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MASTERARBEIT

“Time-course measurements of caffeine and its primary metabolites extracted from fingertips after coffee intake”

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Abstract

Within the scope of this master thesis a rapid, simple as well as efficient method was developed for extracting and quantifying caffeine and its primary metabolites from fingertips, blood and saliva. For this purpose, analyte concentrations from five healthy subjects were monitored before and after coffee intake over a period of five hours. The results show a significant and reproducible increase of caffeine in fingerprints of four out of five volunteers when comparing the caffeine levels before coffee intake with the levels 5 h thereafter. The individual differences were evaluated with respect to metabolism and reproducibility. A microfluidics-based nano-LC system with a hyphenated mass spectrometry platform was the instrumentation of choice for this task. Sample preparation procedure and a total operating time of 10 min per sample allowed a routine throughput of 60 samples per day. The validated methods allow the quantification of caffeine (CF), theobromine (TB) and theophylline/paraxanthine (TP/PX) in the concentration range of 0.5–300 pg/µL (0.25–150 pg on column) with R^2 values >0.999 for fingerprint and >0.998 for whole blood. CF, TB and TP/PX show LOQs of 0.54, 0.68 and 0.42 pg/FP, respectively, while the limits of detection (LOD) were 0.22, 0.28 and 0.20 pg/FP, respectively. The LODs from whole blood were 0.27-0.37 pg/µL with lower limits of quantuitations (LOQs) between 0.6–10.83 pg/µL for the analytes. The overall variation for the fingerprint measurements for five volunteers using three biological and three technical replicates was <22%, while the extraction reproducibility amounted to 7.2% with 3.8% LC-MS variability. Day-to-day variations implicating biological variations, vary for fingerprint (CV <9%) and blood (CV <20%). Therefore, this proof-of-principle study of time course measurements of caffeine, theobromine and theophylline/paraxanthine showed high reproducibility in a cohort of five volunteers.

Keywords: *caffeine, drug screening, fingerprint, metabolite, MRM, nanoChip, pharmacokinetics*

Zusammenfassung

Im Rahmen dieser Masterarbeit wurde eine schnelle, einfache sowie effiziente Methode entwickelt um Koffein und seine primären Metabolite aus dem Fingerabdruck, Vollblut und Speichel zu messen und zu quantifizieren. Für diesen Zweck wurden die Analytkonzentrationen von fünf gesunden Freiwilligen vor und nach der Kaffeeaufnahme über eine Zeitdauer von fünf Stunden kontrolliert. Die Ergebnisse zeigen eine signifikante und reproduzierbare Zunahme von Koffein in Fingerabdrücken in vier von fünf Probanden, wobei die Koffeinkonzentrationen vor der Kaffeeaufnahme mit den Konzentrationen nach 5 h verglichen werden. Die individuellen Unterschiede wurden in Bezug auf Metabolisierung sowie Reproduzierbarkeit bewertet. Ein an ein Massenspektrometer gekoppeltes mikroströmungs-basiertes nano-LC System wurde für diese Experimente verwendet. Die schnelle Probenvorbereitung sowie ein Gesamtaufwand von 10 min pro Probe ermöglichten einen Durchsatz von 60 Proben pro Tag. Die validierten Methoden erlauben die Quantifizierung von Koffein (CF), Theobromin (TB) und Theophyllin/Paraxanthin (TP/PX) zwischen 0.5–300 pg/µL (0.25–150 pg Injektionsmenge) mit R^2-Werten >0.999 für den Fingerabdruck und >0. 998 für das Vollblut. CF, TB und TP/PX zeigen Bestimmungsgrenzen (LOQs) bei 0.54, 0.68 und 0.42 pg/FP beziehungsweise Nachweisgrenzen (LODs) bei 0.22, 0.28 und 0.20 pg/FP. Die LODs vom Vollblut waren 0.27–0.37 pg/µL mit LOQs zwischen 0.61–0.83 pg/µL für die Analyten. Die Gesamtvariation für Fingerabdrücke von fünf Probanden betrug 22%, die Extraktionsreproduzierbarkeit 7.2% und die LC-MS Variation 3.8%. Hierzu wurden je drei biologische und drei technische Proben verwendet. Tägliche Schwankungen, die biologische Schwankungen zur Folge haben, ändern sich innerhalb des Fingerabdrucks (CV <9%) und Blut (CV <20%). Diese Studie zum Nachweis von zeitabhängigen Messungen von Koffein, Theobromin und Theophylline/Paraxanthine zeigte hohe Reproduzierbarkeit in fünf Probanden.

Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

The work has not been submitted previously, the content of the thesis is the result of laboratory work which has been carried out between January – june 2014.

Meinen Eltern

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List of Abbreviations

Ac	Acetate
ACN	Acetonitril
ACTH	Adrenocorticotropic hormone
AMP	Adenosine monophosphate
c	Concentration
cAMP	Cyclic adenosine monophosphate
CF	Caffeine
CF-D9	stable isotope labelled standard - deuterated caffeine
CH	Chloroform
D_2	Deuterium lamp
DCM	Dichloromethane
DEE	Diethyl ether
DESI	Desorption electrospray ionization
e.g.	for example
EA	Ethyl acetate
EE	Extraction efficiency
ESI	Electrospray ionisation
FA	Formic acid
GC/MS	Gas chromatography–mass spectrometry
GFR	Glomerular filtration rate
GMP	Guanosine monophosphate
HETP	height equivalent to a theoretical plate
HPLC	High-pressure liquid chromatography
IMP	Ionosine monophosphate
IR	Infrared
IS	Internal Standard
LC	Liquid Chromatography
LLOQ	Lower limit of quantitation
LOD	Limit of detection

LOQ	Limit of quantitation
m/z	Mass to charge ratio
ME	Methanol/Chloroform
MR	Metabolic ratio
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
p.	page
PX	Paraxanthine
QQQ	Triple Quadrupole
SRM	Selected reaction monitoring
TB	Theobromine
THC	Tetrahydrocanabinol
TIC	Total ion chromatogram
TOF	Time-of-flight
TP	Theophylline
UHPLC	Ultra High Performance Liquid Chromatography
UHR-TOF	Ultra High Resolution Time-of-flight
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet

1 Introduction

Fingerprints have been used in forensic investigations for the identification of individuals since the late 19th century [1]. Importantly, identification by fingerprints is still the cornerstone of forensic evidence. At any time someone touches a surface with bare hands, one may deposit traces of chemicals in their fingerprint sweat which can reveal what this person has eaten, injected or inhaled [2]. Therefore, also orally ingested and metabolized compounds may be excreted in sweat, however, the methods that are used for their detection usually require a large amount of sweat collected over a period of time. A volume of 50 µL secreted sweat can be expected from a fingerprint and therefore, analysis of trace amounts of analytes may be challenging [3]. Apart from this, companies focusing on fingerprint diagnostics use antibody-coated nanoparticles to screen for drug metabolites in the minute traces of sweat from a fingerprint [4]. Obviously, this technique may detect drug metabolites rather than the drugs themselves. In such tasks, it is crucial to differentiate whether a positively detected drug is due to actual intake or by contamination on the fingers or substrate surfaces.

This master thesis is a proof-of-principle study on the possibility of obtaining statistically significant data from coffee consumption by quantifying caffeine and its primary metabolites from fingerprints based on the investigations of Kuwayama *et al.* [5]. Suitable extraction procedures and quantification techniques were developed and validated for this purpose. Additionally, these analytes were also investigated in blood and saliva after the volunteers had ingested coffee. The effect of coffee consumption on blood concentration levels of melatonin and creatinine were evaluated in addition during the time-course measurements. Finally, the pharmacokinetics of these drugs may give a first impression on the individual differences of metabolic activity.

1.1 Caffeine and its Primary Metabolites are the Target Analytes

1.1.1 Caffeine

In the present study, caffeine and its primary metabolites are the target analytes for quantification. Caffeine (1,3,7-trimethylxanthine) is one of the most important naturally occurring xanthine alkaloids. It is a constituent of coffee, tea, chocolate, various energy drinks and it is one of the most widely consumed bioactive substance in the world [6]. Caffeine is a component of coffee beans (Coffee Arabica), tea leaves (Commelia thea), cola accuminata and other plants including the rubiacea, sterculiacea and theacea. Probably the two most important variaties of commercial coffee are *coffea arabica* and *coffea canephora* syn. *coffea robusta* [7]. Caffeine consists of a xanthine core with two fused rings, a pyramidinedione and an imidazole. The pyramidinedione ring contains two amide functional groups, where the nitrogen atoms are double bonded to their conterminal amide carbons atoms [8; 9]. Caffeine is moderately soluble in water at room temperature (1 g/50 mL). It is synthesized in plants from the purine nucleotides AMP, GMP and IMP. The purine nucleotides are then transformed over different pathways into xanthosine and subsequently to theobromine, which is the direct precursor to caffeine (Figure 1). Caffeine is extracted from the plant leaves for commercial use [10]. Caffeine acts as a competitive antagonist of adenosine and inhibits the enzymatic degradation of cyclic adenosine monophosphate (cAMP) by phosphodieserases [11]. Intracellular cAMP is a second messenger and plays an important role in regulating cardiac muscle contraction, amongst others. Increased cAMP may increase the heart contractility (inotropy), rate of heart beat (chronotropy) and conduction velocity (dromotropy) [12; 13].

Studies revealed an increase of daily energy expenditure and a descent of fatigue after caffeine consumption [14]. However, the role of caffeine as a performance enhancing drug is still controversial [15; 16; 17]. Caffeine mobilizes fat stores and stimulates fat lipolysis. Moreover, it may encourage working muscles to use fat as a fuel [14; 18]. Studies revealed that the exercise-associated oxidation of fatty acids is increased by caffeine [19]. Different placebo–controlled studies indicated that it increases alertness, wakefulness, quickens reaction and increases the ability to concentrate and focus. This leads to more correct decisions, a better perceptive comprehension and increases the ability to solve problems requiring reasoning [20; 21; 22]. The amount of caffeine needed to produce these effects varies from person to person and depends most notably on body size and degree of tolerance [23; 24]. Additional factors such as age, liver function, pregnancy, medications, level of liver enzymes, drugs or different hormonal states may influence the rate of caffeine absorption [25]. Usually, caffeine is absorbed from the digestive tract within 45 minutes.

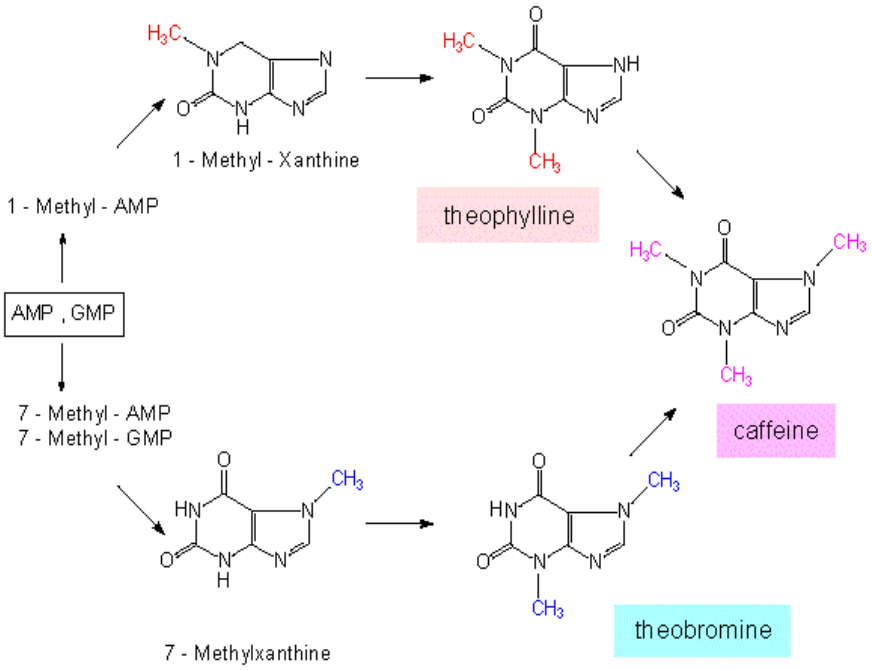


Figure 1 Synthesis of caffeine in plants follows - two different pathways starting from AMP and GMP [26]

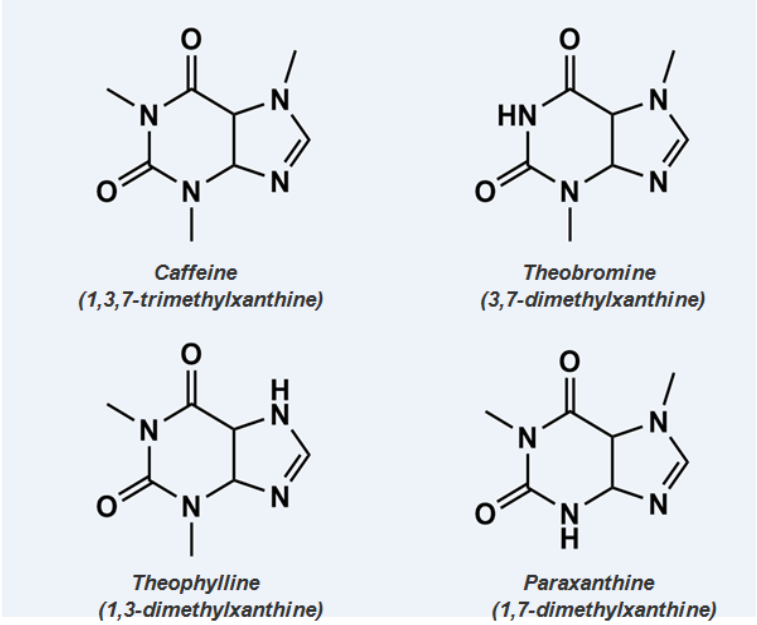


Figure 2 Chemical structures of caffeine and its primary metabolites

1.1.2 Primary Metabolites of Caffeine

Caffeine, a trimethylxanthine, is metabolized by the cytochrome p450 oxidase system in the liver to dimethylxanthines [27]. This demethylation leads to the formation of three primary isomeric metabolites, namely paraxanthine (PX), theobromine (TB) and theophylline (TP), which are then further metabolized [28]. In humans, the major primary metabolite is paraxanthine. Generally 98% of caffeine is metabolized by the CYP450 system of the liver into the primary metabolites, while the remaining 2% are excreted *via* the urine [29].

Paraxanthine. About 84% of caffeine is *N3*-demethylated in the liver to form PX through the catalytic action of cytochrome P450. In contrast, the formation of theobromine and theophylline accounts for only 12% and 4%, respectively [30]. Paraxanthine is a central nervous stimulant with similar activity compared to caffeine. However, it is less toxic and shows less anxiogenic effects and it is generally not produced by plants [30; 31]. Obviously, paraxanthine is a non-selective adenosine receptor antagonist and therefore, increases lipolysis, leading to elevated levels of glycerol and free fatty acid in blood plasma [32].

Theobromine. Theobromine is the predominant methylxanthine found in the cocoa tree (*theobroma cacao)* and therefore, the main xanthine constituent of chocolate. TB levels are higher in dark chocolate (approx. 10 g/kg) than in milk chocolate (1–5 g/kg). It shows similar effects compared to caffeine, although being least potent of all the primary metabolites [28]. It is classified as a mild diuretic, a mild stimulant and relaxes the smooth muscles of the bronchi in the lungs. In the human body, theobromine displays half-lives of 7–10 hours after consumption [33; 34]. Theobromine has been used as a drug for its diuretic effect, particularly in cases where cardiac failure has resulted in an accumulation of body fluid. Because of its ability to dilate blood vessels, theobromine has also been used to treat high blood pressure [35].

Theophylline. Similarly to the other methylxanthine derivatives, theophylline relaxes smooth muscles in the bronchi, stimulates the central nervous system and cardiac muscles and produces dieresis [36]. The potentcy of TP is between that of caffeine and theobromine. Therefore, 1,3-dimethylxanthine is medically used in therapy for respiratory diseases, particularly, to control inflammation in the bronchial tubes [37]. It is important to note that the therapeutic dose of theophylline is a manifold larger than the maximum levels from caffeine metabolism [38].

1.2 Supplementary target analytes for relative quantification

Few studies have focused on whether caffeine may affect serum creatinine or melatonin levels. These molecules are obviously interesting because they may give a first impression on the individual differences of metabolic activity. It is currently no understood if caffeine may alter blood creatinine/melatonin concentrations.

Creatinine. Creatinine is a small molecule waste product of the creatinine phosphate metabolism by skeletal muscle tissue [39]. It is a spontaneously formed cyclic derivative of creatine. Obviously, creatinine production is continuous and proportional to muscle mass [40]. Therefore, men tend to have slightly higher levels of creatinine than women. Serum creatinine is an important indicator of renal health [41] and moreover, creatinine clearance has been used for many decades to estimate the glomerular filtration rate (GFR) [42]. Furthermore, studies revealed that creatinine production during the day remains essentially unchanged [43]. Moreover, animals treated with caffeine show a significantly lower glomerular filtration rate (GFR) and creatinine clearance [44] in contrast to the assumptions that caffeine consumption is associated with increased urine flow rate and creatinine clearance [42].

Melatonin. Melatonin is a derivate of thryptophan and is mainly synthesized in the pineal gland by parenchymatous cells in response to light [45]. Obviously, it functions as a biological modulator of mood, sleep, sexual behavior and circadian rhythm, but plays also critical roles in insomnia, epilepsy, diabetes, obesity, migraine, cancer as well as immune and cardiac disorders [46; 47]. It is mainly metabolized in the liver by cytochrome P450 (CYP2A1) [48] and acts as a strong antioxidant, which may also stimulate the synthesis of glutathione, one of the most important intracellular antioxidants preventing damage to cellular components [45; 49].

1.3 Fingerprints: From Identity to Metabolite Screening

Fingerprints have been used in forensic investigations for the identification of individuals since the late 19th century [1]. It is a fact that fingerprint patterns are unique for an individual. Generally friction skin contains of a series of lines corresponding to ridges and grooves. The pattern of these ridges and grooves unambiguously determines a person and also remains unchanged throughout a person`s lifetime [50]. Ninhydrin is one of the most commonly used reagents for the development of latent fingerprints on paper [51; 52]. A fingerprint contains amino acids and when treated with ninhydrin will result in a purple color change of the fingerprint pattern [53]. Each skin ridge on the fingers is occupied by a single row of pores, through which sweat is excreted and deposited on the surface of the skin. Therefore, also finger sweat can potentially be used to detect and quantify substances a person has ingested [54]. The chemical composition of fingermark residue deposited by sweat differs qualitatively and quantitatively from the general chemical composition of sweat and contains a complex mixture of compounds stemming from different glands [55]. Above all, there are three different types of natural secretion glands in the body. Typically each gland produces a different type of sweat [50]. Natural secretion glands are apocrine glands, eccrine glands and sebaceous glands. The secretions reach the skin surface through epidermal pores [54]. To begin with, the initial composition of fingermark secretions consists of a mixture of numerous substances originating from three different sources: The epidermis, secretory glands in the dermis, as well as extrinsic contaminants.

The epidermis defines the outermost layer of the skin made of the epithelium that is divided into distinct strata (Figure 3). The most external layer of the epidermis is the stratum corneum, which protects the underlying tissue from infections, dehydration, chemicals and mechanical stress [56]. Many lipid compounds comprise a hydrolipidic film which assures protection. Lipids that are found in this film are glycerides and fatty acids (65%), cholesterol (20%) and sterol esters (15%) [57; 58].

The dermis defines the bottom layer of the skin between the epidermis and the subcutaneous tissues [59]. Among other constituents the dermis consists of about five million secretory glands found across the human body (200 sweat glands per cm^2 at an average) including appocrine, eccrine and sebaceous glands [60; 61].

Appocrine glands are found in the genital, breast, linguinal and axillary regions. In mammals, appocrine sweat glands secrete an oily and sometimes smelly substance that acts as a pheromone. Being sensitive to adrenaline, appocrine sweat glands are involved in emotional sweating in humans (e.g. induced by anxiety, stress, fear, sexual stimulation or pain). Because of their localization appocrine gland secretion obviously plays a minor role in fingermark composition [62; 63; 55].

Sebaceous glands are present all over the body except on hands and feet. Sebaceous glands secrete an oily or waxy matter called sebum, which is often found in fingerprints because of the contact of the fingers with other parts of the body [57; 64].

Certainly of great interest are the **Eccrine glands**. They are smaller than appocrine sweat glands in size and distributed all over the body without any exceptions. They are the only glands on fingertips. Eccrine sweat glands secrete hypotonic sweat consisting mostly of water and electrolytes. More precisely it consists of 99% water, various inorganic salts (chloride, bromide, iodide, fluoride, phosphate) and organic materials (amino acids, fatty acids, urea) [65]. Phenol, uric acid and creatinine were all identified in sweat and in fingermark residue in the late 1960s [66; 55]. Regarding vitamins, a study identified B-complex vitamins in fingermark residue in particular Riboflavin [67]. The most abundant group of compounds from eccrine origin present in fingermark residue are various polypeptides or proteins. The main function of eccringe glands is the control of body temperature. Sweat lowers body temperature by dissipation of heat by evaporation. Various studies underlined the presence of an anti-microbial protein called dermicidin in these secretions. As such, eccrine glandsplay an influential role as a part of the innate host defence of the immune system [66; 60].

Compounds	Quant. data/FM
Proteins	384 μg
Polypeptides	All proteins mixed up
Lactic acids	9–10 μg or 154 mM
Chloride	1–15 μg (0.09–1.22 μg/cm^2)
Amino acids	0.2–1 μg
Urea	0.4–1.8 μg
Sodium	0.2–6.9 μg or 54.0 mM
Potassium	0.2–5 μg or 39.9 mM
Ammonia (NH_3)	0.2–0.3 μg or
Ammonium (NH_4^+)	5.13 mM
Phenol	0.06–0.25 μg
Calcium	0.03–0.3 μg or 5.49 mM
Sulphide	0.02–0.2 μg
Magnesium	1.67 mM
Choline	–
Uric acid	150 μM
Vitamins	–
Creatinine	–

Table 1 Chemical composition of sweat from fingers [3]

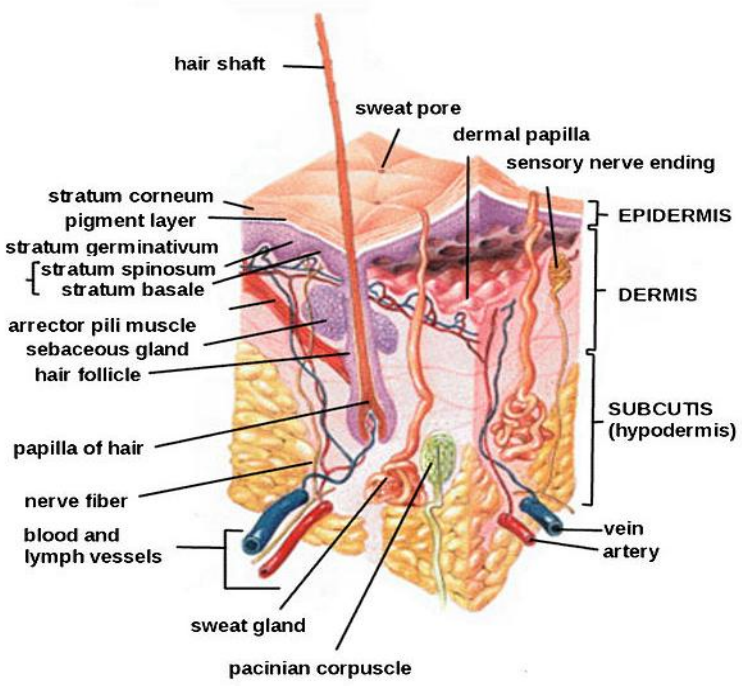


Figure 3 Anatomy of the human skin [68]

1.3.1 Contaminants

Fingermark residue may contain contaminants such as remains from food, dust, bacterial spores, cosmetics (hair products, perfume residue, body cream) etc. Consequently, it may be complicated to differentiate these products from intrinsic residues of fingerprint sweat. For example, cosmetics contain lipid compounds that are also naturally present such as palmitic acid or myristyl myristate) [55]. Importantly, drugs have also been identified in eccrine sweat. Sulfonamides, L-dimethylamphetamine and nicotine seem to enter the eccrine glands through simple diffusion [69; 70; 71]. Therefore, fingerprints maybe used to detect and quantify traces of orally ingested drugs [66]. Of note is the similarity of nicotine to caffeine. It is therefore assumed that caffeine may also diffuse into eccrine glands by passive diffusion and is not actively taken up. Consequently, caffeine kinetics in fingerprints may reflect actual pharmacokinetic behaviour.

1.3.2 Variability of fingermark composition

There are basically five factors that influence the composition of the fingerprint. First, it was shown that the fingerprint of children contains only few fatty acids [72]. In contrast, fingerprints of adults contain squalene, cholesterol, large fatty acids, wax esters as well as glycerides [73]. Earlier research showed that there are even differences in the deposition left by women and men. Consequently, some compounds identified in fingerprint, such as urea and fatty acids, may differ in concentration between males and females. Furthermore, this is eminently interesting because this phenomenon

could be due to different metabolic activity of each individual [74]. It has also been observed that diseases and medications may influence the recovered fingermark residue [55] as well as drug consumption [75; 76].

1.3.3 Deposition conditions influence analyte recovery

The quality of a fingerprint relies heavily on the properties of the surface that the fingerprint is deposited to, e.g. the composition of fingerprints on paper, cotton and wood (porous), waxy surfaces, plastics (semi-porous) or glass and metal (non-porous) may vary substantially [77]. In fact, the more porous the surface is, the higher the adhesion forces and hence the better the quality of the fingerprint [78]. In other words, high porosity favours a faster and more significant penetration of substances from the finger into the matrix. All things considered, the influence of the substrate on the fingerprint composition is dependent on physico-chemical structure, curvature, temperature, electrostatic forces and surface free energy [78; 79]. There are several additional factors, which experimentally influence the composition of the fingerprint. Obviously, the pressure and the contact time between the fingertip and the surface may affect the detected composition of fingerprints [80; 81]. Referring to criminal investigations (where chemical treatments are required to visualize latent fingerprints such as ninhydrine solution or Iodine benzoflavone) the greater the pressure exerted, the higher the amount of compounds that are transferred [81]. The time of the day could have an influence on the composition of fingerprints because of metabolic aspects and the circadian rhythm, e.g. for melatonin. Moreover the rhythmic expression and activity of different compounds can differ during the day [82]. Studies also showed that the finger itself may also influence the fingerprint composition. It is assumed that generally more people are right handed and therefore the fingers of the left hand contained larger amount of chloride than the fingers of the right hand. Intriguingly, it seems that the most commonly used fingers lose their secretions because of frequent contact with different surfaces. Consequently, the less used fingers can build up and keep larger amount of secretions [83]. Another possible influence when preparing fingerprint samples is of course the procedure of washing. Washing the hands with soap or using cosmetics may of course lead to a modification of fingerprint composition [83; 66]. Therefore, a standard washing procedure is mandatory for the experimental setup.

1.4 Saliva

Saliva is stored in secretion granules in the acini of the salivary glands. Its major constituent is water containing electrolytes and proteins. The most abundant salivary electrolytes are sodium, potassium, chloride and bicarbonate [84]. Various proteins also play a key role as antibacterial antifungal agents in saliva (e.g. lysozyme, lactoferrine, cystatins, histadins) [85]. The ionic concentration in the oral fluid is not constant. The oral fluid is hypotonic compared to serum and the stimulation of saliva depends upon the water household of the body and it can be defined as a reflex response controlled by both parasympathetic and sympathetic secretomotor nerves [86]. The oral fluid originates basically from the major **salivary glands**: Glandula parotis, glandula submandibularis and glandula sublingualis (Figure 4). Generally every type of salivary gland produces a typical secretion: A serous fluid, produced by glandula parotis, a sero-mucous secrete salivated by glandula submandibularis and finally the glandula sublingualis, which only secretes mucous saliva [84; 87]. There are many factors which can lead to an increase of the salivary flow, e.g. different olfactory stimuli, taste and mechanical stimulations as well as varying moods (e.g. aggression, fear). Moreover, pregnancy-related hormonal changes and also drugs may influence salivation stimulation [88]. Various conditions may also decrease the salivary flow rate, such as stress hormones, menopausal-related hormonal changes and in addition anti-adrenergic and anticholinergic drugs. Under healthy conditions adults will normally produce about 500–1500 mL saliva per day (6 mL/min). The contribution of the different salivary glands to the total salivary production also depends on the circadian rhythm and the type of stimulation [84].

1.4.1 Detection of Drugs in Saliva

As mentioned above, drugs which interfere on the central and peripheral nervous systems will influence the production of saliva but it is also of great interest to be able to measure drugs in saliva because their detection could indicate recent drug use, similarly to secretions of fingerprint sweat. It was observed that most drugs appear to enter saliva by passive diffusion [89]. Measuring drugs in saliva would provide a non-invasive diagnostic tool for drug monitoring and detection.

