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A caffeinated blend of alternative sampling strategies - from CYP1A2 phenotyping to issues in microsampling

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Ghent, 2015,

The promoter,

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List of abbreviations

ADR	adverse drug reaction
ALAT	alanine aminotransferase
AMI	acute myocardial infarction
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
ASAT	aspartate aminotransferase
AUC	area under the curve
BEH	bridged ethylene hybrid
B/P	blood-to-plasma
CAD	collision-activated dissociation
C_b	total blood concentration
cDNA	complementary deoxyribonucleic acid
CDT	carbohydrate-deficient transferrin
CE	collision energy
CI	confidence interval
C_{max}	concentration found at T_{max} following intake of a given dose
CoA	coenzyme A
C_p	total plasma concentration
CV	coefficient of variation
CXP	collision cell exit potential
CYP	cytochrome P
DBS	dried blood spot
DMPD	dried matrix on paper discs
DMS	dried matrix spotting
DNA	deoxyribonucleic acid
DOB	delta-over-baseline
DP	declustering potential
DPS	dried plasma spot
EDDP	ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	extensive metabolizer
EMA	European Medicines Agency

EP	collision cell entrance potential
ESI	electrospray ionization
EtG	ethyl glucuronide
EtS	ethyl sulfate
FAEEs	fatty acid ethyl esters
FDA	Food and Drug Administration
FIA	flow injection analysis
f_u	unbound fraction in plasma
GC	gas chromatography
GC-MS	gas chromatography coupled to mass spectrometry
GC-MS/MS	gas chromatography coupled to tandem mass spectrometry
GGT	gamma- glutamyltransferase
GHB	γ-hydroxybutyric acid
Hb	hemoglobine
Hct	hematocrit
HPA	hypothalamus-pituitary-adrenal
HPLC	high performance liquid chromatography
HSS	high strength silica
IM	intermediate metabolizer
IP-10	interferon-γ inducible protein 10
IS	internal standard
IU	international units
IUGR	intrauterine growth restriction
LC	liquid chromatography
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LC-QTOF-MS	liquid chromatography coupled to quadrupole time-of-flight mass spectrometry
LLOQ	lower limit of quantification
LoA	limit of agreement
MCV	mean corpuscular erythrocyte volume
MeCN	acetonitrile
MeOH	methanol
MRM	multiple reaction monitoring

mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
NAT	N-acetyltransferase
PCDBS	pre-cut dried blood spots
PCR	polymerase chain reaction
PDBS	perforated dried blood spots
PEth	phosphatidylethanol
PK	pharmacokinetic
PM	poor metabolizer
PSA	prostate-specific antigen
QC	quality control
ρ	blood cell-to-unbound plasma concentration ratio
RBC	red blood cells
RE	relative error
RIA	radioimmunoassay
ROC	receiver operating characteristics
RSD	relative standard deviation
SD	standard deviation
sMRM	scheduled multiple reaction monitoring
SPE	solid phase extraction
TK	toxicokinetic
T_{max}	time when maximum concentration is reached following intake of a given dose
Tris	trishydroxymethylaminomethane
UDP	uridine diphosphate
UGT	UDP-glucuronosyltransferase
UPLC	ultraperformance liquid chromatography
UV	ultraviolet
VAMS	volumetric absorptive microsampling

General background, structure and objectives

Based on

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1. General background

Alternative sampling strategies can be looked at from different viewpoints. First, a classical sample (e.g. blood, plasma, serum or urine) can be sampled in an alternative way. One of the best known examples is dried blood spot (DBS) sampling, in which a drop of blood is deposited on a filter paper, typically following a finger or heel prick. Second, the sample itself can be considered “alternative”. Amongst these are hair and oral fluid, but also sweat, breath, nails and meconium. All these matrices have increasingly received interest in recent years. In this work, we focused on sampling and analysis of dried blood spots and hair.

1.1. Dried blood spots

Since Guthrie and Susi proposed to use newborn DBS to determine phenylketonuria [1], DBS have been extensively used in newborn screening programs. The availability of highly sensitive analytical techniques, mainly liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), resulted in an exponential increase in the interest in DBS for applications in other fields, including therapeutic drug monitoring [2,3], toxicology [4,5] and toxico- and pharmacokinetic studies in (pre-)clinical phases of drug development [6-8]. An interesting, recent application is the use of DBS sampling for phenotyping of drug metabolizing enzymes. The latter is one of the main topics of this thesis.

In general, DBS are prepared by applying a drop of capillary blood, obtained by a finger or heel prick, on a dedicated filter paper card. This can either be performed in a volumetric way, by spotting a defined volume of blood using a microcapillary, or non-volumetrically, by direct application of a drop of blood from the fingertip. Upon application to the filter paper, the resulting spots are dried to the air. This sampling technique offers several advantages over traditional blood sampling by venipuncture. DBS sampling by fingerprick is only minimally invasive and it provides a relatively easy and inexpensive way of taking a representative sample. Sample collection can be performed by an informed patient himself, eliminating the need for a trained phlebotomist and enabling sampling in a non-specialized environment (e.g. at home) [9]. Furthermore, as several compounds showed enhanced stability in DBS [10-13], samples can be transferred via regular mail and stored at room temperature in the laboratory. The reduced risk of infection, due the fact that pathogens are deactivated upon drying [14], further contributes to the convenient and cost-effective transport and storage. Since only small volumes of blood, typically between 10 and 80 µL, are collected, the DBS sampling technique is highly suitable for the collection of samples during preclinical and toxicokinetic studies involving animals. DBS sampling certainly confines to the principles of the 3R’s (replacement, reduction and refinement), as fewer animals are needed and sampling procedures are refined using DBS [15,16]. In addition, the advantage of small blood volumes, combined with the possibility to collect samples by a minimally invasive finger or heel prick, creates a high potential for the use of DBS in pediatric studies as well [17,18]. From an analytical point of view, compounds can often be extracted from DBS cards using straightforward procedures, making this technique particularly suitable for automated processing [19-21]. In a typical workflow for DBS analysis, a 3 to 6 mm diameter disc is punched from the DBS, followed by extraction of the disc with a mixture of water and organic solvent and injection of the (diluted) extract into the analytical system,

typically an LC-MS/MS configuration. Most often, isotopically labeled analogues are used as internal standards, which are either added to the extraction solvent, being the most used approach, or to the DBS punch [22], although other approaches have been described as well, e.g. spraying [23,24] or application to the DBS card prior to spotting [23,25,26].

Despite the many advantages, several issues still surround DBS sampling and analysis. As high quality data can only be derived from high quality samples, good sampling practices are of key importance, especially when non-experienced individuals are involved. Being a microsampling technique, in which small volumes of blood are collected and processed, analytical instruments involved in DBS analysis should provide sufficient sensitivity. Furthermore, when capillary DBS are analyzed, knowledge of the correlation between capillary and venous concentrations of the analyte of interest is needed [27,28]. Obviously, this is an aspect which needs to be evaluated on a case-by-case basis and may depend on the aim of the study (e.g. setting up a toxicokinetic profile vs. measurement of trough levels). In this light, thorough interpretation of DBS results often also requires comparison of DBS results with corresponding data in plasma or serum, as reference intervals or cut-off values are commonly based on the latter [29]. Apart from these issues, some DBS-specific analytical aspects should be addressed for every DBS method, especially when partial punches are analyzed. These parameters are the impact of the volume of blood spotted onto the filter paper, the punch localization and, undoubtedly the most discussed issue in DBS analysis, the influence of hematocrit, which is extensively addressed further in this work (Part B).

1.2. Hair

Hair analysis has become an established discipline in bioanalysis in the last decades with numerous published methods, mainly dealing with qualitative or quantitative determination of drugs of abuse. Collection and analysis of hair has been implemented in various fields, including forensic toxicology, investigation of drug-facilitated crimes, workplace drug testing, doping control in sports, detection of perinatal drug exposure and abstinence monitoring in driving license regranting programs or child custody cases [30,31].

The most important feature of hair analysis is probably the possibility to retrospectively assess drug use. Drugs that are incorporated into hair are no longer subject to biotransformation. As a consequence, many drugs proved to be stable in hair for prolonged periods of time, yielding a window of detection of several months or years, depending on hair length. Determination of drugs in specific hair segments even enables retrospective assessment of drug use that can be assigned to a defined time period in the past, taking into account an average hair growth rate of 1.06 cm/month [32]. Furthermore, being a non-invasive sampling technique, collection of hair samples can be performed by non-specialized personnel, although some expertise is required to avoid variation in the amount of hair left on the scalp [32]. Hair strands are typically collected from the posterior vertex region of the head, as hair growth rate shows less variability there [30,31]. Currently, there are no generic protocols for analysis of hair samples. Depending on the characteristics of the compound of interest, extraction can be performed using organic solvents or aqueous buffers. Alternatively, the hair matrix can be digested using NaOH, acid solutions

or proteolytic enzymes. As compounds are often present in hair in low concentrations, sensitive analytical techniques are required for hair analysis, such as gas chromatography-mass spectrometry (GC-MS) and LC-MS/MS [30].

Although hair analysis provides distinct benefits compared to traditional bioanalytical matrices, it also holds important limitations, especially concerning the interpretation of hair results. One of the main issues in hair analysis is the potential contribution of external contamination or passive drug exposure to measured hair concentrations. Therefore, hair samples are usually decontaminated prior to further processing, although a general consensus on standard wash procedures is currently lacking [33]. Several strategies have been proposed to distinguish active ingestion of a substance from external contamination, such as determining specific metabolites in hair and calculating metabolite/precursor concentration ratios, which can be compared with established cut-off values [34,35]. In addition, while analysis of hair segments has been widely applied to detect changes in drug consumption over time or to allocate drug intake to a certain period, interpretation of segmental hair analysis may be challenged due to external contamination [36]. On the other hand, analytes may also be removed from the hair as a result of damage caused by cosmetic hair treatment, such as bleaching or dyeing, leading to underestimation of hair concentrations [37]. Another factor impeding the interpretation of hair results is the fact that, for many compounds, hair concentrations do not correlate with plasma or serum levels and, consequently, with drug dose.

Research on hair analysis still is expanding and novel applications for hair testing are being actively explored. In this respect, there has been an increased attention for the determination of certain (bio)markers in hair in recent years, including markers of alcohol use, cortisol as a ‘stress’ biomarker and metabolic ratios for investigating drug metabolism. A brief discussion of the former two applications is provided below; the potential use of hair to investigate drug metabolism is extensively discussed in Part A of this thesis.

1.2.1. Markers of alcohol use in hair

Given the widespread use of alcohol and the impact excessive alcohol consumption may have on many aspects of health and society, there is a need for markers of alcohol intake, both from a clinical and forensic point of view. These markers should be able to differentiate alcohol abstinence, non-risk drinking and chronic excessive alcohol consumption. Several indirect markers are currently determined in blood in routine practice, such as carbohydrate-deficient transferrin (CDT), the liver enzymes gamma-glutamyltransferase (GGT), alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT), and mean corpuscular erythrocyte volume (MCV) [38]. However, these tests show overall only moderate specificity and sensitivity for detection of alcohol consumption. Direct products of alcohol metabolism, including ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidylethanol (PEth) and fatty acid ethyl esters (FAEEs), have been proposed as alternative markers, as they are expected to detect alcohol intake with better specificity. Furthermore, assessment of these direct markers in hair (excluding PEth, which has not been demonstrated in hair yet) provides a much wider time window for detection of alcohol

consumption as compared to blood analysis. The most studied direct alcohol markers in hair are EtG and FAEEs.

EtG is a hydrophilic, non-volatile phase II metabolite formed by the conjugation of ethanol with glucuronic acid, a reaction catalyzed by UDP-glucuronosyltransferase (UGT). EtG is a minor alcohol metabolite, since only 0.6 – 1.5 % of an ethanol dose is conjugated with glucuronic acid [38]. Consequently, EtG will be present in hair at low levels (pg/mg range) and, therefore, efficient sample preparation procedures combined with sensitive analytical techniques are required to allow proper quantification. To date, no standardized analytical protocols are available. A wide variety of washing procedures, extraction solvents and clean-up strategies has been applied in combination with GC-MS, GC-MS/MS or LC-MS/MS. In most cases, deuterated EtG (d_5-EtG) is added to the samples as internal standard. For a full overview of analytical methods for the determination of EtG in hair, we refer to Crunelle *et al*. [39].

FAEEs are hydrophobic metabolites formed in several tissues from free fatty acids, triglycerides, lipoproteins and phospholipids by esterification of free fatty acids or fatty acid acyl coenzyme A (CoA) with ethanol. These biotransformation reactions are mediated by FAEE synthase and acyl-CoA/ethanol O-acyl transferase. In hair analysis, four different FAEEs, ethyl myristate, palmitate, oleate and stearate are currently used as alcohol markers. For quantitative purposes, the sum of their concentrations is calculated. FAEEs are often extracted from hair using a mixture of dimethylsulfoxide and *n*-heptane followed by headspace solid phase microextraction and GC-MS. Deuterated ethyl esters are used as internal standards [40].

Although EtG levels measured in hair from social, non-risk drinkers and excessive alcohol consumers display a rather wide variability (giving rise to an overlap between EtG levels in distinct groups of alcohol consumers), a positive correlation with alcohol intake was seen in several studies [41-43]. Moreover, EtG in hair was found to be a better indicator of chronic and excessive alcohol consumption than the traditional markers. ROC (receiver operating characteristics) analysis of EtG in hair and traditional biomarkers (CDT, GGT, ASAT and ALAT) in serum showed a significantly better diagnostic performance of hair EtG in detecting at-risk and heavy alcohol consumption [44]. A correlation between cumulative FAEE concentrations in hair and amount (volume) of alcohol consumed has also been demonstrated [45]. However, data in favor of their use as long-term alcohol markers and agreement with other markers are somewhat less convincing than arguments supporting EtG [46,47]. Therefore, the latter is the preferred marker for assessment of abstinence from alcohol. The Society of Hair Testing proposes to use cut-off values for both EtG and FAEE to discriminate abstinence, repeated alcohol intake and chronic excessive alcohol consumption [48]. An overview of the threshold values is given in Table 1. Although these limits are routinely applied for the interpretation of analytical results, they are still subject to evaluation and discussion and may possibly need to be adjusted in the future [49].

In some cases, the interpretation of data concerning alcohol markers in hair may not be straightforward. In addition to the variability caused by different analytical procedures, their incorporation and stability in hair is affected by several factors. EtG and FAEE are both sensitive to cosmetic treatment of hair. Bleaching, perming and coloring can result in loss of EtG from hair, causing false negative results [50,51]. On the other hand, a false positive EtG hair result was described after using an EtG-containing hair care

product [52], while ethanol-containing hair sprays and lotions can result in elevated FAEE concentrations [53]. Hair pigmentation (i.e. melanin content) has no apparent effect on incorporation of EtG and FAEEs levels in hair [42,46]. Furthermore, ethanol metabolism and incorporation of metabolites in hair is influenced by age and gender. Also certain pathophysiological conditions, such as Gilbert's syndrome, may affect alcohol metabolism and may need to be taken into account. Therefore, the combined use of hair EtG and FAEEs can support data interpretation [50]. In addition, to obtain a comprehensive image of alcohol consumption, hair results are preferably compared with data for traditional alcohol markers in other matrices.

Table 1. Cut-off values for ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs) in scalp hair to strongly suggest repeated alcohol consumption (or assess abstinence from alcohol) and chronic excessive alcohol consumption (> 60 g ethanol/day), as proposed by the Society of Hair Testing [48].

	Repeated alcohol consumption		**Chronic excessive alcohol consumption**	
	Proximal hair segment	**Cut-off**	**Proximal hair segment**	**Cut-off**
EtG	0-3 cm, 0-6 cm	7 pg/mg	0-3 cm, 0-6 cm	30 pg/mg
FAEEs(*)	0-3 cm 0-6 cm	0.2 ng/mg 0.4 ng/mg	0-3 cm 0-6 cm	0.5 ng/mg 1.0 ng/mg

(*) sum of the concentrations of ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate

1.2.2. Cortisol in hair as a biomarker of hypothalamic-pituitary-adrenocortical activity

Cortisol is a glucocorticoid hormone secreted by the adrenal cortex when the hypothalamus-pituitary-adrenal (HPA) axis is stimulated as a reaction to a variety of stressors. Cortisol plays a crucial role in stress reactions and dysregulation of the HPA axis has been associated with numerous stress-related disorders [54]. For many years, cortisol has been determined in traditional matrices, such as blood, saliva and urine. These measurements reflect acute (blood, oral fluid) or short-term (urine) circulating cortisol concentrations. However, acute cortisol levels are subject to considerable intra- and interindividual variations, due to the circadian rhythm and pulsatile secretion of cortisol, which is influenced by a wide variety of factors (e.g. nervousness, nicotine or alcohol consumption, physical exercise, oral contraceptives) as well [54,55]. Consequently, obtaining reliable information on long-term exposure to cortisol would require analysis of multiple samples collected at different time points. A non-invasive, less labor-intensive alternative is offered by methods for the determination of cortisol concentrations in hair. These provide the possibility to retrospectively assess a cortisol status, thereby serving as a measure for integrated, long-term cortisol secretion and baseline activity of the HPA axis.

Different analytical procedures have been used to determine cortisol in hair. Given the hydrophobic nature of cortisol, methanol is often used as extraction solvent. After incubation, the resulting extract is evaporated to dryness and reconstituted in an appropriate solvent. Both immunoassays (ELISA, RIA) and LC-MS/MS methods have been used for quantitation of cortisol [56]. Results obtained by commercially available immunoassays showed overall considerable interassay variability, hampering

the comparability of cortisol levels found in different studies [57]. Until now, no reference ranges for normal cortisol levels in hair have been described.

In the last decade, the number of published studies on hair cortisol has increased rapidly and much effort has been done to investigate the relationship between cortisol concentrations in hair and specific stress-related disorders, as well as other clinical conditions. The most pronounced role of hair cortisol is its application as a biomarker of chronic stress in various settings. Elevated concentrations of cortisol in hair have been measured in patients with severe chronic pain compared to healthy controls [58]. Also long-term unemployed individuals displayed increased hair cortisol levels compared to employed controls [59]. Another study found higher cortisol concentrations in hair of endurance athletes than in controls, suggesting an effect of physical stress on hair cortisol [60]. In a cohort of healthy students, elevated cortisol levels were measured in hair samples of subjects exposed to serious life events (e.g. death of a close relative, serious illness, divorce) compared to participants without such an event [61]. While increased hair concentrations of cortisol are associated with various stressful conditions, as shown in these selected studies, the relationship between hair cortisol and perceived stress or subjective stress-related measures, often assessed by questionnaires, seems less clear, as several studies showed contradicting results [54].

Research has also been conducted on hair cortisol concentrations in the area of psychological diseases. Elevated cortisol levels were found in hair of patients with major depression compared with healthy controls [62]. However, another study found no similar correlation [63]. Also patients with posttraumatic stress disorder showed higher hair cortisol concentrations [64]. On the other hand, lower cortisol concentrations were measured in hair of patients suffering from generalized anxiety disorder than in healthy individuals [65]. A potentially promising clinical application of cortisol in hair is its use as a biomarker to predict the risk of acute myocardial infraction (AMI). In a study with fifty-six patients with AMI and fifty-six controls, hair cortisol proved to be a better predictor for AMI than established risk factors, such age, body mass index, hypertension, blood lipids or diabetes mellitus [66].

Several confounding factors may affect hair cortisol concentrations. A first concern is the stability of cortisol in hair and the potential effect of frequent washing or shampooing. Contradicting data concerning a wash-out effect, resulting in reduced cortisol concentrations in distal segments, have been published. Furthermore, cosmetic hair treatments may also lead to a loss of cortisol from the hair, although results from different studies are inconsistent. These factors, together with the potential influence of age and gender, need to be considered when interpreting hair cortisol results [54,55]. Another important issue is the incorporation of cortisol in hair from other sources than blood capillaries. Ito *et al*. demonstrated that hair follicles display a functional equivalent of the HPA axis that may release cortisol and contribute to the incorporation in hair [67]. A recent study determined cortisol levels in human sweat at concentrations comparable with saliva, and, therefore, the authors suggested that hair cortisol may be increased following intensive exercise [68]. Measurement of cortisol in hair offers an intriguing tool to assess long-term cortisol exposure and hair cortisol levels have been associated with various health-related conditions. However, some studies showed contradictory results and the influence of several confounding factors on hair cortisol concentrations needs further clarification.

2. Structure and Objectives

Alternative sampling strategies, in particular sampling and analysis of DBS and hair, are the common thread throughout this work. The objective of this thesis is twofold; on the one hand we aim at evaluating the utility of DBS and hair as alternative matrices for *in vivo* phenotyping of the cytochrome P (CYP) 450 enzyme CYP1A2 (**Part A**), on the other hand we address a critical issue in the field of dried blood microsampling, being the effect of hematocrit (Hct) (**Part B**). In both parts, caffeine and its major metabolite paraxanthine are used as model compounds.

