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„Evaluation of individual metabolic parameters by the analysis of finger sweat samples using liquid chromatography-mass spectrometry“

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List of Abbreviations

Abbreviation	
137U	1,3,7-Trimethyluric Acid
137X	Caffeine
13U	1,3-dimethyluric acid
13X	Theophylline
17U	1,7-dimethyluric acid
17U	1,7-dimethyluric acid
17X	Paraxanthine
1MU	1-methyluric acid
1MX	1-methylxanthine
1U	1-Methyluric Acid
1X	1-Methylxanthine
37U	3,7-dimethyluric acid
37X	Theobromine
3MU	3-methyluric acid
3MX	3-methylxanthine
7MU	7-methyluric acid
7MX	7-methylxanthine
ACN	Acetonitrile
AFMU	5-acetylamino-6-formylamino-3-methyluracil
AMP	Adenosine monophosphate
CA	Catechin
CF-D9	Caffeine-(trimethyl-D9)/ Caffeine-D9
cAMP	Cyclic Adenosine Monophosphate
CF	Caffeine
CO_2	Carbon dioxide
CV	Coefficient of Variation
CYPXY	Cytochrome PXY
DC	Direct Current
DESI	Desorption Electrospray Ionization
e.g.	For example
EC	Epicatechin
ES	Electrospray
ESI	Electrospray ionisation
FA	Formic Acid
Fig.	Figure
GC-MS	Gas Chromatography-Mass Spectrometry
GMP	Guanosine monophosphate
h	Hour
H_2O	Water

HCD	Higher-energy collisional dissociation
HDL	High-density Lipoprotein
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
IMP	Inosine monophosphate
IPC	ion-pair chromatography
IS	Internal Standard
kg	Kilogram
LC-MS	Liquid Chromatography-Mass Spectrometry
LDL	Low-density Lipoprotein
m/z	Mass-to-charge ratio
MALDI	Matrix-Assisted Laser Desorption/Ionization
MeOH	Methanol
mg	Milligram
min	Minute
mL	Millilitre
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
MS2	Tandem Mass Spectrometry
NAT2	N-Acetyltransferase
ng	Nanogram
NH_3	Ammonia
NMR	Nuclear Magnetic Resonance
PCA	Priniple Component Analysis
pg	pigogram
PLSDA	Partial Least Squares Discriminant Analysis
PX	Paraxanthine
QQQ	Triple Quadrupole
R.t.	Retention Time
RF	Radio Frequency
RP	Reversed Phase
TB	Theobromine
TIC	Total ion chromatogram
TP	Theophylline
UHPLC	Ultra-High-Performance Liquid Chromatography
XDH	Xanthosine Dehydrogenase
XIC	Extracted ion chromatogram
µ	Micro

1. Introduction

Sweat, a transparent, hypotonic biofluid, gets excreted by sweat glands.[1] Due to being marginally more acidic than blood – with a pH range of 4.0 – 6.8 – basic drugs are more likely to gather in sweat than in blood according to pH partition.[2] About 99% of sweat consists of water[2], the rest comprises electrolytes (sodium, potassium and chloride), nitrogenous compounds (amino acids and urea), small metabolites like lactate and pyruvate but also proteins, peptides and metal ions.[1,2] Inhibitors, antigens, cytokines, antibodies and a diversity of xenobiotics such as drugs, cosmetics and ethanol can also be encountered in sweat.[1] In disease state the sweat composition could change by either altering concentrations of components ordinarily present in sweat or by generating new components arising from the disease, which could function as biomarkers.[2] Drug detection in fingerprint samples is already extensively investigated, revealing that some substances are longer detectable in sweat than in blood, proving usefulness for criminal investigations.[3]

If an individual touches a substrate, a fingerprint will remain on the surface. A fingerprint consists of external contaminants on the skin and sweat secreted from sweat glands populating the skin surface.[4] In a fingerprint, traces of molecules that could reveal what a person has eaten, injected or inhaled, may be found. Thus, orally ingested foods and their metabolized products may be excreted in sweat.[5] However, current applications for sweat analysis are still limited since most sweat collecting techniques are time-consuming as they require wearing a sweat patch for several hours.[1,2] Further, detecting trace amounts of target molecules require highly sensitive analytical instruments, which are usually quite expensive.[6]

This master thesis focused on the different metabolic activity of individuals after the ingestion of chocolate and coffee. The diet influences two parts of the human metabolome namely the endogenous as well as the food metabolome. The endogenous metabolome comprises all metabolites derived from the host. The food metabolome is defined as "the sum of all metabolites directly derived from the digestion of foods, their absorption in the gut and biotransformation by the host tissues and microbiota."[7] The food metabolome shows the exposure to certain components present in ingested foods. Yet, a better understanding and a detailed characterization of the food metabolome could lead to the identification of foods that could benefit disease prevention or increase disease risk. Metabolic profiling of urine and plasma samples is already established with the use of mass spectrometry (MS).[7,8] However, urine and blood both have several disadvantages compared to finger sweat analysis. In comparison to blood, fingerprint analysis is non-invasive and set side by side to urine, fingerprints can be taken more easily and quickly.[2] This master thesis shows that it is possible to obtain significant data from the consumption of chocolate and coffee by identifying food

biomarkers and metabolites in finger sweat samples of volunteers. An untargeted metabolomics study using the Q Exactive HF of Thermo Fisher Scientific™ was set up and compounds were identified manually using their fragment spectra and comparing their spectra to an online database (*mzCloud* © 2013 - 2017 HighChem LLC, Slovakia) as well as the data was searched by the Thermo Fisher Scientific™ Compound Discoverer Software. Identified compounds were further verified using analytical standards. The obtained data showed individual differences in metabolic activity as various metabolites could only be detected in some donors and peak concentrations were found at different sampling times. Moreover, these analytes were also investigated in blood after the donors had consumed coffee and chocolate and the data was compared to the sweat data. Both specimens were taken at the same time in order to be able to note the similarities/ differences between them.

2. Theoretical Background

2.1 Food Biomarkers in Chocolate/Cocoa/Coffee

Humans are facing environmental chemicals and molecules that are present in food, water, air and other consumer products on a daily basis. Most of these molecules are absorbed in the gut and get metabolically transformed in tissues or by the microbiota present in the colon.[8] Several metabolites emerging from food have been identified as dietary biomarkers that precisely show the exposure to a specific constituent of the diet such as proline betaine as dietary biomarker for the consumption of citrus fruits. These food biomarkers have been utilized to study the relationship between dietary habit and disease risk (e.g. the identification of choline and its microbial products that could increase the risk for cardiovascular diseases).[7,8]

Variations in the diet have an effect on the human metabolism. Therefore, dietary preferences, lifestyle and genetics affect health status and the possibility to develop a disease.[9] As most food ingredients are broadly scattered among various foods, it is difficult to find unique food biomarkers. A combination of food-derived metabolites should provide a signature for a specific food item and help in a more exact portrayal of food consumption.[10]

While coffee has at least one group of bioactive compounds (methylxanthines), chocolate and cocoa contain at least two groups of bioactive substances: flavan-3-ols (mainly epicatchin (EC), catechin (CA) and their oligo- and polymers) and methylxanthines (theobromine (TB), caffeine (CF) and theophylline (TP). [11, 42]

2.1.1 Methylxanthines

Methylxanthines are purine alkaloids that can be found in chocolate, tea and coffee. Generally, the methylxanthines in coffee and cocoa/chocolate include: CF (1,3,7-trimethylxanthine), TB (3,7-dimethylxanthine) and TP (1,3- dimethylxanthine), which seem to concur to the bitter taste and stimulating effects of coffee and chocolate.[12] Paraxanthine (PX) is the primary metabolite of the human CF metabolism and is formed through the N3-demethylation catalysed by the cytochrome P450 (CYP450) system.[13]

TB is the main methylxanthine encountered in chocolate, whereas CF is only found in low quantities.[11,14] TB and CF stimulate the central nervous as well as the cardiovascular systems and have been connected to physiological effects on other body systems such as the gastrointestinal, respiratory and renal systems. They are quickly absorbed from dietary sources.[15,16] They are pharmacologically active compounds and are believed to contribute to cravings and addiction for chocolate.[11]

2.1.1.1 Caffeine

Naturally, CF is found in the coffee bean, tea, guarana, cola nut, the cocoa bean, mate and cassina. CF is often added to soft drinks and in medicines as stimulating drug (predominantly in pain relievers) either as a natural extract (for example from the cola nut) or as a synthetic chemical. In coffee and tea, the amount of CF ranges from 40 to 130 mg CF per cup.[11,17] A 50g dark chocolate bar contains only about 17-36 mg CF.[11,18]

In plants, CF is generated from the purine nucleotides adenosine monophosphate, guanosine monophosphate and/or inosine monophosphate, with TB being the direct precursor to CF.[19] CF is quickly absorbed from the gastrointestinal tract and distributed fast through the body.[20,21] The plasma levels peak at 15-120 minutes after coffee consumption according to the literature.[21] Only 3%[22] of CF is excreted unchanged via urine, meaning that it is metabolized, mostly in the liver by the CYP45 enzyme system. Some metabolites of CF also have pharmacological effects. Hence, metabolites of CF should not be forgotten regarding the biological properties of caffeinated foods and drinks.[23] Several lifestyle factors are assumed to have an effect on the metabolism and the elimination of CF such acute and chronic exercises, smoking, obesity and dietary preferences.[24]

The three major metabolites of CF are the dimethylxanthines (PX, TP and TB), which are further enzymatically transformed to various xanthines and uric acids (**Fig. 1**).[20] The first metabolic step, which is estimated to be about 80% of the human CF metabolism, is the 3-methyl demethylation resulting in the formation of PX (which is also known as 17X).[21] The kidney is then accountable for the elimination of CF and its metabolites.[24] The enzyme methylxanthine N3-demethylase transforms the three monomethylxanthines (1-methylxanthine, 3-methylxanthine and 7-methylxanthine) emerging from the metabolism of CF, TB and TP into xanthine. Xanthine is further reduced to CO_2 and NH_3 by the oxidative purine catabolic pathway (intermediates are uric acid, allantoin and allantoic acid).[25,26]

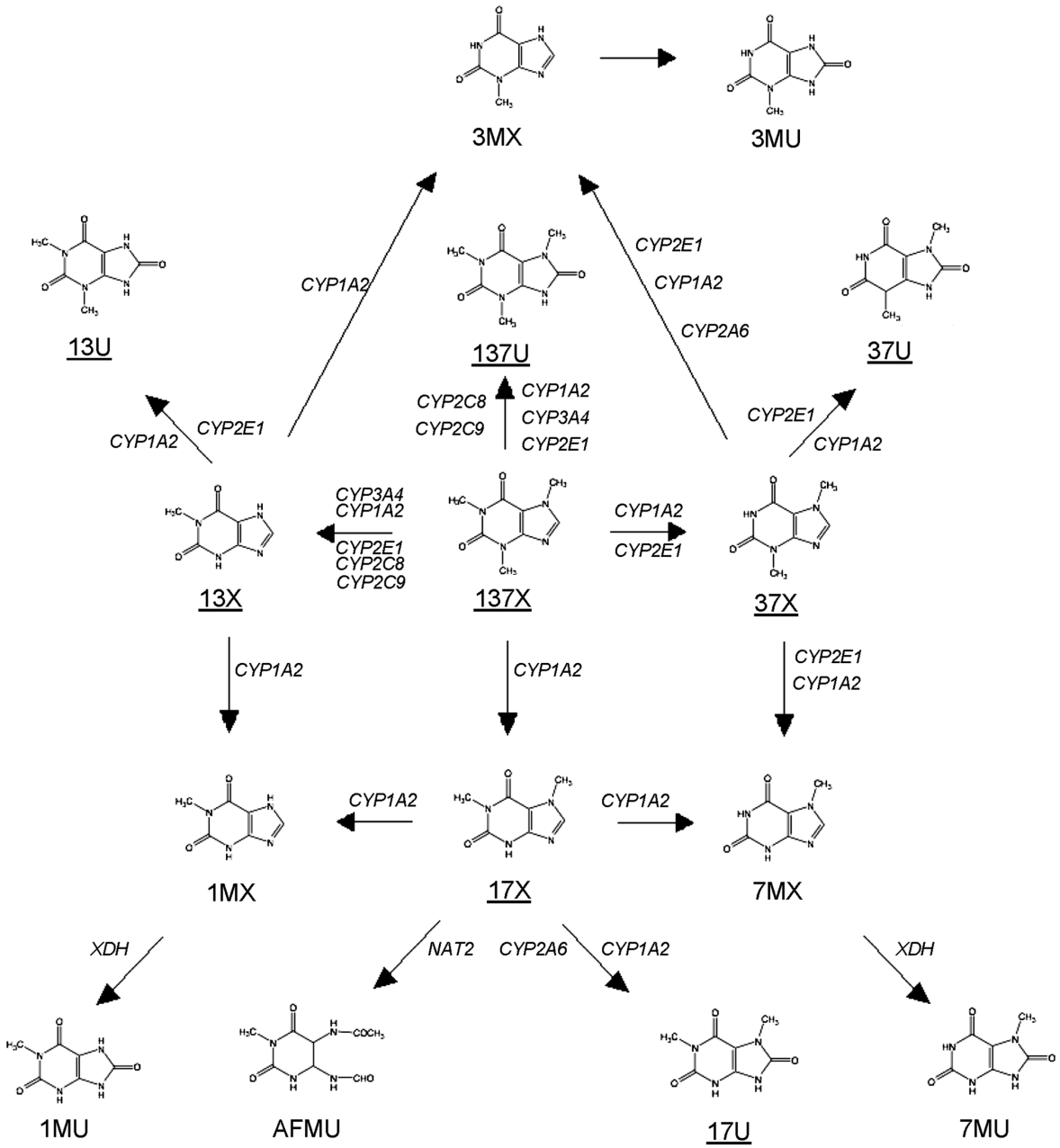

Figure 1: The "current understanding of caffeine metabolism in humans. 137X: 1,3,7-trimethylxanthine (CF); 17X: 1,7-dimethylxanthine (PX); 13X: 1,3-dimethylxanthine (TP); 37X: 3,7-dimethylxanthine (TB); 137U:1,3,7-trimethyluric acid; 17U: 1,7-dimethyluric acid; 13U:1,3-dimethyluric acid; 37U: 3,7-dimethyluric acid; 1MX: 1-methylxanthine; 3MX: 3-methylxanthine; 7MX: 7-methylxanthine; 1MU: 1-methyluric acid; 3MU: 3-methyluric acid; 7MU: 7-methyluric acid; AFMU: 5-acetylamino-6-formylamino-3-methyluracil."[20]

CF has hydrophilic and lipophilic properties. The lipophilic characteristics seem to contribute to CF being able to pass through all biological membranes. Since there is apparently no blood-brain barrier to CF in animal studies, it can be assumed that CF has low protein-binding affinities and thus, rarely binds to proteins (and if it binds, it is reversibly) and is allowed free passage into blood cells. The average half-life of CF is 2.5 to 4.5 hours in adults who were given CF doses lower than 10 mg/kg body weight. However, the half-life can vary depending on the individual. For example, smokers tend to have reduced half-lives by 30-50% compared to nonsmokers.[23]

The lymphatic system also plays a role in the transport and excretion of xenobiotics like CF. The lymphatic system belongs to the circulatory system. The main functions of the lymphatic system include controlling the body's water equilibrium by restoring extracellular fluid, which has escaped into the interstitial space, back to the systemic circulation and carrying immune cells to the lymph nodes. Substances that are absorbed into the lymphatic system are long-chain fatty acids, triglycerides, cholesterol esters, lipid soluble vitamins and xenobiotics.[27] In animal studies, it was shown that radioactive CF enters the left lymphatic duct (about 6.1% of the administered CF). In the left lymphatic duct 82-91% of the radioactive CF have been identified as unchanged CF, however small amounts of TP, 1-methylxanthine and 1,3-dimethyulric acid were also determined.[28] As there are different actions of absorption of xenobiotics including active transport, passive diffusions, pinocytosis, filtration and lymphatic absorption, the lymphatic system seems to play a role in the transport and excretion of xenobiotics (also via sweat). Lymphatic absorption of xenobiotics is not believed to have a major therapeutic effect, however the uptake of toxic chemicals such as benzpyrene could increase their toxicity as they are transported to other organ systems circumventing metabolization in the liver.[29]

CF acts through multiple mechanisms, yet, the most important one is binding to adenosine receptors and thus, acts via adenosine receptor antagonism.[23] As CF blocks adenosine from binding to its receptor, it influences the release of neurotransmitters like norepinephrine, dopamine, acetylcholine, serotonin, glutamate, gamma-aminobutyric acid and possibly neuropeptides. A blockade of these receptors means that the neurotransmitters, which have been recognized to decrease brain activity, can no longer bind and their effects are weakened. Thus, CF is thought to have an effect on mood and performance. CF is a non-selective, not very potent antagonist for adenosine receptors.[23,30,34] "Before" and "after" studies revealed benefits regarding CF consumption, including improved alertness, short-term recall and reaction time. There were also findings for positive mood changes and lower apprehended fatigue. CF-use has been restricted during the Olympic Games until 2004 as it was believed to be a strong performance-enhancing compound, although this role of CF was very disputed.[30] However, in the media it is again discussed whether to allow CF intake during athletic competition and according to "the Washington Post" CF is already on the waiting list for prohibited substances.[31]

Regular CF intake has been related to neuroprotective (against Alzheimer and Parkinson diseases) and even anticancer activity.[32] On the contrary, high CF consumption has been associated with negative effects including agitation, hypertension, dehydration, lowering of the sleep quality and insomnia.[30,32] The advised doses to show maximal benefit and the least risk are around 98 to 400 mg CF per day (for a typical 70 kg person).[30] In animal studies, CF had a lethal dose 50 of 200-400 mg/kg body weight when administered orally, which would mean about 50-100 cups of coffee for a human to reach an equal dose.[23,33]

2.1.1.2 Paraxanthine

The formation of PX and its urinary elimination are believed to be the main route in CF metabolism. PX gets further metabolized in the human body, leading to an increase of following metabolites in urine: 1-methylxanthine (1MX), 1-methyluric acid (1U), 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1,7-dimethyluric acid (17U), which are formed via the enzymes CYP1A2, CYP2A6, N-Acetyltransferase 2 (NAT2) and Xanthosine Dehydrogenase (XDH) proceeding from PX.[22] Like CF, PX enhances diastolic blood pressure, plasma concentrations of adrenaline and free fatty acids.[21] Compared to CF, PX seems to bind more potently to adenosine A_1 and A_{2a} receptors and it shows lower toxicity and less anxiogenic effects.[34] After a single consumption of CF, PX concentrations are comparatively low and are not very likely to concur much to the effects of CF. The consumption of CF on a regularly basis leads to the accumulation of PX and hence, PX contributes certainly to the effects of CF consumption. Furthermore, PX most likely conduces to building up tolerance against CF and withdrawal symptoms.[21] Compared to CF, which levels peaked at 75 minutes after oral consumption, PX levels were the highest at 300 minutes after ingestion.[21] The average plasma half-life of PX is 3.1 hours, which is the shortest of the methylxanthines (CF 4.1, TB 7.2 and TP 6.2 hours).[35]

2.1.1.3 Theobromine

Among various foods containing TB, cocoa and cocoa-products like chocolate have the highest concentration, with dark chocolate containing 237-519 mg[(11)] TB per 50 g bar. However, TB can also occur as metabolite of CF in humans (**Fig 1**). Even though TB is very closely related to CF, the mode of action of TB on the central nervous system is believed to be weak or even lacking all together. TB in the therapeutics is labelled as “diuretic”, “bronchodilator” and “cardio tonic” (according to the Merck Index). Apparently, there are no current therapeutic uses for TB.[11] TB exhibits a lower overall level of relative affinity for adenosine receptors, which is an explanation for the lower potency compared to CF.[36]

2.1.1.4 Theophylline

TP is a metabolite of CF, but also found in tea (*camellia sinensis*) and cocoa (although it is practically not found in dark chocolate[42]). Compared to other xanthine drugs, TP seems to have a different mode of action.[37] It competitively binds to phosphodiesterase, which reduces cyclic AMP (cAMP), and the enhanced levels of cAMP result in the pharmacologic effects of the drug. TP relaxes smooth muscle and is commonly used in the treatment of bronchial asthma. Side effects already appear at plasma concentrations of 20 mg/ml.[38] TP overdoses lead to nausea, vomiting and in severe cases to seizures, cardiac arrhythmias and even death.[39]

2.1.2 Polyphenols

The interest in polyphenols has increased in recent years due to their potential biological and health properties. Polyphenols are believed to have antioxidant capacity as they are hydrogen-donating radical scavengers[40] and they seem to have possible protective effects on human health such as reducing the risk of cardiovascular diseases as they influence blood pressure by lowering it; vascular function; platelet function as they inhibit platelet aggregation and activation; as well as lipid peroxidation in humans. Moreover, they also appear to be anti-carcinogen as studies showed that they inhibit the growth of human cancer cell lines and delayed tumour promotion in mouse. Some of them are antimicrobial, antiviral, and neuro-protective agents.[41,42,43] Other biological effects include the enhancement of endothelial function and the modulation of cytokine transcription in peripheral blood mononuclear cells.[44] Besides, polyphenols could enhance the concentration of HDL cholesterol and modulate the fatty acid composition of LDL, which then becomes less prompt to oxidative damage.[49] In order to have such a variety of biological effects, polyphenols must be bioavailable and efficiently reach target tissues, which makes the full understanding of their absorption, metabolism and elimination from the body that much more important.[41]

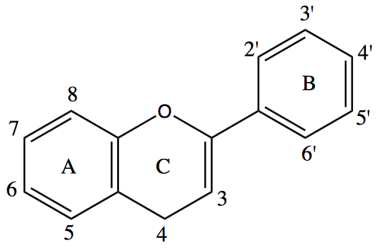

Figure 2: Basic structure of the flavanoids[47]

Currently, over 8000 polyphenolic structures have been identified. Their common structure is an aromatic ring with at least one hydroxyl substituent. According to their structure, they can be classified into: phenolic acids, flavonoids, stilbenes and lignans.[45] Polyphenols have high abundance in the diet since they naturally occur in many plants. They also have large chemical diversity.[8,44] Different foods such as fruits, vegetables and cocoa or chocolate as well as beverages like tea, coffee and red wine are rich in polyphenols.[44] Polyphenols are acknowledged as xenobiotics for the human organism.[51]

The main class of polyphenols present in chocolate and cocoa are the flavonoids (**Fig 2**).[45] The basic structure of flavonoids consists of two aromatic rings (an A-ring and a B-ring) bound by three carbon atoms, which form an oxygenated heterocycle in the middle (C-ring). Their structure is arranged in a way that promotes free radical scavenging since they can donate several electrons or hydrogen atoms to stabilize radical species.[46,47]

Cocoa and chocolate hold one sort of polyphenols namely flavan-3-ols, including monomers (epicatechin and catechin), oligomers and polymers (proanthocyanidins or short procyanidins).[44] The total polyphenol content in chocolate ranges from 5 to 8.4 mg/g chocolate and is even higher for cocoa powder (20 mg/g).[44]

In this study, **epicatechin (EC)** and **catechin (CA)** (**Fig. 3**) were among the biomarkers of chocolate present in sweat. An average 50g dark chocolate bar contains about 10 mg CA and 35 mg EC.[14]

EC and CA are widely spread over a broad range of different foods. CA concentrations are in particular elevated in broad beans, black grapes, apricots and strawberries. EC occurs naturally in apples, blackberries, cherries, pears, raspberries and chocolate.[48] EC is the main monomer present in chocolate and cocoa with a ratio of 10:1 EC: CA. EC and CA are stereoisomers at position 3 of the C-Ring. In addition, the interflavan bond at position 4 is always trans configurated. (+) CA and (-) EC are found in cocoa, yet, their enantiomers occur only rarely in nature. As can be seen from their structure, they possess both free radical trapping and metal-chelating potential which could be important for the protection against iron- and copper-induced free radical reactions.[49,50]

EC and CA are absorbed in the small intestine and are glucuronidated by phase II enzymes. The products are further transformed in the liver to sulfate conjugates and methyl derivates. 33% of the parent compounds stay intact.[51] As their glucuronidated, sulfated, and O-methylated metabolites they are readily absorbed and circulate in human plasma or get excreted via urine.[43,51]

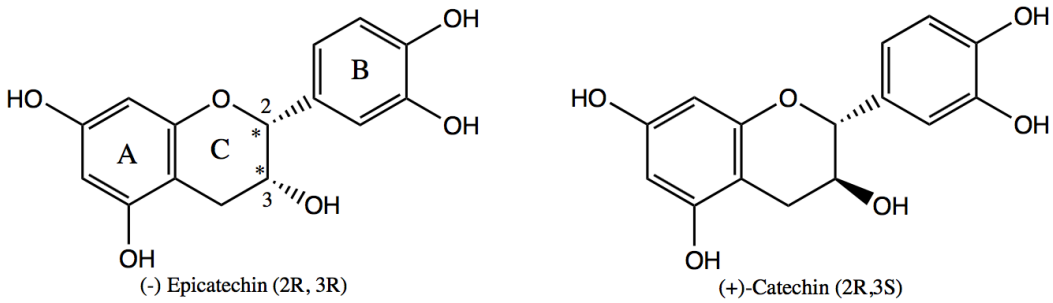

Figure 3: Structure of EC and CA[49]

EC and CA function as building blocks for oligomers and polymers (=procyanidins) in plants.[52] Oligomers and polymers that represent the larger part of polyphenols in chocolate and cocoa are only poorly absorbed in the gut due to their high molecular weight.[44] Hence, the health effects associated with chocolate/cocoa, which have been confirmed in vitro and in vivo studies, seem to be a result of low-molecular-weight metabolites and not caused by a direct action of procyanidins.[44] Studies showed that procyanidins leave the stomach unchanged as they do not get cleaved into monomers when ingested by rats. This leads to the assumption that the microbiome encountered in the colon could be responsible for the formation of low-molecular-weight metabolites.[44] In vitro studies showed that phenolic acids are formed through the human

colon microflora, which then can easily pass the colon barrier. However, these studies also indicated variations in the gut microbial activity of individuals. Moreover, the gut microbiota seems to adapt due to dietary habitualness in order to process compounds present in chocolate and other foods, which explains the individual metabolic activity.[53] Metabolites stemming from microbial degradation are then further absorbed and transformed by phase II enzymes, leading them to either enter the blood flow or be eliminated in urine.[54] **Fig. 4** gives an overview of the proposed metabolism of flavan-3-ols for human beings. The complex metabolism of proanthocyanidins involves reactions like C-ring opening, lactonization, decarboxylation, dehydroxylation, and oxidation reactions.[54] **Fig. 5** shows colonic microbial metabolites identified in urine and plasma after the consumption of chocolate.[55]