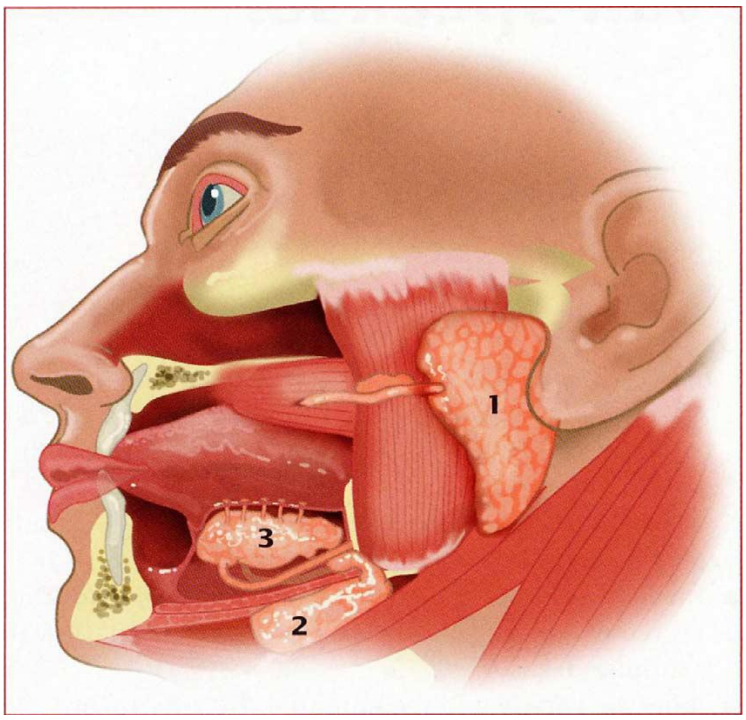


Figure 4 Glandula parotis (1), glandula submandibularis (2) and glandula sublingualis (3) are responsible for the formation of saliva [84; 90]

1.5 Analytical Techniques for Investigating Caffeine and its primary Metabolites in Bodily Fluids

1.5.1 Analytical techniques for fingerprint analysis

There are a few analytical techniques which are used to gain more information about fingermark composition. Quantitative information on amino acid or lipid composition in fingermark residue can be obtained from GC-MS experiments [77]. In order to explore the protein content of fingermarks, more advanced mass spectrometry techniques should be tested. This of course will involve modern ion sources such as ESI, DESI and mass analyzers with higher sensitivity (e.g. Quadrupole, Orbitrap, TOF) [91; 55]. Apart from this, chemical imaging techniques such as Raman or FTIR may also be taken into consideration [72]. Consequently, these chemical imaging techniques have become particularly interesting in the field of forensic science. Earlier studies investigated caffeine in fingerprints using UHPLC-MS and following different sample preparation procedures [92; 5]. To the best of our knowledge, chip-based technologies were not employed for analysing caffeine *in vivo*. It was aimed to quantify caffeine and its primary metabolites together with melatonin and creatinine in extracts from fingerprints, blood and saliva. An efficient separation system is required for this purpose together with a sensitive detection method. The overall setup should also be simple and allowing a rapid sample throughput. Because of these reasons and because the expected analyte concentrations were in the low picomolar range, a liquid chromatography system coupled to triple quadrupole mass spectrometry was the method of choice [36; 93; 94]. In particular, the primary metabolites are rather polar, which may be challenging for the separation of these isomers. UHPLC-UV was employed in initial experiments for the selection of an appropriate extraction solvent. Further experiments with spiked and real samples were mainly performed on a nanoChip-MS system and were compared to a UHPLC-MS setup in some cases.

1.5.2 (Ultra) High Performance Liquid Chromatography - (U) HPLC

Liquid chromatography and in particular high performance liquid chromatography (HPLC) has found widespread use in the development and manufacture of pharmaceuticals, in the analysis of safety and authenticity of food and also in life sciences. HPLC is a separation technique that involves the injection of a small volume of liquid sample into a separation column [95].

The hydrophilic (polar) mobile phase is mostly a mixture of water with organic modifiers (methanol or acetonitrile). The mobile phase passes through the stationary phase (remains fixed in place) usually an apolar chemically modified silica gel. The components ideally equilibrate or partition between the two phases [96] and this distribution between the mobile and stationary phases can be described by the distribution coefficient ($\kappa = C_S / C_M$).

C_s denotes the concentration of solute in the stationary phase and C_M the concentration of the solute in the mobile phase [97]. Different solubilities and affinities of the analytes for the stationary phase result in different migration rates through the system. This leads to the separation of the components of a mixture. The greater the affinity for the mobile phase the more time the analytes spend in the mobile phase and therefore they elute faster [96]. Additionally, lipophilicity can be expressed by the distribution coefficient, which is an important parameterin ADME aspects (absorption, distribution, metabolism, elimination). Obviously, the separation efficiency primarily depends on the choices of column length and particle size, on the organic content of the mobile phase and its viscosity. Polar compounds such as caffeine and its primary metabolites are usually separated by C18-columns, e.g. KINETEX® [95]. The components are finally detected at the exit of the column by a detector.

The separation efficiency of an LC system can be described by means of the van Deemter equation by determining the height equivalent of a theoretical plate (HETP, Figure 5). The van Deemter equation is divided in three different terms: The A term (Eddy-diffusion), the B term (longitudinal diffusion), the C term (mass transfer between stationary phase and mobile phase during separation) using the linear velocity (υ). Therefore, peak broadening is not only due to kinetic effects from mass transfer along the column. High efficiencies in chromatographic systems are characterized by small values of HETP. This can be achieved by minimizing Eddy and longitudinal diffusion and mass transfer [98]. The latter can be achieved by increasing the elution speed.

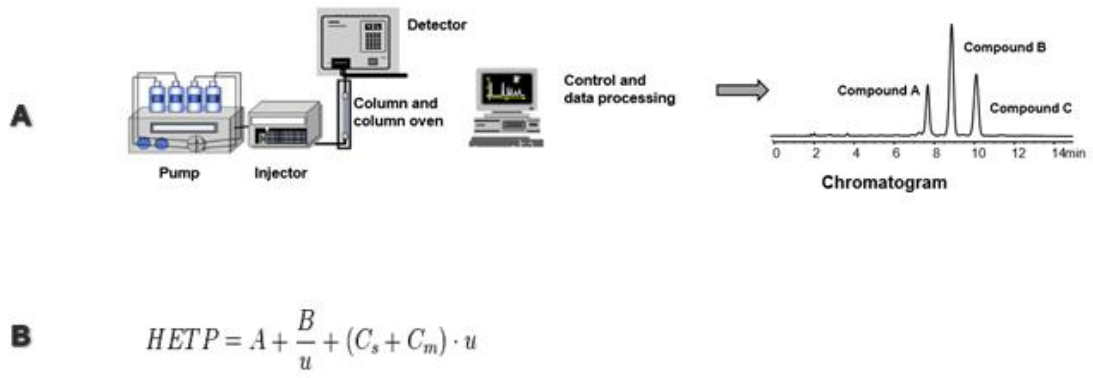


Figure 5 (A) Schematic of an HPLC instrument and (B) the separation efficiency described by the van Deemter equation [98; 99]

Undoubtedly, ultra high performance liquid chromatography (UHPLC) presents the possibility to extend and expand the utility of chromatography. One of the main principles of this evolution was governed by the van Deemter equation as it describes the relationship between linear velocity (flow rate) and plate height (HETP) as outlined above. Increasing the linear velocity increases the separation efficiency, however, this comes at the cost of high backpressures of up to >1000 bar. Higher flow rates in combination with smaller column particles sizes permitted the success of UHPLC systems, which offer significant advantages in resolution or speed and can be coupled to mass spectrometers [99; 100; 101]. Therefore, UHPLC has found applications in drug analysis and serves as a powerful analytical tool for high-throughput analysis [102].

1.5.3 NanoLC (implemented in the Chip Cube)

NanoLC is an alternative to conventional HPLC and benefits from lower flow rates that increase the sensitivity especially for MS hyphenation [103]. For this study, an Agilent Chip-Cube was used, featuring a microfluidic chip-based technology for nanospray LC-MS applications. It is noteworthy that the nanoLC-Chip integrates trapping and analytical columns (both composed of an ientical C_{18} material), capillaries and an ESI nanosprayer directly on the polymer chip. This minimizes peak dispersion and provides chromatographic performance. It significantly reduces the number of fittings, connections and tubing required for nanoflow HPLC [104]. Furthermore HPLC-Chip technology has potential uses across a range of applications including proteomics research, compound analysis, food safety and pharmaceutical development [105].

1.5.4 UV-Vis

In HPLC, the development of photodiode array-based absorbance detectors (early 1980s) added an important second dimension to retention time, namely wavelength. Consequently, it was feasible to obtain information such as analyte identity and peak purity [106]. Three major regions (IR, visible, UV) are used in UV-Vis spectroscopy. Actually UV-Vis detectors are most frequently used to measure components showing an absorption spectrum in the ultraviolet or visible region [107]. The majority of organic compounds can be analyzed by UV-Vis detectors by using a deuterium discharge lamp (D2 lamp) as a light source, with wavelengths ranging from 190–380 nm. Spectrophotometers working in the range from 200–600 nm are widely used as LC detectors. Furthermore almost 70% of published HPLC analyses were performed with UV-Vis detectors. According to this, the relative ease of its operation makes the UV detector one of the most useful and consequently, one of the most widely used LC detectors [109; 110], although it is not nearly as sensitive as a mass spectrometer.

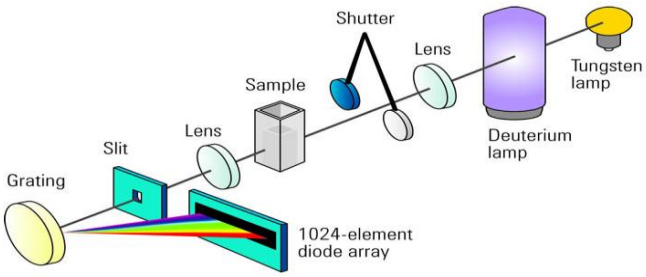


Figure 6 UV-Vis spectroscopy – Schematic of a photodiode array [111]

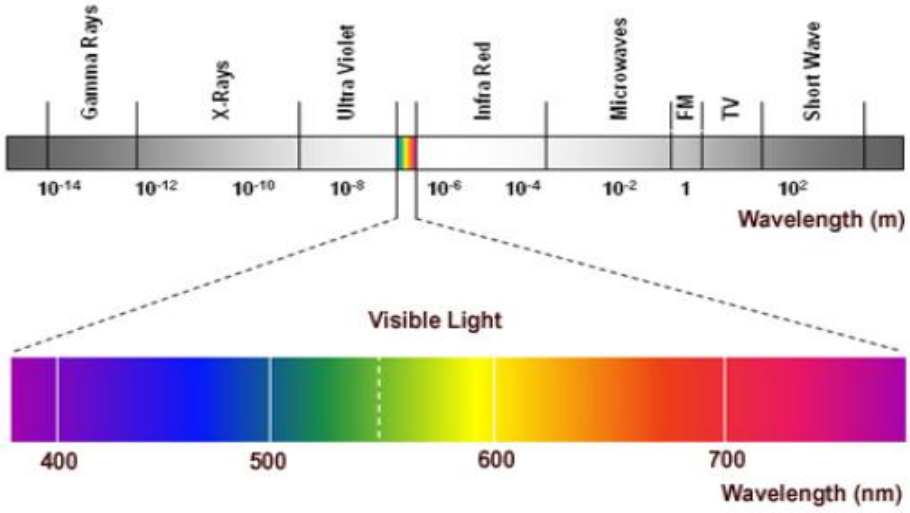


Figure 7 UV-Vis spectroscopy - Wavelengths 100-400 nm (UV) and 400-800 nm (Vis) [108]

1.5.5 Mass Spectrometry

Mass spectrometry is an analytical technique that can measure the masses of ions in the gas phase. From these mass-to-charge ratios it is possible to obtain information on the analytes, e.g. the elemental composition. A mass spectrometer consists of an ion source, a mass analyzer and a detector [112]. The mass analyzer is the component of the mass spectrometer that separates ions according to their mass-to-charge ratios. The mass analyzer then ejects the ions to the detector where they are detected and converted into a digital output. Each mass analyzer has its own benefits and limitations [113]. In particular, triple quadrupoles (linear ion traps) are one of the few mass analyzers that are routinely used for quantitation purposes and are also important in the context of this master thesis as described in the following.

1.5.6 Triple Quadrupole Mass Spectrometer (QqQ)

Triple quadrupole mass spectrometers consist of three aligned quadrupoles Q1, Q2 and Q3 (Figure 8). In Q1 the ions of interest are selected, fragmented in the collision cell (Q2) and finally again selected (Q3) before detection. Each of these quadrupoles consists of 4 conducting metal rods that allow ions of specific *m/z* ratios to pass the quadrupole by applying a distinct combination of direct and alternating voltage. Furthermore, the three quadrupoles can be used in principle in a wide variety of different operation modes (e.g. single reaction monitoring (SRM), multiple reaction monitoring (MRM), MS1 scan, MS2 scan and precursor and product ion scans). Triple-quadrupole mass spectrometers are the "working horse" for quantitative analysis in SRM and MRM modes [114], in which both Q1 and Q3 select certain mass-to-charge ratios and allow only distinct ions to pass. Q2 is thereby used as the fragmenting quadrupole, in which precursor ions are fragmented by collision with inert nitrogen gas. In SRM, the Q1 and Q3 transmit only one precursor ion and one product ion, respectively, whereas several precursor and product ions are followed in MRM.

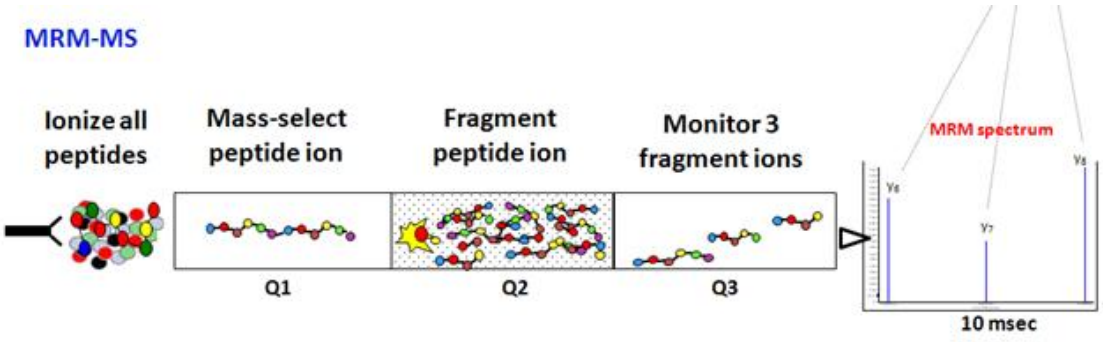


Figure 8 Schematic of a triple quadrupole mass spectrometer [115]

MRM is a tandem mass spectrometric method for rapid, sensitive and selective quantification. It is obviously a powerful method for quantitative measurement of proteins [116; 117; 115]. As explained above, the first step is determined by isolation (preselected in Q1) of a specific precursor ion of interest, followed by a fragmentation step (collision-induced dissociation in q2) and eventually analyzing selected fragment ions in Q3 (product ions). Instead of obtaining full scan MS where all the possible precursor or product ions derived are analyzed, only a small number of sequence-specific fragment ions (transition ions) are analyzed in Q3. This targeted MS analysis allows rapid and continuous monitoring of the specific ions of interest [115; 114] with exceptionally high sensetivity.

Selected ion monitoring (SIM) is frequently used with quadrupole mass spectrometers for method development [118]. In contrast to MRM, selected ion monitoring is a scanning mode without fragmentation in which only a limited *m/z* range is transmitted. This specific *m/z* window contains

only the analyte *m/z* and discards all other species with different mass-to-charge values. However, this mode of operation typically results in reduced sensitivity compared to MRM.

The high selectivity of MS in combination with low-detection limits, the compatibility with LC separation techniques and the ability to deliver quantitative data creates an ideal platform for metabolomics applications [119; 120]. Hyphenated UHPLC-MS has been employed for studies involving toxicity, liver disease, colorectal carcinoma, Alzheimer´s disease, nutritional studies as well as drug metabolite analysis [119]. UHPLC-MS systems use soft ionization methods like Electrospray Ionization (ESI) [121]. To put it in a nutshell, the combination of MS with a liquid chromatography reduces the complexity of the mass spectra due to separation of analytes in time and space. LC-MS is consequently one of the most efficient methods for metabolite identification and quantitation. In other words, it is one of the leading analytical techniques for metabolomics applications [122].

1.6 Experimental Approach for this work

This master thesis is a proof-of-principle study for the quantitation of caffeine and its three primary metabolites in humans after coffee consumption. For this purpose, analyte concentrations were monitored in fingerprints, blood and saliva before and after coffee intake over a period of five hours. The following questions are thereby addressed: Are there individual differences in the metabolism of caffeine? How fast is caffeine metabolized and is there a difference with regard to gender? Is it possible to reproducibly quantify caffeine in fingerprints? Are there significant differences in analyte concentrations over time?

LC-MS was the method of choice for this task because it offers a sensitive, rapid and efficient analysis of the analytes extracted from the different matrices. The quantitation procedures were validated by typical validation characteristics, namely selectivity, linearity and sensitivity, correlation coefficients, accuracies (coefficients of variation), precision, detection limits (LLOD) and quantitation limits (LLOQ).

2 Experimental part

2.1 Materials

- Set of Socorex pipettes (10 µL; 20 µL; 100 µL; 200 µL; 1000 µL)
- Hamilton syringes (100µL, 250µL)
- Erlenmeyer flasks (VWR)
- Tube,Safe-Lock,PP,1.5mL,clear (Eppendorf)
- EPPENDORF Thermomixer comfort 1.5mL
- SONOREX DIGITAL BANDELIN 10P
- SAFETY-LANZETTE (SARSTEDT)
- EDUSCHO-Cafissimo Espresso Classico
- DREITURM (SEIFENCREME Rose pH 6)

2.1.1 Reagents (LC)

- H_2O (MilliQ grade)
- Acetonitrile hypergrade for liquid chromatography (LC/MS) HiPerSolv® CHROMANORM® (VWR)
- Formic acid, for mass spectrometry, ~ 98% (Fluka)
- 2-Propanol LC-MS CHROMASOLV (Sigma Aldrich)
- Nitrogen gas (99.995%)
- Methanol HiPerSolv® CHROMANORM® (VWR)
- Isopropanol HiPerSolv® CHROMANORM® (VWR)

2.1.2 Chemicals

- Caffeine (1,3,7-trimethylxanthine, Fluka)
- Caffeine-D9 (1,3,7-trimethylxanthine-d_9, Sigma Aldrich)
- Paraxathine (1,7-dimethylxanthine, Sigma Aldrich)
- Theobromine (3,7-dimethylxanthin, Sigma Aldrich)
- Theophylline (1,3-dimethylxanthin, Sigma Aldrich)
- Sodium carbonate (Sigma Aldrich)
- Sodium hydroxide (Sigma Aldrich)
- Chloroform (VWR)
- Ammonium bicarbonate (Sigma Aldrich)
- Ethanol (VWR)
- Ethyl acetate (VWR)

2.2 Instrumentation

2.2.1 UHPLC-UV (Agilent 1290)

The UHPLC-UV instrument (Agilent 1290) was equipped with a KINETEX column (1u7 uXB-C18, 50 x 2.1 mm, 100 Å). The injection volume was 5 µL and the chromatograms were recorded at 270 nm using a flow rate of 0.4 mL/min. The autosampler was thermostatted at 4 °C and the column oven at 40 °C. Mobile phase A was aqueous solution (0.1% FA) and mobile phase B was ACN (0.1% FA). A mixture of isopropanol, ACN, MeOH and water (1:1:1:1) was used for backflushing the pistons. Experiments were performed and evaluated using CHemstation B.04.03. SP1 (Agilent). The gradient was applied as outlined in Table 2.

Time (min)	% A (H_2O, 0.1% FA)	% B (ACN, 0.1% FA)
0	100	0
1,5	80	20
3,2	5	95
3,5	100	0
4,5	STOP	STOP

Table 2 Gradient of the UHPLC-UV experiment

2.2.2 NanoChip-MS (Agilent 6490 TripleQuad)

Measurements of fingerprint, whole blood and saliva extracts were performed on an LC Chip-Cube MS system, which consists of a 1260 Infinity LC with a nano- and a cap-pump, as well as an LC-Chip Cube MS Interface combined with a triple quadrupole 6490 mass spectrometer (all Agilent).

Liquid Chromatography. This chip-based setup works on nanoflow and integrates sample enrichment and separation columns, as well as a nanoESI sprayer tip. A small molecule chip was used (UHC-CHIP II, ZORBAX 80SB-C18, 5 µm, 25 mm x 75µm enrichment column and 150 mm x 75 µm separation column, Agilent). The injected sample (0.5 µL) is transferred with the capillary pump onto the trapping column of the nano-Chip. The sample flush (1 µL) and injection path volumes (2 µL) should be tightly controlled when dealing with polar analytes. The autosampler was thermostatted to 4 °C. Mobile phase A was aqueous solution (0.2% FA) and mobile phase B was ACN (0.2% FA). A mixture of isopropanol, ACN, MeOH and water (1:1:1:1) was used for backflushing the pistons. Experiments were performed and evaluated using Mass Hunter B.06.00 and MS-Quantitative, QQQ-Quantitative

and Qualitative Analysis B.06.00 (all Agilent). The gradient was applied as outlined in Table 3 and Table 4 using an overall run time of 25 min.

Table 3 NanoChip - Cap-pump gradient with a total run time of 25 min

Time (min)	% A (H_2O)	% B (ACN)	Flow [µL/min]
0	100	0	3
2	20	80	5
4	20	80	5
5	100	0	5
8	100	0	6
20	100	0	3

Table 4 NanoChip - Nano--pump gradient with a total run time of 25 min

Time (min)	% A (H_2O)	% B (ACN)	Flow [µL/min]
0	100	0	0.4
0.1	92	8	0.4
3	80	20	0.4
5	20	80	0.4
9	20	80	0.4
9.1	100	0	0.4

Mass Spectrometry. The analytes were detected via multiple reaction monitoring (MRM) of three different transitions per molecule as listed in Table 5 with a cycle time of 0.8 s. Typical MS parameters were as follows: capillary voltage –1.7 to –1.9 kV, gas flow 13 L/min, dry gas temperature 200 °C.

Table 5 Parameters of the MRM method

Substance	Precursor Ion	Product Ion	Dwelltime (ms)	Fragmentor/Collision Energy (eV)
Caffeine	195.1	138	50	380/40
		110	50	380/40
		83	50	380/40
Theophylline/ Paraxanthine	181	123.9	50	380/30
		95.9	50	380/30
		69	50	380/30
Theobromine	81	122.2	50	380/30
		107.9	50	380/30
		67	50	380/30
Creatinine	114.1	44.1	50	380/30
Melatonin	233	174.1	50	380/10
		159.2	50	380/30
		130.1	50	380/45
Caffeine-D9	204.2	144.1	50	380/30
		116.2	50	380/30
		89	50	380/30

2.2.3 UHPLC-MS

Extracts from fingerprints and blood were also measured on a UHPLC-MS platform using an Infinity 1290 UHPLC and an 6490 Triple quadrupole mass spectrometer (both Agilent). The chromatographic separation was achieved on a C18 (10 mm × 2.1 mm; 1.7 µm) column using mobile phase consisting of acetonitrile and formic acid (0.2% *w/v*) at a flow rate of 0.4 ml/min. The injection volume was 0.5 µL and the autosampler was thermostatted to 4 °C. Mobile phase A was aqueous solution (0.2% FA) and mobile phase B was ACN (0.2% FA). Experiments were performed and evaluated using Chemstation B.04.03. SP1 (Agilent). The gradient was applied as outlined in Table 6 with an overall run time of 11.5 min.

Table 6 UHPLC Gradient

Time (min)	% A (H_2O)	% B (ACN)
0	100	0
0.1	95	5
3.9	70	30
4.5	20	80
10.5	20	80
11.5	100	0

2.3 Methods

2.3.1 Description of volunteers and the experiment

This study investigates the temporal evolution of caffeine, theobromine, theophylline and paraxanthine in fingerprint, blood and saliva of five volunteers (donors A–E), which is the suggested number of volunteers by power analysis in order to obtain statistical relevant data (calculated using R-studio with a 10% error rate and a significance criterion of 0.05). There were 3 male and 2 women between 25 and 30 of age. All 5 subjects were between non- to moderate caffeine consumers (Table 7). The volunteers were asked to renounce any source of caffeine (e.g. food or beverages containing chocolate/cocoa and caffeine) for 12 hours before the start of each experiment. Subjects presented on 8 AM on the study day before the first cup of coffee. Whole blood, fingerprints and saliva samples were collected before coffee consumption and one, three and five hours after coffee consumption. The extraction procedures of each sample type are outlined below. For this study a measured cup of coffee was provided which contains 80 mg/100 mL caffeine. The experiment was performed on three different days.

Table 7 Donors A-E (3 men and 2 women) at the age of 25 to 30 with different habits in caffeine consumption were asked to eat and drink nothing containing caffeine for 12h before beginning the experiment. Their fingerprints, whole blood as well as saliva were taken just before they drank a measured amount of coffee and these samples were collected again 1, 3 and 5 hours after coffee consumption.

Identifier	Gender	Habitualness
Donor A	female	Non caffeine consumer
Donor B	female	Less moderate caffeine consumer
Donor C	male	Less moderate caffeine consumer
Donor D	male	Moderate caffeine consumer
Donor E	male	Moderate caffeine consumer

2.3.2 Selection of a Suitable Solvent for Extraction

First of all, the extraction efficiency from aqueous samples spiked with CF, TB and TP was evaluated using several extraction solvents. The extraction efficiency was calculated as the ratio of the determined amount of analyte after and before extraction using an UHPLC-UV system. To that effect, six different solvents were used, i.e. chloroform, ethyl acetate, acetonitrile, diethyl ether, dichloromethane and a mixture of methanol/chloroform. Each solvent was mixed 1:1 with the standard spiked aqueous solution except for acetonitrile (1:10) and the methanol/chloroform mixture (2.5:1:1) as listed in Table 8.

Table 8 Extraction solvents, abbreviations and the volume-ratio for extraction

Solvent	*Abbreviation*	*Vol. Ratio for extraction*
Chloroform	CH (Pipette and Hamilton)	1 : 1
Ethyl acetate	EA	1 : 1
Acetonitrile	ACN	1 : 10
Methanol/Chloroform	MC	2.5 : 1 : 1
Diethyl ether	DE	1 : 1
Dichloromethane	DCM (Pipette and Hamilton)	1 : 1

Five concentration levels were determined for a mixture of caffeine, theobromine and theophylline at 1, 3.33, 10, 33.33 and 100 ng/µL dissolved in pure water. The stock solutions were 1 mg/mL for caffeine and theophylline and 0.2 mg/mL for theobromine. These calibration curves were measured in three independent experiments and each level with three technical replicates. The evaluation of the extraction efficiencies were performed similarly in three independent experiments and with three technical replicates. The extraction mixture was vortexed for 1 min and additionally stirred in a Thermomixer at 40 °C and 1400 rpm for 10 min. This process was repeated twice and the extracts were transferred into Eppendorf tubes (1.5 mL), which corresponds to 250 µL organic phase or dilution The extracts were dried under a flow of dinitrogen. The dried residues were reconstituted in 250 µL water and sonicated for 10 min before analysis by UHPLC-UV.

The extraction efficiency for each solvent was calculated for each metabolite by accounting for the respective dilution factors. The measured amounts of each metabolite were divided by the amount from the calibration level and multiplied by the dilution factor (Table 8). The measurements were classified using traffic light logic. Different colors represent caffeine, theobromine and theophylline. A positive result with respect to the extraction efficiency was marked in green and a poor result in red. All these experiments were performed for a mixture of caffeine, theobromine and theophylline dissolved in pure water.

2.3.3 Extraction of CF and primary metabolites from artificial finger sweat

A volume of 50µL secreted sweat can be expected in a fingerprint [3]. In order to evaluate the extraction from sweat, a solution to simulate fingerprint-sweat secretion was prepared according to reference [3]. Table 9 illustrates the composition of the artificial finger sweat, which was mainly composed of lactic acid.

Table 9 Composition of artificial finger sweat

	[µg/mL]	50 mL [mg] 2xSTOCK	M (g/mol)
Lactic acid	200	20 mL	90.08
Aminoacids	100	1	
-Serine	50	5	105.09
-Cysteine	13	1.3	121.16
-Valine	37	3.7	117.15
Urea	20	2	60.06
NaCl	70	7	58.44
KCl	50	5	74.55
ABC (Ammoniumbicarbonate)	5	0.5	79.056
$CaCl_2$	4	0.4	110.98
$MgSO_4$	2	0.2	120.36

An aliquot of 25 µL of this solution was spiked with 25 µL of different concentrations of a mixture containing CF, TB and TP. Concentrations for these measurements ranged from 10 fg/µL to 100 pg/µL and were extracted using 450 µL diethyl ether and acetonitrile, respectively, according to the procedure outlined above. It was attempted to perform a matrix-matched extraction from sweat using 3 biological and technical replicates. Unfortunately, gel-like substances remained on the ground after drying, which were not suitable for further processing. Consequently, the overall process efficiency was evaluated by spiking filter paper with 0.5 and 50 pg/µL CF, TB and TP before the extraction procedure and comparing standard samples in aqueous solution.

2.3.4 Extraction of CF and primary metabolites from human plasma

Plasma from a non-coffee consumer was used to evaluate the lower limit of quantification (LLOQ) and detection (LLOD) of spiked caffeine and primary metabolites. Plasma was spiked with caffeine, theobromine and theophylline from a 10 ng/µL stock solution. Aliquots of 10 µL of the concentration range between 0.05-50 pg/µL were added to 140 µL plasma. Each compound series was then centrifuged for 1 min, stirred in a Thermomixer at 40 °C and 1400 rpm for 10 min and subdivided into 6 samples, 20 µL each. Acetonitrile (480 µL) was added to each sample and extracted (centrifuged for 1 min, stirred in a Thermomixer at 40 °C and 1400 rpm for 10 min). 250 µL of the organic phase or dilution was transferred into Eppendorf tubes and dried by a stream of dinitrogen and analysed by UHPLC-MS. Furthermore the overall process efficiency was performed by spiking 0.5 pg/µL and 50pg/µL CF, TB and TP into the plasma matrix before the extraction procedure, which was then compared with the aqueous standard samples.

