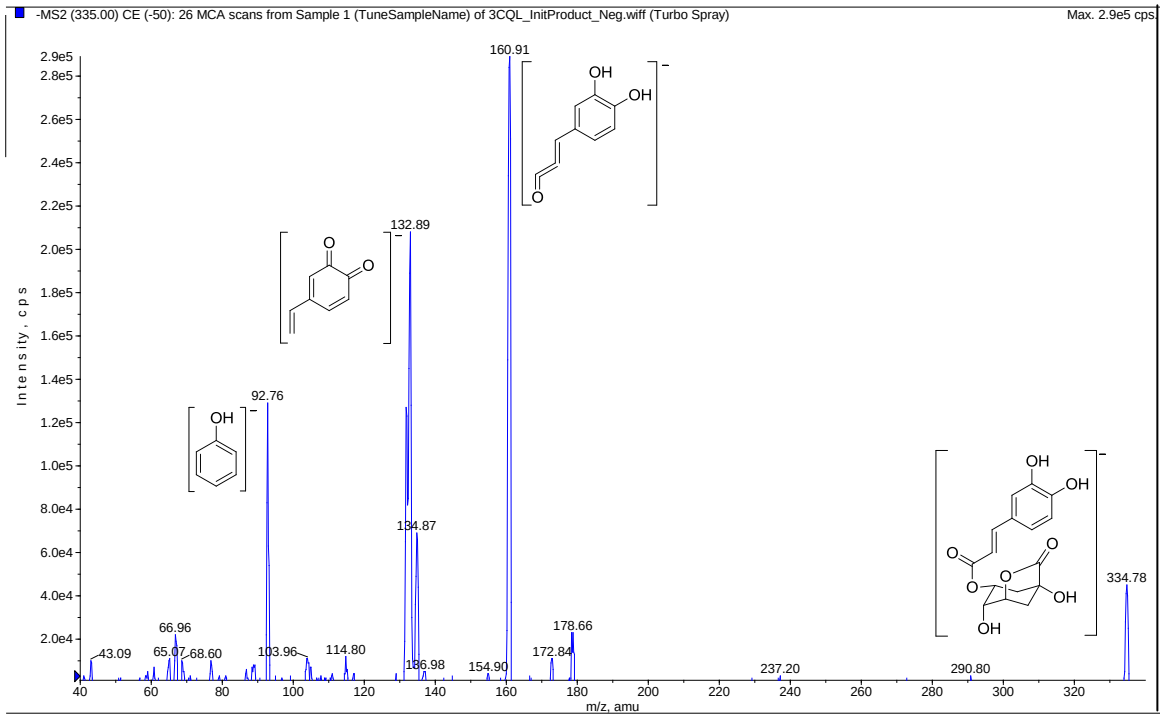


Figure 71 ESI$^-$ production scan of 3-*O*-caffeoylquinic acid-1,5-lactone ([M-H]$^-$, *m/z* 335) at Q3 in mass range of *m/z* 40-360

After dissolving of 3-O-caffeoylquinic acid-1,5-lactone (500 ng/ml) in ethanol/water/formic acid (30/69.9/0.1%; v/v/v) the solution was directly injected via syringe pump into the ion source of a triple quad mass spectrometer (Applied Biosystems, API 3200). The flow rate was set at 10 µl/min and the polarity at the ESI source was negative. The compound was identified by the mass to charge ratio (*m/z*) of 335 and the corresponding fragments (*m/z* 161 caffeic acid derivative, *m/z* 133, *m/z* 93 phenol). After fragmentation in Q2 the compound-dependent parameters of the three fragments with the highest intensity were optimized (see Figure 71). For the analyses the mass transition *m/z* 353 → 161 was used.

On the basis of the correlation of ^{1}H-NMR spectrum by calculation of increment and in comparison with the literature and in the contrast to the production scan, 3-CQA15L was positively identified.

6.3.3.4 Synthesis of 4-*O*-caffeoylquinic acid-1,5-lactone (4-CQA15L)

The alloy of 4- and 5-CQA was dissolved in THF and catalyzed by DCC. Theoretically, 4-CQA should lactonize to 4-CQA15L whereat 5-CQA would be preserved. The aim was to separated 4-CQA15L and 5-CQA via preparative HPLC after lactonization. The above described intramolecular esterification was arranged like the synthesis of 3-CQA15L. For three hours, the reaction mixture was stirred at room temperature. The reaction was monitored by HPLC-DAD based on the retention time. The analysis showed a degradation of 4- and 5-CQA, and unfortunately a reduced intramolecular esterification to the respective lactones. Next to a formation of by-products, an isomerization of 5-CQA was conductive to the formation of 3-CQA and 3-CQA15L, respectively. Frank and coworkers reported the appearance of 5-*O*-caffeoyl-*epi*-δ-quinic acid lactone as well as 5-*O*-caffeoyl-*muco*-δ-quinic acid lactone, 4-*O*-caffeoyl-*epi*-δ-quinic acid lactone and 4-*O*-caffeoyl-*muco*-δ-quinic acid lactone [*Frank et al., 2006*]. Due to the described findings, the separation of 4-CQA15L was not accomplishable.

6.4 Human intervention study

6.4.1 Study design and subjects

This human intervention study was approved by the Ethics Committee of the *Landesärztekammer Rheinland-Pfalz* (837.014.11 (7556), 2011). The study was subdivided into five study days (shown in Figure 72); the first two study days were intended for screening and during the following three days the three different treatments were applied in random order. First (V0 – screening part 1), a general medical screening was conducted to exclude subjects with any known disorders. The body weight, height, and body mass index (BMI) of each participant was monitored, and body fat and body water were analyzed using bioimpedance measurements (BIA 101 analyzer, SMT medical GmbH, Wuerzburg, Germany). In addition, subcutaneous body fat was measured using calipometry (after 7-crinkles-formula of Jackson-Pollock [*Jackson et al., 1980*]). During the medical screening, participants completed questionnaires on various illnesses together with the doctor.

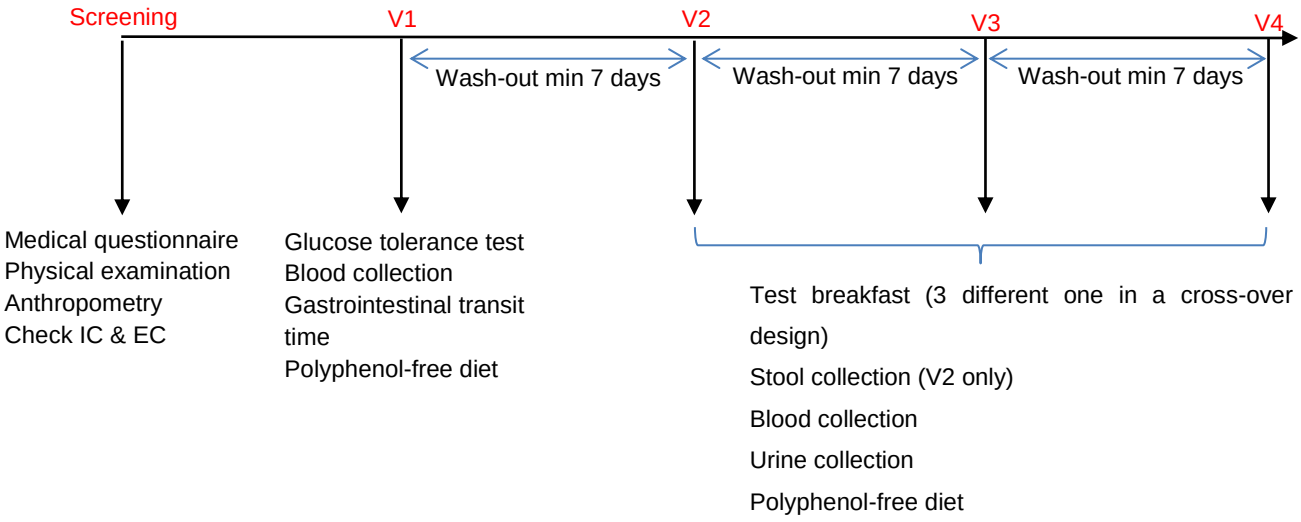


Figure 72 Design of the whole human intervention study.

Before visit 1 (V1 – screening part 2), volunteers followed a polyphenol-free diet for 48 h. On V1, an oral glucose tolerance test (oGTT) was performed. The volunteers consumed glucose syrup (75 g glucose in 200 mL) and peripheral blood glucose concentrations were measured from the fingertip after 0, 30, 60, 120, 180, 240, and 300 minutes. Additionally, the gastrointestinal transit time (GITT) for each subject was measured on a separate day. To determine GITT, subjects drank at least 100 mL of blueberry juice and were asked to note the time at which blue color appeared in their feces (should had been less than 24 h). A urinary pregnancy test was also conducted on the female volunteers (Screening visit).

The study was conducted over three separate days (V2, V3, and V4) and the three different treatments were applied in a random order. Subjects received a polyphenol-free meal 48 h before breakfast time and then fasted for 8 h before breakfast consumption. On each day, the subjects consumed 200 mL of an instant coffee beverage prepared from a commercially-available instant coffee product (3.1 mg chlorogenic acids/kg body weight). The method of instant coffee preparation, as well as consumption time and temperature, was selected according to our previous study [*Erk et al., 2012*]. The beverages were prepared with hot water (60 °C) and consumed without milk or sugar, as quickly as possible. Three different breakfasts were consumed in a cross-over design and in a randomized order. The three breakfasts consisted of: the coffee beverage only (COFFEE), the coffee beverage with a carbohydrate-rich breakfast (two bread rolls and honey; CARB), and the coffee beverage with a fat-rich breakfast (one bread roll and peanut butter; FAT). The carbohydrate-rich and the fat-rich breakfasts were designed to be equicaloric (~626 kcal, based on the nutrition facts of the products). During the progression from V2 to V4, the volunteers followed a

polyphenol-free diet for 48 h before each treatment and at least seven days passed between each visit. For each visit on study days, subject tests were given the same standard polyphenol-free meal. After 5 hours, 2 bread rolls with cheese and after 10 hours, tortellini with cream sauce and yoghurt. See details in Figure 73.

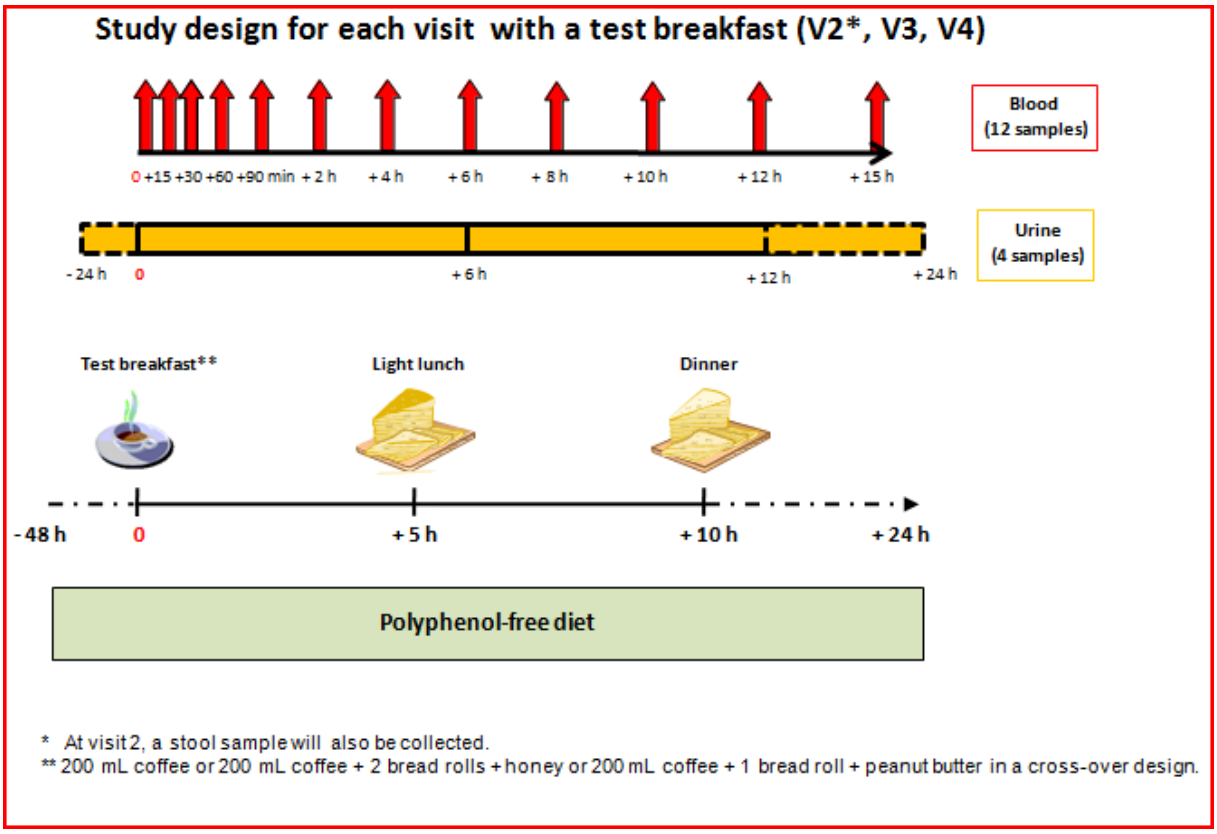


Figure 73 Study design of each visit (V2 to V4).

Twelve blood samples, each a maximum of 7 mL, were collected during each visit at 0 h, 0.25 h, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 15 h after coffee consumption. Additionally, four urine samples were collected; one hours prior to coffee consumption (within the last 24h) and three after coffee consumption (between 0 – 6 h, 6 – 12 h, and 12 – 24 h, respectively). All samples were stored by -80 °C.

Criterion for exclusion from the study was in the event of illnesses requiring medications other than the one authorized, as well as in the case of pregnancy. Moreover, participants who were renouncing for the required dietary restrictions or were intolerance to product consumption were withdrawn from the study.

Finally, the subject collective consisted of seven health men and seven healthy women, 29 ± 8 years old. GIT transit time was 17.4 ± 6.0 h and all other inclusion/exclusion criteria were also achieved. The average of BMI of all probands was 23.0 ± 1.6 kg/m^2.

Bioimpedance measurements

The body composition was determined by bioimpedance measurement of each proband. In order to determine percentage of total body fat and body water a physiological data analyzer (BIA 101 analyzer, SMT medical GmbH, Wuerzburg, Germany) was used and data analyses were carried out by the appropriate software (Bodygram Pro® Version 3.0, Akern bioresearch srl, Pontassieve, Florence, Italy).

Appendixes, Table 51 to Table 53 show the physiological data of the 14 probands. The average of the percental fat free mass was 76.2 ± 7.5%, the average of percental total body water was 55.8 ± 5.5% and the average of percental fat mass was 17.4 ± 6.0 h. After determination of the kinetic data (urine and blood) there is the possibility to detect a relation between these measurements and the physiological data.

Oral glucose tolerance test (oGTT)

Oral glucose tolerance test (oGTT) was performed by a single intake of 75 g glucose. Blood glucose concentrations in the peripheral blood (fingertip) were determined after 0, 30, 60, 120, 180, 240 and 300 minutes. The test is usually used to identify people with impaired glucose tolerance (IGT) (fasting plasma glucose: < 126 mg/dL; 2 h-plasma glucose[1]: >140 mg/dL and <200 mg/dL) [*WHO, 2006*].

Appendix, Table 55 shows the blood glucose concentrations of each proband (01 – 14) up to 300 min after intake of glucose. All probands´ fasting plasma glucose were less than 126 mg/dL and the 2 h-plasma glucose lay between 140 mg/dL and 200 mg/dL. Only the 2 h-plasma glucose of proband 14 was slightly increased in an acceptable magnitude.

Baseline demographic table

	n	mean	sd	min	max
age [years]	14	29.36	8.06	20.0	44.0
BMI [kg/m^2]	14	22.95	1.72	19.5	25.3
height [cm]	14	174.84	9.96	156.0	188.0
weight [kg]	14	70.40	10.02	54.5	88.2

[1] Venous plasma glucose 2 h after ingestion of 75 g oral glucose load

if 2 h-plasma glucose is not measured, status is uncertain as diabetes or IGT cannot be excluded

6.4.3 Data collection, management and validation

Data on CRFs

Data were collected on paper CRFs and stored in locked office space. The CRFs are made of non carbon required paper providing two copies of each page. The original remained on site and a copy was sent to the sponsor for data management.
Laboratory results obtained following blood, urine and stoic analyses were manually uploaded in the database.

Computerized data

Data were entered from the paper CRFs into a computer database (CLINTRIAL^{TM}) at the Nestlé Clinical Development Unit using double blind data entry by two different data entry operators.

Data Management

The data manager prepared a data validation plan and programmed the corresponding edit checks, at least on the primary outcome. The queries highlighted by the edit check were reviewed, and if necessary, sent on data clarification form to the investigator for treatment.

6.4.3 Statistics

The primary goal of the study was to evaluate the influence of breakfast consumption on the bioavailability of CGAs from coffee, because only few data was available. Therefore this study and the outcomes measured were considered exploratory. Based on other ongoing bioavailability studies, a sample size of 12 subjects was considered as sufficient to provide reliable estimates of parameters of interest. Finally 14 subjects were chosen to ensure the data collection of 12 subjects at the end of the study. No “normal power” analysis was done. The estimated effects were the differences between coffee alone or the two food matrix treatments in terms of AUC (area under curve), C_{max} (maximum plasma concentration) and t_{max} (time needed to reach maximum plasma concentration) of the different metabolites in plasma and 24 h urinary excretion. A level of significance of 5% was used. AUCs were calculated using the trapezoidal rule from time 0 to the last time point. The sum of AUCs was then computed by adding the AUCs of all the metabolite by subject and treatment. A linear mixed model, with treatment and baseline as fixed effects and subject as a random effect, was used to analyze the sums of the AUCs. The baseline was computed as the sum of the individual subject´s baseline value. Normality assumptions were checked. A log-

transformations were required and, thus, were applied to the data in order to fulfill statistical assumptions and perform appropriate hypothesis-testing procedures. Similar approaches were used for the plasma t_{max} and C_{max} (for t_{max}, it was not needed to correct for baseline). For urine samples, no baseline correction was applied. For the 24 h urine excretion, values that fell below the detection limit (LOQ) for a given time period were set to zero. A majority of the data was skewed to the right; hence a log-transformation was applied. For this reason, the summary statistics presented show geometric mean defined as *exp(mean(log(x)))*, the lower bound defined as *exp(mean(log(x))-sd(log(x)))* and the upper bound defined as *exp(mean(log(x))+sd(log(x)))*. In our cases, the estimated treatment differences are presented as a ratio between the treatments. The related 95% confidence intervals define a confidence interval for the ratio. The Pearson correlation was used to compute the correlation between the plasma sample and the 24 h urinary excretion. Since the study is exploratory, no adjustments for multiplicity were performed. All the statistical analyses were done with R software (version 3.0.1, Lucent Technologies, Murray Hill, USA).