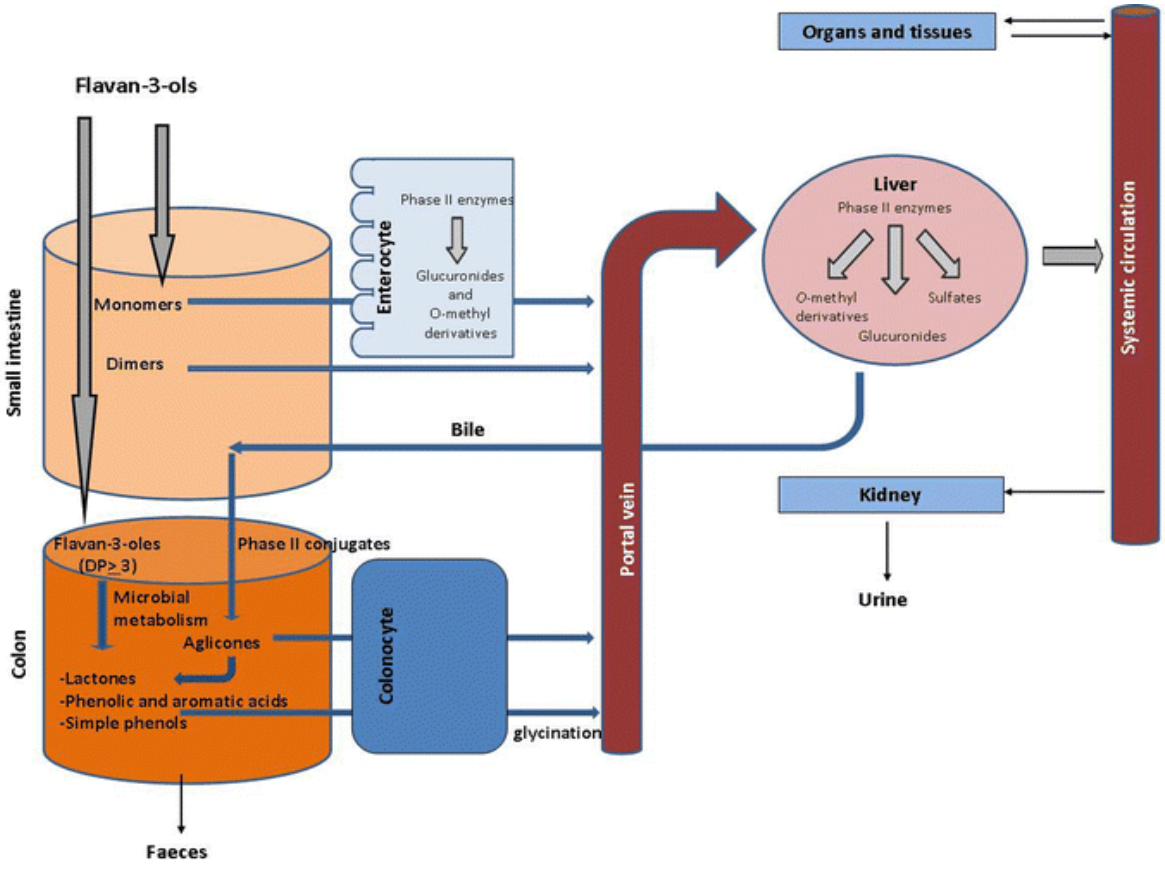

Figure 4: Overview of organs, reactions and agents playing a role in the metabolism and bioavailability of flavan-3-ols. DP stands for degree of polymerization.[54]

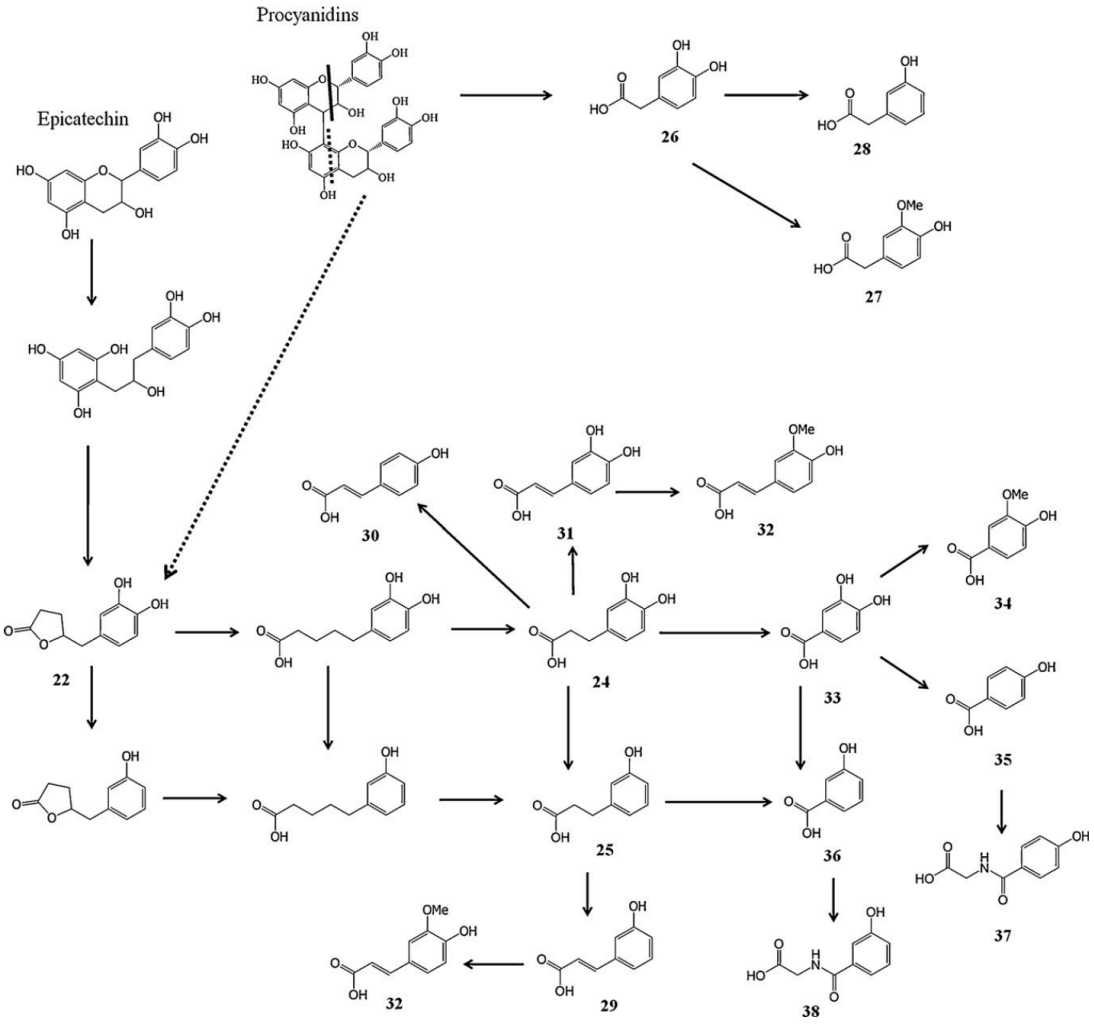

Figure 5: Colonic microbial metabolites identified in urine and plasma (not all were detected in plasma in the study with the reference [55] such as EC) with targeted MS/MS [55]

Table 1: Legend for the compounds in **Fig. 5** corresponding to reference [55]

No.	Colonic Microbial Metabolite	No.	Colonic Microbial Metabolite	No.	Colonic Microbial Metabolite
21	Epicatechin	**27**	3-Methoxy-4-hydroxyphenylacetic acid	**33**	Protocatechuic Acid
	Catechin	**28**	3-Hydroxyphenylacetic acid	**34**	Vanillic Acid
22	5-(3',4'-dihydroxyphenyl)-valerolactone		Phenylacetic Acid	**35**	4-Hydroxybenzoic Acid
23	(5-(3'-methoxy,4'-hydroxyphenyl)-(valerolactone)	**29**	m-Coumaric Acid	**36**	3-Hydroxybenzoic Acid
24	3,4-Dihydroxyphenylpropionic acid	**30**	p-Coumaric Acid	**37**	4-Hydroxyhippuric Acid
25	3-Hydroxyphenylpropionic acid	**31**	Caffeic Acid	**38**	3-Hydroxyhippuric Acid

Other phenolic compounds in cocoa products include syringic acid, chlorogenic acid, clovamide, deoxyclovamide, phloretic acid[49], hippuric acid and isoferulic acid[38].

2.1.3 Organic Acids

The most common organic acids in chocolate and cocoa are citric, oxalic, malic, acetic, lactic and formic acid, which contribute to the taste of chocolate.[56]

2.2 Finger sweat

Fingermarks/Fingerprints and finger sweat are transferred from the fingertips to a surface upon contact. Excreted sweat from the fingertip comprises a combination of external contaminants on the fingertip and secreted sweat.[4] Traditionally, fingerprints have been applied to convict individuals of a crime since the late 19th century through matching of fingerprint ridge patterns.[57] These patterns are extraordinary for each individual and do not change during a person's lifetime. On the surface of the skin ridges several pores are located, which secrete sweat. There are three types of sweat glands in humans: eccrine (on fingertips), apocrine and sebaceous. All of them secrete sweat of a unique composition.[57] In recent years, the interest of the chemical composition of these three secretions has risen since the knowledge of the complexity of the endogenous and exogenous content of fingermarks could prove usefulness for identifying potential biomarkers and target molecules for forensic analysis.[58] Additionally, they may contain evidence if a person has been in contact with explosives or illicit substances.[59]

Eccrine sweat along with contaminants like sebum that have been picked up by the finger, form an impression of the finger's ridge pattern upon contact with a surface. This impression is known as latent fingermark. In criminal investigations, crime officers use a ninhydrin solution or iodine/benzoflavone spray on porous surfaces to visualize latent fingerprints.[60] Since a fingerprint contains amino acids, it turns purple upon reaction with ninhydrin.[61] The composition of fingermark secretions have five different sources: "eccrine sweat, apocrine sweat, sebum secretions, epidermic substances, and external contaminations from the environment".[62]

In this master thesis, the finger sweat was analysed and not the latent fingermark (impression of the ridge pattern).

2.2.1 Skin Anatomy

Skin is the largest organ of the human body and has several functions like "the regulation of body temperature, water retention, protection, sensation, excretion, immunity, blood reservoir and synthesis of vitamin D".[63] For an average adult, the skin has a surface area of about 2 m^2. The skin is divided into two layers: the epidermis and the dermis. The epidermis defines the outer

layer of skin made out of the epithelium and is divided into five subunits or *strata*.[63] Keratinocytes are the main cells present in the epidermis. They produce keratin, which constructs the surface coat (*stratum corneum*). This coat protects the skin and underlying tissue and organs from heat, infections, dehydration, chemicals and mechanical stress.[64] The *stratum corneum* also contains dead cells that are frequently removed through a regular desquamation process, during which proteins needed for skin renewal are expressed. These proteins can migrate to the skin surface. The expressed proteins could then be transferred to the fingerprints of an individual as the *stratum croneum* touches a surface.[65]

The dermis consists primary of collagen, which provides skin tensile strength and resistance to mechanical stress; elastin fibres, which gives the skin its elasticity; and an interfibrillar gel of glycosaminproteoglycans, salts and water. In this layer about five million secretory glands are found, namely the eccrine, apocrine and sebaceous glands.[63] Furthermore, fibroblasts that not only form elastin and collagen but also histiocytes, which are important to the immune response against viral infections, are present in the dermis. The dermis also accommodates hair follicles, lympathic-, nerve - and blood vessels.[66] **Fig. 6** gives an overview of the construction of the human skin.

The three types of sweat glands that are present in the dermis can be distinguished based on their morphology and their mode of secretion.[67]

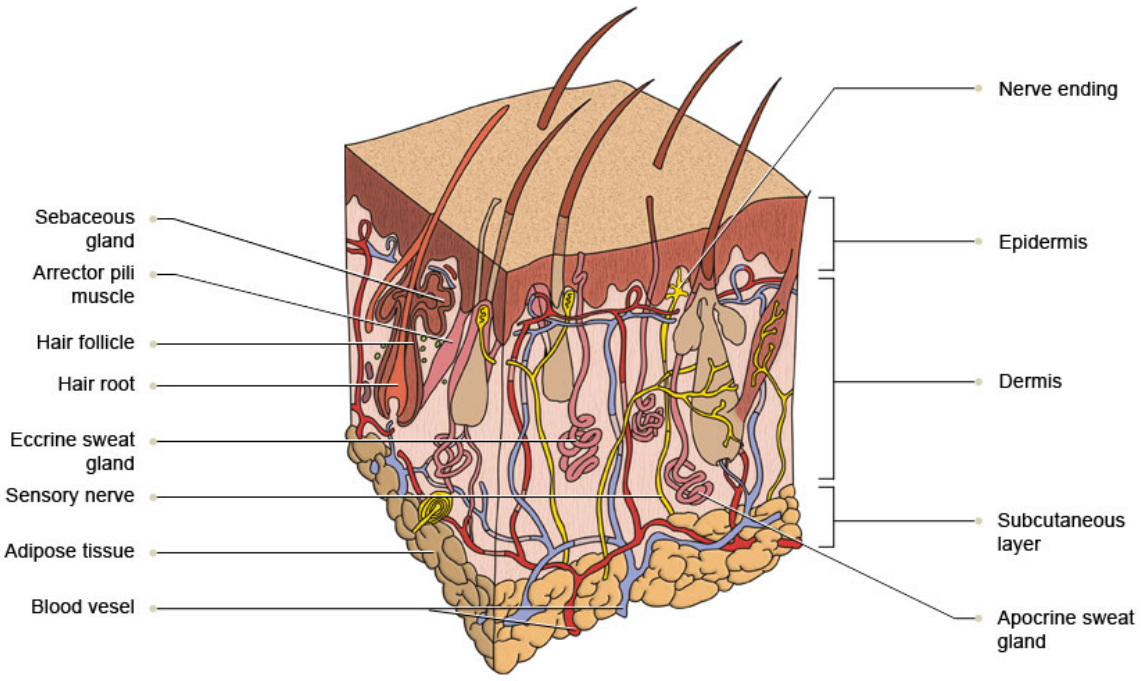

Figure 6: Cross-section of the human skin[68]

Apocrine Sweat Glands. These sweat glands occur in the axilla, mammary, perineal and genital region. Apocrine sweat glands have an excretory duct that opens and secretes into the hair canal. Secretory cells are usually columnar formed although their shape can vary depending on secretory activity.[67] Apocrine sweat glands excrete an oily, odourless substance that contains lipids, steroids and proteins. [67] This substance may also contain sebum since apocrine glands like sebaceous glands open up to hair follicles.[67] Due to the location of the apocrine glands their excreted sweat plays only a minor role in fingerprint composition (only as contamination).

Eccrine Sweat Glands. For fingerprint analysis, eccrine glands provide the main source of sweat. There are about two to four million eccrine sweat glands distributed over the human body surface. These glands can secrete up to 4 litre fluid per hour.[63] Sweat gland density is the highest on the soles and palms (with about 700 sweat glands cm^{-2}) and least abundant on the back (with about 64 sweat glands cm^{-2}).[67] The eccrine sweat glands are coiled tubular systems, which are connected to the skin surface via a sweat duct.[67,69] Their structure is divided in: an intra-epidermal part (called *acrosyringium*), the dermal duct – which is subdivided into a straight and a coiled part – and a secretory portion that coils in a helical way into the dermis layer (**Fig. 7**).[63,67] The dermal duct reabsorbs ions from sweat. This reabsorption of ions decreases the loss of Na+ during perspiration. The secretory portion comprises different cell types: clear cells, dark cells and myoepithelial cells (whose function is not clear yet). Dark cells are osmophilic and seem to be denser than clear cells. Clear cells accommodate a great quantity of mitochondria.[67]

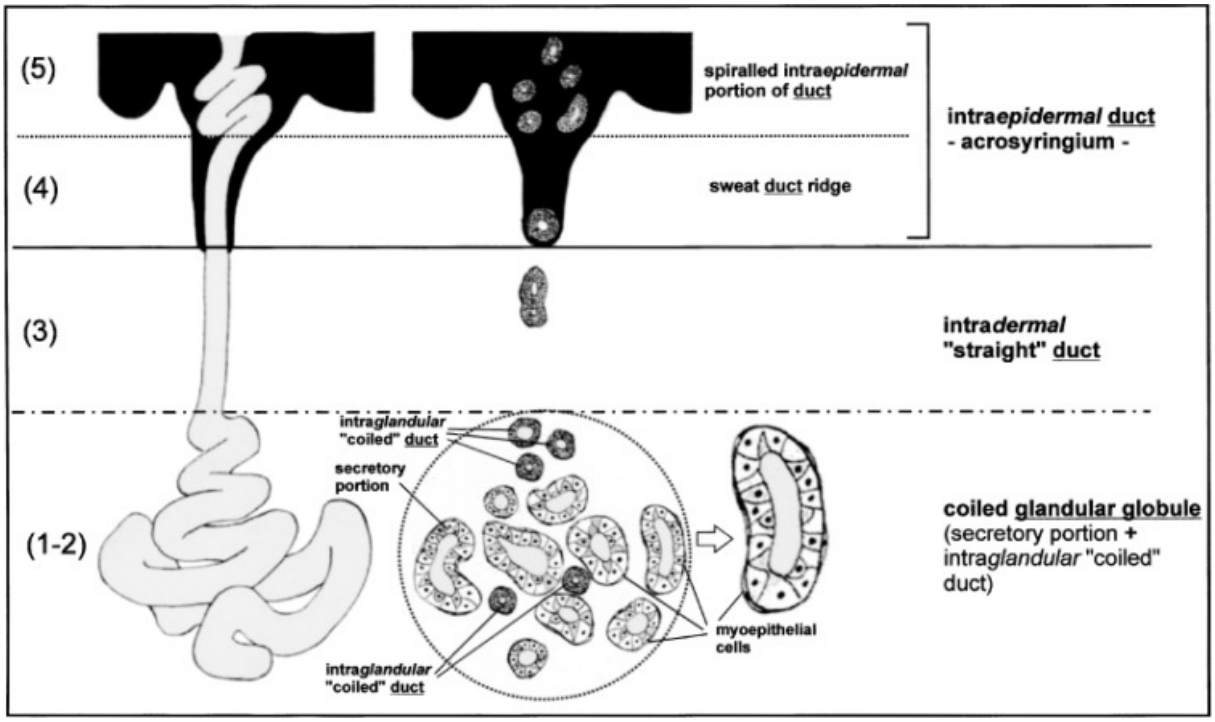

Figure 7: Schematic illustration of the eccrine sweat gland and its main tissue segments.[70]

The eccrine sweat composition varies relying on hydration, exercise, health and location.[67] Next to water (99%) components like sodium, potassium, chloride, lactate, ammonia, amino acids, urea and biocarbonate are present in eccrine sweat. Moreover, proteins and peptides for example DNAse I, cysteine proteinases, lysozyme, cysteine-rich secretory protein, Zn-a2-gylcoprotein and dermcidin (an antimicrobial peptide) have been encountered in eccrine sweat according to the literature. With the presence of dermicidin, an antimicrobial peptide, eccrine glands could have an impact to the innate host defence mechanism.[63,67]

The main function of eccrine sweat glands is thermoregulatory sweating as they decrease the body temperature under heat stress conditions by evaporating water and cooling the body surface in the process. Sweat gland activity is controlled by the central nervous system, which reacts to alterations of the core body temperature as well as to hormones, physical activity and emotional stimuli like stress, anxiety, fear and pain.[67]

Sebaceous glands are located on the whole body, yet, they are absent from the palms and soles. They are especially concentrated on the forehead, scalp and face (up to 800 glands/cm^2).[63] Most of these glands can be found near hair follicles since they open into the hair shaft canal. These glands secret an oily or waxy substance called sebum.[63]

The predominant lipids in human sebum are triglycerides, wax esters and squalene. Sebum contains free fatty acids that are formed from triglycerides through bacterial lipases.[71] Even though palms and feet are free from sebaceous glands, sebum is still transferred onto fingertips since hands and feet often come in contact with other body parts (for example face and hair).[72]

2.2.2 Contaminants present in finger sweat

Fingerprints can have various contaminants arising for example from food, hygiene articles, dust and bacteria spores. Since there may be compounds that are present in sweat endogenously as well as emerging from a contamination, the quantity of these compounds could be overestimated. Moreover, it could be difficult to distinguish whether such a compound might be indeed a contamination or a compound derived from an endogenous source.[65]

Early studies using mainly gas-chromatography mass spectrometry (GC/MS) revealed the presence of cosmetics such as compounds present in shampoos, perfumes and creams in fingerprints. Since cosmetics can also contain lipid compounds, which are naturally found in fingermark secretions such as fatty acids (e.g. palmitic acid) or wax esters (e,g, myristyl myristate), they are very difficult to distinguish from intrinsic fingerprints.[65] Nicotine can also be a contamination of fingerprints. A study wanted to evaluate if the presence of nicotine in the finger sweat could be related to the donor's smoking habit. However, the study revealed cross-contamination from a smoker to a non-smoker through handshakes and touching of other

surfaces. It must be noted that these cross-contamination peaks were at lower intensities than the direct contamination of touching the cigarette.[65]

Drugs such as sulphonamides (antimicrobial, antifungal and antimalarial agents) and L-dimethylamphetamine (stimulant drug) have further been detected in eccrine sweat with concentrations proportional to plasma concentrations. These drugs probably migrate into eccrine glands through simple diffusion, which seems to be the case for caffeine as well.[65]

The study of K. Kuwayama *et. al.* showed the usefulness of fingerprints as alternative biofluids to urine and blood with the help of LC-MS. Healthy donors were given pharmaceutical products containing: acetaminophen, acetylsalicylic acid, allyl isopropyl acetylurea, ibuprofen, dihydrocodeine phosphate, chlorpheniramine maleate, 2,5-methylephedrine hydrochloride, fexofenadine hydrochloride and epinastine hydrochloride. They were able to detect and quantify most of the compounds in finger sweat as well as in whole blood samples. Interestingly, these compounds were often detected at a later sampling time in fingerprints than e.g. in blood.[6] With their study, they have also proven that fingerprints can be used to detect traces of illicit drugs since drugs and their metabolites are excreted via sweat.

2.2.3 Variability of fingermark composition

According to the literature, the fingerprint constitutions (e.g. lipids) differ among fingerprints of the same individual and between different people. However, the extent of these differences is still unclear.[73] There are different variables that significantly influence fingerprint composition. These factors include: donor properties (diet, age, gender, contact with personal hygiene items, metabolic disorders and skin pathology), the deposition conditions (pressure, contact duration) and the nature of the substrate the fingerprint is transferred to (glass or paper).[65] Another important role plays the transpired time between the sampling of the fingerprint and the time of analysis. Storage conditions like humidity, light exposure, temperature, dust and so on influence the finger sweat composition if the samples are stored for long periods.[65]

As an example for age as a factor of different fingermark composition, sebum production increases at the beginning of puberty and thus, has an effect on the lipid composition of fingermarks deposited by adults in comparison to young children.[74] The fingermark composition of children mainly consists of aqueous saline constituents and fewer free fatty acids. These aqueous saline molecules are volatile and disappear quickly which explains why the secretions from children evaporate much faster after deposition on a surface than those of adults. The residues of adults contain squalene, cholesterol, large fatty acid esters, wax esters and glycerides, which are less volatile and therefore, remain on substrates.[75] Hence, age plays an important role in the finger sweat composition regarding lipid composition.

There are different studies on the influence of gender, which have led to contradictory results. One study showed no significant differences in the fingerprint composition of males and females,

while another study exhibited that compounds such as urea and fatty acids vary between the genders. These differences could be a result of distinct metabolism regarding glands and hormones.[76,77]

Diseases and medications can further influence the sweat composition. An example is the measurement of sweat electrolytes for the screening of cystic fibrosis. Due to defective chloride channels, the concentration of this electrolyte is elevated in the sweat of individuals with cystic fibrosis.[78]

The finger itself appears to be a parameter having an impact on sweat composition. A study observed chloride levels in finger sweat samples and found that the fingerprint of the left hand contained larger amounts of chloride compared to the right hand. A reason for this could be that most people are right handed and hence, the fingers of the right hand constantly lose their secretions due to a more regular contact with different substrates.[65] Washing hands could also influence the fingerprint composition. Emulsification of the lipid compounds could take place and therefore, a decrease in concentration of these compounds could be notable in the finger sweat composition. [65]

2.2.4 Advantages and Disadvantages of Fingerprints as an Analyte Source

Blood, urine, saliva and sweat are the biofluids used in clinical applications. Some advantages of using sweat for clinical analysis are: Taking fingerprint samples is a non-invasive procedure whereas blood is an invasive sampling technique and a physician is required to draw blood of patients or suspects. Additionally, patients who must undergo frequent analysis of their blood are at a higher risk of infection. Urine can also be sampled non-invasively, however suspects need to be escorted to a lavatory and must be observed by a police officer in order to avoid falsification of the sample. For medical analysis, patients often require a catheter for the collection of urine. Another advantage of fingerprint analysis is the simple sample preparation due to the small amount of matrix components present in the fingerprints compared to the other biofluids. Sweat samples are less likely to get altered and thus, components in fingerprints such as drugs are more stable in sweat since sweat contains fewer enzymes compared to blood. Hence, the samples can be transported to laboratories at room temperature and can be stored for long periods. Fingerprint samples can be taken easily, quickly and no specialist is required. For example, patients could sample themselves at home and would not need to go to the hospital every day. Moreover, they could be taken directly at the crime scene, which is important to convict a suspect as biological specimens need to be taken as soon as possible in order to prove drug intake since drugs are metabolised and excreted constantly. [2,6,79]

Disadvantages of using sweat for clinical uses are high expenses and a lot of expertise is required for running the sensitive LC-MS/MS instruments that are needed to detect trace amounts of drugs, metabolites and other molecules.[6] However, the maintenance- and basic costs

are relatively little. Most of the sweat sampling methods currently used are time-consuming since they require wearing a sweat patch for several hours. The greatest disadvantage of fingerprint samples is the need to normalize the sweat volume and finding a suitable endogenous component to compensate variations in the different sweat amounts among individuals.[2] Because of this disadvantage finger sweat analysis is currently rather semi-quantitative. Other disadvantages include that most large molecules (for example proteins) are diluted and thus, are only present in sub-picomolar ranges, that some analyte concentrations only represent the metabolism of eccrine sweat gland itself and that large pH changes (4.5-7.0) are possible, which could misrepresent concentrations of weak acids and bases.[80]

2.3 Analytical Techniques for Investigating Finger Sweat Composition

The majority of sweat constituents of interest are small molecules stemming from metabolic pathways. Thus, sweat analysis usually focuses on metabolomics, the "omics" of small molecules typically below 1000 Da. It was defined in 2002 as "a comprehensive analysis in which all the metabolites of a biological system are identified and quantified" in context of physiological stimuli or disease state.[1]

Modern analytical methods for the investigation of fingerprints include immunodetection, spectroscopy among with Raman spectroscopy and infrared spectroscopy, nuclear magnetic resonance spectroscopy (NMR) as well as MS along with GC-MS and liquid-chromatography MS (LC-MS).[81] With the help of MS pico- and nanomolar concentrations of metabolites can be detected. NMR allows the quantification of metabolites in the micromolar range. Mass spectrometry can online be coupled to chromatographic separation, reducing matrix effects and complexity of biological samples. The advantage of NMR is that it is non-destructive compared to most MS techniques.[82] One disadvantage of MS techniques is the loss of the original sample.[81] In MS two different approaches can be used: targeted and untargeted. In untargeted experiments, all molecules within a specific range of mass values are measured. These signals then require annotation using either in silico and online libraries or experimental identification with analytical standards. The advantage of untargeted measurements is that the experiments offer a broader coverage of different compounds and could lead to the identification of unexpected metabolites, whereby novel biomarkers could be found. The Q Exactive HF (Thermo Fisher Scientific™) allows untargeted experiments. In targeted experiments predefined specific metabolites are measured, typically focusing on a few pathways of interest or a group of structurally related metabolites. Targeted approaches offer a better quantitation due to a higher degree of sensitivity compared to untargeted approaches and easier identification of compounds. A triple quadrupole mass analyzer is generally used for targeted, quantitative analysis.[1,83,84,85]

The goal of this thesis was to uncover as many metabolites as possible present in fingerprints in relation to chocolate and/or coffee consumption. Therefore, a sufficient separation system is required, together with the possibility of an untargeted approach. The overall setup was aimed to be simple and should allow high sample throughput. Due to these reasons and because the concentrations of expected metabolites were in the picomolare range, a liquid chromatography system (Vanquish UHPLC) coupled with the Q Exactive HF – a hybrid instrument including a filtering quadrupole and an orbitrap mass analyzer – was the method of choice.