2.3.5 Extraction of CF and primary metabolites from blood

Blood was drawn by pricking any one of the fingers except for index fingers with a lancet for self-collection. The handling turned out to be easy and simple: The protective cap was removed held against the finger and the triggering button was squeezed. A volume of 20 µL of whole blood was taken with a small pipette and transferred into an Eppendorf tube containing 480 µL ACN. The mixture was vortexed for 1 min and stirred in a Thermomixer at 40 °C and 1400 rpm for 10 min. This process was repeated twice and the extraction mixture was centrifuged for 10 min at about 20000 rpm. The solutions (250 µL) were transferred into Eppendorf tubes. The solutions were dried under a flow of dinitrogen and were reconstituted in 250 µL water containing 0.2% FA. The extracted blood samples were sonicated for 10 min and again stirred in a Thermomixer at 30 °C (1400 rpm) for 10 min before transfer 96 well plates for analysis by nanoChip-MS and UHPLC-MS.

Table 10 Extraction procedure for CF and primary metabolites from blood

Blood *Extraction procedure with ACN*	20µL blood were added to 480 µL ACN 3x 10min Thermomixer 40°C,1400rpm
	3x 1min mixed Centrifuged for 10min, 20000 rpm
	250µL transferred Dried and concentrated by Dinitrogen
	Dissolved in 250 µL water (0.2% FA) Sonicated for 10 min 10min Thermomixer 30°C, 1400rpm

2.3.6 Extraction of CF and primary metabolites from fingerprints

A filter paper (1 cm x 1 cm) was wetted with 50 µL H_2O. The hands were washed with water and soap for one minute to completely remove external contaminants. Then, the index fingers were pressed on the wetted filter paper for 1 min. The filter paper was transferred into an Eppendorf tube and CF and metabolites were extracted with acetonitrile. First, the extraction solution was vortexed for 1 min, stirred in a Thermomixer at 40 °C and 1400 rpm for 10 min. Similar to the extraction form blood, this process was repeated twice and the mixture was centrifuged for 10 min at 20000 rpm. After centrifugation the small filter paper was removed by a small pincette. Afterwards 250 µL of the organic phase or the dilution were lifted and transferred into Eppendorf tubes. The tubes were dried under a flow of dinitrogen. The dried residues were reconstituted in 250 µL water containing 0.2% formic acid. The extracted samples were sonicated (10 min) and transferred into 96 well plates for analysis by nanoChip-MS and UHPLC-MS.

Table 11 Extraction procedure for CF and primary metabolites from Fingerprint

Fingerprint *Extraction procedure with ACN*	500µL ACN was added
	3 x 10 min Thermomixer 40°C, 1400 rpm
	3 x 1 min mixed (Vortex)
	Centrifugated for 10 min, 20000 rpm
	Filter paper removed
	250 µL lifted and transferred
	Dried and concentrated by Dinitrogen
	Dissolved in 250 µL water (0.2% FA)
	Sonicated for 10 min

2.3.7 Extraction of CF and primary metabolites from saliva

Saliva was retrieved by spitting into a single-use small bowl. An aliquot of 20 µL of saliva was recovered and transferred into an Eppendorf tube which contained 480 µL of ACN. The solution was vortexed for 1 min, stirred in a Thermomixer at 40 °C and 1400 rpm for 10 min. This process was repeated twice and the extraction mixture was centrifuged (10 min at 20000 rpm). 250 µL were transferred into Eppendorf tubes (1.5 mL). The solutions were evaporated under a flow of dinitrogen and the residue was reconstituted in 250 µL water containing 0.2% FA. Eventually, the samples were sonicated for 10 min and transferred into 96 well plates for analysis by nanoChip-MS and UHPLC-MS.

Table 12 Extraction procedure for CF and primary metabolites from Saliva

Saliva *Extraction procedure with ACN*	480 µL ACN was added
	3 x 10 min Thermomixer 40°C,1400 rpm
	3 x 1 min vortexed
	Centrifugated for 10 min, 20000 rpm
	250 µL transferred
	Dried and concentrated by Nitrogen
	Dissolved in 250 µL water (0.2% FA)
	Sonicated for 10 min

2.3.8 Internal Standard

The stable isotope-labelled standard caffeine-D9 was used as the internal standard (IS) and was spiked to the calibration solvents. It was used for calibration by plotting the ratio of the analyte signal to the internal standard. The IS was also spiked to all biological samples (fingerprint sweat, saliva and whole blood) in a concentration of 10 pg/µL. Additionally a 100 pg/µL standard mixture containing CF, TB and TP was measured every thirtieth sample as a quality control.

3 Results and Discussion

LC-MS based methods were evaluated for their suitability of quantifying caffeine and its primary metabolites in human body fluids, e.g. sweat of fingerprint, saliva and blood. An extraction method was developed and the recoveries of extraction from several organic solvents were investigated by UHPLC-UV. Spiked samples for validation purposes and real samples were analyzed mainly by nano-LC combined with a triple quadrupole mass spectrometer in the MRM mode. The performance of the nano-LC is finally compared with that of an UHPLC instrument. From these studies, it is aimed at deriving information on the individual metabolic activity and whether it is possible to reproducibly quantify the analytes after consumption of a cup of coffee as already described in the experimental approach.

3.1 Selection of a Suitable Solvent System for Extraction by UHPLC-UV

One of the main initial tasks was the evaluation of suitable extracting conditions that allow efficiently extracting CF and its primary metabolites with one single extraction. Six different organic solvent systems were evaluated, namely chloroform (CH), ethyl acetate (EA), acetonitrile (ACN), methanol/chloroform (MC), diethyl ether (DE) and dichloromethane (DCM). Chloroform is widely used as an extraction agent [123], but also acetonitrile [124] or methanol/chloroform [125] were reported. An UHPLC-UV method was set up with a short and flat gradient for separating CF, TB and TP using an Agilent KINETEX 1u7 uXB-C18 column (100 Å, 50 x 2.1 mm, Table 2. The analytes were simply identified by their retention times and their peak areas were used for calculating the recovery of extraction. For the extraction, an equimolar mixture of CF, TB and TP was prepared in aqueous solution at five concentrations ranging from 1–100 ng/µL. This mixture was then combined with the organic solvent in a 1 : 1 ratio with CH, EA, DE and DCM, in a 1 : 10 ratio with ACN and in a 1 : 1 : 2.5 ratio with MC (Table 8). After extraction of the metabolites, the organic phase was dried under a nitrogen stream. Before HPLC measurements, the dried samples were reconstituted in water (0.1% FA). For each solvent system, the detected amount of CF, TB and TP was calculated and the recovery of extraction of each metabolite was determined by comparison with the respective standard calibration curves. The calibration curves were determined by three independent experiments and each level by three technical replicates (Figure 9). The equations of the calibration curves and the respective correlation coefficients (R^2) were as follows: Caffeine ($y = 6.836x$, $R^2 = 0.9998$), theobromine ($y = 5.71x$, $R^2 = 0.9998$) and theophylline ($y = 7.5829x$, $R^2 = 0.9996$). Therefore, the calibration curves featured overall correlation coefficients of >0.999 over the concentration range of two orders of magnitude. Under the conditions explained in the experimental part, the retention times of TB, TP and CF were 1.347, 1.513, 1.748 min, respectively, as indicated in Figure 10, which shows the UV chromatogram of a standard mixture of CF, TB and TP at 1 ng/µL.

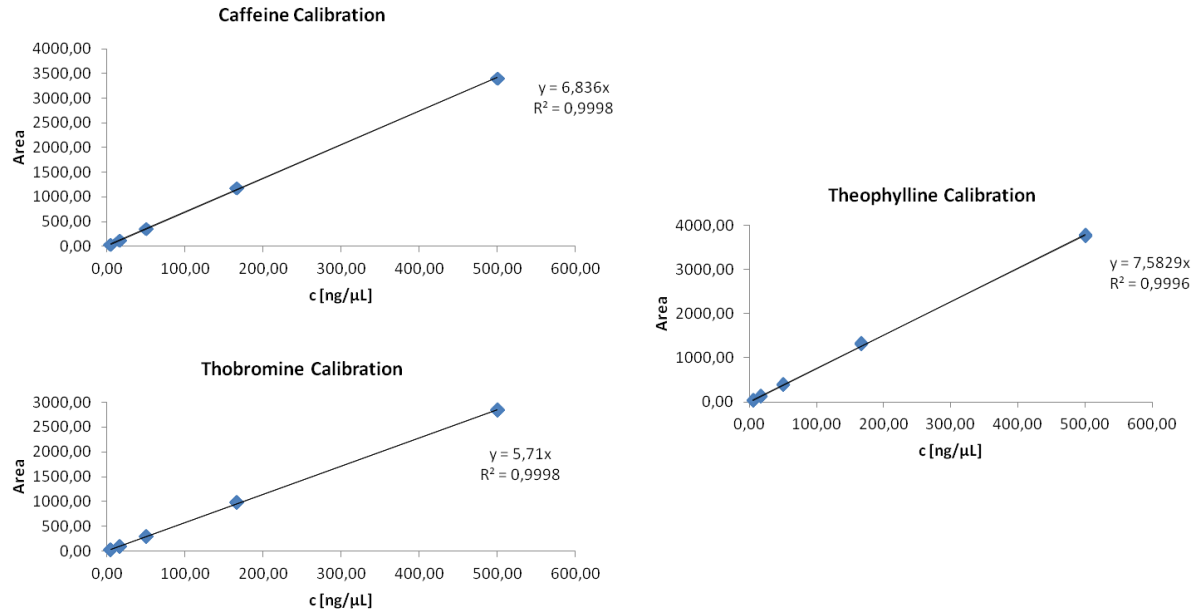


Figure 9 Calibration curves for caffeine, theobromine and theophyline at five concentration levels ranging from 1-100ng/µL

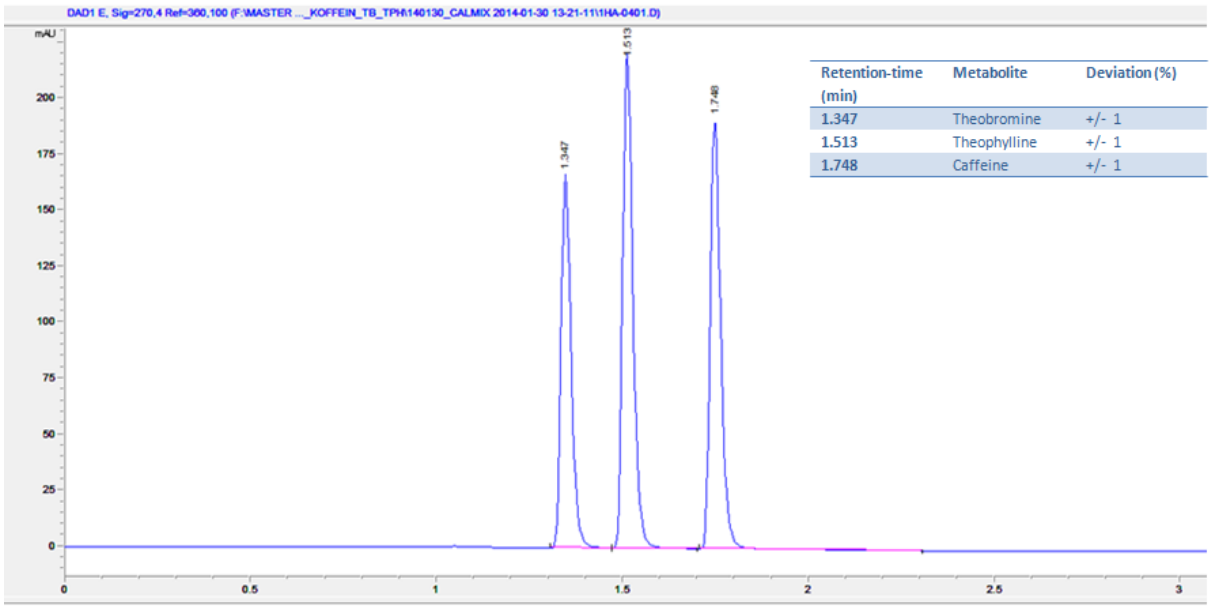


Figure 10 UHPLC-UV measurements of a 1ng/µL calibration solution featuring a mixture of TB, TP, CF with respective retention times at 1.347, 1.513, 1.748 min

The extractions of five concentration levels for each of the six solvent systems were performed in three independent experiments and by measuring three technical replicates. Additionally, the extractions with chloroform and dichloromethane were performed by two operators with a pipette and a Hamilton syringe, respectively. These measurements were made in order to estimate possible

errors during the collection of the organic phase. Overall, this matrix-free evaluation of the six different extraction solvents showed that the recovery of extraction varied greatly among the three metabolites depending on the lipophilicity of the extraction solvent. This is to be expected since the primary metabolites of caffeine are highly polar. Accordingly, the measurements were classified using traffic light logic (Table 13). Different colors represent caffeine, theobromine and theophylline. A high recovery of extraction was marked in green and a poor result in red.

At first view, it is noticeable that the recovery of extraction of caffeine is relatively high independently of the extraction solvent with the exception of ethyl acetate. This stands in contrast to the recoveries of extraction of theophylline and theobromine, which show acceptable results only in acetonitrile and diethyl ether. Acetonitrile is a solvent of medium-polarity and has the advantage that it is not volatile compared to diethyl ether. The high recoveries of extraction in acetonitrile are not too surprising since it is miscible with water and leads to a dilution of the analytes, in contrast to diethyl ether, which allows a “true” liquid-liquid extraction. The lowest concentration of 1 ng/µL (5 ng on column) is close to the LOQ of the HPLC-UV method, which may explain the frequent occurrence of zero values. The extraction with the mixture of methanol/chloroform apparently led to a false positive result for caffeine. This may be due to the fact that these samples were stored at 4°C for more than twelve hours after dissolving. Therefore, it is believed that some solvent is evaporated, which would lead to an increased sample concentration. Stability tests of the three analytes up to 48 hours proved a solvent loss of 5 µL in 24 hours at 4 °C in the autosampler (Table 14). This represents a concentration increase of 4% in 24 hours, which however, cannot completely account for the intensity gain of the detected signals. Ethyl acetate displayed generally low recoveries of extraction although some selectivity for TP was observed. The chloroform, dichloromethane and methanol/chloroform extractions delivered equally poor results.

Performing the extraction experiments with either a pipette or a Hamilton syringe did not show considerable differences with respect to the recoveries of extraction. Similarly, also the effect of different operators on the recovery of extraction was negligible, as well as the effect of concentration in the tested range. So far, the results suggest that acetonitrile and diethyl ether are the most suitable solvents for the simultaneous extraction of CF, TB and TP.

Amount = 0
0,01 < CF < 0,69
0,01 < TP < 0,69
0,01 < TB < 0,69
0,7 < Amount< 0,99
Amount > 1,0

Table 13 Recoveries of extraction for selected solvent extraction systems determined by UHPLC-UV. Mix denotes the standard calibration mixture containing CF, TB and TP. Solvent systems are abbreviated as DE (Diethyl ether), ACN (Acetonitrile), MC (Methanol/chloroform), CH (Chloroform), DCM (Dichloromethane) and EA (Ethyl acetate). The extractions with chloroform and dichlormethane were performed with a pipette (Operator 1) and a Hamilton syringe (Operator 2). The levels refer to the injected amount in ng/µL. The color code represents the recoveries of extraction categorized using traffic light logic. Red indicates zero amounts of recoveries of extraction. The colors orange to yellow describe the amounts of CF, TP and TB between 0.01 and 0.69. The green color represents the recovery of extraction between 0.7 and 0.99 and the beige color amounts the recovery of extraction above 1.0. The coefficients of variations for each concentration and analyte are shown in brackets.

			Recovery of Extraction TB		Recovery of Extraction TP		Recovery of Extraction CF	
Extracting Solvent	Level (ng/µL)	Dilution	Operator 1	Operator 2	Operator 1	Operator 2	Operator 1	Operator 2
Mix	1	1,00	0,76 *(0.34%)*		0,66 *(0.88%)*		0,82 *(0.53%)*	
	3.3	1,00	0,95 *(0.16%)*		0,93 *(0.19%)*		0,96 *(0.09%)*	
	10	1,00	1,00 *(0.10%)*		1,00 *(0.10%)*		1,00 *(0.32%)*	
	33.3	1,00	1,03 *(0.16%)*		1,04 *(0.57%)*		1,03 *(0.20%)*	
	100	1,00	1,00 *(0.04%)*		1,00 *(0.05%)*		1,00 *(0.02%)*	
DE	1	1,00	0,70 *(0.8%)*		0,46 *(19 %)*		0,60 *(13.5%)*	
	3.3	1,00	0,95 *(5%)*		0,89 *(5%)*		0,91 *(5.5%)*	
	10	1,00	1,00 *(2.5%)*		0,88 *(2.2%)*		0,86 *(9.3%)*	
	33.3	1,00	1,09 *(4.6%)*		0,95 *(4.6%)*		0,93 *(6.7%)*	
	100	1,00	1,11 *(2.3%)*		0,95 *(3%)*		0,94 *(2%)*	
ACN	1	10,00	0,00 *(-%)*		0,00 *(-%)*		0,00 *(-%)*	
	3.3	10,00	0,42 *(6.2%)*		0,00 *(-%)*		0,64 *(14.2%)*	
	10	10,00	0,94 *(18.5%)*		0,70 *(25%)*		0,85 *(20.8%)*	
	33.3	10,00	1,00 *(10.4%)*		0,86 *(10.9%)*		0,90 *(10.4%)*	
	100	10,00	1,13 *(3.4%)*		1,00 *(3.9%)*		1,00 *(3.9%)*	
MC	1	4,17	0,00 *(-%)*		0,00 *(-%)*		0.86 *(0.4%)*	
	3.3	4,17	0,10 *(17%)*		0,04 *(28%)*		2,23 *(8.4%)*	
	10	4,17	0,17 *(12.9%)*		0,25 *(13.5%)*		2,26 *(7.5%)*	
	33.3	4,17	0,23 *(13.5%)*		0,34 *(17.7%)*		2,20 *(17.8%)*	
	100	4,17	0,22 *(18.2%)*		0,40 *(12.1%)*		2,34 *(13.1%)*	
CH	1	1,00	0,19 (2.7%)	0,21 *(6.4%)*	0,00 *(-%)*	0,00 (-%)	0,83 *(0.4%)*	0,84 *(23%)*
	3.3	1,00	0,34 *(0.3%)*	0,4 *(2.3%)*	0,11 (6.1%)	0,10 *(3.6%)*	0,99 *(4.7%)*	1,2 *(7.6%)*
	10	1,00	0,34 *(7.3%)*	0,36 *(4.5%)*	0,17 *(0.5%)*	0,19 *(3.1%)*	0,98 *(2.2%)*	1,05 *(4.4%)*
	33.3	1,00	0,31 *(4.6%)*	0,39 *(5.1%)*	0,20 *(2.9%)*	0,21 *(1.7%)*	1,01 *(3.4%)*	1,06 *(2.2%)*
	100	1,00	0,39 *(1%)*	0,4 *(0.3%)*	0,21 *(0.5%)*	0,22 *(6.9%)*	1,00 *(1.3%)*	1,05 *(6.6%)*
DC	1	1,00	0,02 *(10.2%)*	0,02 *(10%)*	0,00 (- %)	0,00 (- %)	0,97 *(1.3%)*	0,90 *(8.2%)*
	3.3	1,00	0,14 *(4.3%)*	0,08 *(9.5%)*	0,03 *(0.6%)*	0,06 *(0.04%)*	0,91 *(1.2%)*	0,89 *(8.7%)*
	10	1,00	0,08 *(9.7%)*	0,06 *(12.4%)*	0,13 *(0.04%)*	0,11 *(0.15%)*	0,96 *(0.28%)*	0,86 *(3.9%)*
	33.3	1,00	0,10 *(2.1%)*	0,16 *(0.8%)*	0,08 *(0.06%)*	0,14 *(0.04%)*	0,93 *(0.89%)*	0,90 *(17%)*
	100	1,00	0,25 *(6.9%)*	0,26 *(5.4%)*	0,16 *(0.09%)*	0,16 *(5.8%)*	0,96 *(3.7%)*	0,97 *(3.6%)*
EA	1	1,00	0,00 *(-%)*		0,16 *(10.8%)*		0,27 *(1.6%)*	
	3.3	1,00	0,13 *(1.6%)*		0,25 *(4.5%)*		0,42 *(4.1%)*	
	10	1,00	0,14 *(13.6%)*		0,30 *(5.5%)*		0,44 *(5.9%)*	
	33.3	1,00	0,19 *(0.4%)*		0,35 *(0.3%)*		0,50 *(0.4%)*	
	100	1,00	0,20 *(0.1%)*		0,37 *(9.9%)*		0,51 *(1%)*	

Table 14 Stability studies of the analytes in the calibration mixture at five different concentration levels. The first series was measured after 12 hours and the second series after 42 hours. An increase in recovery can be detected, which is due to evaporation of the solvent and accounts for a signal increase of approximately 4% in 24 h. The coefficients of variation in three independent experiments measuring three technical replicates are denoted in brackets.

	Recovery of Extraction TB		Recovery of Extraction TP		Recovery of Extraction CF	
Level (ng/µL)	**12 h**	**42 h**	**12 h**	**42 h**	**12 h**	**42 h**
1	0,76 *(0.34%)*	0,86 *(0.88%)*	0,66 *(0.88%)*	0,76 *(2.1%)*	0,82 *(0.53%)*	0,92 *(1.4%)*
3.3	0,95 *(0.16%)*	1,03 *(2%)*	0,93 *(0.19%)*	1,01 *(0.6%)*	0,96 *(0.09%)*	1,04 *(0.9%)*
10	1,00 *(0.10%)*	1,09 *(0.25%)*	1,00 *(0.10%)*	1,09 *(0.5%)*	1,00 *(0.32%)*	1,09 *(0.1%)*
33.3	1,03 *(0.16%)*	1,10 *(3.4%)*	1,04 *(0.57%)*	1,11 *(0.7%)*	1,03 *(0.20%)*	1,10 *(1.9%)*
100	1,00 *(0.04%)*	1,07 *(0.5%)*	1,00 *(0.05%)*	1,07 *(0.7%)*	1,00 *(0.02%)*	1,07 *(0.4%)*

3.2 Chip-based Microfluidics LC-MS

The UHPLC-UV results served to evaluate the most suitable solvent for the simultaneous extraction of CF and its primary metabolites and acetonitrile and diethyl ether turned out to be suitable extracting solvents. For biological samples however, a more sensitive and selective detection method is required than UV. Therefore, a mass spectrometric method was selected based on MRM and equipped with a Nano-LC system (Agilent 6490 Triple quadrupole equipped with a Chip Cube). This setup was mainly used for quantifying caffeine and its primary metabolites in whole blood, oral fluid from salivary glands and sweat secretion from fingerprints. Earlier studies investigated caffeine in fingerprints using UHPLC-MS and following different sample preparation procedures [5; 92]. To the best of our knowledge, chip-based technologies were not employed for analyzing caffeine *in vivo* up to date.

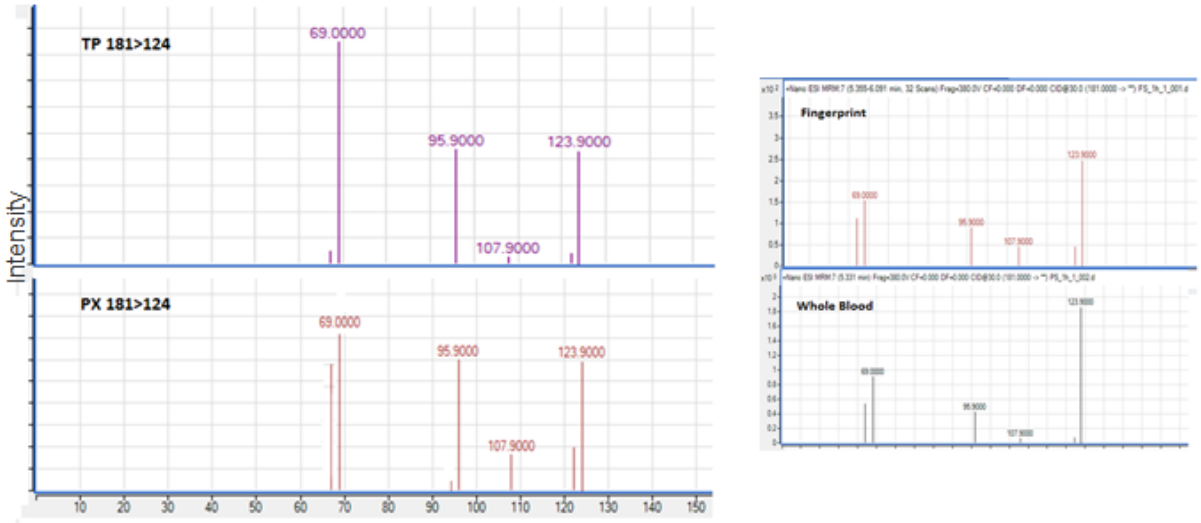
3.2.1 Final Chip LC-MS Method

The development of methods in analytical chemistry is based on the evaluation of concentrations using fundamental physical constants and quantities. The analytical signal depends not only on the properties of the analyte species involved but also on the instrumental parameters. It is obvious that experimental conditions should be chosen in order to permit the prediction of the analytical signal.

The aim of this work was to develop a sensitive and selective method to evaluate and quantify small amounts of caffeine, theobromine, theophylline and paraxanthine *in vivo*. Caffeine and its primary metabolites share the same xanthine structure with just small differences in structure, whereas the primary metabolites are isomers differing only in the position of the demethylation, i.e. the absence of a methyl group at position 1, 3 or 7, respectively. This results in many shared MS/MS transitions in particular for TB, TP and PX (Figure 11) [126]. Theophylline and paraxanthine have virtually identical product ion spectra and their reported retention times are also very similar [5], although it was possible to separate them using LC [127; 91; 128]. There is generally great interest in monitoring these two analytes simultaneously. Fact is that more and more studies focus on paraxanthine because roughly 80% of caffeine is *N*3-demethylated to form paraxanthine (PX) [30]. Trapping the primary metabolites turned out to be challenging using the nanoChip Cube setup. The injected sample is transferred with the capillary pump onto the trapping column of the nano-Chip. It turned out that the sample flush (1 µL) and injection path volumes (2 µL) must be tightly controlled in order not to lose any of the polar analytes. Although starting 100% aqueous, the metabolites tended to diffuse into the trapping column, which is underlined by the broad peaks of TB and TP/PX (Figure 13). The peak shape of caffeine indicates an efficient trapping. Therefore, the high polarity of the primary metabolites seems to constitute the working limits of the nanoChip with regard to separation and

trapping efficiency. Due to this reason, it was not possible to separate TP and PX and consequently, these two metabolites will be evaluated together in the following discussion. Retention times of 5.5, 6.1 and 7.1 min for TB, TP/PX and CF were determined, respectively using a three minute flat gradient from 8 to 20% organic modifier (Figure 12). Additionally, it turned out that the equilibration of the trapping and the separation column on the nano-Chip is crucial for obtaining reproducible results after employing high percentages of organic mobile phase. Therefore, the system was equilibrated for 16 min in 100% aqueous phase after each run, which amounted to a total chromatographic run time of 25 min.

Figure 11 Product ion scans of a standard solution of theobromine (TP) and paraxanthine (PX) showing their similar fragment mass spectra (left). The fragments show equal product ions at *m/z* 69, 95.9 and 123.9 but with a different ratio between product ions *m/z* 67 and 69. Therefore, a more abundant mass fragment at *m/z* 67 is indicative of paraxanthine. Two further product ion scans from fingerprint and whole blood indicate an overlap of PX and TP (right).



The final MRM parameters are reported in Table 15. A total of 16 transitions amount to an overall cycle time of 800 ms. The following transitions were used as quantifiers for the MRM-analysis: CF (195.1 – 138.0), TB (181.0 – 67.0), TP/PX (181.0 – 69.0), CF-D9 (204.2 – 144.1). Three transitions were used per analyte with the exception of creatinine for which SRM was applied. Stable isotope labelled caffeine (CF-D9) was used as an internal standard (IS) to optimize extraction procedures, to correct for the loss of analytes during sample preparation and to account for spray instabilities. It was spiked to a final concentration of 10 pg on column to each sample. It is important to note that any factor that influences the analyte signal of CF should also affect the signal of the internal standard to a similar degree.