A blind data review was executed to address and document the following points:

- Protocol deviations, definition of minor and major ones, in order to define per protocol (PP) and intention to treat (ITT) data sets
- Statistical analyses planned for the primary outcome abd main secondary outcomes

6.4.4 Randomization

The subjects were randomly allocated to one of the six possible sequences. There were no stratification factors. The six sequences are given bye:

Treatment 1	Treatment 2	Treatment 3
A	B	C
A	C	B
B	A	C
B	C	A
C	A	B
C	B	A

Where A is coffee (COFFEE), B is coffee + 2 bread rolls + honey (CARB) and C is coffee + 1 bread roll + peanut butter (FAT).

6.4.5 Area under the curve (AUC)

Area under the curves (AUCs) were calculated using the trapezoidal rule from time 0 to the last time point. The sum of AUCs was then computed by adding the AUCs of all the metabolites by subjects and treatments. A linear mixed model with treatment and baseline as fixed effects and subjects as random was used for the primary outcome (sum of AUCs). The baseline was computed as the sum of the individual baseline value. Normality assumptions were checked and the use of log-transformations were needed and applied to the data in order to fulfill statistical assumptions and perform appropriate hypothesis and testing procedures.

6.5 Analysis of coffee

0.5 g instant coffee used for the study was dissolved in 50 mL boiled water and after cooling samples were centrifuged for 5 min at 5,000 x g. Thereafter, 2 mL of supernatant was filtered (membrane filter 0.45 µm PFDV), diluted (20-fold, EtOH/H_2O/HCOOH, 30/69.9/0.1, v/v/v) and 20 µL was injected to the HPLC-DAD system (Agilent Technologies 1200 Series). Parameters and solvent gradient are illustrated in Table 39.

Table 39 HPLC parameters for determination of instant coffee chlorogenic acids [*Witt, 2012*].

Column	Phenomenex Synergie Polar		
Material	RP C18, 4 µm, 250x4.6 mm		
Mobile phase	A: water (0.1% HCOOH) B:acetonitrile		
oven	40 °C		
wavelength	325 nm		
Injection volume	40 µl		
Time	**Flow [ml/min]**	**Solvent A [%]**	**Solvent B [%]**
0.1	0.8	98	2
5	0.8	88	12
20	0.8	70	30
21	0.8	10	90
24	0.8	10	90
25	0.8	98	2
29	0.8	98	2

Limit of quantification (LOQ)

For determination of 5-CQA, 3-CQA, 4-CQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, 4-FQA, 3-FQA and 5-FQA using the HPLC-DAD, the LOQ was defined with the signal-to-noise ratio of 1:10. Based on this, for 5-CQA a LOQ of 8.8 pg on column was calculated (same LOQ was assumed for 3- and 4-CQA). For 3,4-diCQA a LOQ of 8.5 pg on column was calculated

(same LOQ was assumed for 3,5- and 4,5-diCQA). For 4-FQA a LOQ of 9.8 pg on column was calculated (same LOQ was assumed for 3- and 5-FQA) [*Witt,* 2012].

6.6 Determination of chlorogenic acids in instant coffee (commercially available Nescafé® Green blend) and chlorogenic acids and metabolites in plasma

All preparation steps and solutions were performed and prepared on ice to avoid any stability issue. The 1 mM solution of $d^{13}C_2$-CA was prepared by dissolving appropriate amounts of the compound in methanol and for 1 mM labeled DHiFA3S disodium salt and labeled DHiFA3G with water/acetonitrile (50/50, v/v). For obtaining of 200 µM standard solutions the stock solutions were diluted in 1% acetic acid in water/acetonitrile (1/49.5/49.5, v/v/v). A further dilution with mentioned solvent offer the working solution at the concentration of 2 µM. Stock solutions of CA, FA, iFA, DHCA, DHFA, MeFA and MeDHFA(10 mM) and 3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA (10 mM) were prepared by dissolving appropriate amounts of the compounds in methanol or 1% acetic acid water, respectively. Finally stock solutions of pCoA, mCoA, oCoA, 3PPA, CinAc, 4MCA, 34MPPA, DHiFA, mDHCoA, oDHCoA, pDHCoA, 4CQA15L, 3CQA15L, 3FQA15L, 4FQA15L, DHFA4S, FA4S, CA4S, DHCA4S, DHCA3S, CA3S, iFA3S, DHiFA3S, 5CQA4S, 5CQA3S, 5FQA4S, pCoAS, mCoAS, mDHCoAS, pDHCoAS, CA4G, DHCA4G, DHFA4G, CA3G, FA4G, DHCA3G, iFA3G, DHiFA3G, 5FQA4G, mDHCoAG, pDHCoAG, pCoAG and mCoAG (5 mM) were prepared by dissolving appropriate amounts of the compounds in 1% acetic acid water/acetonitrile (1/49.5/49.5, v/v/v). The intermediate dilution achieve a concentration of 250 µM by dilution of the stock solutions in appropriate amounts with 1% acetic acid in water (for phenolic acids and glucuronides) and of 1% acetic acid in water/acetonitrile (1/49.5/49.5, v/v/v) (for sulfates and lactones). These solutions were further diluted corresponding to 0.1, 0.2, 0.5, 1, 2, 5, 10, 15, 20 µM and to yield the standard curves in plasma concentrations corresponding to 5, 10, 25, 50, 100, 250, 500, 750 and 1000 nM were prepared. In order to compensate errors occurring during the extraction procedure and the matrix effect, also the standard curve samples were extracted in the same way as the plasma samples [*Marmet et al., 2014*].

Sample preparation was performed according to Marmet *et al.* [*Marmet et al., 2014*]. After spiking of 100 µl plasma with 5 µl labeled standard solution (2 µM) samples were vortexed and diluted with 500 µl ethanol for protein precipitation and mixed for 5 minutes. Samples were centrifuged (17,500 x g, 5 min, 4 °C), supernatants were transferred into 1.5 ml tubes and the solvent was evaporated under nitrogen gas. The residues were secondly extracted

and after centrifugation the supernatants were pooled. After evaporation of the solvent, the residue were added with 100 µl of water/acetonitrile/acetic acid (93/6/1, v/v/v) and 5 µl were injected into the LC-ESI-MS/MS system. The HPLC conditions and source parameters for determination of instant coffee ingredients and metabolites are shown in Table 40.

Table 40 HPLC conditions and source parameters (API 5500 QTrap) for determination of chlorogenic acids in instant coffee (commercially available Nescafé® Green blend) and chlorogenic acids and metabolites in plasma. CUR: curtain gas, CAD: collision gas, IS: ion spray voltage, TEM: temperature; GS1: gas 1, GS2: gas 2.

Column	Waters Acquity UHPLC BEH		
Material	C18, 1.7 µm, 150x2.1 mm		
Mobile phase	A: water (1% H_3CCOOH) B:acetonitrile (1% H_3CCOOH)		
Injection volume	5 µl		
TCC	40 °C		
Time	**Flow [ml/min]**	**Solvent A [%]**	**Solvent B [%]**
0	0.35	97	3
2	0.35	976	3
10	0.35	85	15
20	0.35	70	30
25	0.35	10	90
30	0.35	10	90
35	0.35	97	3
40	0.35	97	3
source			
CUR [psi]		45	
CAD		medium	
IS [V]		-4500	
TEM [°C]		550	
GS1 [psi]		70	
GS2 [psi]		80	
parameter quadrupol			
Dwell [msec]		40	
EP [V]		-11	

MultiQuant™ integration algorithm details

For integration and data processing MultiQuant™ (version 3.0.2, Sciex, Darmstadt, Germany) with the MQ4 integration algorithm was used. The integration parameters were as follows: Gauddian smooth width: 1.0 points, RT half window: 30.0 sec, min. peak width: 3 points, min peak hight: 0.00 cps, noise percentage: 99%, baseline sub. window: 0.20 min,

peak splitting factor: 2 points. Following regression options were used: Regression parameter: area, regression type: linear, weighting type: 1/x.

Limit of quantification (LOQ)

FDA defines the LOQ as the lowest concentration of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. According to the FDA, the lowest concentration of the calibration curve should be accepted as the LOQ if the analyte response is at least 5 times higher than the blank response and if this response is reproducible with a precision of at least 20% [*FDA, 1996*].
The LOQs of coffee ingredients and metabolites determined in plasma samples are shown in Appendix, Table 60.

6.7 Determination of creatinine

Creatinine is a product of a non-enzymatic reaction of creatinine phosphate in organism and is preferred excreted via kidney. In this context, a direct relationship between muscular mass and creatinine coefficient in adult is observed and the daily creatinine excretion for a given individual is constant. The daily output is not influenced by the food intake and independent of diuresis [*Hobson, 1939*].
Thus the metabolites in urine could be determined and compared independent of the excreted urine volume.

Creatinine in study samples was determined according to an established method of Watzek and coworkers [*Watzek et al., 2012*]. Urine samples were diluted (1:1000) and spiked with 10 µL of isotope stable standard solution of D_3-creatinine (100 µg/mL) for stable isotope dilution analysis (SIDA). An aliquot of 50 µL was directly injected into a HPLC-ESI-MS/MS system (Perkin Elmer Series 200 HPLC system coupled to a SCIEX API 2000 triple quadrupole mass spectrometer). Determination was performed using single ion monitoring (SIM) mode in positive ionization mode, the mass to charge (*m/z*) ratio measured for D_3-creatinine was 116.9 and for creatinine 114.0. Solvent gradient and optimized source and compound dependent parameters are illustrated in Table 41 and 42.

Table 41 HPLC conditions for determination of creatinine according to [*Watzek et al., 2012*]

Column	Phenomenex Luna		
Material	C8, 3 µm, 150x4.6 mm		
Mobile phase	A: water (0.05% HCOOH) B:methanol		
Injection volume	50 µl		
Time	**Flow [ml/min]**	**Solvent A [%]**	**Solvent B [%]**
0.1	0.3	99	1
1	0.3	99	1
20	0.3	80	20
22	0.3	99	1
25	0.3	99	1

Table 42 Source and compound dependent parameter for determination of creatinin in urine of humans via SIDA (API 2000) [*Watzek et al., 2012*]. DP: declustering potential, FP: focusing potential, EP: entrance potential, CEP: cell entrance potential, CUR: curtain gas, IS: ion spray voltage, TEM: temperature; GS1: gas 1, GS2: gas 2

	creatinine	D_3-creatinine
Q1 [amu]	114.0	116.9
Dwell[ms]	200	200
parameter quadrupole		
DP [V]	31	21
FP [V]	250	370
EP [V]	11	10
CEP [V]	8	9
source		
CUR [psi]	20	
IS [V]	5000	
TEM [°C]	200	
GS1 [psi]	30	
GS2 [psi]	30	

Table 43 shows the ratios of amounts of creatininev to D_3-creatinine as well as the ratios of the corresponding areas. Figure 74 shows the corresponding standard curve. The data offer a clear proportionality between creatinine and D_3-creatinine with a correlation factor of $R > 0.999$. Due to the determined linearity, the standard curve is usable for quantification of creatinine.

Table 43 Concentrations and quotients for calculation of the standard curve of creatinine (analyte) and D_3-creatinine (IS) [*Watzek et al., 2012*].

creatinine [ng/ml]	7.5	10	12.5	25	50	75	100	250	500	750
D_3-creatinine [ng/ml]	100	100	100	100	100	100	100	100	100	100
area analyte / area IS	0.10	0.13	0.15	0.22	0.33	0.64	0.77	2.01	3.76	5.23
amount analyt / amount IS	0.08	0.10	0.13	0.26	0.51	0.77	1.03	2.56	5.13	7.69

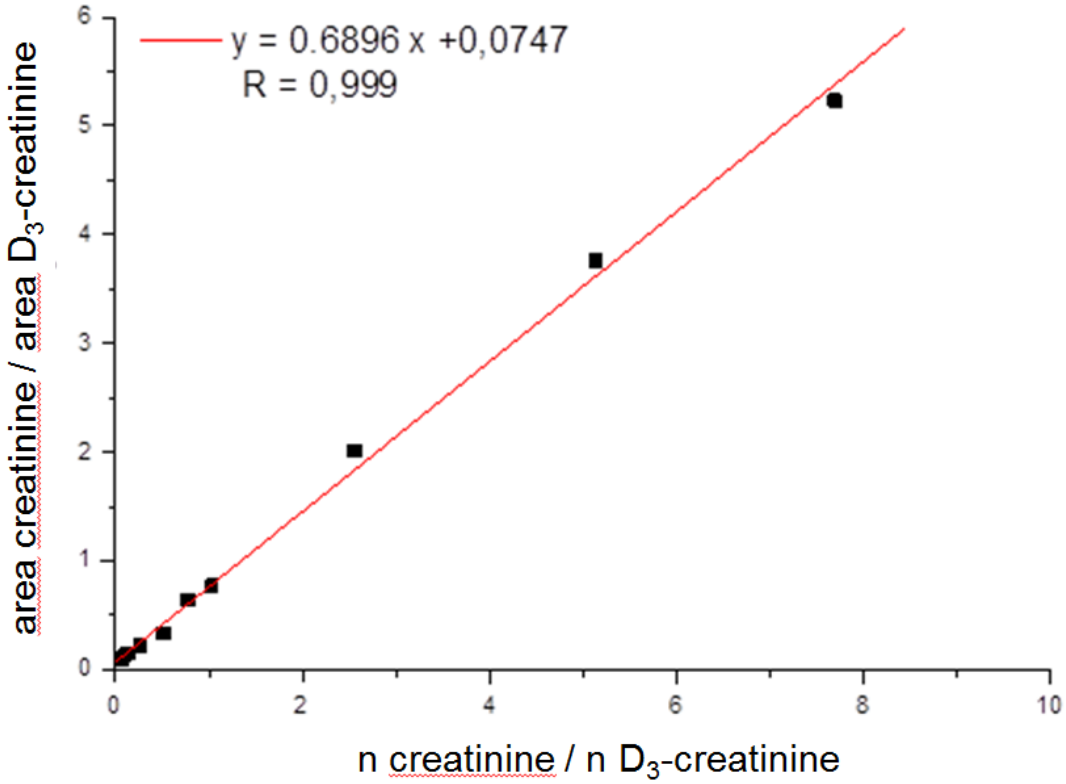


Figure 74 Standard curve for quantification of creatinine via SIDA [*Watzek et al., 2012*]

Limit of quantification (LOQ) and intraday/interday variability

Using the regression line of plotting the concentration of creatinine against the peak high (y = 6934.4666 x; R = 0.996) and subsequent transposition to x with the corresponding background gives the ability to calculate the LOQ [*Watzek et al., 2012*]:

$$LOQ = x = \frac{6 \cdot background}{gradient} = \frac{60}{6934.4666} = 8.65 \frac{ng}{ml}$$

Calculating with a injection volume of 50 µl standard solution, the absolute quantifiable amount was 2.8 pmol [*Watzek et al., 2012*].

For the intraday variability < 2% and for the interday < 1% was determined [*Watzek et al., 2012*].

6.8 Determination of chlorogenic acids in instant coffee (commercially available Nescafé® Green blend) and chlorogenic acids and metabolites in urine

6.8.1 Validation of the determination method for chlorogenic acids in instant coffee (commercially available Nescafé® Green blend) and chlorogenic acids and metabolites in urine

For determination of the linearity the standard curves with the correlation factors were used [*Qualitätssicherung, 2004*].

For determination of the accuracy/recovery three different concentrations of standard solution (25, 50 and 75 ng/ml) were used. All solutions were prepared three times and each solution was injected three times (see) [*Qualitätssicherung, 2004*].

For determination of the interday and intraday variability different standard solutions (25, 50 and 75 ng/ml) were at different days and serially injected (see) [*Qualitätssicherung, 2004*].

Table 44 Accuracy/recovery [%] as well as interday and intraday variability [%] of determined coffee ingredients and metabolites in urine samples.

	recovery [%] (n = 3)				interday variability (n = 2)	intraday variability (n = 3)
	25 ng/ml	50 ng/ml	75 ng/ml	average	variation coefficient [%]	variation coefficient [%]
5CQA	95	94	95	95	3,69	3,52
3CQA	98	98	101	99	2,08	4,27
4CQA	96	97	99	97	1,91	3,43
CA	96	97	97	96	3,06	2,01
FA	97	97	100	98	2,08	2,69
DHCA3G	90	82	73	82	2,12	6,96
FAG	101	99	98	99	1,63	2,59
FA4S	102	97	95	98	2,65	4,00
DHCA	98	97	95	96	3,77	4,86
DHIFA	96	96	95	96	2,50	1,41
IFA	97	95	95	96	2,49	2,93
4FQA	96	98	99	97	2,07	2,92
CQA3S	85	76	63	75	3,71	4,99

	recovery [%] (n = 3)				interday variability (n = 2)	intraday variability (n = 3)
	25 ng/ml	50 ng/ml	75 ng/ml	average	25 ng/ml	50 ng/ml
FQA4G	85	76	73	78	3,58	5,99
FQA4S	88	78	73	80	3,64	4,26
CA3G	84	76	77	79	2,38	5,57
3FQA	96	100	104	100	2,21	2,50
5FQA	98	96	95	96	2,03	2,47
CA4G	98	100	103	100	2,36	4,99
CA4S	88	79	73	80	2,91	4,91
DHCA3S	98	85	77	86	2,89	6,93
DHCA4S	103	101	101	102	3,92	4,84
IFA3G	96	96	96	96	1,66	2,66
IFA3S	102	96	95	98	2,40	3,89
DHFA4S	99	97	96	97	2,38	4,15
CA3S	98	96	96	96	2,79	3,98
DHFA4G	99	98	99	98	1,68	3,98
DHIFA3G	100	102	103	102	2,43	4,44
DHFA	96	95	95	96	2,69	1,48
5CQA4S	86	77	61	75	3,32	4,80
DHCA4G	98	82	76	85	3,23	4,33
CQL	94	97	98	97	3,55	3,51
CQLG	96	95	97	96	1,79	2,54
CQA3G	98	96	86	93	6,13	15,39

Using the regression line of plotting the concentration of analyte against the peak high and subsequent transposition to x with the corresponding background gives the ability to calculate the LOQs:

$$LOQ = x = \frac{6 \cdot background}{gradient}$$

The LOQs of coffee ingredients and metabolites determined in urine samples are shown in Appendix, Table 61 to Table 63.