2.3.1 HPLC – High performance Liquid Chromatography

A separation step prior to mass spectrometry analysis can be performed in order to decrease the complexity of the samples and thus, enhance the possibility of finding low abundant molecules such as biomarkers in complex samples, as well as reducing problems associated with ion-suppression/enhancement effects.[86] For metabolomics approaches, usually GC or LC techniques are used. For metabolic profiling, high performance liquid chromatography (HPLC) is often the first choice since samples such as urine and serum/plasma can often be measured with minimal sample preparation.[86] HPLC is a chromatographic separation technique based on the distribution of analytes between a liquid mobile and a solid stationary phase.[88] HPLC does not require complex pre-treatment and chemical derivatization to obtain volatile and thermally stable compounds as needed in GC-MS experiments.[87] LC-MS methods for metabolomics are rapid and a great variety of structural diverse classes and physicochemical properties can be studied simultaneously.[86] Compared to normal liquid phase HPLC uses smaller particles (micron-sized) of high mechanical strength as support for column packing materials, therefore enabling liquid to flow through the column at high pressure. Conventional HPLC columns have an inner diameter of 4.6 mm and are using flow rates of 0.4 to 2 mL/min for the mobile phase.[88] Generally, in LC-MS experiments investigating metabolomics reversed-phase (RP) is the most employed separation technique.[86] In RP-LC analytes are distributed between an apolar stationary phase (mostly octadecylsilane - C18 or octasilane - C8) and a polar mobile phase. RP-LC involves hydrophobic interactions between the analyte and the stationary phase.[88] The most common substrate for RP columns is silica, where the stationary phases are based on siloxanes. The silanol groups on silica support get chemically derivatised with organosilane reagents. These reagents contain the functional groups like chloro-, alkoxy, alkylamino or other functions. An advantage of silica is that it does not shrink or swell when organic solvents are used as mobile phases. Furthermore, the production of silica as well as its bonding chemistry is well understood and is also very reproducible.[89,90] Commonly used weak mobile phases for RP are water or a combination of water and organic solvents (MeOH, ACN, THF), while strong mobile phases usually contain isopropanol or THF together with solvents like ACN, MeOH or H_2O. Mobile-phase additives can be used to improve LC separation and MS detection. Examples for

such additives are ammonium formate, formic acid, ammonium acetate and so on. Buffer salts are added in concentrations ranging from 5-10 mM, whereas acids are used at 0.05-0.2%.[91] The retention times of the analytes depend on the intensity of the interaction of the compound with the stationary phase, the solubility in the mobile phase and other influences like column temperature. The retention time for molecules that are less polar is longer, whereas polar molecules are only weakly retained and elute more readily. Less polar components elute with increasing organic solvent in order of enhanced relative surface hydrophobicity. RP-LC is optimal for coupling with ESI since it does not need a high ionic strength for elution or an increasing salt concentration.[88]

Hydrophilic interaction chromatography (HILIC) and ion-pair chromatography (IPC) are becoming more popular due to the limitations of RP-LC regarding the separation of the polar fraction of the metabolome and thus, HILIC and IPC offer orthogonality to RP-LC. For maximum coverage, the literature advises to measure the same samples with HILIC and RP-LC since HILIC uses an opposite retention principle and therefore, different selectivity allowing a greater coverage of the metabolome.[92]

HILIC uses a polar stationary phase (bare silica or polar bonded silica) and a highly organic mobile phase (e.g. acetonitrile) as the eluent, which is modified with water as the strong eluting solvent. In this mode of separation, lipophilic compounds are sparsely attracted to the stationary phase and elute with weak retention. On the other hand, hydrophilic compounds are retained and only elute when the water concentration is increased. However, the retention mechanism in HILIC is more complex, involving secondary electrostatic, hydrophobic, and hydrogen-bonding interactions relying upon the conditions applied like mobile phase additives. HILIC is often used for samples like urine.[93]

Nanoliquid chromatography (nano-LC) was first introduced by Karlsson and Novotny in 1988. Up until now no general definition for nano-LC has been found, however most “nano” applications use capillaries with an inner diameter of 10 to 100 µm and a particle size of 3 - 5 µm (or even 1,5 - 1,8 µm for ultra-high-performance liquid chromatography – UHPLC). Compared to conventional HPLC columns, miniaturized columns offer a higher separation efficiency in a shorter separation time, together with less mobile phase consumption. Additionally, less sample volume is needed, which is desirable for biological samples that are often limited in availability. Needing less amount of stationary phase is also an advantage when it comes to the use of expensive packing material. The reduced flow rates (of 40-600 nL/min) lead to a reduction of the chromatographic dilution, thus providing higher sensitivity. However, using smaller particle sizes leads to higher pressure in the column.[94]

2.3.2 Mass Spectrometry

Mass spectrometry allows the measurement of mass-to-charge ratio of ions in the gas phase to obtain information on the analytes. A mass spectrometer is constructed of an ion source, a mass analyser and a detector. The ion source ionizes the analytes in the sample, which get transported via magnetic or electric fields to the mass analyzer. The mass analyzer separates the ions according to their mass-to-charge ratio (*m/z* value). The mass analyzer then ejects the ions to the detector, where they are registered and converted into a digital signal.[96,129]

2.3.2.1 Electrospray Ionization

Electrospray Ionization (ESI) has become one of the most popular ionization techniques as it allows direct coupling of HPLC systems with mass spectrometers. It permits the analysis of both small and large molecules of various polarities even in complex biological matrices.[95,96] The ESI source generates gas-phase ions from a solution and transfers them to the mass selective units.

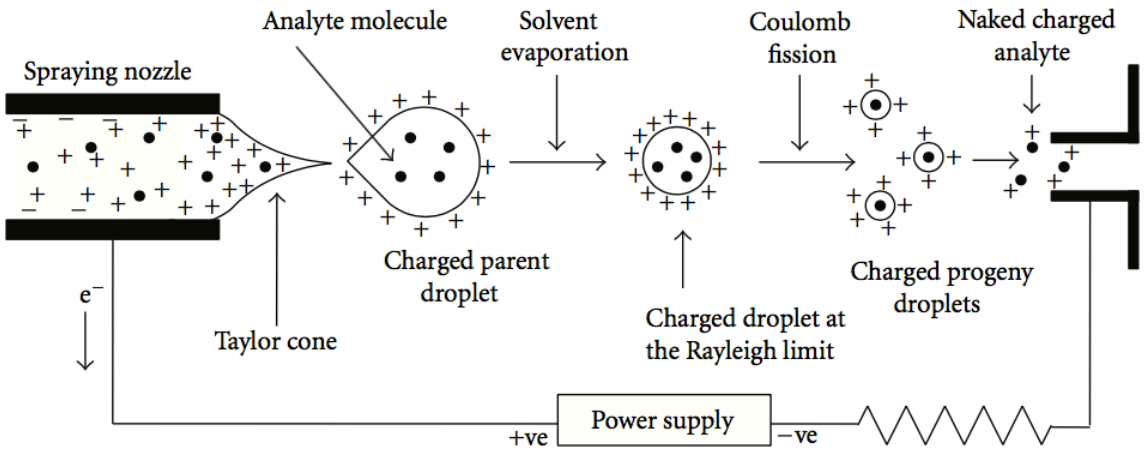

Figure 8: Electrospray ionization process. Charged droplets generated by the spraying capillary decrease in size due to the solvent evaporation. At the Rayleigh limit, the droplet deforms as the surface tension can no longer sustain the droplet shape since electrostatic repulsion of like-wise charges is too high. Multiple coulomb explosions occur until the charges are transferred to the analyte molecules.[96]

The ionization itself takes place under atmospheric pressure and ions are not fragmented. Thus, ESI is considered a "soft" ionization technique. Large molecules result in ions carrying multiple charges, which reduces their mass-to-charge ratio compared to a single charged species and allows their detection.[96, 129]

For the electrospray process, generally the analyte is diluted with a volatile solvent to concentrations typically between 10^{-6} and 10^{-4} M. The HPLC system delivers the samples to the ESI source and serves as the sample inlet. The liquid sample is then injected through a capillary and is nebulized under atmospheric pressure by a spray needle. At the tip of the needle a high voltage (3-6 kV) is applied, which leads to the dispersion of the sample solution into an aerosol of highly charged electrospray droplets – among them the analytes. As the droplets move towards the vacuum system, the solvent evaporates. The aerosol expands into a counter current

stream of hot nitrogen gas, which serves as a heat supply for the vaporization of the solvent. The evaporation of the solvent leads to an enhanced charge density on the surface of the droplets, the droplets become unstable, reaching the Rayleigh limit. At this point, the droplet deforms since the surface tension can no longer sustain the droplet shape as electrostatic repulsion of like-wise charges is too great, resulting in coulomb explosion. The parent droplet breaks into much smaller offspring droplets. The solvent droplets keep reducing their size (to about 1-2 µm in diameter) by repeating this process (**Fig 8**)[95,96,129]

Oxidation of the solvent takes place in positive ion mode and reduction happens in the negative ion mode. Therefore, depending on the polarity of the emitter electrode these redox reactions supply positively or negatively charged ions. Most of the gas arising from the desolvating aerosol is pumped off. Only a small proportion passes through the opening of a skimmer into the high vacuum (10^{-3} – 10^{-4} Pa) at the beginning of the mass spectrometer.[96, 97, 129]

2.3.2.2 Q Exactive HF

The Q Exactive HF is a hybrid instrument consisting of a quadrupole as a mass filter and an orbitrap as a mass analyzer. The orbitrap mass analyzer was commercialized by Thermo Fisher Scientific™ in 2005 and hybrid instruments based on the orbitrap have become popular ever since.[129]

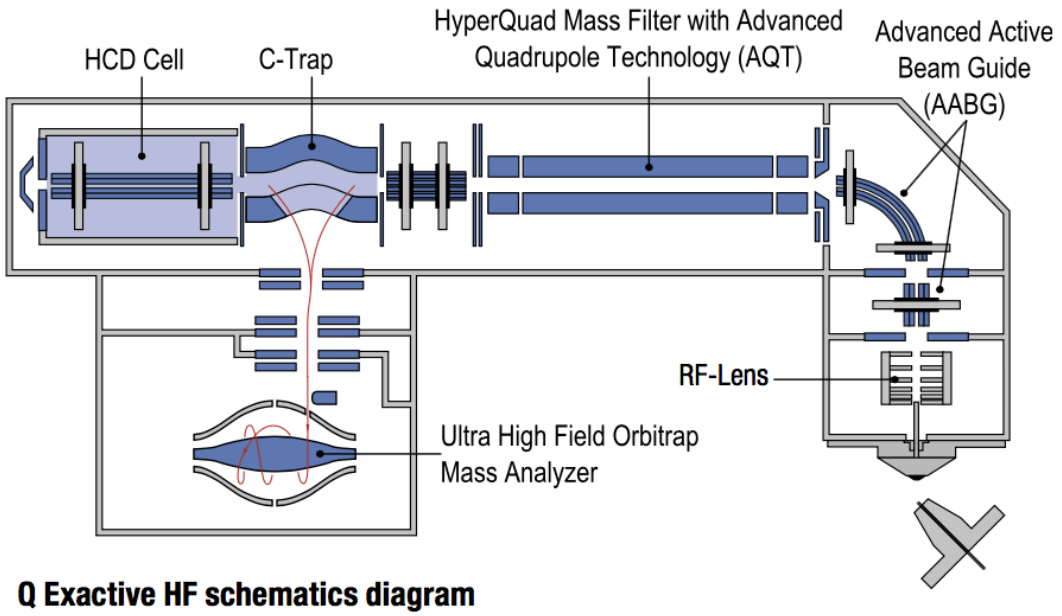

Figure 9: Construction details of the Q Exactive HF [104]

The Q Exactive HF is composed of an ion source, a stacked-ring ion guide (so-called S-lens) and an injection flatapole, a bent flatapole, a quadrupole mass filter, a C-trap, a higher energy

collisional dissociation (HCD) cell and an orbitrap mass analyzer (**Fig. 9**).[99,104] The S-lens comprises stainless steel apertures to which a RF voltage is applied and which are spaced by a steadily increasing gap. The apertures are divided into two sets with different diameters: The first set has an inner diameter of 7.5 mm to capture the entire expansion from the transfer tube, the second set has a smaller diameter of 5.0 mm, which efficiently focuses the ions into a tight beam through an exit lens. This construction increases the ion transmission from the atmosphere into the mass spectrometer.[98] The injection flatapole is supplied with ion selection properties and acts as a filter of unpreferred ions even before the higher resolution selection. The bent flatapole ensures no neutral species get ejected in order to hinder them from getting further into the instrument. Collisional cooling takes place in the bent flatapole.[99] Ions enter the hyperbolic quadrupole through a lens.[102]

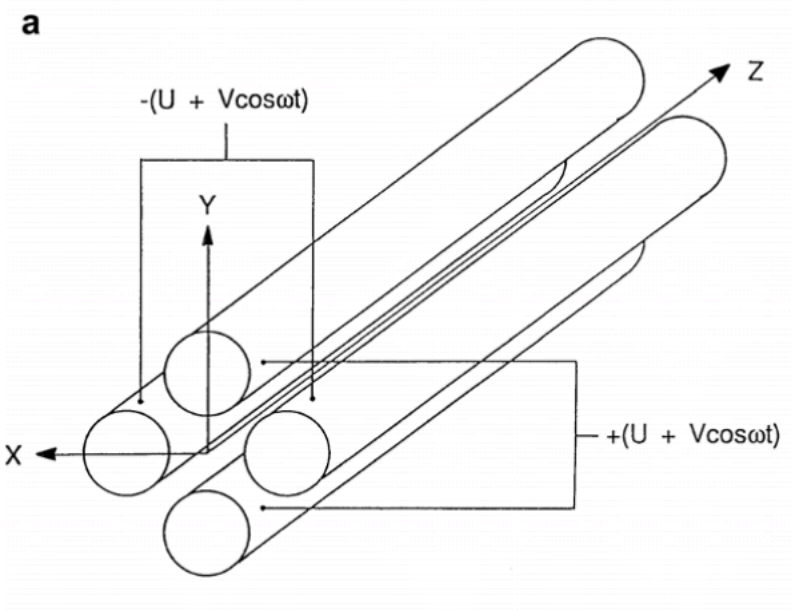

Figure 10: Schematic linear quadrupole mass analyzer [129]

A linear quadrupole (**Fig. 10**) consists of four hyperbolically shaped metal rods, which are kept at equal distance. Each pair of opposite rods are connected electrically and kept at the same potential -which consists of a DC (direct current) and an RF (radio frequency) component - but with opposite polarity. Due to the resulting electrical field the ions travel forward in the z-direction with an oscillatory movement in the x-y-plane. The amplitude of oscillation is connected to the mass-to-charge ratio of molecules and can be modulated by altering the DC and RF voltages. The amplitudes can be changed in such a manner that only desirable *m/z* ratios are "stable" leading these ions to travel along the z-axis without hitting the quadrupole rods and further arriving at the detector. The oscillatory amplitude of unpreferred ions is large and therefore the ions are "unstable" resulting in them hitting the metal rods, getting neutralized and

not being able to get to the detector or in this case the C-trap. Quadrupoles have high ion transmission, are not very heavy, compact and relatively inexpensive and they allow high scan speeds since scanning is achieved by only varying electric potentials. [100, 129]

In case of the Q Exactive HF, the entrance segment of the quadrupole is driven by RF-only in order to guarantee entering ions to stay well focused.[99,102]

Moreover, the combination of a quadrupole mass filter and an orbitrap mass analyzer allows to separate "in space" meaning that isolation and fragmentation occur at separate areal regions. In comparison, "in time" selection is found in trapping instruments like the quadrupole ion trap, where sequential steps are studied in the same physical space, only separated from each other by different timing.[101]

A short octapole then leads the ions into the C-Trap. The C-trap is a bent RF-only quadrupole that accumulates, stores and thermalizes ions before they get ejected into the orbitrap mass analyzer.[99] The C-trap can hold up to a million of ions. Through high-voltage electric pulses ions get transferred from the C-trap into the orbitrap. The HCD cell is filled with gas and is directly connected with the C-trap via an ion lens. Ions can get fragmented in the HCD cell by altering the offset of the RF rods and the axial field to achieve the necessary collision energy. The ion population then gets transferred back into the C-Trap from where the ions are transferred into the orbitrap.[102, 129]

The orbitrap mass analyzer consists of a central spindle-shape electrode, with ion attracting voltage, and an outer electrode that is cut in two pieces by an insulating ceramic ring (**Fig. 11**).[129]

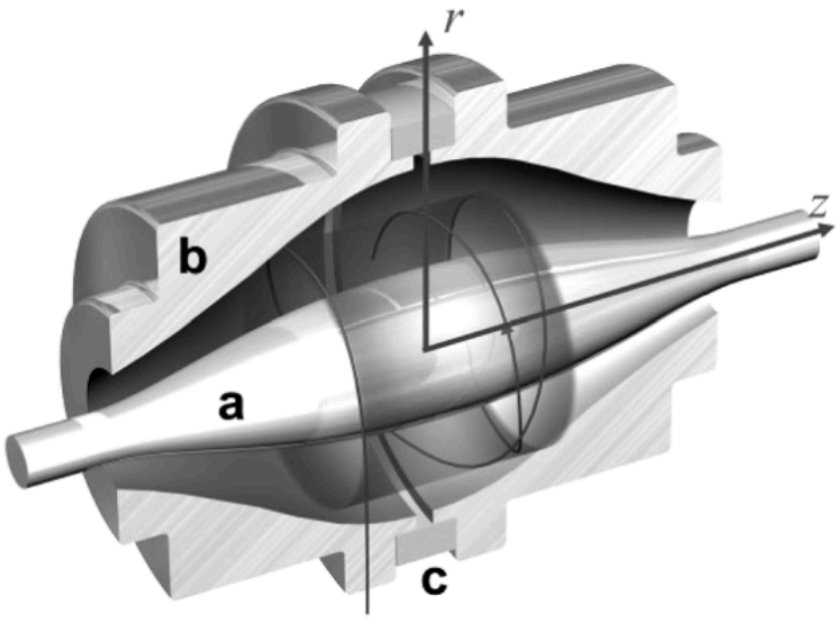

Figure 11: Schematic representation of the orbitrap mass analyzer. Where (a) is the central electrode (b) the outer electrode (c) an insulating ceramic ring that splits (b) into two halves.[129]

A differential amplifier is attached to the halves of the outer electrode and detects the image current stemming from moving ions. Ions entering with a high specific kinetic energy will be forced to move around the wire in complex spiral patterns due to the electric field and their tangential velocity keeps them from hitting into the electrode. In addition to the oscillation around the wire, the electric field traps the ions on an axial movement. The frequency of the harmonic axial oscillation in the direction of the z-axis - ω_z - can be concluded from the image current detection. For each *m/z* value a sine wave is generated. ω_z is given as:

$$\omega_z = \sqrt{\frac{k}{m/z}}$$

where k is an instrumental constant.[129]

Since all ions have the same amplitude upon being injected tangentially into the orbitrap analyzer, the ions movement only varies in their frequency of axial motion, depending on their *m/z* values. Via Fast Fourier Transformation the image current signal is converted into the frequency domain and further to accurate *m/z* values. The longer the transient signal is recorded, the higher is the resolution of the obtained mass spectrum. Orbitrap instruments only use static electric fields and operate without magnetic fields or frequency excitements. The frequency of the axial oscillation is inversely proportional to the square root of the *m/z* values and proportional to the acquisition time [129], and thus, it is proportional to $(q/m)^{1/2}$, where q is the ion charge and m the mass. The mass resolution R can be calculated as:[129]

$$R = \frac{m}{\Delta m} = \frac{1}{2\Delta\omega_z}\sqrt{\frac{kq}{m}}$$

meaning, that the resolution drops less for molecules with increasing *m/z* values as compared to ion cyclotron mass spectrometers.[129]

The covered mass range in the Q Exactive HF (orbitrap mass analyzer) is *m/z* 50-6000. The maximal resolving is 240000 at *m/z* 200.[103]

2.3.3 Tracefinder™

This commercial software (Thermo Fisher Scientific™) is designed for routine data acquisition and the qualitative screening and quantification of target molecules in measured data. Depending on the purpose, different methods can be chosen from given templates or one can quickly set up a new method. In the method development section of the software, a list of target compounds can be submitted to create an in-house compound database in order to screen for or quantitate target molecules. Therefore, the user feeds the database with information like *m/z* values, retention time, polarity and species e.g. $[M+H]^+$. Moreover, peak detection settings can be specified in the "Method Development" section. The user can set certain criteria the target molecules within a sample need to meet. A green flag then means all user-set criteria have been met, whereas a red flag demonstrates that the sample exceeds or fails the user-defined criteria. Therefore, positive and negative hits in the batch are highlighted.[104,105]

2.3.4 Compound Discoverer™

Thermo Fisher Scientific™ Compound Discoverer™ software offers many tools to analyse mass spectrometry-based large-scale untargeted metabolomics data. The software allows the identification via online or local database searches of MS^2 spectra, pathway mapping and interactive statistic tools like PCA, PLSDA and differential analysis. The online database search uses mzCloud, Chemspider, KEGG and BioCyc. The workflows are modular and the included workflow templates can be modified in order to address different applications.[106]

3. Materials and Methods:

3.1 Materials

3.1.1 Material

- 10 µl Graduated Tips (TipOne)
- 200µl and 1000 µl Tips (Greiner Bio-One)
- Chocolate Bar, 70% Cocoa (Lindt Excellence)
- Chocolate Bar, 85% Cocoa (Hofer Choceur)
- Cocoa Powder, heavily de-oiled (Naturata)
- Coffee Capsules (Tchibo "Espresso kräftig")
- Eppendorf Safe-Lock Tubes 0.5 ml (Polypropylen)
- Eppendorf Tubes 1.5 ml (Polypropylen)
- Falcon Tubes 15 ml (High clarity Polypropylene Conical Tube)
- Falcon Tubes 50 ml (High clarity Polypropylene Conical Tube)
- FreeStyle Lancets (Abbott)
- Glass Vials, 8 ml (Scherf Präzision Europa GmbH)
- Glass Vials, N9, 1.5 ml, Screw Neck (Macherey-Nagel)
- Inserts, 15 mm tip (Macherey-Nagel)
- Kinetex LC-column (2.6 µm XB-C18;100 x 2.1 mm; 100 Å)
- Plastic Bags (VWR Chemicals)
- Precision Wipes, fuzz-free, 11x21 cm (Kimtech Science)
- Saftey-Lancet (Sarstedt)
- Screw Caps, Butyl/PTFE, (Kimble)
- Screw caps, center hole, PTFE (Macherey-Nagel)
- Set of Gilson Pipetman Pipettes (10 µl, 20µl, 100 µl, 200µl, 1000µl)

3.1.2 Reagents

- Acetonitrile HiPerSolv Chromanorm, LC-MS Grade (VWR Chemicals)
- Formic Acid (Sigma Aldrich)
- Formic Acid, LC-MS Grade (VWR Chemicals)
- Methanol HiPerSolv Chromanorm, LC-MS Grade (VWR Chemicals)
- N_2-gas
- Water (MilliQ Grade)

3.1.3 Standards

- 1-Methylxanthine(Aldrich)
- 1,7-Dimethyluric acid (Aldrich)

- 1,7-Dimethylxanthine (PX)(Sigma)
- 2-Hydroxycinnamic Acid (Aldrich)
- 3-(3-hydroxyphenyl)propionic acid (Sigma Aldrich)
- 3-Hydroxybenzoic Acid (Sigma Aldrich)
- 3-Methylcatechol (Aldrich)
- 3-Methylxanthine (Aldrich)
- 3,4-Dihydroxybenzoic Acid (Aldrich)
- 4-Ethylcatechol (Sigma Aldrich)
- 4-Hydroxybenzaldehyde (Fluka)
- 4-Hydroxybenzoic Acid (Sigma Aldrich)
- Caffeic Acid (Sigma)
- CF anhydrous (Fluka)
- CF-(trimethyl D9) (Aldrich)
- CA(Sigma Aldrich)
- Chlorogenic Acid (Sigma Aldrich)
- Gallic Acid (Sigma)
- Histamine (Sigma Aldrich)
- Melatonin
- N-Acetyl-Tryptophan
- Nicotinamide
- Nicotinic Acid (Fluka)
- p-Coumaric Acid (Fluka)
- Phenylacetic Acid (Sigma Aldrich)
- Pyrocatechol
- Pyrogallol
- Salicylic Acid (Sigma Aldrich)
- Syringic Acid
- TB (Sigma)
- TP (Sigma)
- Vanillic Acid (Fluka)
- Vanillin (Sigma Aldrich)
- Xanthine (Sigma Aldrich)

3.1.4 Instruments

- Centrifuge 5424 (Eppendorf)
- Duo Plus Package with miVac Duo, miVac Duo Pump and miVac SpeedTrap (Genevac SP Scientific)

- Microcentrifuge (Starlab)
- Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific)
- Thermomixer Comfort 1.5 ml (Eppendorf)
- Vanquish UHPLC System (Thermo Scientific)
- Vortex Genie 2 (Scientific Industries, Inc.)

3.1.5 Software

- Compound Discoverer 2.0 (Thermo Scientific)
- ChemDraw Professional 15.0 (Perkin Elmer)
- Prism 7.0 (Graphpad)
- Tracefinder 4.1 (Thermo Scientific)
- Xcalibur 4.0 (Thermo Scientific)

3.2 Methods

Extracts of coffee, cocoa powder and chocolate were prepared. Analytes were separated via HPLC with gradient elution on a reversed phase column, which was coupled online to an orbitrap mass spectrometer using an ESI source for positive and negative ionization and the mass-to-charge ratios of the analytes were detected with an orbitrap mass analyzer. Major compounds were identified using their fragment spectra and the raw data was loaded into the Thermo Fisher Scientific™ Compound Discoverer Software, compounds were manually checked using the online database *mzCloud* and verified using purchased analytical standards. Volunteers consumed coffee, cocoa powder and chocolate. Fingerprint samples were taken before and after coffee/cocoa powder/chocolate consumption. **Fig. 12** gives an overview about the order of the sample collection, sample preparation and analysis for finger sweat sample.

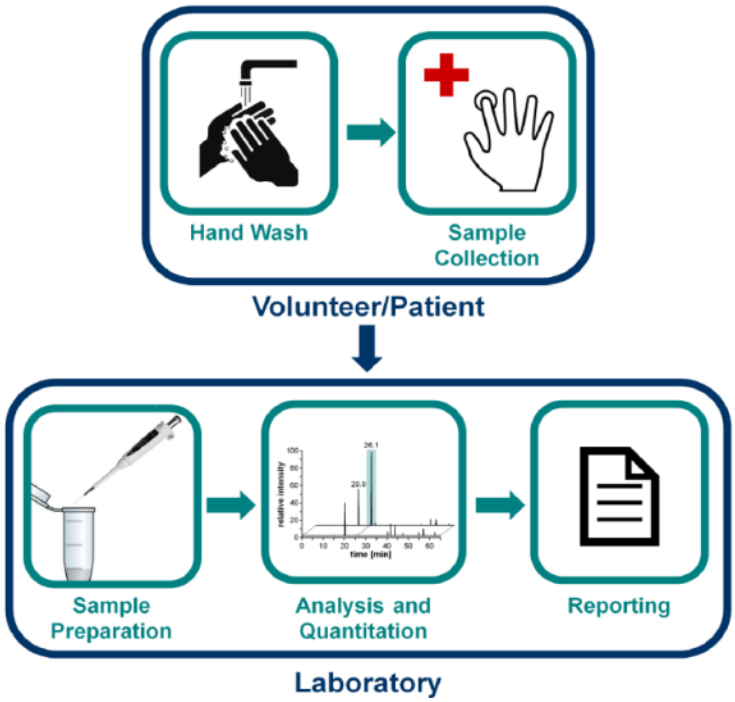

Figure 12: Workflow from taking fingerprint samples to getting the results

Finger sweat and whole blood samples were investigated for xenobiotics and their metabolites associated with chocolate and coffee consumption. Untargeted screening was conducted to identify as many features as possible. Of each individual both hands were sampled and analysed separately. Whole blood samples were taken at the same time points as the finger sweat samples and these two specimens were compared to each other. Again, compounds were identified using their fragment spectra, searching them against a database, controlled manually or confirmed using analytical standards. Moreover, the time-courses of the xenobiotics and their metabolites were studied.