Figure 12 Comparison of the Cap-Pump Gradient (3.0-6.0 µL flow) and Nano-Pump Gradient (0.4 µL flow). The trapping column was switched from the nano Pump to the capillary pump 9 min after injection. The total run time was 25 min

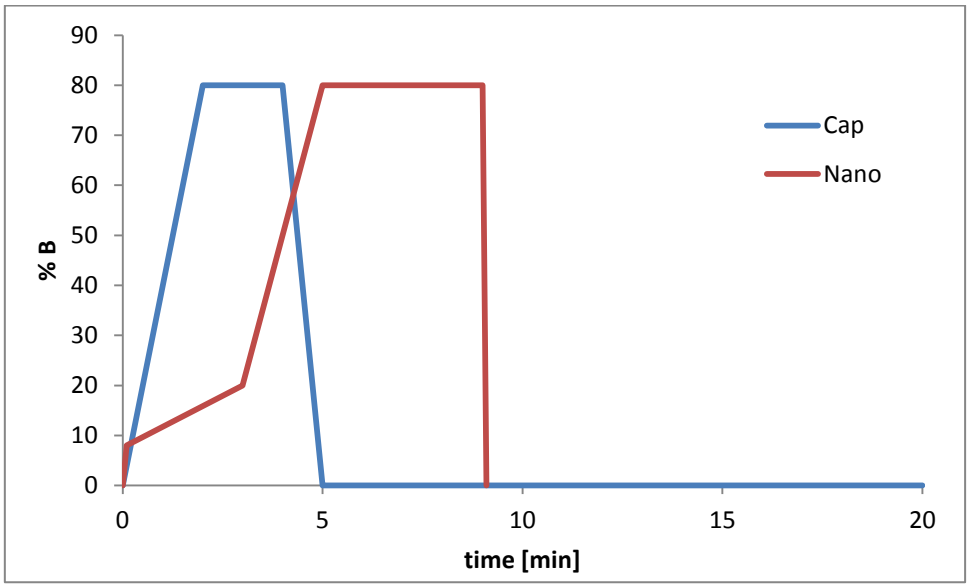


Figure 13 LC-MRM Chromatogram of Theobromine (TB, t_R 5.5 min, blue), Theophylline/Paraxanthine (TP/PX, t_R 6.1 min, yellow) and Caffeine (CF, t_R 7.1 min, green). The data was obtained from a calibration solution (50 pg/µL). The horizontal axis defines the retention time in minutes and the vertical axis the relative abundance of the signal of the product ion during MRM. The following transitions were monitored for each compound: TB t_R 5.5 (181.0 – 67.0), TP/PX t_R 6.1 (181.0 – 69.0), CF t_R 7.1 (195.1 – 138.0). Three technical replicates are displayed.

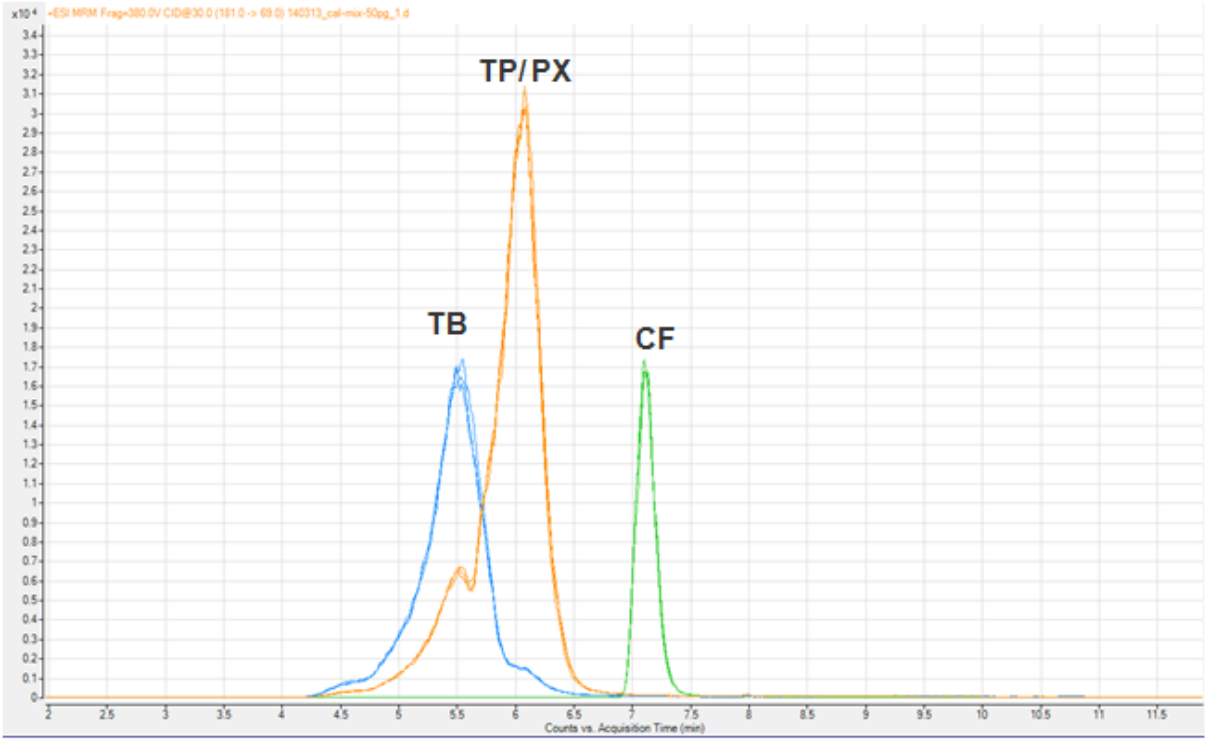


Table 15 MRM-transitions of CF, TP, TB, CF-D9 (IS), creatinine and melatonin. The following transitions were used as quantifiers: CF (195.1 – 138.0), TB (181.0 – 67.0), TP (181.0 – 69.0), CF-D9 (204.2-144.1). The retention times for each metabolite are shown in brackets.

Substance	Precursor Ion	Product Ion	Dwelltime (ms)	Fragmentor/Collision Energy (eV)
Caffeine	195.1	138	50	380/40
		110	50	380/40
		83	50	380/40
Theophylline/ Paraxanthine	181	123.9	50	380/30
		95.9	50	380/30
		69	50	380/30
Theobromine	81	122.2	50	380/30
		107.9	50	380/30
		67	50	380/30
Creatinine	114.1	44.1	50	380/30
Melatonin	233	174.1	50	380/10
		159.2	50	380/30
		130.1	50	380/45
Caffeine-D9	204.2	144.1	50	380/30
		116.2	50	380/30
		89	50	380/30

3.3 Quantitation of Caffeine and its Primary Metabolites in Fingerprints

3.3.1 Sample Preparation

Small filter papers (1cm x 1cm) wetted with H_2O were used for fingerprint measurements. The fingers were washed thoroughly with water and soap for one minute to ensure that the fingertips were not contaminated e.g. by dust, analytes or sebum. After one minute, the index fingers were pressed on the wetted filter papers for 60 s. The extraction procedure was performed according to chapter "Extraction of CF and primary metabolites from fingerprints" using acetonitrile. Finally, the dried residues were reconstituted in 250 µL water containing 0.2% formic acid. The straightforward sample preparation procedure allowed a routinely achieved throughput of up to 60 samples per day.

3.3.2 Method Validation

For the quantitation of caffeine and its primary metabolites in fingerprints, typical validation parameters were evaluated, notably selectivity, linearity, sensitivity, correlation coefficients, accuracies (by coefficients of variation), lower limit of quantification (LLOQ) and the limit of detection (LOD) of caffeine and its primary metabolites. Obviously, specificity in liquid chromatography is obtained by choosing optimal columns and chromatographic conditions. LODs and LOQs for nanoChip MS- and UHPLC-MRM methods were validated according to FDA guidelines [129].

The concentration range was established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision. Eight calibration standards with concentrations ranging from 0.25–150 pg on column were used by injecting 0.5 µL. The calibration curves were obtained by plotting the peak ratios of caffeine and its metabolites to the internal standard (CF-D9) against the nominal concentrations of the calibration standards at 0.5, 1, 10, 30, 50, 80, 100, and 300 pg/µL (Figure 14). The peak areas were calculated from the quantifiers of caffeine, theobromine, theophylline/paraxanthine, and the internal standard in the positive ion mode. The ratio of the peak areas of the analytes with respect to the internal standard was linear over the entire range. The calibration model was selected based on the analysis of the data by linear regression. The fit for the calibration was obtained with the linear equation y=mx.

Caffeine:	y = 0.0607x	(R^2 = 0.9994)
Theobromine:	y = 0.0261x	(R^2 = 0.9993)
Theophylline:	y = 0.0277x	(R^2 = 0.9995)

The extraction from fingerprints was validated from three technical replicates and three extraction replicates for caffeine, theobromine and theophylline spiked on filter paper. The lower limits of quantitation (LLOQs) of the analytes were in the range of the lowest calibration standard. The LLOQ

for caffeine and its metabolites was defined as the lowest concentration giving signal-to-noise ratio of at least 10. The lowest concentration that can be detected, with a signal-to-noise ratio of 3:1, is specified as the limit of detection (LOD). CF, TB and TP show LOQs of 0.54, 0.68 and 0.42 pg/FP, respectively, while the limits of detection (LOD) were 0.22, 0.28 and 0.20 pg/FP, respectively. These values obtained with a microfluidic nanoChip are >100 lower than recently reported with a conventional HPLC-MS setup [5]. The precision of the method is expressed as percent coefficient of variation (% CV) and covers three technical replicates at concentrations of 0.5 and 50 pg/µL, respectively. The higher concentration yields an improved precision over the lower concentration. This is evident by considering that the lower concentration is in the range of the LOQ of the analytes. The precision of the analytes is <5%, with the exception of caffeine at 0.5 pg/µL for which 15.9% was obtained. However, this is in accordance with the FDA guidance for bioanalytical method validation for which a 20% CV near the LOD are acceptable [130]. Filter paper was spiked with CF, TB and TP before the extraction procedure and compared with standard samples in aqueous solution for evaluating the overall process efficiency. The measured overall process efficiencies for the three analytes were between 88–92%.

It was attempted to perform a matrix-matched extraction from sweat. A solution to simulate fingerprint-sweat secretion was prepared according to reference and based on the fact that 50 µL sweat is expected in a fingerprint [3]. Calibration solutions from 0.1–100 pg/µL were prepared. Moreover, full MS scans were recorded for assessing the complexity of the sample matrix. However, during the concentration step via nitrogen small amounts of condensed water as well as gel-like substances remained on the ground and therefore, it was not possible to fully dry the samples impeding quantitative analysis. It is obviously assumed that the gel-like substance stems from urea and lactic acid which are the major constituents of the artificial sweat matrix.

Furthermore, the performance of the real-life experiments from the five volunteers was evaluated in particular with respect to the time point of 5 h after coffee consumption (Figure 16). Both the LC-MS variation and the extraction variation show CV < 5%. The CV of the extraction reproducibility and intriguingly, the CV of the biological variations were both <10%, which amounts to an overall variation of 20.4%. The overall variation corresponds to the CV of the concentrations of caffeine determined from fingerprints after 5 h of coffee intake including separately all technical and biological replicates of the five donors.

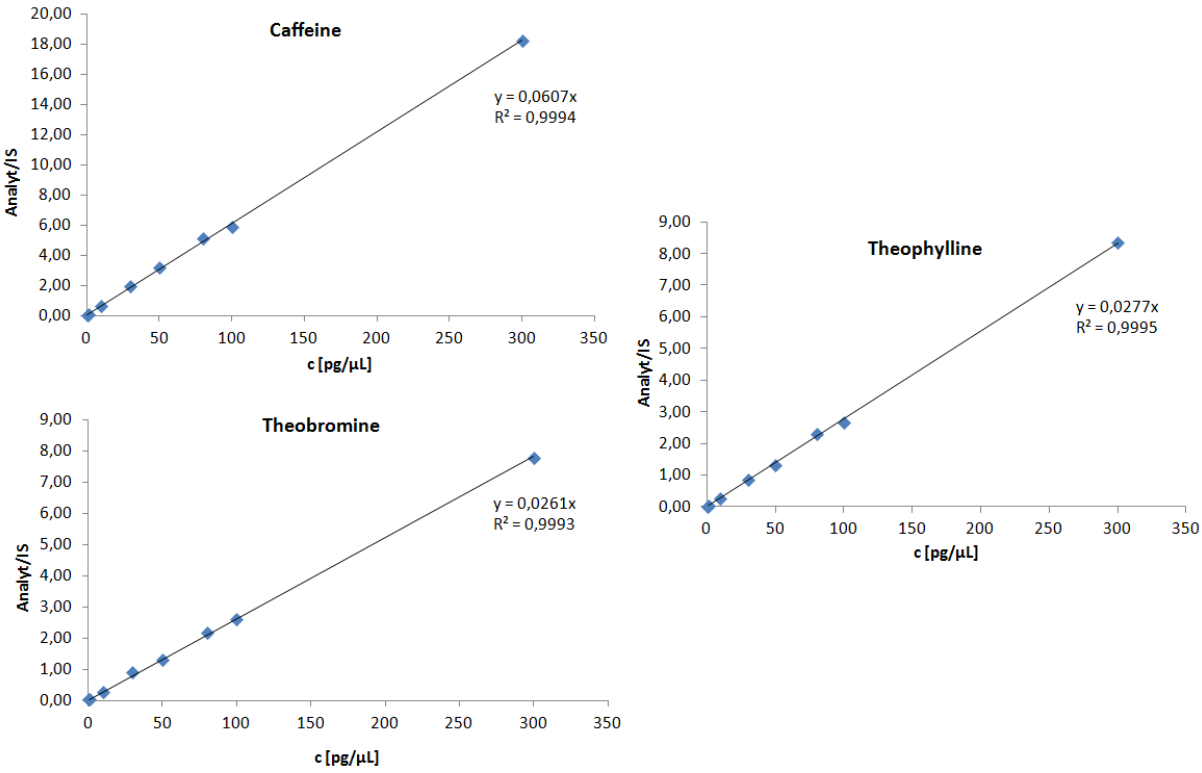


Figure 14 Calibration curves for caffeine, theobromine and theophylline in 8 concentration levels ranging from 0.5–300 pg/µL (0.25–150 pg/µL on column) with overall correlation coefficients >0.999 over the concentration range.

Table 16 Analytical validation of CF and its metabolites: The average areas of the matrix blanks are 17, 9.6, and 78.2 for CF, TB and TP, respectively. The precision for CF, TB and TP is between 0.03 and 15.6% with overall process efficiency from 88.4–92%. The LLOQ for caffeine and its metabolites was defined as the lowest concentration giving signal-to-noise ratio of at least 10. The lowest concentration that can be detected, with a signal-to-noise ratio of 3:1, is specified as the limit of detection (LOD).

Compound	**Spiked (pg/FP)**	Precision (%)	LLOQ (pg/FP)	LOD (pg/FP)	**Overall Process Efficiency**
CF	0.5	15.9	0.54	0.22	
	50	0.03			88.4 (7.2%)
TB	0. 5	4.1	0.68	0.28	
	50	0.7			92.0 (5.9%)
TP	0. 5	1.9	0.42	0.20	
	50	0.2			89.9 (7.5%)

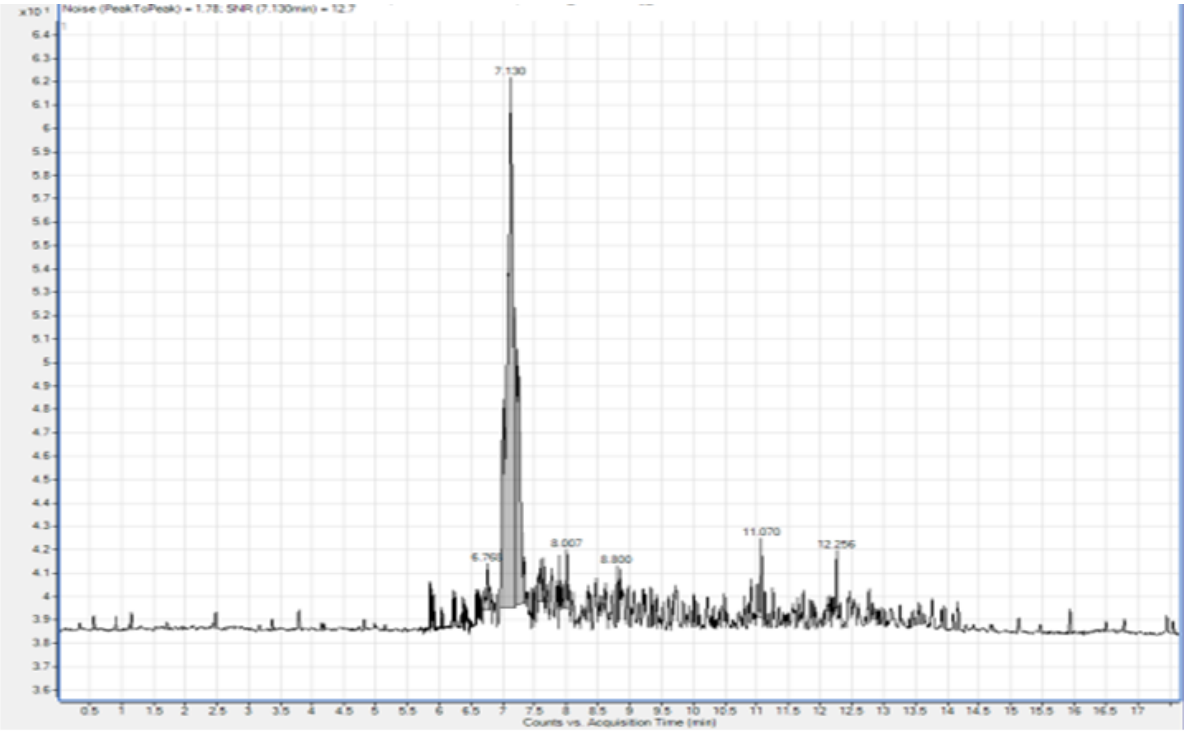


Figure 15 LLOQ for caffeine (0.54/FP, 0.01 pg on column) at t_R 7.1 min with a signal-to-noise ratio of 12.7

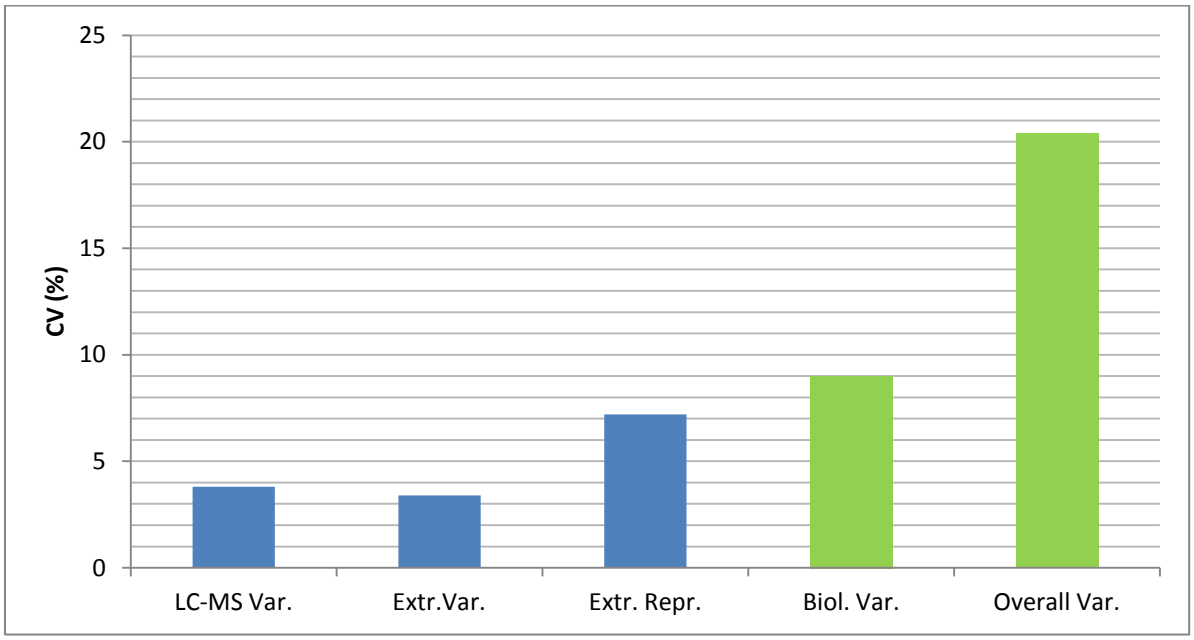


Figure 16 Coefficients of Variation for LC-MS Variation.[a] Extraction Variation[b] , Extraction Reproducibility[c] , Biological Variation[d] and Overall Variation[e] of caffeine found in fingerprint.

[a] The LC-MS variation represents the coefficients of variations of 3 technical replicates
[b] The extraction variation represents the coefficients of variations of 3 average extractions
[c] Extraction reproducibility: The coefficient of variation of 3 biological extractions, each with 3 technical replicates
[d] The biological variance indicates the coefficients of variations of the average caffeine amount after 5 h of coffee intake of all the donors
[e] The overall variation represents the coefficients of variations of all technical and biological replicates of 5 Donors after 5 h of coffee intake

3.3.3 Evaluation of the Quantitation of Caffeine and its Metabolites in Fingerprints

The main part of the master thesis relates to the evaluation of time-course measurements of caffeine and its primary metabolites. A cohort of 3 male and 2 women at the age of 25 to 30 were recruited for the purpose of obtaining statistically validated data. These time-course measurements may give a first impression on the individual metabolism and consequently, the activity of CYTP450 enzymes in the liver. The response of caffeine and its metabolites in fingerprints were evaluated on three different days and each at 4 different time points, i.e. before coffee intake (0 h) and 1, 3 and 5 h after coffee consumption. The development of the analyte concentrations and the coefficients of variation (in brackets) for each donor are shown in Table 17. The average CF, TB, TP/PX and the respective CVs are denoted from three technical replicates. By the means of a student t-test, significances (p–values) were calculated for three states: 0 vs. 1 h, 0 vs. 5 h and 1 vs. 5 h. These states were chosen due to the observed pharmacokinetics of the analytes in blood and sweat. The null hypothesis for the test was that there is no significant difference in amounts of analyte found in fingerprints between time point 1 and time point 2. A p-value of >0.05 was rejected.

Table 17 Time-course measurements of caffeine and its metabolites from fingerprints of three independent experiments of donors A-E. The average amounts (pg/µL) of CF, TB and TP/PX of three technical replicates at 4 different time points with the coefficient of variations in brackets are displayed.

[a] determines the sample variance of three biological triplicates with the calculated CVs in brackets.
[b] The p-value greater than 0.05 (p > 0.05) determines that the probability for observing a specific result is due to random chances. This will be the case if calculated t-value is below the t-critical value.

			Donor A			
Time [h]	Average CF (pg/µL)	Biol. Average[a]	Average TB (pg/µL)	Biol. Average[a]	Average TP/PX (pg/µL)	Biol. Average[a]
0	6,64 (2.98%)		3,2 (2.65%)		1,5 (11.09%)	
	6,82 (3.52%)	5,46(40.1%)	2,69 (14.16%)	2,53 (29.93%)	1,30 (2.97%)	1.08 (52.94%)
	2,93 (6.62%)		1,71 (4.42%)		0,43 (6.89%)	
1	29,88 (4.02%)		5,37 (6.8%)		3,14 (10.11%)	
	26,25 (6.85%)	27,38 (7.92%)	3,9 (8.82%)	3,91 (36.98%)	2,48 (11.18%)	2,35 (37.09%)
	26,00 (4.69%)		2,47 (6.94%)		1,42 (7.77%)	
3	11,24 (6.53%)		2,31 (0.75%)		1,85 (4.44%)	
	15,25 (10.49%)	17,7 (45.02%)	2,66 (2.33%)	2,98 (29.41%)	2,08 (6.21%)	2,29 (25.22%)
	26,60 (1.3%)		3,98 (16.24%)		2,94 (2.14%)	
5	16,96 (1.24%)		5,24 (10.81%)		5,31 (16.51%)	
	18,93 (3.51%)	16,97 (11.5%)	7,84 (16.39%)	6,46 (20,26%)	6,04 (2.85%)	5.39 (10.51%)
	15,03 (9.03%)		6,30 (5.29%)		5,00 (11.19%)	
	CF		**TB**		**TP/PX**	
p-value[b] (0 vs 1h)	0.00012		0.11983		0.05614	
p-value (0 vs 5h)	0.00129		0.00887		0.00032	
p-value (1 vs 5h)	0.00181		0.04373		0.00509	

			Donor B			
Time [h]	Average CF (pg/µL)	Biol. Average[a]	Average TB (pg/µL)	Biol. Average[a]	Average TP/PX (pg/µL)	Biol. Average[a]
0	31,34 (0.15%)		7,67 (0.08%)		0.70 (32.26%)	
	48,35 (5.39%)	38,68 (22,6%)	9,68 (0.91%)	9,12 (13.93%)	0.42 (18.13%)	0,54 (26,77%)
	36,34 (2.90%)		10,03 (2.74%)		0,50 (16.06%)	
1	51,11(2.13%)		9,49 (1.26%)		0.64 (14.13%)	
	53,98 (3.22%)	53,69 (4,56%)	8,77 (8.41%)	9,42 (6,59%)	0.40 (0.33%)	0,59 (30,11%)
	55,98 (0.57%)		10.0 (1.89%)		0.74 (12.97%)	
3	29,73 (3.36%)		9.59 (15.59%)		1.04 (5.45%)	
	25,42 (7.62%)	25,35 (17,4%)	11.04 (5.64%)	10,48 (7,5%)	1.18 (0.33%)	0,92 (36,34%)
	20,91 (9.95%)		10.83 (5.24%)		0.54 (3.52%)	
5	21,31 (0.15%)		11.39 (5.36%)		2.64 (6.04%)	
	19,90 (12.89%)	20,06 (5,87%)	11.37 (2.21%)	10,91 (7,43%)	1.51 (38.14%)	1,66 (55,69%)
	18,97 (5.04%)		9.97 (8.41%)		0.81 (10.56%)	
	CF		**TB**		**TP/PX**	
p-value [b] (0 vs 1h)	0.04373		0.11981		0.05614	
p-value (0 vs 5h)	0.03191		0.06139		0.08459	
p-value (1 vs 5h)	0.00014		0.03474		0.09069	
			Donor C			
0	1,96 (18,38%)		2,77 (0.08%)		0,39(32.26%)	
	5,02 (4,08%)	4,12 (45,67%)	6,30 (0.91%)	4,35 (41,20%)	2,17(18.13%)	1,32 (67,54%)
	5,38 (2,80%)		3,98 (2.74%)		1,39(16.06%)	
1	6,62 (0,89%)		1,86 (1.26%)		0,79(14.13%)	
	9,50 (4,7%)	9,39 (28,90%)	1,95 (8.41%)	1,89 (2,59%)	1,48 (0.33%)	1,10 (31,95%)
	12,04 (7,84%)		1,87 (1.89%)		1,02(12.97%)	
3	16,70 (1,69%)		1,79 (15.59%)		2,15 (5.45%)	
	18,0 (6,27%)	18,06 (7,71%)	4,74 (5.64%)	3,04 (50,38%)	4,82 (0.33%)	3,18 (45,12%)
	19,49 (3,98%)		2,58 (5.24%)		2,57 (3.52%)	
5	17,01 (1,04%)		1,44 (5.36%)		1,82 (6.04%)	
	16,30 (9,71%)	16,50 (2,64%)	5,49 (2.21%)	2,95 (74,75%)	6,99 (38.14%)	3,40 (91,53%)
	16,2 (4,72%)		1,94 (8.41%)		1,39 (10.56%)	
	CF		**TB**		**TP/PX**	
p-value (0 vs 1h)[b]	0.02881		0.07048		0.36068	
p-value (0 vs 5h)	0.00273		0.22296		0.00032	
p-value (1 vs 5h)	0.02100		0.24600		0.16420	

			Donor D			
Time [h]	Average CF (pg/µL)	Biol. Average[a]	Average TB (pg/µL)	Biol. Average[a]	Average TP/PX (pg/µL)	Biol. Average[a]
0	9,63 (0,24%)		1,34 (7,1%)		2,28 (4,29%)	
	0,32 (9,47%)	8,19 (88,6%)	0,10 (8,37%)	1,27 (89,74%)	0,07 (8,91%)	2,09 (92,12%)
	14,63 (0,42%)		2,38 (1,30%)		3,90 (2,74%)	
1	11,48 (2,32%)		1,14 (4,02%)		2,11 (2,17%)	
	23,09 (2,39%)	17,25 (33,7%)	1,37 (1,04%)	1,28 (9,69%)	3,48 (4,24%)	2,68 (26,63%)
	17,18 (2,54%)		1,33 (3,30%)		2,45 (1,17%)	
3	10,54 (1,98%)		0,93 (7,30%)		1,95 (2,24%)	
	21,79 (3,20%)	20,08 (43,9%)	2,31 (1,85%)	1,61 (42,67%)	3,43 (1,25%)	3,01 (30,77%)
	27,92 (3,57%)		1,61 (3,58%)		3,66 (3,73%)	
5	14,35 (0,52%)		1,21 (3,32%)		3,17 (3,64%)	
	21,25 (4,01%)	17,51 (19,9%)	1,39 (2,28%)	1,33 (7,98%)	2,92 (0,92%)	3,81 (35,04%)
	16,93 (3,84%)		1,39 (0,30%)		5,35 (1,09%)	
	CF		**TB**		**TP/PX**	
p-value (0 vs 1h)[b]	0.08515		0.49640		0.32730	
p-value (0 vs 5h)	0.07143		0.46972		0.13871	
p-value (1 vs 5h)	0.47540		0.30989		0.14210	
			Donor E			
0	6.26 (1,02%)		0,53 (16,74%)		2,22 (2,79%)	
	3.38 (4,96%)	7,47 (65,2%)	0,43 (2,31%)	0,89 (80,21%)	1,48 (6,81%)	3,22 (74,69%)
	12.77 (2,01%)		1,71 (4,55%)		5,97 (2,92%)	
1	18.32 (2,40%)		0,71 (6,43%)		3,38 (1,68%)	
	10.68 (4,19%)	17,83 (38,8%)	0,59 (3,90%)	0,97 (57,56%)	2,10 (5,82%)	3,66 (46,90%)
	24.48 (1,01%)		1,61 (3,63%)		5,50 (1,82%)	
3	31.14 (0,98%)		1,35 (3,80%)		3,30 (1,16%)	
	11.86 (4,86%)	16,76 (75,5%)	0,47 (7,63%)	0,84 (54,76%)	2,22 (4,13%)	2,45 (30,93%)
	7.28 (1,60%)		0,70 (1,46%)		1,84 (1,92%)	
5	25.27 (0,47%)		7,03 (5,32)		7,03 (1,92%)	
	13.65 (0,16%)	19,73 (29,5%)	3,27 (1,63%)	1,08 (46,91%)	3,27 (5,22%)	5,62 (36,55%)
	20.28 (0,32%)		6,58 (4,51%)		6,58 (1,45%)	
	CF		**TB**		**TP/PX**	
p-value (0 vs 1h)[b]	0.05426		0.44301		0.40601	
p-value (0 vs 5h)	0.01211		0.02256		0.13010	
p-value (1 vs 5h)	0.28820		0.02534		0.13701	

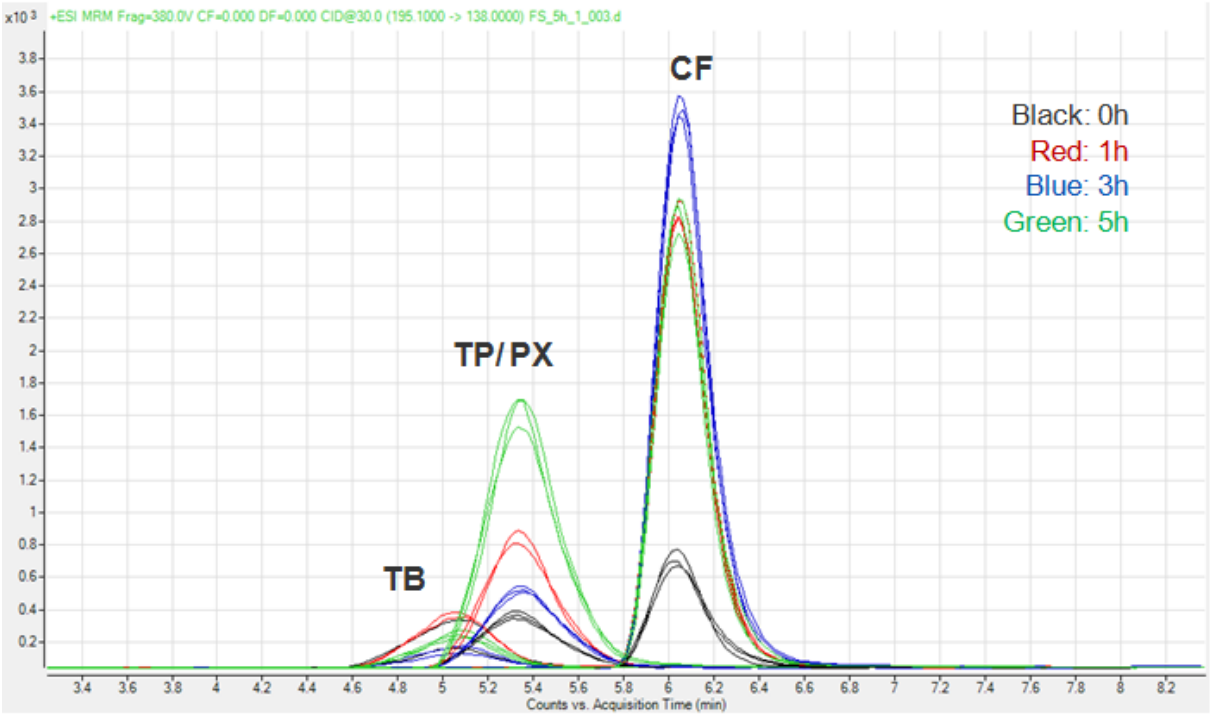


Figure 17 Graphically illustrated LC-MS analyte kinetics of 3 technical replicates at 4 different time points of TB, TP/PX and CF from donor E. The x-axis determines the retention time and the vertical axis denotes the intensity.