6.8.2 Sample preparation for determination of chlorogenic acids in instant coffee (commercially available Nescafé® Green blend) and chlorogenic acids and metabolites in urine

All preparation steps and solutions were performed on ice to avoid any stability issue. The 1 mg/ml solutions of $D^{13}C_2$-CA, D_3-DHiFA3S and D_3-DHiFA3G were prepared by dissolving appropriate amounts of the compounds in methanol. For obtaining of 10 µg/ml standard solutions the stock solutions were diluted in 1% acetic acid in water/acetonitrile (1/49.5/49.5, v/v/v). A further dilution with mentioned solvent offer the working solution at the concentration of 1 µg/ml. Stock solutions of 3CQA, 4CQA, 5CQA, 3FQA, 4FQA, 5FQA, CA, CA3G, CA4G, CA3S, CA4S, CQA3G, CQA3S, CQA15L, CQA15LG, DHCA, DHCA3G, DHCA4G, DHCA3S, DHCA4S, DHFA, DHFA4G, DHFA4S, DHiFA, DHiFA3G, DHiFA3S, FA, FA4S, FAG, 5FQA4G, 5FQA4S, iFA, iFA3G and iFA3S (1 mg/ml) were prepared by dissolving appropriate amounts of the compounds in methanol. The intermediate dilution achieved a concentration of 10 µg/ml by dilution of the stock solutions in appropriate amounts with 1% acetic acid in water/acetonitrile (1/49.5/49.5, v/v/v). These solutions were further diluted to achieve 100 ng/ml and to prepare the standard curves in urine corresponding to 0.5, 1, 2.5, 5, 10, 25, 40 and 50 ng/ml. In order to compensate errors occurring during the extraction procedure and the matrix effect, also the standard curve samples were extracted in the same way as the urine samples below-mentioned.

After spiking of 100 µl plasma with 25 µl labelled standard solution (100 ng/ml) samples were vortexed and diluted with 500 µl ethanol for protein precipitation and mixed for 5 minutes at 4 °C. Samples were centrifuged (17,500 x g, 5 min, 4 °C), supernatants were transferred into 1.5 ml tubes and the solvent was evaporated in a vacuum centrifuge. The residues were afterwardsextracted and after centrifugation the supernatants were pooled. After evaporation of the solvent, the residue was added with 1 ml of water/acetonitrile/acetic acid (49.5/49.5/1, v/v/v) and 10 µl were injected into the LC-ESI-MS/MS system. The HPLC conditions and

source parameters for determination of chlorogenic acids and metabolites are shown in Table 45.

Table 45 HPLC conditions and source parameters (API 5500 QTrap) for determination of chlorogenic acids in instant coffee (commercially available Nescafé® Green blend) and chlorogenic acids and metabolites in urine. CUR: curtain gas, CAD: collision gas, IS: ion spray voltage, TEM: temperature; GS1: gas 1, GS2: gas 2.

Column	Phenomenex Synergie Polar		
Material	RP C18, 4 µm, 250x4.6 mm		
Mobile phase	A: water (0.1% H_3CCOOH) B:acetonitrile		
Injection volume	10 µl		
Time	**Flow [ml/min]**	**Solvent A [%]**	**Solvent B [%]**
0.05	0.9	95	5
15	0.9	76	24
21	0.9	72	28
23	0.9	72	28
23.1	0.9	1	99
28	0.9	1	99
28.1	0.9	95	5
33	0.9	95	5
source			
CUR [psi]		45	
CAD		medium	
IS [V]		-4500	
TEM [°C]		550	
GS1 [psi]		70	
GS2 [psi]		80	
parameters quadrupol			
Dwell [msec]		40	
EP [V]		-10	

MultiQuant™ integration algorithm details

For integration and data processing MultiQuant™ (version 3.0.2, Sciex, Darmstadt, Germany) with the MQ4 integration algorithm was used. The integration parameters were as follows: Gauddian smooth width: 1.0 points, RT half window: 30.0 sec, min. peak width: 3 points, min peak hight: 0.00 cps, noise percentage: 99%, baseline sub. window: 0.20 min, peak splitting factor: 2 points. Following regression options were used: Regression parameter: area, regression type: linear, weighting type: 1/x.

6.8.3 Sample preparation for determination of quinic acid (QA) in instant study coffee and urine

The determination of QA was performed after a method developed by Erk and coworkers [*Erk et al.,* 2009].

For determination of quinic acid (QA) in instant study coffee, coffee samples were dissolved in ethanol/water/formic acid (29.9/70/0.1, v/v/v) to obtain a final concentration of 1 mg/ml. After subsequent centrifugation (5 min, 5000 rpm, RT), the solutions were filtered with a membrane filter (0.45 µm PVDF), diluted 400-fold with an ethanol/water/formic acid (29.9/70/0.1, v/v/v) solution, and 50 µl of each sample were spiked with 50 µl of the labeled standard (200 ng/ml) and analyzed by HPLC-ESI-MS/MS in the MRM method.

For determination of QA in urine, urine samples were dissolved 20-fold with ethanol/water/formic acid (29.9/70/0.1, v/v/v). Subsequent, 40 µl of each sample was spiked with 56 µl of the labeled standard (200 ng/ml) and acidified with 16 µl of methanolic hydrochloric acid (0.25 M). Finally, the samples were analyzed by HPLC-ESI-MS/MS in the MRM method.

Table 46 HPLC conditions for determination of quinic acid modified according to [*Erk et al., 2009*]

Column	Waters Atlantis		
Material	RP C18, 3 µm, 150x4.6 mm		
Mobile phase	A: water (0.05% HCOOH) B:acetonitrile		
Injection volume	20 µl		
Time	**Flow [ml/min]**	**Solvent A [%]**	**Solvent B [%]**
0.1	0.8	95	5
6	0.8	95	5
6.1	0.8	1	99
11	0.8	1	99
11.1	0.8	95	5
16	0.8	95	5

Table 47 Source and compound dependent parameter for determination of Quinic acid in instant study coffee and urine of humans via SIDA (API 3200) [*Erk et al., 2009*]. DP: declustering potential, CE: collision energy, EP: entrance potential, CEP: cell entrance potential, CUR: curtain gas, CAD: collision gas, IS: ion spray voltage, TEM: temperature; GS1: gas 1, GS2: gas 2.

	D-(-)-quinic acid	U-^{13}C-D-(-)-quinic acid
Q1 [amu]	191	198
Q3 [amu]	85	89
Dwell[ms]	150	150
parameter quadrupole		
DP [V]	-55	
CE [V]	-30	
EP [V]	-10.5	
CEP [V]	-28	
CXP [V]	0	
source		
CUR [psi]	30	
CAD	6	
IS [V]	-4500	
TEM [°C]	450	
GS1 [psi]	60	
GS2 [psi]	50	

Limit of quantification (LOQ)

For determination of QA using the LC-MS/MS, the LOQ was defined with the signal-to-noise ratio of 1:10. Based on this, a LOQ of 195.4 pg (on column) was calculated [*Erk et al.,* 2009].

6.9 Fecal sample incubation

6.9.1 Preparation of reagents

6.9.1.1 PBS – 0.1% L-cystein and 10% glycerol

L-cysteine	100 mg
glycerol	100 mL
Final volume in PBS	1 L

Autoclaved at 121 °C for 15 min. This solution was stable 3 months at room temperature.

6.9.1.2 Growth medium: gut microbiota medium (GMM)

Preparation of stock solutions of ingredients for the preparation of the GMM was as follows.

- Potassium dihydrogen phosphate: stock solution at 1 M, pH 7.2

KH_2PO_4	68.1 g
Adjust pH with NaOH at	2
Final volume in distilled water	1 L

- calcium chloride: stock solution at 0.8%

$CaCl_2$	0.8 g
Final volume in distilled water	100 mL

- vitamin K_3 (menadione): stock solution at 1 mg/mL

menadione	10 mg
Volume final ethanol 95%	10 mL
Can be kept one month in a dark flask at 4 °C.	

- iron(II)-sulfate: stock solution at 0.4 mg/mL

$FeSO_4$	4 mg
Final volume in distilled water	10 mL

- Tween 80: stock solution at 25%

Tween 80	25 g
Final volume in distilled water	100 mL
The solution was warmed up slowly to mix the ingredients.	

- resazurin: stock solution at 0.25 mg/mL

resazurin	25 mg
Final volume in distilled water	100 mL

- hemin: stock solution at 1 mg/mL

hemin	25 mg
NaOH 1 N	5 mL
Final volume in distilled water	25 mL

- VFA mix: stock solution

acetic acid (glacial) 100%	17 mL
propionic acid	6 mL
valeric acid	1 mL
isovaleric acid	1 mL
isobutyric acid	1 mL
The 5 acids were mixed and adjust to a pH of ± 6.7 with NaOH (drop directly pellets into the solution).	
Final volume in distilled water	50 mL

Except vitamin K_3, all these solutions were kept for one year at 4 °C.

- **Gut microbiota medium preparation (GMM)**

Ingredients for one liter medium.

Component	Amount/L	comments
tryptycase peptone	2 g	weigh
yeast extract	1 g	weigh
D-glucose	0.4 g	weigh
L-cysteine	0.5 g	weigh
cellobiose D+	1 g	weigh

Component	Amount/L	comments
maltose	1 g	weigh
fructose	1 g	weigh
meat extract	5 g	weigh
KH_2PO_4	100 mL	1 M stock solution pH 7.2
$MgSO_4$ (-7 H_2O)	0.002 g	weigh
$NaHCO_3$	0.4 g	weigh
NaCl	0.08 g	weigh
$CaCl_2$	1 mL	0.8 mg/100 mL stock solution
vitamin K_3 (menadione)	1 mL	stock solution 1 mg/mL
$FeSO_4$	1 mL	stock solution 0.4 mg/mL
hemin	1 mL	stock solution 1 mg/mL
Tween 80	2 mL	stock solution 25%
ATCC vitamin mix	10 mL	commercial solution
ATCC trace mineral mix	10 mL	commercial solution
acetic acid (glacial) propionic acid valeric acid isovaleric acid isobutyric acid	5.5 mL	VFA mix (p. 167)
resazurin	4 mL	0.25 mg/mL stock solution

6.9.1.3 Stock and working solution of genistein (internal standard)

Stock solution of 10 mM: 2.7 mg genistein was dissolved in 1 mL of DMSO. Storage of stock solution for maximal one year at -20 °C in aliquots of 25 µL in 0.5 mL Eppendorf tubes.

Working solution of 100 µM: 10 µL of stock solution were diluted in 990 µL of buffer A for CoulArray.

6.9.1.4 Faeces master mix preparation

A pool of 0.6 g of faeces from the 14 different subjects was prepared in 30 mL of PBS supplemented with 0.1% L-cystein hydrochloride and 10% glycerol.

Faeces were throughly mixed and stored at 4 °C for 2 h and then at -20 °C until further use.

6.9.1.5 Preparation of the solvents

Solvent A – NaH_2PO_4 50 mM pH 1.5

Initially, 6.9 g of NaH_2PO_4 were diluted in 0.75 L of Milli-Q water and the pH adjusted to 1.5 using *ortho*-phosphoric acid (85%). Afterwards, the solution were filed up to a volume of 1.0 L and filtered at filtration pyrex millipore (0.2 µm) system.

Solvent B – Solution NaH_2PO_4/acetonitrile 50:50

Solvent A was diluted with the same volume of acetonitrile and filter at filtration pyrex millipore (0.2 µm) system.

Both solutions can be kept for one week at room temperature and should be filtered every two days.

6.9.2 Incubation of the fecal samples, sample preparation and chromatographic conditions

Incubations of the fecal samples of the 14 volunteers were operated as described in the scheme of incubation (Table 48).

Table 48 Scheme of incubation.

	Replicat 1	Replicat 2	Replicat 3	Positive control	Negative control
GMM	19 mL	19 mL	19 mL	20 mL	19 mL
Master Mix	1 mL	1 mL	1 mL	-	1 mL
	Allow the system to equilibrate for 1 min				
bioactive compound 10 mM (5-CQA)	20 µL	20 µL	20 µL	20 µL	20 µL DMSO
aliquots	1.5 mL at the timepoints 0, 0.5, 1, 2 and 4 h Centrifuged the aliquots at 17,400 x g for 5 min. Pipetted supernatant into separate 1.5 mL Eppendorf amber tubes. Stored the Eppendorf at -40 °C until further use.				

The standard curve was prepared using aliquots of the fecal negative control of each subject. The controls were spiked with standards to obtain final concentrations of 0.15, 0.3, 0.6, 1.25, 2.5, 5, 10, 20 and 40 µM. Therefor, 4 µL of each stock solution (10 mM 5-CQA, 10 mM CA, 10 mM DHCA) were pipetted to 988 µL of the fecal negative control. Thus a standard point of

40 µM was achieved. By following dilution steps of 350 µL of each standard point with 350 µL of fecal negative control the individual calibration curves were achieved.

After incubation samples were prepared by solide phase extraction (Table 49) as performed in former studies [*Renouf and Hendrich, 2011*].The same solid phase extraction procedure was used to prepare the calibration curve.

Table 49 Solide phase extraction of bioactive compounds.

conditioning	1 mL methanol 1 mL distilled water
sample	300 µL acidified sample (pH 2) 15 µL genistin (IS; at 100 µM)
rinse	4 x 1 mL acidified water (pH 2)
Dry the column using the vacuum.	
eluation	0.8 mL methanol
Dry the column using the vacuum.	
evaporation	
Add the initial volume (315 µL) of solvent A	

Table 50 shows the chromatographic conditions which were used to determine either, 5-CQA, CA or DHCA.

Table 50 HPLC conditions for determination of bioactive compounds.

Column	Macherey-Nagel, Nucleodur
Material	C18, 5 µm, 250x3 mm
Mobile phase	A: NaH_2PO_4 (50 mM, pH 1.5); B: NaH_2PO_4 (50 mM)/acetonitrile (50/50, v/v)
Injection volume	50 µl
Wavelength	325 nm

Time	Flow [ml/min]	Solvent A [%]	Solvent B [%]
0.1	0.5	85	15
4	0.5	85	15
12	0.5	80	20
19	0.5	78	22
40	0.5	0	100
45	0.5	0	100
48	0.5	85	15
55	0.5	85	15

For determination of the linearity the standard curves with the correlation factors were used

Limit of quantification (LOQ)

For determination of redoxactive compounds using the CoulArray® detector, the LOQ was fitted to the lowest concentration of the standard curve (0.15 µM for 5-CQA, CA and DHCA).

7 References

ABSciex. (**2008**) *API 3200/4000/5000 LC-MS/MS System Basic Training*. Darmstadt.

Adamson, R.H., Bridges, J.W. Evans, M.E., Williams, R.T. (**1970**) Species differences in the aromatization of quinic acid *in vivo* and the role of gut bacteria. *Biochemistry. 116*, 437-443.

Ader, P., Grenacher, B., Langguth, P., Scharrer, E., and Wolffram, S. (**1996**) Cinnamate uptake by rat small intestine: transport kinetics and transepithelial transfer. *Exp Physiol*. *81*, 943-955.

Adom, K. K., Sorrells, M. E., and Liu, R. H. (**2005**) Phytochemicals and Antioxidant Activity of Milled Fractions of Different Wheat Varieties. *J Agr Food Chem*. *53*, 2297-2306.

Adzet, T., Camarasa, J., Escubedo, E., and Merlos, M. (**1988**) In vitro study of caffeic acid — bovine serum albumin interaction. *Eur J Drug Metab Pharmacokinet*. *13*, 11-14.

Arion, W. J., Canfield, W. K., Ramos, F. C., Schindler, P. W., Burger, H. J., Hemmerle, H., Schubert, G., Below, P., and Herling, A. W. (**1997**) Chlorogenic acid and hydroxynitrobenzaldehyde: New inhibitors of hepatic glucose 6-phosphatase. *Arch Biochem Biophys*. *339*, 315-322.

Azuma, K., Ippoushi, K., Nakayama, M., Ito, H., Higashio, H., and Terao, J. (**2000**) Absorption of chlorogenic acid and caffeic acid in rats after oral administration. *J Agr Food Chem*. *48*, 5496-5500.

Bakuradze, T., Boehm, N., Janzowski, C., Lang, R., Hofmann, T., Stockis, J.-P., Albert, F. W., Stiebitz, H., Bytof, G., Lantz, I., Baum, M., Eisenbrand, G. (**2011**) Antioxidant-rich cofee reduces DANN damage, elevates glutathione status and contributes to weight control: Results from an intervention study. *Mol Nutr Food Res. 55*, 793-797.

Bakuradze, T., Lang, R., Hofmann, T., Eisenbrand, G., Schipp, D., Galan, J., Richling, E. (**2015**) Consumption of a dark roast coffee decreases the level of spontaneous DNA strand breaks: a randomized controlled trial. *Eur J Nutr. 54*, 149-156.

Bakuradze, T., Lang, R., Hofmann, T., Schipp, D., Galan, J., Eisenbrand, G., Richling, E. (**2016**) Coffee consumption rapidly redues background DANN strand breaks in healthy humans: Results of a short-term repeated uptake intervention study. *Mol Nutr Food Res. 60*, 682-686.

Balaban, R. S., Nemoto, S., and Finkel, T. (**2005**) Mitochondria, oxidants, and aging. *Cell*. *120*, 483-495.

Bassoli, B. K., Cassolla, P., Borba-Murad, G. R., Constantin, J., Salgueiro-Pagadigorria, C. L., Bazotte, R. B., da Silva, R. S. D. F., and de Souza, H. M. (**2008**) Chlorogenic acid reduces the plasma glucose peak in the oral glucose tolerance test: effects on hepatic glucose release and glycaemia. *Cell Biochem Funct*. *26*, 320-328.

Belitz, H. D., Grosch, W., and Schieberle, P. (**2009**) *Food Chem*. 4th rev. and extended ed., Springer, Berlin.

Bennat C; Engelhardt UH; Kiehne A; Wirries F; Maier HG (**1994**) HPLC Analysis of chlorogenic acid lactones in roasted coffee. *Z Lebensm Unters Forsch.*199, 17-21.

Biesalski, H.K.,Grimm, P., Biesalski, U., Baum, K., Nowitzki-Grimm, S. (**2007**) Taschenatlas der Ernährung. Thieme; Auflage: 4., überarb. u. erw. Aufl. ISBN-13: 978-3131153548

Bohn, T. (**2014**) Dietary factors affecting polyphenol bioavailability. *Nutrition Reviews*. *72*, 429-452.