In vivo phenotyping of a drug metabolizing enzyme is commonly performed by administration of a selective enzyme substrate, followed by the determination of a specific phenotyping metric in traditional matrices (plasma, serum or urine). In **Part A**, caffeine is applied as probe drug to assess the phenotype of CYP1A2, an enzyme involved in the metabolism of several clinically used drugs. As the metabolic pathway resulting in the formation of paraxanthine from caffeine is uniquely catalyzed by CYP1A2, the paraxanthine:caffeine molar concentration ratio determined in plasma at a defined time point following intake of a caffeine test dose is generally accepted as a valid measure of CYP1A2 activity. The first chapter of Part A, **Chapter A.1.**, provides a comprehensive overview of alternative sampling strategies applied for *in vivo* CYP450 phenotyping. In addition to DBS and hair-based procedures, the role of oral fluid, exhaled breath and sweat in this context is discussed. The following chapters describe the development and evaluation of alternative CYP1A2 phenotyping procedures. **Chapter A.2.** presents the development and validation of LC-MS/MS methods for the determination of caffeine and paraxanthine in 3-mm DBS punches and small volumes (50 µL) of whole blood and plasma. Special attention is paid to the influence of Hct on several indices of DBS method performance, such as accuracy, precision, recovery and matrix effects. The developed methods were applied in a small-scale phenotyping study in healthy volunteers. The results of this study served as a basis to set up a large comparative phenotyping study, described in **Chapter A.3.** In this study, a 150-mg caffeine test dose was administered to 73 healthy volunteers. Six hours post-administration, capillary DBS were obtained by direct application of a drop of blood onto a filter paper card. Along with these samples, venous whole blood was collected for the preparation of venous DBS and plasma. Comparison of caffeine and paraxanthine concentrations and resulting paraxanthine:caffeine ratios, i.e. the actual CYP1A2 phenotyping indices, in the various matrices allows to thoroughly investigate the usefulness of DBS as an alternative matrix for CYP1A2 phenotyping. Furthermore, as partial punches of non-volumetrically applied DBS are analyzed, the impact of specific parameters, being Hct, volume spotted and punch location, is assessed. A DBS-based CYP1A2 phenotyping procedure may provide a minimally invasive, convenient and patient-friendly alternative for the classical plasma-based approach that requires venipuncture. The development and validation of an LC-MS/MS method for the determination of caffeine and paraxanthine in hair is presented in **Chapter A.4.** All steps of the hair extraction protocol, involving enzymatic digestion of the hair matrix, and the subsequent clean-up step using solid phase extraction (SPE) were optimized. As external contamination and the influence of wash steps are important issues in hair analysis, the effect of the applied hair decontamination procedure is evaluated as well. The resulting method, being the first for the determination of both caffeine and paraxanthine in hair, allowed

to evaluate the potential of hair analysis for CYP1A2 phenotyping. Given the widespread consumption of caffeinated beverages and food products, a CYP1A2 phenotyping method involving collection and analysis of hair samples would eliminate the need to administer a caffeine test dose. Furthermore, a suchlike procedure may enable to assess a phenotype when conducting a standard phenotyping protocol is impossible (e.g. postmortem). Therefore, caffeine and paraxanthine concentrations were measured in 3-cm proximal hair segments of 60 healthy volunteers and the resulting paraxanthine:caffeine ratios were correlated with reference CYP1A2 phenotyping metrics determined in plasma. The influence of several factors that may have an effect on CYP1A2 activity, such as oral contraceptives and smoking, or on the incorporation of caffeine and paraxanthine in hair, such as hair color and cosmetic treatment of hair, is evaluated as well. The results of this study are described in **Chapter A.5.** As this is the first study to compare metabolic ratios in hair and plasma, it may provide useful information concerning the potential of hair analysis for phenotyping purposes.

Experiments described in Chapters A.2. and A.3. revealed that caffeine and paraxanthine concentrations in fixed-size DBS punches are affected by a Hct-dependent bias. Therefore, **in Part B**, these compounds were selected as model compounds to further explore the Hct effect. First, the nature of this effect, which is generally considered as the most important factor hampering a more widespread use of DBS as an alternative for venous blood, is outlined in **Chapter B.1.**, along with a detailed overview of currently available strategies to cope with the Hct problem in quantitative DBS analysis. A new approach to correct for the Hct-induced bias of caffeine, using an algorithm based on potassium concentrations in DBS, is presented in **Chapter B.2.** To construct this algorithm, caffeine concentrations were determined in DBS and whole blood samples from 100 healthy volunteers and hospital patients with a wide Hct range. The usefulness of this approach was further examined by applying the same algorithm to paraxanthine, determined in 103 samples. In the last chapter of part B, **Chapter B.3.**, the potential of a new sampling technique, volumetric absorptive microsampling (VAMS), to overcome the Hct bias is evaluated. This technique allows to collect a fixed volume of blood by dipping an absorbent polymeric tip, attached to a plastic handle, into a pool or drop of blood. The obtained blood microsample is subsequently dried. Using a validated LC-MS/MS method, caffeine and paraxanthine concentrations were determined in over 80 VAMS samples originating from hospital patients with varying Hct values. The results, and those of DBS samples, are compared with corresponding whole blood concentrations. This set-up, being the first in-human VAMS study involving incurred samples, allows an in-depth evaluation of this new sampling technique.

Finally, a general conclusion of this work, together with future perspectives, is given.

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Part A

Alternative sampling strategies for CYP1A2 phenotyping

Chapter A.1.

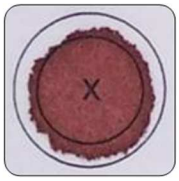
Alternative sampling strategies for CYP450 phenotyping

Based on

De Kesel PM, Lambert WE, Stove CP. Alternative sampling strategies for cytochrome P450 phenotyping. In revision (Clin Pharmacokinet).

Abstract

Interindividual variability in the expression and function of drug metabolizing cytochrome P (CYP) 450 enzymes, determined by a combination of genetic, non-genetic and environmental parameters, is a major source of variable drug response. Phenotyping by administration of a selective enzyme substrate, followed by the determination of a specific phenotyping metric, is an appropriate approach to assess the *in vivo* activity of CYP450 enzymes, as it takes into account all influencing factors. A phenotyping protocol should be as simple and convenient as possible. Typically, phenotyping metrics are determined in traditional matrices, such as blood, plasma or urine. Several sampling strategies have been proposed as an alternative for these traditional sampling techniques. In this chapter, we provide a comprehensive overview of available methods using dried blood spots, hair, oral fluid, exhaled breath and sweat for *in vivo* CYP450 phenotyping. We discuss the relation between phenotyping metrics measured in these samples and those in conventional matrices, along with the advantages and limitations of the alternative sampling techniques. Reliable phenotyping procedures for several clinically relevant CYP450 enzymes, including CYP1A2, CYP2C19 and CYP2D6, are currently available for oral fluid, breath or dried blood spots, while additional studies are needed for other CYP450 isoforms, such as CYP3A4. The role of hair analysis for this purpose remains to be established. Being non- or minimally invasive, these sampling strategies provide convenient and patient-friendly alternatives for classical phenotyping procedures, which may contribute to the implementation of CYP450 phenotyping in clinical practice.



dried blood spots

hair

oral fluid

exhaled breath

A.1.1. Introduction

Enzymatic biotransformation is one of the main routes of elimination of drugs and other xenobiotics from the human body. These enzymatic processes can be divided into two phases: compounds are modified by oxidation, reduction or hydrolysis in phase I, while conjugation reactions in phase II typically increase the hydrophilic character of substrates by adding endogenous molecules, such as glucuronic acid or gluthathion. Phase I metabolism, being the rate-limiting step of enzymatic biotransformation, is mainly catalyzed by cytochrome P (CYP) 450 enzymes [1]. CYP450 is a superfamily of heme-containing enzymes located primarily in the endoplasmatic reticulum of liver cells and enterocytes in the epithelium of the small intestine. On the basis of increasing amino acid similarity, this group of enzymes is divided into families and subfamilies, denoted by numbers and letters, respectively. Individual enzymes within a subfamily are given an additional number. While 57 functional human *CYP450* genes have been identified, only a limited number of the resulting proteins plays a role in the metabolism of drugs. The latter enzymes mainly belong to the CYP1, CYP2 and CYP3 families [2].

Many CYP450 enzymes display a considerable interindividual variability in their expression and function, which is determined by a combination of genetic, epigenetic and non-genetic host factors (e.g. sex, age, pathophysiological conditions) as well as environmental influences (e.g. diet, tobacco smoke, drug intake) [3]. Consequently, each individual has its own CYP450 metabolic profile. As it is estimated that the major CYP450 enzymes are involved in 70-80 % of phase I reactions metabolizing clinically used drugs [4], variability in enzyme activity may result in variability in bioavailable drug levels and, consequently, variable response to drug therapy. When the parent drug is pharmacologically active, impaired enzyme function may increase the risk of developing adverse drug reactions (ADRs). For example, Phillips *et al.* found that 59 % of drugs cited in ADR studies are, at least partially, metabolized by enzymes with reduced function [5]. Likewise, when metabolization results in inactivation of a parent drug, higher than normal enzyme activity may cause standard drug therapy to fail. In these cases, increasing the drug dose is needed to obtain the same level of efficacy [3,6].

Therefore, estimating the metabolic status of an individual for a given pathway can be a useful tool to explain unexpected drug plasma concentrations, identify causes of ADRs, guide therapy or even predict the appropriate drug dose prior to the start of a therapy. Some CYP450 enzymes, in particular CYP2D6, CYP2C19 and CYP2C9, are encoded by highly polymorphic genes and distinct subpopulations can be identified based on the genetic signature [4,7,8]. For many drugs metabolized by these enzymes, clinical outcome could be correlated with a certain genotype and genotyping a patient has proven to be a reliable strategy to assess the metabolic phenotype [3,6,8]. This approach holds the advantages that is has to be performed only once for each individual and that intake of test drugs is not required. Also for other important CYP450 enzymes, such as CYP1A2 and CYP3A4, a wide interindividual variability in enzyme activity has been described. Although multiple polymorphisms of the respective genes have been identified, variant alleles giving rise to altered enzyme function are very rare and genetic factors alone are unable to fully explain the variation in enzyme function [2,9]. These enzymes are highly influenced by environmental factors, such as inhibitor or inducer drugs, diet components and tobacco smoke [3]. Therefore, phenotyping by administration of a selective substrate is the preferred approach to assess

the *in vivo* activity of these enzymes, as it takes into account genetic, non-genetic and environmental influences. In addition, even for those enzymes whose function is mainly genetically controlled, individuals that were genotyped as extensive metabolizers may display a (transient) poor metabolizer phenotype, due to intake of enzyme-inhibiting drugs or certain comorbidities influencing enzyme activity, such as HIV, liver disease and cancer. This phenomenon, called phenoconversion, has been observed mainly for CYP2D6 and CYP2C19 [10]. Also here, phenotyping can be applied in conjunction with genotyping to obtain an integrated image of enzyme function.

In general, phenotyping for a drug metabolizing enzyme is performed by the administration of a selective substrate of the enzyme, followed by the determination of a specific phenotyping metric. The latter may comprise various pharmacokinetic parameters, such as the systemic clearance of the probe drug, single point concentrations or metabolite/parent drug concentration ratios (or parent drug/metabolite ratios), typically obtained from plasma or urine samples. Multiple probe drugs and phenotyping protocols have been described for different CYP450 enzymes, amongst which several have been validated to provide a reliable estimate of the actual enzyme activity. We refer to the work of Streetman *et al.* [11] and Fuhr *et al.* [12] for an overview of available phenotyping procedures. Fuhr *et al.* also discussed the requirements for appropriate phenotyping procedures. Apart from the use of selective substrates and metrics and the need of probe drugs with an acceptable safety profile, reliable analytics and low intraindividual variability of the metric, the authors stated that phenotyping procedures ideally should be simple and convenient. The use of alternative samples strategies instead of traditional blood/plasma or urine sampling may fulfill this requirement. The major drawback of venous blood sampling in the context of phenotyping is probably the inherent invasiveness, which may specifically pose a problem when repeated sampling is needed to construct a full concentration-time profile. The latter is, for example, required in drug-drug interaction studies as this approach takes into account the influence of various factors on the kinetics of the probe drug, including processes other than enzyme activity. In addition, venipuncture requires specialized staff. Concerning urine sampling, the susceptibility to non-specific noise caused by urinary flow or pH is a matter of concern. For example, urinary metabolic ratios of the probe drugs caffeine and dextromethorphan for phenotyping of CYP1A2 and CYP2D6, respectively, showed high intraindividual variability caused by these variables [13,14]. Furthermore, phenotyping protocols often require that urine is collected over a period of several hours. Apart from being inconvenient for the patient, this impedes efficient transport of samples.

This chapter provides an overview of alternative sampling strategies for *in vivo* CYP450 phenotyping. More specifically, the use of dried blood spots (DBS), hair, oral fluid, exhaled breath and sweat for this purpose will be discussed. Special emphasis was placed on how phenotyping metrics measured in these alternative samples relate to those in conventional matrices, as we believe that this is a key point to evaluate the validity and usefulness of alternative sampling strategies for CYP450 phenotyping. A discussion on the actual therapeutic usefulness of phenotyping for individual enzymes was out of the scope of this chapter. A structured literature search was performed using PubMed (U.S. National Library of Medicine) and ISI Web of Science™ databases. Keywords were cytochrome P (CYP) 450, all major CYP450 isoforms, phenotyping, enzyme activity and the above mentioned matrices. Only articles in English were selected and references of relevant articles were screened. Although similar sampling

procedures as discussed in this chapter can also be used for phenotyping other drug metabolizing enzymes, such as conjugative phase II enzymes, these methods are not covered here.

A.1.2. Dried blood spots

As mentioned in the General Background section, owing to several distinct advantages over traditional samples, DBS have been applied in various fields, such as newborn screening, therapeutic drug monitoring, toxicology and pharmacokinetic studies in drug development. In the last few years, several groups evaluated the utility of DBS for CYP450 phenotyping. By validating five individual LC-MS/MS methods for the determination of caffeine, flurbiprofen, midazolam, omeprazole and rosiglitazone, being substrates of CYP1A2, CYP2C9, CYP3A4, CYP2C19 and CYP2C8, respectively, in 3 mm DBS punches, Lad *et al.* illustrated the idea of using DBS sampling for this purpose. However, specific metabolites of these probe drugs, enabling assessment of metabolic ratios, were not included and the developed methods were not applied in a phenotyping study [15].

Since then, DBS-based phenotyping of single CYP450 isoforms has been investigated in several studies, summarized in Table A.1.1. In a CYP3A4 phenotyping study, de Boer *et al.* determined midazolam concentrations in plasma, whole blood and volumetrically applied venous and capillary DBS of 12 healthy volunteers at different time points following administration of a single 7.5 mg midazolam test dose. This approach allowed to construct pharmacokinetic (PK) profiles for the different matrices, from which area under the curve (AUC) values were derived as CYP3A4 phenotyping metric [16]. Interestingly, while mean midazolam AUC values for venous and capillary DBS punches were comparable, mean AUC obtained from whole blood analysis was considerably lower. Although, based on high regression coefficients for venous DBS, whole blood and plasma, the authors concluded that the results for the evaluated sampling techniques were strongly correlated, we believe that this 'DBS-effect' needs further examination before accepting the presented approach for CYP3A4 phenotyping. Furthermore, strongly deviating results for capillary DBS in some cases negatively impacted the correlation with the other matrices.

More clear findings in favor of DBS sampling were described for other CYP450 enzymes. Déglon *et al.* developed an automated system for on-line desorption of DBS combined with LC-MS/MS analysis. This platform was used for the determination of the CYP2C9 probe substrate flurbiprofen and its metabolite 4-hydroxyflurbiprofen in whole, volumetrically applied 5 µL DBS [17]. The results of a PK study, involving administration of 50 mg flurbiprofen to 10 healthy volunteers, were described in a separate report by Daali *et al.* [18]. Concentration ratios of 4-hydroxyflurbiprofen to flurbiprofen, being measures of CYP2C9 activity, in capillary DBS collected at multiple time points post-administration were in good agreement with corresponding ratios in plasma and urine and with flurbiprofen clearances, as evidenced by Spearman rank correlation coefficients. In addition, it was demonstrated that metabolic ratios in DBS and plasma changed in a similar way following CYP2C9 induction and inhibition. Murphy *et al.* measured cotinine and *trans* 3'-OH-cotinine levels in plasma and non-volumetrically applied capillary DBS of smokers and non-smokers exposed to second hand smoke. As CYP2A6 mediates cotinine conversion

to *trans* 3'-OH-cotinine, the resulting metabolite/precursor ratio is often used as CYP2A6 phenotyping metric. Ratios in DBS punches and corresponding plasma samples of 81 subjects were highly correlated, with a Spearman rank correlation coefficient of 0.94 ($p < 0.001$) [19]. As described in Chapters A.2. and A.3., we investigated whether DBS were suited for CYP1A2 phenotyping using the established probe drug caffeine and its major metabolite paraxanthine [20]. Six hours after the intake of a 150 mg oral caffeine dose, paraxanthine/caffeine ratios were determined in plasma, whole blood and 3 mm punches of venous and non-volumetrically applied capillary DBS of 73 healthy volunteers. While capillary DBS concentrations were significantly lower than venous whole blood and plasma concentrations and DBS concentrations were influenced by hematocrit and blood volume spotted, both compounds were similarly affected. As a consequence, Bland-Altman and Passing-Bablok plots showed that paraxanthine/caffeine ratios, i.e. the CYP1A2 phenotyping metric, were highly comparable in all matrices [21].

In addition to these single enzyme phenotyping studies, DBS have also been used to measure the activity of multiple CYP450 enzymes simultaneously by the administration of a cocktail of probe drugs. Donzelli *et al.* applied a new phenotyping cocktail, called the Basel cocktail, consisting of low doses of losartan (12.5 mg, for CYP2C9), omeprazole (10 mg, for CYP2C19), midazolam (2 mg, for CYP3A4), caffeine (100 mg, for CYP1A2), metoprolol (12.5 mg, for CYP2D6) and efavirenz (50 mg, for CYP2B6). This cocktail was administered to 16 healthy volunteers followed by non-volumetric collection of capillary DBS at different time points along with saliva samples and whole blood for plasma preparation. As a result of the low drug doses and the moderate sensitivity of the applied LC-MS/MS method, only the pairs caffeine - paraxanthine and omeprazole - 5-OH-omeprazole could be quantified in 3 mm DBS punches. As a measure of CYP1A2 activity, paraxanthine/caffeine ratios at 8 h in DBS correlated with corresponding AUC ratios in plasma. Similar findings were obtained for omeprazole/5-OH-omeprazole ratios at 2 h in DBS, being the CYP2C19 phenotyping metric [22]. Bosilkovska *et al.* conducted a phenotyping study in which another combination of probe drugs, the Geneva cocktail, containing bupropion (25 mg, for CYP2B6), flurbiprofen (25 mg, for CYP2C9), omeprazole (5 mg, for CYP2C19), dextromethorphan (10 mg, for CYP2D6) and midazolam (1 mg, for CYP3A4), was administered to 10 healthy volunteers together with a cup of coffee (100 mg caffeine, for CYP1A2) or coke (25 mg caffeine). Also here, capillary and venous blood were collected at several time points after intake of the drug cocktail. DBS were prepared by spotting 10 µL of capillary blood on DBS cards and whole spots were analyzed. In contrast to Donzelli *et al.*, the higher volume, combined with the use of more sensitive equipment, allowed quantification of all probe drugs and corresponding metabolites in DBS. Single point metabolite/parent drug ratios at 2 h for CYP1A2 and CYP3A4 and at 3 h for CYP2B6, CYP2C9 and CYP2D6, and the $AUC_{2,3,6}$ metabolite/parent drug ratio for CYP2C19 showed acceptable Spearman rank correlation coefficients with AUC_{last} ratios in plasma, both when the cocktail was administered alone or in combination with CYP450 inhibitor and inducer drugs [23,24].

For almost all CYP450 enzymes evaluated in the cited studies, a good agreement between phenotyping metrics measured in DBS and conventional, mostly plasma-based, metrics was found. Therefore, DBS sampling seems to represent a minimally-invasive and patient-friendly alternative for traditional venous blood sampling to obtain high-quality phenotyping data. Given the ease of the sampling technique and

the convenient transport, DBS are highly suitable to be implemented in large-scale phenotyping studies or even in home-sampling scenarios. To further accept DBS-based CYP450 phenotyping, other CYP450 isoforms should be investigated and the results of the above-mentioned studies need to be confirmed in other, sufficiently large study populations. Future studies may also consider to evaluate the impact of known issues, mainly hematocrit and blood volume, on DBS-based phenotyping metrics.