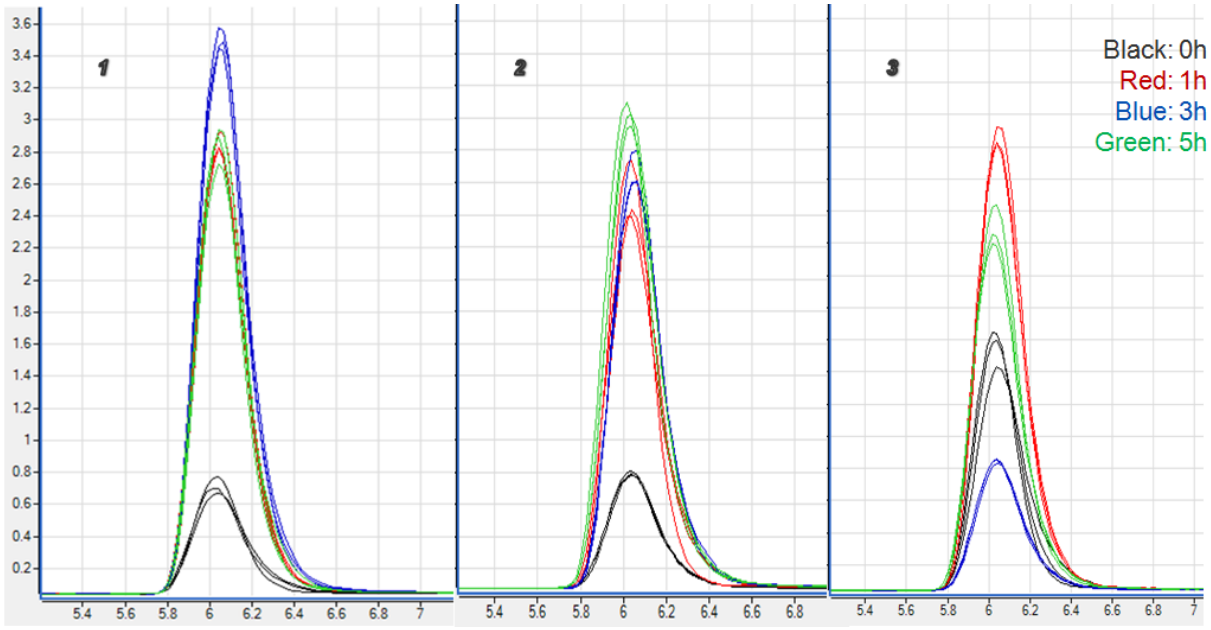


Figure 18 Analyte kinetics for CF of donor E on 3 different days (1-3) and each day featuring 4 different time points. Each time point is displayed by three technical replicates.

The measured technical replicates show correlations of variations (CVs) <15% except for Donor C for whom higher CVs were obtained. In contrast, the biological replicates vary quite dramatically not only among individuals, but also from day-to-day experiments of the same volunteer, which is probably due to the individual differences in absorption and response to caffeine [131]. When comparing the caffeine concentrations determined in fingerprints after 1 h of coffee consumption with respect to the concentrations before intake, a significant increase of the analyte is observed for donors A–C. The p-values in this case are below $p < 0.05$. Donors D and E did not display a significant immediate CF increase. A similar picture is observed when comparing the time point 5 h after coffee intake with the state before consumption. In this case, only donor D did not show a significant increase in CF concentration. However, this seems mainly due to day-to-day variations of the CF concentration before coffee intake, which features concentrations of 9.63 (0.24%), 0.32 (9.47%) and 14.63 (0.42%) pg/µL that amount to an overall CV of 89%. Equally, one may point out that there is a significant decrease of the average concentration of caffeine found between 1 and 5 hours after coffee consumption for donors A and B (p-value 1 vs 5h) and a significant increase over the same period for donor C, while again, donors D and E did not show significant concentration changes in CF. Intriguingly, the caffeine concentrations after 5 h of coffee consumption were very similar for all five donors over all experiments. The biological variation at this time point is <9% and the overall variation including instrumental and extraction variations is slightly over 20%. It is noteworthy that both donor D and E regard themselves as regular coffee consumers in contrast to donors A and B which hardly consume coffee that could account for the observed differences in response to coffee intake. A decisive factor in analysing caffeine habituation is the difference between “fast and slow metabolism”. In this context, “slow metabolizers” do not process caffeine effectively, whereas “fast metabolizers” do so.

By the means of time dependent line graphs of the five test subjects in Figure 19 it appears that CF metabolization may be separated into these two groups. There are on the one hand the relatively “fast metabolizers“ (donors C-E) and on the other hand the comparatively “slow metabolizers” (donors A and B). Slow metabolizers are characterized by a CF peak concentration after 1 h of coffee intake, whereas fast metabolizers lack this CF maximum and only slowly increase CF over time. The reason for different CF profiles in the present study may be related to the activity of the CYP450 enzymes in the liver, particularly CYP1A2 or even to differences in gender. Earlier studies revealed that individuals who are homozygous for the CYP1A2*1A allele do metabolize faster than carriers with the CYP1A2*1F allele [132]. Another publication shows that “slow metabolizers” who consume multiple cups of coffee or energy drinks may have an increased risk of hypertension and a significantly increased risk of a non-fatal heart attack [132] . The danger for slow metabolizers arises from the fact that upon multiple coffee consumption, the caffeine concentrations rapidly build up in

the bloods and the liver may not be able to detoxify. In parallel, slow metabolizers also show higher levels of epinephrine in their urine upon caffeine consumption. Hence caffeine is known to stimulate the release of hormones including epinephrine [132; 133].

Furthermore, the individual response to caffeine is likely influenced by various factors including demographic and environmental factors such as age, circadian factors, sleep, drugs, absorption and metabolization [131]. Studies proved that the clearance of caffeine is subject to individual daily changes and therefore, also between individuals [134; 135]. It has also been demonstrated that CYP1A2 showed distinctive inter-individual variations which means that the amount of caffeine cleared from the body is proportional to the amount of CYP1A2. At the same time, this indicates that variable activity of CYP1A2 will lead to highly variable half–lives for caffeine among individuals [136]. Actually, studies revealed that individual differences in caffeine response may also be attributed to genetic factors. Therefore, even genes may be able to change the body`s adaptive response to long-term caffeine intake [131]. Additionally, people can develop caffeine-tolerance by up-regulating adenosine A1 or A2 receptors, which are members of the adenosine receptor group of G-protein-coupled receptors in the central nervous system [137; 138; 139]. Moreover, mice lacking functional A2A receptors did not show increased vigilance in response to caffeine administration [140]. There is actually no proven study on humans that confirms that the lack of functional A2A receptors leads to actually no biological response to caffeine. In addition to these geno- and phenotypic factors, also experimental conditions may influence the measurement of CF in fingerprints, i.e. by the pressure towards the filter paper or the amount of sweat secreted in a fingerprint that may vary among individuals as described earlier. The average daily amount of sweat without exercise amounts to approximately 100-200 ml sweat per day [141].

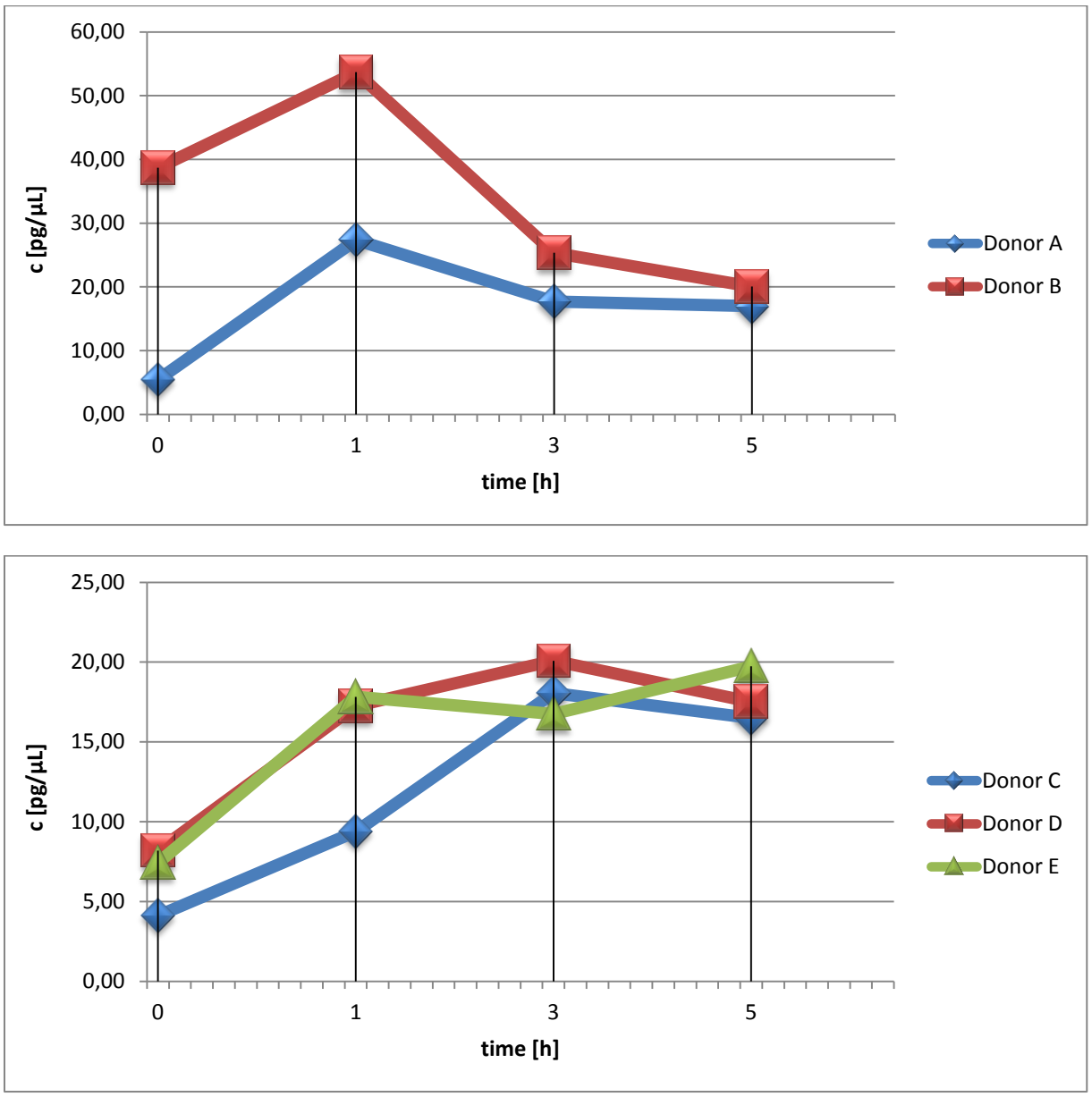


Figure 19 Typical concentration profiles of slow metabolizers (*top*) and fast metabolizers (*bottom*). The time courses show the biological average (3 technical replicates on three different days) of CF found in fingerprints of the donors before coffee intake and 1, 3 and 5 h afterwards. The horizontal axis determines the time in hours. The y-axis denotes the concentration found in fingerprint in pg/µL.

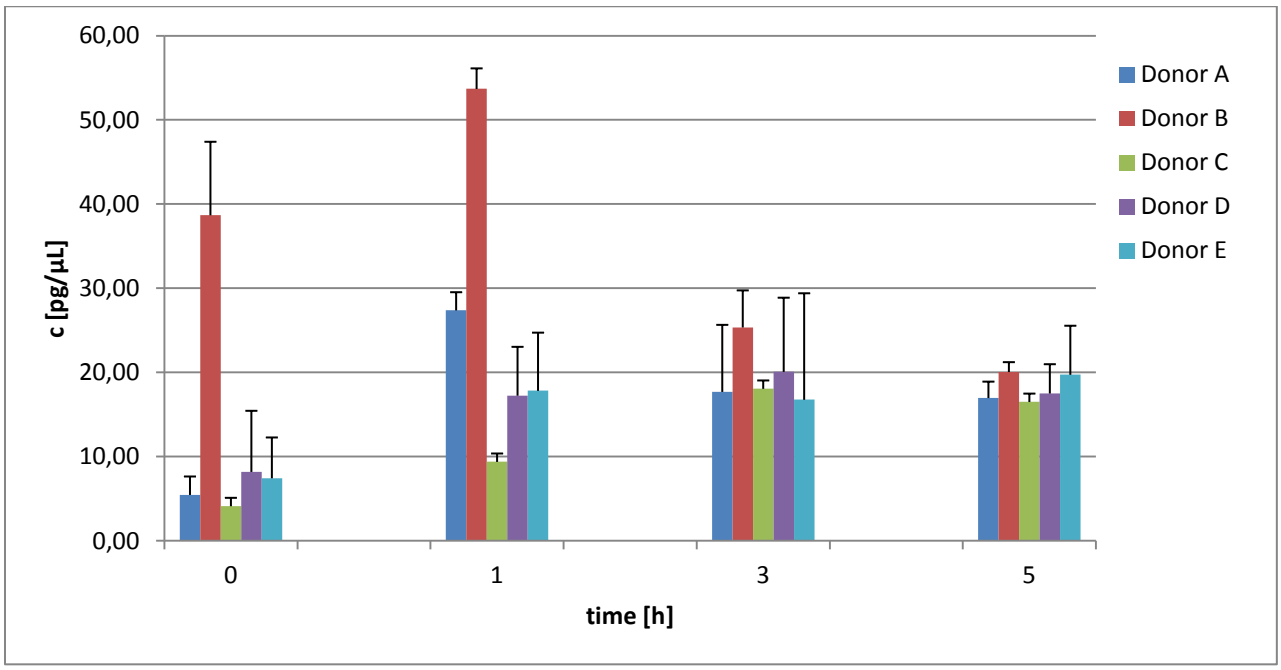


Figure 20 Time course measurements of caffeine found in fingerprint of donors A-E (blue-turquoise). The bars represent the biological average (3 technical replicates on three different days) including standard deviations of caffeine measured at 4 different time points in pg/µL.

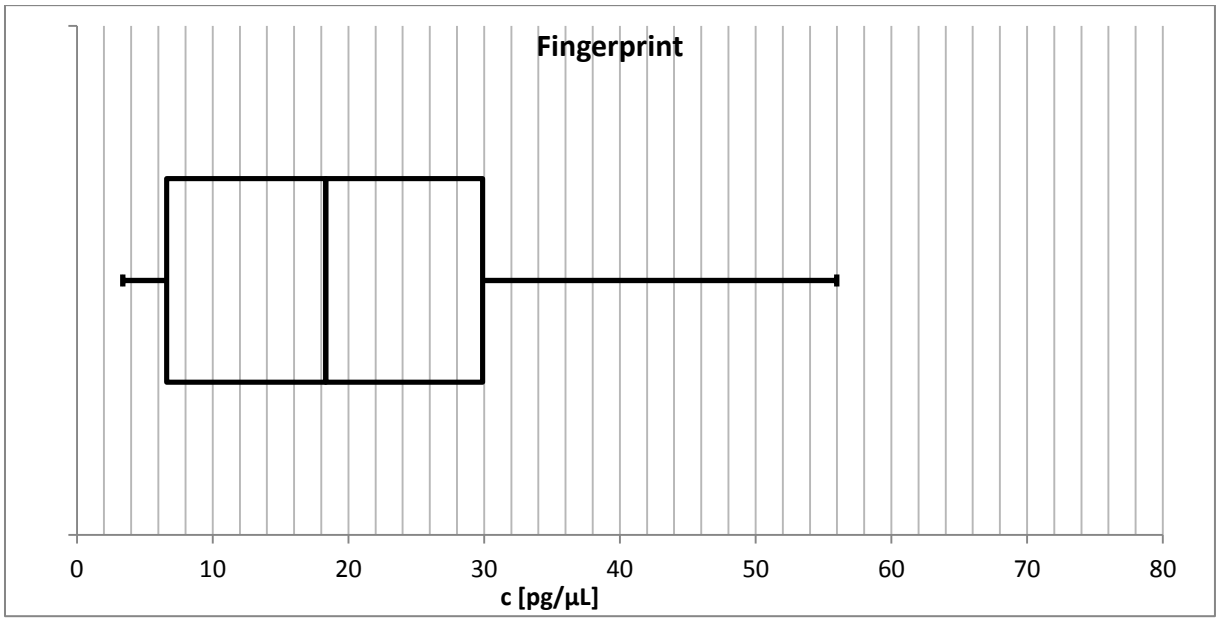


Figure 21 Measurements of fingerprint are graphically illustrated using a Box Plot to visualize key statistical measures (median, mean quartiles). A box plot diagram for the concentrations of 5 donors after caffeine consumption (1h) in fingerprint is shown. It indicates a median of 18.32 pg/µL and Q_1 = 6.62 pg/µL and Q_3 = 29.88 pg/µL.

Box plots are useful to compare distributions among several sets of data. In particular the measurements made after 1 h of caffeine intake are displayed accordingly for fingerprint measurements (Figure 21). It shows that the CF concentrations in fingerprints lie very close together with comparatively small fluctuations. This is actually impressive because it should not be forgotten that these measurements include analysis of five volunteers on three different days including three technical replicates for each day. It indicates a median of 18.32 pg/µL and a Q_1 = 6.62 pg/µL and Q_3 = 29.88 pg/µL. As mentioned above differences in caffeine metabolism are probably related to the hepatic p450 system [142; 143]. Caffeine was even under discussion as a possible marker for testing p450 activity and in particular, for assessing the activity of the CYP1A2 enzymes [143]. The metabolic activity should not only be assessed by CF profiling alone, but also by the appearance of the primary metabolites of CF, namely TB and PX/TP. With regard to these metabolites a trend towards a general increase can be observed after coffee consumption. These metabolites were detected in relatively small amounts in fingerprints compared to CF even after 5 h of coffee intake. This may be due to the polar nature of the primary metabolites that reduces their ability to passively diffuse into secreting glands. Metabolite levels which significantly increase can be found in donors A, B, C and E although no uniform trend could be deduced.

In the present study, the volunteers started the experiment deprived of caffeine sources for at least 12 h. However, certain substances may additionally alter the rate of caffeine metabolism. Substances may slow the metabolism of caffeine and therefore inhibit the clearance of caffeine from the body activating the production of CYP1A2 enzymes and moreover increase their activity [144]. Table 18 shows different substances and genetic factors which on the one hand may increase (e.g. cruciferous vegetables, coffee, grilled meat, tobacco smoke) and on the other hand decrease the rate of caffeine metabolism (e.g. grapefruit juice or alcohol) [145].

Table 18 Influence of various substances and genetic factors on caffeine metabolism according to Ref. [145]

Increases Speed of Caffeine Metabolism	Decreases Speed of Caffeine Metabolism
Cruciferous vegetables (broccoli, cauliflower, cabbages, radishes)	Grapefruit juice
Coffee	Alcohol
Grilled meat	Pregnancy
Tobacco smoke	Liver disease
Lean people	Obese people
Younger people	Older people

Finally, this study proves that coffee consumption can be monitored by measuring caffeine and its primary metabolites from fingerprint sweat using a nanoChip triple quadrupole MS system. The applied procedure is fast, non-invasive and shows acceptable reproducibility. Significantly altered amounts of caffeine can be found in fingerprint sweat in four out of five volunteers when comparing CF levels before and 5 h after coffee intake. Interestingly, after five hours of coffee consumption, the CF concentrations show an inter-individual variation of <9%. The procedure allows to differentiate between slow and fast metabolizers, which is indicative of the metabolic activity in hepatocytes or may be caused by gender.

3.4 Quantitation of Caffeine and its Primary Metabolites in Whole Blood

3.4.1 Sample Preparation

Blood was drawn by pricking any one of the fingers except for index fingers with a lancet for self-collection. The protective cap was removed held against the finger, the triggering button was squeezed and a volume of 20 µL of whole blood was taken with a pipette and transferred into an Eppendorf tube containing the extraction solvent, i.e. acetonitrile or diethyl ether. The extraction procedure was performed according to chapter Extraction of CF and primary metabolites from blood and the dried residues were reconstituted in 250 µL water containing 0.2% formic acid.

3.4.2 Method Validation

The method validation of caffeine and its metabolites in whole blood was performed using an UHPLC-MS instrumentation in the positive MRM mode. Plasma from a non-coffee consumer was used to evaluate the lower limit of quantification (LLOQ) and the limit of detection (LOD) of caffeine and its primary metabolites. The validation characteristics for this method were selectivity, linearity and sensitivity, correlation coefficients, precision (coefficients of variation), detection limits (LOD) and lower limit of quantitation (LLOQ). A concentration range of 0.1–100 pg/µL was selected using five calibration standards, but without internal standard. The peak areas were calculated from the transitions of caffeine, theobromine and theophylline by MRM. The calibration model was selected based on the analysis of the data by linear regression. The linear relationship was calculated between the peak-area ratio and the amount of analytes.

Caffeine:	y = 3966.2x	(R^2 = 0.9997)
Theobromine:	y = 11163x	(R^2 = 0.9986)
Theophylline:	y = 15689x	(R^2 = 0.9989)

The validation parameters for the quantitation of caffeine and its metabolites in whole blood were obtained from three technical and three extraction replicates. The overall process efficiency was performed by spiking CF, TB and TP into the plasma matrix before the extraction procedure, which was then compared with the aqueous standard samples. The measured overall process efficiencies of caffeine, theobromine and theophylline were between 80–85%.

The lower limits of quantitation (LLOQs) of the analytes were above the range of the lowest calibration standard. CF, TB and TP show LOQs of 0.65, 0.73 and 0.88 pg/µL, respectively, while the limits of detection (LOD) were 0.21, 0.24 and 0.29 pg/µL, respectively. The precision of the method is expressed as percent coefficient of variation (%CV) and covers three technical replicates at

concentrations of 0.5 and 50 pg/µL, respectively. Similar to fingerprint validation, the higher concentration yields an improved precision over the lower concentration. The precision of the analytes is <5%, with the exception of caffeine at 0.5 pg/µL for which 16.78% was obtained (Table 19). In addition, the performance of the real-life experiments from five volunteers was evaluated on the nanoChip-MS platform and in particular with respect to the time points of 1h and 5 h after coffee consumption (Figure 22). Both the LC-MS variation and the extraction variation show CVs < 9%. In this case the biological and overall variance was calculated for both 1h and 5h after The CV of the extraction reproducibility and intriguingly, the CV of the biological variations after 1h were both <20%, which amounts to an overall variation of 35.7%. The CV of the biological variation after 5h was 44.2% which amounts to an overall variance of 48.2%.

Table 19 Analytical validation of CF, TB and TP in whole blood using UHPLC-MS. The average matrix blanks show areas at 184.4, 191.4 and 197.6 for CF, TB and TP respectively. The precision for CF, TB and TP is between 0.21 and 16.78% with overall process efficiency from 79.8–84.7%.