Booth, A. N., Emerson, O. H., Jones, F. T., and DeEds, F. (**1957**) Urinary metabolites of caffeic and chlorogenic acids. *J Biol Chem*. *229*, 51-59.

Booth, A. N., Williams, R. T. (**1963**) Dehydroxylation of Caffeic Acid by Rat and Rabbit Cæcal Contents and Sheep Rumen Liquor. *Nature. 198*, 684-685.

Brazier, J. S. (**1982**) Appraisal of Anotox, a new anaerobic atmospheric detoxifying agent for use in anaerobic cabinets. *J Clin Pathol*. *35*, 233-238.

Budryn, G., Zaczynska, D., Rachwal-Rosiak, D. (**2015**) Changes of free and nanocapsulated hydroxycinnamic acids from green coffee added to different food products during processing and in vitro enzymatic digestion. *Food Res Int. 89*, 1004-1014.

Cammann, K. (**2000**) *Instrumentelle analytische Chemie, Verfahren, Anwendung, Qualitätssicherung*. 1st ed., Spektrum Akademischer Verlag.

Campa, C., Doulbeau, S., Dussert, S., Hamon, S., and Noirot, M. (**2005**) Qualitative relationship between caffeine and chlorogenic acid contents among wild Coffea species. *Food Chem*. *93*, 135-139.

Cermak R., Breves G. M. (**2006**) In vitro degradation of the flavonol quercetin and of quercetin glycosides in the porcine hindgut. *Arch Anim Nutr*. 60, 180–189.

Chaube, S., and Swinyard, C. A. (**1976**) Teratological and toxicological studies of alkaloidal and phenolic compounds from Solanum Tuberosum L. *Toxicol Appl Pharm*. *36*, 227-237.

ChemSpider a, Royal Society of Chemistry: The free chemical database http://www.chemspider.com/Chemical-Structure.7975151.html?rid=d1da1a72-cdcc-49b7-b72f-4ea3164dbd6f&page_num=0, retrieved 20.06.2015

ChemSpider b, Royal Society of Chemistry: The free chemical database http://www.chemspider.com/Chemical-Structure.4444237.html?rid=db0ca06a-fec8-4a21-9b51-8874f7afddf6, retrieved 20.06.2015

ChemSpider a, Royal Society of Chemistry: The free chemical database http://www.chemspider.com/Chemical-Structure.22912767.html, retrieved 21.05.2017

Chesson, A., Provan, G. J., Russell, W. R., Scobbie, L., Richardson, A. J., and Stewart, C. (**1999**) Hydroxycinnamic acids in the digestive tract of livestock and humans. *J Sci Food Agr*. *79*, 373-378.

Ciolino, H. P., Daschner, P. J., and Yeh, G. C. (**1998**) Resveratrol Inhibits Transcription of CYP1A1 in Vitro by Preventing Activation of the Aryl Hydrocarbon Receptor. *Cancer Res*. *58*, 5707-5712.

Ciolino, H. P., Daschner, P. J., and Yeh, G. C. (**1999**) Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem J*. *340*, 715-722.

Clifford, M. N. (**1999**) Chlorogenic acids and other cinnamates - nature, occurrence and dietary burden. *J Sci Food Agr*. *79*, 362-372.

Clifford, M. N. (**2000**) Chlorogenic acids and other cinnamates - nature, occurrence, dietary burden, absorption and metabolism. *J Sci Food Agr*. *80*, 1033-1043.

Clifford, M. N., Johnston, K. L., Knight, S., and Kuhnert, N. (**2003**) Hierarchical scheme for LC-MSn identification of chlorogenic acids. *J Agr Food Chem*. *51*, 2900-2911.

Clifford, M. N., and Knight, S. (**2004**) The cinnamoyl-amino acid conjugates of green robusta coffee beans. *Food Chem*. *87*, 457-463.

Coffee Review, http://www.coffeereview.com/coffee-glossary, retrieved 30.09.2016

Couteau, D., McCartney, A. L., Gibson, G. R., Williamson, G., and Faulds, C. B. (**2001**) Isolation and characterization of human colonic bacteria able to hydrolyse chlorogenic acid. *J Appl Microbiol*. *90*, 873-881.

Crozier, T. W., Stalmach, A., Lean, M. E., and Crozier, A. (**2012**) Espresso coffees, caffeine and chlorogenic acid intake: potential health implications. *Food Funct*. *3*, 30-33.

deutscher Kaffeeverband, http://www.kaffeeverband.de, retrieved 21.05.2017

Dixon, R. A., Achnine, L., Kota, P., Liu, C. J., Reddy, M. S. S., and Wang, L. J. (**2002**) The phenylpropanoid pathway and plant defence - a genomics perspective. *Mol Plant Pathol*. *3*, 371-390.

dos Santos, M. D., Almeida, M. C., Lopes, N. P., de Souza, G., oacute, ria, E., iacute, and lia, P. (**2006**) Evaluation of the Anti-inflammatory, Analgesic and Antipyretic Activities of the Natural Polyphenol Chlorogenic Acid. *Biol Pharma Bull*. *29*, 2236-2240.

Dragovic-Uzelac, V., Pospišil, J., Levaj, B., and Delonga, K. (**2005**) The study of phenolic profiles of raw apricots and apples and their purees by HPLC for the evaluation of apricot nectars and jams authenticity. *Food Chem*. *91*, 373-383.

Dziki, D., Gawlik-Dziki, U., Pecio, L., Rozylo, R., Swieca, M. (**2014**) Current trends in the enhancement of antioxidant activity of wheat bread by the addition of plant materials rich in phenolic compounds. *Trends Food Sci Technol. 40*, 48-61.

Ehala, S., Vaher, M., and Kaljurand, M. (**2005**) Characterization of Phenolic Profiles of Northern European Berries by Capillary Electrophoresis and Determination of their Antioxidant Activity. *J Agr Food Chem*. *53*, 6484-6490.

Eisenbrand, G., Metzler, M., Hennecke, F.J. (**2005**) *Toxikologie für Naturwissenschaftler*. 3 ed., Wiley-VCH, Weinheim.

Eisenbrand, G., Schreier, P. (**2006**) *RÖMPP Lexikon Lebensmittelchemie*. 2 ed., Georg Thieme Verlag, Stuttgart.

Eklund, A. (**1975**) Effect of chlorogenic acid in a casein diet for rats. Nutritional and pathological observations. *Nutr Metab*. *18*, 258-264.

Encarnacao, J., A. da, Farrell, T., L., Ryder, A., Kraut, N., U., Williamson, G. (**2015**) In vitro enzymic hydrolysis of chlorogenic acids in coffee. *Mol Nutr Food Res. 59*, 231.239.

Erk, T., Bergmann, H., and Richling, E. (**2009**) A Novel Method for the Quantification of Quinic Acid in Food Using Stable Isotope Dilution Analysis. *J Aoac Int*. *92*, 730-733.

Erk, T., Hauser, J., Williamson, G., Renouf, M., Steiling, H., Dionisi, F., and Richling, E. (**2013a**) Structure– and dose–absorption relationships of coffee polyphenols. *Biofactors*. 40, 103-112.

Erk, T., Renouf, M., Williamson, G., Melcher, R., Steiling, H., and Richling, E. (**2013b**) Absorption and isomerization of caffeoylquinic acids from different foods using ileostomist volunteers. *Eur J Nutr*. 53(1).159-166.

Erk, T., Williamson, G., Renouf, M., Marmet, C., Steiling, H., Dionisi, F., Barron, D., Melcher, R., and Richling, E. (**2012**) Dose-dependent absorption of chlorogenic acids in the small intestine assessed by coffee consumption in ileostomists. *Mol Nutr Food Res*. *56*, 1488-1500.

Farah, A., de Paulis, T., Moreira, D. P., Trugo, L. C., and Martin, P. R. (**2006**) Chlorogenic acids and lactones in regular and water-decaffeinated arabica coffees. *J Agr Food Chem*. *54*, 374-381.

Farah, A., De Paulis, T., Trugo, L. C., and Martin, P. R. (**2005**) Effect of roasting on the formation of chlorogenic acid lactones in coffee. *J Agr Food Chem*. *53*, 1505-1513.

Farah, A., and Donangelo, C. M. (**2006**) Phenolic compounds in coffee. *Braz J Plant Physiol*. *18*, 23-36.

Farah, A., Monteiro, M., Donangelo, C. M., and Lafay, S. (**2008**) Chlorogenic Acids from Green Coffee Extract are Highly Bioavailable in Humans. *J Nut*. *138*, 2309-2315.

Farrell, T. L., Dew, T. P., Poquet, L., Hanson, P., and Williamson, G. (**2011**) Absorption and Metabolism of Chlorogenic Acids in Cultured Gastric Epithelial Monolayers. *Drug Metab Dispos*. *39*, 2338-2346.

FDA (**1996**) Guidance for Industry, Q2B Validation of Analytical Procedures: Methodology

Ferrero-Miliani, L., Nielsen, O.H., Andersen, P. S., Girardin, S. E. (**2007**) Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. *Clin Exp Immunol.* 147, 227-235.

Ferruzzi M. G. (**2010)** The influence of beverage composition on delivery of phenolic compounds from coffee and tea. *Physiol Behav*.100, 33–41.

Fischer, E. (**1902**) *Über die Ester der Aminosäuren*. De Gruyter.

Frank, O., Zehentbauer, G., and Hofmann, T. (**2006**) Bioresponse-guided decomposition of roast coffee beverage and identification of key bitter taste compounds. *Eur Food Res Technol A*. *222*, 492-508.

Frankel, E. N., Waterhouse, A. L., and Teissedre, P. L. (**1995**) Principal Phenolic Phytochemicals in Selected California Wines and Their Antioxidant Activity in Inhibiting Oxidation of Human Low-Density Lipoproteins. *J Agr Food Chem*. *43*, 890-894.

Gest, H. (**2004**) The discovery of microorganisms by Robert Hooke and Antoni van Leeuwenhoek, Fellows of The Royal Society. Notes Rec R Soc Lond. 58, 187-201.

Gonthier, M. P., Remesy, C., Scalbert, A., Cheynier, V., Souquet, J. M., Poutanen, K., and Aura, A. M. (**2006**) Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota in vitro. *Biomed Pharmaco*. *60*, 536-540.

Gonthier, M. P., Verny, M. A., Besson, C., Remesy, C., and Scalbert, A. (**2003**) Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. *J Nutr*. *133*, 1853-1859.

Griffiths, L. A. (**1964**) Studies on flavonoid metabolism. Identification of the metabolities of (+)-catechin in rat urine. *Biochem J*. *92*, 173-179.

Gumbinger, H. G., Vahlensieck, U., and Winterhoff, H. (**1993**) Metabolism of caffeic acid in the isolated perfused rat liver. *Planta Med*. *59*, 491-493.

Guy, P. A., Renouf, M., Barron, D., Cavin, C., Dionisi, F., Kochhar, S., Rezzi, S., Williamson, G., and Steiling, H. (**2009**) Quantitative analysis of plasma caffeic and ferulic acid equivalents by liquid chromatography tandem mass spectrometry. *J Chromatogr B*. *877*, 3965-3974.

Hach, B., and Heim, F. (**1971**) Comparative studieson the central stimulating effects of caffeie and chlorogenic acid in white mice. *Arzneimittelforschung*. *2*, 23-25.

Hagiwara, A., Hirose, M., Takahashi, S., Ogawa, K., Shirai, T., and Ito, N. (**1991**) Forestomach and Kidney Carcinogenicity of Caffeic Acid in F344 Rats and C57BL/6N × C3H/HeN F1 Mice. *Cancer Res*. *51*, 5655-5660.

Hagl, S., Deusser, H., Soyalan, B., Janzowski, C., Will, F., Dietrich, H., Albert, F. W., Rohner, S., and Richling, E. (**2011**) Colonic availability of polyphenols and D-(-)-quinic acid after apple smoothie consumption. *Mol Nutr Food Res*. *55*, 368-377.

He, W., Liu, X., Xu, H., Gong, Y., Yuan, F., and Gao, Y. (**2010**) On-line HPLC-ABTS screening and HPLC-DAD-MS/MS identification of free radical scavengers in Gardenia (Gardenia jasminoides Ellis) fruit extracts. *Food Chem*. *123*, 521-528.

Heber, D., Blackburn, G. L., Liang, V. W. G., Milner, J. (**2006**) Nutrional Oncology. *Academic Press.* p. 314

Hirose, M., Kawabe, M., Shibata, M., Takahashi, S., Okazaki, S., and Ito, N. (**1992**) Influence of caffeic acid and other o-dihydroxybenzene derivatives on N-methyl-N'-nitro-N-nitrosoguanidine-initiated rat forestomach carcinogenesis. *Carcinogenesis*. *13*, 1825-1828.

Hirose, M., Masuda, A., Imaida, K., Kagawa, M., Tsuda, H., and Ito, N. (**1987**) Induction of forestomach lesions in rats by oral administrations of naturally occurring antioxidants for 4 weeks. *Jpn J Cancer Res*. *78*, 317-321.

Hirose, M., Shirai, T., Takahashi, S., Ogawa, K., and Ito, N. (**1993**) Organ-Specific Modification of Carcinogenesis by Antioxidants in Rats. *Antimutagenesis and Anticarcinogenesis Mechanisms Iii*. *61*, 181-188.

Hobson, W. (**1939**) Urinary output of creatine and creatinine associated with physical exercise, and its relationship to carbohydrate metabolism. *Biochem J*. *33*, 1425-1431.

Hoelzel, C., Knasmuller, S., Wagner, K., H., Elbling, L., Huber, W., Kager, N., Ferk, F., Ehrlich, V., Nersesyan, A., Neubauer, O., Desmarchelier, A., Marin-Kuan, M., Delatour, T., Verguet, C., Bezencon, C., Besson, A., Grathwohl, D., Simic, T., Kundi, M., Schilter, B., Cavin, C. (**2010**) Instant coffee with high chlorogenic acid levels protects humans against oxidative damage of macromolecules. *Mol Nutr Food Res. 54*, 1722-1733.

Huang, M. T., Smart, R. C., Wong, C. Q., and Conney, A. H. (**1988**) Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res*. *48*, 5941-5946.

IARC. (**1993**) Monographs on the Evaluation of Carcinogenic Risks to Humans Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, (IARC, W., Ed.) pp 115-134.

ICD-10-WHO Version **2016** Internationale statistische Klassifikation der Krankheiten und verwandter Gesundheitsprobleme. 10. Revision, Version 2016 (with updates of the

21.12.2015); http://www.dimdi.de/static/de/klassi/icd-10-who/kodesuche/onlinefassungen/htmlamtl2016/index.htm (05.06.2016)

Ito, N., Hirose, M., and Takahashi, S. (**1993**) Cell proliferation and forestomach carcinogenesis. *Environ Health Perspect*. *101 Suppl 5*, 107-110.

IUPAC. (**1969**) IUPAC Commission on the Nomenclature of Organic Chemistry (CON) and IUPAC-IUB Commission on Biochemical Nomenclature (CBN). The nomenclature of cyclitols. Tentative rules. *Biochem J*. *112*, 17-28.

Jacobsen, C., Schwarz, K., Stöckmann, H., Meyer, A.S., Adler-Nissen, J. (**1999**) Partitioning of Selected Antioxidants in Mayonnaise. *J Agric Food Chem. 47*, 3601-3610

Kagawa, M., Hakoi, K., Yamamoto, A., Futakuchi, M., and Hirose, M. (**1993**) Comparison of reversibility of rat forestomach lesions induced by genotoxic and non-genotoxic carcinogens. *Jpn J Cancer Res*. *84*, 1120-1129.

Kampa, M., Alexaki, V. I., Notas, G., Nifli, A. P., Nistikaki, A., Hatzoglou, A., Bakogeorgou, E., Kouimtzoglou, E., Blekas, G., Boskou, D., Gravanis, A., and Castanas, E. (**2004**) Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Res*. *6*, R63-R74.

Karr, R. M., Lehrer, S. B., Butcher, B. T., and Salvaggio, J. E. (**1978**) Coffee worker's asthma: A clinical appraisal using the radioallergosorbent test. *J Allergy Clin Immun*. *62*, 143-148.

Karthikeyan, S., Kanimozhi, G., Prasad, N. R., and Mahalakshmi, R. (**2011**) Radiosensitizing effect of ferulic acid on human cervical carcinoma cells in vitro. *Toxicol in Vitro*. *25*, 1366-1375.

Kasai, H., Fukada, S., Yamaizumi, Z., Sugie, S., and Mori, H. (**2000**) Action of chlorogenic acid in vegetables and fruits as an inhibitor of 8-hydroxydeoxyguanosine formation in vitro and in a rat carcinogenesis model. *Food Chem Toxicol*. *38*, 467-471.

Kaye, M., and Freedman, S. O. (**1961**) Allergy to raw coffee-an occupational disease. *Can Med Assoc J*. *84*, 469-471.

Kern, M., Tjaden, Z., Ngiewih, Y., Puppel, N., Will, F., Dietrich, H., Pahlke, G., and Marko, D. (**2005**) Inhibitors of the epidermal growth factor receptor in apple juice extract. *Mol Nutr Food Res*. *49*, 317-328.

Kern, S. M., Bennett, R. N., Mellon, F. A., Kroon, P. A., and Garcia-Conesa, M. T. (**2003**) Absorption of hydroxycinnamates in humans after high-bran cereal consumption. *J Agr Food Chem*. *51*, 6050-6055.

Kitts, D. D., and Wijewickreme, A. N. (**1994**) Effect of dietary caffeic and chlorogenic acids on in vivo xenobiotic enzyme systems. *Plant Foods Hum Nutr*. *45*, 287-298.

Kivilompolo, M., and Hyötyläinen, T. (**2007**) Comprehensive two-dimensional liquid chromatography in analysis of Lamiaceae herbs: Characterisation and quantification of antioxidant phenolic acids. *J Chromatogr A*. *1145*, 155-164.

Konishi, Y., Hitomi, Y., Yoshida, M., and Yoshioka, E. (**2005**) Absorption and Bioavailability of Artepillin C in Rats after Oral Administration. *J Agr Food Chem*. *53*, 9928-9933.

Konishi, Y., and Kobayashi, S. (**2004**) Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabolites in intestinal Caco-2 cell monolayers. *J Agr Food Chem*. *52*, 2518-2526.

Konishi, Y., Zhao, Z., and Shimizu, M. (**2006**) Phenolic Acids Are Absorbed from the Rat Stomach with Different Absorption Rates. *J Agr Food Chem*. *54*, 7539-7543.