A.1.3. Hair

Apart from several well-established applications of hair analysis, mentioned in the General Background section, such as determination of drugs of abuse or alcohol biomarkers in forensic toxicology, workplace drug testing, doping control in sports and abstinence monitoring in driving license regranting programs or child custody cases, hair has also been proposed as an alternative matrix for the assessment of drug metabolism [25,26]. In 1996, Mizuno *et al.* determined caffeine in hair strands of 6 healthy individuals and 6 patients with liver cirrhosis. Significantly higher caffeine concentrations were found in hair samples of liver cirrhosis patients. In a separate group of volunteers, the authors showed that elimination of a defined caffeine dose, based on saliva concentrations, was slower in patients with liver disease compared to healthy individuals. This led the authors to suggest that caffeine concentrations in hair could be used as an indicator of liver metabolic capacity. It should be noted, however, that no data were available on caffeine intake of the individuals whose hair samples were analyzed and no metabolites of caffeine were measured in hair to make a more conclusive statement on metabolic capacity [27]. To date, 2 studies evaluated the usefulness of hair analysis for the assessment of CYP450-mediated metabolism by correlating metabolic ratios in hair with enzyme genotypes or reference phenotyping metrics (Table A.1.1). Thieme *et al.* analyzed hair samples of 23 children for which illegal, prolonged administration of the antidepressant amitriptyline was suspected. The *N*-demethylation of amitriptyline to nortriptyline is mediated by CYP2C19. While nortriptyline/amitriptyline ratios did not change significantly along individual hairs, large interindividual variations in these ratios were observed. The authors found a clear correlation between nortriptyline/amitriptyline ratios in hair and the number of functional alleles of CYP2C19. In particular, CYP2C19 poor metabolizers, carrying 2 dysfunctional alleles, had significantly lower hair ratios compared to intermediate or extensive metabolizers. Apart from CYP2C19, also CYP2D6 is involved in amitriptyline and nortriptyline metabolism by mediating the hydroxylation of both compounds. Despite wide interindividual variability, no correlation between hydroxylation of amitriptyline and nortriptyline and CYP2D6 polymorphisms was found. This was probably due to the fact that only intermediate and extensive, and no poor or ultrarapid CYP2D6 metabolizers were present in the study population [28]. As described in Chapter A.4. and A.5., we evaluated the potential of hair analysis for CYP1A2 phenotyping. Paraxanthine/caffeine concentration ratios were determined in proximal 3-cm hair segments of 60 healthy volunteers. Hair ratios of all subjects were compared with a reference CYP1A2 phenotyping metric, being paraxanthine/caffeine ratios in plasma 6 hours after intake of a caffeine test dose (150 mg). While ratios in both matrices showed a similar range and a statistically significant correlation, large differences between hair and plasma ratios were seen in individual cases. These deviations could not be attributed to factors

potentially affecting the incorporation or retention of small molecules in hair, such as hair pigmentation and cosmetic hair coloring, or affecting CYP1A2 activity, such as oral contraceptives, smoking, gender, age and caffeine consumption. On the basis of these results, we concluded that hair analysis is not (yet) suitable for CYP1A2 phenotyping, as interpreting paraxanthine/caffeine hair ratios on an individual basis was difficult [29].

Hair analysis has also been used as a tool to retrospectively document changes in CYP450-mediated metabolism over time by determining metabolic ratios in consecutive hair segments. In particular, this approach has been applied in several studies to detect pregnancy-induced altered metabolism. Klein *et al.* collected hair samples of 28 smoking women during the postpartum period and divided the obtained samples into segments corresponding to the trimesters of pregnancy. All volunteers reported a consistent smoking behavior throughout pregnancy and concentrations of nicotine and its major metabolite cotinine were determined in all segments. Nicotine/cotinine ratios in the third trimester were significantly decreased compared to those in the first trimester. According to the authors, these findings illustrated that nicotine metabolism to cotinine, mainly catalyzed by CYP2A6, increases as pregnancy advances [30], which was in agreement with previous findings [31]. In a more recent study by the same group, similar results for nicotine/cotinine ratios in hair of 74 smoking women were obtained [32]. O'Brien *et al.* applied an analogous approach to examine changes in antidepressant metabolism during pregnancy. Citalopram/norcitalopram ratios in hair segments corresponding to the first and third trimester of pregnancy were significantly lower than ratios in the postpartum period [33]. Although only 4 women were included, these findings could confirm the elevated metabolism of citalopram [34] and increased CYP2D6 activity during pregnancy [35-37]. Finally, Himes *et al.* analyzed hair samples from 29 opioid-dependent pregnant women enrolled in methadone-assisted therapy. Methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), the major metabolite of methadone, were measured in proximal 3-cm hair segments collected throughout pregnancy. In addition, cumulative methadone doses taken during the time interval corresponding to the analyzed hair segments were calculated. A positive linear trend between this cumulative dose and EDDP/methadone ratios in hair was observed [38]. This could illustrate auto-induction of methadone metabolism, mainly mediated by CYP3A4 [39], or, again, elevated CYP450 activity during pregnancy [35-37]. These studies suggest that applying segmental hair analysis to retrospectively assess drug metabolism may hold promise as an alternative phenotyping approach. Here, hair analysis, due to its distinct characteristics, provides a tool to gain information on a subject's metabolic status that could not have been obtained by analyzing traditional matrices (blood or urine), given their much shorter time window of detection. On the other hand, although altered drug metabolism during pregnancy is a generally recognized concept, it should be noted that in none of the above mentioned studies the metabolic ratios measured in hair were compared with reference phenotyping indices determined in other matrices to support their validity in reflecting changed metabolism.

In general, in contrast to traditional phenotyping procedures, the probe drugs used to estimate enzyme function in the cited hair methods were not specifically administered for this purpose. To determine metabolic ratios in hair, these methods relied on intake or administration of therapeutic drugs or intake of enzyme substrates via cigarette smoke or nutrition, leading to variation in the duration of exposure

and the amount of ingested or inhaled compounds between individuals. Another factor that needs to be taken into account when metabolic ratios in hair are evaluated, is the possibility that metabolites and precursors may be incorporated into hair to a different extent. It is generally accepted that compounds are incorporated into hair from the bloodstream, sweat or sebum or through external contamination [40]. Several compound-dependent factors affect these processes, such as their physicochemical properties and affinity for binding to melanin in hair. In addition, analytical hair decontamination procedures or external influences, such as regular hair washing, influence of UV-light and cosmetic treatment of hair, may potentially cause differential loss of metabolites and precursors from the hair [25]. Therefore, as many variables may impact analyte concentrations in hair, the usefulness of hair metabolic ratios needs to be evaluated on a case by case basis and should be supported by controls in other matrices or data on enzyme genotypes. Futhermore, as hair results are likely to provide an average image of the enzyme phenotype, short-term changes in enzyme activity will not be detected by hair analysis, in contrast to DBS- or oral fluid-based phenotyping procedures.

A.1.4. Oral fluid

Collection of oral fluid as an alternative to plasma has been applied in diverse areas. For example, as concentrations in oral fluid reflect the free, non-protein bound, pharmacologically active fraction of a drug in plasma, oral fluid sampling has gained attention as a tool for therapeutic monitoring of selected drugs [41]. Currently, the best established applications of oral fluid are probably roadside and workplace drug testing [42,43]. Rapid, simple and non-invasive sample collection are important advantages of oral fluid sampling. As a consequence, samples may be collected by non-experienced individuals and repeated sampling can be done more easily. Compared to blood sampling, the risk of infection is lower. Oral fluid is usually obtained by drooling or spitting in a dedicated collection device or by absorbent swabs. To hasten sample collection and increase the sampled volume, oral fluid flow can be stimulated, e.g. by citric acid or chewing paraffin wax. However, stimulation may affect drug concentrations due to the dilution effect and an altered pH of the oral fluid. Other challenges associated with oral fluid analysis are adsorption of analytes to collection devices, interferences from food or drinks and contamination with orally ingested or inhaled drugs left in the mouth [44-46].

Oral fluid sampling has been evaluated in phenotyping studies involving several CYP450 enzymes (Table A.1.2.). The CYP1A2 substrate caffeine is by far the most investigated and applied probe drug in oral fluid-based phenotyping procedures. Starting from the 1980's, collection of oral fluid has been used for the measurement of caffeine clearance as an indicator of liver function, with several studies showing an excellent agreement between PK parameters derived from oral fluid and serum or plasma samples [47-53]. Fuhr *et al.* evaluated the usefulness of caffeine as CYP1A2 phenotyping probe drug by performing a meta-analysis of 4 studies in which caffeine and paraxanthine had been measured in plasma, urine and oral fluid samples of 78 subjects. It was concluded that caffeine clearance from oral fluid and 3-7 h post-dose paraxanthine/caffeine ratios in both oral fluid and plasma were highly correlated with systemic caffeine plasma clearance, providing a better estimate of CYP1A2 activity than

urinary ratios [54]. Furthermore, in a study involving 25 patients undergoing hepatectomy, significant correlations were found between *in vivo* parameters of CYP1A2 activity, being caffeine clearance and paraxanthine/caffeine ratios (3 and 6 h post-dose) in oral fluid or plasma, and *in vitro* parameters, being caffeine 3-demethylation and relative CYP1A2 content in liver microsomes [13]. Since then, the validity of oral fluid for CYP1A2 phenotyping was corroborated by several other studies [55-61] and, consequently, this approach has been widely applied to assess CYP1A2 activity in various settings [35,59,60,62-72].

As urinary dextromethorphan metabolic ratios for CYP2D6 phenotyping are affected by considerable intraindividual variability, Hou *et al.* evaluated the use of oral fluid for this purpose. Dextromethorphan/dextrorphan ratios in oral fluid samples of 61 healthy volunteers, measured 3 h following dextromethorphan administration, showed a significant correlation with 0-8 h urinary ratios and enabled to differentiate all CYP2D6 poor metabolizers from intermediate and extensive metabolizers identified by urinary metabolic ratios. The latter 2 groups, however, could not be separated on the basis of oral fluid results [73]. The applicability of this method and its potential to provide an alternative for urine-based CYP2D6 phenotyping was demonstrated in 100 anuric patients with renal failure [74]. However, in a study with repetitive dextromethorphan administration, Hu *et al.* observed poor repeatability of 3, 4, and 5 h oral fluid dextromethorphan/dextrorphan ratios. The authors proposed to use 6 h ratios instead, as these showed good repeatability and correlated significantly with urinary phenotyping metrics [75]. In addition, Lutz *et al.* were able to differentiate CYP2D6 poor metabolizers in a population of 170 volunteers using 2 h oral fluid dextromethorphan/dextrorphan ratios. Comparison with other phenotyping metrics or genotypic data was not included in this study [76].

While the latter procedures were based on the CYP2D6-mediated O-demethylation of dextromethorphan to dextrorphan, Kuo *et al.* proposed to use another metabolic route of dextromethorphan, N-demethylation to 3-methoxymorphinan, for CYP3A phenotyping. However, dextromethorphan/3-methoxymorphinan ratios in oral fluid of 3 volunteers showed a poor correlation with corresponding urinary ratios [77]. Based on good correlations between midazolam concentrations in plasma and oral fluid, determined in 8 subjects at multiple time points, Link *et al.* suggested that midazolam may provide a suitable probe drug for oral fluid-based CYP3A phenotyping. However, it should be noted that, due to the high plasma protein binding of midazolam, the common CYP3A phenotyping metrics AUC and clearance were considerably lower and higher, respectively, in saliva compared to plasma [78]. As mentioned in the section on DBS, Donzelli *et al.* tested a cocktail of probe drugs to simultaneously measure the activity of multiple CYP450 enzymes. Along with DBS, oral fluid samples were collected from 16 volunteers. Similar to DBS results, oral fluid concentrations of most probe drugs were below the lower limit of quantification (LLOQ). Paraxanthine/caffeine ratios at 8 h in oral fluid, being the CYP1A2 phenotyping metric, showed a significant correlation with corresponding AUC ratios in plasma. As a potential measure of CYP2C19 activity, omeprazole/5-OH-omeprazole ratios at 2 h in oral fluid correlated poorly with corresponding AUC ratios in plasma [22].

The role of nicotine metabolites and corresponding metabolic ratios in various matrices, including oral fluid, as biomarkers of exposure to tobacco smoke, smoking cessation or to guide treatment of nicotine

dependence has been thoroughly explored, as indicated in several studies and recent review papers [79-84]. Here, we will discuss studies that specifically evaluated the usefulness of nicotine metabolic ratios in oral fluid for CYP2A6 phenotyping. A first proof was provided by Dempsey *et al.*, who administered deuterium labeled nicotine and cotinine to 62 healthy volunteers. Resulting *trans* 3'-OH-cotinine/cotinine ratios in oral fluid 6 h post-intake were highly correlated with several indices of CYP2A6 activity, including 6 h ratios in plasma and oral clearance of both nicotine and cotinine [85]. CYP2A6 phenotyping based on *trans* 3'-OH-cotinine/cotinine ratios originating from nicotine in tobacco smoke was examined by St. Helen *et al.* Regression analysis revealed high correlations between ratios in oral fluid and both whole blood and plasma of smokers and nonsmokers exposed to tobacco smoke. Bland-Altman analysis confirmed the agreement between oral fluid and whole blood, while this was not the case for the oral fluid-plasma comparison. Potential causes of the latter discrepancy were not further addressed. Overall, the authors concluded that all matrices provided similar measures of nicotine metabolism [86]. In another study, Lea *et al.* addressed the intraindividual variation of oral fluid *trans* 3'-OH-cotinine/cotinine ratios in 6 regular daily smokers and observed that ratios were not affected by the time of sampling during the day [87].

In conclusion, oral fluid may provide a reliable alternative for plasma-based CYP450 phenotyping. Given the easy, rapid and non-invasive sample collection, this technique seems particularly suited for large epidemiological phenotyping studies. From the many available studies it can be stated that oral fluid has been established as a valid alternative sampling method for CYP1A2 phenotyping, with caffeine clearance or the paraxanthine/caffeine ratio as metrics. Although promising data were obtained for other CYP isoforms, such as CYP2D6 and CYP2A6, further studies are needed to confirm the results with these enzymes.

A.1.5. Exhaled breath

In the past 40 years, breath analysis has been extensively explored as a diagnostic tool to examine liver function. Typically, these breath tests are based on oral or intravenous administration of an isotopically labeled test compound. While radioactive ^{14}C-labeled compounds were initially used, probe drugs carrying a functional group in which a stable ^{13}C-isotope replaces a ^{12}C-atom have now become standard practice, thereby providing a safer alternative. Upon administration, the probe drug is subject to metabolism and the labeled functional group is enzymatically cleaved. Following additional oxidation reactions, $^{13}CO_2$ is eventually formed. The latter is mixed with the body bicarbonate pool and exhaled in the breath. The $^{13}CO_2/^{12}CO_2$ ratio in breath samples, measured by isotope ratio mass spectrometry or isotope-selective nondispersive infrared spectrometry, has been used as an indicator of liver function. Usually, the ^{13}C-enrichment of breath following administration of the probe drug is compared to the baseline enrichment before ingestion, as several factors (e.g. diet) may cause variations in the natural occurrence of $^{13}CO_2$. Therefore, the final results are expressed as delta-over-baseline (DOB) values [88-90].

Apart from their use to investigate liver function, ^{13}C breath tests have been applied to quantitatively assess the activity of metabolic enzymes. For many probe drugs, the initial metabolic reaction, often a dealkylation step, is catalyzed by CYP450 enzymes and, therefore, the amount of $^{13}CO_2$ in breath should reflect enzyme activity. For a full overview of published breath tests, available probe drugs and their clinical utility, we refer to several recent reviews on this topic [91-95]. Here, we highlight a number of selected studies in which the potential of breath tests for CYP450 phenotyping has been evaluated by comparison with other indices of enzyme activity (Table A.1.3.). The N-demethylation of aminopyrine, the first probe drug used in breath tests, is mediated by several CYP450 enzymes, with CYP2C19 being the most important isoform [96]. Kodaira *et al.* found a significant correlation between $AUC_{0\text{-}3h}$ of DOB $^{13}CO_2/^{12}CO_2$ ratios and both plasma [^{13}C]-aminopyrine $AUC_{0\text{-}3h}$ and clearance. [97]. [^{13}C]-pantoprazole has been proposed as an alternative CYP2C19 phenotyping probe, as several studies found significantly lower DOB values in CYP2C19 poor metabolizers compared to intermediate or extensive metabolizers [98-103]. In addition, plasma [^{13}C]-pantoprazole AUC correlated significantly with the AUC of DOB $^{13}CO_2/^{12}CO_2$ ratios [98,101,103].

Leeder *et al.* developed a new breath test for CYP2D6 phenotyping using [^{13}C]-dextromethorphan as probe drug. Single 40 min DOB $^{13}CO_2/^{12}CO_2$ ratios proved equally effective as urinary [^{13}C]-dextromethorphan/dextrorphan ratios in distinguishing between CYP2D6 poor and extensive metabolizers [104]. The utility of this approach was evaluated by Opdam *et al.* in a study involving breast cancer patients taking tamoxifen, a prodrug that is converted to endoxifen by CYP2D6. The correlation between CYP2D6 phenotype, determined by the [^{13}C]-dextromethorphan breath test, and serum endoxifen levels was comparable with the correlation between the latter and sequence-based CYP2D6 activity prediction [105]. For the CYP1A2 substrate [^{13}C]-caffeine, Park *et al.* observed a significant correlation between 1 h single DOB $^{13}CO_2/^{12}CO_2$ ratios and plasma [^{13}C]-caffeine clearance [106]. These results were in agreement with previous studies, in which excellent correlations between caffeine breath test results and caffeine clearance were found [107-110].

One of the most widely applied phenotyping procedures is the erythromycin breath test, although several studies revealed conflicting results concerning its ability to reflect CYP3A4 phenotype, especially in relation to the established CYP3A4 phenotyping probe drug midazolam. While Lown *et al.* found a significant correlation between erythromycin breath test results, expressed as % ^{14}C exhaled in 1 h, and body weight-adjusted clearance of midazolam [111], these results could not be confirmed in other studies applying identical PK parameters [112,113]. Franke *et al.* used $1/T_{max}$ as an alternative erythromycin breath test parameter, but found no correlation with midazolam clearance either [114]. The fact that, in addition to CYP3A4, P-glycoprotein is involved in erythromycin metabolism, whereas this is not the case for midazolam, may explain the discordant results for both probe drugs [115].

In general, breath tests based on the administration of isotopically labeled probe drugs seem to provide an interesting, non-invasive alternative to assess the activity of several CYP450 enzymes. Breath samples are easily collected and do not require extensive sample work-up prior to analysis. However, widespread routine use of breath tests for phenotyping purposes has been limited to date, due to the

high cost of isotopically labeled compounds and the highly specialized equipment required for analysis of breath samples.

A.1.6. Miscellaneous matrices

In addition to the above-mentioned matrices, many endogenous compounds and xenobiotics are, to a limited extent, secreted into sweat as well. Several applications of sweat testing have been described, including measurement of chloride ions as a biomarker of cystic fibrosis [116] and detection of drugs of abuse in forensic toxicology, roadside and workplace drug testing [117-119]. Sweat for drug testing is commonly collected using transdermal absorptive sweat patches attached to the skin, representing a non-invasive sampling technique. In most cases, analytes can be removed from these patches using relatively simple extractions protocols, resulting in clean extracts that are subsequently analyzed by GC- or LC-MS. Sweat patches can be worn for several days, enabling continuous drug testing over an extended period of time compared to urine, saliva or blood. Limitations of this sampling strategy are the potential influence of external contamination of the devices or the skin and the unknown collected volume, as sweat production may vary in function of ambient temperature or physical activity [119]. Delahunty *et al.* suggested that sweat could potentially be used as an alternative for urine or plasma to estimate CYP1A2 activity. Following administration of caffeine, the latter compound and its metabolites were determined in transdermal absorptive sweat patches worn for several days by healthy volunteers. Caffeine, paraxanthine and theobromine were readily detected in the patches, allowing to calculate metabolite/parent drug ratios [120]. However, comparison of these ratios with corresponding ratios in plasma or urine, to illustrate their actual usefulness for CYP1A2 activity assessment, was not included in the study. To the best of our knowledge, no other studies on the use of sweat for CYP450 phenotyping are available to date. Kuwaya *et al.* developed a method that allowed to determine caffeine and parxanthine in fingerprints at several time points following consumption of a cup of coffee. Although this concept may hold promise for CYP1A2 phenotyping, paraxanthine/caffeine ratios were not calculated [121].

Another example of using an alternative matrix for measuring CYP450 activity was described by Grosso *et al.* In a study to investigate the association between intrauterine growth restriction (IUGR) and fetal caffeine exposure, caffeine and its primary metabolites were quantified in umbilical cord serum samples of 1606 women. As higher paraxanthine/caffeine ratios were positively correlated with an increased risk of IUGR, it was suggested that CYP1A2 activity may have an effect on fetal growth [122].

A.1.7. Conclusion

A substantial number of studies has evaluated the usefulness of DBS, hair, oral fluid or exhaled breath as alternative sampling strategies for CYP450 phenotyping. A large body of evidence supports the validity of oral fluid for CYP1A2 phenotyping, using caffeine as a probe drug. While this method has already been implemented in several phenotyping studies, promising results were also obtained for

other oral fluid-based phenotyping approaches, especially for nicotine metabolic ratios as a measure of CYP2A6 activity and the CYP2D6 probe drug dextromethorphan. CYP3A phenotyping in oral fluid following midazolam or dextromethorphan administration needs further investigation. Concerning the utility of exhaled breath as an alternative matrix for CYP450 phenotyping, the available data are, in some respect, comparable to those of oral fluid. While [^{13}C]-caffeine and [^{13}C]-dextromethorphan breath tests proved to be reliable procedures for CYP1A2 and CYP2D6 phenotyping, respectively, conflicting results were obtained for the assessment of CYP3A4 activity using the widely applied probe drug [^{14}C]-erythromycin. [^{13}C]-pantoprazole-based breath tests, on the other hand, showed promise to assess CYP2C19 metabolizer status. Recently, several groups proposed DBS sampling as an alternative strategy for CYP450 phenotyping. Procedures for several clinically relevant CYP450 isoforms, including CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19 and CYP2D6, revealed good agreement between phenotyping metrics determined in DBS and in plasma. Also here, inconclusive data were obtained for CYP3A4 phenotyping using midazolam as probe drug. Although hair sampling may provide an interesting tool in case a standard phenotyping procedure is not feasible (e.g. post-mortem), the actual value of hair analysis in this field remains to be established, given the limited number of studies available to date.