Compound	Spiked (pg/µL)	Precision (%CV)	LLOQ (pg/µL)	LOD (pg/µL)	Overall Process Efficiency (%)
CF	0.5	16.78	0.65	0.21	
	50	0.27			79.8 (15.1%)
TB	0.5	2.10	0.73	0.24	
	50	0.39			84.7 (7.8%)
TP	0.5	4.10	0.88	0.29	
	50	0.43			81.5 (9.3%)

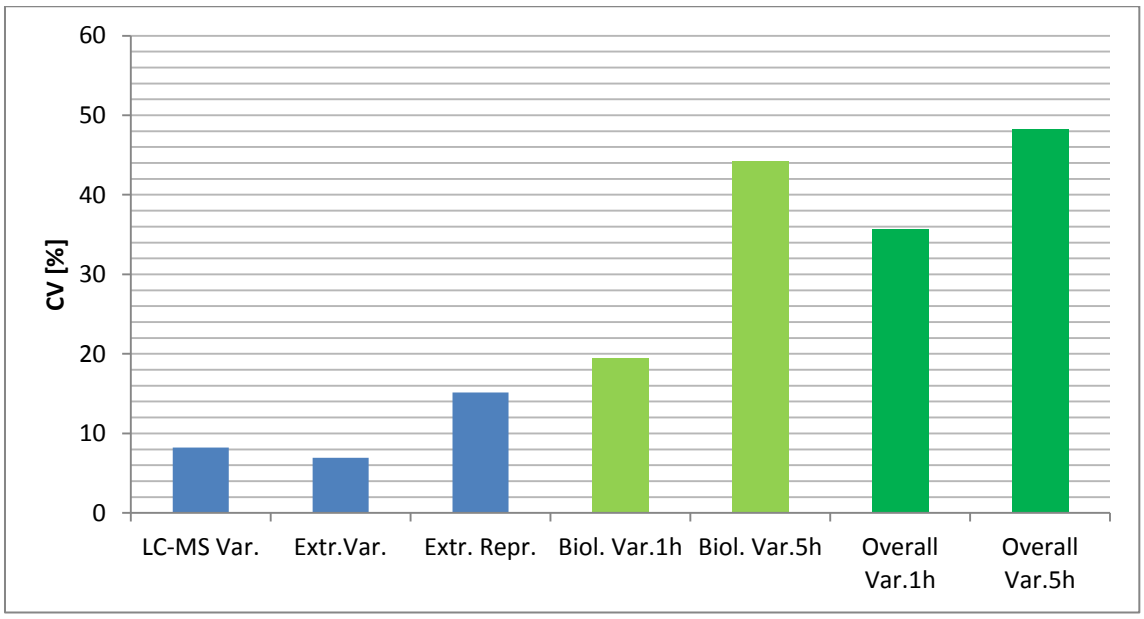
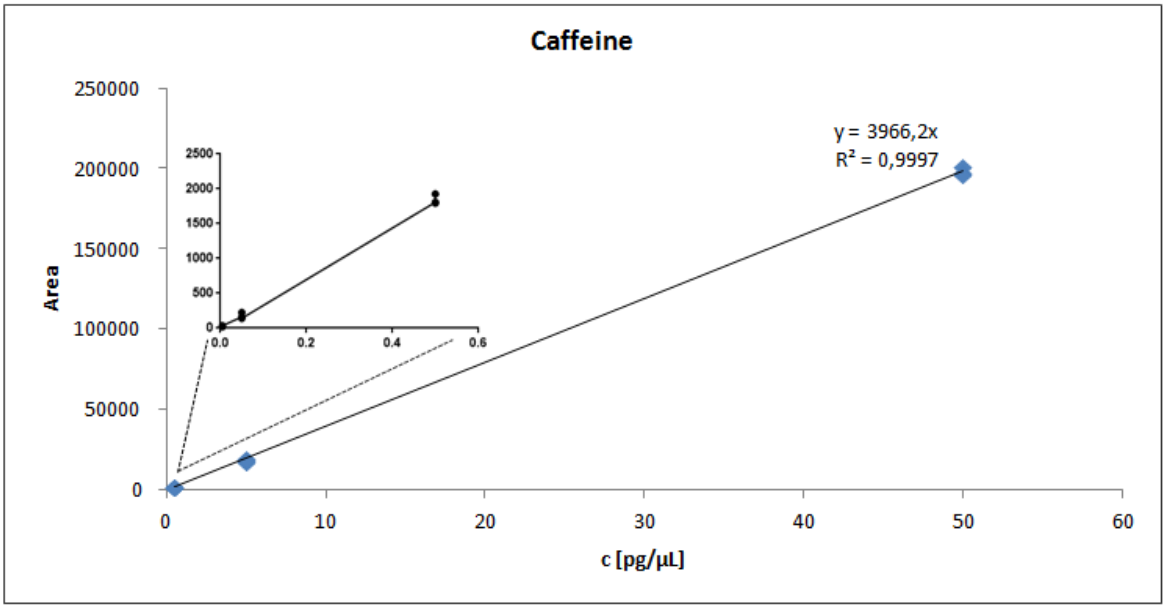
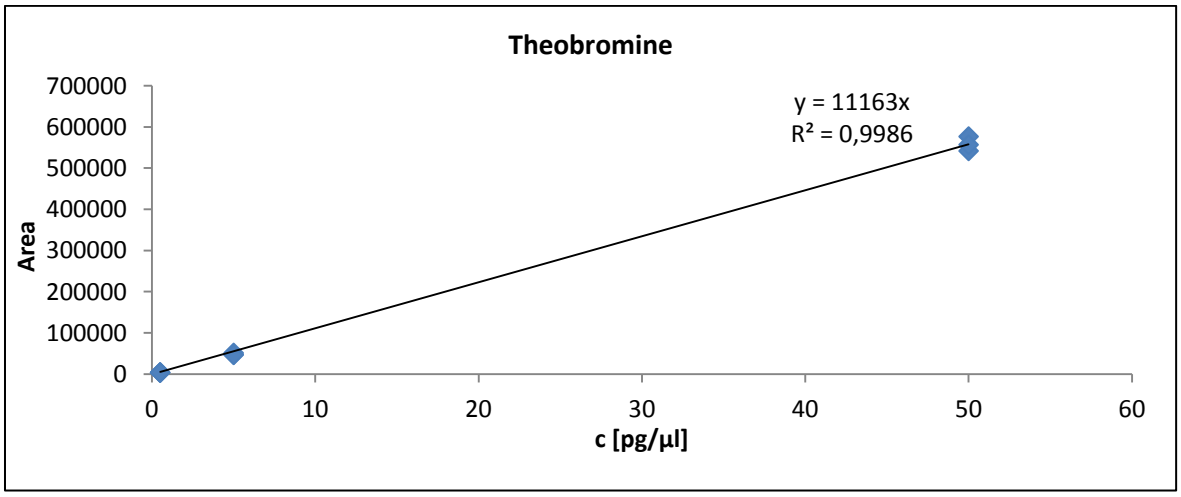


Figure 22 Coefficients of Variation for LC-MS Variation[a] , Extraction Variation[b] , Extraction Reproducibility[c] , Biological Variation after 1h[d] Biological Variation after 5h[e] and Overall Variation (1h)[f] and Overall Variation (5h)[g] of caffeine detected in whole blood measurements using the nanoChip-MS platform.

[a] The LC-MS variation represents the coefficients of variations of 3 technical replicates
[b] The extraction variation represents the coefficients of variations of 3 average extractions
[c] Extraction reproducibility: The coefficient of variation of 3 biological extractions, each with 3 technical replicates
[d] The biological variance indicates the coefficients of variations of the average caffeine amount after 1 h of coffee intake of all the donors
[e] The biological variance indicates the coefficients of variations of the average caffeine amount after 5 h of coffee intake of all the donors
[f] The overall variation represents the coefficients of variations of all technical and biological replicates of 5 donors after 1 h of coffee intake
[g] The overall variation represents the coefficients of variations of all technical and biological replicates of 5 donors after 5 h of coffee intake





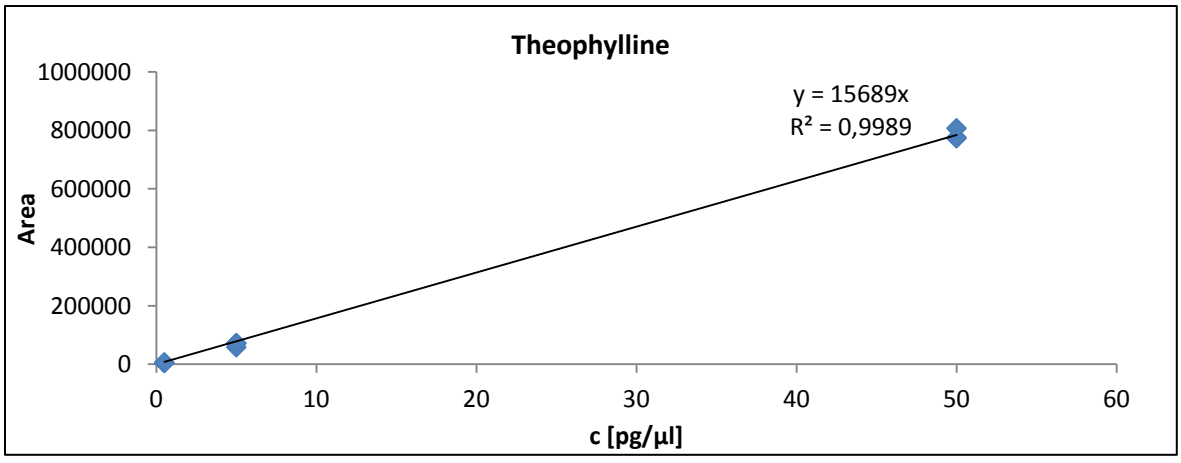


Figure 23 Calibration curves for caffeine, theobromine and theophylline from 4 calibration levels ranging from 0.1–100 pg/µL (0.5–50 pg/µL on column) with overall correlation coefficients >0.998 over the concentration range.

3.4.3 Evaluation of the Quantitation of Caffeine and its Metabolites in Whole Blood

The response of caffeine and its primary metabolites in whole blood were evaluated using the nanoChip-MS platform on three different days at 4 different time points similarly as for fingerprints in the same volunteers. The analyte concentrations and the coefficients of variation for each donor are shown in Table 20. The Average amounts of CF, TB and TP/PX and the CVs are denoted from three technical replicates each. By the means of a student t-test, significances (p–values) were calculated for three states similarly to the evaluation in fingerprints (0 vs. 1 h; 0 vs. 5 h and 1 vs. 5 h). These states were chosen due to the observed pharmacokinetics of the analytes in blood and sweat form fingerprints. The null hypothesis for the test was that there is no significant difference in amounts of analyte found in fingerprints between time point 1 and time point 2. A p-value of > 0.05 was rejected.

Table 20 Time-course measurements of caffeine and its primary metabolites from whole blood in three inter-day experiments of donors A-E. The average amounts (pg/µL) at 4 different time points of CF, TB and TP/PX are displayed with the coefficients of variation in brackets.

[a] determines the sample variance of three biological triplicates with the calculated CVs in brackets.
[b] The p-value greater than 0.05 ($p > 0.05$) determines that the observed result is due to random chances. This will be the case if the calculated t-value is below the t-critical value.

			Donor A			
Time [h]	Average CF (pg/µL)	Biol. Average[a]	Average TB (pg/µL)	Biol. Average[a]	Average TP/PX (pg/µL)	Biol. Average[a]
0	14,95 (11.53%)		59,60 (41,15%)		7,20 (10,67%)	
	36,82 (5,43%)	28,04 (41,22%)	72,16 (22,38%)	63,33 (12,12%)	8,99 (4,01%)	7,34 (21,53%)
	32,35 (1,10%)		58,24 (8,76%)		5,84 (31,60%)	
1	88,22 (4,81%)		24,30 (5,84%)		2,06 (7,10%)	
	63,72 (6,77%)	64,49 (36,21%)	18,58 (31,39%)	21,31 (13,47%)	3,63 (17,07%)	2,64 (32,69%)
	41,54 (3,23%)		21,04 (3,10%)		2,23 (7,71%)	
3	63,84 (3,46%)		16,22 (41,08%)		3,08 (9,78%)	
	43,82 (3,44%)	57,22 (20,29%)	16,72 (5,41%)	16,14 (3,90%)	4,50 (17,18%)	3,95 (19,25%)
	64,00 (4,69%)		15,47 (38,13%)		4,26 (7,64%)	
5	64,30 (2,14%)		21,35 (3,66%)		3,51 (12,98%)	
	78,17 (2,21%)	63,91 (22,62%)	23,53 (13,35%)	18,03 (42,86%)	8,37 (7,95%)	7,02 (43,63%)
	49,26 (2,91%)		9,19 (21,55%)		9,17 (11,51%)	
	CF		**TB**		**TP/PX**	
p-value[b] (0 vs 1h)	**0.0480**		**0.0028**		**0.0094**	
p-value (0 vs 5h)	**0.0153**		**0.0010**		**0.2904**	
p-value (1 vs 5h)	**0.4864**		**0.2739**		**0.0577**	

			Donor B			
Time [h]	Average CF (pg/µL)	Biol.Average[a]	Average TP (pg/µL)	Biol.Average[a]	Average TP/PX (pg/µL)	Biol. Average[a]
0	4,01 (6,42%)		21,20 (3,64%)		8,46 (11,47%)	
	7,34 (0,95%)	7,73 (50,93%)	21,20 (7,34%)	19,40 (16,10%)	15,07 (16,10%)	13,17 (31,14%)
	11,85 (2,71%)		15,79 (1,98%)		15,97 (3,62%)	
1	55,54 (1,79%)		26,15 (1,66%)		2,42 (0,86%)	
	67,03 (0,35%)	60,13 (10,11%)	19,27 (5,46%)	19,26 (35,79%)	3,22 (2,64%)	2,93 (15,35%)
	57,83 (1,11%)		12,36 (2,61%)		3,16 (1,09%)	
3	70,15 (1,67%)		30,45 (3,27%)		3,78 (3,38%)	
	90,34 (1,39%)	82,48 (13,11%)	19,90 (1,88%)	22,75 (29,61%)	4,26 (1,84%)	4,24 (10,69%)
	86,96 (2,52%)		17,91 (1,35%)		4,69 (2,60%)	
5	76,54 (0,89%)		35,30 (4,11%)		5,65 (2,08%)	
	89,66 (3,71%)	80,0 (10,59%)	22,22 (5,47%)	24,13 (42,88%)	5,69 (3,89%)	5,72 (1,46%)
	73,80 (2,23%)		14,87 (7,07%)		5,81 (2,92%)	
	CF		**TB**		**TP/PX**	
p-value [b] (0 vs 1h)	**0.0003**		**0.4886**		**0.0240**	
p-value (0 vs 5h)	**0.0006**		**0.2583**		**0.0439**	
p-value (1 vs 5h)	**0.0173**		**0.2700**		**0.0034**	
			Donor C			
0	-		-		-	
	25,15 (20,58%)	15,29 (107,52%)	0,22	0,13 (91,78%)	0,21	1,02 (112,27%)
	3,42 (16,97%)		0,04		1,83	
1	70,90 (11,19%)		-		1,04	
	39,02 (6,40%)	48,90 (39,02%)	2,70	3,67 (37,34%)	1,08	0,98 (14,26%)
	36,78 (4,74%)		4,63		0,82	
3	30,05 (12,63%)		0,9		1,97	
	66,08 (0,55%)	48,07 (53%)	8,52	4,71 (114,48%)	2,97	2,47 (28,6%)
	-		-		-	
5	74,67 (1,50%)		4,84		5,65	
	69,36 (1,32%)	54,97 (53,94%)	24,12	10,41 (114,82%)	5,91	4,99 (27,58%)
	20,87 (3,15%)		2,25		3,40	
	CF		**TB**		**TP/PX**	
p-value (0 vs 1h)[b]	**0,0607**		**0,0835**		**0,2250**	
p-value (0 vs 5h)	**0,0697**		**0,1374**		**0,0163**	
p-value (1 vs 5h)	**0,3915**		**0,2161**		**0,0181**	

			Donor D			
Time [h]	Average CF (pg/µL)	Biol.Average[a]	Average TP (pg/µL)	Biol.Average[a]	Average TP/PX (pg/µL)	Biol. Average[a]
0	9,18 (0,65%)		20,46 (3,0%)		15,49 (0,96%)	
	11,50 (0,28%)	8,99 (29,07%)	19,87 (2,9%)	20,65 (4,33%)	19,28 (3,79%)	18,40 (14,06%)
	6,29 (2,64%)		21,63 (0,97%)		20,43 (2,55%)	
1	12,63 (0,56%)		4,78 (3,75%)		3,72 (1,55%)	
	58,07 (3,59%)	41,01 (60,33)	20,77 (6,09%)	14,87(59,03%)	4,86 (2,16%)	4,04 (17,79%)
	52,31 (0,86%)		19,05 (2,18%)		3,54 (1,98%)	
3	39,04 (0,90%)		12,99 (3,76%)		5,46 (1,84%)	
	56,42 (1,58%)	45,14 (21,66%)	26,77 (1,24%)	19,03 (37,01%)	5,80 (1,27%)	5,58 (3,48%)
	39,96 (2,67%)		17,33 (4,94%)		5,47 (3,03%)	
5	17,43 (2,94%)		12,00 (8,33%)		5,37 (10,26%)	
	16,33 (7,07%)	16,88 (4,61%)	12,23 (9,82%)	12,12 (17,26%)	4,40 (15,75%)	4,88 (14,0%)
	-		-		-	
	CF		**TB**		**TP/PX**	
p-value (0 vs 1h)[b]	**0,0764**		**0,1859**		**0,0029**	
p-value (0 vs 5h)	**0,0124**		**0,0016**		**0,0037**	
p-value (1 vs 5h)	**0,1166**		**0,3205**		**0,2559**	
			Donor E			
0	1,76 (1,75%)		2,55 (0,98%)		29,47 (4,51%)	
	1,27 (6,79%)	3,39 (96,16%)	4,62 (3,72%)	6,28 (76,17%)	24,64 (8,94%)	26,23 (10,69%)
	7,14 (0,32%)		11,67 (1,21%)		24,58 (1,97%)	
1	39,33 (1,73%)		4,54 (9,58%)		4,33 (5,44%)	
	75,64 (1,10%)	66,63 (36,15%)	7,35 (1,71%)	11,98 (88,10%)	3,38 (0,68%)	4,45 (25,61%)
	84,90 (3,02%)		24,07 (0,21%)		5,65 (2,19%)	
3	60,19 (2,69%)		10,43 (5,73%)		7,37 (1,51%)	
	70,31 (2,06%)	70,44 (14,65%)	9,42 (6,46%)	14,54 (55,09%)	5,53 (3,33%)	6,80 (16,21%)
	80,83 (3,06%)		23,78 (0,40%)		7,51 (2,06%)	
5	40,84 (1,97%)		9,27 (5,14%)		9,72 (6,09%)	
	49,93 (0,39%)	48,35 (15,18%)	8,33 (4,12%)	10,96 (34,50%)	8,20 (2,27%)	8,96 (9,62%)
	54,29 (7,56%)		15,30 (24,48%)		9,67 (3,75%)	
	CF		**TB**		**TP/PX**	
p-value (0 vs 1h)[b]	**0,0215**		**0,2304**		**0,0010**	
p-value (0 vs 5h)	**0,0012**		**0,1286**		**0,0027**	
p-value (1 vs 5h)	**0,1592**		**0,4433**		**0,0028**	

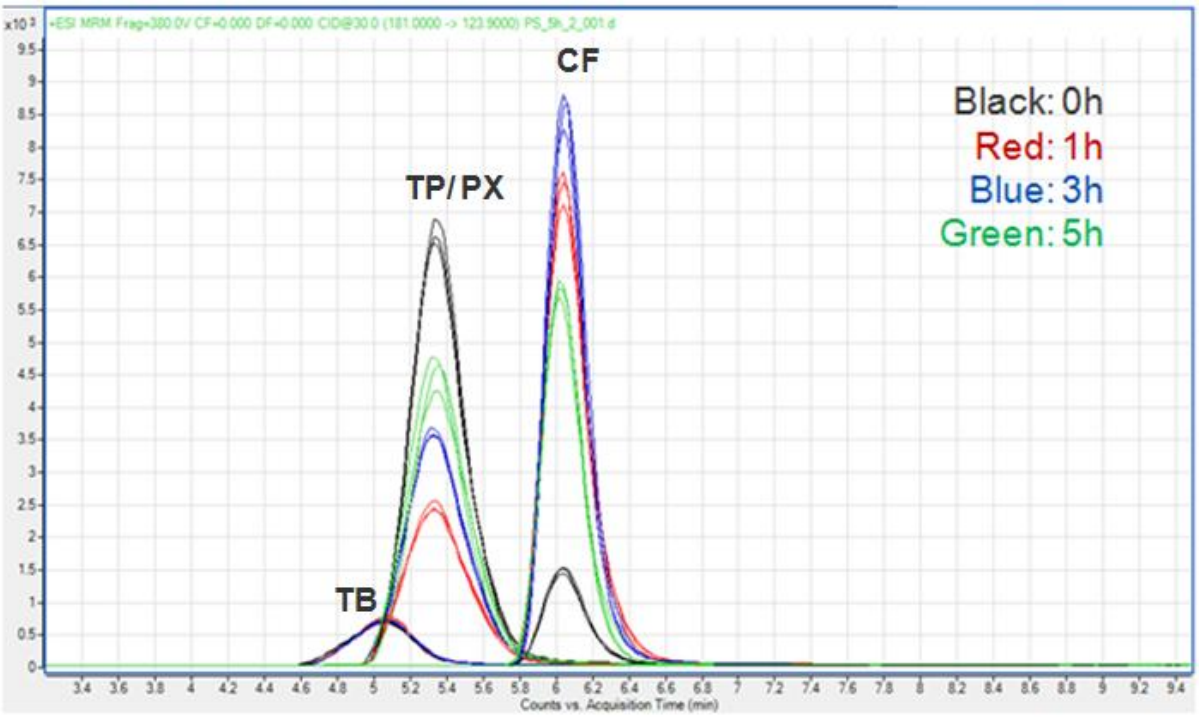


Figure 24 Illustrated LC-MS analyte kinetics of 3 technical replicates at 4 different time points of TB, TP/PX and CF of donor E. The x-axis determines the retention time and the y-axis denotes the intensity of the quantifier-MRM transition.

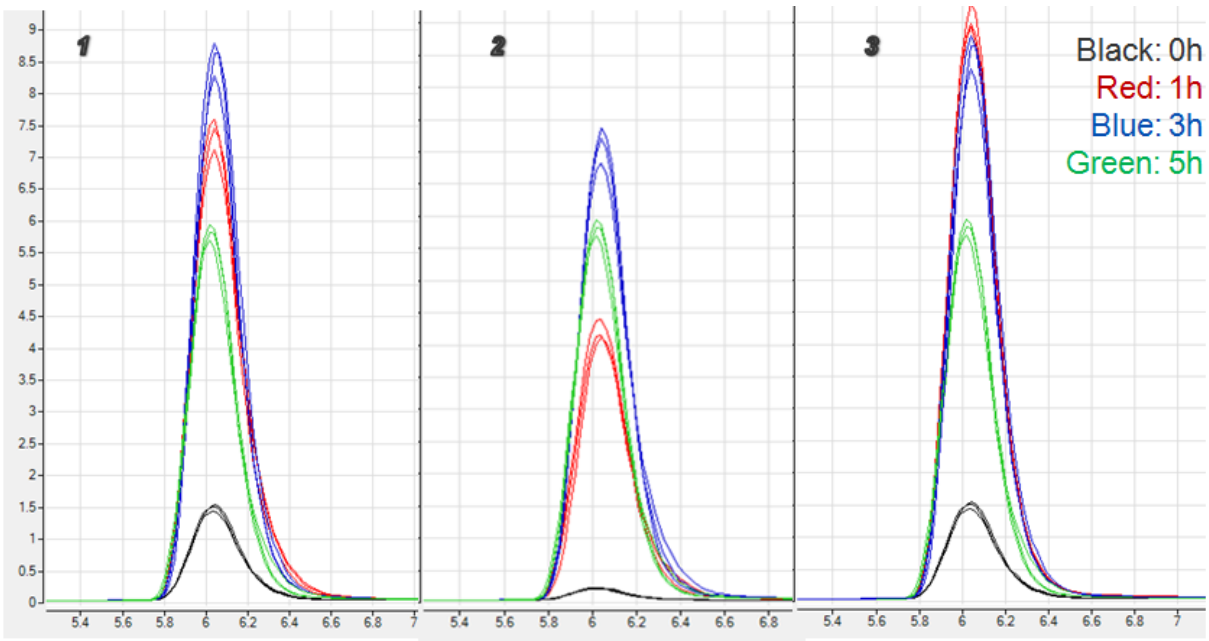


Figure 25 Analyte kinetics for CF of donor E on three different days (1–3) featuring 4 different time points. Each time point is displayed by three technical replicates.

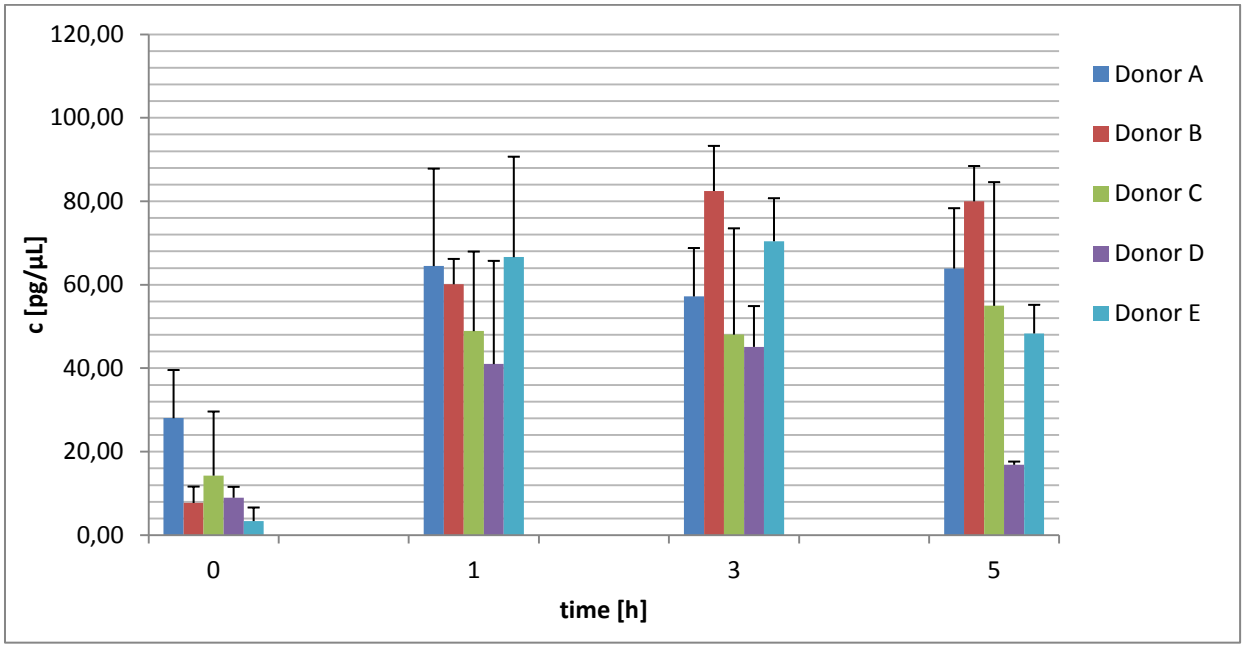


Figure 26 Time course measurements of caffeine in whole blood of donors A-E (blue-turquoise). The x-axis defines the different time points whereas the vertical axis determines the concentration found in pg/µL. The bars represent the biological average (including technical triplicates measured on each of the three days). The standard deviations of caffeine are given measured at 4 different time points on three different days.

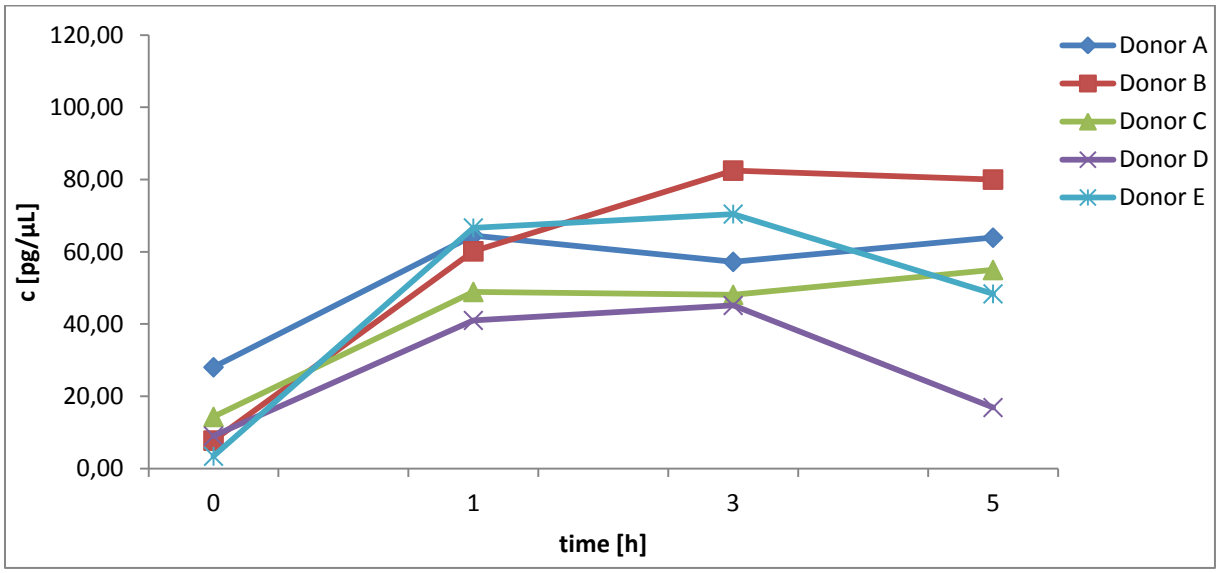


Figure 27 Concentration profiles for CF that were detected in blood before coffee intake and 1, 3 and 5 h afterwards for donors A-F (blue-turquoise). The x-axis defines the different time points whereas the vertical axis determines the concentration found in pg/µL. It shows the biological average including technical triplicates measured on each of the three days.

The measured technical replicates show correlations of variations (CVs) of <15% except for donor C, which caused problems during sample preparation due to the relatively fast coagulation. The biological replicates varied among individuals and also in day-to-day experiments of the same volunteer. Accordingly, method validation suggested that the biological variation is the main parameter responsible for the large overall variation of the measurement series in blood. A significant increase of caffeine can obviously be clarified for Donor A, B and E. The shown p-values are below $p < 0.05$. By means of Figure 26, it a clear trend of CF increase can be observed over the course of 5 h after coffee consumption. By the means of a box plot the key statistical measures (median 57.83 pg/µL, Q_1 = 39.3 pg/µL, Q_3 = 70.9 pg/µL with a maximum of 88.22 pg/µL) clearly show broader range in experiments from five volunteers compared to fingerprint measurements. This also translates to larger biological and overall variations of the measurements in blood compared to fingerprints.

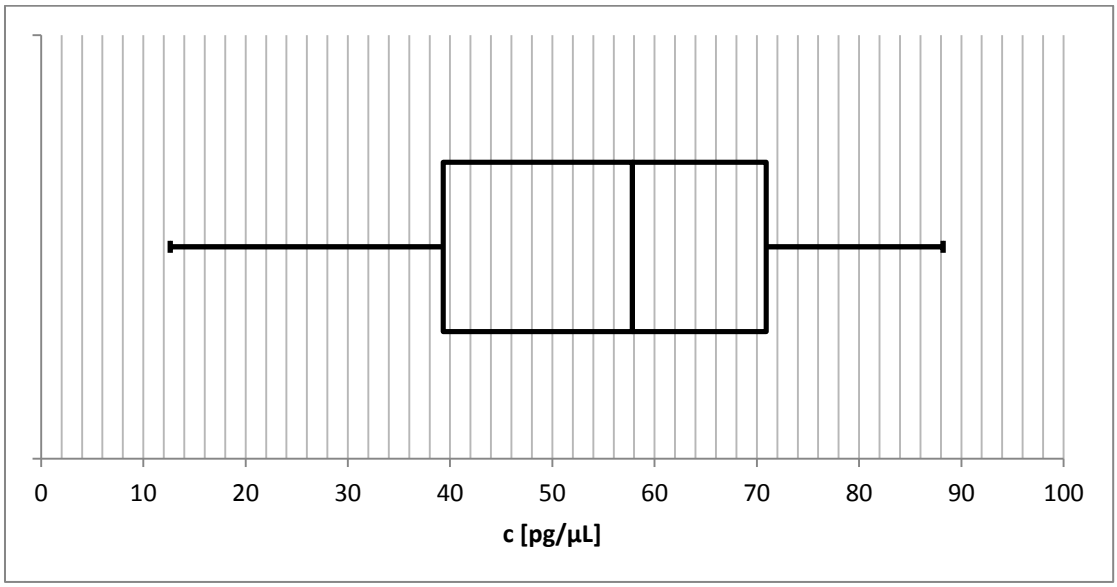


Figure 28 Measurements of whole blood are graphically illustrated using a Box Plot to visualize key statistical parameters (median, mean quartiles). This box plot represents the concentrations of 5 donors after caffeine consumption (1 h) in whole blood with a median of 57.83 pg/µL, Q1 = 39.3 pg/µL, Q_2 = 70.9 pg/µL and a maximum of 88.22 pg/µL.