Kromidas, S. (**2017**) Das HPLC-MS-Buch für Anwender, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany

Lang, R., Dieminger, N., Beusch, A., Lee, Y.-M., Dunkel, A., Suess, B., Skurk, T., Wahl, A., Hauner, H., and Hofmann, T. (**2013a**) Bioappearance and pharmacokinetics of bioactives upon coffee consumption. *Anal Bioanal Chem*. *405*, 8487-8503.

Lang, R., Yagar, E. F., Wahl, A., Beusch, A., Dunkel, A., Dieminger, N., Eggers, R., Bytof, G., Stiebitz, H., Lantz, I., Hofmann, T. (**2013b**) Quantitative studies on roast kinetics for bioactives in coffee. *J Agr Food Chem.* 61, 12123-12128

Laurent, C., Besançon, P., and Caporiccio, B. (**2007**) Flavonoids from a grape seed extract interact with digestive secretions and intestinal cells as assessed in an in vitro digestion/Caco-2 cell culture model. *Food Chem*. *100*, 1704-1712.

Layton, L. L., Greene, F. C., and Panzani, R. (**1965a**) Allergy to Green Coffee. Failure of Patients Allergic to Green Coffee to React to Chlorogenic Acid, Roasted Coffee, or Orange. *J Allergy*. *36*, 84-91.

Layton, L. L., Panzani, R., and Cortese, T. A. (**1968**) Coffee-Reaginic Human Sera Tested in Human Volunteers and Macaque Monkeys. *Int Arch Allergy Immunol*. *33*, 417-427.

Layton, L. L., Panzani, R., Greene, F. C., and Corse, J. W. (**1965b**) Atopic hypersensitivity to a protein of the green coffee bean and absence of allergic reactions to chlorogenic acid, low-molecular-weight components of green coffee, or to roasted coffee. *Int Arch Allergy Appl Immunol*. *28*, 116-127.

Lekse, J. M., Xia, L., Stark, J., Morrow, J. D., and May, J. M. (**2001**) Plant catechols prevent lipid peroxidation in human plasma and erythrocytes. *Mol Cell Biochem*. *226*, 89-95.

Lemanska, K., Szymusiak, H., Tyrakowska, B., Zielinski, R., Soffers, A. E., Rietjens, I. M. (**2001**) The influence of pH on antioxidant properties and the machanism of antioxidant action of hydroxyflavones. *Free Radic Biol Med.* 31, 869-881.

Lempereur, I., Rouau, X., and Abecassis, J. (**1997**) Genetic and Agronomic Variation in Arabinoxylan and Ferulic Acid Contents of Durum Wheat (Triticum durum L.) Grain and Its Milling Fractions. *J Cereal Sci*. *25*, 103-110.

Lepelley, M., Cheminade, G., Tremillon, N., Simkin, A., Caillet, V., and McCarthy, J. (**2007**) Chlorogenic acid synthesis in coffee: An analysis of CGA content and real-time RT-PCR expression of HCT, HQT, C3H1, and CCoAOMT1 genes during grain development in C. canephora. *Plant Sci*. *172*, 978-996.

Liang, N, Kitts, D., D. (**2015**) Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutr. 8*, 1-20.

Llorach, R., Garrido, I., Monagas, M., Urpi-Sarda, M., Tulipani, S., Bartolome, B., Andres-Lacueva, C. (2010) Metabolomics study of human urinary metabolome modifications after intake of almond (Prunus dulcis (Mill.) D.A. Webb) skin polyphenols. *J Proteomic Res. 9*, 5869-5867

Lottspeich, F., Engels, J. W., Zettlmeier Lay, S. (**2008**) *Bioanalytik*. 2 ed., Spektrum Akademischer Verlag, Germany.

Ludwig, I. A., Paz de Peña, M., Concepción, C., and Alan, C. (**2013**) Catabolism of coffee chlorogenic acids by human colonic microbiota. *Biofactors*. 39, 623-632.

Marmet, C., Actis-Goretta, L., Renouf, M., and Giuffrida, F. (**2014**) Quantification of phenolic acids and their methylates, glucuronides, sulfates and lactones metabolites in human plasma by LC–MS/MS after oral ingestion of soluble coffee. *J Pharma Biomed Anal*. *88*, 617-625.

Matei, M. F., Jaiswal, R., and Kuhnert, N. (**2012**) Investigating the Chemical Changes of Chlorogenic Acids during Coffee Brewing: Conjugate Addition of Water to the Olefinic Moiety of Chlorogenic Acids and Their Quinides. *J Agr Food Chem*. *60*, 12105-12115.

Mateos, R., Goya, L., and Bravo, L. (**2006**) Uptake and metabolism of hydroxycinnamic acids (chlorogenic, caffeic, and ferulic acids) by HepG2 cells as a model of the human liver. *J Agr Food Chem*. *54*, 8724-8732.

Matsui, Y., Nakamura, S., Kondou, N., Takasu, Y., Ochiai, R., and Masukawa, Y. (**2007**) Liquid chromatography-electrospray ionization-tandem mass spectrometry for simultaneous analysis of chlorogenic acids and their metabolites in human plasma. *J Chromatogr B*. *858*, 96-105.

Mattila, P., and Hellstrom, J. (**2007**) Phenolic acids in potatoes, vegetables, and some of their products. *J Food Comp Anal*. *20*, 152-160.

Mattila, P., Hellstrom, J., and Torronen, R. (**2006**) Phenolic acids in berries, fruits, and beverages. *J Agric Food Chem*. *54*, 7193-7199.

Mattila, P., and Kumpulainen, J. (**2002**) Determination of Free and Total Phenolic Acids in Plant-Derived Foods by HPLC with Diode-Array Detection. *J Agr Food Chem*. *50*, 3660-3667.

McKinney, R. E. (**2004**) Environmental Pollution Control Microbiology. *Marcel Dekker Ins.,* New York

Minussi, R. C., Rossi, M., Bologna, L., Cordi, L. v., Rotilio, D., Pastore, G. M., and Durán, N. (**2003**) Phenolic compounds and total antioxidant potential of commercial wines. *Food Chem*. *82*, 409-416.

Mølgaard, P., and Ravn, H. (**1988**) Evolutionary aspects of caffeoyl ester distribution In Dicotyledons. *Phytochemistry*. *27*, 2411-2421.

Möller, B., and Herrmann, K. (**1983**) Quinic acid esters of hydroxycinnamic acids in stone and pome fruit. *Phytochemistry*. *22*, 477-481.

Montavon, P., Duruz, E., Rumo, G., and Pratz, G. (**2003**) Evolution of Green Coffee Protein Profiles with Maturation and Relationship to Coffee Cup Quality. *J Agr Food Chem*. *51*, 2328-2334.

Monteiro, M., Farah, A., Perrone, D., Trugo, L. C., and Donangelo, C. (**2007**) Chlorogenic acid compounds from coffee are differentially absorbed and metabolized in humans. *J Nutr*. *137*, 2196-2201.

Moon, J.-K., Yoo, H. S., and Shibamoto, T. (**2009**) Role of Roasting Conditions in the Level of Chlorogenic Acid Content in Coffee Beans: Correlation with Coffee Acidity. *J Agr Food Chem*. *57*, 5365-5369.

Moreira, D. P., Monteiro, M. C., Ribeiro-Alves, M., Donangelo, C. M., and Trugo, L. C. (**2005**) Contribution of Chlorogenic Acids to the Iron-Reducing Activity of Coffee Beverages. *J Agr Food Chem*. *53*, 1399-1402.

Moridani, M. Y., Scobie, H., Jamshidzadeh, A., Salehi, P., and O'Brien, P. J. (**2001**) Caffeic acid, chlorogenic acid, and dihydrocaffeic acid metabolism: Glutathione conjugate formation. *Drug Metabol Dispos*. *29*, 1432-1439.

Müntnich, S. (**2013**) *In vitro* Studien zur intestinalen Absorption von Hydroxyzimtsäurederivaten im Ussing-Kammer-Modell, Diplomarbeit, *Technische Universität Kaiserslautern*, Kaiserslautern.

Muralidhara, B. K., and Prakash, V. (**1995**) Interaction of 3'-O-caffeoyl D-quinic acid with human serum albumin. *Int J Pept Protein Res*. *46*, 1-8.

Murota, K., and Terao, J. (**2003**) Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. *Arch Biochem Biophys*. *417*, 12-17.

Mutschler, E. (**1975**) *Arzneimittelwirkungen : ein Lehrbuch der Pharmakologie für Pharmazeuten, Chemiker und Biologen : mit einführenden Kapiteln in die Anatomie und Physiologie*. 3., vollständig überarb. und erw. Aufl. ed., Wissenschaftliche Verlagsgesellschaft, Stuttgart, Germany.

Nardini, M., Cirillo, E., Natella, F., and Scaccini, C. (**2002**) Absorption of phenolic acids in humans after coffee consumption. *J Agr Food Chem*. *50*, 5735-5741.

Natella, F., Nardini, M., Belelli, F., and Scaccini, C. (**2007**) Coffee drinking induces incorporation of phenolic acids into LDL and increases the resistance of LDL to ex vivo oxidation in humans. *Am J Clin Nutr*. *86*, 604-609.

Natella, F., Nardini, M., Giannetti, I., Dattilo, C., and Scaccini, C. (**2002**) Coffee drinking influences plasma antioxidant capacity in humans. *J Agr Food Chem*. *50*, 6211-6216.

Neises, B., and Steglich, W. (**1978**) Simple Method for the Esterification of Carboxylic Acids. *Angewandte Chemie International Edition in English*. *17*, 522-524.

Nogueira M; Trugo LC (**2003**) Distribuição de isômeros de ácido clorogênico e teores de cafeína e trigonelina em cafés solúveis Brasileiros. *Ciênc Tecnol Alim*. 23, 296-299.

Nyambe-Silavwe, H., Williamson, G. (**2017**) Polyphenol- and fibre-rich dried fruits with green tea attenuate starch-derived postprandial blood glucose and insulin: a randomised, controlled, single-blind, cross-over intervention. *Br J Nutr.* 116, 443-460

Ohnishi, M., Morishita, H., Iwahashi, H., Toda, S., Shirataki, Y., Kimura, M., and Kido, R. (**1994**) Inhibitory effects of chlorogenic acids on linoleic acid peroxidation and haemolysis. *Phytochemistry*. *36*, 579-583.

Olthof, M. R., Hollman, P. C., Buijsman, M. N., van Amelsvoort, J. M., and Katan, M. B. (**2003**) Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. *J Nutr*. *133*, 1806-1814.

Olthof, M. R., Hollman, P. C. H., and Katan, M. B. (**2001**) Chlorogenic acid and caffeic acid are absorbed in humans. *J Nutr*. *131*, 66-71.

Ortega, N., Reguant, J., Romero, M.P., Macià, A., Motilva, M.J. (**2009**) Effect of fat content on the digestibility and bioaccessibility of cocoa polyphenol by an in vitro digestion model. *J Agr Food Chem. 57*, 5743-5749.

Ovaskainen, M. L., Torronen, R., Koponen, J. M., Sinkko, H., Hellstrom, J., Reinivuo, H., and Mattila, P. (**2008**) Dietary intake and major food sources of polyphenols in Finnish adults. *J Nutr*. *138*, 562-566.

Peng, B. J., Zhu, Q., Zhong, Y. L., Xu, S. H., Wang, Z. (**2015**) Chlorogenic Acid Maintains Glucose Homeostasis through Modulating the Expression of SGLT-1, GLUT-2, and PLG in Different Intestinal Segments of Sprague-Dawley Rats Fed a High-Fat Diet. *Biomed Environ Sci. 28*, 894-903.

Perez-Silva, G., Rodriguez, D. (**1966**) Dehydroxylation of Caffeic Acid by a Bacterium isolated from Rat Faeces. *Nature*. *212*, 303-304.

Phillips, K. D., and Willis, A. T. (**1981**) Appraisal in the diagnostic laboratory of three commercially available anaerobic cabinets. *J Clin Pathol*. *34*, 1110-1113.

Pietta, P. G. (**2000**) Flavonoids as antioxidants. *J Nat Prod*. *63*, 1035-1042.

Ploemen, J. H. T. M., van Ommen, B., de Haan, A., Schefferlie, J. G., and van Bladeren, P. J. (**1993**) In vitro and in vivo reversible and irreversible inhibition of rat glutathione S-transferase isoenzymes by caffeic acid and its 2-S-glutathionyl conjugate. *Food Chem Toxicol*. *31*, 475-482.

Plumb, G. W., Garcia-Conesa, M. T., Kroon, P. A., Rhodes, M., Ridley, S., and Williamson, G. (**1999**) Metabolism of chlorogenic acid by human plasma, liver, intestine and gut microflora. *J Sci Food Agr*. *79*, 390-392.

Poquet, L., Clifford, M. N., and Williamson, G. (**2008**) Transport and Metabolism of Ferulic Acid through the Colonic Epithelium. *Drug Metabol Dispos*. *36*, 190-197.

Price, V.F., Jollow, D.J. (**1989**) Effect of glucose and gluconeogenic substrates on fasting-induced suppression of acetaminophen glucuronidation in the rat. Toxicology and Appl Pharmacol. 101, 356-69.

Qualitätssicherung. (**2004**) *Qualitätssicherung – Handbuch der fiktiven Firma „Muster“ – Validierung analytischer Verfahren*. 2 ed., Bundesverband der Arzneimittelhersteller e.V., Germany.

Quick, A. J., Cooper, A. (**1931**) The conjugation of benzoic acid in man. *J Biol Chem*. *92*, 65-85.

RADANAL. *CoulArray® Detector, operating manuel*. Chelmsford, UK.

Radtke, J., Linseisen, J., and Wolfram, G. (**1998**) Phenolic acid intake of adults in a Bavarian subgroup of the national food consumption survey. *Zeitschrift fur Ernahrungswissenschaft*. *37*, 190-197.

Rahman, B., Schneider, H.-P., Bröer, A., Deitmer, J. W., and Bröer, S. (**1999**) Helix 8 and Helix 10 Are Involved in Substrate Recognition in the Rat Monocarboxylate Transporter MCT1. *Biochem*. *38*, 11577-11584.

Rechner, A. R., Bremner, P., Bourne, L. C., Hubbard, G. P., and Rice-Evans, C. A. (**2001a**) Novel biomarkers of the biotransformation of polyphenols in vivo. *Free Radical Bio Med*. *31*, 1213-1222.

Rechner, A. R., Spencer, J. P. E., Kuhnle, G., Hahn, U., and Rice-Evans, C. A. (**2001b**) Novel biomarkers of the metabolism of caffeic acid derivatives in vivo. *Free Radical Bio Med*. *30*, 1213-1222.

Renouf, M., Guy, P., Marmet, C., Longet, K., Fraering, A. L., Moulin, J., Barron, D., Dionisi, F., Cavin, C., Steiling, H., and Williamson, G. (**2010a**) Plasma appearance and correlation between coffee and green tea metabolites in human subjects. *Br J Nutr*. *104*, 1635-1640.

Renouf, M., Guy, P. A., Marmet, C., Fraering, A. L., Longet, K., Moulin, J., Enslen, M., Barron, D., Dionisi, F., Cavin, C., Williamson, G., and Steiling, H. (**2010b**) Measurement of caffeic and ferulic acid equivalents in plasma after coffee consumption: small intestine and colon are key sites for coffee metabolism. *Mol Nutr Food Res*. *54*, 760-766.

Renouf, M. and Hendrich, S. (**2011**) *Bacteroides uniformis* is a putative bacterial species accociated with the degradation of the isoflavone genistein in human feces. *J Nutr. 141*, 1120-1126.

Renouf, M., Marmet, C., Guy, P., Fraering, A. L., Longet, K., Moulin, J., Enslen, M., Barron, D., Cavin, C., Dionisi, F., Rezzi, S., Kochhar, S., Steiling, H., and Williamson, G. (**2010c**) Nondairy creamer, but not milk, delays the appearance of coffee phenolic acid equivalents in human plasma. *J Nutr*. *140*, 259-263.

Renouf, M., Marmet, C., Guy, P. A., Beaumont, M., Lepage, M., Williamson, G., and Dionisi, F. (**2013**) Dose-response plasma appearance of green tea catechins in adults. *Mol Nutrition Food Res*. *57*, 833-839.

Renouf, M., Marmet, C., Giuffrida, F., Lepage, M., Barron, D., Beaumont, M., Williamson, G., Dionisi, F. (**2014**) Dose-response plasma appearance of coffee chlorogenic and phenolic acids in adults. *Mol Nutr Food Res.* 85. 301-309

Richelle, M., Tavazzi, I., and Offord, E. (**2001**) Comparison of the antioxidant activity of commonly consumed polyphenolic beverages (coffee, cocoa, and tea) prepared per cup serving. *J Agr Food Chem*. *49*, 3438-3442.

Roche, M., Dufour, C., Mora, N., and Dangles, O. (**2005**) Antioxidant activity of olive phenols: mechanistic investigation and characterization of oxidation products by mass spectrometry. *Org Biomol Chem*. *3*, 423-430.

Rodríguez-Delgado, M. A., Malovaná, S., Pérez, J. P., Borges, T., and García Montelongo, F. J. (**2001**) Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. *J Chromatogr A*. *912*, 249-257.

Romani A., Pinelli P., Cantini C., Cimato A., Heimler D. (**200**6) Characterization of Violetto di Toscana, a typical Italian variety of artichoke (Cynara scolymus L.). *Food Chem.* 95, 221-225

Römpp, H., Falbe, J., Regitz, M. (**1996**) *Römpp*. 10 ed., Thieme, Stuttgart.

Rychlik, M., and Asam, S. (**2008**) Stable isotope dilution assays in mycotoxin analysis. *Anal Bioanal Chem*. *390*, 617-628.

Sato, Y., Itagaki, S., Kurokawa, T., Ogura, J., Kobayashi, M., Hirano, T., Sugawara, M., and Iseki, K. (**2011**) In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid. *Int J Pharmaceut*. *403*, 136-138.

Sawa, T., Nakao, M., Akaike, T., Ono, K., and Maeda, H. (**1998**) Alkylperoxyl Radical-Scavenging Activity of Various Flavonoids and Other Phenolic Compounds: Implications for the Anti-Tumor-Promoter Effect of Vegetables. *J Agr Food Chem*. *47*, 397-402.

Scheline, R. R. (**1968**) Metabolism of phenolic acids by the rat intestinal microflora. *Acta Pharmacol Toxicol (Copenh)*. *26*, 189-205.