Based on the studies discussed in this chapter, it can be concluded that reliable phenotyping procedures for selected CYP450 enzymes are currently available for either oral fluid, breath and DBS. Important to note is that it should be decided on a case-by-case basis what phenotyping metric is to be used. While in many of the described alternative procedures metabolic ratios at one given time point have been used as phenotyping metric, other metrics (e.g. AUC, clearance, etc.) may be more appropriate in other scenarios. Also here -especially when repeated sampling is to be performed- these alternative matrices offer the benefit that they can be collected in a non- or minimally invasive way, causing minimal discomfort for the patient. Table A.1.4. summarizes selected advantages and limitations of the alternative sampling strategies. To date, phenotyping of drug metabolizing enzymes has been mainly applied in research settings, for example in drug-drug interaction studies or to assess the impact of various factors, such as gender, age, diet or cigarette smoke, on enzyme activities. The availability of simple, convenient and patient-friendly protocols may hasten the acceptance and routine application of CYP450 phenotyping in clinical practice as well. There, phenotyping could play a role in predicting optimal drug dose prior to the start of the therapy or to guide therapy in case of unexpected changes in plasma levels of (or response to) a given drug. Examples of therapeutic drugs for which phenotyping using alternative strategies may be applied are the CYP2D6 substrates imipramine, nortriptyline (tricyclic antidepressants), haloperidol, risperidone (antipsychotics), codeine and tramadol (opioid analgesics), the CYP2C19 substrates omeprazole, lansoprazole (proton-pump inhibitors) and clopidogrel (platelet aggregation inhibitor), the CYP1A2 substrates clozapine and olanzapine (antipsychotics) and the CYP2C9 substrate warfarin (anticoagulant) [3,6,8]. For several of these, it remains to be fully established if and to what extent phenotyping (and resulting dose adaptation) indeed results in a better clinical outcome.

Furthermore, in contrast to venous blood sampling, collecting DBS, oral fluid or breath does not require a phlebotomist and can be performed by instructed patients themselves. In this light, a phenotyping

scenario involving home-sampling may be envisaged, in which samples are transferred to the laboratory by mail, provided that the analytes of interest are stable under the transport conditions. In our opinion, DBS sampling may be the preferred approach for this purpose, given the ease of transport and storage of DBS cards compared to oral fluid and, especially, breath samples collected in dedicated bags. The required analytical instrumentation is another factor that may determine which alternative sampling strategy is best suited to allow a more widespread use of phenotyping. While DBS and oral fluid can be analyzed using techniques that have become standard in the modern clinical laboratory, such as LC-MSMS, measurement of $^{13}CO_2/^{12}CO_2$ ratios in breath requires highly specialized equipment not commonly available in such laboratories. In recent years, microdosing of probe drugs has been applied in phenotyping studies to reduce the risk of adverse drug reactions or interferences with other drugs. Since doses are at least 100-fold lower than regular doses, highly sensitive, accurate and precise analytical procedures are required [123]. While this already represents a challenge for microdosing approaches using plasma, it may especially pose a problem when alternative sampling strategies are to be applied. DBS methods, for example, typically analyze only a few µL of dried blood. For oral fluid, other issues, such as drug adsorption to sampling devices, may play a role. Alternative sampling strategies have -to the best of our knowledge- not yet been applied in microdosing studies. This may, however, be an interesting future application taking into account the increasing sensitivity of the latest generations of LC-MS/MS instruments. Finally, for alternative sampling techniques to become fully accepted as valid phenotyping strategies, the results obtained to date need to be confirmed in future studies, including trials in subjects with deviating enzyme activity and large population studies, allowing to define reference ranges of phenotyping metrics in the various matrices.

Table A.1.1. CYP450 phenotyping procedures using dried blood spots (DBS) and hair.

Matrix	CYP450 isoform	Probe drugs	Phenotyping metric	Comments	References
DBS	CYP3A4	Midazolam	Midazolam $AUC_{0\text{-}12h}$	Unexplained difference between DBS and whole blood AUC	[16]
	CYP2C9	Flurbiprofen	OH-flurbiprofen/flurbiprofen ratio (multiple time points)	Significant correlation with plasma and urine ratios and flurbiprofen clearance	[17,18]
	CYP2A6	Nicotine (from tobacco smoke)	*trans* 3'-OH-cotinine/cotinine ratio	Significant correlation with plasma ratios	[19]
	CYP1A2	Caffeine	6 h paraxanthine/caffeine ratio	Significant correlation with whole blood and plasma ratios, examination of DBS-specific parameters	[20,21]
	CYP2C19, CYP1A2 (CYP3A4, CYP2C9, CYP2D6, CYP2B6)	Omeprazole, caffeine (midazolam, losartan metoprolol, efavirenz)	2h omeprazole/OH-omeprazole & 8 h paraxanthine/caffeine ratio (other probe drugs or metabolites < LLOQ)	Significant correlation with plasma AUC ratios	[22]
	CYP3A4, CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP2C19	Midazolam, caffeine, bupropion, flurbiprofen, dextromethorphan, omeprazole	2 h OH-midazolam/midazolam & paraxanthine/caffeine ratio, 3 h OH-bupropion/bupropion, OH-flurbiprofen/flurbiprofen & dextrorphan/dextromethorphan ratio, $AUC_{2,3,6h}$ OH-omeprazole/omeprazole ratio	Significant correlation with plasma AUC ratios	[23,24]
Hair	CYP2C19	Amitriptyline	Nortriptyline/amitriptyline ratio in entire hair samples	Significant differences between PM and IM/EM	[28]
	CYP1A2	Caffeine	Paraxanthine/caffeine ratio in proximal 3-cm segments	Overall significant correlation with 6 h paraxanthine/caffeine plasma ratios, but large deviations in individual cases	[29]

AUC: area under the curve, LLOQ: lower limit of quantification, PM: poor metabolizer, IM: intermediate metabolizer, EM: extensive metabolizer

Table A.1.2. CYP450 phenotyping procedures using oral fluid.

Matrix	CYP450 isoform	Probe drugs	Phenotyping metric	Comments	References
Oral fluid	CYP1A2	Caffeine	Caffeine clearance and 3-10 h paraxanthine/caffeine ratios	Significant correlation with caffeine plasma/serum clearance, paraxanthine/caffeine plasma ratios and *in vitro* CYP1A2 activity	[13,54,56-61]
	CYP2D6	Dextromethorphan	3 or 6 h dextromethorphan/dextrorphan ratio	Significant correlation with urinary dextromethorphan/dextrorphan ratios and differentiation between PM and IM/EM	[73,75]
	CYP3A	Dextromethorphan	8 & 12 h dextromethorphan/3-methoxymorphinan	Poor correlation with urinary dextromethorphan/3-methoxymorphinan ratios	[77]
		Midazolam	Midazolam AUC and clearance	Large differences with plasma AUC and clearance	[78]
	CYP2C19, CYP1A2 (CYP3A4, CYP2C9, CYP2D6, CYP2B6)	Omeprazole, caffeine (midazolam, losartan metoprolol, efavirenz)	2h omeprazole/OH-omeprazole & 8 h paraxanthine/caffeine ratio (other probe drugs or metabolites < LLOQ)	Correlation with plasma AUC ratios significant for CYP1A2, poor for CYP2C19	[22]
	CYP2A6	Nicotine-d_2, cotinine-d_4	6 h *trans* 3'-OH-cotinine-d_4/cotinine-d_4 ratio	Significant correlation with plasma ratios and oral clearance of nicotine and cotinine	[85]
		Nicotine (from tobacco smoke)	*trans* 3'-OH-cotinine/cotinine ratio	High correlation with whole blood ratios, not consistently similar with plasma ratios	[86]

AUC: area under the curve, LLOQ: lower limit of quantification, PM: poor metabolizer, IM: intermediate metabolizer, EM: extensive metabolizer

Table A.1.3. CYP450 phenotyping procedures using exhaled breath.

Matrix	CYP450 isoform	Probe drugs	Phenotyping metric	Comments	References
Exhaled breath	CYP2C19	[^{13}C]-aminopyrine	$AUC_{0\text{-}3h}$ of DOB $^{13}CO_2/^{12}CO_2$ ratios	Significant correlation with plasma [^{13}C]-aminopyrine $AUC_{0\text{-}3h}$ and clearance	[97]
		[^{13}C]-pantoprazole	DOB $^{13}CO_2/^{12}CO_2$ ratio (multiple time points & AUC)	Significant differences between PM and IM/EM and correlation with plasma [^{13}C]-pantoprazole AUC	[98-103]
	CYP2D6	[^{13}C]-dextromethorphan	Single 40 min DOB $^{13}CO_2/^{12}CO_2$ ratio	Comparable with urinary [^{13}C]-dextromethorphan/dextrorphan ratios to distinguish between PM and EM	[104]
	CYP1A2	[^{13}C]-caffeine	Single 1 h DOB $^{13}CO_2/^{12}CO_2$ ratio	Significant correlation with plasma [^{13}C]-caffeine clearance	[106]
	CYP3A4	[^{14}C]-erythromycin	% ^{14}C exhaled in 1 h or $1/T_{max}$	Poor correlation with midazolam clearance	[111-114]

AUC: area under the curve, DOB: delta-over-baseline, PM: poor metabolizer, IM: intermediate metabolizer, EM: extensive metabolizer

Table A.1.4. Author's view on main advantages and limitations of dried blood spot (DBS), oral fluid and exhaled breath sampling.

	DBS	Oral fluid	Exhaled breath
Patient comfort (incl. ease of sampling, invasiveness)	++	++	+++
Sampling in absence of specialized	+++	+++	+++
Transport & storage	+++	++	-
Home-sampling potential	+++	++	+
Analysis using standard analytical equipment	+++	+++	-

A.1.8. References

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Chapter A.2.

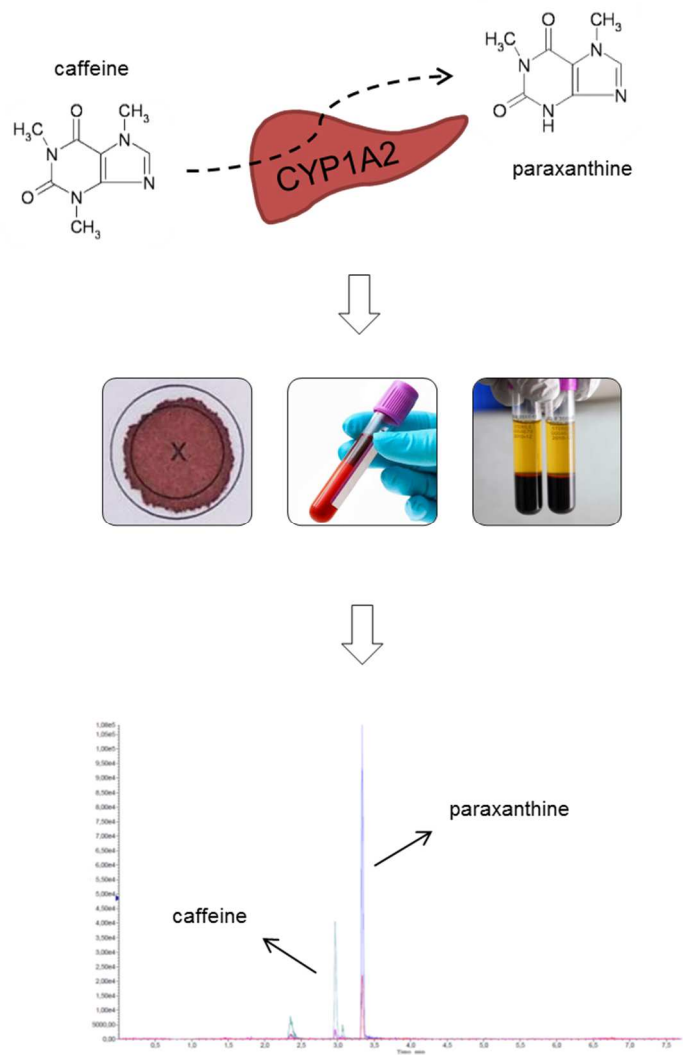
Development and validation of LC-MS/MS-based procedures for CYP1A2 phenotyping in dried blood spots, whole blood and plasma

Based on

De Kesel PM, Lambert WE, Stove CP. CYP1A2 phenotyping in dried blood spots and microvolumes of whole blood and plasma. Bioanalysis. 2014;6(22):3011-24.

Abstract

Phenotyping, using caffeine as probe substrate, is a proper method to assess CYP1A2 activity. The utility of dried blood spots (DBS) for CYP1A2 phenotyping was evaluated. Therefore, LC-MS/MS methods were developed and validated for quantitation of caffeine and its metabolite paraxanthine in DBS, whole blood and plasma. All parameters met the pre-established criteria. While recovery, matrix effects and precision were unaffected by Hct, there was a Hct-effect on accuracy, although for the evaluated Hct interval (0.36-0.50) it remained within acceptable limits. The phenotyping methods were successfully applied in healthy volunteers. In conclusion, excellent method performance and highly comparable phenotyping indices in DBS, whole blood and plasma, combined with the benefits of DBS sampling, illustrate the suitability of DBS-based CYP1A2 phenotyping.



A.2.1. Introduction

The cytochrome P450 (CYP450) family of enzymes is involved in the oxidative biotransformation of many drugs and xenobiotics. CYP450 enzymes play a predominant role in approximately 80 % of phase I drug metabolism reactions and are responsible for almost 50 % of drug clearance [1]. Consequently, variability in the expression and function of these enzymes may cause altered drug plasma levels and effects [2]. Therefore, methods to adequately determine the *in vivo* enzyme activity can be very helpful, for example, to guide therapy with enzyme substrates. While genotyping is a suitable approach to evaluate an individual's enzyme activity for particular CYP isoforms whose activity is mainly influenced by genetic polymorphisms (e.g. CYP2D6) [3], a phenotyping approach is preferred for other enzymes. CYP1A2 is an example of the latter: apart from genetic factors, its activity is determined by nongenetic host factors and environmental influences [2,4]. Concerning the latter, the *CYP1A2* gene is sensitive to induction or inhibition by tobacco smoke, brassicaceae vegetables or therapeutic drugs (e.g. omeprazole, carbamazepine, oral contraceptives, fluvoxamine) [4-8]. Phenotyping takes into account the combined effect of all above mentioned factors on enzyme activity. Several therapeutic drugs, including the antipsychotics clozapine and olanzapine, are CYP1A2 substrates. As the enzyme shows a large interindividual variability in activity, a large variability in response to these substrates may be expected [9]. Indeed, a wide range of serum levels of clozapine after administration of fixed doses has been described, potentially leading to suboptimal therapy or increased risk of toxicity [10].

Phenotyping is typically performed by controlled administration of a selective substrate [11]. For determining CYP1A2 activity, the N3-demethylation of caffeine to paraxantine has been widely applied as this metabolic route is solely mediated by CYP1A2 [12]. The metabolic pathways of caffeine (1,3,7-trimethylxanthine) to its primary metabolites paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine), together with the major CYP450 enzymes catalyzing these biotransformation reactions, is shown in Figure A.2.1. A phenotyping index can be obtained by determining the paraxanthine:caffeine molar concentration ratio in plasma, 5-7h after controlled caffeine administration (100–200 mg) [11,13]. In this chapter, we have evaluated the utility of dried blood spots (DBS) as an alternative matrix for the determination of caffeine and its major metabolite paraxanthine in the context of CYP1A2 phenotyping. As capillary blood to prepare DBS is generally obtained by a simple finger or heel prick, DBS sampling provides a less invasive alternative compared to the classical venipuncture required for plasma-based phenotyping. Furthermore, collection of DBS represents a convenient and inexpensive sampling technique and several compounds and metabolites showed enhanced stability in DBS compared to liquid samples [14-17]. Hence, representative samples can be collected by home sampling, without the need for specialized personnel, and transported at ambient temperature to the laboratory [18]. There, DBS offer the benefit of straightforward and automatable sample processing and analysis [19,20].

DBS sampling has been successfully applied for the determination of a wide range of compounds in different fields, such as toxicology and therapeutic drug monitoring [21,22]. Recently, DBS have also been used for phenotyping individual CYP450 enzymes, such as CYP3A4 [23] and CYP2C9 [24,25], or multiple enzymes, including CYP1A2, simultaneously using a cocktail of probe drugs [26,27]. In this

chapter, we describe the development, validation and application of LC-MS/MS-based procedures for CYP1A2 phenotyping, using caffeine as a model substrate, in DBS, whole blood and plasma. The developed methods are fully validated based on international guidelines [28, 29]. In contrast to other validated methods for DBS-based phenotyping, in this study the influence of the hematocrit (Hct), an important parameter potentially impacting DBS-based quantification (as further outlined in part B) [30], is investigated in-depth by analyzing quality control (QC) samples prepared from blood with different Hct levels for the evaluation of accuracy, precision, stability, matrix effects and recovery. The applicability of the developed phenotyping methods is evaluated by comparing phenotyping indices, i.e. paraxanthine:caffeine molar concentration ratios, in capillary DBS (obtained by non-volumetric, direct application of a drop a blood from the fingertip onto the filter paper), venous DBS, whole blood and plasma obtained from healthy volunteers.

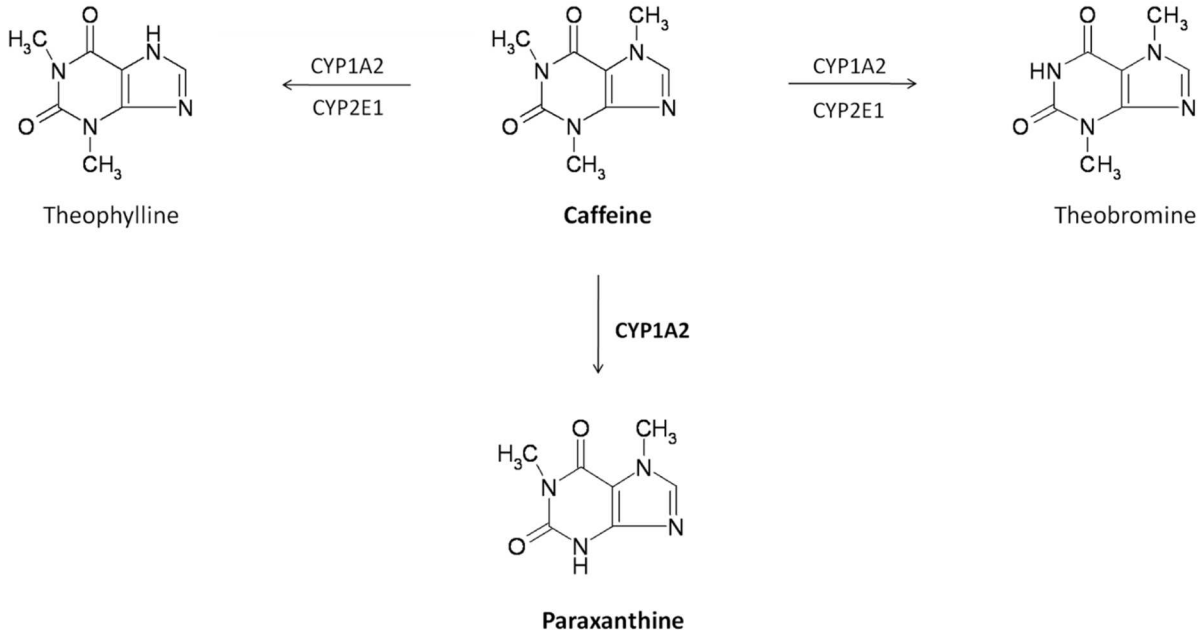


Figure A.2.1. Metabolic pathways of caffeine and the major CYP450 enzymes involved.

A.2.2. Material and methods

A.2.2.1. Chemicals and stock solutions

Caffeine, paraxanthine, theophylline, theobromine, the internal standards (IS) caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$, formic acid, acetic acid, ammonium formate and ammonium acetate were obtained from Sigma-Aldrich (Diegem, Belgium). LC-MS quality methanol and acetonitrile were purchased from Biosolve (Valkenswaard, The Netherlands). Ultrapure water was provided by a Synergy® Water Purification System (Merck Millipore, Overijse, Belgium).

Stock solutions (1 mg/mL) were prepared by dissolving 10 mg of the compounds in 10 mL of water. To prepare calibrators and QCs, independently prepared stock solutions were used. For the IS, 100 µg/mL stock solutions were prepared by appropriate dilution of a commercially available 1 mg/mL solution of caffeine-$^{13}C_3$ in methanol or by dissolving 2 mg of paraxanthine-$^{13}C_4$-$^{15}N_3$ in 20 mL of methanol. All stock solutions were stored at -20 °C. Working solutions were prepared the day of analysis by diluting the stock solutions in water.