3.2.1 Study design

In three different studies, donors consumed either coffee or chocolate/cocoa or both. This master thesis focuses on the xenobiotics present in coffee/chocolate consumption and therefore, mainly on methylxanthines (CF, TB, TP) and flavan-3-ols (CA and EC) as well as their metabolites (PX and monomethylxanthines) after the ingestion of coffee and chocolate. **Table 2** sums up the three studies.

Table 2: Overview of the three different studies during this master thesis

	Name	Short description
1.	Preliminary study	• Donors consumed either chocolate or coffee • One person was sampled without having consumed anything (control) • Sampling time was performed over a time span of 180 min
2.	Cocoa Powder/ Chocolate study	• Donors received either chocolate or cocoa powder • Sampling time was performed over a time span of 42 hours
3.	Simultaneous Coffee and Chocolate Consumption	• Donors consumed both coffee and chocolate • Sampling time was performed over a time span of 120 min

The preliminary study revealed the presence of these metabolites in fingerprints. A method was developed for the extraction from fingerprint and whole blood samples as well as for the separation and analysis of these compounds (by other bachelor and master students).

In the second study, four volunteers (two males and two females, between the age of 24-28) consumed either 50 g dark chocolate (Hofer Choceur 85%) or 30 g cocoa powder (Naturata) dissolved in water on two subsequent days in order to screen for compounds and metabolites that are linked with the ingestion of cocoa and cocoa products as well as to study the long-term time-courses of these compounds in fingerprint and whole blood samples of the volunteers. Samples were taken at 0; 0,25; 1; 2; 7; 18; 19,25; 21; 22; 24; 26; 30 and 42 hours after cocoa/chocolate consumption.

In the third study, eleven healthy volunteers (as the data was made anonymous: donors A-K) participated. Among the volunteers, there were six men and five women between the age of 24 and 50. All of the volunteers were between non- to moderate coffee consumers and one was a non-chocolate consumer. The volunteers were asked to fast any source of CF (for example coffee and energy drinks) as well as chocolate/cocoa for 12 hours before the start of the experiment. Fingerprint- and whole blood samples were taken before coffee and chocolate consumption. Whole blood samples were drawn only from four donors. Each volunteer was asked to consume

a cup of coffee with two capsules (Tchibo Espresso kräftig) à la 7,5 g coffee and 50 g of a chocolate bar (Lindt Excellence 70%). Sampling time points were 0, 15, 30, 45, 60, 90 and 120 minutes after coffee/chocolate consumption. **Table 3** gives an overview of the donors and their different habits in coffee and chocolate consumption.

Table 3: Donors A-K (6 men and 5 women) between the age of 24 and 50 with different habits in coffee and chocolate consumption

Identifier	Gender	Habitualness Coffee	Habitualness Chocolate	took part in 1st study	Whole blood in 2nd study
Donor A	female	moderate coffee consumer	moderate chocolate consumer	no	no
Donor B	male	moderate coffee consumer	moderate chocolate consumer	no	no
Donor C	male	moderate coffee consumer	moderate chocolate consumer	yes	yes
Donor D	male	moderate coffee consumer	moderate chocolate consumer	no	yes
Donor E	female	moderate coffee consumer	moderate chocolate consumer	no	no
Donor F	female	non-coffee consumer	moderate chocolate consumer	yes	yes
Donor G	female	moderate coffee consumer	non-chocolate consumer	no	no
Donor H	male	moderate coffee consumer	moderate chocolate consumer	yes	no
Donor I	male	moderate coffee consumer	moderate chocolate consumer	no	no
Donor J	female	moderate coffee consumer	moderate chocolate consumer	yes	yes
Donor K	male	non-coffee consumer	moderate chocolate consumer	no	no

3.2.2 Sample preparation

3.2.2.1 Extraction of coffee/chocolate/cocoa

Coffee. From one cup coffee (about 250 ml) of one capsule Tschibo "Espresso kräftig" 1 ml solution was transferred into an Eppendorf tube. The sample was centrifuged for 10 min at 14680 rpm. After that, 10µl of the coffee solution were added to 990µl of an aqueous solution of 1pg/µl caffeine-D9 (CF-D9). Next, the sample was centrifuged again for 10 min at 14680 rpm. Finally, the coffee extract was diluted 1:100 and 1:1000.

Cocoa/Chocolate. 1g of the cocoa powder and chocolate were dissolved in 5 ml water. The samples were sonicated for an hour at 80°C. Samples were vortexed in between. The samples were stored at 4°C over night (to cure the fat). With a pipette, 1 ml fat free suspension was transferred into an Eppendorf tube and the samples were centrifuged for 10 min at 14680 rpm. After that, 100 µl of this solution were added to 900 µl of an aqueous solution of 1pg/µl CF-D9.

Next, the sample was centrifuged again for 10 min at 14680 rpm. The solution was again diluted 1:10.

3.2.2.2 Extraction from blood

Blood was drawn by pricking one's finger on the left hand with a lancet for self-collection. The protective cap was removed, the lancet was held against one's finger and then pressed into the skin. A volume of 20 µl of whole blood was collected with a pipette and transferred into an Eppendorf tube filled with 480 µl ACN. The mixtures were vortexed and then shaken on a Thermomixer at 40°C at 1400 rpm for 10 minutes. Then they were vortexed again. This procedure was repeated two more times. The samples were then centrifuged for 10 minutes at 14680 rpm.

250 µl of each sample were transferred into a new Eppendorf tube and the samples were dried using a SpeedVac. The dried samples were reconstituted in an aqueous solution of 250 µl 1 pg/µl deuterated CF-D9 containing 0.2% FA. The extracted blood samples were sonicated for 10 min and again shaken in a Thermomixer at 30°C at 1400 rpm for 10 min before they were transferred into glass vials with inserts. The whole extraction process for the blood samples took about 1 ½ hours. Samples were stored at 4°C prior to analysis.

3.2.2.3 Extraction from fingerprint samples

Filters were stamped out of a fuzz-free paper (Kimtech Science) through gouge and hammer. In order to avoid contamination, the fuzz-free paper was placed into a plastic bag before the filters were cut out. The diameter of the filters was about 1 cm (**Fig. 13**). Exactly one filter was placed into a 0.5 ml safe-lock Eppendorf tube. The filters were pre-wetted with 3 µl of water. The volunteers had to clean their hands using lukewarm water and were not allowed to use soap. They were asked to dry their hands with disposable paper towels. After that, they were sweating for 1 minute before the filters were placed between their index finger and thumb with a pincette (cleaned with 70% EtOH). They pressed their fingers onto the filter paper for another minute. Afterwards, the filters were transferred into the Eppendorf Tubes again. Each filter was extracted with an aqueous solution of 120 µl 1 pg/µl deuterated CF-D9 containing 0.2% FA. The extraction solution was pipetted up and down about 15 times prior to pelleting the filter paper with the pipet tip. The extracted samples were conveyed into glass vials holding inserts. The whole sample preparation and sample collection for a single fingerprint sample took only several minutes. Samples were stored at 4°C before analysis.

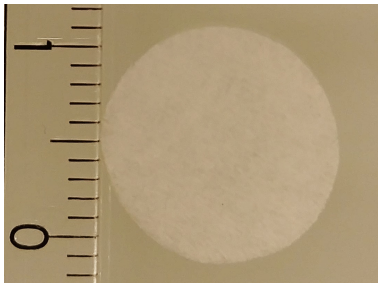

Figure 13: Dimensions of the filter paper used for fingerprint analysis

3.2.2.4 Internal Standards

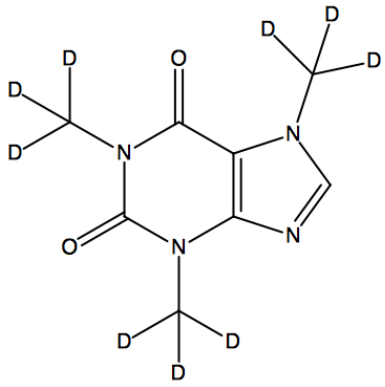

Figure 14: Structure for CF-(trimethyl-D9)

The stable isotope-labelled standard caffeine-(trimethyl-D9) (CF-D9)(**Fig. 14**) was used as the internal standard (IS) for the positive ion mode and was either spiked into the reconstitution solution (blood) or in the extraction solution (fingerprint) with a concentration of 1 pg/µl. It was used for normalization of the compounds. Moreover, a 1 pg/µl solution was injected into the LC-MS system every 4-5th sample as quality control. In the negative ionization mode, N-Acetyl-Tryptophan was used as the internal standard, however only in the 3rd study.

3.2.2.5 Standard Solutions:

A list of molecules of interest was created through literature research, leading to a dozen possible metabolites associated with the ingestion of coffee, chocolate and cocoa. Some of these molecules were purchased as analytical standards to test if these compounds could be analysed with the given gradient (**Section 3.3**). Other molecules have already been identified in sweat and standard solutions were used to confirm these compounds. Stock solutions of each standard were prepared with concentrations of 1 µg/µl. The standards were first dissolved in MeOH to give the stock solutions. After that, they were further diluted down to concentrations of 1pg/µl with water and only the last dilution step was performed with H_2O and 0.2%FA. Some of the standards (xanthine, 1-methylxanthine, 3-methylxanthine, 1,7-dimethyluric acid) did not dissolve in water very well and hence, they were sonicated and 0.2% FA had been added before the last dilution step. 10 µl of each standard were injected into the LC-MS. **Table 4** shows the standard solutions, their concentration and the amount of pg on column that could be detected.

Table 4: Purchased standards, that were dissolved in MeOH upon their arrival. They were further dissolved with water. The table lists their detectable concentrations and whether they could be detected in positive, negative or in both modes. N.d. stands for not detected.

Standard	Concen-tration [pg/µl]	pg on column	pos/neg mode	Standard	Concen-tration [pg/µl]	pg on column	pos/ neg mod e
1-Methylxanthine	10	100	both	**Histamine**	1	10	pos
1,7-Dimethyluric Acid	10	100	both	**Nicotinamide**	1	10	pos
1,7-Dimethylxanthine	1	10	pos	**Nicotinic Acid**	1	10	pos
3-(3-Hydroxyphenyl)-propionic Acid	10	100	neg	**o-Coumaric Acid (2-Hydroxy-cinnamic Acid)**	1	10	neg
3-Hydroxybenzoic Acid	10	100	neg	**p-Coumaric Acid**	1	10	neg
3-Methylcatechol	10	100	neg	**Paraxanthine**	1	10	pos
3-Methylxanthine	10	100	both	**Phenylacetic Acid**			n.d.
4-Ethylcatechol	10	100	neg	**Protocatechoic Acid**	1	10	neg
4-Hydroxybenzaldehyde	1	10	neg	**Pyrocatechol**	10	100	neg
4-Hydroxybenzoic Acid	10	100	neg	**Pyrogallol**	10	100	neg
Caffeic Acid	10	100	both	**Salicylic Acid**	1	10	neg
Caffeine	1	10	pos	**Syringic Acid**	10	100	pos
Catechine	1	10	both	**Theobromine**	1	10	pos
Chlorogenic Acid	10	100	pos	**Theophylline**	1	10	pos
Gallic Acid	10	100	neg	**Vanillic Acid**			n.d.
Gentisic Acid	10	100	neg	**Vanillin**	10	100	pos
Histamine	1	10	pos	**Xanthine**	10	100	neg

3.3 UHPLC-MS:

The UHPLC instrument (Thermo Fischer Scientific™ Vanquish) was equipped with a LC column from KINETEX (2.6 µm XB-C18;100 x 2.1 mm; 100 Å). The stationary phase of the column had iso-butyl side chains and a TMS endcapping. The solid support of this column was core-shell silica.[128] The injection volume was 10 µL and a flow-rate of 0.5 ml/min was used. The autosampler was thermostatted at 4°C and the column chamber, the post column cooler as well as the pre-heater were held at 40°C. Mobile phase A consisted of water with 0.2% FA and mobile phase B was methanol (MeOH) with 0.2% FA. A 10% MeOH solution was applied for needle washing as well as for backflushing the pistons. The gradient for the experiments is given in **Fig. 15** and outlined in **Table 5**, constituting of an overall run time of 7.5 min.

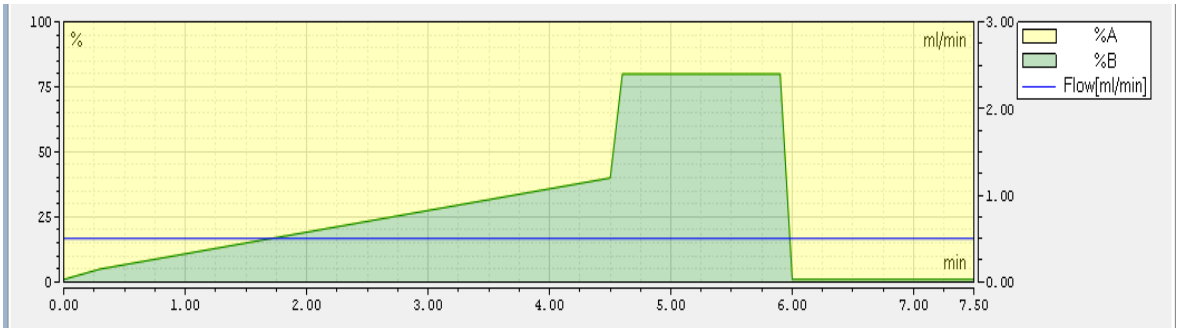

Figure 15: The gradient that was applied for all samples of this master thesis.

Table 5: Gradient for the separation of fingerprint- and blood samples

Time [min]	% A (H_2O, 0.1% FA)	% B (MeOH, 0.1%FA)	Flow [µl/min]	Curve
0.00	99.0	1.0	500	5
0.30	95.0	5.0	500	5
4.50	60.0	40.0	500	5
4.60	20.0	80.0	500	5
5.90	20.0	80.0	500	5
6.00	99.0	1.0	500	5
7.50	**Stop Run**			

The UHPLC was directly coupled to Thermo Fisher Scientific™ Q Exactive HF – a hybrid quadrupole-orbitrap mass spectrometer using an ESI source for ionization, applying positive as well as negative ion mode. Ions with *m/z* values between 100 and 1000 passed through the quadrupole mass filter incorporated in the Q Exactive HF unobstructed. First, a full scan of the ions was made by the orbitrap mass analyzer, resulting in MS^1 spectra. The four most abundant ions (top 4 method) of the full scan were selected for further fragmentation in a HCD collision cell applying 30 eV collision energy. In order to measure lower abundant molecules a dynamic exclusion for 6 seconds was applied. The resolution was set to 60.000 for the full MS scan and 15.000 for the MS^2. **Table 6** and **7** contain detailed MS-settings. In the 3rd study, for each sample two technical replicates were measured. In the other studies, only every 4-5 sample was measured twice as quality control.

Table 6: General Q Exactive HF mass spectrometer settings for the analysis of extracts from fingerprint and blood samples (MS1)

Properties of the method	
Use lock mass	best
Chromatographic peak width (FWHM)	3 s
Method duration	7.50 min

Full MS	
Runtime	0 to 7.5 min
Polarity	1. Positive 2. Negative
Resolution	60.000
AGC target	3e6
Maximum IT	50 ms
Scan range	100 to 1000 m/z

Table 7: General Q Exactive HF mass spectrometer settings for the MS^2 methods

Properties of the method	
Use lock mass	best
Chromatographic peak width (FWHM)	3 s
Method duration	7.5 min
Full MS/ dd-MS2	
Runtime	0 to 7.5 min
Polarity	1. Positive 2. Negative
Default charge	1
Inclusion	On
Full MS	
Resolution	30.000
AGC target	1e6
Maximum IT	50 ms
Scan Range	1. 100 to 260 m/z 2. 250 to 1000 m/z
dd-MS2/dd-SIM	
Resolution	15.000
AGC target	1e5
Maximum IT	50 ms
Loop Count	4
TopN	4
Isolation window	1.2 m/z
NCE	30
Apex trigger	1.6e4
Charge Exclusion	2-8, >8
Exclude isotopes	On
Dynamic exclusion	6.0 s
If idle ...	Pick others

3.4 Data Acquisition and Interpretation:

The raw data of measured samples was loaded into the Compound Discoverer Software (Thermo Fisher Scientific™). The used workflow tree was an untargeted metabolomics approach, through which compounds present in finger sweat samples and whole blood samples could be identified via an online database search (*mzCloud* Copyright © 2013 - 2017 HighChem LLC, Slovakia and *Chemspider* © Royal Society of Chemistry 2015). **Fig. 16** displays the employed workflow tree.

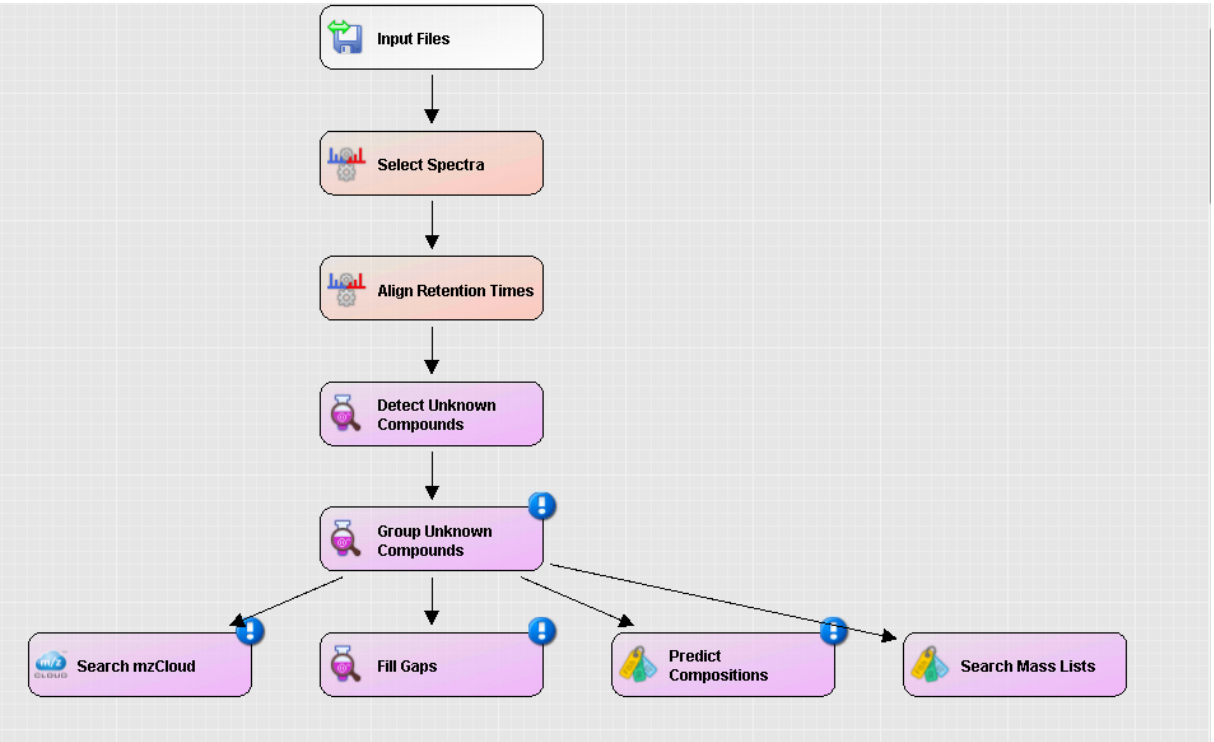

Figure 16: Workflow tree for the untargeted metabolomics analysis of the samples using the Compound Discoverer Software. Description: untargeted Metabolomics workflow: Retention time alignment, Component Detection, Grouping, Elemental Composition Prediction, Gap Filling, ID using *mzCloud* (needs MS/MS) and ChemSpider (using Formula); KEGG Pathway Mapping and Differential Analysis (ANOVA, adjusted p-values, fold change, CV, etc.)

The general settings for the Compound Discoverer Software are summed up in **Table 8**.

Table 8: General settings for the Compound Discoverer Software

Properties of the method	
Precursor Selection	Use MS(n-1) Precursor
Min. Precursor Mass	100 Da
Max. Precursor Mass	5000 Da
Mass Analyzer	Is FTMS
S/N Threshold (FT – only)	1.5
Alignment Model	Adaptive Curve
Maximum Shift [min]	1
Mass Tolerance	20 ppm

Each found compound was manually checked. Files were opened in the Xcalibur Software (Thermo Fisher Scientific™), using a general setting of 5-10 ppm mass tolerance and a mass precision of four decimals. All used reference spectra were found on "*mzCloud* – Advanced Mass Spectral Database, https://www.mzcloud.org" (effective November 2017).

The Tracefinder Software (Thermo Scientific) screened for the compounds included in the self-created compound database containing sweat constituents as well as metabolites associated with the consumption of coffee and chocolate. Peak areas were automatically obtained. Again, the peak picking of the software was checked manually. The data was exported as excel-files. **Table 9** sums up the settings used for the integration of the peaks of the analytes.

Table 9: General settings for the Tracefinder software

Tracefinder 4.1 software settings		
Method Type	Quan by Selecting compounds from CDB	
Compound Database	Self-created databases	
MS^1/MS^2	Only MS^1 with retention time alignment	
Feature	**Parameter**	**Value Setting**
Peak	Detection Algorithm	ICIS
	Peak Detection Strategy	Nearest RT
	Window (sec)	6.00
	Peak threshold type	Area
	Area to noise factor	5
	Peak noise factor	10
	Mass precision	5
	Mass tolerance	10 ppm

3.5 External Project:

During this master project, external fingerprint samples received via mail have also been analysed. The external project, which studies caffeine-induced effects on sleep and cognitive performance, is in cooperation with the Centre of Chronobiology of the Psychatric Hospital of the University of Basel (CH) headed by Prof. Dr. Christian Cajochen. Individuals were asked to either consume CF-tablets or nothing. The sampling was divided into two parts: The donors took their samples themselves at home once a day or were stationed at the sleeping laboratory in Basel, where they were sampled more frequently. Individuals were sampled over the course of 28 days. The sample preparation and extraction procedure are identical as described in section **3.2.2.3**. Samples were also measured with the UHPLC Q Exactive HF system as described above. Since the main goal of this external project was to measure and quantify CF and its main

metabolites a shorter gradient that can be seen in **Fig. 17** and that is outlined in **Table 10** was used.

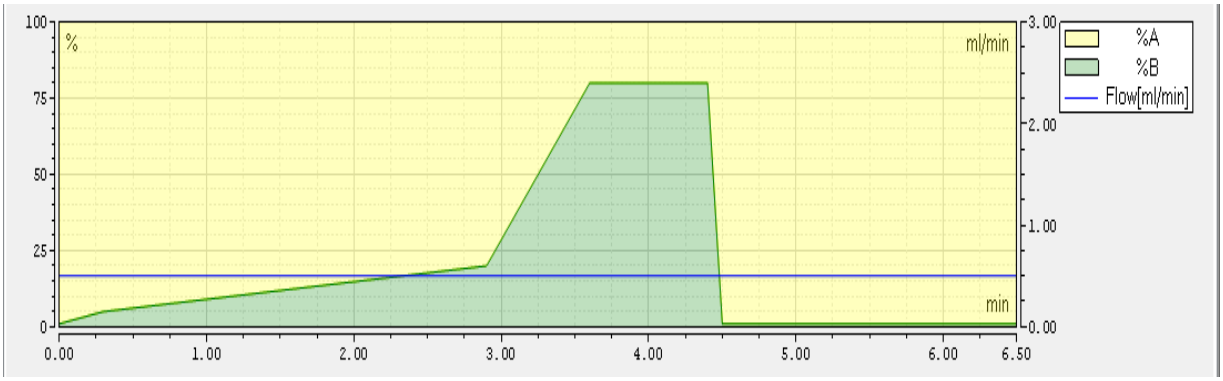

Figure 17: Gradient for the external samples

Table 10: Applied gradient for the external samples

Time [min]	% A (H_2O, 0.1% FA)	% B (MeOH, 0.1%FA)	Flow [µl/min]	Curve
0.00	99.0	1.0	500	5
0.30	95.0	5.0	500	5
2.90	60.0	20.0	500	5
3.60	20.0	80.0	500	5
4.40	20.0	80.0	500	5
4.50	99.0	1.0	500	5
6.50	**Stop Run**			

In order to quantify the amount of CF, TB, PX and TP on the filters, a calibration curve using standard solutions was generated. Stock solutions were prepared with concentrations of 1 µg/µl in H_2O and 0.2% FA. This stock solution was further diluted to give following concentrations: 500, 250, 200, 125, 100, 50, 25, 15, 10, 5, 1 and 0,1 pg/µl. These dilutions were each spiked with CF-D9 (1 pg/µl concentration in each solution) to which CF, TB, PX and TP were normalized to. The results were constituted in ng on the filter (as they were extracted with 120 µl of extraction solution). Data Acquisition and Interpretation were equal to section **3.4**. However, samples were only measured in MS1 mode. The only differences were that the method duration was set to 6.50 min and the scan range was from 150 to 1000 *m/z.*

4. Results and Discussion

In this master thesis extracts of coffee and chocolate were investigated regarding their composition using LC-MS. After compounds that were found in coffee and chocolate have been identified, healthy volunteers were asked to consume coffee and chocolate and their finger sweat and whole blood samples were analysed with an untargeted approach to find as many features as possible, mainly focusing on the compounds associated with coffee and chocolate intake. All compounds that have been detected in finger sweat and blood of the donors as well as in the extracts and that have been manually or experimentally checked are presented in this section. Moreover, the time-courses of xenobiotics (methylxanthines and flavan-3-ols) and their metabolites in the different biological specimens of individuals were analysed. Blood and finger sweat samples were compared as well as the fingerprint samples of the left and the right hand. CF and its primary metabolites were (semi-)quantified in finger sweat samples with the help of a calibration curve. From these studies, it is investigated if individual metabolic parameters can be drawn from finger sweat samples and whether it is possible to reproducibly show the significant increase of the xenobiotics and their metabolites after the consumption of coffee and chocolate in all donors in their finger sweat.