Scherbl, D., Muentnich, S., Richling, E. (**2014**) *In vitro* studies of chlorogenic acids from coffee using the Ussing chamber model. *Food Res Int. 63c*, 456-463

Schmidt, R. F., Lang, F., Heckmann, M. (**2007**) Physiologie des Menschen. *Springer.* p.957ff

Selinheimo, E., Kruus, K., Buchert, J., Hopia, A., and Autio, K. (**2006**) Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. *J Cereal Sci*. *43*, 152-159.

Selma, M. a. V., Espín, J. C., and Tomás-Barberán, F. A. (**2009**) Interaction between Phenolics and Gut Microbiota: Role in Human Health. *J Agr Food Chem*. *57*, 6485-6501.

Silva, F. A. M., Borges, F., Guimarães, C., Lima, J. L. F. C., Matos, C., and Reis, S. (**2000**) Phenolic Acids and Derivatives: Studies on the Relationship among Structure,

Radical Scavenging Activity, and Physicochemical Parameters†. *J Agr Food Chem*. *48*, 2122-2126.

Sirota, R., Gibson, D., Kohen, R. (**2015**) The role of the catecholic and the electrophilic moieties of caffeic acid in Nrf2/Keap 1 pathway activation in ovarian carcinoma cell lines. *Redox Biol. 4*, 48-59.

Sisson, P. R., Ingham, H. R., and Byrne, P. O. (**1987**) Wise Anaerobic Work Station: an evaluation. *J Clin Pathol*. *40*, 286-291.

Stalmach, A., Mullen, W., Barron, D., Uchida, K., Yokota, T., Cavin, C., Steiling, H., Williamson, G., and Crozier, A. (**2009**) Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. *Drug Metab Dispos*. *37*, 1749-1758.

Stalmach, A., Mullen, W., Nagai, C., and Crozier, A. (**2006**) On-line HPLC analysis of the antioxidant activity of phenolic compounds in brewed, paper-filtered coffee. *Braz Journal Plant Physiol*. *18*, 253-262.

Stalmach, A., Steiling, H., Williamson, G., and Crozier, A. (**2010**) Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. *Arch Biochem Biophys*. *501*, 98-105.

Steinmetz, K. A., and Potter, J. D. (**1996**) Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc*. *96*, 1027-1039.

Stryer, L. (**1995**) *Biochemistry*. 4th ed., W.H. Freeman, New York.

Tanaka, T., Kojima, T., Kawamori, T., Wang, A., Suzui, M., Okamoto, K., and Mori, H. (**1993**) Inhibition of 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis by the naturally occurring plant phenolics caffeic, ellagic, chlorogenic and ferulic acids. *Carcinogenesis*. *14*, 1321-1325.

Teissedre, P.-L., and Landrault, N. (**2000**) Wine phenolics: contribution to dietary intake and bioavailability. *Food Res Inter*. *33*, 461-467.

Thom, E. (**2007a**) The effect of chlorogenic acid enriched coffee on glucose absorption in healthy volunteers and its effect on body mass when used long-term in overweight and obese people. *J Int Med Res*. *35*, 900-908.

Thom, E. (**2007b**) The effect of chlorogenic acid enriched coffee on glucose absorption in healthy volunteers and its effect on body mass when used long-term in overweight and obese people. *J Int Med Res*. *35*, 900-908.

Tice, R. (**1998**) Chlorogenic Acid [327-97-9] and Caffeic Acid [331-39-5]. Review of Toxicological Literature., North Carolina.

Tinttunen, S., and Lehtonen, P. (**2001**) Distinguishing organic wines from normal wines on the basis of concentrations of phenolic compounds and spectral data. *Eur Food Res Technol*. *212*, 390-394.

Trugo, L. C., and Macrae, R. (**1984**) Chlorogenic Acid Composition of Instant Coffees. *Analyst*. *109*, 263-266.

van der Sluis, A. A., Dekker, M., de Jager, A., and Jongen, W. M. F. (**2001**) Activity and Concentration of Polyphenolic Antioxidants in Apple: Effect of Cultivar, Harvest Year, and Storage Conditions. *J Agr Food Chem*. *49*, 3606-3613.

van der Sluis, A. A., Dekker, M., Skrede, G., and Jongen, W. M. F. (**2002**) Activity and Concentration of Polyphenolic Antioxidants in Apple Juice. 1. Effect of Existing Production Methods. *J Agr Food Chem*. *50*, 7211-7219.

van Dijk, A. E., Olthof, M. R., Meeuse, J. C., Seebus, E., Heine, R. J., and van Dam, R. M. (**2009**) Acute Effects of Decaffeinated Coffee and the Major Coffee Components Chlorogenic Acid and Trigonelline on Glucose Tolerance. *Diabetes Care*. *32*, 1023-1025.

Vinson, J. A., Dabbagh, Y. A., Serry, M. M., and Jang, J. (**1995**) Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease. *J Agr Food Chem*. *43*, 2800-2802.

Vrhovsek, U., Rigo, A., Tonon, D., and Mattivi, F. (**2004**) Quantitation of Polyphenols in Different Apple Varieties. *J Agr Food Chem*. *52*, 6532-6538.

Wang, H., Provan, G. J., and Helliwell, K. (**2004**) Determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC. *Food Chem*. *87*, 307-311.

Watzek, N., Scherbl, D., Feld, J., Berger, F., Doroshyenko, O., Fuhr, U., Tomalik-Scharte, D., Baum, M., Eisenbrand, G., and Richling, E. (**2012**) Profiling of mercapturic acids of acrolein and acrylamide in human urine after consumption of potato crisps. *Mol Nutr Food Res*. *56*, 1825-1837.

Watzl, B., Rechkemmer, G. (**2001**) Basiswissen aktualisiert: Phenolsäuren. *Ernährungs-Umschau*. *48,* 413-416.

Weidel, E., SchantzM., Richling, E. (**2014**) A rapid method for quantifying chlorogenic acid levels in potato samples. *J AOAC Int.*97, 902-907.

Wen, X., Walle, T. (**2006**) Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug Metab Dispos. 34*, 1786-1792.

Welsch, C. A., Lachance, P. A., and Wasserman, B. P. (**1989a**) Dietary Phenolic-Compounds - Inhibition of Na+-Dependent D-Glucose Uptake in Rat Intestinal Brush-Border Membrane-Vesicles. *J Nutr*. *119*, 1698-1704.

Welsch, C.,Lachance,, P.A.,Wasserman, B.P. (**1989b**) Effects of Native and Oxidized Phenolic Compounds on Sucrase Activity in Rat Brush Border Membrane Vesicles1. *Amer Inst Nutr. 119*, 1737-1740.

Wenk, G. L., McGann-Gramling, K., Hauss-Wegrzyniak, B., Ronchetti, D., Maucci, R., Rosi, S., Gasparini, L., and Ongini, E. (**2004**) Attenuation of chronic neuroinflammation by a nitric oxide-releasing derivative of the antioxidant ferulic acid. *Journal of Neurochemistry*. *89*, 484-493.

WHO (**2006**) Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia : report of a WHO/IDF consultation*.*, *World Health Organization*.

William, A. (**2012**) Tetanus Epidemiology and Prevention of Vaccine-Preventable Diseases (12 ed.). *Public Health Foundation.* 291–300.

Williamson, G., and Clifford, M. N. (**2010**) Colonic metabolites of berry polyphenols: the missing link to biological activity? *Brit J Nutr*. *104*, S48-S66.

Williamson, G., Day, A. J., Plumb, G. W., and Couteau, D. (**2000**) Human metabolic pathways of dietary flavonoids and cinnamates. *Biochem Soc Trans*. *28*, 16-22.

Williamson, G., Dionisi, F., and Renouf, M. (**2011**) Flavanols from green tea and phenolic acids from coffee: Critical quantitative evaluation of the pharmacokinetic data in humans after consumption of single doses of beverages. *Mol Nutr Food Res*. *55*, 864-873.

Williamson, G. **(2013)** Possible effects of dietary polyphenols on sugar absorption and digestion. *Mol Nutr Food Res. 57*, 48-57.

Williamson, G., Clifford, M. N. (**2017**) Role of the small intestine, colon and microbiota in determining the metabolic fate of polyphenols. *Biochem Pharmacol.* doi: 10.1016/j.bcp.2017.03.012.

Witt, A. (**2012**) Methodenoptimierung zur Untersuchung von Chlorogensäuren und deren Metabolite in Urin nach Kaffeeverzehr, Diplomarbeit, *Technische Universität Kaiserslautern*, Kaiserslautern.

Wren, M. W. D. (**1977**) The Culture of Clinical Specimens for Anaerobic Bacteria: A Comparison Of Three Regimens. *J Med Microbiol*. *10*, 195-201.

Zhang, W., Lopez-Garcia, E., Li, T. Y., Hu, F. B., van Dam, R. M. (**2009**) Coffee consumption and risk of cardiovascular diseases and all-cause mortality among men with type 2 diabetis. *Diabetes Care.* 32, 253-262.

Zhao, Z., Egashira, Y., and Sanada, H. (**2004**) Ferulic Acid Is Quickly Absorbed from Rat Stomach as the Free Form and Then Conjugated Mainly in Liver. *J Nutr*. *134*, 3083-3088.

8 Appendices

Table 51 Anthropometric data determined for proband 01 – 05; RZ: resistance [Ω], Xc: reactance [Ω], FFM [kg]: fat free mass, TBW [L]: total body water, ECW [L]: extra cellular water, BCM [kg]: body cell mass, FM [kg]: fat mass, PA [degrees]: phase angle, FM [%]: percental fat mass, FFM [%]: percental fat free mass, TBW [%]: percental total body water, ECW [%]: percental extra cellular water, ICW [%]: percental intra cellular water, BCM [%]: percental body cell mass, BMI [kg/m^2]: body mass index, ECM [kg]: extra cellular mass, ECM [%]: percental extra cellular mass.

Proband	01	02	03	04	05
Gender	f	f	m	m	f
Body weight [kg]	74.0	58.7	80.7	73.7	64.5
Age [a]	40	22	31	24	24
Body hight [cm]	171.0	169.5	179.9	180.6	168.0
RZ [Ω]	602	626	449	493	670
Xc [Ω]	52	64	55	50	53
FFM [kg]	48.3	44.5	66.8	61.6	43.2
TBW [L]	35.3	32.5	48.9	45.1	31.6
ECW [L]	18.1	15.1	20.3	21.0	17.0
BCM [kg]	23.1	23.5	38.9	32.5	19.6
FM [kg]	25.7	14.2	13.9	12.1	21.3
PA [degrees]	4.9	5.8	7.0	5.8	4.5
FM [%]	34.7	24.3	17.3	16.4	33.0
FFM [%]	65.2	75.7	82.8	83.6	67.0
TBW[%]	47.7	55.4	60.6	61.2	49.1
ECW [%]	51.1	46.5	41.6	46.7	53.7
ICW [%]	48.8	53.6	58.4	53.3	46.4
BCM [%]	47.9	52.9	58.3	52.7	45.3
BMI [kg/m^2]	25.3	20.4	24.9	22.6	22.9
ECM [kg]	25.1	20.9	27.8	29.2	23.7
ECM [%]	52.1	47.1	41.7	47.3	54.7

Table 52 Anthropometric data determined for proband 06 – 10; RZ: resistance [Ω], Xc: reactance [Ω], FFM [kg]: fat free mass, TBW [L]: total body water, ECW [L]: extra cellular water, BCM [kg]: body cell mass, FM [kg]: fat mass, PA [degrees]: phase angle, FM [%]: percental fat mass, FFM [%]: percental fat free mass, TBW [%]: percental total body water, ECW [%]: percental extra cellular water, ICW [%]: percental intra cellular water, BCM [%]: percental body cell mass, BMI [kg/m^2]: body mass index, ECM [kg]: extra cellular mass, ECM [%]: percental extra cellular mass.

Proband	06	07	08	09	10
Gender	f	m	m	f	f
Body weight [kg]	60.6	78.7	80.2	54.5	60.8
Age [a]	26	20	25	24	44
Body hight [cm]	156.0	185.0	186.7	156.0	176.6
RZ [Ω]	636	493	481	766	650
Xc [Ω]	53	52	51	75	51
FFM [kg]	41.1	64.7	66.8	35.4	46.1
TBW [L]	30.1	47.4	48.9	25.9	33.7
ECW [L]	15.7	21.6	22.2	12.4	18.2
BCM [kg]	19.2	34.9	36.1	18.3	20.7
FM [kg]	19.5	14	13.4	19.1	14.7
PA [degrees]	4,8.	6	6.1	5.6	4.5
FM [%]	32.2	17.7	16.7	35.0	24.3
FFM [%]	67.8	82.3	83.3	65.0	75.8
TBW[%]	49.6	60.2	61.0	47.6	55.5
ECW [%]	52.2	45.6	45.5	47.6	53.9
ICW [%]	47.8	54.4	54.5	52.4	46.1
BCM [%]	46.8	53.9	54.0	51.5	45.0
BMI [kg/m^2]	24.0	23.0	23.0	22.4	19.5
ECM [kg]	21.8	29.9	30.7	17.1	25.3
ECM [%]	53.2	46.1	46.0	48.4	55.0

Table 53 Anthropometric data determined for proband 11 – 14; RZ: resistance [Ω], Xc: reactance [Ω], FFM [kg]: fat free mass, TBW [L]: total body water, ECW [L]: extra cellular water, BCM [kg]: body cell mass, FM [kg]: fat mass, PA [degrees]: phase angle, FM [%]: percental fat mass, FFM [%]: percental fat free mass, TBW [%]: percental total body water, ECW [%]: percental extra cellular water, ICW [%]: percental intra cellular water, BCM [%]: percental body cell mass, BMI [kg/m^2]: body mass index, ECM [kg]: extra cellular mass, ECM [%]: percental extra cellular mass.

Proband	11	12	13	14
Gender	f	m	m	m
Body weight [kg]	66.3	88.1	67.2	77.0
Age [a]	44	34	25	28
Body hight [cm]	167	187	177.5	183.0
RZ [Ω]	522	465	474	583
Xc [Ω]	50	44	51	57
FFM [kg]	49.6	70.4	60.0	57.4
TBW [L]	36.3	51.5	43.9	42.0
ECW [L]	17.5	25	19.8	20.0
BCM [kg]	25.3	35.6	32.7	29.6
FM [kg]	16.7	17.7	7.2	19.6
PA [degrees]	5.5	5.4	6.1	5.6
FM [%]	25.2	20.1	10.7	25.4
FFM [%]	74.8	79.9	89.3	74.6
TBW[%]	54.7	58.5	65.4	54.6
ECW [%]	48.3	48.6	45.1	47.7
ICW [%]	51.7	51.4	55.0	52.3
BCM [%]	51.0	50.6	54.5	51.6
BMI [kg/m^2]	23.8	25.2	21.3	23.0
ECM [kg]	24.3	34.8	27.3	27.8
ECM [%]	49.0	49.3	45.6	48.4

Table 54 Microbiota analysis in faecal samples of each proband (01 - 14) presented in colony forming units per g faeces [CfU/g].

Proband	01	02	03	04	05	06	07	08	09	10	11	12	13	14
E. coli	$5 \cdot 10^7$	$1 \cdot 10^6$	$3 \cdot 10^3$	$3 \cdot 10^3$	$2 \cdot 10^3$	$3 \cdot 10^7$	$1 \cdot 10^6$	$2 \cdot 10^3$	$3 \cdot 10^5$	$3 \cdot 10^3$	$<10^4$	$4 \cdot 10^5$	$1 \cdot 10^5$	$2 \cdot 10^5$
E. coli-variant	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$2 \cdot 10^5$	$<10^4$	$<10^4$	$1 \cdot 10^3$	$4 \cdot 10^3$
Enterobacteriaceae	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$<10^4$
Enterococcus sp.	$<10^4$	$3 \cdot 10^7$	$<10^4$	$1 \cdot 10^5$	$2 \cdot 10^5$	$5 \cdot 10^3$	$2 \cdot 10^3$	$1 \cdot 10^6$	$2 \cdot 10^7$	$3 \cdot 10^5$	$<10^4$	$<10^4$	$2 \cdot 10^3$	$1 \cdot 10^5$
other aerobic	$<10^4$	10^4	$<10^4$	$3 \cdot 10^3$	$3 \cdot 10^3$	$<10^4$	$<10^4$	$<10^4$	$<10^4$	$5 \cdot 10^3$	$4 \cdot 10^3$	$<10^4$	x·10	$<10^4$
Bacteroides sp.	$1 \cdot 10^9$	$2 \cdot 10^9$	$3 \cdot 10^3$	$<10^8$	$2 \cdot 10^3$	$1 \cdot 10^9$	$5 \cdot 10^9$	$5 \cdot 10^3$	$4 \cdot 10^9$	$<10^8$	$1 \cdot 10^3$	$1 \cdot 10^3$	$<10^8$	$1 \cdot 10^9$
Clostridium sp.	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$3 \cdot 10^3$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$
Bifidobacterium sp.	$1 \cdot 10^9$	$1 \cdot 10^9$	$1 \cdot 10^8$	$3 \cdot 10^7$	$2 \cdot 10^3$	$1 \cdot 10^5$	$1 \cdot 10^9$	$3 \cdot 10^7$	$1 \cdot 10^8$	$2 \cdot 10^6$	$1 \cdot 10^7$	$1 \cdot 10^7$	$1 \cdot 10^8$	$1 \cdot 10^9$
Lactobacillus sp.	$1 \cdot 10^6$	$1 \cdot 10^6$	$1 \cdot 10^5$	$1 \cdot 10^4$	$2 \cdot 10^5$	$1 \cdot 10^5$	$1 \cdot 10^6$	$5 \cdot 10^5$	$5 \cdot 10^5$	$<10^2$	$1 \cdot 10^4$	$1 \cdot 10^3$	$1 \cdot 10^5$	$2 \cdot 10^3$
other anaerobic	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$	$<10^6$
Candida sp.	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$1 \cdot 10^2$	$6 \cdot 10^3$	$1 \cdot 10^4$	$6 \cdot 10^3$	$1 \cdot 10^3$	$5 \cdot 10^3$	$1 \cdot 10^3$	$<10^2$	1·102	$<10^2$
Geotrichum sp.	$<10^2$	$<10^2$	$4 \cdot 10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$
other fungi	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$2 \cdot 10^3$	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$

Table 55 Data of oral glucose tolerance (oGTT) test (0 to 300 min) of each proband (01 – 14) after ingestion of 75 g glucose.