A.2.2.2. UPLC®-MS/MS procedure

A.2.2.2.1. Optimization of LC-MS/MS parameters

A Waters Acquity UPLC® system (Waters, Milford, MA, USA) coupled to an AB SCIEX API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) was used for all experiments. The LC-MS/MS system was controlled by AB SCIEX Analyst 1.5.2 and Waters Acquity console software. The mass spectrometer was equipped with an electrospray ionization (ESI) (TurboIonSpray®) and an atmospheric pressure chemical ionization (APCI) source.

First, the influence of the type of ion source, the ionization mode and the solvent composition on the ionization efficiency of caffeine and paraxanthine was examined. Therefore, standard solutions of both compounds (100 ng/mL) in methanol/water (MeOH/H_2O; 75/25, v/v) or acetonitrile/water (MeCN/H_2O; 75/25, v/v) were introduced into the ion source by direct continuous infusion using a syringe pump operating at low flow rate (20 µL/min). For both solvents, the effect of different acidic additives, being formic acid (0.1 %), acetic acid (0.1 %), ammonium formate (5 mM) and ammonium acetate (5 mM), was evaluated. Molecular ion signal intensities were obtained using the automatic quantitative optimization function provided by the Analyst software. This function also allowed to perform fragmentation experiments by optimizing compound-dependent MS parameters (declustering potential (DP), collision cell entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP)). Source-dependent MS parameters (ion spray voltage, source temperature, nebulizer, heater and curtain gas pressure), as well as vacuum settings for collision-activated dissociation (CAD), were optimized using flow injection analysis (FIA). In this experiment, 10 µL of a mix of the analytes is injected by the autosampler into the mobile phase, which is directly delivered to the ion source at a constant flow rate. No column is placed between the LC and MS system. Preliminary parameters were obtained using a standard solution of caffeine and paraxanthine (100 ng/mL) in water and a methanol/water mixture (75/25, v/v) as mobile phase, pumped at 1 mL/min. Final parameters were assessed using spiked extracts of DBS, whole blood or plasma and the optimized LC conditions.

To optimize chromatographic conditions, two reversed phase C18 columns were tested. In the first column, a Waters Acquity UPLC® BEH-C18 column, the C18 phase is bonded onto hybrid particles (BEH; Bridged Ethylene Hybrid), while in the second, a Waters Acquity UPLC® HSS-T3 column, the C18 chains are bonded onto silica particles (HSS; High Strength Silica). Both columns have identical dimensions (150 mm x 2.1 mm; 1.8 µm particle size) and are equipped with corresponding Waters VanGuard pre-columns (5 mm x 2.1 mm; 1.8 µm particle size). These columns were used in combination with the eluents methanol and water, containing formic acid or ammonium formate, to optimize

chromatographic separation by varying different parameters, such as gradient elution program, column temperature and flow rate.

A.2.2.2.2. Final method

In the final method, the Waters Acquity UPLC® HSS-T3 column equipped with a Waters VanGuard pre-column (respectively 150 mm and 5 mm x 2.1 mm; 1.8 µm particle size) was used. The column oven was set at 60 °C, the autosampler at 4 °C. The mobile phase, pumped at a flow rate of 0.4 mL/min, consisted of 0.01 % formic acid in water (solvent A) and 0.01 % formic acid in methanol (solvent B), with the following proportions of solvent B in the 8-min gradient elution program: 15 % for 1.5 min, linearly increased to 60 % in 1 min, followed by a short decline to 50 % in 0.6 min, a fast increase to 95 % in 0.4 min, maintained for 2 min and finally, reversal to starting conditions. The API 4000™ mass spectrometer was equipped with an ESI source, operating in positive mode. The interface heater was on (100 °C) and nitrogen was used as nebulizer (gas 1), heater (gas 2), curtain and CAD gas. The source temperature was set at 300 °C, the ion spray voltage at 3800 V for DBS analysis and 4000 V for whole blood and plasma samples. Gas pressure settings respectively for DBS and whole blood/plasma analysis were 55 and 40 psi for gas 1; 70 and 80 psi for gas 2; 10 and 40 psi for the curtain gas. Arbitrary vacuum settings for CAD were 9 and 12 for DBS and whole blood/plasma samples, respectively. Analytes were detected in the scheduled multiple reaction monitoring (sMRM) mode. Two characteristic precursor to product ion transitions were monitored per compound, except for the IS for which one transition was analyzed. All MRM transitions, together with compound-specific MS parameters, are listed in Table A.2.1 (see further).

A.2.2.3. Sample collection

After making finger pricks with an automated lancet (VAC366594, Becton Dickinson, Franklin Lakes, NJ, USA), blood was directly applied from the fingertip onto Whatman 903 filter paper cards (WHA10334885, GE Healthcare, Dassel, Germany) to generate capillary DBS. Ethylenediaminetetraacetic acid (EDTA) tubes (Venosafe® 4 mL VF-054SDK, Terumo, Leuven, Belgium) were used to collect venous blood, which was used to generate plasma and to prepare venous DBS by spotting 25 µL of blood onto Whatman 903 filter paper using a calibrated pipette. Whole blood and plasma samples were stored at -20 °C, DBS were dried for at least 2 hours and stored at ambient temperature in zip-closure plastic bags, containing two 5-g packets of desiccant (Minipax® absorbent packets, Sigma-Aldrich), until analysis. For method development and validation purposes, venous whole blood from a caffeine abstinent healthy, male volunteer was collected in EDTA tubes (Venosafe® 9 mL VF-109SDK, Terumo).

A.2.2.4. Sample preparation

For analysis of DBS, 3-mm discs were punched from the center of a DBS using a Harris micro-punch and cutting mat (Sigma-Aldrich). Punches taken from four different types of blank filter paper (Whatman 903, Munktell 2460, Ahlstrom 226 and 237) were analyzed and chromatograms were examined for possible interferences with caffeine and paraxanthine. Subsequently, DBS extraction conditions were

optimized. Different organic solvents (methanol, acetonitrile and a methanol/acetonitrile mixture (50/50, v/v)) were compared and the influence of addition of water to the extraction solvent was evaluated. Different volumes of extraction solvent, varying from 30 to 100 µL, were tested. Extraction times ranged from 5 to 60 minutes. For each condition, spiked DBS were analyzed in triplicate and the peak areas of the test compounds were compared. As IS are preferably added as early as possible in the sample preparation, we evaluated two different approaches. The IS were either dissolved in extraction solvent, or 2 µL IS solution was spotted onto the dried punch.

In the final procedure, 70 µL of a methanol/water (80/20, v/v) mixture, containing 0.01 % formic acid and both IS, was added to the 3-mm punches in 2-mL cups. Samples were shaken for 10 minutes at 1000 rpm and 22 °C on a Thermomixer® comfort (Eppendorf, Hamburg, Germany) and subsequently centrifuged at ambient temperature for 10 minutes at 10000 x *g*. After mixing 45 µL of the resulting supernatants with 195 µL of water, containing 0.01 % formic acid, 200 µL was transferred to a vial with plastic insert, of which 10 µL was injected onto the UPLC® column.

A simple protein precipitation procedure was used for plasma and whole blood samples, starting from 50 µL to which 10 µL of IS working solution was added. Subsequently, 100 µL methanol, containing 0.01 % formic acid, was added. Samples were then treated in an identical way as DBS samples, with the only exception that, after centrifugation, supernatants (40 µL) were diluted with 130 µL of water, containing 0.01 % formic acid.

A.2.2.5. Preparation of calibrators and QCs

Quantitative analyses were performed for caffeine and paraxanthine. Theophylline and theobromine, being metabolites of caffeine with the same molecular mass as paraxanthine, were qualitatively monitored in every analytical run to ensure selectivity. Calibrators were prepared at eight concentration levels in blank whole blood with intermediate Hct (0.43 ± 0.006) and in plasma. For caffeine, 0.050, 0.075, 0.25, 0.50, 1.0, 2.5, 5.0 and 10.0 µg/mL solutions were made; for paraxanthine, 0.025, 0.050, 0.100, 0.25, 0.50, 1.0, 2.5 and 5.0 µg/mL solutions were prepared. QCs at four concentration levels were made in blank whole blood with low (0.36 ± 0.007), intermediate (0.43 ± 0.006) and high (0.50 ± 0.008) Hct. For analysis of whole blood and plasma, only blood with intermediate Hct (and resulting plasma) was used. The nominal concentrations of QCs for caffeine and paraxanthine were respectively 0.05 and 0.025 (lower limit of quantification, LLOQ), 0.12 and 0.06 (low), 4.0 and 2.0 (medium), 8.0 and 4.0 (high) µg/mL. The proportion of non-matrix solvents used for spiking blank blood or plasma never exceeded 5 % of the total sample volume. Blood with varying Hct levels was prepared by centrifuging an aliquot of the blood in glass tubes for 5 min at 1000 x *g* and by removing or adding plasma. The obtained Hct was measured in duplicate using a Sysmex XE-5000 hematology analyzer (Sysmex Corporation, Kobe, Japan). The mean of these duplicate measurements was considered as the actual Hct [31].

A.2.2.6. Validation

Validation of the developed methods was performed based on U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [28,29]. For that purpose, selectivity, carry-over, LLOQ, linearity, precision, accuracy, matrix effect, recovery, stability and incurred sample reanalysis were evaluated. Importantly, this validation involved the evaluation of QC's at different Hct levels.

Selectivity was assessed by analyzing blank DBS, blood and plasma from six individual donors. To evaluate cross-interferences with caffeine or paraxanthine, samples spiked with caffeine, paraxanthine, theophylline or theobromine at 10 µg/mL (n=5) were analyzed. In addition, blank and zero samples (IS-spiked blank DBS, whole blood or plasma) were included in every analytical run to evaluate the purity of the isotopically labeled IS. Interferences were considered unacceptable if peak areas were higher than 20 % of the LLOQ for the analytes and 5 % for the IS [28]. Identical criteria were applied for carry-over, which was examined by injecting blank samples after the highest calibrators (n=5 or 6) [29].

The LLOQ was defined as the lowest concentration of caffeine and paraxanthine which could be measured with %relative standard deviation (%RSD) and %bias below 20 %. To determine linearity, eight-point calibration lines for caffeine and paraxanthine were constructed on five non-consecutive days (DBS) or in duplicate on three non-consecutive days (blood, plasma). Homoscedasticity of the data was evaluated by performing the F-test at the 99 % confidence level and by plotting residuals vs. nominal concentrations [32]. Slopes and intercepts of the calibration lines were calculated using weighted (1/x, $1/x^2$, $1/\sqrt{x}$, 1/y, $1/y^2$ and $1/\sqrt{y}$) and unweighted linear regression. To select the most appropriate model, the sum% relative error (RE) was calculated and %RE was plotted against nominal concentrations [32]. To test the selected linear model for goodness of fit, the deviation of the obtained concentrations from the nominal values was calculated. Mean concentrations should be within ± 15 % of the nominal value and within ± 20 % for the LLOQ in order to accept the chosen model [28].

For the evaluation of precision and accuracy, QCs were prepared and analyzed in duplicate on five (DBS) or three (blood, plasma) non-consecutive days. Intra- and interbatch precision (%RSD) were calculated using a single factor analysis of variance (ANOVA), according to Wille *et al.* [33]. Accuracy (%bias) was calculated by dividing the difference between the obtained concentration and the nominal value by the nominal value and multiplying it by 100. %RSD and %bias should be within ± 15 % (20 % for the LLOQ) [28].

Matrix effect and recovery were determined at four (DBS) or two (blood, plasma) concentration levels using blank whole blood and plasma from six individual donors (Hct range 0.36 - 0.46), spiked before (C) or after (B) extraction, and using standard solutions of caffeine and paraxanthine in mobile phase at the same concentrations (A). Absolute matrix effect was calculated by the percent ratio of peak areas of (B) to those of (A), absolute recovery by the percent ratio of peak areas of (C) to those of (B). Relative matrix effect and recovery were expressed as %RSD of absolute matrix effect and recovery values [34]. Relative matrix effect should not exceed 15 % [28]. Further in-depth evaluation of a possible impact of Hct was done by assessing recovery and matrix effects in DBS prepared from blood with strongly

deviating Hct values (0.19 and 0.63). These blood samples were prepared starting from a sample with normal Hct (0.42). To accurately measure matrix effect and recovery, all DBS analyses were performed on 3-mm pre-punched discs, onto which 3.5 µL of blank or spiked blood was spotted.

Short- and long-term stability of DBS were assessed by analyzing low and high QCs prepared in blood with low, intermediate and high Hct in sixfold at time point zero and after 4 days of storage at 50 °C, 324 days at room temperature, 53 days at -20 °C and 36 days at 4 °C. DBS were stored in zip-closure plastic bags with desiccant, regardless of storage temperature. Stability of caffeine and paraxanthine in whole blood and plasma was assessed by analyzing low and high QCs in triplicate at time point zero and after 172 days of storage at -20 °C and 7 days at 4 °C. To evaluate processed sample stability, extracts of low and high QCs (n=3) were re-injected after 24 h in the autosampler (i.e. autosampler stability) and after storage for 7 (DBS) or 6 (blood and plasma) days at -20 °C. At every time point, stability samples were analyzed against a freshly prepared calibration curve. The mean concentration of the QCs at a given time point should not deviate more than 15 % from time point zero and from the nominal concentration [28].

Finally, incurred sample reanalysis with an interval of 31 days was performed on 36 capillary DBS samples, originating from healthy volunteers who received an oral dose of 150 mg caffeine (see below). To fulfill the acceptance criterion, for two-thirds of the samples the %difference between the results should be within ± 20 % [29].

A.2.2.7. CYP1A2 phenotyping study

A small scale CYP1A2 phenotyping study, approved by the Ethics Committee of Ghent University Hospital (B670201111655), was conducted in healthy volunteers after obtaining written informed consent from each subject. A 36-hour caffeine abstinence period was asked from the participants prior to oral administration of a 150 mg caffeine test dose. Capillary DBS were collected before intake of the caffeine test dose and approximately 1, 2, 3, 4, 5 and 6 hours thereafter. At the last time point, also venous whole blood samples were collected. From the latter, we prepared plasma samples and venous DBS within 1 hour after sampling. In all matrices (capillary DBS, venous DBS, whole blood and plasma), we determined the concentrations of caffeine and paraxanthine, as well as the corresponding paraxanthine:caffeine molar concentration ratios.

A.2.3. Results and discussion

A.2.3.1. LC-MS/MS parameters

Signal intensities of the molecular ions of caffeine and paraxanthine obtained by continuous infusion of standard solutions in various solvents into the ESI source are displayed in Figure A.2.2., while Figure A.2.3. shows analogous data for the APCI source. For both compounds, highest ionization efficiency was observed using the ESI source in positive ionization mode, as commonly used for determination of caffeine and its dimethylxanthine metabolites paraxanthine, theophylline and theobromine [35-37]. Concerning solvent composition, methanol as organic solvent yielded higher responses compared to

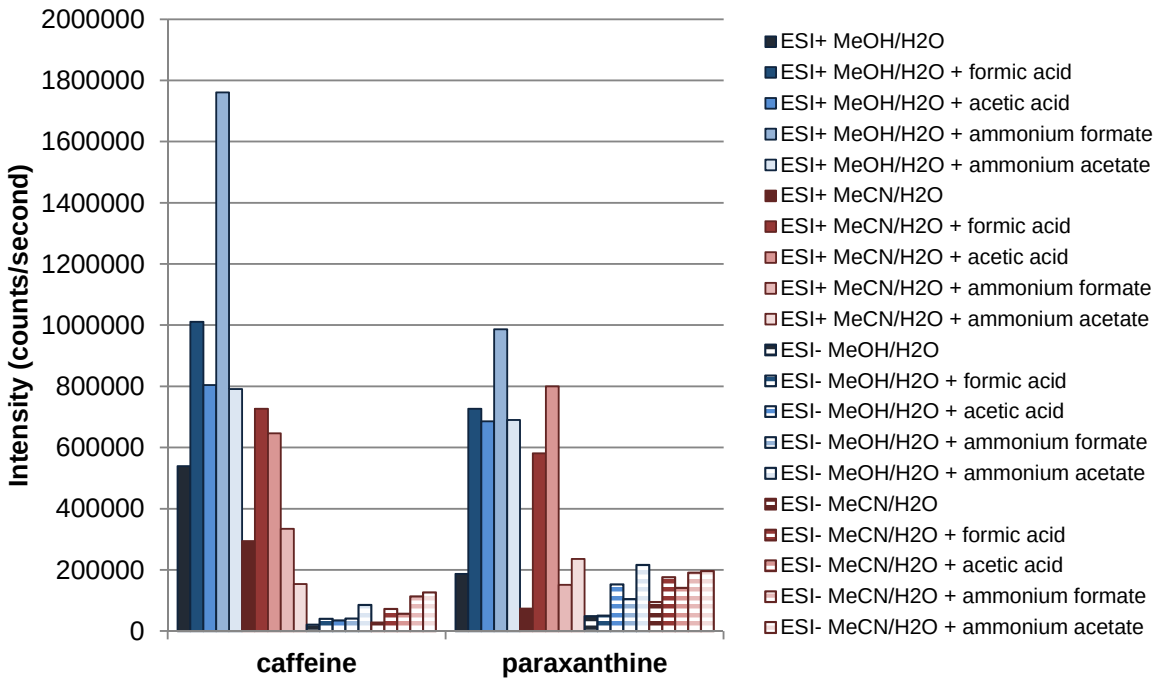


Figure A.2.2. Influence of ionization mode and solvent composition on the signal intensity of molecular ions of caffeine and paraxanthine following continuous infusion into the electrospray ionization (ESI) source.

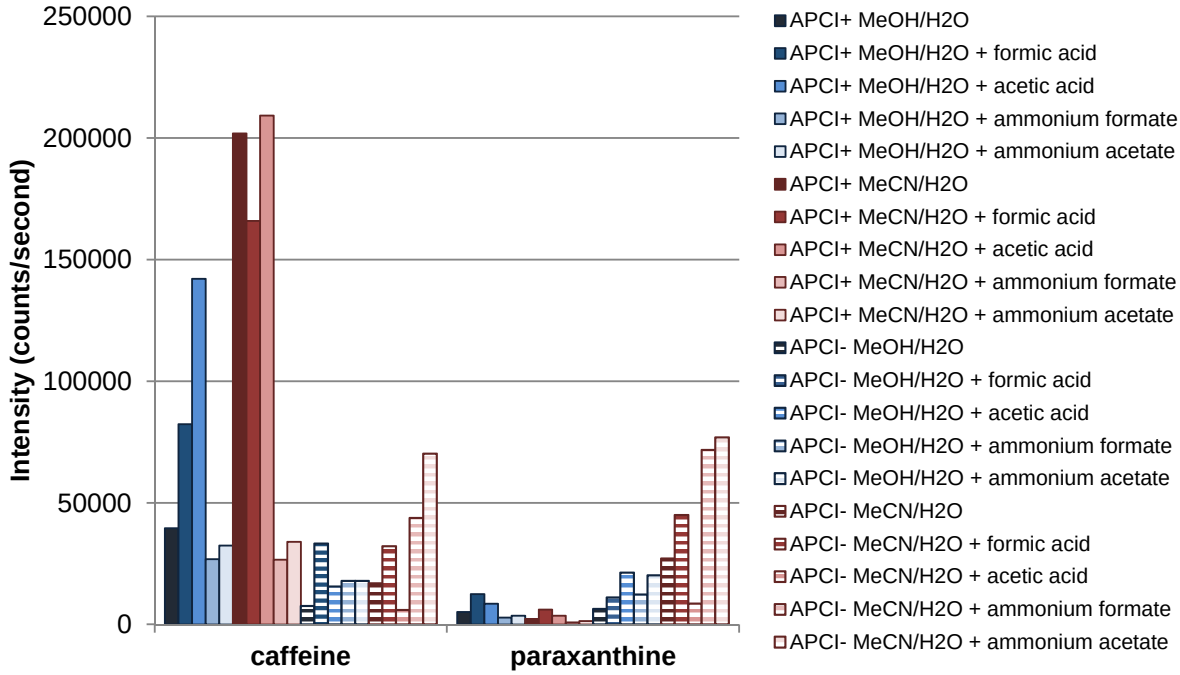


Figure A.2.3. Influence of ionization mode and solvent composition on the signal intensity of molecular ions of caffeine and paraxanthine following continuous infusion into the atmospheric pressure chemical ionization (APCI) source.

acetonitrile, while acidic additives overall further increased MS responses. Based on these findings, methanol/water (MeOH/H_2O; 75/25, v/v), containing ammonium formate (5 mM) or formic acid (0.1 %), in combination with ESI in positive mode was selected for further experiments. Following optimization of the compound-dependent MS parameters, fragmentation patterns were determined. For each compound, 2 product ions were selected based on selectivity and abundance. Resulting MRM transitions and compound parameters are listed in Table A.2.1.

Table A.2.1. Multiple reaction monitoring transitions and compound-specific MS parameters for caffeine, paraxanthine, theophylline, theobromine and internal standards.