4.1 Compounds Present in Coffee/Chocolate

The first step of this master thesis was to identify compounds that appeared in coffee and chocolate. **Figure 18** and **19** show the chromatograms of coffee and chocolate (measured in the positive ion mode) using the Xcalibur Software and applied layouts (extracted ion

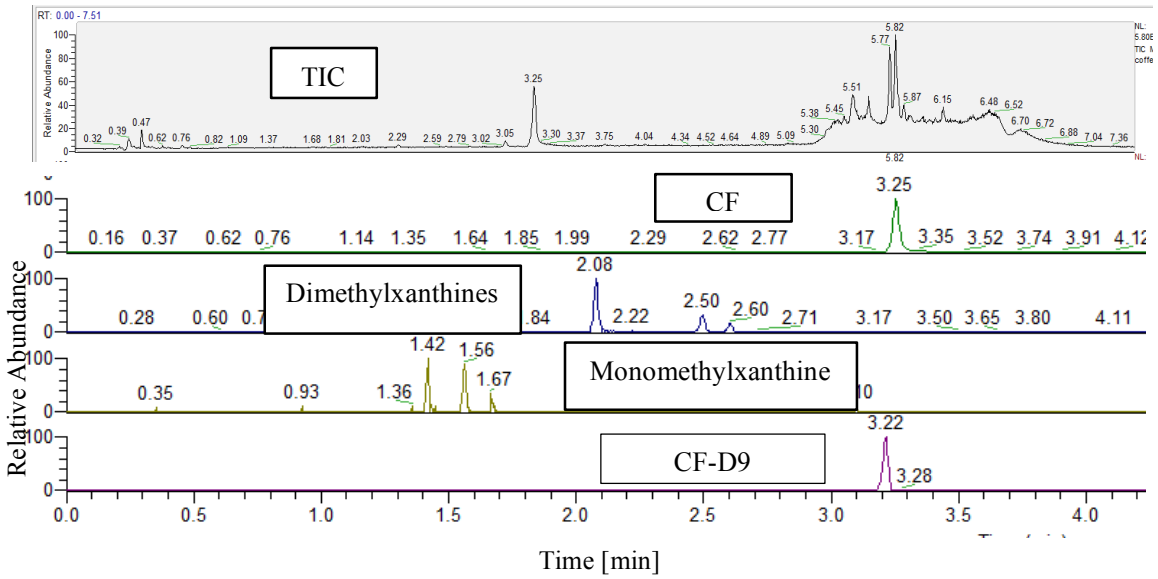

Figure 18: Chromatogram of the coffee extract measured in positive mode. The raw data was opened in Xcalibur, showing the total ion chromatogram as well as extracted ion chromatograms for CF, dimethylxanthines (TB, PX and TP), monomethylxanthines (1-, 3- and 7-methylxanthine) and the internal standard (CF-D9)

chromatograms). **Table 11** lists the compounds present in coffee and chocolate with their

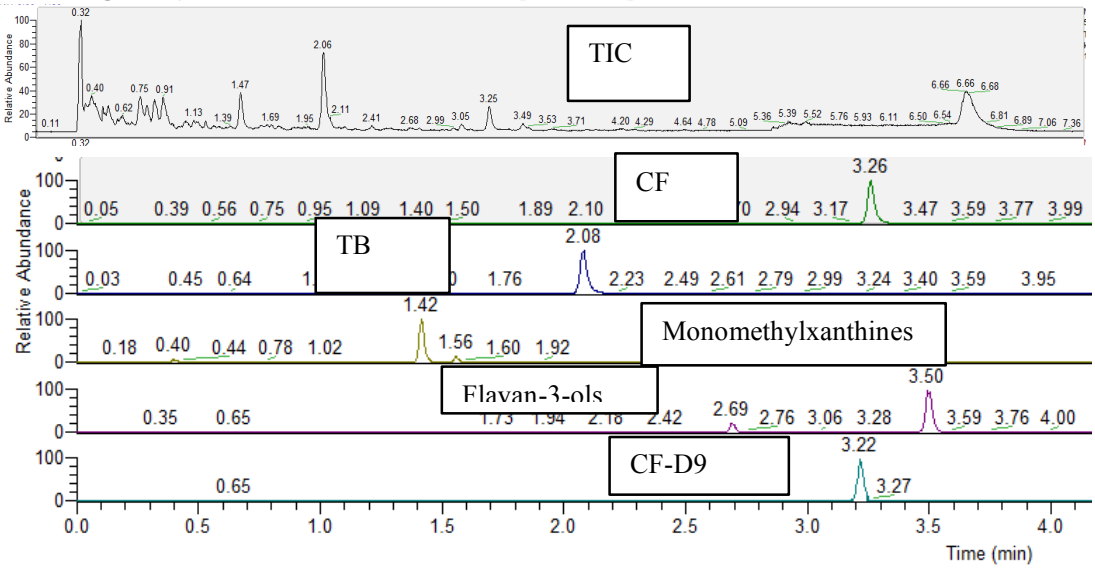

Figure 19: Chromatogram of the chocolate extract (which looked very similar to the cocoa extract) measured in positive mode. Applied layouts were for CF, TB, the monomethylxanthines (3- and 7-methylxanthine), the flavan-3-ols (CA and EC) and the internal standard (CF-d9)

corresponding retention times (which have been confirmed with standards or the online database mzCloud).

Table11: Compounds found in coffee/chocolate and their retention times

Compound:	Retention time	Coffee/ Chocolate	Compound:	Retention time	Coffee/ Chocolate
2-Hydroxycinnamic Acid	0,86	Chocolate	**Leucine/ Isoleucine**	0,91	Chocolate
1-Methylxanthine	1,67	Coffee	**Leucine/ Isoleucine**	0,85	Chocolate
3-Methylxanthine	1,56	Both	**Lysine**	0,34	Chocolate
7-Methylxanthine	1,42	Both	**Paraxanthine**	2,50	Coffee
Arginine	0,37	Chocolate	**Phenylalanine**	1,47	Both
Asparagine	0,38	Chocolate	**Proline**	0,44	Chocolate
Aspartic Acid	0,39	Chocolate	**Serine**	0,37	Chocolate
Caffeine	3,26	Both	**Serotonin**	1,04	Chocolate
Catechin	2,69	Chocolate	**Theobromine**	2,06	Both
Chlorogenic Acid	3,05	Coffee	**Theophylline**	2,60	Coffee
Choline	0,37	Both	**Threonine**	0,39	Chocolate
Epicatechin	3,47	Chocolate	**Tryptophan**	2,13	Chocolate
Glutamine	0,80	Chocolate	**Valine**	0,50	Chocolate
Lactose	0,48	Chocolate			

As an example, the MS^2- and the reference spectrum (taken from https://www.mzcloud.org/DataViewer#/Main/reference$23/T53%23Standard/Recalibrated/10523) for EC can be seen in **Figure 20**. In the same manner, the compounds derived from the Compound Discoverer Software were manually confirmed.

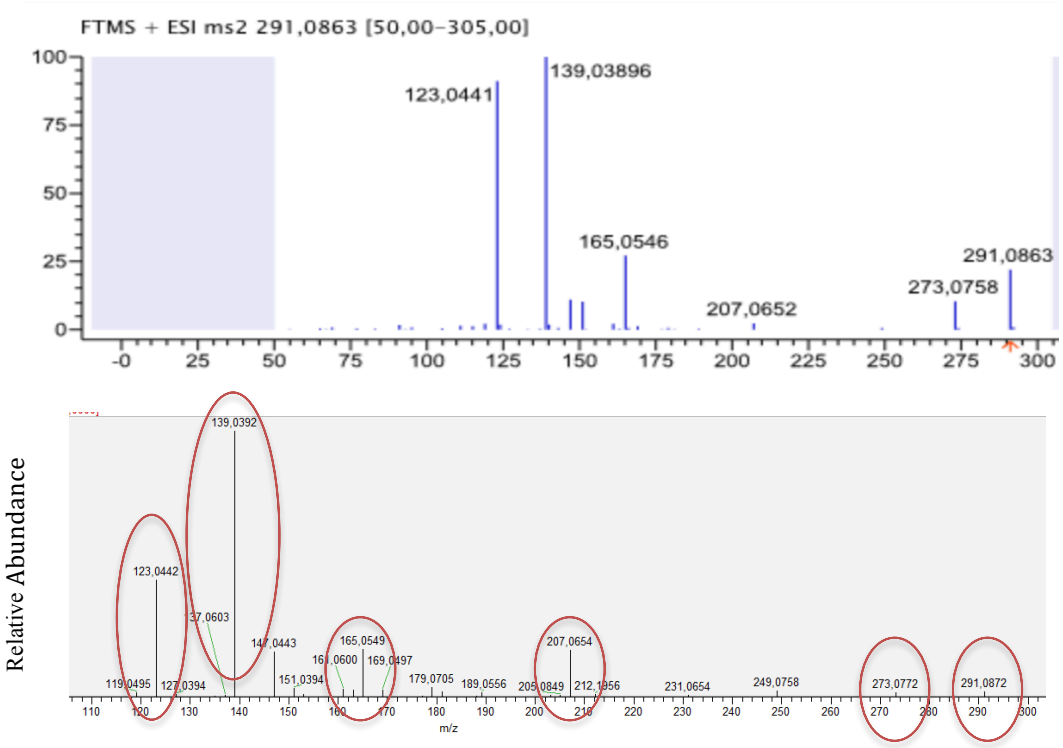

Figure 20: The reference spectrum on the top was taken from *mz*Cloud. On the bottom is the measured fragment spectrum of the chocolate extract. Consistent fragments are highlighted in red.

The chocolate extract showed also several compounds in the negative ion mode. The result can be seen in **Table 12**.

Table 12: Compounds present in chocolate measured in the negative ion mode and their retention times

Compound	Retention Time	Compound	Retention Time
2-Hydroxycaproic acid	3,64	**Fumaric acid**	0,99
3-Hydroxy-3-methylglutaric acid	1,10	**Glutamic acid**	0,40
3-Phenyllactic acid	4,02	**L-(+)-Tartaric acid**	0,45
Aspartic acid	0,38	**Malic Acid**	0,52
Catechin	2,69	**Protocatechuic acid**	1,84
Citraconic acid	1,49	**Succinic acid**	0,89
Citric acid	0,75	**Uric Acid**	0,70
Epicatechin	3,50	**Xanthine**	0,87

4.2 Compounds identified in Fingerprints corresponding to eccrine sweat:

Since samples in this master thesis were measured using an untargeted approach, not only compounds associated with food intake (chocolate and coffee ingestion) but also constituents commonly present in sweat were identified via the Compound Discoverer Software. Most of the compounds according to the literature (amino acids, small metabolites, DNA/RNA, small peptides and so on) could be found in finger sweat samples and contaminations arising from cosmetics, food and other sources were detected. All identified compounds, manually confirmed or verified with analytical standards, are summed up in **Table 13**.

Table 13: List of compounds found in human sweat with the described methods in Chapter 3. With the aid of the precursors, it can be seen whether the compound could be measured in positive, negative or both mode/s. R.t. stands for retention time.

Compound	Chemical Formular	Mono-isotopic Mass	pos Pre-cursor [M+H]+	neg Pre-cursor [M-H]-	R.t. [min]
Amino Acids					
Arginine	$C_6H_{14}N_4O_2$	174,1117	175,1195		0,38
Aspartic Acid	$C_4H_7NO_4$	133,0375	134,0453	132,0297	0,40
Carnitine	$C_7H_{15}NO_3$	161,1052	162,1130		0,39
Histidine	$C_6H_9N_3O_2$	155,0695	156,0773	154,0617	0,36
Isoleucine/Leucine/ Norleucine	$C_6H_{13}NO_2$	131,0946	132,1025		0,85 0,92 0,95
L-(+)-Citrulline	$C_6H_{13}N_3O_3$	175,0957	176,1035		0,42
L-Glutamic Acid	$C_5H_9NO_4$	147,0532	148,0610	146,0454	0,41
L-Phenylalanine	$C_9H_{11}NO_2$	165,0790	166,0868	164,0712	1,52
Lysine	$C_6H_{14}N_2O_2$	146,1055	147,1134		0,37
Methionine	$C_5H_{11}NO_2S$	149,0511	150,0589		0,63
Ornithine	$C_5H_{12}N_2O_2$	132,0899	133,0977		0,36
Pipecolic Acid	$C_6H_{11}NO_2$	129,0790	130,0868		0,59
Proline	$C_5H_9NO_2$	115,0633	116,0711		0,42
Serine	$C_3H_7NO_3$	105,0426	106,0504	104,0348	0,37
Taurine	$C_2H_7NO_3S$	125,0147	126,0225	124,0068	0,40
Threonine	$C_4H_9NO_3$	119,0582	120,0660	118,0504	0,40
Tryptophan	$C_{11}H_{12}N_2O_2$	204,0899	205,0977	203,0821	2,13
Tyrosine	$C_9H_{11}NO_3$	181,0739	182,0817	180,0661	0,89
Valine	$C_5H_{11}NO_2$	117,0790	118,0868		0,54
Amino Acid Derivatives/PTMs					
L-Pyrogluatmic Acid	$C_5H_7NO_3$	129,0426	130,0504		0,82
Methionine Sulfoxide	$C_5H_{11}NO_3S$	165,0460	166,0538		0,41
Amino Acid Breakdown Products					
L-Kynurenine	$C_{10}H_{12}N_2O_3$	208,0847	209,0925		1,33
Urocanic Acid	$C_6H_6N_2O_2$	138,0429	139,0508		0,55 0,70
Dipeptides					
Glycl-L-Leucine	$C_8H_{16}N_2O_3$	188,1161	189,1239		1,48
N-Acetyl-DL-serine	$C_5H_9NO_4$	147,0532	148,0610	146,0453	0,55
Sugars / Sugar alcohols					
Arabinose	$C_5H_{10}O_5$	150,0528		149,0450	0,43
Galactose	$C_6H_{12}O_6$	180,0634		179,0556	0,41
Glucose	$C_6H_{12}O_6$	180,0634		179,0556	0,42
L-Iditol	$C_6H_{14}O_6$	182,0790	183,0868		0,43

Maltol	$C_6H_6O_3$	126,0317	127,0395		0,40
Organic Acids					
Citric Acid	$C_6H_8O_7$	192,0270		191,0192	0,80
Fumaric Acid	$C_4H_4O_4$	116,0110		115,0031	
Malic Acid	$C_4H_6O_5$	134,0215		133,0137	0,54
Quinic Acid	$C_7H_{12}O_6$	192,0634		191,0556	
Sorbic Acid	$C_6H_8O_2$	112,0524	113,0603		4,62
Tartaric Acid	$C_4H_6O_6$	150,0164		149,0086	0,44
Dicarboxylic Acids					
Azelaic Acid	$C_{12}H_{22}O_4$	230,1518		229,1440	5,32
Dodecanedioic acid	$C_9H_{16}O_4$	188,1049		187,0970	5,55
Pimelic Acid	$C_7H_{12}O_4$	160,0736		159,0657	3,32
Sebacic Acid	$C_{10}H_{18}O_4$	202,1205	203,1283		5,41
Suberic Acid	$C_8H_{14}O_4$	174,0892		173,0814	4,54
DNA/RNA					
Adenine	$C_5H_5N_5$	135,0545	136,0623		0,51
Adenosine	$C_{10}H_{13}N_5O_4$	267,0968	268,1046		1,14
Dihydrothymine	$C_5H_8N_2O_2$	128,0586	129,0664		1,22
Metabolites involved in purine nucleosides degradation					
Hypoxanthine	$C_5H_4N_4O$	136,0385	137,0463		0,73
Inosine	$C_{10}H_{12}N_4O_5$	268,0808	269,0886		1,28
Steroids					
Progesterone	$C_{21}H_{30}O_2$	314,2246	315,2324		5,95
Vitamins					
Nicotinamide	$C_6H_6N_2O$	122,0480	123,0558		0,64
Nicotinic Acid	$C_6H_5NO_2$	123,0320	124,0398		0,61
Exogenous Compounds					
Acesulfam	$C_4H_4NO_4S$	161,9861	162,9939	160,9783	1,86
Butylparaben	$C_{11}H_{14}O_3$	194,0943		193,0865	5,52
Caffeine	$C_8H_{10}N_4O_2$	194,0804	195,0882		3,25
Catechin	$C_{15}H_{14}O_6$	290,0790	291,0868	289,0712	2,69
Climbazole	$C_{15}H_{17}ClN_2O_2$	292,0979	293,1057		5,37
Cyclamic Acid	$C_6H_{13}NO_3S$	179,0616		178,0538	3,54
D-(+)-Camphor	$C_{10}H_{16}O$	152,1201	153,1279		5,77
Epicatechin	$C_{15}H_{14}O_6$	290,0790	291,0868	289,0712	3,47
Nicotine	$C_{10}H_{14}N_2$	162,1157	163,1235		0,57
Nobiletin	$C_{21}H_{22}O_8$	402,1315	403,1393	401,1236	5,65
Panthenol	$C_9H_{19}NO_4$	205,1314	206,1392	204,1236	2,03
Paracetamol	$C_8H_9NO_2$	151,0633	152,0712		1,91
Rutin	$C_{27}H_{30}O_{16}$	610,1534	611,1612	609,1456	5,18
Saccharin	$C_7H_5NO_3S$	182,9990		181,9912	2,81
Salicylic Acid	$C_7H_6O_3$	138,0317		137,0239	5,03
Tangeritin	$C_{20}H_{20}O_7$	372,1209	373,1287		5,76
Theobromine	$C_7H_8N_4O_2$	180,0647	181,0726		2,06
Theophylline	$C_7H_8N_4O_2$	180,0647	181,0726		2,60
Triethanolamine	$C_6H_{15}NO_3$	149,1052	150,1130		0,38
Vanillin	$C_8H_8O_3$	152,0473	153,0551		3,52
Xylitol	$C_5H_{12}O_5$	152,0685	153,0763		0,44

Metabolites involved in CF/TB degradation					
1-Methylxanthine	$C_6H_6N_4O_2$	166,0491	167,0569	165,0413	1,69
3-Methylxanthine	$C_6H_6N_4O_2$	166,0491	167,0569	165,0413	1,59
1,7-Dimethyluric Acid	$C_7H_8N_4O_3$	196,0596	197,0674	195,0518	2,45
7-Methylxanthine	$C_6H_6N_4O_2$	166,0491	167,0569		1,45
Paraxanthine	$C_7H_8N_4O_2$	180,0647	181,0726		2,47
Uric Acid	$C_5H_4N_4O_3$	168,0283		167,0205	0,70
Xanthine	$C_5H_4N_4O_2$	152,0334		151,0256	0,92
Metabolites involved in EC/CA/Procyanidins degradation					
4-Hydroxybenzaldehyde	$C_7H_6O_2$	122,0368		121,0290	2,97
4-Hydroxybenzoic Acid	$C_7H_6O_3$	138,0317		137,0239	2,54
Caffeic Acid	$C_9H_8O_4$	180,0423	181,0501	179,0345	3,17
Chlorogenic Acid	$C_{16}H_{18}O_9$	354,0951	355,1029		3,03
Gentisic Acid	$C_7H_6O_4$	154,0266		153,0188	2,55
Hippuric Acid	$C_9H_9NO_3$	179,0582	180,0661		2,85
m-Coumaric Acid*	$C_9H_8O_3$	164,0473	165,0551		0,89
Protocatechuic Acid	$C_7H_6O_4$	154,0266		153,0188	1,84
Contaminants					
Dibutyl phthalate	$C_{16}H_{22}O_4$	278,1518	279,1596		6,07
PEG n5	$C_{10}H_{22}O_6$	238,1416	239,1495		2,65
PEG n6	$C_{12}H_{26}O_7$	282,1679	283,1757		3,13
PEG n7	$C_{14}H_{30}O_8$	326,1941	327,2019		3,57
PEG n8	$C_{16}H_{34}O_9$	370,2203	371,2281		0,75
Other compounds					
10-Hydroxydecanoic acid	$C_{10}H_{20}O_3$	188,1412		187,1334	5,68
2-Aminonicotinic acid	$C_6H_6N_2O_2$	138,0429	139,0507		0,550,70
3-tert-butylhexanedioic acid	$C_{10}H_{18}O_4$	202,1205		201,1127	5,40
6-Hydroxypicolinic acid	$C_6H_5NO_3$	139,0269		138,0191	0,74
Betaine	$C_5H_{11}NO_2$	117,0790	118,0868		0,40
Caprolactam	$C_6H_{11}NO$	113,0841	114,0919		2,58
Carbendazim	$C_9H_9N_3O_2$	191,0695	192,0773		2,40
Choline	$C_5H_{14}NO$	104,1075	105,1153	103,0997	0,38
Creatine	$C_4H_9N_3O_2$	131,0695	132,0773		0,41
Creatinine	$C_4H_7N_3O$	113,0589	114,0667		0,39
Decanamide	$C_{10}H_{21}NO$	171,1623	172,1701		5,71
Histamine	$C_5H_9N_3$	111,0796	112,0875		0,34
Melatonin	$C_{13}H_{16}N_2O_2$	232,1212	233,1290		4,65
Sphingosine	$C_{18}H_{37}O_2N$	299,2824	300,2903		5,56
Internal Standards					
N-Acetyl-Tryptophan	$C_{13}H_{14}N_2O_3$	246,1004	247,1077	245,0926	4,36
CF d9	$C_8D_9HN_4O_2$	203,2464	204,1441		3,20

* *Purchased analytical standards of p- and o-coumaric acid with corresponding retention times of 3,95 and 4,97 were measured. Thus, it is very unlikely that m-coumaric acid would elute at 0,89 even though the fragment spectrum agrees with the reference spectrum.*

Highlighted in green in **Table 13** are compounds that are present both in the extracts of coffee/chocolate and in fingerprint samples. Overall, they have 26 compounds in common, which can also be seen from the Venn diagram in **Fig. 21**.

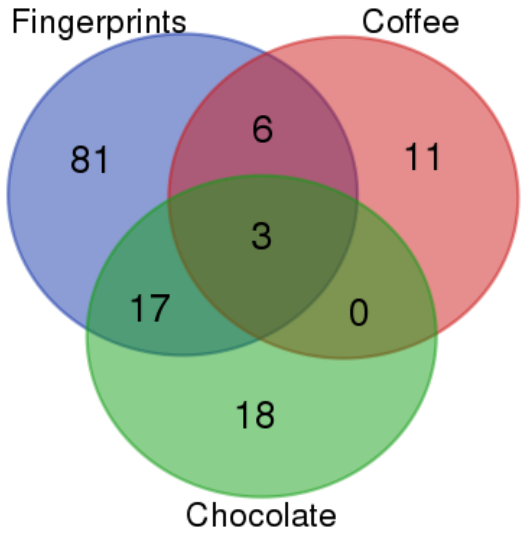

Figure 21: Venn diagram showing the number of mutual and unique compounds in fingerprint samples as well as the coffee and chocolate extracts (http://bioinformatics.psb.ugent.be/webtools/Venn/)

Not all compounds could be detected in every sweat sample and the detection highly depended on whether the donor had consumed certain products (for example: paracetamol – a drug to reduce pain and fever or nobiletin – a flavonoid found in citrus fruits) or not. However, the amino acids that elute at the beginning of the method (for the gradient see **Chapter 3.3**) were detectable in all sweat samples. Since most amino acids eluted in a very narrow retention time range it would be best to measure the samples on a HILIC LC-column in order to achieve better separation. Additionally, there was a great interest in measuring histamine in sweat samples as it is a prominent contributor to allergic diseases. Elevated levels of histamine in plasma and in tissue have been associated with anaphylaxis and allergic reactions of the skin, nose and airways.[107] Therefore, the fingerprint method would be a quick and easy way to determine if a person reacts allergic to a certain food, drug or cosmetic constituent.

The only smoker was identified through elevated nicotine levels in finger sweat samples. However, the found nicotine could also be due to direct contact with the cigarette. Nicotine is most likely a contamination rather than actually being present in fingerprints.

Melatonin was further detected in donors, however only in the morning and not during the day. Melatonin is a derivative of tryptophan and its production increases during darkness and decreases during light, which is also supported by the findings in this master thesis.[108] It modulates mood, sleep, sexual behaviour and circadian rhythm. Moreover, it is also involved in insomnia, migraine, obesity, epilepsy, cancer, diabetes as well as immune and cardiac disorders.[109,110]

Glucose and other sugars could be detected in the negative ion mode (**Table 13**). Studies revealed that sweat glucose levels can actually mirror blood glucose levels when collected properly (as skin surface glucose could distort the results of sweat glucose).[111] Sweat analysis would be more comfortable (as it is non-invasive) for diabetes patients who constantly have to stab themselves with lancets to measure their blood glucose levels.

Salicylic acid (SA) could be detected in all fingerprint samples even before coffee/chocolate consumption. SA is part of everyday life as it is a constituent in many daily necessities like

shampoos.[6] Similar to salicylic acid, pantothenol has also been detected in all samples. Pantothenol is used in cosmetics as humectant, emollient and moisturizer and thus, it is a frequent constituent of shampoos and hair conditioners. Vanillin is used as a flavouring agent in foods, beverages, and pharmaceuticals and hence, was also identified in all samples. Xylitol is used as a diabetic sweetener and yet, it is found naturally in many fruits such as strawberries and vegetables for example cauliflower. Consequently, xylitol was also found in many of the donors without having consumed products containing the synthetic sweetener.[112] It can be assumed that these compounds (SA, vanillin, pantothenol and xylitol) are more likely contaminants than arising from metabolism.

Donor J received cocoa powder with two sweetener tablets. These tablets contained saccharin and natriumcyclamate, both could be detected in the fingerprint samples of Donor J and concentrations peaked at 15 minutes after consumption both times. The finger sweat analysis seems to be a reliable tool to check if a person ingested synthetic sweeteners. For example, aspartame, which was also detected in fingerprint samples in the preliminary study (as described in the experimental section), has been in relation with harmful effects and finger sweat samples could be used to check for doses corresponding to toxic properties in patients.

Other contaminations arose from plastics among them dibutyl phthalate which is frequently used as plasticizer.[112]

Moreover, in the MS1 spectra ions with multiple charged states (+3, +4, +5) were found and it can be assumed that these ions are most likely small peptides, which need further investigation.

4.3 Methylxanthines and Flavan-3-ols in whole blood and fingerprint samples

In **Table 13** all compounds that have been identified in finger sweat samples are shown. As this master thesis focused on methylxanthines (CF, TB, TP) and flavan-3-ols (EC and CA), **Fig. 22** displays a fingerprint sample after chocolate consumption.

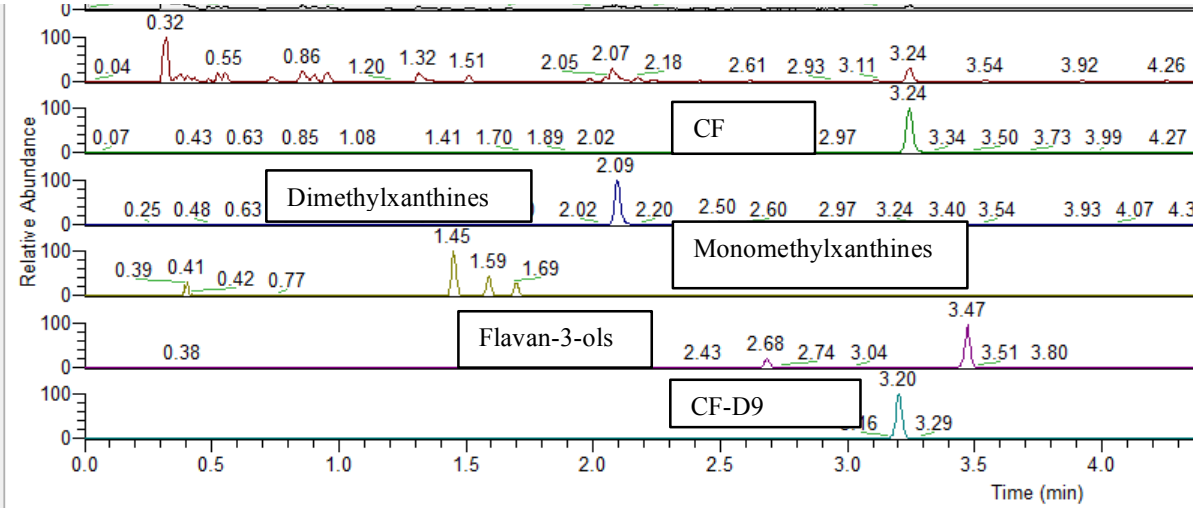

Figure 22: Chromatogram of a finger sweat sample after chocolate consumption. Major compounds that have been found in the extracts of coffee/chocolate could also be identified in fingerprints

4.3.1 Preliminary study

In order to demonstrate the significant increase in methylxanthines, flavan-3-ols as well as their metabolites, in the preliminary study Donor C was sampled at 0, 20, 40, 60, 90, 120 and 180 minutes without having consumed coffee or chocolate. Donor C was asked to skip food containing CF and TB for 12 hours and samples were taken after 12 hours of fasting. The time-course measurement of different metabolites of Donor C in finger sweat as well as in whole blood samples can be seen in **Fig. 23**. For the fingerprint samples the not dominant hand was chosen for display as the dominant hand often excreted less metabolites (which is consistent to the literature[65]). With two out of eleven donors (in the second study) the right hand excreted more metabolites, yet, for uniformity the left hand was displayed for all donors.