Time point Proband	0min [mg/dL]	30min [mg/dL]	60min [mg/dL]	120min [mg/dL]	180min [mg/dL]	240min [mg/dL]	300min [mg/dL]	AUC
01	83	178	132	109	82	82	76	31185
02	59	101	106	90	81	83	59	25695
03	90	181	127	78	74	94	92	30015
04	89	173	182	58	87	86	89	31245
05	91	168	189	98	65	74	82	31590
06	84	167	168	113	70	78	81	31920
07	84	149	147	75	62	70	81	27195
08	90	165	156	58	79	78	87	28830
09	92	154	138	104	78	76	66	29670
10	98	171	135	112	74	82	95	31605
11	95	119	105	112	81	87	85	29070
12	72	154	127	56	85	83	84	27375
13	88	165	116	76	83	84	92	28830
14	86	155	231	117	95	84	89	36765

Table 56 Amount of administered instant coffee dependent on body weight [mg/kg bw] of each proband (01 – 14) at the appropriate study days.

Proband	Gewicht [kg]	Kaffee [g]	CGA [mg]	CGA [mg/kg bw]
		V2 - 05.11.2011		
1	74.0	2.64	242	3.28
2	58.7	2.11	194	3.30
3	80.6	2.89	265	3.29
4	73.7	2.65	243	3.30
5	64.5	2.32	213	3.30
6	60.6	2.19	201	3.32
7	78.7	2.82	259	3.29
8	80.8	2.89	265	3.28
9	54.5	1.96	180	3.30
10	60.8	2.18	200	3.29
11	66.3	2.36	217	3.27
12	88.2	3.14	288	3.27
13	67.2	2.39	219	3.26
14	77.0	2.75	252	3.28
		V3 - 19.11.2011		
1	73.6	2.65	243	3.31
2	58.8	2.11	194	3.29
3	80.3	2.85	261	3.26
4	72.1	2.57	236	3.27
5	63.4	2.25	207	3.26
6	60.3	2.13	196	3.24
7	79.2	2.82	259	3.27
8	78.8	2.83	260	3.30
9	54.7	1.96	180	3.29
10	60.1	2.13	196	3.25
11	65.3	2.33	214	3.28
12	88.4	3.15	289	3.27
13	66.1	2.35	216	3.26
14	77.1	2.75	252	3.27
		V4 - 03.12.2011		
1	73.3	2.62	241	3.28
2	58.8	2.11	194	3.29
3	81.1	2.89	265	3.27
4	73.3	2.61	240	3.27
5	63.5	2.29	210	3.31
6	59.2	2.11	194	3.27
7	79.1	2.82	259	3.27
8	79.6	2.87	263	3.31
9	55.6	2.00	184	3.30
10	59.0	2.11	194	3.28
11	67.0	2.39	219	3.27
12	88.4	3.14	288	3.26
13	66.1	2.36	217	3.28
14	77.5	2.79	256	3.30

Table 57 Data of creatinine [g/L] (mean ± SD) of each proband (1 - 14) of the three study days (V2 – V4) of four time periods (-24 – 0 h, 0- 6 h, 6 – 12 h, 12 – 24 h) (n = 2).

	Creatinine [g/L] (mean ± SD)			
	V2 - 05.11.2011			
	-24 - 0h	**0 - 6h**	**6 - 12h**	**12 - 24h**
1	1.48 ± 0.32	0.69 ± 0.05	0.74 ± 0.01	1.13 ± 0.43
2	0.28 ± 0.02	0.44 ± 0.16	0.41 ± 0.00	0.47 ± 0.09
3	0.73 ± 0.00	0.88 ± 0.11	0.74 ± 0.06	1.23 ± 0.07
4	0.83 ± 0.01	0.66 ± 0.02	1.60 ± 0.07	1.81 ± 0.03
5	0.07 ± 0.01	0.76 ± 0.01	0.35 ± 0.00	0.70 ± 0.04
6	0.47 ± 0.02	0.33 ± 0.03	0.75 ± 0.00	0.80 ± 0.05
7	1.09 ± 0.03	1.29 ± 0.20	2.47 ± 0.11	3.84 ± 0.07
8	1.52 ± 0.01	0.74 ± 0.05	1.09 ± 0.04	0.80 ± 0.02
9	0.57 ± 0.04	0.30 ± 0.05	1.91 ± 0.05	1.25 ± 0.06
10	0.39 ± 0.01	0.45 ± 0.00	0.58 ± 0.11	1.36 ± 0.00
11	0.47 ± 0.01	0.95 ± 0.27	0.50 ± 0.08	0.79 ± 0.21
12	2.56 ± 0.24	0.87 ± 0.18	1.92 ± 0.11	2.34 ± 0.10
13	1.90 ± 0.12	1.90 ± 0.29	0.60 ± 0.12	1.40 ± 0.13
14	0.65 ± 0.02	0.79 ± 0.09	0.58 ± 0.03	1.42 ± 0.17
	V3 - 19.11.2011			
	-24-0h	**0-6h**	**6-12h**	**12-24h**
1	1.56 ± 0.26	0.91 ± 0.12	0.63 ± 0.03	0.65 ± 0.15
2	0.37 ± 0.01	0.25 ± 0.02	0.27 ± 0.00	0.41 ± 0.01
3	0.56 ± 0.06	0.72 ± 0.00	0.71 ± 0.02	1.04 ± 0.00
4	0.75 ± 0.01	0.47 ± 0.01	1.47 ± 0.05	1.99 ± 0.06
5	0.31 ± 0.01	0.54 ± 0.05	0.64 ± 0.02	1.06 ± 0.10
6	0.32 ± 0.07	0.45 ± 0.02	0.40 ± 0.03	0.92 ± 0.03
7	2.05 ± 0.02	0.65 ± 0.04	1.09 ± 0.01	1.98 ± 0.07
8	1.98 ± 0.16	0.94 ± 0.05	0.75 ± 0.05	2.34 ± 0.32
9	1.31 ± 0.02	0.38 ± 0.03	1.04 ± 0.05	1.82 ± 0.11
10	0.84 ± 0.05	0.98 ± 0.11	0.56 ± 0.07	0.60 ± 0.01
11	0.41 ± 0.00	0.54 ± 0.01	0.37 ± 0.01	0.85 ± 0.01
12	2.23 ± 0.06	1.21 ± 0.06	0.65 ± 0.05	0.97 ± 0.15
13	2.22 ± 0.03	0.87 ± 0.02	0.69 ± 0.02	2.41 ± 0.17
14	0.88 ± 0.12	0.43 ± 0.00	0.68 ± 0.07	1.79 ± 0.15
	V4 - 03.12.2011			
	-24-0h	**0-6h**	**6-12h**	**12-24h**
1	1.95 ± 0.35	0.46 ± 0.02	1.26 ± 0.09	0.83 ± 0.02
2	0.41 ± 0.04	0.24 ± 0.02	0.30 ± 0.02	0.62 ± 0.01
3	0.36 ± 0.01	0.32 ± 0.01	1.05 ± 0.11	1.13 ± 0.04
4	1.01 ± 0.01	0.47 ± 0.01	1.00 ± 0.06	1.78 ± 0.16
5	0.86 ± 0.05	0.37 ± 0.00	1.20 ± 0.10	0.85 ± 0.06
6	1.23 ± 0.22	0.28 ± 0.01	0.69 ± 0.01	0.75 ± 0.03
7	1.36 ± 0.05	0.72 ± 0.07	2.06 ± 0.01	2.04 ± 0.01
8	1.91 ± 0.16	0.42 ± 0.01	0.87 ± 0.14	1.13 ± 0.04
9	1.04 ± 0.05	0.27 ± 0.01	1.15 ± 0.04	0.85 ± 0.06
10	0.96 ± 0.07	0.60 ± 0.01	0.96 ± 0.13	0.72 ± 0.03
11	0.68 ± 0.08	0.60 ± 0.10	0.52 ± 0.05	0.70 ± 0.05
12	2.06 ± 0.38	1.26 ± 0.19	1.46 ± 0.04	1.68 ± 0.17
13	2.34 ± 0.20	1.61 ± 0.19	0.85 ± 0.04	0.74 ± 0.01
14	0.90 ± 0.03	0.45 ± 0.02	0.57 ± 0.02	1.12 ± 0.07

Table 58 Compound dependent parameters (QTrap 5500) for determination of instant coffee ingredients and metabolites in plasma [*Marmet et al., 2014*]. DP: declustering potential, CE: collision energy, CXP: cell exit potential, RT: retention time; NA: not available.

Compound	Parent mass	Product 1	Product 2	DP [V]	CE1 [V]	CE2 [V]	CXP1 [V]	CXP2 [V]	RT [min]
34MPPA	179	135.2	NA	−80	−14	NA	−9	NA	18,70
3CQA15L	335	161	133	−95	−28	−54	−9	−9	10,90
3CQA	353	190.9	179	-70	-22	-20	-9	-9	5,00
3FQA15L	349	174.9	160	-115	-26	-42	-9	-9	14,20
3FQA	367	192,9	133,9	-5	-22	-40	-9	-9	8,10
3PPA	149	105	NA	-65	-14	NA	-7	NA	18,50
4CQA15L	335	161	133	-105	-30	-56	-9	-13	11,70
4CQA	353	173,1	179,1	-10	-20	-22	-9	-9	7,40
4FQA15L	349	174,8	160	-70	-24	-50	-7	-9	14,70
4FQA	367	173	NA	-80	-20	NA	-9	NA	10,90
4MCA	176,9	133	117	-60	-14	-42	-9	-7	19,90
5CQA3S	432,9	190,8	353	-25	-32	-24	-9	-15	7,20
5CQA4S	432,9	191	179	-100	-38	-32	-9	-7	6,10
5CQA	353	190,9	85,1	-60	-34	-50	-9	-5	8,00
5FQA4G	543,1	191	366,9	-120	-38	-24	-5	-17	5,90
5FQA4S	446,9	190,8	193,1	-30	-32	-30	-11	-9	7,50
5FQA	367	190,8	172,9	-25	-20	-24	-9	-11	10,80
CA	179,1	135	134,1	-85	-22	-30	-7	-5	7,80
CA3G	354,9	178,8	134,8	-25	-24	-52	-11	-7	7,10
CA3S	259	179,1	134,9	-70	-22	-32	-9	-9	7,20
CA4G	354,9	178,9	134,8	-25	-24	-52	-11	-7	4,70
CA4S	259	178,9	135	-60	-22	-40	-9	-9	6,40
CinAc	147	103	NA	-20	-14	NA	-9	NA	19,40
DHCA	181,1	137	109	-65	-16	-20	-7	-7	7,50
DHCA3G	356,9	112,8	180,9	-90	-22	-24	-7	-11	7,20
DHCA3S	261	137	180,8	-5	-36	-32	-7	-47	6,60
DHCA4G	356,9	113	181	-80	-22	-26	-7	-11	6,70
DHCA4S	261	136,8	180,8	-50	-30	-24	-9	-11	6,30
DHFA	195,1	136,1	120,9	-105	-20	-36	-7	-11	11,60
DHFA4G	370,9	112,9	175	-25	-22	-18	-17	-9	8,30
DHFA4S	275	195	79,9	-75	-22	-58	-11	-11	8,10
DHiFA	195,1	135,9	150,9	-70	-18	-14	-19	-7	12,80
DHiFA3G	371	112,9	195	-35	-24	-26	-7	-9	9,90
DHiFA3S	275	195,1	135,8	-70	-24	-32	-9	-11	9,10
FA	193,1	134	177,8	-65	-20	-14	-7	-9	12,10
FA4G	368,9	113	193	-85	-16	-18	-5	-11	6,50
FA4S	273	193	133,9	-70	-20	-38	-9	-9	8,10
iFA	193,1	178	133,9	-90	-16	-22	-9	-9	13,00
iFA3G	369	112,8	193	-20	-20	-22	-7	-11	9,30
iFA3S	273	177,9	134	-70	-26	-48	-9	-9	9,60
mCoA	163,1	118,9	90,8	-60	-20	-30	-9	-13	12,70
mCoAG	339	112,9	119	-55	-22	-56	-13	-11	8,20
mCoAS	242,9	163	119	-50	-24	-42	-7	-9	9,00
mDHCoA	165	121	105,9	-70	-16	-30	-7	-5	11,60
mDHCoAG	341	113	121	-45	-24	-42	-5	-5	8,50
mDHCoAS	244,9	165	121	-90	-24	-32	-5	-9	8,20
MeDHFA	209	150	165,1	-120	-16	-16	-11	-9	16,00
MeFA	207,2	103	163,1	-80	-18	-12	-7	-9	17,00
oCoA	163,1	119,1	116,9	-55	-16	-30	-7	-11	14,60
oDHCoA	165	121	106,1	-30	-18	-30	-9	-5	13,10
pCoA	163,1	118,9	93	-55	-20	-38	-9	-11	10,50
pCoAG	339	113	118,9	-80	-18	-54	-5	-15	4,80
pCoAS	242,9	119	163	-55	-38	-20	-7	-7	7,30
pDHCoA	165	121	59	-80	-14	-16	-9	-9	10,10
pDHCoAG	341	113	59	-75	-22	-54	-5	-7	6,90
pDHCoAS	244,9	165	58,9	-65	-24	-36	-7	-9	7,40
CA13	182,1	137	136	-30	-20	-34	-9	-11	7,80
DHiFAd3G	374	113	198	-60	-24	-28	-7	-13	9,80
DHiFAd3S	277,8	198,1	136,2	-15	-24	-36	-5	-9	9,10

Table 59 Compound dependent parameters (QTrap 5500) for determination of instant coffee ingredients and metabolites in plasma. DP: declustering potential, CE: collision energy, CXP: cell exit potential, RT: retention time.

Compound	Parent mass	Product	DP [V]	CE [V]	CXP [V]	RT [min]
5-CQA	353,1	191	-70	-24	-11	11.58
3-CQA	353,2	191	-40	-40	-11	8.90
4-CQA	353,2	173	-10	-44	-11	11.20
CA	178,9	135	-85	-18	-9	14.38
FA	192,9	134	-35	-24	-9	19.99
DHCA-3-Gluc	357,1	181	-45	-28	-9	19.25
CQL	335	132,9	-85	-56	-13	17.06
FAG	250	133,9	-85	-30	-11	14.63
FA-4-Sulf	273	192,9	-40	-20	-13	10.81
DHCA	180,9	137	-25	-24	-7	12.47
DHIFA	194,9	136	-105	-20	-7	18.79
IFA	192,9	134	-35	-24	-9	20.39
4-FQA	367,1	172,9	-50	-22	-11	14.88
CQA-3-Sulf	433,1	191	-75	-36	-11	9.43
FQA-Gluc	543	190,9	-120	-38	-15	8.48
FQA-Sulf	447	191	-50	-36	-11	9.60
CA-3-Gluc	355,2	178,8	-25	-46	-9	10.83
3-FQA	367	192,9	-45	-24	-17	12.47
5-FQA	367	191	-60	-22	-13	15.99
CA-4-Gluc	355,2	178,9	-30	-24	-11	8.44
CA-4-Sulf	259	179	-25	-22	-11	9.66
DHCA-3-Sulf	261	180,8	-45	-12	-15	9.11
DHCA-4-Sulf	261	180,8	-45	-12	-15	8.74
IFA-3-Gluc	369	178	-80	-36	-9	12.47
IFA-3-Sulf	272,9	193	-50	-22	-13	11.54
DHFA-4-Sulf	275	195	-35	-24	-13	9.77
CA-13C3	181,9	136,9	-20	-22	-15	14.35
CA-3-Sulf	258,9	179	-45	-22	-11	10.18
IFA-d3-3-Gluc	372	178	-30	-38	-11	12.40
IFA-d3-3-Sulf	275,9	196	-25	-22	-9	11.53
DHFA-4-Gluc	370,9	112,9	-25	-22	-17	10.45
DHIFA-3-Gluc	371	119	-30	-36	-9	11.89
DHFA	195,1	136,1	-105	-20	-7	17.86
5CQA-4-Sulf	432,9	191	-100	-38	-9	9.42
5FQA-4-Sulf	446,9	190,8	-30	-32	-11	9.58
5FQA-4-Gluc	543,1	191	-120	-38	-5	8.46
DHCA-4-Gluc	357	181	-45	-28	-9	8.52
4-CQL	335	161	-50	-32	-11	17.03
CQL-Gluc	511,1	132,9	-60	-94	-2	13.08
CQA-3-Gluc	528,9	191	-75	-36	-11	7.42

Table 60 Limits of quantification for determination of coffee ingredients and metabolites in plasma samples.

Analyte	LOQ [nM]	Analyte	LOQ [nM]
34MPPA	25	DHCA3G	5
DHCA	5	iFA3G	5
FA	5	pCoA	5
CA	5	DHiFA3G	5
DHFA	5	iFA3S	5
4-CQA	5	DHiFA3S	5
MeFA	5	4CQA15L	5
iFA	5	5CQA4S	5
3-CQA	5	5CQA3S	5
5-CQA	5	3CQA15L	5
MeDHFA	5	5FQA4G	25
DHiFA	5	5FQA4S	5
mCoA	5	3FQA15L	5
oCoA	5	4FQA15L	5
3-FQA	5	pCoAS	5
5-FQA	5	mDHCoAG	5
4-FQA	5	mCoAS	5
DHFA4S	5	pDHCoAG	5
CA4G	5	pCoAG	5
FA4S	5	mDHCoAS	5
DHCA4G	5	mCoAG	5
CA4S	5	pDHCoAS	5
DHFA4G	5	oDHCoA	5
DHCA4S	5	3PPA	25
CA3G	5	mDHCoA	25
DHCA3S	5	pDHCoA	50
FA4G	5	CinAc	5
CA3S	5	4MCA	25

Table 61 Limits of quantification for determination of coffee ingredients and metabolites in urin of proband 01 to 05.