	Precursor ion (*m/z*)	**Product ion (*m/z*)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
caffeine	195.2	138.2	31	10	27	10
		110.1	61	10	33	8
paraxanthine	181.2	124.1	36	8	29	8
		142.2	61	10	25	12
theophylline	181.2	124.1	31	10	27	10
		96.1	61	10	33	6
theobromine	181.2	138.3	66	10	27	12
		110.3	66	10	31	8
caffeine-$^{13}C_3$	198.2	140.2	66	10	27	10
paraxanthine-$^{13}C_4$-$^{15}N_3$	188.2	129.2	51	10	29	8

DP: declustering potential, EP: collision cell entrance potential, CE: collision energy, CXP: collision cell exit potential

A fragmentation pathway of caffeine and paraxanthine is shown in Figure A.2.4. For both compounds, the most intense product ions, at *m/z* 138.2 and 124.2 for caffeine and paraxanthine, respectively, originate from the neutral loss of methyl isocyanate (O=C=NCH_3, 57 Da) from the protonated molecular ions due to a retro-Diels Alder rearrangement, as proposed by Bianco *et al.* [37]. Identical fragmentation reactions are responsible for the formation of the product ions of the isotopically labeled IS of caffeine and paraxanthine. For caffeine, further loss of CO (28 Da) from the ion at *m/z* 138.2 results in the formation of the second product ion (*m/z* 110.1). A product ion for paraxanthine at *m/z* 142.2 has also been observed by Bianco *et al.*, although the corresponding fragmentation reaction was not identified.

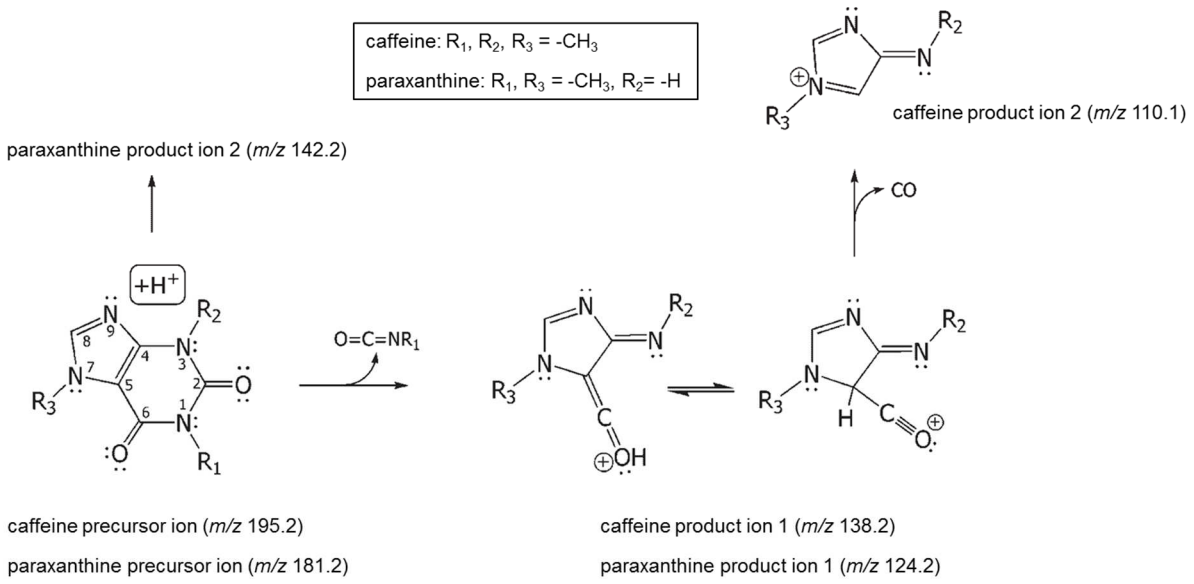


Figure A.2.4. Proposed fragmentation pathway of the protonated precursor ions of caffeine and paraxanthine. Partly adapted from [37].

As can be seen in Table A.2.1., paraxanthine, theophylline and theobromine are isomeric compounds with an identical molecular weight. In addition, especially paraxanthine and theophylline showed very similar fragmentation patterns. To unequivocally quantify these compounds, they need to be chromatographically separated. For this purpose, two different C18 columns (Waters Acquity UPLC® BEH-C18 and HSS-T3) were used in combination with methanol and water, containing ammonium formate (5 mM) or formic acid (0.1 %), as mobile phases. On the BEH-C18 column, paraxanthine and theophylline could not be separated using various gradient elution conditions. Better results were obtained with the HSS-T3 column using an uncommon gradient profile in which the linearly increasing proportion of the organic constituent of the mobile phase was briefly reverted. In addition, formic acid as mobile phase additive gave rise to better peak shapes compared to ammonium formate. Therefore, the influence of different concentrations of formic acid in methanol and water on the response of caffeine and paraxanthine was evaluated. As shown in Figure A.2.5., lower concentrations had no effect on the response of caffeine, while even slightly higher peak areas of paraxanthine were obtained when 0.01 % formic acid was used. In the final optimized method, the mobile phase, consisting of 0.01 % formic acid in water (solvent A) and 0.01 % formic acid in methanol (solvent B), was pumped at a flow rate of 0.4 mL/min. The HSS-T3 column was set at 60°C and the following 8-min gradient elution program was applied: 0.0-1.5 min, isocratic 15 % B; 1.5-2.5 min, linear increase to 60 % B; 2.5-3.1 min, linear decrease to 50 % B; 3.1-3.5 min, linear increase to 95 % B; 3.5-5.5 min, isocratic 95 % B; 5.5-5.8 min, linear decrease to 15 % B; 5.8-8.0 min, isocratic 15 % B. A resulting chromatogram of theobromine, paraxanthine, theophylline and caffeine in a capillary DBS taken from a healthy volunteer six hours after the intake of 150 mg of caffeine, together with the applied gradient, is shown in Figure A.2.6.

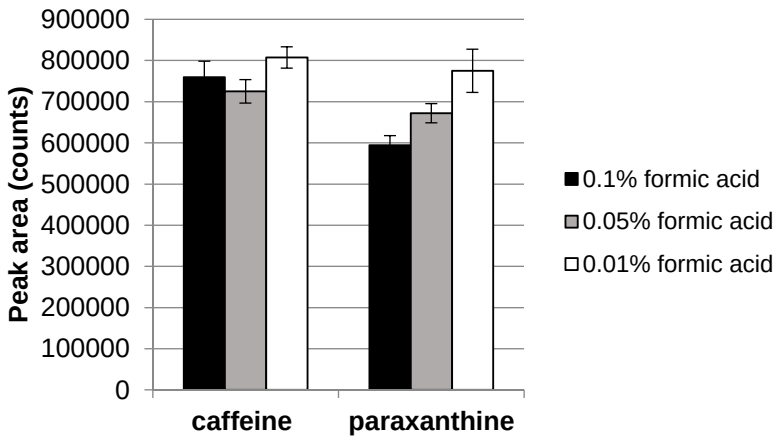


Figure A.2.5. The influence of different concentrations of formic acid in the mobile phase on the response of caffeine and paraxanthine. Mean (n=3) peak areas are shown, with standard deviations.

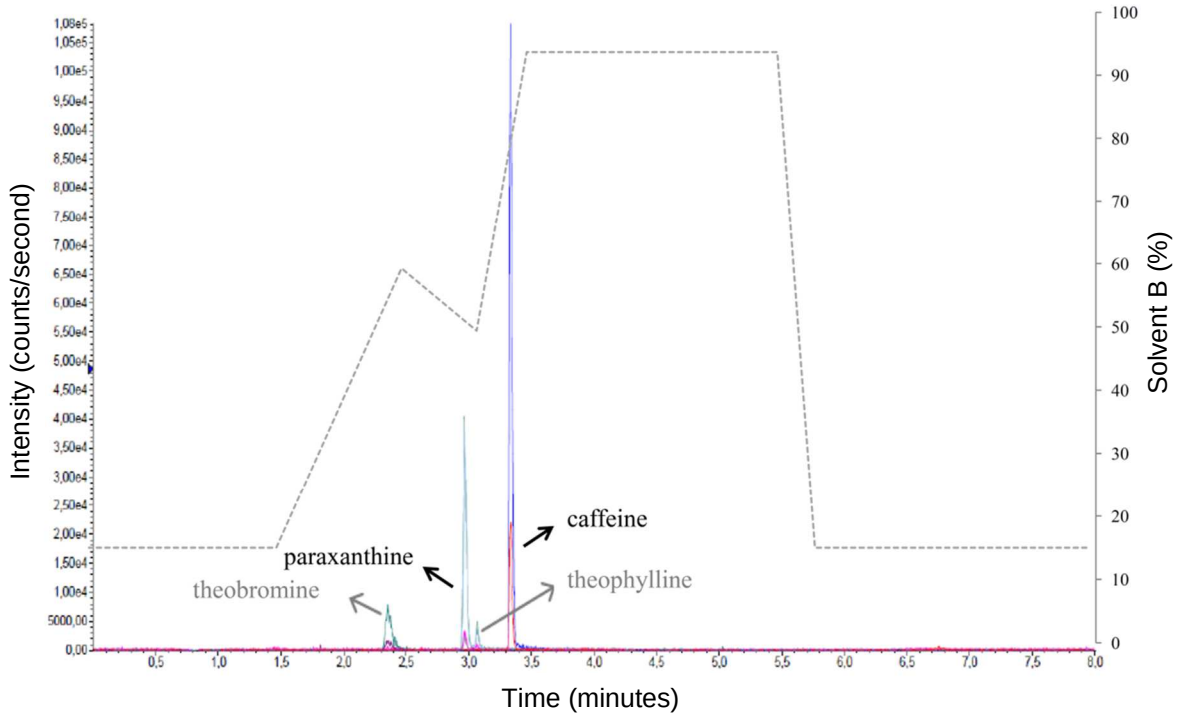


Figure A.2.6. Chromatogram of theobromine, paraxanthine, theophylline and caffeine in a capillary DBS taken from a healthy volunteer six hours after the intake of 150 mg of caffeine. The broken line indicates the proportion of solvent B (methanol containing 0.01% formic acid) in the mobile phase.

A.2.3.2. Filter paper selection and DBS extraction conditions

Whatman 903 filter paper was selected as only extracts from this paper yielded a response for caffeine below 20 % of the LLOQ. Methanol and a methanol/acetonitrile mixture (50/50, v/v) gave comparable results when tested as extraction solvents (Figure A.2.7.). Because of compatibility with the mobile phase, methanol was selected.

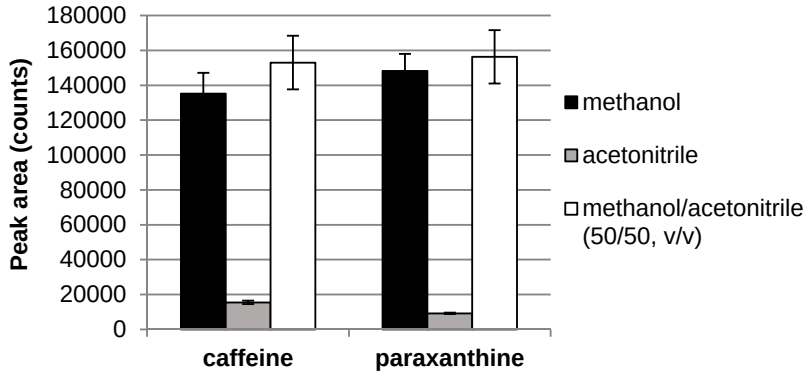


Figure A.2.7. Different solvents to extract caffeine and paraxanthine from DBS. Mean (n=3) peak areas of caffeine and paraxanthine in DBS extracts are shown, with standard deviations.

Addition of water to the extraction solvent resulted in higher extraction efficiencies (Figure A.2.8.), however, when 30 % water was added, extracts became colored and turbid.

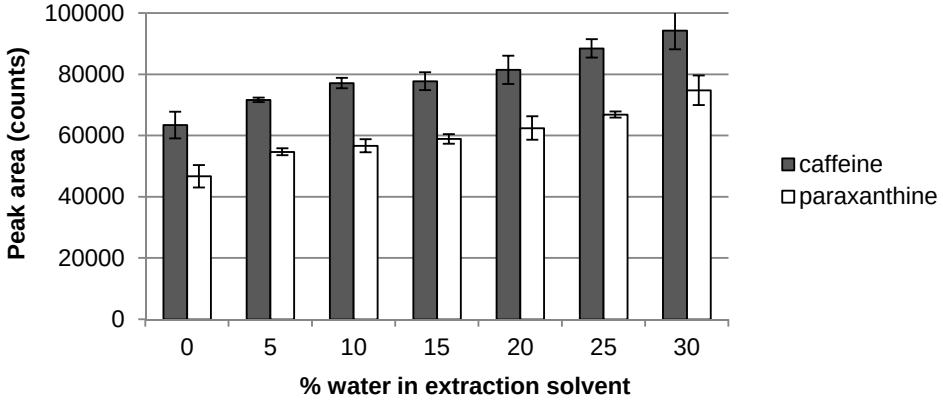


Figure A.2.8. Incremental proportions of water in the solvent (methanol) to extract caffeine and paraxanthine from DBS. Mean (n=3) peak areas of caffeine and paraxanthine in DBS extracts are shown, with standard deviations.

No significant differences were observed when higher volumes of extraction solvent were used or when extraction time was increased (Figure A.2.9. and A.2.10.). We opted to include the IS in the extraction solvent for reasons of convenience, resulting in a straightforward and fast procedure, yet yielding an excellent and reproducible recovery with minimal matrix effects (see further). In the final optimized

procedure, DBS punches were extracted for 10 minutes with 70 µL of a methanol/water (80/20, v/v) mixture, containing 0.01 % formic acid and the IS. This simple manual DBS extraction protocol allows conversion to an automated, online extraction procedure. Combined with the advantages of DBS sampling, this may offer the potential to implement the developed DBS approach in clinical practice or for large population phenotyping studies.

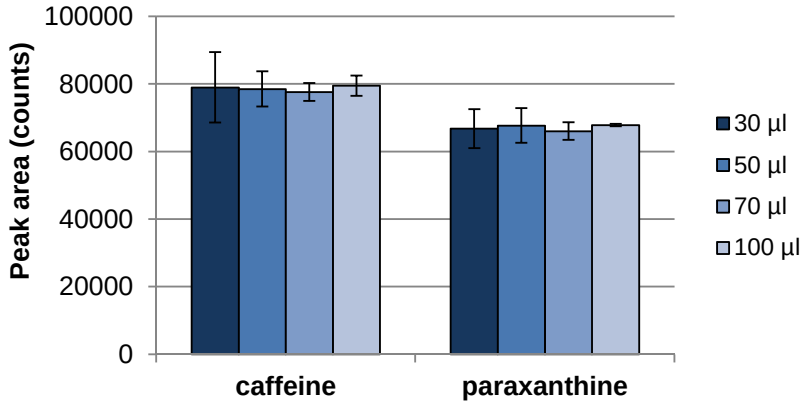


Figure A.2.9. Increasing volumes of extraction solvent (methanol/water (80/20, v/v), containing 0.01% formic acid) to extract caffeine and paraxanthine from DBS. Mean (n=3) peak areas of caffeine and paraxanthine in extracts of DBS are shown, with standard deviations.

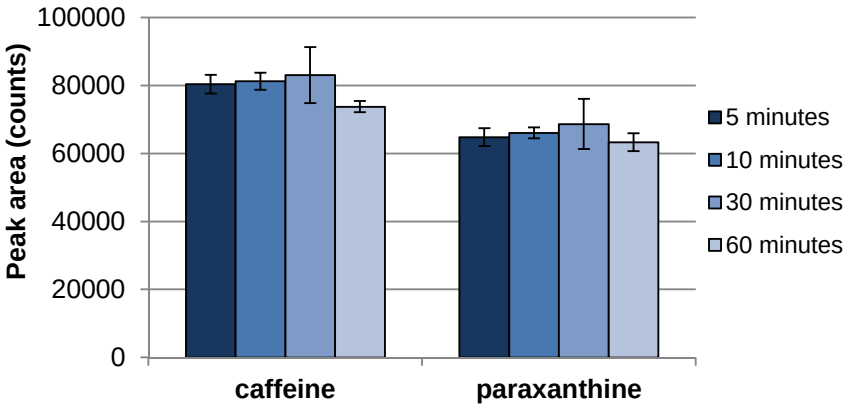


Figure A.2.10. Increasing extraction times for extraction of caffeine and paraxanthine from DBS. Methanol/water (80/20, v/v) with 0.01% formic acid, was used as extraction solvent. Mean (n=3) peak areas of caffeine and paraxanthine in extracts of DBS are shown, with standard deviations.

A.2.3.3. Validation

Although simple and fast extraction procedures were applied for DBS, whole blood and plasma, no unacceptable interferences, cross-interferences or carry-over were observed for any of the evaluated

matrices. As the calibrator data were heteroscedastic, weighted linear regression was applied. The weighting factors, as well as calibration and sensitivity data, are summarized in Table A.2.2. Back-calculated concentrations of the calibrators deviated less than 5 % (DBS), 9 % (whole blood) and 4 % (plasma) from the nominal concentrations, hence, the chosen models met the acceptance criteria and the calibration lines were linear.

Table A.2.2. Calibration and sensitivity data for the determination of caffeine and paraxanthine in DBS (n=5), whole blood and plasma (n=6) using LC-MS/MS.

	Weighting	Slope, mean ± SD [95 % CI]	Intercept, mean ± SD [95 % CI]	R^2	Standard deviation of residuals	Range (µg/mL)*
Caffeine						
DBS	$1/y^2$	0.654 ± 0.071 [0.591 – 0.716]	0.014 ± 0.009 [0.006 – 0.022]	0.998	0.040	0.050 – 10.0
Whole blood	$1/x^2$	0.560 ± 0.069 [0.505 – 0.615]	0.003 ± 0.003 [0.001 – 0.005]	0.997	0.040	0.050 – 10.0
Plasma	$1/x^2$	0.549 ± 0.053 [0.507 – 0.591]	0.003 ± 0.002 [0.001 – 0.005]	0.998	0.014	0.050 – 10.0
Paraxanthine						
DBS	$1/y$	1.688 ± 0.207 [1.507 – 1.870]	0.024 ± 0.012 [0.013 – 0.035]	0.998	0.054	0.025 – 5.0
Whole blood	$1/y^2$	1.336 ± 0.132 [1.230 – 1.441]	0.006 ± 0.003 [0.004 – 0.009]	0.996	0.073	0.025 – 5.0
Plasma	$1/x^2$	1.497 ± 0.100 [1.417 – 1.577]	0.005 ± 0.003 [0.003 – 0.008]	0.998	0.128	0.025 – 5.0

*: The lower end of the range corresponds to the LLOQ. SD: standard deviation, CI: confidence interval, DBS: dried blood spots

As shown in Table A.2.3., also intra- and interbatch precision and accuracy for QCs, analyzed in duplicate on five (DBS), respectively three (whole blood and plasma) different days, fulfilled the predefined criteria (< 15 % RSD and bias; < 20 % RSD and bias for the LLOQ). To further ensure the validity of the DBS procedure, the Hct levels of blood samples used for the preparation of QCs (0.36, 0.43 and 0.50) were chosen to cover the reference ranges for women and men, lying at approximately 0.36 – 0.44 and 0.41 – 0.50, respectively [38]. As the accuracy data indicate, quantification of caffeine and paraxanthine in DBS is overall subject to a Hct effect, with lower concentrations measured at low Hct and higher concentrations at high Hct. As caffeine and its metabolites are able to enter red blood cells but do not bind to cell constituents nor to plasma proteins, blood-to-plasma concentration ratios of these compounds are expected to lie around 0.85 (slightly lower than 1 given the fraction of solid constituents in blood) [39,40]. Therefore, the observed effect of Hct on assay bias is likely to be caused by differential spreading of blood with different Hct on the filter paper [30]. However, %bias remained within ± 15 % for all QCs at the evaluated Hct levels.

Table A.2.3. Intra- and interbatch precision and accuracy for QCs of caffeine and paraxanthine at four concentration levels: (a) in DBS (n=5x2) prepared with whole blood with low (0.36 ± 0.007), intermediate (0.43 ± 0.006) and high (0.50 ± 0.008) Hct; (b) in whole blood (n=3x2); (c) in plasma (n=3x2).

QC	Hct	Intrabatch precision (%RSD)		Interbatch precision (%RSD)		Accuracy (%bias)	
		caffeine	paraxanthine	caffeine	paraxanthine	caffeine	paraxanthine
(a) DBS							
LLOQ	**0.36**	6.56	11.31	8.85	13.26	-8.98	-7.33
	0.43	6.51	11.40	6.51	14.79	2.68	0.82
	0.50	8.64	7.55	8.64	14.32	7.68	-2.92
Low	**0.36**	4.40	4.31	7.53	8.21	-6.79	-11.66
	0.43	4.81	8.48	4.81	8.48	-2.83	-3.38
	0.50	2.90	6.97	7.15	5.47	5.34	-1.37
Medium	**0.36**	3.06	3.21	4.48	7.20	-5.81	-7.21
	0.43	2.91	4.21	3.83	6.11	0.49	0.62
	0.50	5.43	6.21	5.60	8.16	3.57	4.39
High	**0.36**	3.68	3.49	4.64	7.06	-3.86	-7.59
	0.43	2.87	3.20	5.41	6.29	-2.52	-1.69
	0.50	4.59	6.17	6.45	9.40	5.78	7.01
(b) Whole blood							
LLOQ		4.14	5.44	16.12	13.27	-4.58	-8.49
Low		4.55	2.64	7.33	8.50	-2.43	-4.60
Medium		2.97	0.96	4.38	6.01	-1.01	-3.00
High		1.96	1.19	5.13	8.52	-3.98	-2.54
(c) Plasma							
LLOQ		3.02	5.13	10.39	10.09	2.34	2.17
Low		5.47	2.44	5.79	9.18	-1.51	-4.10
Medium		1.21	1.03	3.95	7.52	-0.63	-1.86
High		3.10	1.67	3.30	8.04	-0.48	-0.41

QC: quality control, Hct: hematocrit, RSD: relative standard deviation, DBS: dried blood spots, LLOQ: lower limit of quantification

The absolute matrix effect, evaluated in six different lots of whole blood, plasma and DBS prepared from blood with a Hct range of 0.36 – 0.46, approximated 100 % at all tested concentration levels, even without IS compensation (Table A.2.4.). In addition, relative matrix effects fell within 15 % RSD predefined limits. Using the same six lots of whole blood, plasma and DBS, the recovery of caffeine and paraxanthine was evaluated. An excellent (> 90 %) and reproducible recovery (RSD < 9 %) was observed in DBS. Also in plasma the recovery of both compounds was high (> 90 %) and reproducible (RSD < 9 %). The recovery of caffeine in whole blood ranged from 81.93 to 83.33 % and was found to be reproducible (RSD < 8 %). The recovery of paraxanthine in whole blood ranged from 72.14 to 73.31

% and was reproducible as well (RSD < 14 %). All recovery data approximated 100 % when compensation by the IS was taken into account (Table A.2.4.).