As pointed out in the chapter on the detection of caffeine and its primary metabolites from fingerprints, caffeine is metabolized by cytchrome P450 enzymes in the liver, which represents the rate-limiting step for plasma clearance. In particular the isoenzyme CYP1A2 is responsible for demethylation of caffeine and the generation of the primary metabolites [146; 147]**.** Studies also proved that the clearance of caffeine is subject to individual daily changes and therefore, also between individuals [134; 135]. It has been further demonstrated that CYP1A2 showed distinctive inter-individual variations which means that the amount of caffeine cleared from the body is proportional to the amount of CYP1A2 [136]. Generally, the clearance of caffeine is affected by

exogenous factors (i.e. drugs, medications, smoking status [148] as well as caffeine itself [149]), but also endogenous factors (i.e. pregnancy, ethnicity and genetics). For example, Asian and African populations do metabolize caffeine at slower rate than Caucasians [144].

When comparing the caffeine concentrations determined in blood after 1 h of coffee consumption with respect to the concentrations before intake, a significant increase of the analyte can be observed for donors A, B and E. The p-values in this case are below $p < 0.05$. Donors C and D did not display a significant immediate CF increase. The p-values 0 vs 5 h show significant increase concerning caffeine level in donor B, D and E. There is no significant decrease of the average concentration of caffeine found between 1 and 5 hours after coffee consumption, except for donor D. It is noteworthy that donor B shows even a significant increase in caffeine concentration found in blood (1 vs. 5 h). It is evident that in fingerprint measurements donor D and donor E did not show significant increase in caffeine levels found before and 1 hour after consumption. In addition, p-values 3 vs. 5 h calculated for blood highlighted that only donor D and E show significant decrease of caffeine after coffee consumption (p-values 0.018 and 0.022 for donor D and E, respectively, Figure 27). This may confirm that donor D and donor E can be regarded as “fast metabolizers”.

The concentration profiles for the primary metabolites were not as straight-forward as for caffeine. The profiles of theobromine were very inconsistent. Donor B, C and E showed a possible but not significant trend of theobromine to slowly increase after caffeine consumption up to 5 h. High initial theobromine levels can be an artefact from possible consumption of chocolate or tea 10–12 h before the experiment because of the relatively long half-life of TB of 7–10 h [33; 34]. Surprisingly, the initial response to coffee consumption was primarily characterized by a significant decrease in TP/PX levels (except for donor C), followed by an increase up to 5 h after coffee consumption for donors B and E. In particular, there are different factors which may affect theophylline elimination in general: smokers metabolize theophylline twice as fast as non-smokers and actually elderly people show slower TP clearance [150]. Using metabolites specific to caffeine as marker substance was described already by Obase et al. 2003 where theophylline has been used as a marker substance for CYP1A2 activity [151].

It can be summarized that significant changes of caffeine before and after coffee intake can be determined in blood using the nanoChip LC-MS system. Generally, higher concentrations were determined in blood compared to fingerprints. The extracting procedure was validated in plasma and it seems that the biological variation is the main factor for the relatively high overall coefficients of variation. Moreover, it seems possible to categorize fast and slow metabolizers from profiling the caffeine concentration in blood and these results parallel the findings from fingerprints. Profiling the metabolites is less straight-forward due to dramatic inter-individual differences.

3.4.4 Creatinine and Melatonin

Measuring whole blood allowed the simultaneous measurements of creatinine and melatonin in parallel to CF, TB and TP/PX in donors B, D and E. These molecules were neither validated, nor calibrated. Effects of caffeine on the blood levels of these molecules were evaluated by direct comparison of the MRM-areas before and after coffee consumption.

Serum creatinine is an important indicator of renal health. Measuring serum creatinine is inexpensive and creatinine clearance is being used for many decades to estimate the glomerular filtration rate (GFR) [42]. Actually, creatinine is a waste product of muscle tissue and a healthy kidney will excrete creatinine in the urine. Consequently, kidney damage is directly proportional to increased blood creatinine levels [152]. Creatinine production is continuous and proportional to muscle mass [40]. Therefore, men tend to have higher blood levels of creatinine than women. Reference values for serum creatinine are 0.7–1.3 mg/dL (70–130 ng/µL) for men and 0.6–1.1 mg/dL (60–110 ng/µL) for women [153]. The concentration range is quite narrow and it was revealed that creatinine production during the day remains essentially unchanged [43]. The creatinine concentrations in blood of the donors B, D and E are displayed in Figure 29 as averages of three independent experiments including each three technical replicates. It can be observed that the creatinine concentrations remain largely constant over the course of the experiment. Consequently, caffeine does not seem to influence creatinine levels. Only donor D seems to be slightly out of the range, especially the level before coffee consumption. Creatinine may therefore be used as a control marker in healthy individuals to account for experimental variations.

Melatonin is a biological modulator of mood, sleep, sexual behaviour and circadian rhythm [46; 47]. Its production is mainly stimulated in the dark and inhibited by light [154]. It was demonstrated that an interaction of melatonin with caffeine increased the total protein and brain tryptophan levels but decreased total brain cholesterol. Moreover, the effects of caffeine on protein levels could be attributed to inhibition of glutamate release due to blockage of adenosine receptors [155; 156]. This study also revealed that caffeine may interact with melatonin to promote not only the synthesis of proteins, but may also stimulate hormone levels (e.g. gonadotrophin release, estrogen and androgen levels). Hence the interference of melatonin and caffeine also may stimulate tryptophan metabolism and accumulation of xanthurenic acid [156]. Fact is that only few studies have focused on whether caffeine may affect blood melatonin levels [156]. Melatonin is generally metabolized in the liver via hydroxylation by CYP450 [48]. It was discovered that the enzyme CYP1A2 may also be of importance for the metabolism of human melatonin [157] and this implies that caffeine and melatonin are metabolized by the same enzyme. Melatonin was measured in blood of donors B, D and E and MRM-Areas <500 were determined, which remained largely constant over time (Figure not shown). It was

beyond the scope of this thesis to investigate the effect of caffeine intake on these molecules in detail and additional investigations would be required to clarify these relationships.

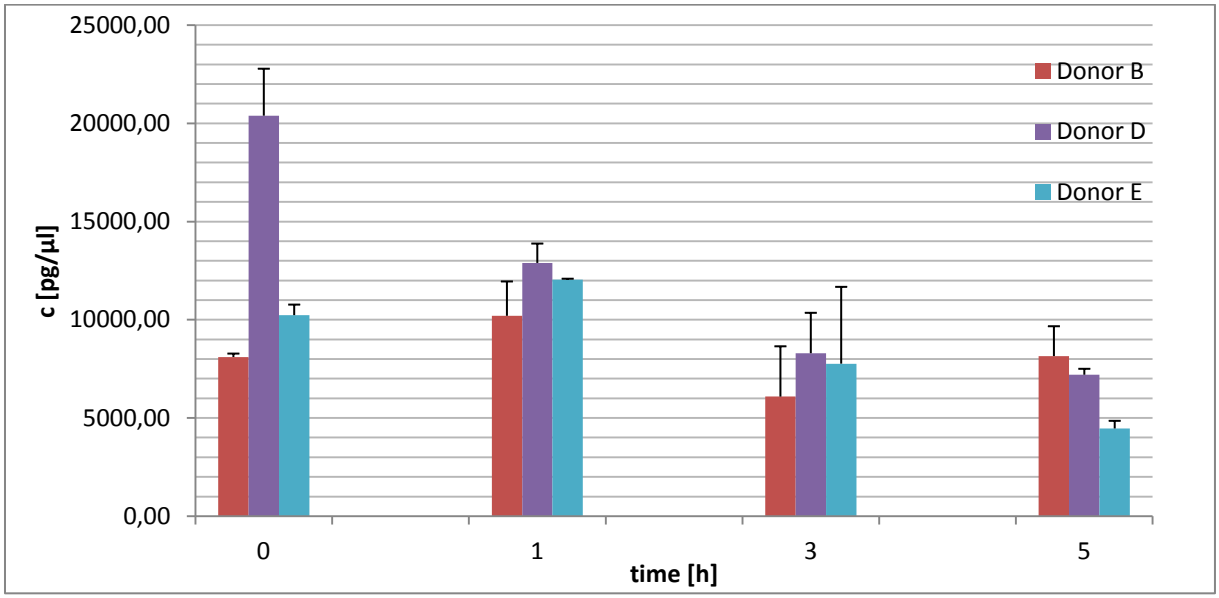


Figure 29 Time course measurements of serum creatinine of donors B, D and E (blue-turquoise). The x-axis defines the different time points (0–5 h) and the vertical axis determines the area of the transition 144.1 -> 44.1. The bars represent the biological average (of technical triplicates) and standard deviations of creatinine measured at 4 different time points on three different days. Each bar includes 9 measurements.

3.5 Quantitation of Caffeine and its Primary Metabolites in Saliva

3.5.1 Sample Preparation

Saliva was retrieved by spitting into a falcon tube for single-use. A volume of 20 µL of saliva was taken with a pipette and transferred into an Eppendorf tube. The extraction procedure was performed according to the chapter ”Extraction of CF and primary metabolites from saliva”. The dried extracts were reconstituted in 250 µL water containing 0.2% formic acid.

3.5.2 Method Validation

Similarly to extracts form blood, extracts from saliva were not validated, nor (matrix-matched) calibrated. The concentration profiles of CF and its primary metabolites were relatively quantified by comparing the state before coffee consumption with the states after consumption. Therefore, MRM-areas of the quantifier transitions of each analyte were directly compared offering relative quantitation. The overall measurement coefficients of variation were divided into instrument, extraction and biological variations, similarly to the fingerprint and blood chapters.

The performance of the real-life experiments from five volunteers was evaluated in particular with respect to the time point 5 h after coffee consumption (Figure 30). The LC-MS variation and the extraction variation show CVs < 17% which both amounts to an extraction reproducibility of 30.8%. The CV of the biological variations after 5h was <110%, which finally amounts to an overall variation of 148.3%.

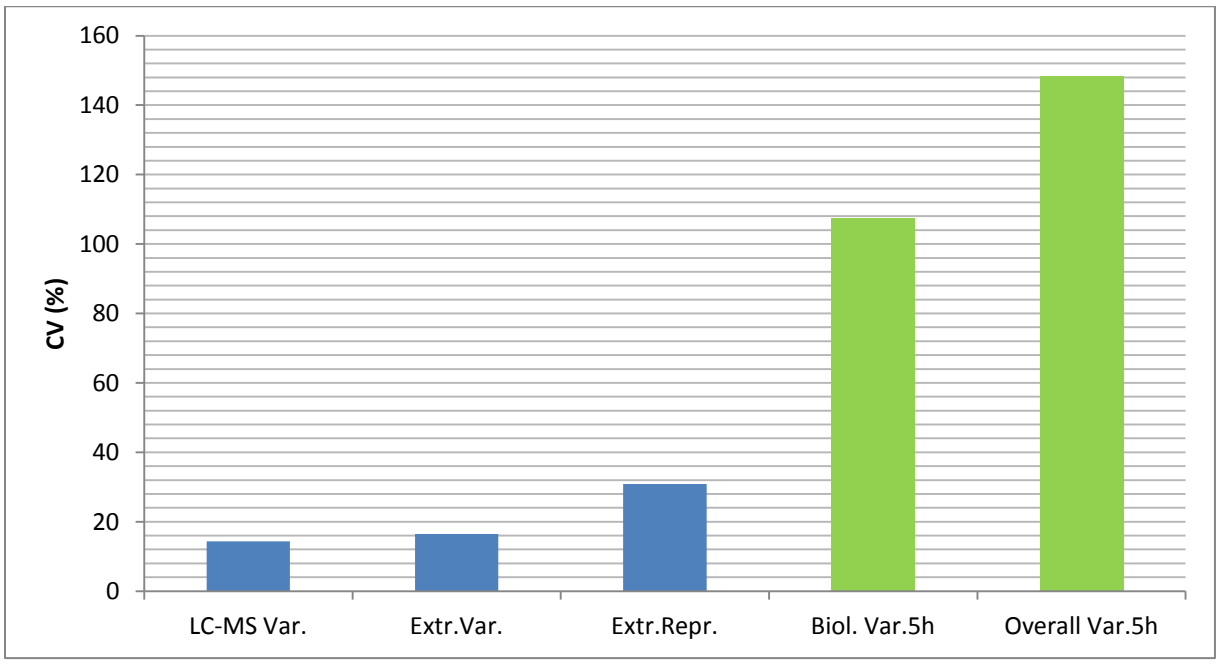


Figure 30 Coefficients of Variation for LC-MS Variation.[a] Extraction Variation[b], Extraction Reproducibility[c] , Biological Variation after 5[d] and Overall Variation (5h)[e] of caffeine detected in saliva.

[a] The LC-MS variation represents the coefficients of variations of 3 technical replicates
[b] The extraction variation represents the coefficients of variations of 3 average extractions
[c] Extraction reproducibility: The coefficient of variation of 3 biological extractions, each with 3 technical replicates
[d] The biological variance indicates the coefficients of variations of the average caffeine amount after 5 h of coffee intake of all the donors
[e] The overall variation represents the coefficients of variations of all technical and biological replicates of 5 donors after 5 h of coffee intake

3.5.3 Evaluation of the Quantitation of Caffeine and its Metabolites in Saliva

The response of caffeine and its metabolites in saliva was evaluated on three different days at 4 different time points similarly as for fingerprints and whole blood. Areas of the quantifier of each analyte were compared in different states with calculated CVs in brackets. The Average direct areas of CF, TB and TP/PX and the CVs are denoted from three technical replicates each (Table 21). By the means of a student t-test, significances (p–values) were calculated for three states similarly to the evaluation in fingerprints and blood (0 vs. 1 h; 0 vs. 5 h and 1 vs. 5 h). The null hypothesis for the test was that there is no significant difference in areas of analyte found in saliva between time point 1 and time point 2. A p-value of >0.05 was rejected.

Table 21 Time-course measurements of caffeine and its metabolites from saliva at three different days from donors A-E. In this table the areas of each analyte at 4 different time points of three technical replicates of CF, TB and TP are displayed with the coefficient of variations in brackets.

[a] determines the sample variance of three biological replicates with the calculated CVs in brackets.
[b] The p-value greater than 0.05 (p > 0.05) determines that the observed results are due to random chances. This will be the case if calculated t-value is below the t-critical value.

			Donor A			
Time [h]	Area CF (Area)	Average Area[a]	Area TB (Area)	Average Area	Area TP/PX (Area)	Average Area[a]
0	1074,67 (23,6%)		647,3 (90,2%)		5254,0 (36,79%)	
	20145,33 (30,75%)	10425,44 (29,51%)	2136,6 (34,59%)	1079,4 (69,95%)	17308,3 (47,07%)	7695,3 (46,43%)
	387,33 (34,11%)		454,3 (85,06%)		523,67 (55,42%)	
1	49495,0 (39,1%)		264,0 (25,7%)		7247,3 (51,69%)	
	32080,0 (38,53%)	34852,0 (30,11%)	512,3 (83,53%)	296,3 (64,95%)	6543,0 (54,05%)	4846,56 (73,96%)
	22981,0 (12,86%)		112,67 (85,63%)		749,3 (116,16%)	
3	22708,3 (7,64%)		-		-	
	13915,3 (17,9%)	15556,11 (11,77%)	-	-	-	-
	10044,67 (10,59%)		-		-	
5	6695,3 (54,69%)		-		-	
	28664,0 (0,96%)	17125,78 (19,55%)	5457,3 (4,5%)	4539,83 (5,57%)	18834,0 (15,63%)	15871,5 (16,03%)
	16018,0 (2,99%)		3622,3 (6,65%)		12909,0 (16,44%)	
	CF		**TB**		**TP/PX**	
p-value[b] (0 vs 1h)	**0.0271**		**0.1429**		**0.3194**	
p-value (0 vs 5h)	**0.1679**		**0.0514**		**0.1280**	
p-value (1 vs 5h)	**0.0777**		**0.0614**		**0.0471**	

			Donor B			
Time [h]	Area CF (Area)	Average Area[a]	Area TB (Area)	Average Area[a]	Area TP/PX (Area)	Average Area[a]
0	11893,3 (0,09%)		48313,0 (4,21%)		26817,67 (1,89%)	
	12875,67 (0,94%)	13813,33 (1,03%)	17077,0 (1,58%)	26790,56 (2,61%)	30700,67 (2,69%)	33871,22 (2,48%)
	16671,0 (2,06%)		14981,67 (2,03%)		44095,33 (2,86%)	
1	150668,67 (0,91%)		36130,0 (3,80%)		50896,0 (3,36%)	
	109595,33 (5,01%)	127307,78 (4,08%)	15708,67 (2,17%)	127307,78 (2,36%)	45534,0 (0,80%)	50338,0 (2,07%)
	121659,3 (6,33%)		12424,33 (1,11%)		54584,0 (2,07%)	
3	196355,67 (2,30%)		38443,33 (2,68%)		94765,67 (2,40%)	
	119371,3 (1,41%)	166363,33 (1,33%)	11484,0 (3,63%)	166363,33 (2,33%)	61158,67 (1,97%)	84748,11 (1,59%)
	183363,0 (0,27%)		14030,33 (0,68%)		98320,0 (0,4%)	
5	128077,67 (1,14%)		31132,33 (4,31%)		99599,33 (1,73%)	
	25559,67 (2,68%)	88116,67 (2,46%)	2312,33 (18,5%)	88116,67 (8,75%)	18378,67 (4,40%)	68655,67 (2,81%)
	110712,67 (3,57%)		10879,33 (3,45%)		87989,0 (2,30%)	
	CF		**TB**		**TP/PX**	
p-value [b] (0 vs 1h)	**0.0053**		**0.3525**		**0.0343**	
p-value (0 vs 5h)	**0.0717**		**0.2170**		**0.1512**	
p-value (1 vs 5h)	**0.1720**		**0.2945**		**0.2729**	
			Donor C			
Time [h]	Area CF (Area)	Average Area[a]	Area TB (Area)	Average Area[a]	Area TP/PX (Area)	Average Area[a]
0	143,0 (10,49%)		263,0 (49,05%)		351,0 (38,25%)	
	819,33 (4,27%)	1698,0 (6,29%)	2829,0 (64,63%)	3176,78 (42,66%)	1474,67 (24,66%)	3092,78 (21,6%)
	4131,67 (4,12%)		6438,3 (14,28%)		7452,67 (1,89%)	
1	25091,0 (5,55%)		1259,67 (25,33%)		6502,67 (2,21%)	
	25100,67 (1,21%)	21821,56 (3,34%)	7090,0 (24,04%)	3861,78 (22,06%)	10833,67 (22,11%)	7465,67 (9,42%)
	15273,0 (3,26%)		3235,67 (16,81%)		5060,67 (3,92%9	
3	22755,0 (1,32%)		1624,67 (7,42%)		10913,67 (1,96%)	
	30887,0 (0,47%)	26025,22 (1,1%)	8920,0 (15,89%)	4833,56 (13,77%)	19816,33 (1,95%)	13729,33 (1,82%)
	24433,67 (1,50%)		3956,0 (17,99%)		10458,0 (1,55%)	
5	21671,33 (2,91%)		1999,33 (2,79%)		16222,0 (1,79%)	
	-	21690,67 (4,4%)	-	4394,50 (31,56%)	-	14081,83 (5,72%)
	21710,0 (5,89%)		6789,67 (60,33%)		11941,67 (9,65%)	
	CF		**TB**		**TP/PX**	
p-value (0 vs 1h)[b]	**0.0080**		**0.3980**		**0.0989**	
p-value (0 vs 5h)	**0.0019**		**0.3608**		**0.0219**	
p-value (1 vs 5h)	**0.4859**		**0.4365**		**0.0619**	

			Donor D			
Time [h]	Area CF (Area)	Average Area[a]	Area TB (Area)	Average Area[a]	Area TP/PX (Area)	Average Area[a]
0	5522,33 (4,20%)		9617,0 (1,62%)		15178,0 (0,76%)	
	2132,67 (2,58%)	3449,0 (2,95%)	2848,33 (6,22%)	6086,33 (4,14%)	6639,0 (2,27%)	9882,0 (2,66%)
	2692,0 (2,07%)		5793,67 (4,57%)		7829,0 (4,94%)	
1	32493,33 (0,84%)		6782,33 (1,42%)		17854,67 (1,44%)	
	97069,67 (1,21%)	54904,22 (1,0%)	17391,0 (1,46%)	11063,56 (2,78%)	56821,67 (3,26%)	31144,0 (2,03%)
	35149,67 (0,94%)		9017,33 (5,45%)		18755,67 (1,38%)	
3	127,50 (6,10%)		317,33 (7,26%)		132,67 (7,74%)	
	75179,0 (2,51%)	55367,61 (3,49%)	21355,67 (1,74%)	29753,22 (3,59%)	41535,67 (81,48%)	35204,11 (2,80%)
	90796,33 (1,87%)		67586,67 (1,77%)		63944,0 (0,61%)	
5	33391,33 (0,78%)		8208,67 (4,90%)		36978,67 (2,16%)	
	22100,33 (1,55%)	27054,33 (1,68%)	8282,33 (0,98%)	8252,89 (3,85%)	21527,67 (3,33%)	28441,56 (1,92%)
	25671,33 (2,71%)		8267,67 (5,67%)		26818,33 (0,27%)	
	CF		**TB**		**TP/PX**	
p-value (0 vs 1h)[b]	**0.0673**		**0.1358**		**0.1183**	
p-value (0 vs 5h)	**0.0065**		**0.1920**		**0.0171**	
p-value (1 vs 5h)	**0.1584**		**0.2379**		**0.4289**	
			Donor E			
Time [h]	Area CF (Area)	Average Area[a]	Area TB (Area)	Average Area[a]	Area TP/PX (Area)	Average Area[a]
0	119 (4,2%)		54,67 (12,93%)		513,67 (1,57%)	
	-	127,1 (4,91%)	-	88,1 (8,55%)	99,0 (8,63%)	504,89 (7,33%)
	262,3 (5,62%)		209,67 (4,17%)		902,0 (11,80%)	
1	1787,3 (5,57%)		102,67 (8,73%)		1214,67 (10,76%)	
	1565,3 (9,24%)	1842,56 (5,61%)	-	102,2 (9,27%)	489,67 (9,01%)	983,78 (7,3%)
	2175,0 (2,0%)		204,0 (9,8%)		1247,0 (2,13%)	
3	1616,0 (2,75%)		112 (8,79%)		1481,3 (1,94%)	
	1323,3 (7,09%)	1560,78 (3,93%)	-	92,2 (7,27%)	844,0 (3,87%)	1264,2 (4,46%)
	1743,0 (1,95%)		164,67 (5,74%)		1467,3 (7,57%)	
5	1132,0 (17,73%)		57,0 (2,48%)		1270,67 (24,77%)	
	980,67 (5,07%)	1151,78 (9,52%)	-	48,56 (5,45%)	737,3 (25,72%)	1030,44 (18,32%)
	1342,67 (5,77%)		107,67 (8,43%)		1083,3 (4,47%)	
	CF		**TB**		**TP/PX**	
p-value (0 vs 1h)[b]	**0.0029**		**0.4217**		**0.1154**	
p-value (0 vs 5h)	**0.0024**		**0.2916**		**0.0717**	
p-value (1 vs 5h)	**0.0198**		**0.1673**		**0.4412**	

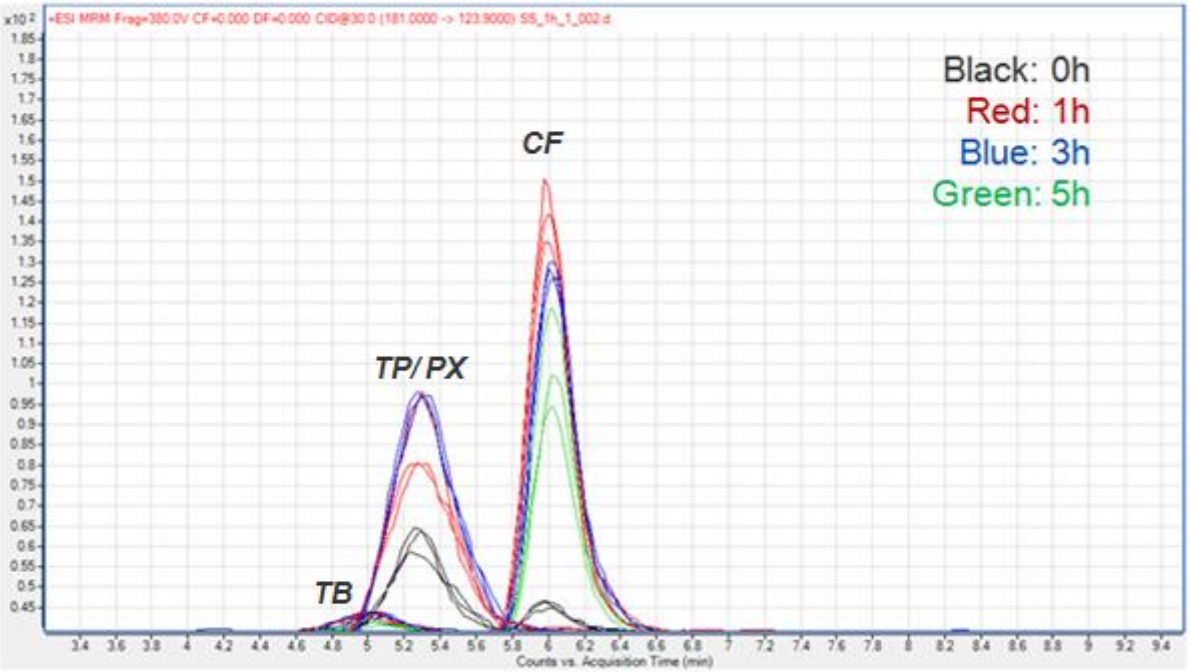


Figure 31 Analyte kinetics of CF, TB and TP/PX of donor E found in saliva covering 3 technical replicates at 4 different time points (before coffee intake and 1, 3 and 5 h thereafter).

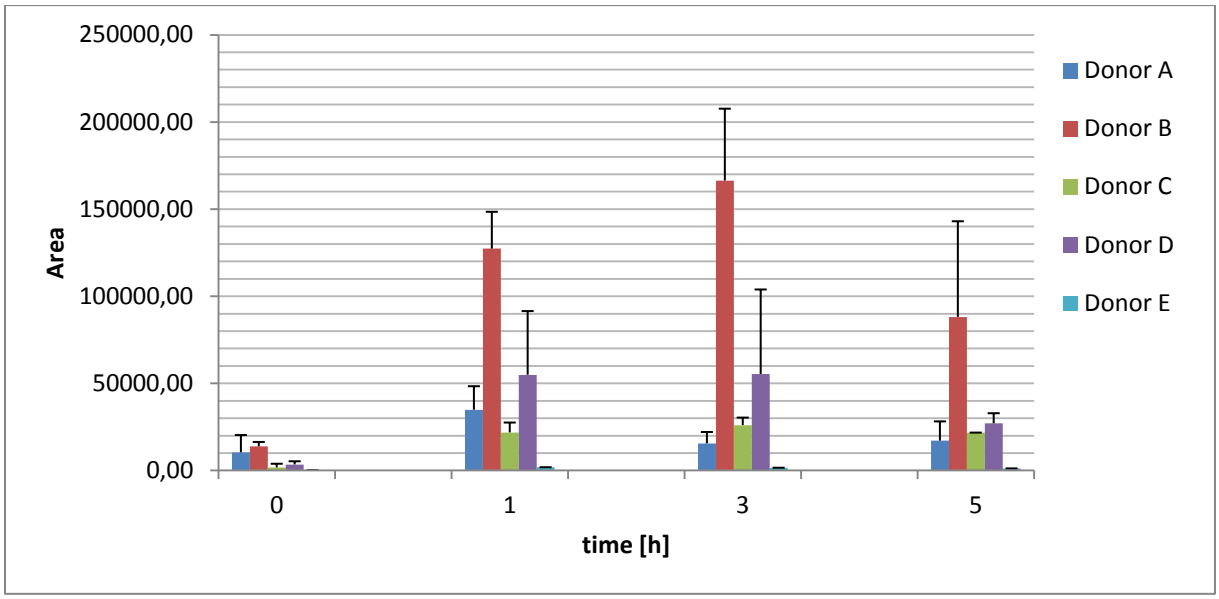


Figure 32 Time course measurements of caffeine found in saliva of donors A-E (blue-turquoise). The x-axis defines the different time points whereas the vertical axis determines the area of the MRM-quantifiers. The bars represent the biological average (technical triplicates) and standard deviations of caffeine measured at 4 different time points on three different days.