All fingerprint samples (from all three studies) were normalized to the internal standard (IS) (CF-D9 or N-Acetly-Tryptophan) as well as to creatinine. The normalization to the IS is necessary in order to compensate losses during sample preparation or variability during the measurement. The normalization to creatinine should eliminate variations in sweat rate and sweat amount. Creatinine is a waste product and the last step of creatine phosphate metabolism by skeletal muscle tissue. Its production is proportional to muscle mass and as a consequence, men tend to have higher levels of creatinine than women.[113] In healthy donors, creatinine excretion is quite constant and remains unchanged throughout the day as it is not affected by the diet.[114,115,116] In healthy individuals, the kidney is responsible for the elimination of creatinine in urine. Creatinine excretion is an indicator for kidney damage as blood creatinine levels increase when the kidney does not function properly.[117]

Creatinine was chosen as endogenous normalization factor as based on the literature it was assumed that the sweat creatinine excretion was constant within an individual and different across individuals. It has to be noted that urinary biomarkers are often expressed as the ratio of the biomarker concentration divided by the creatinine concentration and the normalization to creatinine is used to control different urinary flow rates.[118] Variations of creatinine secretion across individuals arise through weight and diet.[117]

Blood samples were only normalized to the internal standard as variations in sample volumes did not have to be compensated.

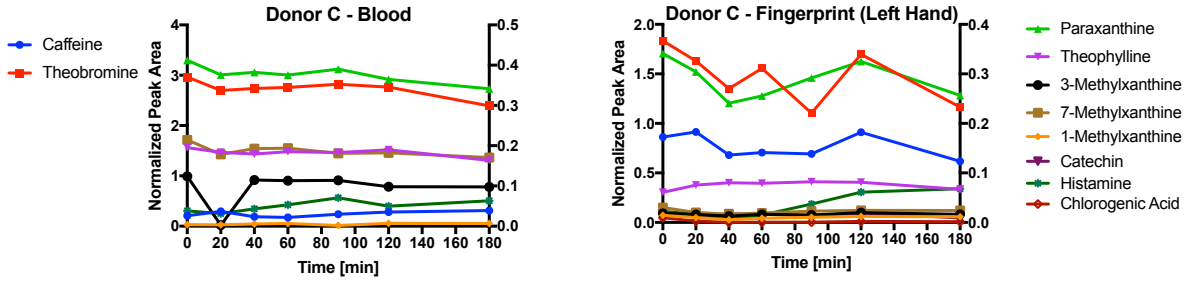

Figure 23: Donor C's whole blood and fingerprint samples over time without the consumption of coffee or chocolate. Only CF and TB are on the left y-axis, the other metabolites are displayed on the right y-axis.

As can be seen from **Fig. 23** blood as well as fingerprint levels of the metabolites are quite constant over time. In comparison **Fig. 24** and **25** show the time-course measurement of blood and finger sweat samples after the consumption of either coffee (donor D) or chocolate (donor K) sampled at 0, 20, 40, 60, 90, 120 and 180 min after ingestion.

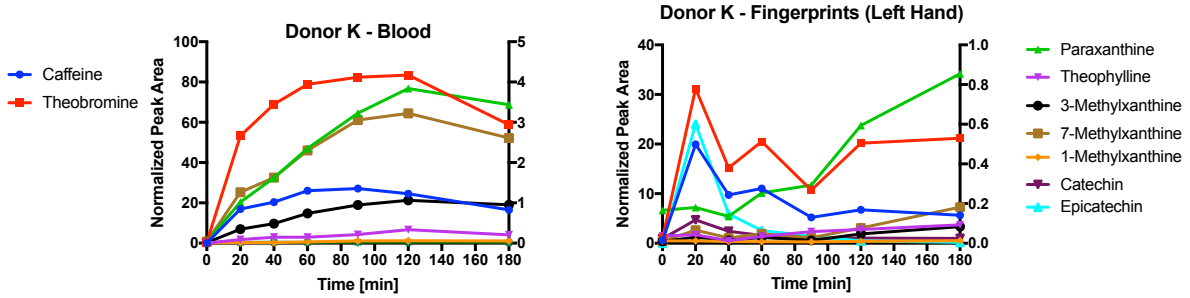

Figure 24: Donor K's whole blood and finger sweat samples after the consumption of chocolate. Only CF and TB are on the right y-axis, the other metabolites are displayed on the right y-axis.

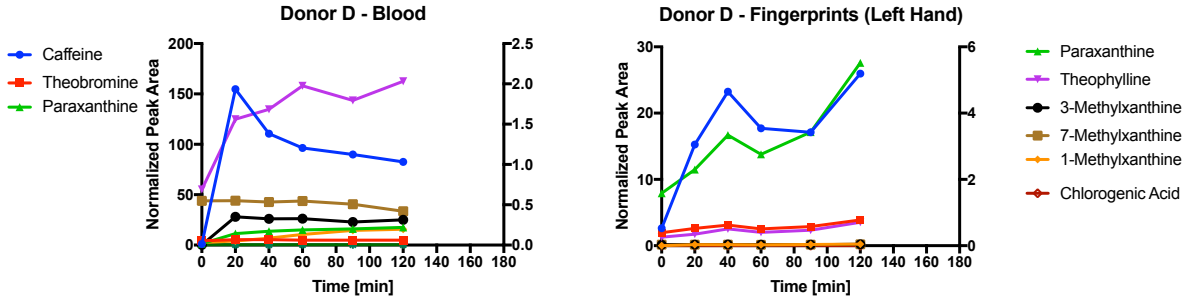

Figure 25: Donor D's whole blood and fingerprint samples after the consumption of coffee. CF and TB are displayed on the left y-axis.

Compared to Donor C, Donor K and Donor D have a significant increase of metabolite levels. In **Fig. 25** it can be seen quite clearly that as CF decreases, PX that is the primary metabolite of CF in the liver, increases. Moreover, having consumed chocolate, there is also an enhancement of 3- and 7-methylxanthine, which are also quite constant in Donor D, which leads to the assumption that 3- and 7-methylxanthine are more likely to arise from either being present in chocolate or from the metabolism of TB in the liver or gut microbiota. In addition, it was also clear from this

preliminary study that not all compounds present in finger sweat could also be detected in blood (e.g. EC and CA as well as chlorogenic acid) with the chosen extraction method.

4.3.2 Cocoa Powder/ Chocolate study over a time span of 42 hours

The experiment lasted for 42 hours. Donors received two doses of chocolate or cocoa powder: at the beginning of the first day and on the second day. From **Fig. 26** to **29** it can be seen that all chocolate related xenobiotics increase after ingestion of cocoa powder and chocolate, then decrease until the second dose of cocoa/chocolate was received. Moreover, the blood time-courses of all four donors look very similar as the metabolites increase and decrease parallel. Yet, fingerprint time-course analysis of the four donors vary more strongly in contrast to the blood samples. In addition, not all metabolites could be found in all four donors indicating different metabolism of the individuals. For example, EC levels in Donor C and F enhance with the consumption of chocolate/cocoa powder, whereas in donor H and J there is no accumulation. As 3- and 7-methylxanthine are found in the extracts of chocolate, it could be the case that they do not stem from the metabolism of the liver or gut but get absorbed like CF. However, in Donor F, it can be seen that 3- as well as 7-methylxanthine are not very likely to only arise from the ingestion of chocolate but also as a product of TB metabolism as there is a second enhancement of the two at the same time as TB decreases (indicated in the red circle in **Fig 27**). On the bottom of each figure, there is also the time-course of uric acid and xanthine, two metabolites that arise from the metabolism of CF and TB. Uric acid and xanthine could only be measured in negative ion mode and therefore, there was no normalization to creatinine which could only be measured in positive ion mode and to the internal standard (CF-D9). Hence, different sweat amounts could not be controlled. However, it can be seen that uric acid and xanthine enhance when TB, PX and CF decline, indicating that these two emerge from the metabolism as CF, PX and TB have first to be metabolized in order for xanthine and uric acid to show up. Additionally, longer sampling times were necessary in consideration of xanthine and uric acid to elevate significantly since in the other two studies performed during this thesis where the sampling times was only up to two hours their time-courses look nearly constant with fluctuations based on different sweat amounts.

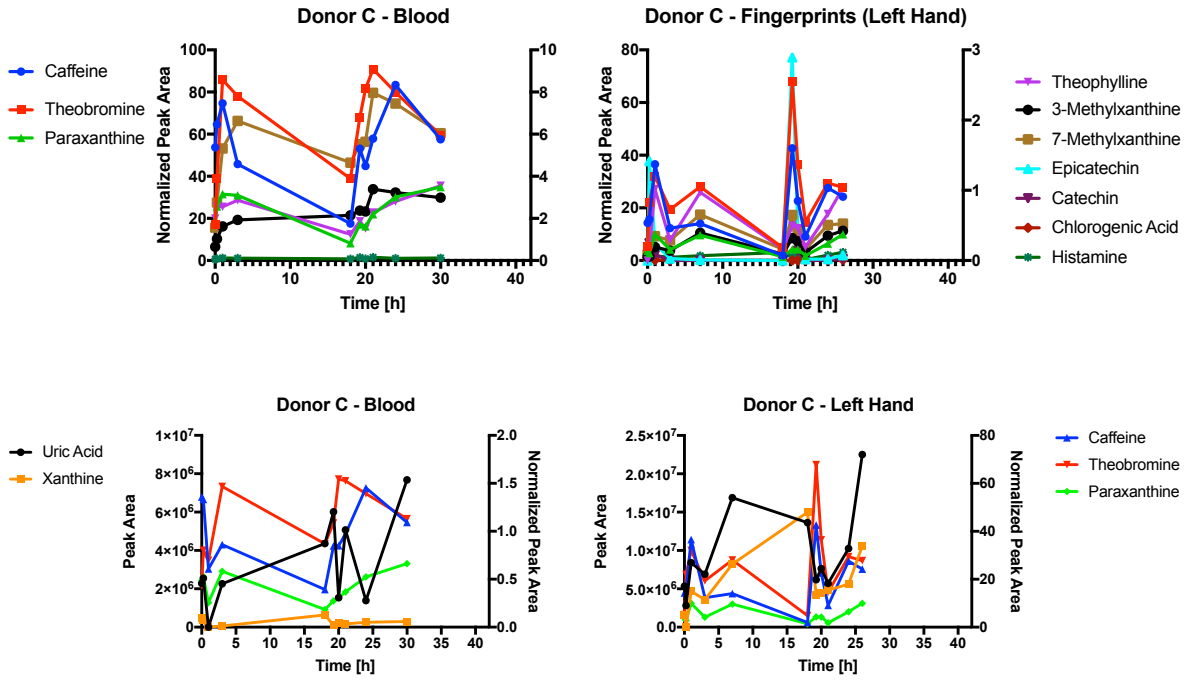

Figure 26: Donor C has received 50g of a chocolate bar. However, the experiment had to be cancelled as Donor C had health problems on the second day of the experiment, which were not related to this study. Top: CF, PX and TB are on the right y-axis. Bottom: uric acid and xanthine are on the left y-axis.

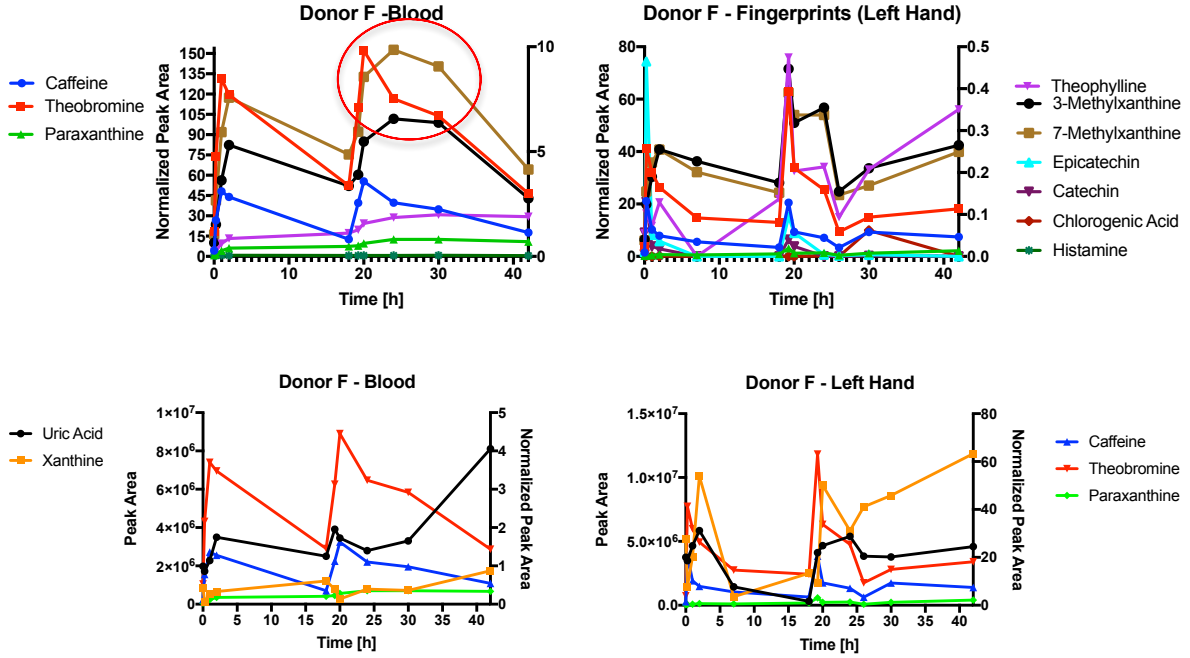

Figure 27: Donor F consumed 30g cocoa powder dissolved in water. Top: CF, PX and TB are on the right y-axis. Bottom: uric acid and xanthine are displayed on the left y-axis.

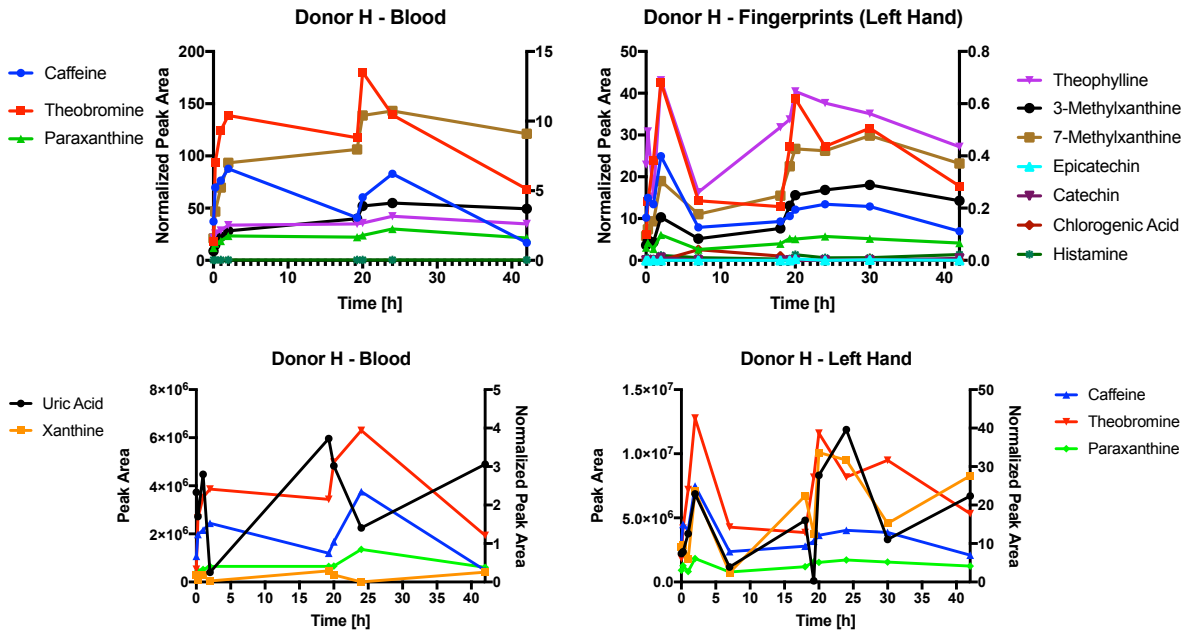

Figure 28: Donor H was asked to drink 30g cocoa powder dissolved in water. Top: CF, PX and TB are on the right y-axis. Bottom: uric acid and xanthine are on the left y-axis.

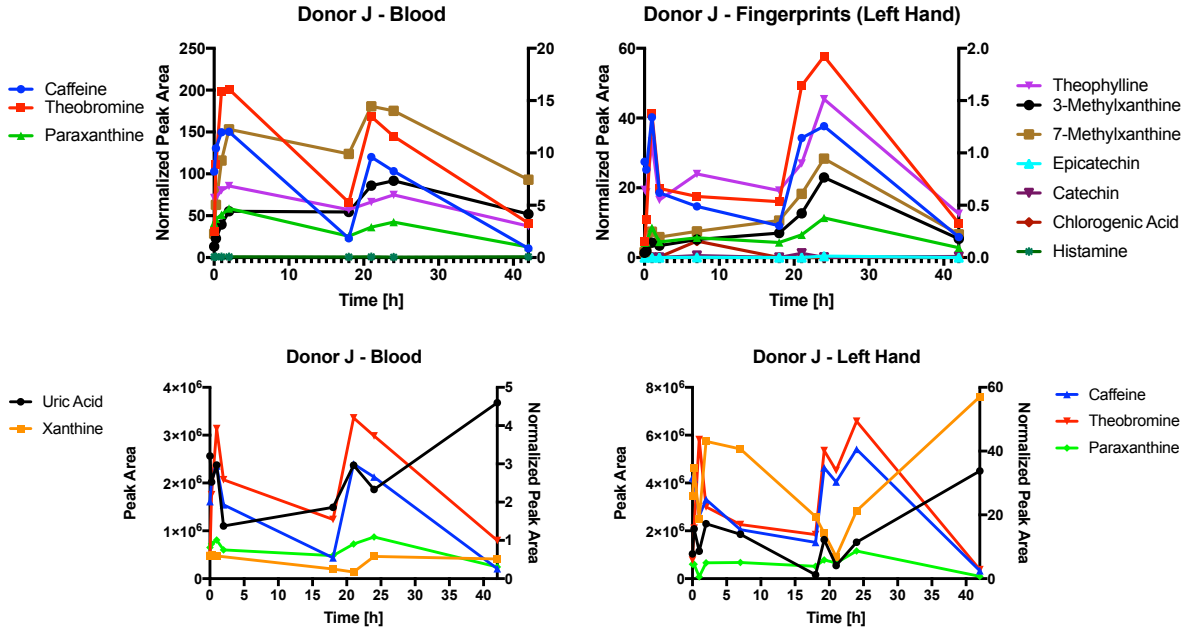

Figure 29: Donor J consumed 30g cocoa powder dissolved in water. Top: CF, PX and TB are on the right y-axis. Bottom: uric acid and xanthine are displayed on the left y-axis.

In the figures (**26-29**) above, it can be seen clearly that there is a significant increase of the metabolites after both doses of cocoa powder/chocolate, proving that the increase is associated with the cocoa/chocolate consumption and does not arise from other sources as in this study, donors were allowed to eat and drink anything before the study and during the study. Metabolite levels consequently elevated at each dose of chocolate/cocoa.

4.3.3 Simultaneous Coffee and Chocolate Consumption

In this study, all 11 donors (A-K) received two capsules of coffee and 50g of a chocolate bar (with the exception of Donor G who only received the coffee).

4.3.3.1 Analysis of Fingerprint samples and comparison of left-/right-hand samples

Fig. 30 to **40** show the left- and right-hand fingerprint samples of the 11 Donors. The bars indicate the average of the two technical replicates including error ranges of the two measurements. Error bars of the two technical replicates are displayed but are predominantly smaller than the height of the symbol.

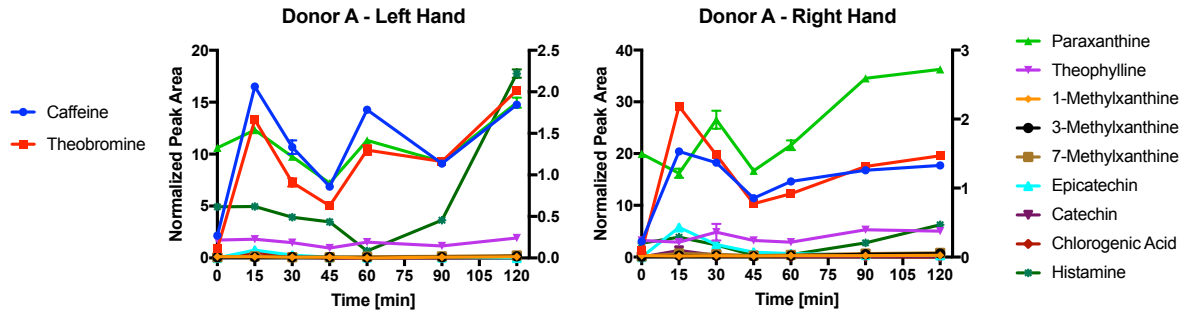

Figure 30: Left-and right-fingerprint samples of Donor A. Only CF and TB are displayed on the left y-axis.

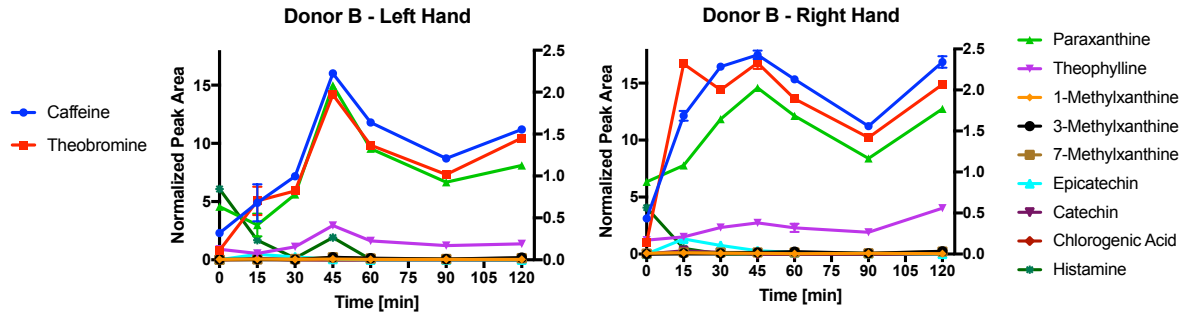

Figure 31: Left-and right-fingerprint samples of Donor B. Only CF and TB are displayed on the left y-axis.

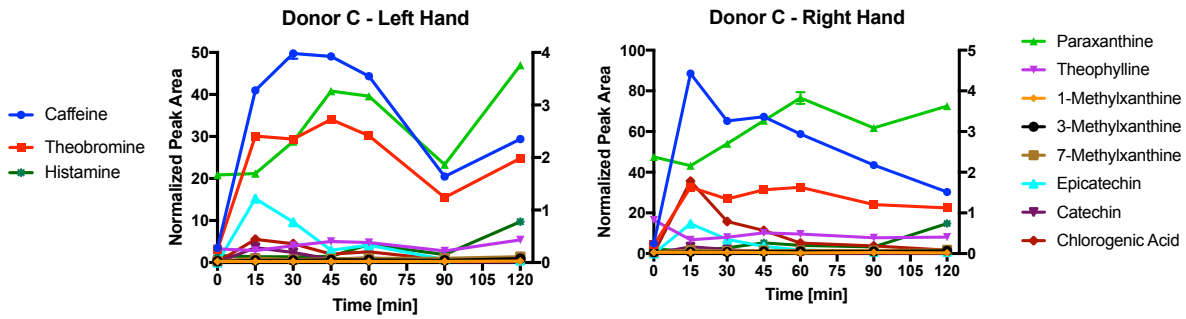

Figure 32: Left-and right-fingerprint samples of Donor C. Only CF, histamine and TB are displayed on the left y-axis.

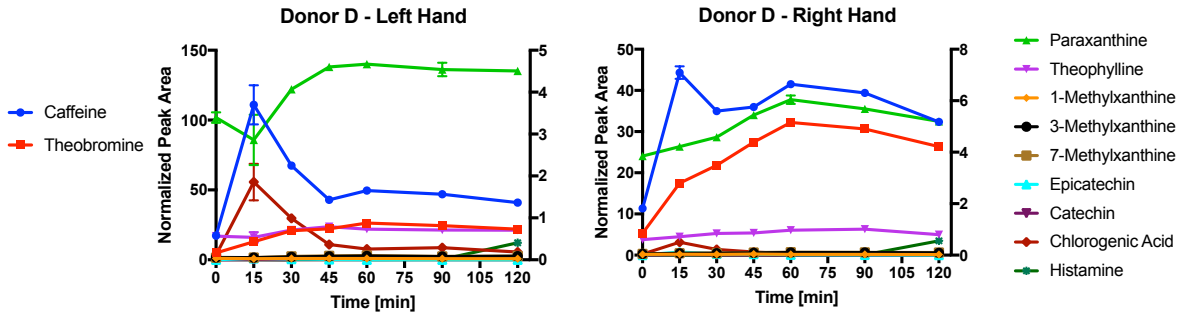

Figure 33: Left-and right-fingerprint samples of Donor D. Only CF, histamine and TB are displayed on the left y-axis.

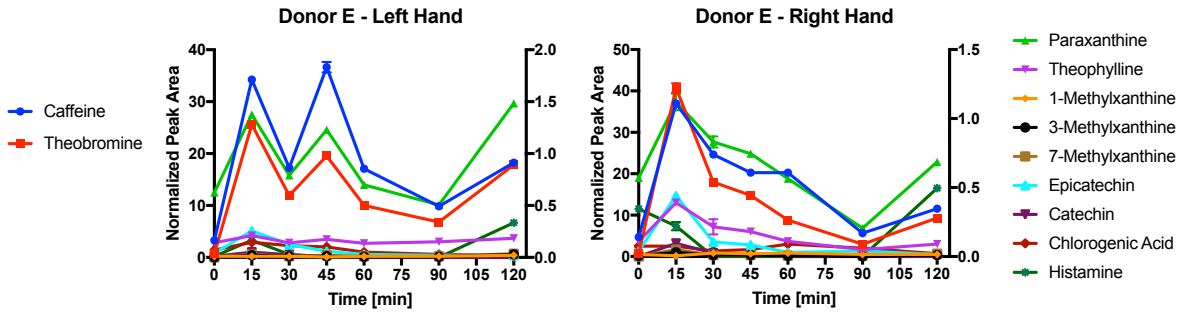

Figure 34: Left-and right-fingerprint samples of Donor E. Only CF and TB are displayed on the left y-axis.

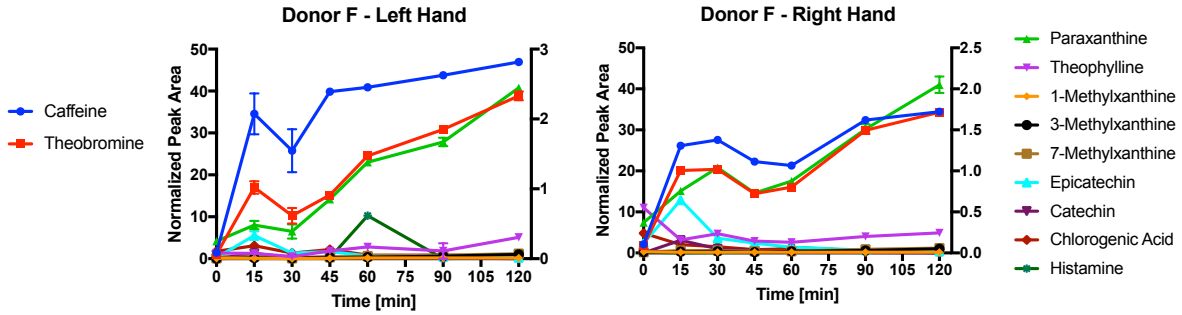

Figure 35: Left-and right-fingerprint samples of Donor F. Only CF and TB are displayed on the left y-axis.