Table A.2.4. Absolute and relative matrix effect and recovery data (n=6) for caffeine and paraxanthine: (a) in DBS (four concentration levels); (b) in whole blood; (c) in plasma originating from six different lots of whole blood (Hct range 0.36 – 0.46).

QC	Absolute matrix effect (mean ± SD, %)		Relative matrix effect (%RSD)		Absolute recovery (mean ± SD, %)		Relative recovery (%RSD)	
	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS
Caffeine								
(a) DBS								
LLOQ	99.10 ± 6.02	98.60 ± 4.52	6.07	4.59	95.36 ± 5.45	100.18 ± 4.64	5.72	4.63
Low	101.27 ± 2.35	99.40 ± 2.13	2.33	2.15	97.03 ± 1.48	101.56 ± 2.05	1.53	2.02
Medium	98.42 ± 0.84	99.73 ± 1.34	0.85	1.34	93.71 ± 2.40	96.98 ± 2.35	2.56	2.43
High	98.07 ± 1.05	99.48 ± 2.19	1.07	2.20	94.91 ± 3.67	99.56 ± 4.03	3.87	4.04
(b) Whole Blood								
Low	103.57 ± 3.16	96.78 ± 1.78	3.05	1.83	83.33 ± 3.47	100.52 ± 5.00	4.17	4.98
High	102.55 ± 2.60	102.04 ± 5.01	2.53	4.91	81.93 ± 4.58	103.94 ± 7.67	5.59	7.38
(c) Plasma								
Low	95.41 ± 5.24	98.78 ± 4.10	5.49	4.15	97.60 ± 8.00	99.30 ± 7.03	8.20	7.08
High	98.57 ± 4.08	102.29 ± 3.29	4.14	3.21	95.87 ± 4.63	98.40 ± 4.59	4.83	4.66
Paraxanthine								
(a) DBS								
LLOQ	106.32 ± 4.31	101.83 ± 3.53	4.06	3.46	92.90 ± 4.68	101.33 ± 5.35	5.04	5.28
Low	101.37 ± 2.05	98.46 ± 2.47	2.02	2.51	92.60 ± 1.95	100.20 ± 3.00	2.11	2.99
Medium	99.82 ± 1.69	98.17 ± 1.26	1.69	1.29	91.02 ± 2.95	97.21 ± 2.73	3.24	2.81
High	100.35 ± 1.40	102.61 ± 1.13	1.40	1.10	92.21 ± 7.56	100.64 ± 4.08	8.20	4.05
(b) Whole Blood								
Low	104.00 ± 4.33	98.73 ± 1.44	4.17	1.46	73.31 ± 7.38	98.44 ± 3.94	10.06	4.00
High	104.98 ± 2.26	99.63 ± 1.60	2.15	1.60	72.14 ± 9.59	101.43 ± 5.39	13.29	5.32
(c) Plasma								
Low	98.06 ± 2.41	101.58 ± 1.23	2.45	1.21	95.41 ± 7.36	97.68 ± 7.95	7.71	8.14
High	96.27 ± 3.55	99.68 ± 2.14	3.68	2.15	94.05 ± 3.61	98.35 ± 4.74	3.84	4.82

Hct: hematocrit, QC: quality control, SD: standard deviation, RSD: relative standard deviation, IS: internal standard, DBS: dried blood spots, LLOQ: lower limit of quantification

Apart from the impact on assay bias (and precision), the Hct may also affect recovery or matrix effect in DBS analysis, as described by Youhnovski *et al.*, who observed a lower recovery of naproxen at high Hct levels (no change in matrix effect was seen) [41]. We therefore also evaluated whether a broad Hct range (0.19 – 0.63), covering over 99% of the Hct values observed in a hospital population (see Chapter

B.1., Figure B.1.1.), might have an impact on absolute recovery and matrix effects when quantifying caffeine and paraxanthine concentrations in DBS. As can be deduced from Figure A.2.11. and A.2.12., no Hct impact was present. Noteworthy is that -owing to the efforts that were put in optimizing chromatography and developing a robust DBS extraction procedure- even without IS compensation, matrix effects were minimal and recovery was excellent, also at extreme Hct values.

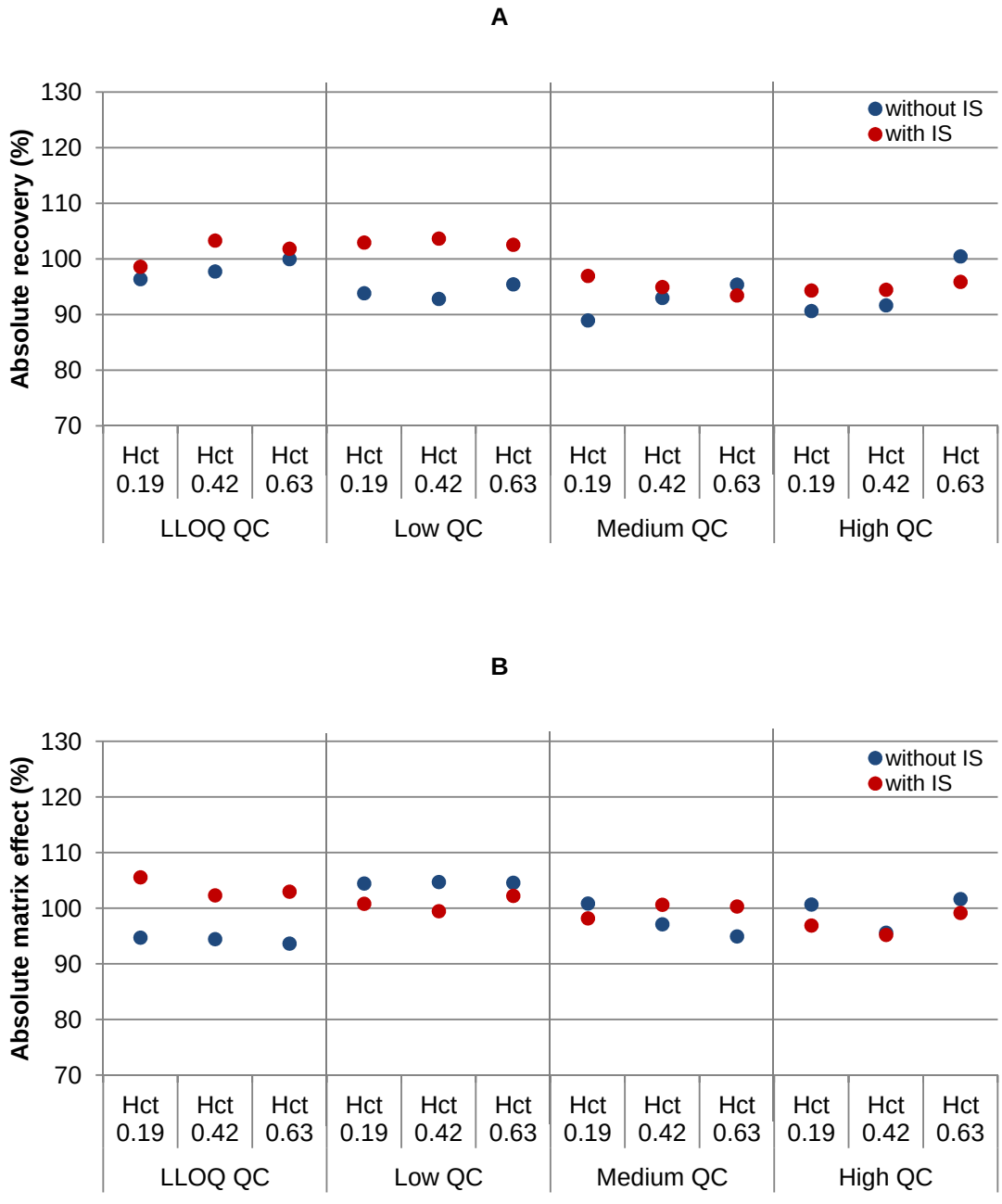


Figure A.2.11. Absolute recovery and matrix effects for caffeine (A and B, respectively) at four concentration levels in DBS prepared from whole blood with varying Hct levels (0.19; 0.42; 0.63).

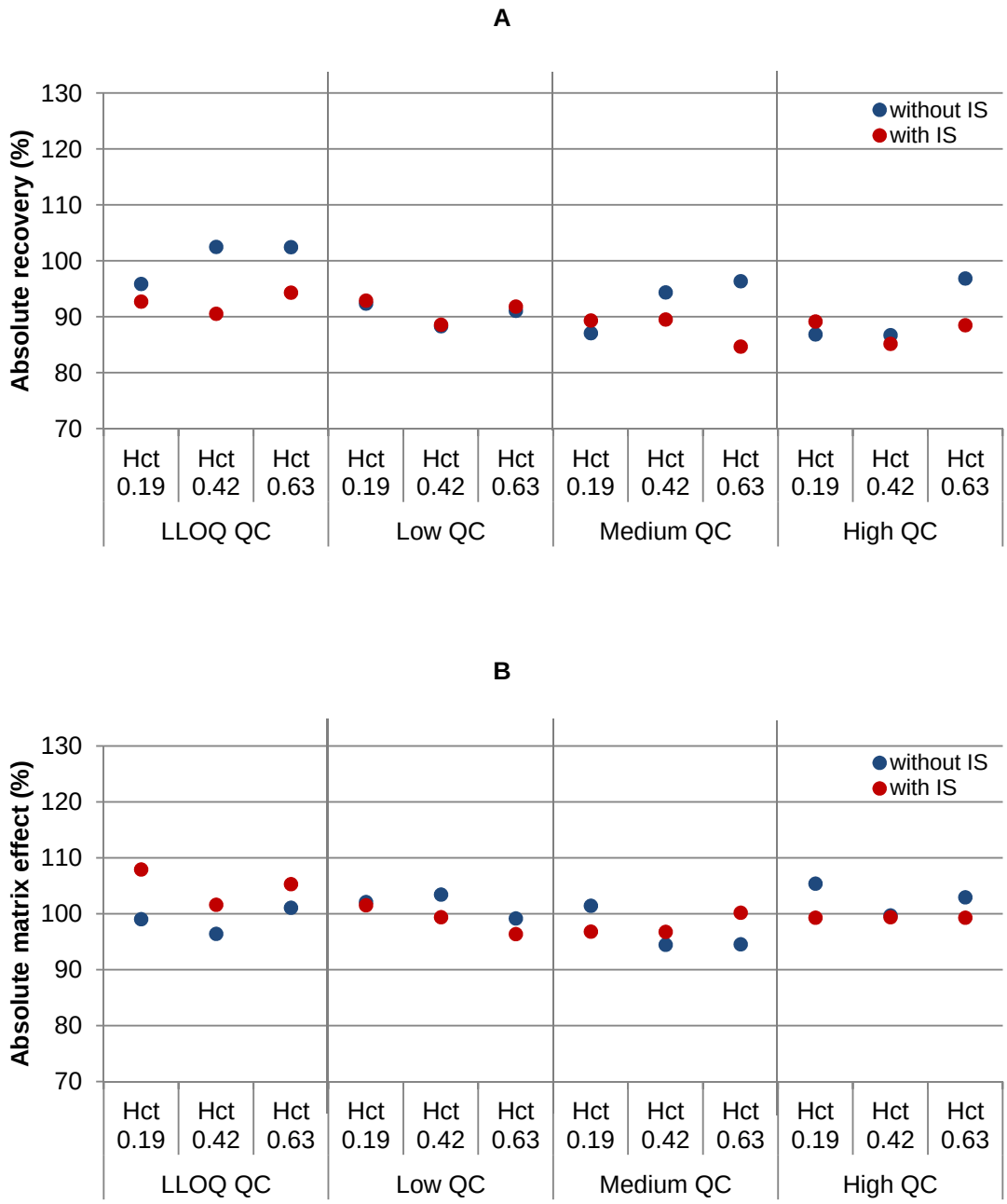


Figure A.2.12. Absolute recovery and matrix effects for paraxanthine (A and B, respectively) at four concentration levels in DBS prepared from whole blood with varying Hct levels (0.19; 0.42; 0.63).

Short- and long-term stability data of caffeine and paraxanthine in DBS at three Hct levels for low and high QCs are shown in Table A.2.5. DBS were stable for at least 4 days when stored at 50 °C and for at least 324, 53 and 36 days at room temperature, -20 and 4 °C, respectively. These results indicate that DBS can be transported and stored for long periods (up to 324 days) at ambient temperature and even will remain stable for up to 4 days under more extreme conditions (50 °C), as can be encountered during shipping. Additionally, the stability data demonstrate that extraction efficiency was consistent irrespective of the age and Hct of DBS under the storage conditions tested. Also in whole blood and plasma, both compounds were stable for at least 172 days when stored at -20 °C and for at least 7 days

when stored at 4 °C (Table A.2.6.). Processed samples, re-injected after 24-h storage in the autosampler and after 6- (whole blood, plasma) or 7-day (DBS) storage at -20 °C, were stable as well. Stock solutions of the analytes and the IS stored at -20 °C were stable for at least 6 months.

Table A.2.5. Short- and long-term stability of low and high QC samples of caffeine and paraxanthine in DBS (n=6) prepared using whole blood with low (0.36 ± 0.007), intermediate (0.43 ± 0.006) and high (0.50 ± 0.008) Hct levels. Samples were stored at 50 °C, room temperature, 4 °C and -20 °C. Data are presented as the percentage of the concentrations measured at time point zero.

QC	Hct	50 °C 4 days (mean ± SD, %)		Room temperature 324 days (mean ± SD, %)	
		caffeine	paraxanthine	caffeine	paraxanthine
low	**0.36**	100.69 ± 5.75	101.78 ± 3.51	99.67 ± 4.14	98.43 ± 4. 78
	0.43	97.96 ± 5.39	102.90 ± 6.02	103.95 ± 2.89	101.91 ± 9 .15
	0.50	98.55 ± 4.43	97.10 ± 7.29	97.98 ± 6.19	98.60 ± 9.17
high	**0.36**	102.37 ± 3.20	95.68 ± 3.54	98.99 ± 3.18	94.83 ± 4.3 3
	0.43	98.43 ± 5.14	96.92 ± 3.60	101.20 ± 0.99	95.10 ± 6.9 5
	0.50	94.43 ± 3.59	92.21 ± 4.99	100.19 ± 6.89	97.13 ± 6.5 8

QC	Hct	-20 °C 53 days (mean ± SD, %)		4 °C 36 days (mean ± SD, %)	
		caffeine	paraxanthine	caffeine	paraxanthine
low	**0.36**	100.40 ± 8.35	103.15 ± 0.14	104.06 ± 4.88	102.00 ± 4.28
	0.43	101.38 ± 6.78	101.93 ± 5.91	103.92 ± 7.54	105.12 ± 5.14
	0.50	95.71 ± 4.59	100.91 ± 10.14	100.64 ± 1.94	101.93 ± 6.02
high	**0.36**	97.80 ± 3.31	100.90 ± 5.90	99.67 ± 5.75	98.12 ± 4.6 0
	0.43	99.45 ± 4.59	98.61 ± 5.03	100.47 ± 3.72	98.11 ± 4.1 1
	0.50	94.28 ± 4.51	99.03 ± 3.95	101.22 ± 3.50	97.91 ± 3.2 0

QC: quality control, Hct: hematocrit, SD: standard deviation

Table A.2.6. Stability of low and high QC samples of caffeine and paraxanthine in whole blood and plasma (n=3). Samples were stored at -20 °C and 4 °C. Data are presented as the percentage of the concentrations measured at time point zero.

QC	-20 °C 172 days (mean ± SD, %)		4 °C 7 days (mean ± SD, %)	
	caffeine	paraxanthine	caffeine	paraxanthine
Whole blood				
Low	103.06 ± 1.07	101.80 ± 8.41	101.00 ± 5.23	101.58 ± 4.24
High	102.54 ± 4.56	99.14 ± 2.52	100.78 ± 8.41	100.41 ± 1 6.74
Plasma				
Low	100.43 ± 0.41	103.01 ± 6.13	98.39 ± 4.56	100.25 ± 1 .55
High	101.35 ± 1.73	103.83 ± 5.81	98.37 ± 0.80	104.98 ± 2 .01

Incurred sample reanalysis, performed on 36 capillary DBS samples from study participants (see further), revealed a significant positive difference between repeated and original caffeine and paraxanthine concentrations. However, for only one sample the paraxanthine concentration measured during repeated analysis deviated more than 20 % from the original measurement (Figure A.2.13. A,B). Therefore, the acceptance criterion was met. For the paraxanthine:caffeine molar ratios, no significant difference was found, as these were evenly distributed around 0 %, with all data points lying within the ± 20 % acceptance limits (Figure A.2.13. C).

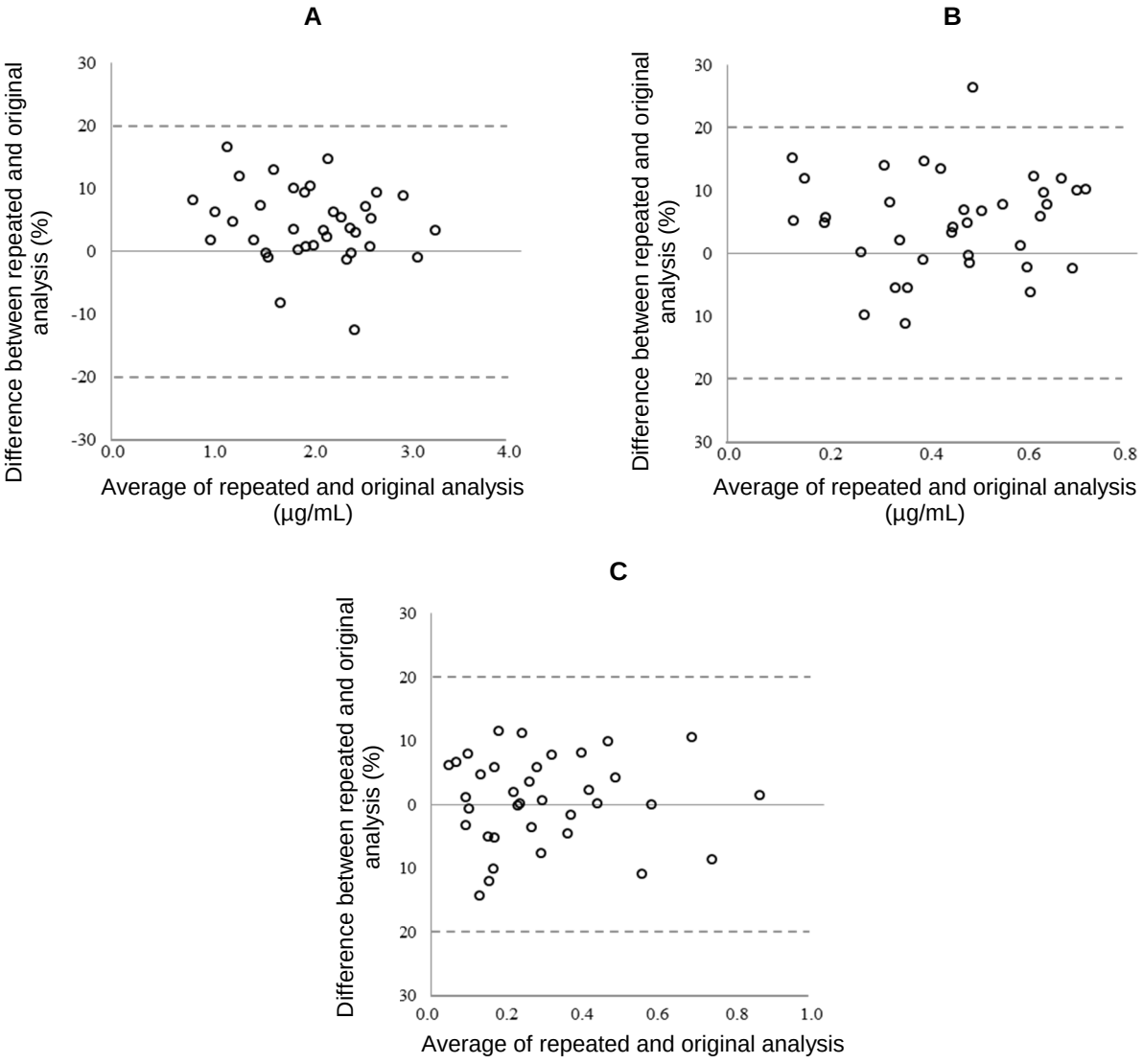


Figure A.2.13. Incurred sample reanalysis for caffeine (A), paraxanthine (B) and the paraxanthine:caffeine molar concentration ratio (C) performed on 36 capillary DBS samples of volunteers who received 150 mg caffeine. The % difference between the repeated and the original analysis is plotted against the average of concentrations (µg/mL) or molar concentration ratios. Broken lines indicate the ± 20 % difference acceptance limits.