	S01			S02			S03			S04			S05		
Substance	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]
5CQA	600	7114,6	0,001	900	11180	0,001	300	8776,5	0,001	600	7311,4	0,001	400	6467,4	0,001
3CQA	800	3118,3	0,004	600	4096,6	0,002	600	3175,6	0,003	600	2393,8	0,004	400	2431	0,003
4CQA	250	2149,4	0,002	300	2856,3	0,002	200	2354,1	0,001	200	1779,6	0,002	200	1676,3	0,002
CA	25000	14932	0,056	25000	16825	0,050	14000	9639,2	0,048	25000	11874	0,070	12000	11209	0,036
FA	500	29687	0,001	600	42003	0,000	200	19607	0,000	300	29016	0,000	200	26710	0,000
DHCA3G	4000	2380,8	0,028	3000	7343,1	0,007	2000	2647,7	0,013	1000	1073,6	0,016	1400	2074	0,011
FAG	150	6157,6	0,001	200	10514	0,000	150	4870,5	0,001	100	9154,6	0,000	100	10159	0,000
FA4S	10000	27371	0,008	2500	44930	0,001	2500	71545	0,001	1500	60067	0,001	1800	52823	0,001
DHCA	6000	1967,7	0,101	5000	2284,2	0,072	4000	1632,6	0,081	6000	1694	0,117	4500	3006,1	0,049
DHIFA		3141,3	0,000	600	4353,3	0,004	1400	2121,9	0,020	300	2851,1	0,003	100	7803,7	0,000
IFA	500	13246	0,001	2000	16677	0,004	2000	7562,7	0,008	4000	15913	0,008	2200	13116	0,005
4FQA	1000	18920	0,001	250	24081	0,000	500	16132	0,001	300	15926	0,000	300	14570	0,000
CQA3S	600	3686,2	0,002	600	5682,3	0,001	500	4731,9	0,001	600	3077,1	0,003	700	2775,6	0,003
FQA4G	140	793,46	0,002	100	812,47	0,001	100	816,06	0,001	70	271,61	0,003	100	278,72	0,004
FQA4S	600	7092,7	0,001	400	8839,7	0,001	300	8213,6	0,000	250	4675,8	0,001	350	4298,9	0,001
CA3G	1000	353,57	0,048	600	520,01	0,019	500	349,86	0,024	350	277,72	0,021	350	528,25	0,011
3FQA	1200	64385	0,000	2000	87372	0,000	1400	77918	0,000	1000	54462	0,000	1200	54033	0,000
5FQA	1500	8008,8	0,003	900	9686,4	0,002	900	7734,6	0,002	600	7366,9	0,001	500	6666,5	0,001
CA4G	3000	5641,2	0,009	1500	10038	0,003	2000	8464,3	0,004	500	5392,2	0,002	1500	5300,3	0,005
CA4S	8000	25429	0,007	2500	31976	0,002	6000	20540	0,007	15000	15829	0,022	1400	16092	0,002
DHCA3S	2000	2066	0,022	600	907,09	0,015	800	1549	0,012	400	746,21	0,012	150	528,65	0,006
DHCA4S	5000	2082	0,055	800	1319,4	0,014	600	2000,7	0,007	300	976,37	0,007	150	716,63	0,005
IFA3G	250	7439,2	0,001	100	7327,9	0,000	200	6840,2	0,000	400	4433,2	0,001	100	3978	0,000
IFA3S	8000	24923	0,007	2500	38673	0,001	3000	78306	0,001	5000	24097	0,005	1000	27026	0,001
DHFA4S	3000	20267	0,003	6000	31205	0,004	10000	27508	0,008	1000	20076	0,001	2000	28509	0,002
CA3S	8000	38811	0,005	2000	33666	0,001	3000	35320	0,002	2500	23490	0,002	100	26806	0,000
DHFA4G	7000	13785	0,008	1400	7451,5	0,003	2500	8390	0,005	2000	4443,5	0,007	800	3945,5	0,003
DHIFA3G	250	1465,2	0,003	120	895,85	0,002	150	879,83	0,003	100	576,35	0,003	150	603,11	0,004
DHFA	3000	44162	0,002	2000	29588	0,002	2000	19250	0,003	5000	3097,5	0,049	2700	3215,2	0,026
5CQA4S	1000	2078	0,007	400	3145,7	0,002	500	2482,8	0,003	600	2446,4	0,003	500	8279,7	0,001
DHCA4G	6000	2307,2	0,044	2500	3800,2	0,011	1500	2933,7	0,009	200	2075,2	0,002	1500	2015,2	0,012
4CQL	900	108572	0,000	800	112626	0,000	700	68898	0,000	400	98389	0,000	150	83910	0,000
CQLG	160	2159,9	0,001	100	3126,4	0,000	150	2372,1	0,001	200	1736,9	0,001	100	1685,6	0,001
CQA3G	150	31,085	0,055	100	33,883	0,033	60	34,183	0,020	100	18,94	0,060	100	19,794	0,057

Table 62 Limits of quantification for determination of coffee ingredients and metabolites in urin of proband 06 to 10.

	S06			S07			S08			S09			S10		
Substance	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]
5CQA	400	5741,6	0,001	200	6120,3	0,001	200	6371,7	0,001	150	6226,3	0,000	250	8086,3	0,001
3CQA	250	2276	0,002	600	2295,8	0,004	250	2705,1	0,002	250	2892,6	0,001	200	3639,5	0,001
4CQA	200	1606,2	0,002	250	1830	0,002	400	1988,6	0,003	100	1954,7	0,001	300	2280,7	0,002
CA	14000	8386,1	0,056	10000	6616,7	0,050	5000	11328	0,015	10000	12578	0,027	9000	12573	0,024
FA	150	27637	0,000	400	14042	0,001	300	28756	0,000	150	30712	0,000	200	32006	0,000
DHCA3G	1500	1882,9	0,013	2000	2203	0,015	5000	2932,9	0,029	2000	2262,6	0,015	2000	2447,4	0,014
FAG	100	4429,3	0,001	150	4069	0,001	150	3922,4	0,001	100	4542,3	0,001	150	5443,6	0,001
FA4S	2000	26047	0,002	3000	13143	0,005	12000	24061	0,011	1500	22010	0,001	3500	47957	0,002
DHCA	4500	1810,4	0,082	3000	1041,6	0,095	4500	1385,4	0,107	2500	897,42	0,092	3000	2056,6	0,048
DHIFA	200	2787,6	0,002	150	1539,7	0,003	200	2885,1	0,002	100	2924,5	0,001	100	2972	0,001
IFA	2500	12759	0,006	1600	NA	NA	2000	11283	0,005	1200	11711	0,003	1800	11448	0,005
4FQA	250	13330	0,000	400	11407	0,001	300	16987	0,000	200	18362	0,000	150	19229	0,000
CQA3S	900	2861,8	0,004	600	2784,8	0,003	600	3750	0,002	700	3751	0,003	700	3808,6	0,003
FQA4G	100	306,37	0,004	200	409,29	0,005	100	343,58	0,003	100	305,07	0,004	100	416,87	0,003
FQA4S	350	4595,4	0,001	1000	6118,7	0,002	600	6597,2	0,001	400	6592	0,001	300	6512,6	0,001
CA3G	350	269,43	0,022	400	257,2	0,026	600	303,59	0,033	700	280,11	0,042	400	326,15	0,021
3FQA	800	46876	0,000	900	57544	0,000	900	55215	0,000	700	54036	0,000	500	62289	0,000
5FQA	500	6250,5	0,001	700	4904,7	0,002	2000	7785,9	0,004	400	6059,8	0,001	700	8613,9	0,001
CA4G	900	5400,6	0,003	2000	6755,8	0,005	4000	6051,3	0,011	1500	5588,3	0,005	1000	6429,8	0,003
CA4S	1000	15098	0,002	3000	10156	0,007	3000	17483	0,004	2500	17607	0,003	5000	18775	0,006
DHCA3S	200	542,11	0,008	400	368,77	0,025	500	435,62	0,026	150	517,66	0,007	400	752,98	0,012
DHCA4S	100	722,07	0,003	300	835,33	0,008	200	585,67	0,008	250	655,64	0,009	300	933,84	0,007
IFA3G	150	4102,4	0,001	300	4584,7	0,001	1000	5521,8	0,003	450	5698,4	0,001	100	5318,8	0,000
IFA3S	4000	22037	0,004	1400	18307	0,002	10000	24661	0,009	2000	24038	0,002	2500	27061	0,002
DHFA4S	2000	15131	0,003	1500	11011	0,003	3000	24885	0,003	800	16516	0,001	100	18985	0,000
CA3S	2000	20051	0,002	5000	11079	0,010	3000	22794	0,003	2000	17837	0,003	2000	16214	0,003
DHFA4G	1500	3551,4	0,007	9000	3600,9	0,040	4500	6291,3	0,012	1500	3129,2	0,008	5000	1292,2	0,062
DHIFA3G	200	516,6	0,006	200	545,92	0,006	200	572,38	0,006	250	683,26	0,006	150	677,73	0,004
DHFA	2500	17105	0,004	2000	1541,5	0,040	2500	20181	0,004	2000	29916	0,002	4000	21364	0,006
5CQA4S	600	2340,5	0,004	600	2346,3	0,004	300	2948,2	0,001	500	2615,6	0,003	700	2643,6	0,004
DHCA4G	1500	1968,3	0,013	4000	2337,8	0,029	10000	2312,6	0,072	2000	2311,3	0,015	6000	2462,6	0,041
4CQL	300	85158	0,000	300	58055	0,000	250	98005	0,000	350	109921	0,000	100	115217	0,000
CQLG	150	1581,6	0,001	150	1672,3	0,001	150	1789,1	0,001	350	1549,2	0,003	150	1871,7	0,001
CQA3G	200	22,754	0,100	150	23,806	0,071	150	17,217	0,099	100	14,239	0,080	150	17,092	0,099

Table 63 Limits of quantification for determination of coffee ingredients and metabolites in urin of proband 11 to 14.

	S11			S12			S13			S14		
Substance	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]	background	gradient	LOQ [nmol/ml]
5CQA	150	7570	0,000	250	7038,8	0,001	300	9840,8	0,001	250	7715,7	0,001 €
3CQA	200	3060,5	0,001	150	3159,7	0,001	300	4013,6	0,001	250	3043,2	0,001 €
4CQA	200	2152,7	0,002	100	2307,8	0,001	200	2964,7	0,001	200	2264,2	0,001 €
CA	12000	12293	0,033	4000	12037	0,011	6000	17694	0,011	11000	11828	0,031 €
FA	200	29057	0,000	300	25882	0,000	300	33778	0,000	150	31172	0,000 €
DHCA3G	6000	2295,7	0,044	4000	2390,3	0,028	4000	3144,5	0,021	2000	2282,4	0,015 €
FAG	100	5200,8	0,000	300	7963,4	0,001	200	6203,2	0,001	300	5517,8	0,001 €
FA4S	4000	68371	0,001	4000	13942	0,006	5000	27322	0,004	1600	26369	0,001 €
DHCA	5000	1920,2	0,086	2500	1874,5	0,044	2500	1	82,418	2000	1879,6	0,035 €
DHIFA	120	2991,9	0,001	100	2908,2	0,001	500	3173,4	0,005	800	2964	0,008 €
IFA	1400	11672	0,004	1200	11032	0,003	1000	11412	0,003	100	11398	0,000 €
4FQA	100	16871	0,000	150	14779	0,000	150	19981	0,000	100	18629	0,000 €
CQA3S	500	3444,3	0,002	600	3832,6	0,002	300	5425,4	0,001	350	4108,9	0,001 €
FQA4G	150	375,51	0,004	100	338,82	0,003	150	549,98	0,003	100	435,16	0,003 €
FQA4S	400	5964,4	0,001	400	7131,8	0,001	200	10907	0,000	300	7644	0,001 €
CA3G	400	322,08	0,021	500	307,76	0,027	500	429,32	0,020	400	327,86	0,021 €
3FQA	700	53462	0,000	700	49148	0,000	500	65971	0,000	500	60556	0,000 €
5FQA	1000	7648,3	0,002	600	6697,9	0,001	800	9889,5	0,001	1000	8422,1	0,002 €
CA4G	1500	6192,4	0,004	3000	6367,4	0,008	1600	7492,9	0,004	1000	6929,2	0,002 €
CA4S	4000	18388	0,005	7000	20912	0,008	4000	30070	0,003	3500	20998	0,004 €
DHCA3S	200	767,14	0,006	400	613,73	0,015	500	810,51	0,014	150	709,9	0,005 €
DHCA4S	300	857,33	0,008	300	653,61	0,011	500	960,21	0,012	150	1059,8	0,003 €
IFA3G	400	5517,6	0,001	300	4940,2	0,001	250	7717,6	0,000	250	6024,9	0,001 €
IFA3S	6000	26428	0,005	7000	28228	0,005	10000	30641	0,007	2000	25841	0,002 €
DHFA4S	2000	17942	0,002	1500	15789	0,002	1500	17473	0,002	2000	25984	0,002 €
CA3S	4500	23890	0,004	7000	241193	0,001	5000	35214	0,003	3000	21671	0,003 €
DHFA4G	2000	5170,2	0,006	2000	3382,5	0,010	2000	5215,1	0,006	2000	5138,4	0,006 €
DHIFA3G	200	640,17	0,005	150	642,85	0,004	200	1216	0,003	250	1000,4	0,004 €
DHFA	2000	25985	0,002	1600	3535,5	0,014	1000	33887	0,001	1000	27700	0,001 €
5CQA4S	500	2472,9	0,003	400	2724,2	0,002	300	4038,5	0,001	300	3242,6	0,001 €
DHCA4G	4000	2337,5	0,029	3000	2426,4	0,021	3000	2683,5	0,019	3000	2371,3	0,021 €
4CQL	150	105206	0,000	200	109519	0,000	250	98040	0,000	150	96513	0,000 €
CQLG	200	1721,4	0,001	200	1769,1	0,001	100	2499,5	0,000	150	2088,9	0,001 €
CQA3G	100	18,489	0,061	100	20	0,057	100	29,408	0,038	100	20,7	0,055 €

Danksagung

Ich danke allen, die durch ihre Unterstützung zum Gelingen dieser Arbeit beigetragen haben.

- Prof. Dr. Elke Richling für die Überlassung des interessanten Themas, die Diskussionsbereitschaft und das entgegengebrachte Vertrauen.
- Prof. Dr. Dr. Dieter Schrenk für die Erstellung des Zweitgutachtens.
- Prof. Dr. Helmut Sitzmann für die Übernahme des Prüfungsvorsitzes.
- Dem Nestlé Research Center (NRC) für die finanzielle Unterstützung.
- Dr. Mathieu Renouf, Dr. Laure Poquet und Cyntia Marmet für die Unterstützung der Plasma- und Urinmetabolitmessungen sowie der Unterstützung bei den Inkubationsexperimenten.
- Isabelle Cristiani, Julien Sauser, Samir Dahbane und Shahram Emady-Azar für das Datenmanagement und die statistische Auswertung.
- Dr. Jens Galan und Dr. Dirk Galan für die Beantragung der Humanstudie bei der Ethikkomission, die Durchführung der medizinischen Untersuchungen und die Blutabnahmen an den Studientagen.
- Prof. Dr. Gerhard Eisenbrand für die zahlreichen Diskussionen und Hilfestellungen.
- Dr. Tamara Bakuradze, Dolores Müller, Meike Rünz, Teresa Röhrig, Annika Witt, Lena Künzler, Sylvia Schmitt und Dr. Gina Montoya Para für die Unterstützung während der Humanstudie und die netten Abende.
- Prof. Dr. Melanie Esselen und ihrem Arbeitskreis für die stete Hilfsbereitschaft und zur Verfügungstellunng der Labore.
- Sabrina Müntnich und David Liesenfeld für die Synthesen von Standards.
- Ingrid Hemm für ihre Zuvorkommenheit, Herzlichkeit und Hilfsbereitschaft und die Unterstützung bei den alltäglichen Problemen außerhalb des Labors.
- Aribert Vetter, Eva Gorgus und Rainer Scheuermann für ihren Einsatz bei technischen Problemen aller Art sowie die allmorgendliche Diskussionsbereitschaft.
- Den Mitarbeitern des Arbeitskreises von Prof. Dr. Dr. Dieter Schrenk, im Speziellen Dr. Karl Heinz Merz, für die technische, fachliche und materielle Unterstützung.
- Dr. Nico Watzek, Dr. Julia Ziegler, Nadine Böhm, Dr. Franz Berger, Dr. Max Hemgesberg und Dr. Alexander Cartus für die entgegengebrachte Freundschaft, das stets offene Ohr und die vielen fachlichen Hilfestellungen.
- Christian Baumann, Dr. Axel Besa, Dr. Loïc Beyet, Dr. Kristin von Ciapiewzki, Dr. Quentin Enjalbert, Julia Jasak, Dr. Stefianie Kreppenhofer, Dr. Jasmin Meltretter, Alexandre Paccou und Dr. Wolfgang Schütz für die stetige Ermutigung und freundschaftliche Hilfe.
- Lilia Wild, Caroline Dechant, Rebecca Leiner und Lilli Weidel, Freunde, die mich ganz genau kennen, trotzdem immer zu mir halten und stets ein offenes Ohr haben.
- Dominik Weber, der in schweren Zeiten eine gute Stütze war und mit schönen Ausflügen und leckeren Weinen freie Momente versüßt hat.
- Der gesamten Familie Press, weil sie sind wie sie sind und mich immer bestärken.
- Meinen Neffen, die auch in den schwersten Lebenslagen ein Lächeln auf mein Gesicht zaubern können.
- Meinen Geschwistern, die immer an mich glauben, mich unterstützen und auf die ich mich in jeder Lebenslage verlassen kann.

- Und ganz besonders meinen Eltern, die mir mein Studium und meine Promotion ermöglicht haben und in jeder Lebenslage hinter mir standen, stehen und stehen werden.

Lebenslauf

Angaben zur Person

Name	Denise Scherbl

Berufstätigkeit

seit 10.2015	Sciex, Applications Support Specialist kleine Moleküle, ansässig in Frankreich
01.2014 – 10.2015	Sciex, Applications Support Specialist für den klinischen und forensischen Bereich, ansässig in Deutschland

Hochschulausbildung

05.2011 – 01.2014	Promotion im Fachbereich Chemie, Fachrichtung Lebensmittelchemie und Toxikologie an der Technischen Universität Kaiserslautern, Prof. Dr. Elke Richling Thema: „Influence of breakfast consumption on chlorogenic acid metabolism in humans“
10.2006 – 03.2011	Studium der Lebensmittelchemie und Toxikologie an der Technischen Universität Kaiserslautern Abschluss: Diplom-Lebensmittelchemiker
10.2010 – 03.2011	Diplomarbeit im Fachbereich Chemie, Fachrichtung Lebensmittelchemie und Toxikologie an der Universität Kaiserslautern, Prof. Dr. Elke Richling Thema: „Entwicklung von Methoden zur Bestimmung von Acrylamid- und Glycidamid-Glutathionaddukten in biologischen Proben“

Weiterbildung / Spezielle Qualifikationen

seit 06.2011	Weiterbildung zum Fachtoxikologen der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie (DGPT) Kurse: molekulare Zelltoxikologie**,** Grundlagen der Organtoxikologie und -pathologie I und II, Lebensmittel-toxikologie, klinische Toxikologie

Schulbildung

1997 – 2006	Gymnasium am Rittersberg Kaiserslautern, Allgemeine Hochschulreife
1993 – 1997	Grundschule Wolfstein

Erklärung zur eigenständigen Durchführung

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

Kaisersautern, den 23.10.2017

Denise Scherbl