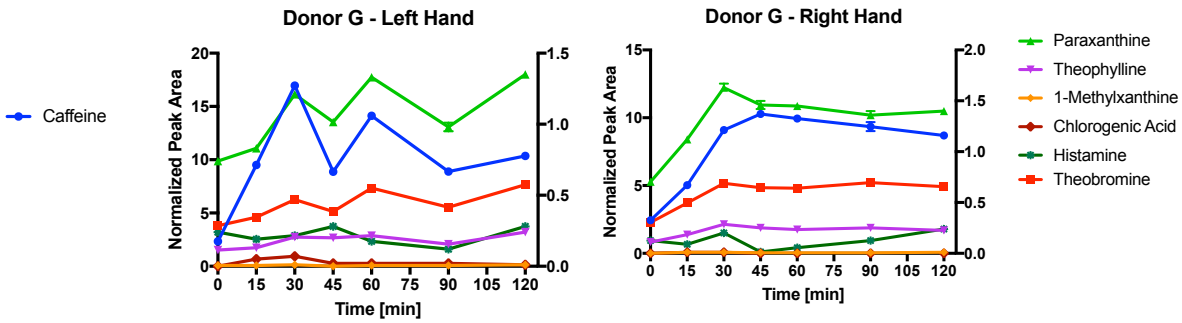

Figure 36: Left-and right-fingerprint samples of Donor G. Only CF is displayed on the left y-axis.

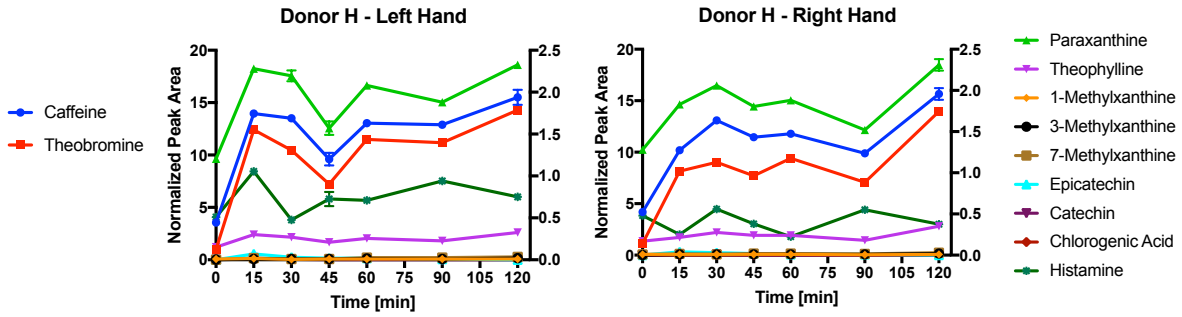

Figure 37: Left-and right-fingerprint samples of Donor H. Only CF and TB are displayed on the left y-axis.

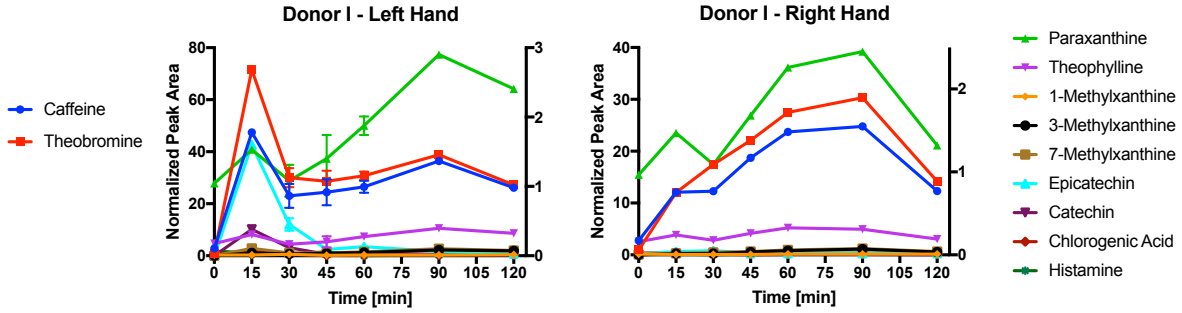

Figure 38: Left-and right-fingerprint samples of Donor I. Only CF and TB are displayed on the left y-axis.

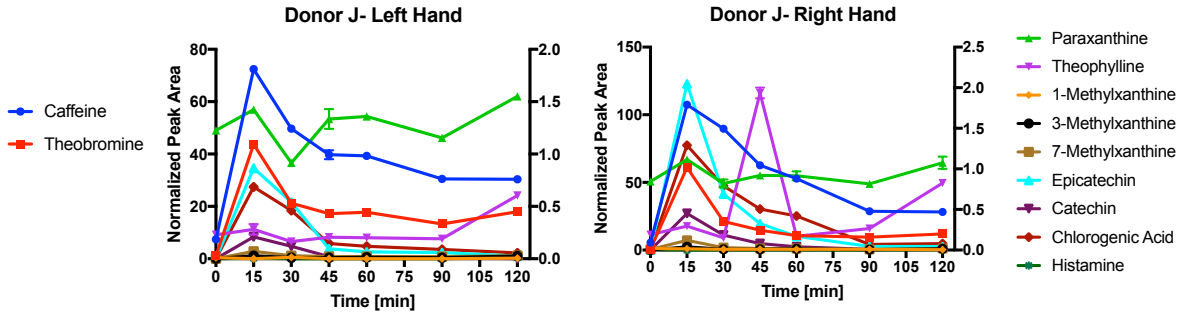

Figure 39: Left-and right-fingerprint samples of Donor J. Only CF and TB are displayed on the left y-axis.

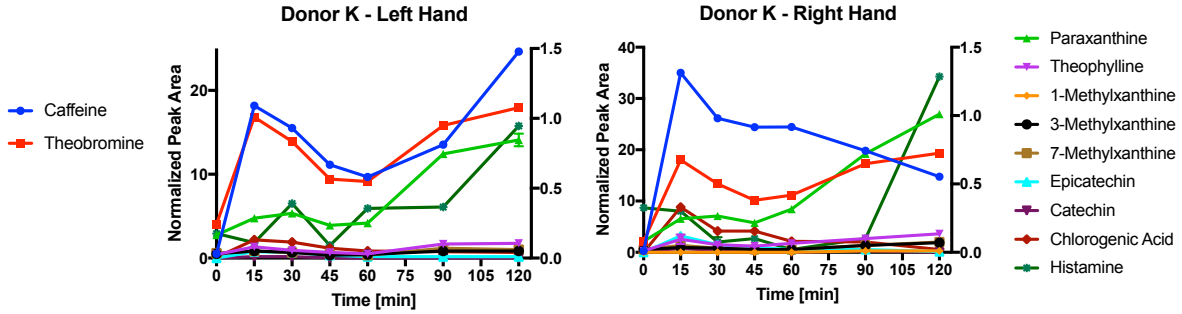

Figure 40: Left-and right-fingerprint samples of Donor K. Only CF and TB are displayed on the left y-axis.

All of the donors received the same amount of coffee and chocolate and hence, should absorb similar quantities of the metabolites. However, in every donor different amounts of metabolites were present in the finger sweat samples, which could be based on dietary pattern, health status or other lifestyle factors (e.g. regular sport activities). **Fig. 41** shows the time-course of CF and TB of various donors.

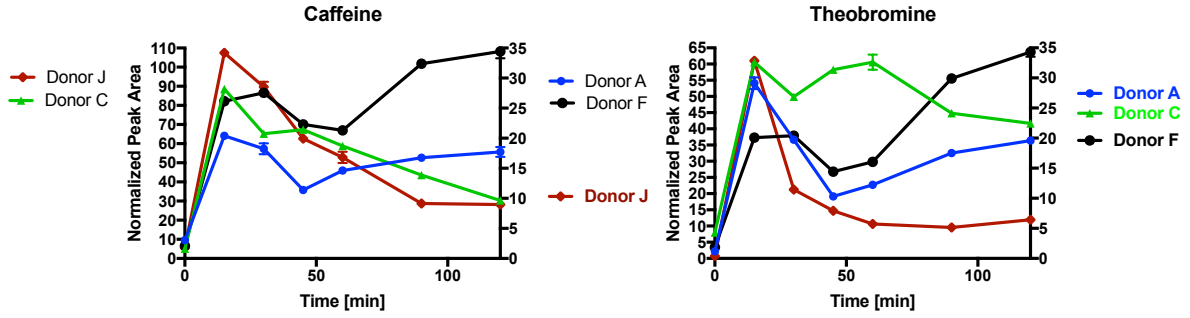

Figure 41: Displaying the different time-course of CF and TB from different donors. The legend in colour belongs to the graph on the right (where only Donor J is on the left y-axis).

Fig. 41 exhibits the different amounts that individuals absorb after coffee/chocolate consumption. The differences in CF metabolism are greater than with TB. A possible explanation could be that for example Donor F is a non-coffee consumer and therefore, is not as used to CF uptake and has a different metabolism based on dietary habitualness. Donor C and J are regular coffee consumers and have higher CF values and seem to absorb CF more efficiently, but also metabolise it more quickly.

In addition, CF and TB were chosen as an example to show a significant difference before and after coffee/chocolate consumption as they are the main constituents (of interest) present in coffee and chocolate. However, as one donor did not eat chocolate and hence the TB level of this donor remained almost constant over time, this person was not contemplated in the following graphics. **Fig. 42** and **43** show that CF and TB enhance significantly after coffee/ chocolate consumption in all 10 individuals. A t-test was performed for before (time point 0 min) and after coffee/ chocolate ingestion (time point 15 min) in order to determine the statistical significance.

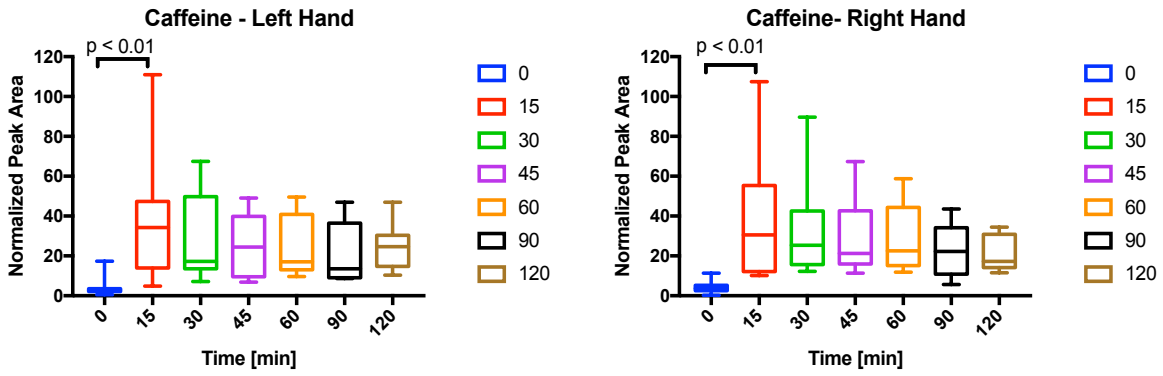

Figure 42: After coffee and chocolate intake, CF increases significantly in all 10 individuals. The t-test resulted in a value of p<0.01, meaning that column 0 (before consumption) and 15 (after consumption) are significantly different from each other.

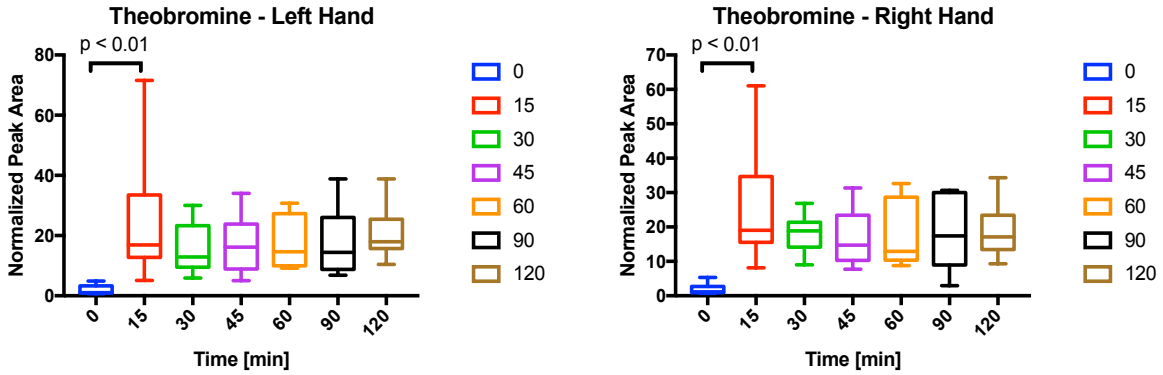

Figure 43: TB has an even higher concentration than CF in dark chocolate. This figure shows that TB increased significantly in all 10 donors. Moreover, a t-test was performed resulting in a calculated value of p <0.01, which means column 0 and column 15 are significantly different from each other.

During this master thesis, it became quite clear that the sweat compartment of the left and the right side differed from each other in spite of expectation. Looking at **Fig. 30** to **40**, the distinct hands do not only vary in their time-course but also in the normalized peak areas, meaning that one hand excretes higher levels of metabolites as the other (in most cases the non-dominant hand). What all donors have in common is that the peak concentrations of metabolites are reached very early on (around 15 minutes). Some donors exhibit a second peak at a later sampling time. However, this difference occurred in all 11 donors without exception. At first, it was believed that there was a factor that could adjust the right- to the left fingerprint values. Different factors were evaluated: the most promising had been dividing the average of all of the amino acids found in the left hand by the average of the right amino acid values to create factor x (formula below) for each sampling time point:

$$Left\ (respectively\ the\ recalculate\ Right) = Right * x$$

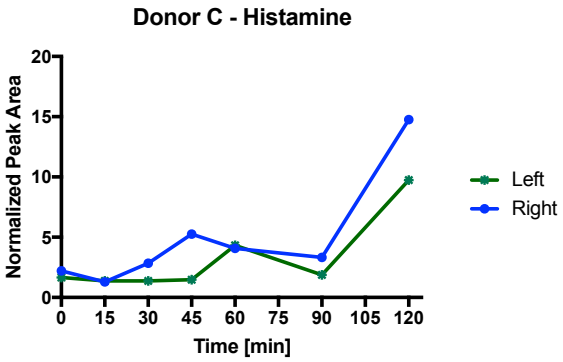

Figure 44: Histamine time-course analysis of Donor C

After multiplication, the time-course of both hand-sides looked more similar and yet, still very distinct from one another. Therefore, the conclusion can be drawn that the left and right fingerprint samples are different from each other, which could be due to a distinct number of sweat glands present on the fingers, different gland activity on each hand or could be an effect of the lymphatic system where the right-hand lymph fluid would drain into the right lymphatic duct and the left-hand lymph fluid would drain into the left lymphatic duct (thoracic duct).[119]

Another major goal of this master thesis was to determine how far individual metabolic differences can be drawn from fingerprint data. The data presented in the figures above prove that there are significant varieties across the individuals, which could be due to health status, age, sex, dietary preferences and gut microbiota that would arise through a specific dietary pattern. For example, donor G does not consume anything with more than 5% sugar in it and thus, does not consume chocolate, which can also be seen as the TB levels that only arise from CF metabolism (in the liver via CYP1A2 and CYP2E1) and are much lower compared to the other donors. Moreover, donor G lacks metabolites associated with chocolate intake (3-methylxanthine, 7-methylxanthine, EC and CA), proving once more that these metabolites stem from chocolate consumption or metabolized products of compounds present in chocolate. Another example of distinct metabolic parameters is the increase of histamine concentrations. Chocolate does not contain histamine but many other biogenic amines like tyramine, octopamine and phenylethylamine[18] and hence, chocolate is ranked in histamine intolerance advisers as indigestible and should be avoided by people who have a diagnosed histamine intolerance. As for instance, donor H has problems with histamine and his histamine level rises after the consumption of chocolate, whereas donor J's histamine concentration is unchanged and very low to begin with. Donor C is another volunteer that reported complications regarding histamine and the data shows that the histamine levels are up to 10 times higher than of the other individuals. Donor C was one donor that addressed nausea and dizziness during the experiment which could be connected to the increase of histamine concentration. **Fig. 44** shows only the histamine normalized peak areas of Donor C. It has to be noted that histamine increases in donors that react to histamine at around 120 minutes after coffee/chocolate ingestion. Hence, to investigate the time-course of histamine further, it would be necessary to sample longer. Donor J also reported dizziness and a feeling of sickness at about 50 minutes after the beginning of the experiment. Looking at the time-course of donor J, it can be seen that at this point the TP

level peaks, which could explain the associated health problems as high concentrations of TP are associated with nausea.[39] Comparing donors that regularly consume coffee (for example donor D) and donors that do not consume coffee (for example donor K), it is notable that PX peak areas start at nearly 0 and only rise slightly after consumption in case of the non-consumers. Regular coffee consumers appear to have a ground level of PX, meaning that it accumulates, which has already been reported in the literature.[21]

Moreover, there seem to be two types of "metabolizers": slow and fast. Donor D metabolizes CF very quickly as CF levels decrease and at the same time PX levels increase significantly 15 minutes after coffee/chocolate consumption. On the other hand, slow metabolizers have a "broader" CF peak as CF concentrations only slowly increase CF over time. The reason for this distinct metabolism could be based on the activity of CYP450 (in particular CYP1A2) enzymes in the liver, gender, age, dietary habitualness, genes, sleep and other drug consume.[120] Studies revealed that "poor metabolizers" have an increased risk of a non-fatal heart attack and hypertension after the consumption of multiple cups of coffee. Therefore, slow metabolizers may be more sensitive to the negative health effects associated with CF and are unable to build up tolerance against it. For slow metabolizers, it becomes dangerous as upon multiple coffee consumption, CF concentrations quickly rise in blood and thus, the liver can no longer perform detoxification properly.[121]

Individual responses to metabolites are influenced by a variety of factors including age, sleep, drug intake, absorption and metabolization.[126] For example: The clearance of CF changes within an individual on a daily basis and is also different across individuals.[122] Another factor that influences CF clearance is the activity of CYP1A2. Among individuals the amount of CYP1A2 (the more CYP1A2, the more clearance) and its activity leads to variable half-lives for CF.[123] **Table 14** gives an overview of the factors that positively or negatively affect CYP1A2 activity and thus, influences the speed of CF metabolism (not mentioning the various drugs with which CF interacts).[124]

Table 14: Impact of various substances and genetic factors on CF/TB metabolism according to reference [124]

Factors that increase the activity of CYP1A2	Factors that decrease the activity of CYP1A2
Lean people	Obesity
Coffee consumption	Alcohol
Cruciferous vegetables (e.g. broccoli)	Grapefruit juice
Grilled meat	Liver disease
Males	Females
Smoking	pregnancy

Younger people	Older people
	Luteal cycle of the menstrual cycle

However, the factors listed above are not the only properties that influence the measurement of metabolites in fingerprints, but also e.g. the deposition pressure on the substrate or the amount of sweat secreted in a fingerprint, which varies across individuals and makes absolute quantification not possible at the moment.

4.3.3.2 Comparison of finger sweat and whole blood samples

As both whole blood as well as fingerprint samples have been analysed in parallel, these two specimens can be examined in contrast. **Fig. 45** to **48** shows the fingerprints of the left hand and the blood samples of Donor C, D, F and J (from study 3 where they consumed coffee and chocolate). In case of the blood samples, CF, TB and PX are plotted on the left y-axis while only CF and TB are displayed on the y-axis regarding the fingerprint samples.

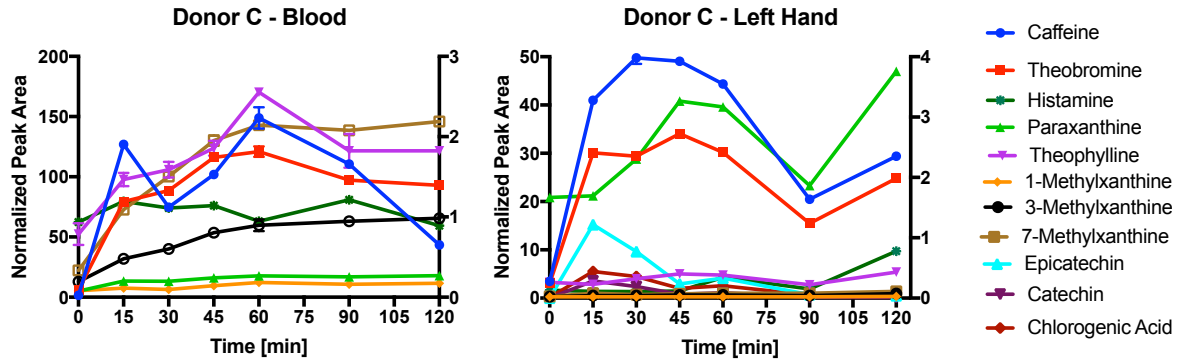

Figure 45: Blood and fingerprint samples of Donor C. In case of the fingerprint data, histamine is also displayed on the left y-axis.

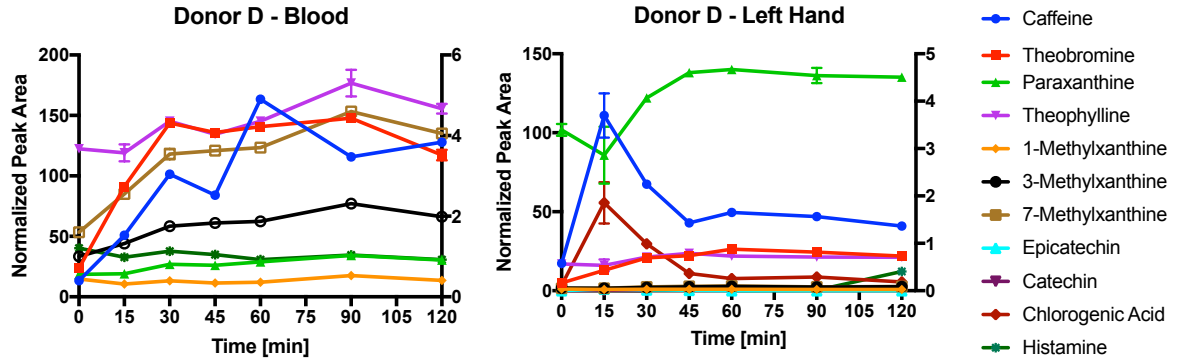

Figure 46: Blood and fingerprint samples of Donor D.

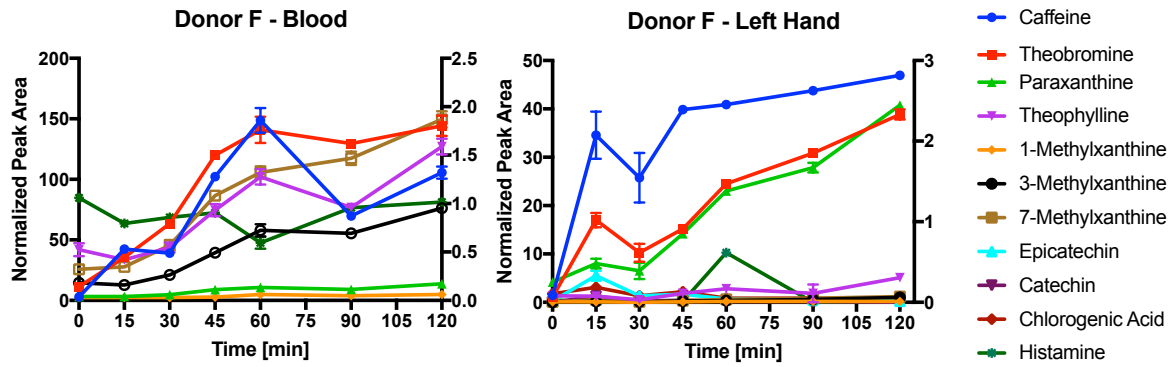

Figure 47: Blood and fingerprint samples of Donor F.

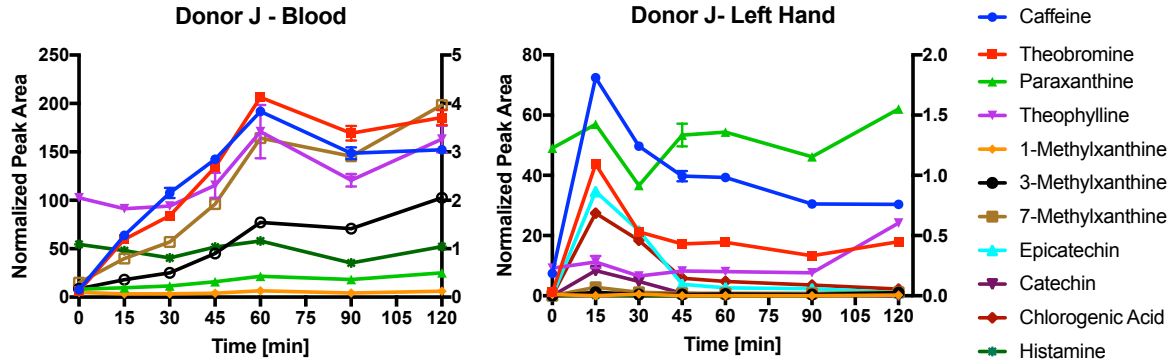

Figure 48: Blood and fingerprint samples of Donor J.

As can be seen from the figures above, not all metabolites found in fingerprints could also be detected in blood. EC, CA and chlorogenic acid could only be observed in finger sweat samples without exception. EC and CA were often not detected in plasma samples according to literature.[55] EC and CA are believed to form complexes with human serum albumin (HSA) and therefore, are not freely available. Moreover, it seems to be the case that different affinities towards HSA affect the bioavailability of these flavan-3-ols and hence, also their biological effects. As binding to HSA influences not only the transport of these compounds, also their metabolism is affected. Studies revealed that HSA-complexes with polyphenols are formed via hydrophobic interactions, hydrogen-binding and electrostatic forces, meaning they are not covalent.[125] Catechins are generally known to have strong interactions with proteins. These interactions can lead to alterations of the protein conformation and thus, block the interactions with the natural substrate. Catechins can restrain enzymes like trypsin, decarboxylase, squalene epoxidase, ribonuclease, xanthine oxidase and histidine/dopa decarboxylase.[126] The binding affinity of catechins to trypsin is in the order of EGCG ≈ ECG > EC/CA ≈ EGC.[126] Similar to the catechins, chlorogenic acid is thought to have antiviral, antibacterial and antifungal properties due to its capability to bind to proteins and enzymes. HSA is believed to be the most likely binding partner for chlorogenic acid.[127] Hence, binding to proteins makes the detection in blood more difficult than in finger sweat that does not contain as many proteins and enzymes as blood.

The kinetics of blood and fingerprints seem to be different as they do not proceed parallel. The fingerprint samples have a fast increase at the beginning (peak at around 15 minutes) of the experiment in all donors. Some of them exhibit a second peak at later sampling times. On the other hand, in blood samples the peak concentrations are found at a later sampling time than in fingerprint samples. Looking at CF and TB it is the case that there is an increase in their blood concentrations at the same time as their fingerprint levels are already decreasing. Blood time-courses of the four donors look more alike in comparison to the great variations of the finger sweat samples. However, comparing the blood samples of this study, where donors ingested both coffee and chocolate to the preliminary study, where donors only consumed one of them, the time-course of the preliminary study looks more homogenous (without a second peak in their blood time-course: **Fig. 49**).