The measured technical replicates (Table 21) show large coefficients of variation (CVs) and also the inter-day measurements vary greatly for the same individual and among individuals. The main determinant responsible for overall variation in measurement series was again the biological variation (107.4%). Figure 32 displays the time-dependent profile of caffeine measured in saliva of the 5 donors. It appears that saliva, produced in salivary glands, actually shows a significant CF increase for 4 out of 5 donors before compared to 1 h after coffee intake. There is no doubt that the saliva volume as well as its composition varies within every individual and during the day, which would amount to dilution effects of the analyte. Such fluctuations are observed among the individuals but the trend is similar for all. Normally adults will approximately produce 500-1500 mL saliva per day [158]. Salivary production depends on different olfactory stimuli, taste, mechanical stimulations and varying moods. In other words it depends on psychological-status, health-status, oral hygiene, medication and also the time of the day, hence it is expected that salivary production during sleep drops to almost zero [88].

Direct areas of the quantifier of each analyte compared in different states show significant increase of CF before coffee consumption and 5 h thereafter for donor C-E. Furthermore donor E shows significant changes concerning areas of caffeine for all specified times. It is evident that CF after 1h remains relatively constant for 4 out of five individuals. Significant changes concerning the metabolite levels can be observed for the theophylline/paraxanthine pair for donors A (1 vs. 5 h), B (0 vs. 1 h), C (0 vs. 5 h) and D (0 vs. 5 h). Therefore, over the course of 5 h the amount of TP/PX slowly increases upon coffee consumption at least for donors A-D. In contrast, theobromine actually shows great individual differences without clear trends in the concentration profile.

A box plot is used in order to compare distributions between several sets of data. However the data of CF and its primary metabolites were relatively quantified and the amount of caffeine graphically illustrated (1 h after coffee consumption). The mean CF concentrations in saliva after 1 h lies in the areas 14684,72 and 54904,22 including 2 outliers at 127307 and 166363.

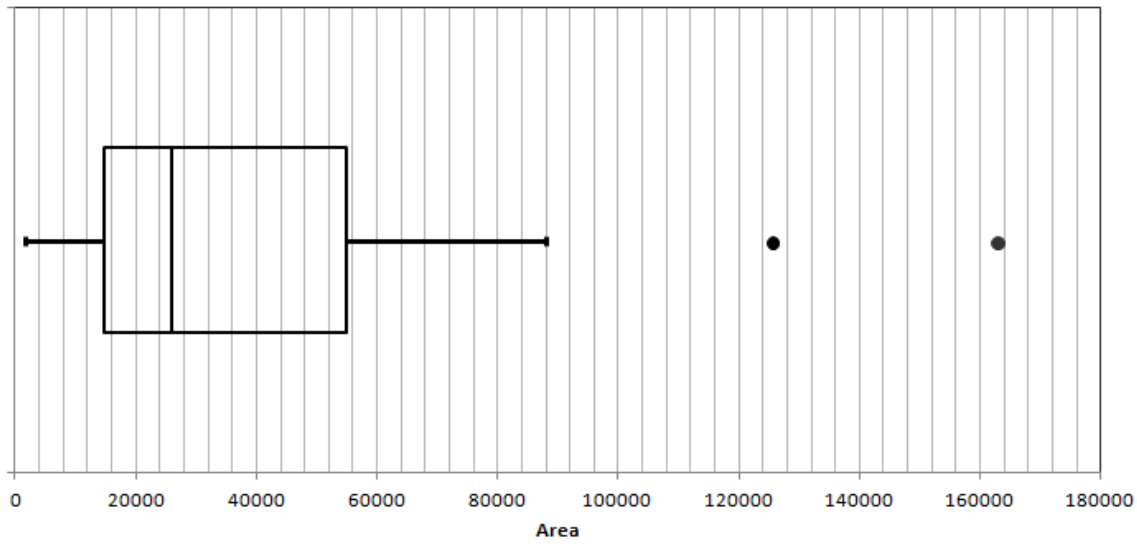


Figure 33 Measurements of saliva are graphically illustrated using a Box Plot to visualize key statistical measures (median, mean quartiles) of direct areas of the quantifier of caffeine found 1 h after coffee consumption. It indicates outliers at 127307 and 166363 with a median of 26025,22 and mean quartiles Q_1 = 14684,72 and Q_3 = 54904,22.

Although salivary composition varies continuously, both quantitatively and qualitatively as described above [88], identifying drugs in saliva is of great interest as a non-invasive diagnostic tool for drug detection and monitoring. It seems that the determination of CF profiles is feasible, whereas metabolite screening requires further optimization particularly with respect to sample retrieval.

3.6 Comparison of UHPLC and nanoChip-LC-MS with the respect to the Quantitation of Caffeine

An additional series of three donors (F–H) was recruited for comparing the UHPLC- with the nanoChip-LC-MS systems with respect to the quantitation of caffeine in blood. Calibration curves were constructed for the concentration range of 0.5–50 pg/µL on both platforms, respectively, which are displayed in Figure 34. The comparison of the calibration curves reveals a slightly better regression coefficient for the nanoChip compared to the UHPLC setup. However, large differences in analyte MRM-areas were observed when comparing both setups. The nanoChip system delivered for areas arount 1800 for 0.5 pg/µL of CF and 198400 for 50 pg/µL of CF. In contrast, the UHPLC system featured 345 and 12840 for 0.5 pg/µL and 50 pg/µL of CF, respectively. Consequently, the calibration curve derived from the nanoChip system is approximately 15-fold steeper than that of the UHPLC, which indicates a better sensitivity for the former setup. The carry-over was surveyed after injection of 500 fg on column and resulted in similar amounts of 0.0011% for the UHPLC and 0.00091% for Chip-LC. The LOQ for both setups was calculated according to FDA guidelines [129] and was found to be 0.48 and 0.26 pg/µL for the UHPLC- and the nanoChip-MS systems, respectively.

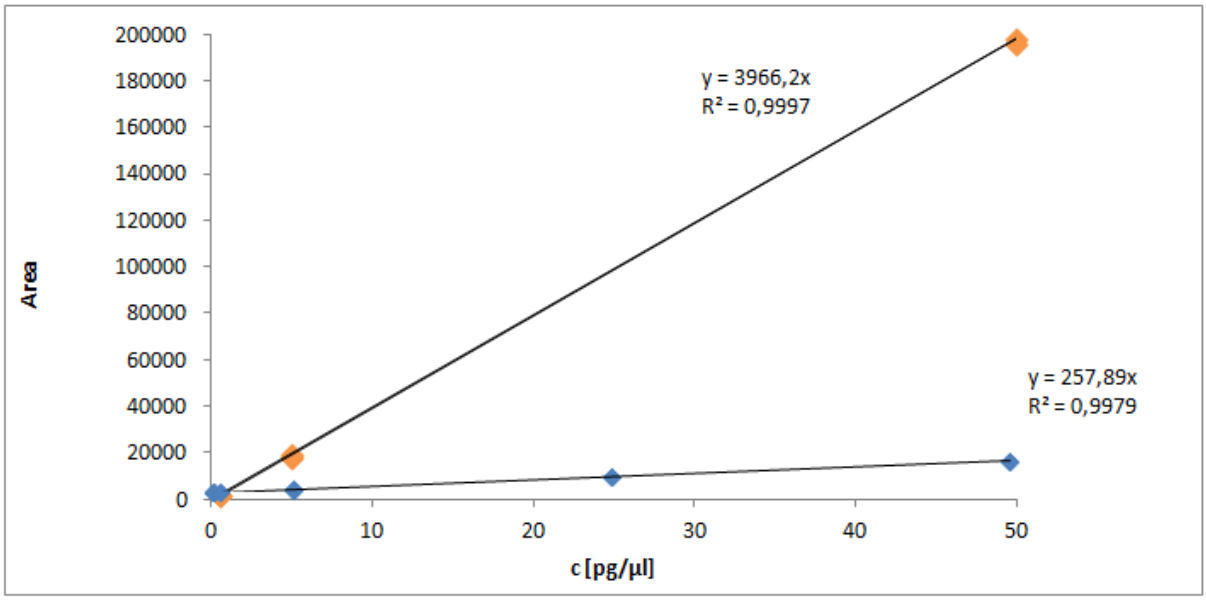


Figure 34 Calibration curves for caffeine obtained from Chip-LC (yellow) in comparison with UHPLC (blue) with concentrations ranging from 0.05–50 pg/µL. Average areas of matrix blanks were measured and the calculated LOQ was 0.48 pg/µL for the UHPLC- and 0.26 pg/µL for the nanoChip-LC systems.

Time course measurements of the caffeine concentrations in whole blood were performed once similarly to the previous chapters, including a cohort of 3 donors (F-H). A graphical illustration of the findings is given in Figure 35. The bars represent the average amount of caffeine for each donor from one experiment with standard deviations including three technical replicates. The single biological experiment also explains the generally low standard deviations. Both instrument setups deliver identical trends for profiling the individual concentrations of caffeine in blood. A CF maximum is reached after 1 h for donors A and B and after 3 h for donor C on both instruments. Apparently, the nanoChip yields slightly higher concentrations compared to the UHPLC system, although being in the same range. NanoChip and UHPLC measurements varied among standard deviations and correlations of variation: NanoChip measurements from 0.5–50 pg/µL from three technical replicates display CVs from 1.3–4.17% compared with 18.5–1.3% in UHPLC measurements for 0.5–50 pg/µL, respectively. Therefore, nanoChip provides slightly better sensitivity and lower CVs for analyzing CF while UHPLC allows for shorter run times.

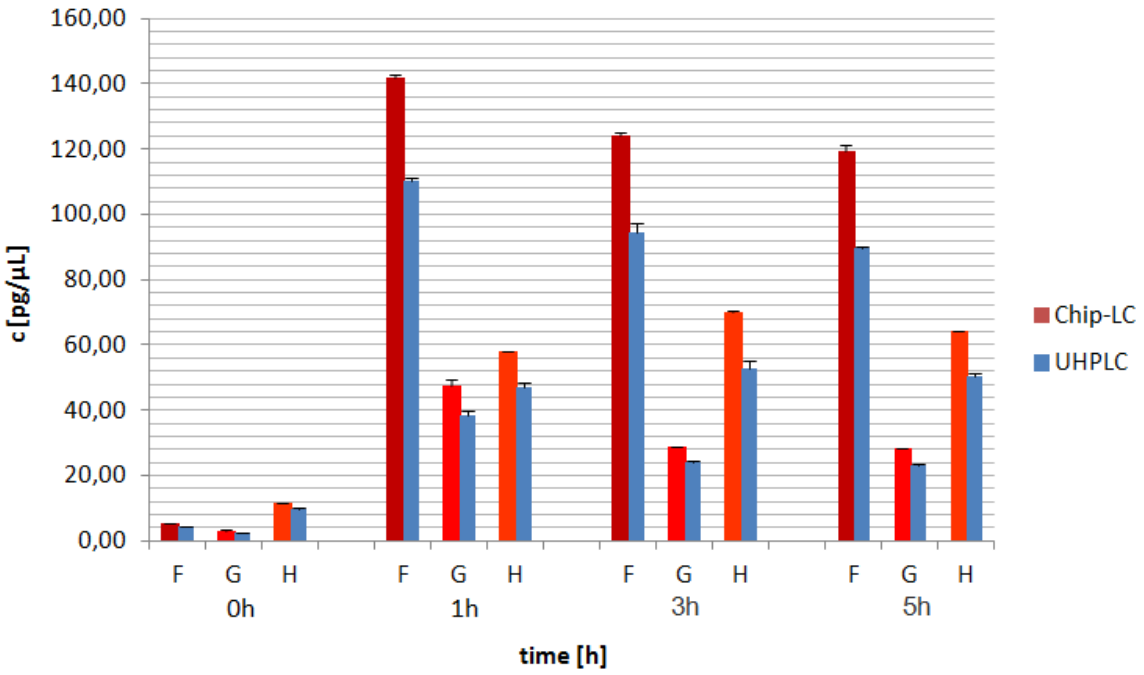


Figure 35 Time course measurements of caffeine from whole blood samples of 3 donors measured with nanoChip-LC-MS (red – orange) compared to UHPLC-MS (blue) at 4 different time points respectively. The values represent the average amount of caffeine with the standard deviation of three technical replicates.

4 Discussion of the Findings from Fingerprints, Blood and Saliva

Analytical Aspects. The method of choice for this study was a mass spectrometric method based on MRM. The instrument was equipped with a microfluidic-based Chip Cube nanoLC system. Caffeine and its primary metabolites were quantified in sweat secretion from fingerprints and whole blood and relatively quantified in saliva. Not only their similar retention times but also virtually identical product ion spectra [5] created a challenge in separation and identification of the primary metabolites, which are isomeric forms of dimethylxanthine. The high polarity of the metabolites seems to constitute the working limits of the nanoChip with regard to separation. It turned out that this is mainly caused by a low trapping efficiency of the primary metabolites (theobromine, theophylline/paraxanthine), which tended to diffuse into the trapping column during loading. Both sample flush and injection path volumes must be tightly controlled in order to minimize this effect. These problems were not observed for caffeine. Moreover, the equilibration of the trapping and the separation column requires 16 min in 100% aqueous phase after each run. Theophylline and paraxanthine were not separated and are evaluated together. The following transitions were used as quantifiers for the MRM-analysis: CF (195.1 – 138.0), TB (181.0 – 67.0), TP/PX (181.0 – 69.0), CF-D9 (204.2-144.1).

The most suitable extraction agents have been determined by ultra- high-performance liquid chromatography with UV detection (UHPLC-UV). Out of five solvent systems diethyl ether and acetonitrile showed the best results for simultaneously extracting caffeine, theobromine and theophylline. Results showed that the recovery of extraction obviously varied greatly among the three metabolites depending on the lipophilicity of the extraction solvent. In particular, extracting the highly polar metabolites was not efficient with most solvents and only showed acceptable results in acetonitrile and diethyl ether. Acetonitrile was selected as the final extracting agent. It is polar, miscible with water and has the advantage of being non-volatile compared to diethyl ether.

The internal standard (CF-D_9) was always added after drying the samples during reconstitution in water containing 0.2% formic acid and was used to correct for spray fluctuations and to account for the extraction procedure. The validated methods allow quantification of caffeine, theobromine and theophylline/paraxanthine in the concentration range of 0.5–300 pg/µL (0.25–150 pg on column) with good linearity for all analytes. The calibration curve R^2 values were >0.999 for fingerprint and >0.998 for whole blood. The measured overall process efficiency of caffeine, theobromine and theophylline were between 88.4–92% and 79.8–84.7% for fingerprint and whole blood, respectively.

The sensitivity of the method was assessed by calculating LODs for each analyte. The lower limits of quantitation (LLOQs) of the analytes were in the range of the lowest calibration standard. CF, TB and

TP show LLOQs of 0.54, 0.68 and 0.42 pg/FP, respectively, while the limits of detection (LOD) were 0.22, 0.28 and 0.20 pg/FP, respectively. The detection and quantitation limits in whole blood were 0.27-0.37 pg/µL (LOD) and the LOQ was determined between 0.61-0.83 pg/µL revealing that only a slight impact of the matrix on these parameters. The overall variation for the fingerprint measurements of all technical and biological replicates was found to be less than 22%. The extraction reproducibility amounted to 7.2% with 3.8% LC-MS variability for the fingerprint series and a biological variability of 8.9%. Whole blood showed higher biological and overall variance (42.3–48.1%) with an extraction reproducibility of 15.1% and LC-MS variability of 8.2%. The sample preparation procedure was straightforward and fast with a total operating time of 10 min per sample, which allowed a routine throughput of 60 samples per 8 h. However, the sample preparations of whole blood and saliva turned out to be more challenging due to difficulties of collecting precise blood volumes resulting from coagulation or different quantities obtained from salivary glands e.g. which can actually can be due to foaming. This may partially account for higher coefficients of variation of the extraction reproducibility. Consequently, caffeine and its primary metabolites are preferentially quantified from fingerprints due to low coefficients of variation from methodical, instrumental and biological parameters.

Biological Aspects. Caffeine is a substance regularly consumed by humans in coffee, tea and chocolate. The amount of caffeine needed to produce biological effects varies from person to person and differences in caffeine metabolism are probably related to the hepatic P450 system as well as other factors [142; 143]. Moreover, individuals can develop a tolerance for caffeine which can be associated with up-regulation of adenosine A1 or A2 receptors [137; 138]. Detection of caffeine had already been reported by Rowell et al. [92] determining the amount of caffeine in fingerprints without removing external contaminants and also by Kuwayama et al. [5] testing fingerprints and blood of three subjects. In this study caffeine, theobromine and theophylline/paraxanthine from fingerprint, blood and saliva of five subjects were evaluated, which is the calculated amount of test subjects needed for statistical relevant data using power-analysis. Therefore, the whole experiment was performed in three inter-day experiments with all five donors. In each experiment, fingerprints, blood and saliva samples were collected before coffee consumption and 1, 3, and 5 h thereafter. By means of profiling caffeine concentrations in fingerprints, blood and saliva, one may estimate on the one hand the intake through the gastrointestinal tract. Additional profiling of the three primary metabolites may allow assessing the activity of the metabolic CYP450 system. Obviously, this study proves that the simple and easy-to-use analytical procedure allow to differentiate between slow and fast metabolizers, to evaluate the metabolic activity of hepatocytes in individuals and to profile the time dependent progress of caffeine in the body.

Concerning caffeine detected in fingerprint sweat, a significant increase can be observed for donors A-C (0 vs. 1 h), however, no CF increase is observed for donors D and E in the same time period. Noteworthy, a significant decrease of the the average concentrations of caffeine found between 1 and 5 hours is observed for donors A and B while donors D and E showed no significant reduction. Comparing the time point before with 5 h after consumption shows a significant increase in CF concentration in for 4 out of 5 donors. Surprisingly the measurements showed high degree of similarity concerning caffeine concentrations after 5 h of coffee consumption for all donors.

Concerning caffeine concentrations determined in blood, significant increase before and 1 h after consumption can be observed for donors A, B and E. Donor B however is the only participant who shows a significantly increase in caffeine concentration between 1 and 5 h. The lack of a CF peak combined with slow CF decrease over time obviously demonstrated the relatively fast metabolization rate of donors D and E. Moreover donors D and E turned out to be the only participants who show significant decrease of caffeine after absorption (p-values 3 vs. 5 h).

Similarly to fingerprints and whole blood the response of caffeine and its metabolites was evaluated in saliva on three different days and at 4 different time points. Compared to the measurements of fingerprints and blood, the MRM-areas of the quantifier of each analyte (CF, TB and TP/PX) were compared in different states and therefore, were relatively quantified. A significant increase of CF was found before coffee consumption and 5 h thereafter for donors C-E. The amount of CF between 1 and 5 h remains relatively constant for each individual (4 out of 5).

Figure 36 displays box plots for fingerprints, blood and saliva and compares the states 1 h and 5 h after caffeine intake. Comparison of the measurements of caffeine in fingerprint (FP) with whole blood (B) shows distinctive differences concerning concentration distribution of CF. In contrast to extracts from fingerprints and blood, extracts from saliva were neither validated, nor calibrated. The concentration distributions of CF in saliva were calculated with respect to aqueous calibration solutions, which may lead to a general overestimation of these concentrations (Figure 36). It emerges that broader distributions are found in blood and saliva compared to fingerprint. Blood measurements clearly show larger variations in experiments from five volunteers compared to fingerprint measurements, which is actually due to the larger biological variance. It is interesting that the distribution of values after 5 h in fingerprint is very narrow, which allows quantification experiments under FDA requirements. Actually, after five hours the CF concentrations measured in fingerprint show just a biological variation of CV <9% in contrast to blood (CV<45%) and saliva (CV<108%).

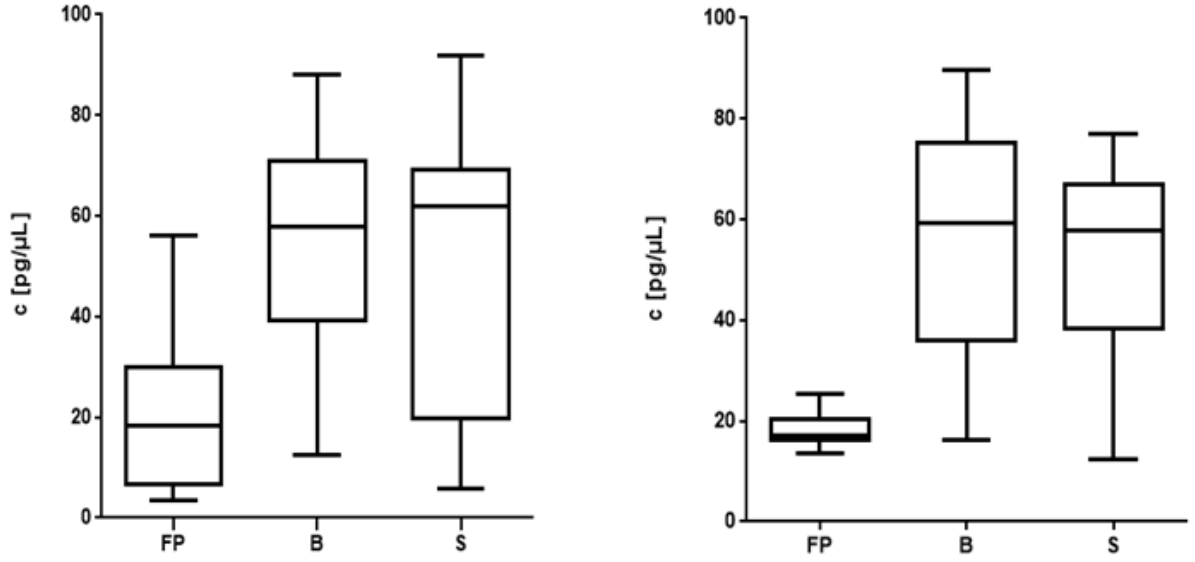


Figure 36 Measurements after 1 h of caffeine intake (left) in comparison with measurements 5 h afterwards (right) are displayed accordingly for Fingerprints (FP), Blood (B) and Saliva (S), respectively. The CF concentration found in saliva was calculated with respect to aqueous calibration solutions.

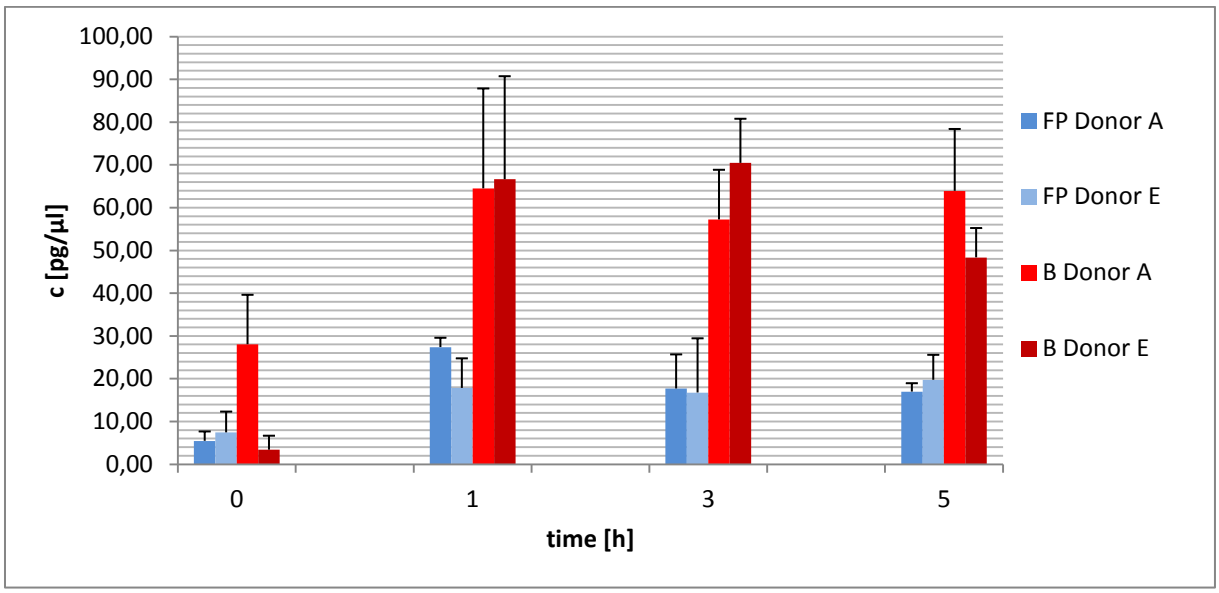


Figure 37 Time course measurements of caffeine in fingerprint (FP) and whole blood (B) of donors A and E). The x-axis defines the different time points whereas the vertical axis determines the concentration found in pg/µL. The bars represent the biological average (including technical triplicates measured on each of the three days). The standard deviations of caffeine are given measured at 4 different time points on three different days (i.e. each standard deviations contains 9 experiments).

Significant concentration changes were also detected for the primary metabolites. However, it seems that these significances are essentially affected by individual differences, i.e. the biological variations were large from day-to-day experiments as well as from inter-individual CF concentrations. In fingerprints, significant changes were observed for TB in donors A, B and E, while significant changes for TP/PX were observed for donors A and C. The low abundance of metabolites in fingerprints may stem from the fact that these molecules do not diffuse efficiently into the appocrine glands due to their high polarity. In blood, however, significant changes in metabolite levels were detected for TB in donors A and D and for TP/PX in all five donors, which is reasonable because PX constitutes the major metabolite of CF *in vivo* [146; 159].Therefore, it may be possible to use TB or TP/PX as markers for the metabolism of CF and for testing p450 activity. Moreover, it may be beneficial to monitor these metabolites over longer time periods for reducing the overall coefficients of variation. Significant changes in the metabolite levels were detected in saliva for the TP/PX pair for donors A (1 vs. 5 h), B (0 vs. 1 h), C (0 vs. 5 h) and D (0 vs. 5 h). In contrast, theobromine actually shows great individual differences without clear trends in the concentration profile.

A striking observation was the possible correlation between slow/fast metabolizers and gender. The hypothesis that women may have a greater response than men which can be due to different detoxification in the body [150] cannot be confirmed on the basis of the available data. Furthermore, the clearance of caffeine can be affected by exogenous factors, demographic and environmental, as well as genetic factors or drugs [160; 161]. Differences in CF half-lives may also be related to different caffeine clearances and hence to hormonal differences.

All things considered coffee consumption can be monitored by measuring caffeine and its primary metabolites from fingerprint sweat. It was shown that it is possible to reproducibly quantify caffeine in fingerprints. Moreover, the simple and easy-to-use procedure may even allow to differentiate between slow and fast metabolizers and furthermore, a statement about individual metabolic activity in hepatocytes can be made. This of course can lead to further applications to screen other metabolites found in fingerprint sweat, blood or saliva. Significant and reproducible increases of caffeine in fingerprints were obtained for a cohort of five individuals in particular when comparing the caffeine levels before coffee intake with the levels 5 h thereafter.

5 Conclusion

Mass spectrometry based methods incorporating microfluidic systems for the quantitation of caffeine and its primary metabolites were developed and validated in the course of this master thesis. Various extracting methods and techniques were highlighted that have been developed for the detection and analysis of CF and its primary metabolites fingerprint, whole blood and saliva. The most suitable extraction agents have been determined by ultra- high-performance liquid chromatography with UV detection (UHPLC-UV). Diethyl ether and acetonitrile have shown potential as solvents for simultaneously extracting caffeine, theobromine and theophylline. Greater imbalances based on the volatility of diethyl ether led to the selection of acetonitrile as the final extracting solvent. In analytical chemistry there is a trend toward miniaturization to minimize on the one hand costs and on the other hand to simplify the system. It turned out that using the nanoChip-LC-MS method compared to UHPLC ensured a higher sensitivity and improved CVs. The separation of theophylline and paraxanthine were not separated and therefore, they were finally evaluated together. In fact it is actually possible to separate theophylline and paraxanthine using (U)HPLC [127; 91; 128]. However, the high polarity of these primary metabolites actually outbid the working limits of the nanoChip.

To summarize, an analytical method based on nanoChip-MS was developed that is suitable for the absolute quantitation of caffeine and its primary metabolites in fingerprint sweat, whole blood and for the relative quantitation of the same analytes in oral fluid from salivary glands. Overall, this proof-of-principle study of time course measurements of caffeine, theobromine and theophylline/paraxanthine showed that the biological response and metabolism can be reproducibly determined in a cohort of five individuals and that the method allows differentiating fast- from slow metabolizers. Especially, fingerprint sweat secretion emerged as an efficient method for quantifying the ingested drugs. The analysis of a person`s sweat is rapid, simple and non-invasive to determine the presence or absence and the concentration profiles of drugs and metabolites with reproducible biological variances according to FDA guidelines [130].

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Education

2012 –now **MSc "Biological Chemistry" with specialization in "Chemical Biology"**

University of Vienna

MSc project (Univ.-Prof. Mag. Dr. Gerner): Time-course measurements of caffeine and its primary metabolites extracted from fingertips after coffee intake

Modules: Biochemistry, Biophysical Chemistry, Bioinorganic Chemistry, Microbiology and Genetics, Immunology

2009-2012 **BSc "Bachelor of Nutritional Sciences"**

BSc project (Univ.-Prof Dr. Haber): The importance of proteins in performance sports – an overview concerning drug abuse in performance sports

School Adalbert Stiftergymnasium, Linz, 12.6.07, graduated with exceptional degree

Practical Experience

Aug 2013 – Feb 2014 **Analytical Laboratory - QuantaRed**

Research Assistant in the field of characterisation and interpretation of organic materials via FTIR and UV/Vis spectroscopy

Other Qualifications

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English (advanced level), French (basic)

Experienced use of Windows and MS Office

Hobbies: Sports, Music, Travelling