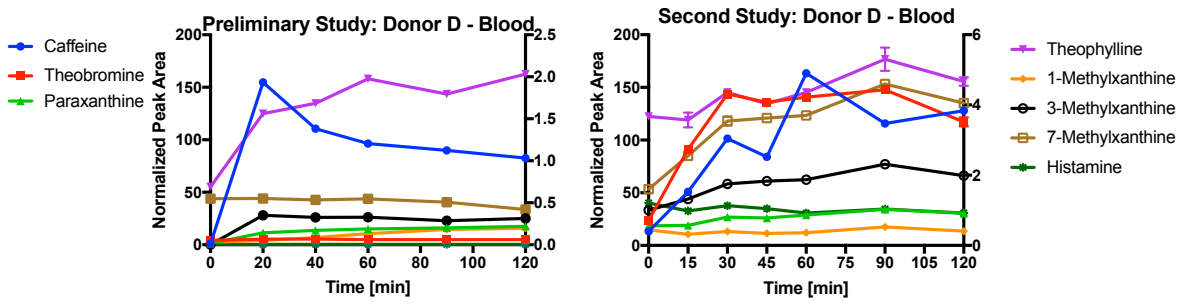

Figure 49: Donor D. Left showing the blood time-course measurement of the preliminary study, where Donor D received only coffee and right showing the time-course measurement of the second study, where Donor D consumed coffee as well as chocolate

The reason could be that in this study, the first metabolites (predominantly CF) come from coffee and are already in their dissolved form. CF from chocolate could get absorbed delayed as chocolate has a high fat content and hence, the time-course could be different from only consuming either coffee or chocolate.

It appears further that higher concentrations of the monomethylxanthines are detectable in blood than in finger sweat samples (when comparing their unnormalized peak areas). The reason for this could be that the lymphatic system plays a role in the excretion of xenobiotics via sweat, leading to the xenobiotics bypassing the liver and therefore, not being metabolized. Metabolized products would first have to circulate in blood, before entering the sweat compartment, possibly explaining the low concentrations of monomethylxanthines present in sweat.

4.3.3.3 Reproducibility and LC-Variability

All measured samples had the same retention time without a shift greater than 0.01 min. Blood, fingerprint as well as the extracted coffee/chocolate samples were measured with the methods describe in **chapter 3**. Therefore, the retention time was stable throughout all the experiments.

The LC-MS variation show predominantly coefficient of variations (CV) <5%. The CV is described as the standard deviation divided by the average. The technical replicates were investigated after their LC-MS variation. **Fig. 50** shows the number of samples that have CV's below 1 and 5% and above 5%, calculated for the internal standard as well as for CF and TB.

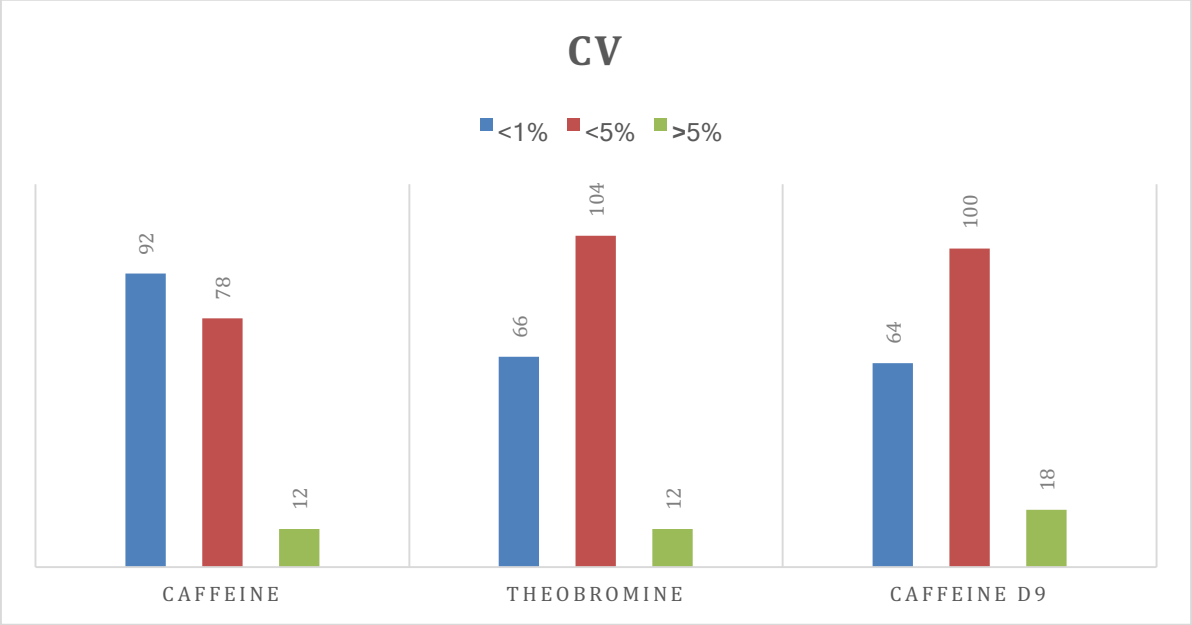

Figure 50: Number of samples with cv values <1%, <5% and >5%

4.3.3.4: Quantifying Caffeine, Paraxanthine and Theobromine:

The calibration curve that was used to quantify the concentrations of the metabolites at each sampling point can be seen in **Fig. 51**.

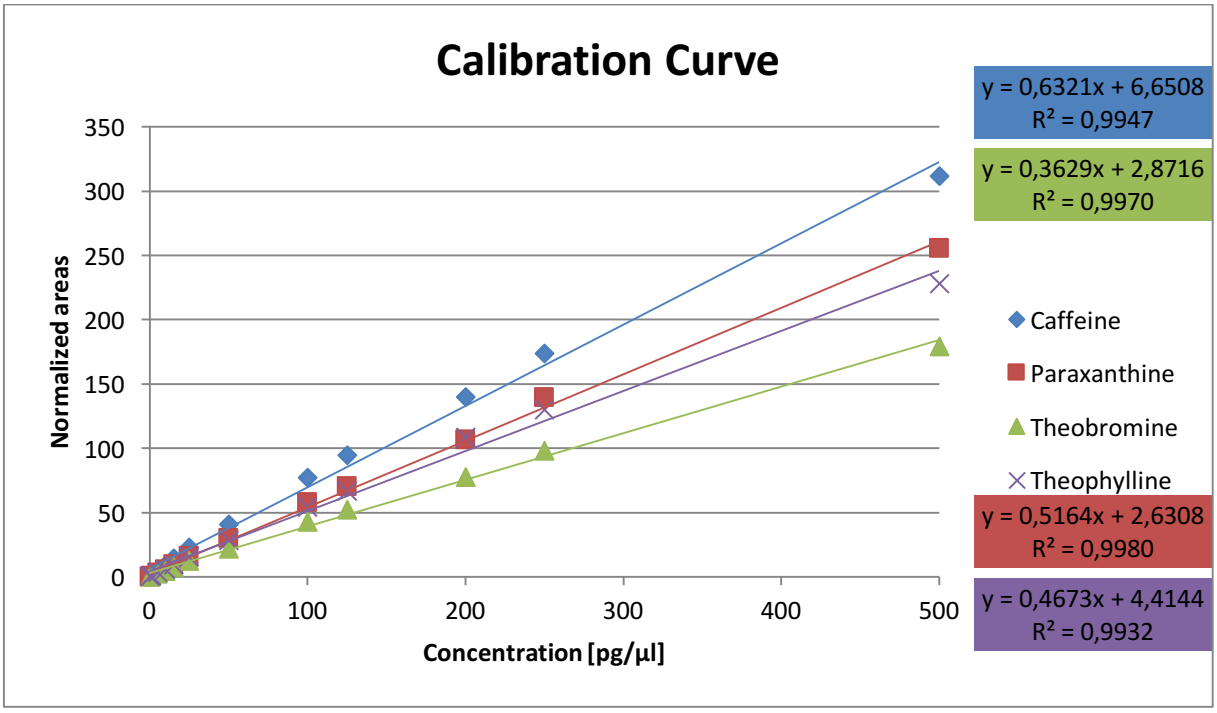

Figure 51: Calibration curves for CF, PX, TB and TP. The areas were normalized to the areas of the internal standard (CF-D9)

Table 15 lists all concentrations in pg/µl of each compound in the finger sweat samples of each individual.

Table 15: Concentrations [pg/µl] for CF, TB, and PX for each individual at the sampling time points 0, 15, 30, 45, 60, 90 and 120 minutes after ingestion

			0	15	30	45	60	90	120
Donor A	CF	Left	11,50	97,64	65,14	42,20	35,52	74,22	59,55
		Right	9,57	152,46	109,30	46,31	49,01	82,52	62,03
	PX	Left	11,05	7,43	5,71	3,44	0,53	8,51	6,08
		Right	7,14	6,99	4,15	2,50	3,39	12,34	8,58
	TB	Left	8,56	144,78	82,53	58,72	50,37	143,03	125,96
		Right	5,91	397,84	211,84	81,54	78,66	161,00	131,48
Donor B	CF	Left	-1,52	19,24	8,80	72,79	28,51	7,89	18,05
		Right	3,95	25,62	26,61	34,97	28,04	7,14	25,40
	PX	Left	-2,06	-2,03	-2,52	8,18	0,27	-2,71	-1,58
		Right	-0,10	-1,14	-0,55	1,35	0,09	-2,85	-0,48
	TB	Left	-2,43	47,41	19,83	120,64	48,78	19,07	38,60
		Right	0,39	78,81	48,76	68,12	51,80	20,11	47,36
Donor C	CF	Left	24,69	392,96	313,83	243,20	324,50	215,58	250,67
		Right	25,88	510,30	296,84	352,53	256,61	231,87	274,79
	PX	Left	15,67	15,31	13,27	15,57	24,22	20,11	35,85
		Right	15,78	10,48	10,50	16,49	16,17	15,94	36,68
	TB	Left	46,17	507,99	325,96	298,75	389,57	290,09	376,06
		Right	45,95	326,31	212,88	286,70	250,11	225,91	359,82
Donor D	CF	Left	104,09	1004,08	334,57	315,75	266,33	154,98	169,59

		Right	37,12	235,07	162,50	211,29	189,68	128,11	98,63
	PX	Left	22,35	26,36	20,40	37,74	26,83	14,48	19,13
		Right	14,64	23,53	22,62	35,98	30,54	19,38	16,37
	TB	Left	48,10	196,37	177,80	286,55	248,67	141,94	160,07
		Right	30,86	160,99	180,06	286,76	262,57	179,85	147,00
Donor E	CF	Left	15,16	98,17	49,04	80,86	33,32	25,92	66,09
		Right	11,42	76,76	28,47	17,87	26,90	10,41	24,09
	PX	Left	0,91	0,24	-1,75	-1,34	-2,90	-2,81	2,54
		Right	-1,87	-1,87	-3,49	-3,82	-3,82	-4,16	-2,58
	TB	Left	1,74	133,78	63,81	76,92	36,81	35,98	122,68
		Right	-2,09	159,62	41,73	28,03	20,41	11,26	40,76
Donor F	CF	Left	2,96	275,81	318,96	224,69	229,21	270,46	252,45
		Right	1,30	126,88	133,41	115,10	143,58	140,73	152,68
	PX	Left	-2,23	-0,22	0,93	1,07	4,80	8,01	11,70
		Right	-2,44	-0,23	1,56	-0,05	2,64	3,52	6,80
	TB	Left	9,68	239,78	221,40	148,24	242,74	337,24	371,25
		Right	11,33	176,03	177,75	133,62	194,42	235,36	275,32
Donor G	CF	Left	44,55	143,22	201,72	86,89	89,58	76,62	85,13
		Right	29,68	58,20	81,70	37,48	48,10	48,21	54,78
	PX	Left	16,24	11,43	13,42	8,49	6,42	6,62	10,14
		Right	9,15	13,60	15,13	3,24	5,35	5,37	7,77
	TB	Left	3,82	1,71	2,38	-0,56	-1,10	-0,79	1,35
		Right	0,79	3,90	4,31	-2,70	-1,38	-0,31	0,65
Donor H	CF	Left	14,74	57,68	46,59	53,91	69,28	42,01	83,82
		Right	12,67	56,27	46,02	47,76	56,88	38,24	63,29
	PX	Left	5,40	8,55	6,26	7,79	10,50	4,30	12,25
		Right	3,58	9,58	5,78	6,13	8,07	4,09	8,26
	TB	Left	4,63	97,72	68,99	76,59	114,57	71,40	143,84
		Right	2,94	85,29	59,98	60,40	85,84	52,86	106,71
Donor I	CF	Left	4,66	224,02	115,48	133,64	173,96	229,99	131,71
		Right	5,06	49,61	44,22	81,87	152,79	130,40	58,79
	PX	Left	1,78	4,17	2,12	4,94	10,95	18,35	10,90
		Right	1,67	3,83	0,87	5,08	13,96	11,96	4,02
	TB	Left	1,43	608,65	283,37	291,21	366,56	438,71	250,12
		Right	1,63	96,21	127,69	182,13	321,82	292,25	131,09
Donor J	CF	Left	14,74	212,07	139,59	113,90	96,49	83,37	87,97
		Right	13,07	485,00	181,93	201,08	233,40	78,21	115,28
	PX	Left	0,01	0,28	-1,72	0,01	-0,55	-0,74	1,05
		Right	-0,72	1,20	-2,94	-1,29	0,09	-2,01	0,78
	TB	Left	-1,01	226,08	103,81	85,78	76,27	63,53	93,52
		Right	-2,49	482,42	71,51	78,77	77,63	43,71	85,21
Donor K	CF	Left	-7,49	52,52	66,94	35,71	39,34	77,50	108,54
		Right	-6,71	207,54	112,95	97,12	110,02	172,64	81,57
	PX	Left	-3,88	-3,88	-3,15	-3,88	-3,51	0,82	-0,08
		Right	-3,79	-3,24	-3,58	-3,93	-3,19	3,02	2,63
	TB	Left	34,80	93,79	113,06	60,37	74,08	171,29	143,29
		Right	41,66	187,70	101,92	70,02	87,60	270,37	202,36

Calculated concentrations for CF spanned from 0.00 to 1000 pg/µl, for PX from 0.00 to 40 pg/µl and for TB from 0.00 to 600 pg/µl. **Table 15** shows again the great variations in the different amounts of xenobiotics each donor absorbs and even differ in concentrations at time point 0 before the consumption of coffee/chocolate.

According to the literature, a single cup of coffee (about 250 mL) contains between 40 to 130 mg of CF.[11] With this calibration curve a concentration of 154 mg per cup (Tchibo Espresso kräftig) was calculated. A 50g chocolate bar contains between 237-519 mg TB and 17-36 mg CF, 218 mg

TB and 45 mg CF were calculated for the Chocheur chocolate (85%), which is also consistent to the literature.[11]

4.4: External Project: Quantifying CF and its primary metabolites:

The calibration curve used for the quantitation can be seen in **Fig. 51**. During the duration of this master thesis, more than 900 samples were measured. As an example for the data evaluation, the time-courses of two different donors and two different conditions (CF consumption and non-CF consumption) are displayed in **Fig. 52** and **53**.

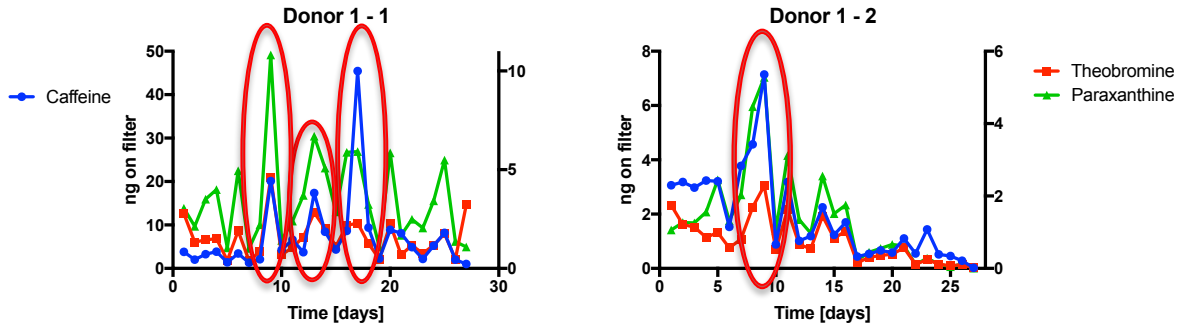

Figure 52: Time-course measurement of CF and its primary metabolites of donor 1 displayed as the amount (in ng) on the filter. Samples were taken over 28 days. Condition 1 is showed on the left, where the donor has consumed CF tablets, which can be seen through the high amounts of CF (up to 45 ng) present on the filter. Condition 2 is where the donor did not receive any CF, which can be seen on the low amounts of CF present on the filter, however, at day 9 it looks like the donor has consumed something containing CF and therefore, has a higher CF peak than his/her ground state level.

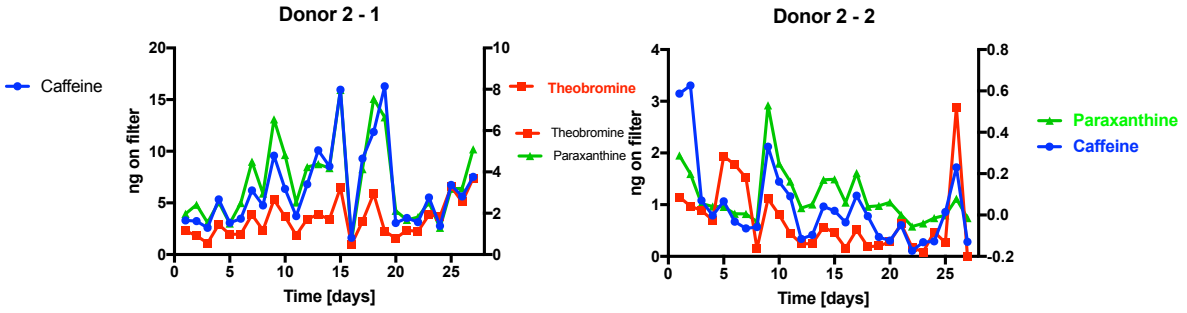

Figure 53: Time-course measurement of donor 2. The two conditions are more easily distinguishable with donor 2, as the amount of CF and its primary metabolite PX is ranging from 0.0 to 0.8 at condition 2, meaning that the donor has not consumed any CF product. In condition 1, the individual has ingested coffee and therefore, the values for CF range from about 4.5 to 15, which is significantly different from the ground state level shown in condition 2 (where CF is below 1 ng on the filter). The legend in colour belongs to figure on the right.

As can be seen from the figures above, the two states (having consumed CF or not) can be distinguished quite easily as the ground state levels of CF and its main metabolites are significantly lower when the donors have not consumed any CF-containing products (e.g. coffee, tea, energy drinks and chocolate).

As CF being an illicit drug to enhance one's performance during the Olympic Games is again discussed, this method could be a valuable tool to quantify CF from fingerprints if CF should be again prohibited during competition.

5. Summary and Outlook

Coffee and chocolate are two of the most consumed beverages/ foods that are related to health effects as they contain bioactive substances (methylxanthines and in case of chocolate further polyphenols – mainly flavan-3-ols). Some of these bioactive substances have been identified as food biomarkers and have been used to study the relationship between regular consumption and health benefits and/or disease risk. Studying the presence and amount of metabolites arising from certain constituents found in food would help to get a better understanding of metabolism in the body itself as well as of differences in the metabolism of individuals.

Fingerprint analysis is becoming more popular regarding identifying suspects at a criminal scene or proving drug intake. Compared to blood and urine, finger sweat analysis offers many advantages. It is a quick and easy method to detect trace amounts of drugs or other compounds (e.g. emerging from food metabolism or personal hygiene articles) in finger sweat without being invasive (like blood) and without the need of a specialist taking the samples (for example: patients that need frequent analysis could sample themselves at home and their samples could be transported to the laboratory at room temperature). Urine is also a non-invasive sampling technique; however, suspects need to be observed in order to avoid falsification of the sample. Additionally, fingerprint samples need very little sample preparation and they have less matrix components in comparison to blood and urine, which have more proteins and digestive enzymes. Yet, fingerprint analysis still lacks a normalization factor to compensate different sweat volumina collected from an individual and between individuals. Therefore, absolute quantification is not possible at the moment. Moreover, highly sensitive analytical instruments that are quite expensive are required as the molecules of interest are in the picomolar range. In this study, the main focus lies on showing that components found in coffee and chocolate as well as their metabolites can be detected in fingerprints after the consumption of the drink and the food. The sample preparation and collection is very easy compared to the whole-blood samples and takes only several minutes for each sample. The chromatographic separation and detection methods of choice are a UHPLC (RP) coupled directly to an orbitrap mass spectrometer via an ESI source, which provides an optimal tool for the separation and detection of most metabolites of interest. However, as also the sweat composition itself is of interest, which includes many amino acids and other more polar substances separating them on a HILIC column would be the better choice. This master thesis shows that not only can xenobiotics and their metabolites be detected in finger sweat samples but also that there are great differences among individuals regarding the amount of xenobiotics that get absorbed as well as a different time-courses and metabolism across individuals. Further, some of the individuals have different metabolites (e.g. lack of 3- and 7-methylxanthines in Donor G), indicating different metabolic

speed and activity, which could be based on several factors including age, gender, genes, dietary habit and other lifestyle factors. Surprisingly, a factor that also influences the amount of excreted metabolites was the *hand* itself, since fingerprint samples from the left- and the right-hand varied strongly. A reason for this could be the delivery to the sweat compartment from the left- or the right lymphatic duct. Moreover, this master thesis proves that blood and sweat show distinct kinetics and that some metabolites are absent from blood as for example EC, which has high binding affinity to HSA. As a conclusion, this master thesis confirms that individual metabolic parameters can be drawn from fingerprint analysis.

Detection of drugs in fingerprint samples could become more useful in the future than only showing illicit use. For example, the necessary determination of patient compliance, which means monitoring the intake of medicine of patients e.g. suffering from Alzheimer's disease, could be massively improved by analysing their sweat samples instead of using their blood or urine. Moreover, patients that have to undergo regular examination of their blood are at a higher risk of infection, which could be avoided if their data could be obtained from finger sweat samples. Previous studies already revealed, that diabetics could measure sweat glucose levels as they are proportional to their blood glucose levels. Diseases could change the sweat composition by either changing the concentration of commonly found constituents or by generating new compounds, which could function as biomarkers. These biomarkers could then be used to confirm or rule out if a person suffers from a specific disease, solely from their fingerprint sample without the need of an invasive procedure.

Furthermore, this master thesis show that histamine levels enhance extremely in certain individuals who have reported problems with histamine. Fingerprint samples could possibly show if an individual is allergic to a certain food constituent. The individual would only have to consume the product and finger sweat samples would need to be taken after consumption. This would provide an easy and inexpensive (neglecting the unique expenses of purchasing the analytical instruments) to detect if people had a food allergy.

Finally, if xenobiotics and their metabolites in finger sweat samples are investigated more extensively, it may provide a better understanding of the individual absorption, distribution and metabolism of these external substances in the human body, which could also lead to a better characterization of the individual gut microbiome and a more "personalized" medication.

6. Abstract

The aim of this master thesis was to study individual metabolic parameters via extracting and analysing xenobiotics and their metabolites from finger sweat and whole blood samples. The xenobiotics this master thesis focused on were emerging from coffee and chocolate and were mainly methylxanthines (caffeine, theobromine, and theophylline) and flavan-3-ols (epicatechin and catechin). Both of these bioactive groups are associated with health effects and have become food biomarkers. Thus, a more detailed characterization of their absorption and metabolism could lead to a better understanding of their beneficial properties regarding disease prevention and other health factors. For this purpose, eleven healthy donors were asked to consume coffee and chocolate and analyte levels of the xenobiotics and their metabolites were monitored before and after coffee/chocolate consumption in fingerprint as well as in blood samples. The results show a significant and reproducible increase of the xenobiotics in fingerprints in all donors when comparing the xenobiotics and metabolite levels before coffee and chocolate intake with the levels 15 min thereafter. The method of choice was an untargeted screening method, using a reversed-phase UHPLC system coupled to the Q Exactive HF (Thermo Fisher Scientific™) which is a hybrid instrument with a quadrupole as a mass filter and an orbitrap as a mass analyzer. Compounds were identified by loading the raw data into the Compound Discoverer Software (Thermo Fisher Scientific™) and checked manually by comparing the measured spectra to an online database. A sample preparation procedure of about 5 minutes for each fingerprint sample and a total run time of the UPHLC-MS of 7.5 min per sample allowed high sample throughput. This study revealed differences in the amount of absorbed metabolites, as well as different speeds in metabolic activity, differences between the left- and right-hand fingerprint samples and distinct kinetics of blood and sweat. Furthermore, not all compounds found in sweat could also be detected in blood possibly due to high protein binding affinities of these xenobiotics/ metabolites. Therefore, this study is a proof-of-principle that individual metabolic parameters can be drawn from secreted sweat of the fingers.

7. Zusammenfassung

Im Rahmen dieser Masterarbeit wurde untersucht, ob individuelle metabolische Parameter durch Extraktion und Analyse von Xenobiotika und deren Metaboliten aus Fingerschweiß und Blut gewonnen werden können. Die Xenobiotika, auf die sich diese Masterarbeit konzentrierte, stammen aus Kaffee und Schokolade und waren hauptsächlich Methylxanthine (Koffein, Theobromin und Theophyllin) und Flavan-3-ole (Epicatechin und Catechin). Beide bioaktiven Gruppen stehen in Zusammenhang mit gesundheitlichen Effekten und sind zu Biomarkern für Lebensmittel geworden. Daher könnte eine genauere Charakterisierung ihrer Absorption und ihres Metabolismus zu einem besseren Verständnis ihrer gesundheitsfördernden Eigenschaften in Bezug auf Krankheitsprävention und andere Gesundheitsfaktoren führen. Zu diesem Zweck wurden elf gesunde Spender gebeten, Kaffee und Schokolade zu konsumieren, und die Analytkonzentrationen der Xenobiotika und ihrer Metaboliten wurden vor und nach dem Kaffee- / Schokoladenkonsum sowohl in Fingerschweiß- als auch in Blutproben gemessen. Die Ergebnisse zeigen eine signifikante und reproduzierbare Zunahme der Xenobiotika in den Fingerscheißproben bei allen Freiwilligen, wenn der Metabolitspiegel vor der Kaffee-/Schockoladeaufnahme mit den Metabolitkonzentration von 15 Minuten nach Verzehr verglichen wurde. Die Methode der Wahl war ein untargeted Screening-Verfahren, bei dem ein Umkehrphasen-UHPLC-System mit der Q Exactive HF (Thermo Fisher Scientific™) verwendet wurde, das ein Hybridinstrument mit einem Quadrupol als Massenfilter und einer Orbitrap als Massenanalysator ist. Verbindungen wurden identifiziert mit Hilfe der Compound Discoverer Software (Thermo Fisher Scientific™), manuell überprüft durch Abgleich der aufgenommenen Spektren mit einer online Datenbank und mittels analytischer Standards verifiziert. Ein Probenvorbereitungsverfahren von etwa 5 Minuten für jede Fingerschweißprobe und einer Gesamtlaufzeit des UPHLC-MS-Systems von 7,5 Minuten pro Probe ermöglichten einen hohen Probendurchsatz. Diese Studie zeigt Unterschiede in der Menge an absorbierten Metaboliten, sowie individuelle Geschwindigkeiten in der metabolischen Aktivität, Unterschiede zwischen den linken und rechten Fingerschweißproben und unterschiedliche Kinetiken von Blut und Schweiß. Darüber hinaus konnten nicht alle im Schweiß vorkommenden Verbindungen im Blut nachgewiesen werden, möglicherweise aufgrund von hohen Proteinbindungsaffinitäten dieser Xenobiotika /Metaboliten. Daher ist diese Studie ein Beweis dafür, dass individuelle Stoffwechselparameter aus dem sezernierten Schweiß der Finger gewonnen werden können.

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