A.2.3.4. CYP1A2 phenotyping in healthy volunteers

Capillary DBS were collected from six healthy volunteers (age 24 - 36 years; 2 women; 1 smoker; more data are listed in Table A.2.7.) before oral intake of a 150-mg caffeine capsule and after approximately 1, 2, 3, 4, 5 and 6 hours, allowing the construction of pharmacokinetic profiles of caffeine and paraxanthine. These concentration-time profiles in capillary DBS for all participants are displayed in Figure A.2.14. Concentrations of both compounds, measured in 3-mm DBS punches using the developed method, fell into the linear concentration ranges over the entire sampling period. At the 6 hour time point, also venous blood was collected, which was used for generating venous DBS and plasma. Figure A.2.14. A illustrates that this time point was beyond T_{max} of caffeine for all participants, indicating that absorption of the caffeine dose was completed. From all samples, paraxanthine:caffeine molar concentration ratios, i.e. the CYP1A2 phenotyping metric, were calculated (Table A.2.7.). Highly comparable ratios were measured in the different matrices. Paired samples t-tests ($\alpha = 0.01$) revealed no significant differences between the phenotyping metrics in capillary DBS, venous DBS, whole blood and plasma. These results are in accordance with other phenotyping studies, in which good correlations were found between concentrations of caffeine and paraxanthine [27] or paraxanthine:caffeine ratios [26] in DBS and plasma, the latter being the reference matrix for CYP1A2 phenotyping. These findings have served as a basis for the set-up of a large comparative phenotyping study, the results of which are described in Chapter A.3.

Table A.2.7. Paraxanthine:caffeine molar concentration ratios of six healthy volunteers (including data on sex, age, smoking habits and daily caffeine consumption) measured in capillary DBS, venous DBS, whole blood and plasma six hours after the intake of a 150-mg oral dose of caffeine.

Volunteer	Sex	Age (years)	Smoker	Caffeine consumption*	Paraxanthine:caffeine molar concentration ratio			
					Capillary DBS	Venous DBS	Whole blood	Plasma
1	male	36	no	medium	0.28	0.28	0.29	0.28
2	male	27	no	high	0.43	0.45	0.47	0.43
3	male	25	no	medium	0.63	0.62	0.72	0.60
4	male	26	no	none	0.83	0.86	0.89	0.81
5	female	24	no	low	0.42	0.40	0.42	0.38
6	female	29	yes	medium	0.23	0.22	0.23	0.19

DBS: dried blood spots

* Caffeine consumption was defined as follows: none = 0 caffeine containing drinks/day; low = 1 – 3 caffeine containing drinks/day; medium = 4 – 6 caffeine containing drinks/day; high = 7 or more caffeine containing drinks/day.

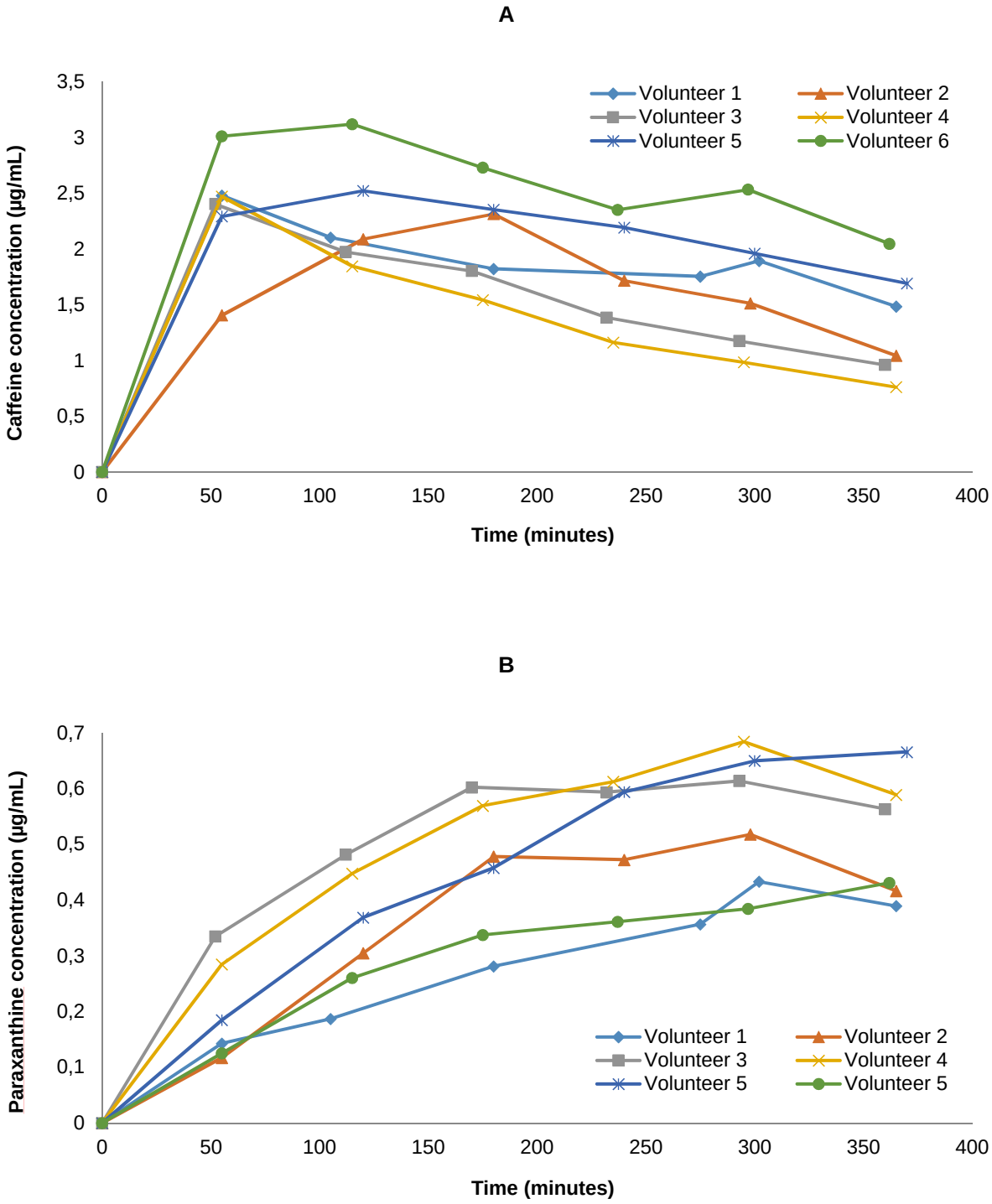


Figure A.2.14. Concentration-time profiles of caffeine (A) and paraxanthine (B) in capillary DBS from 6 healthy volunteers who received an oral 150-mg dose of caffeine. It should be noted that the scale of the y-axes of graph A and B is different.

A.2.4. Conclusion

In this study, caffeine and paraxanthine were determined in DBS and microvolumes of whole blood and plasma using LC-MS/MS-based methods in the context of CYP1A2 phenotyping. The developed methods were fully validated based on EMA and FDA guidelines on bioanalytical method validation [28, 29]. All evaluated parameters met the pre-established criteria. Straightforward and fast extraction procedures were applied for the different matrices, yielding excellent extraction efficiencies and no matrix effects. For DBS-based quantitation of caffeine and paraxanthine, special attention was paid to the impact of Hct, a widely recognized challenging factor in DBS analysis. An effect of Hct on assay bias was observed, although deviations were within acceptable limits of ± 15 %. Varying Hct levels had no apparent influence on the precision of the method, nor on recovery or matrix effects. The validated methods were applied in a phenotyping study in which 6 healthy volunteers received a 150-mg oral dose of the test probe caffeine. No significant differences were observed between CYP1A2 phenotyping indices, i.e. paraxanthine:caffeine molar concentration ratios, determined in capillary DBS, venous DBS, whole blood and plasma 6 hours post-administration, suggesting the applicability and usefulness of DBS-based CYP1A2 phenotyping as a convenient alternative for the classical plasma-based approach. As mentioned above, the results of the described CYP1A2 phenotyping study, conducted in 6 healthy volunteers, need to be confirmed on the basis of data obtained from a larger test group, allowing thorough statistical evaluation of the correlation of the paraxanthine:caffeine ratio in capillary DBS and plasma, the reference matrix for CYP1A2 phenotyping (see Chapter A.3.).

A.2.5. References

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Chapter A.3.

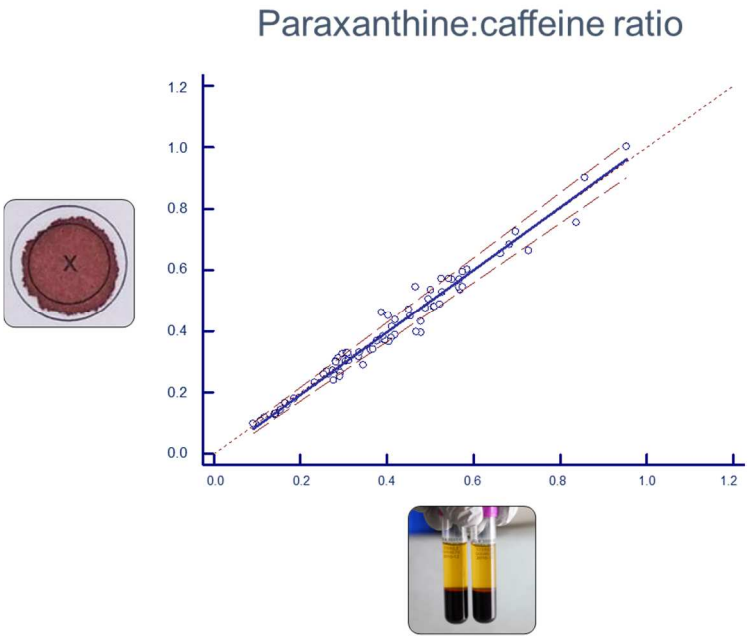
CYP1A2 phenotyping in healthy volunteers using dried blood spots and microvolumes of whole blood and plasma

Based on

De Kesel PM, Lambert WE, Stove CP. Why dried blood spots are an ideal tool for CYP1A2 phenotyping. Clin Pharmacokinet. 2014;53(8):763-71.

Abstract

Dried blood spot (DBS) sampling has gained wide interest in bioanalysis during the last decade. Also in pharmacokinetic and phenotyping studies, DBS-based sampling has already been successfully applied. However, all available phenotyping studies used small data sets and did not include a systematic evaluation of DBS-specific parameters. The latter is important since several of these factors still challenge the breakthrough of DBS in routine practice. In this study, caffeine and paraxanthine are determined in capillary DBS, venous DBS, whole blood and plasma for CYP1A2 phenotyping. The aim of the study described in this chapter was to explore the usefulness of DBS as a tool for CYP1A2 phenotyping. Therefore, a CYP1A2 phenotyping study was conducted in seventy-three healthy volunteers, who received a 150 mg oral dose of caffeine. Six hours post-administration, caffeine and paraxanthine concentrations and paraxanthine:caffeine molar concentration ratios, i.e. the actual CYP1A2 phenotyping indices, were determined in capillary DBS (obtained by non-volumetric application, direct from the fingertip), venous DBS, whole blood and plasma. Furthermore, the impact of DBS-specific parameters, including hematocrit, volume spotted and punch location, was evaluated. Concentrations of caffeine and paraxanthine in capillary DBS were on average 12.7 respectively 13.8 % lower than those in venous DBS and 31.5 respectively 33.1 % lower than those in plasma. While these differences were statistically significant ($p < 0.001$), no significant difference was observed between the paraxanthine:caffeine molar ratios in the distinct evaluated matrices ($p \geq 0.053$). This ratio also alleviated the impact of hematocrit and volume spotted. In conclusion, we have demonstrated that CYP1A2 phenotyping in capillary DBS is a valid and convenient alternative for the classical plasma-based approach. Additionally, we have provided an objective basis as to *why* DBS are an ideal tool for CYP1A2 phenotyping.



A.3.1. Introduction

During the last decade, dried blood spot (DBS) sampling has become a widely used microsampling technique in bioanalysis, owing to the development of more sensitive analytical techniques and various benefits offered by the technique itself [1-5]. Among the advantages are minimal invasiveness, easy sample collection and improved compound stability. This allows the collection of representative samples by an informed patient himself in his home-environment, followed by convenient and cost-effective transfer, e.g. via regular mail, to the analytical laboratory [6].

However, despite the many advantages, several issues concerning DBS analytics and the potential to fully replace venous sampling still remain. First of all, being a capillary sampling technique, the correlation between venous and capillary concentrations is one of the key points that needs to be evaluated when setting up DBS-based methods [7, 8]. Furthermore, adequate interpretation of DBS results requires comparison with plasma or serum concentrations, as clinical reference intervals are commonly based on the latter [9]. Finally, potential bias introduced by the spotting technique itself must be excluded, for example by comparing concentrations measured in venous whole blood samples with concentrations in venous DBS. From an analytical perspective, the (combined) influence of hematocrit (Hct), blood volume spotted and punch localization on a DBS result are important challenges to be addressed during method development and validation [2, 3, 10-12]. The degree of bias caused by these parameters needs to be evaluated on a case-by-case basis since it may be influenced by several factors, such as compound characteristics, filter paper type or extraction conditions.

CYP1A2, an enzyme whose activity is subject to large inter-individual variability, is involved in the metabolism of several therapeutic drugs, including the antipsychotics clozapine and olanzapine. Consequently, the response to these drugs will be influenced by CYP1A2 status [13]. Since CYP1A2 activity is determined by genetic, environmental and nongenetic endogenous factors [14-16], phenotyping with a selective substrate rather than genotyping is commonly used to assess enzyme activity. Caffeine is widely accepted as model substrate of CYP1A2, owing to its safety profile and since the N3-demethylation to paraxanthine is almost uniquely controlled by CYP1A2. Measuring caffeine clearance via a full concentration-time profile is considered as the gold standard for CYP1A2 activity determination [17]. However, as this approach is inconvenient, the paraxanthine:caffeine ratio in plasma, measured 5-7h after controlled caffeine administration (100–200 mg), is a generally accepted metric for this purpose [18, 19].

A proof-of-principle of DBS-based phenotyping has already been shown for CYP3A4 [20] and CYP2C9 [21]. Very recently, two groups reported on the use of a cocktail approach to simultaneously phenotype multiple enzymes, including CYP1A2, in DBS [22, 23]. However, all these studies were conducted in a very limited number of volunteers ($n \leq 16$), hampering thorough statistical evaluation. In all cases, except for the study by Donzelli *et al*. [23], blood spots were volumetrically applied to the filter paper in a controlled environment [20-22]. Moreover, no systematic investigation of parameters potentially influencing the phenotyping index has been undertaken, yet. Therefore, the main objective of the study described in this chapter was to explore the usefulness of DBS as a matrix for CYP1A2 phenotyping.

A.3.2. Material and methods

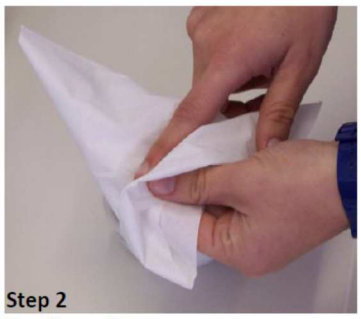
A.3.2.1 CYP1A2 phenotyping study

A CYP1A2 phenotyping study was conducted in healthy volunteers. The study was approved by the Ethics Committee of Ghent University Hospital (B670201111655). Sample size was selected based on the following factors: i) feasibility of the study; ii) the possibility to conduct statistical analyses, such as Bland-Altman comparison and Passing-Bablok regression analysis; iii) estimation of the required sample size to investigate differences between caffeine and paraxanthine concentrations and paraxanthine:caffeine ratios at a significance level of 0.05 and power of 90%. The latter calculation was based on data of the preliminary CYP1A2 phenotyping study in dried blood spots, whole blood and plasma described in Chapter A.2. [24]. Relevant differences between caffeine and paraxanthine concentrations in the different matrices were chosen at 0.08 and 0.04 µg/mL respectively, being 10% of the lowest concentrations measured in the preliminary study. For the paraxanthine:caffeine ratio, a difference of 0.05 was considered relevant. The highest required sample size for the intended comparisons between the different matrices resulting from this calculation was 59. Based on these data, a sample size of 73 individuals was considered adequate for the planned study. Written informed consent was obtained from each subject. Participants were asked to cease caffeine ingestion for 36 hours prior to intake of a capsule containing 150 mg caffeine. Capillary DBS were collected 5 minutes before and 6 hours after administration. At the 6-hour time point (± 5 minutes), venous whole blood samples were collected as well. Plasma samples and venous DBS were prepared within 1 hour after blood sampling. Concentrations of caffeine and paraxanthine (and the corresponding paraxanthine:caffeine molar concentration ratios) were determined in capillary DBS, venous DBS, whole blood and plasma.

A.3.2.2. Sample collection

Capillary DBS were obtained by direct application of a drop of blood from the fingertip onto Whatman 903 filter paper cards (WHA10334885, GE Healthcare, Dassel, Germany). Instructions on good sampling practices were given to all participants by means of a hands-on demonstration and an illustrated flyer (Figure A.3.1.). Following disinfection of the fingertip, finger pricks were made with an automated lancet (VAC366594, Becton Dickinson, Franklin Lakes, NJ, USA). The first drop of blood was wiped off and the following drops, at least four for every participant, were collected on the DBS cards, thereby ensuring that a single drop was used for every spot and avoiding direct contact between filter paper and fingertip. DBS of which both sides of the filter paper were colored were analyzed. Venous blood, collected in EDTA tubes (Venosafe® 4 mL VF-054SDK, Terumo, Leuven, Belgium), was used for preparing venous DBS by spotting 25 µL of blood onto Whatman 903 filter paper using a calibrated pipette and for preparing plasma. Liquid samples were stored at -20 °C, DBS were dried for at least 2 hours and stored at ambient temperature in zip-closure plastic bags, containing two 5-g packets of desiccant (Minipax® absorbent packets, Sigma-Aldrich, Diegem, Belgium), until analysis. For evaluating DBS-specific parameters, venous whole blood from a caffeine abstinent, healthy, male volunteer was collected in EDTA tubes (Venosafe® 9 mL VF-109SDK, Terumo).

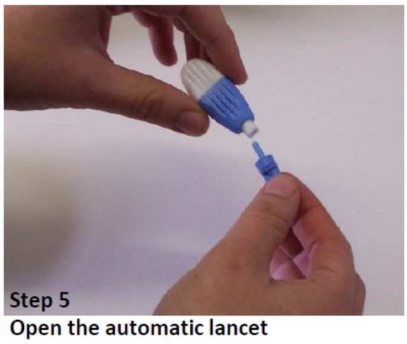
Step 1
Submerge fingertip in warm water bath and/or keep arm downwards



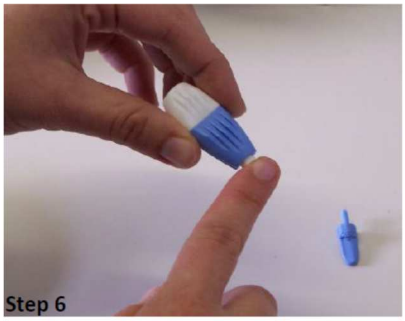
Step 2
Dry fingertip

Step 3
Disinfect the fingertip

Step 4
Dry fingertip

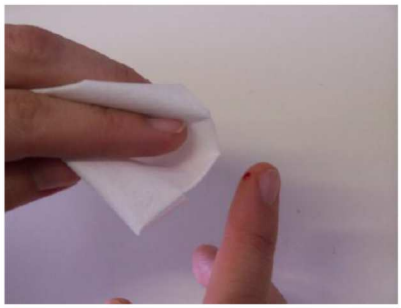


Step 5
Open the automatic lancet



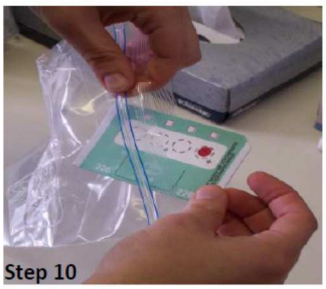
Step 6
Punch the lancet against the fingertip, which will be pricked automatically

Step 7
Wipe off the first drop of blood



Step 8
Collect the following drops on the DBS cards in the preprinted circles
Apply one drop per spot and avoid direct contact between finger and DBS card

Step 9
Disinfect the fingertip



Step 10
Dry for 2 h at room temperature before storing the DBS card in a zip-closure plastic bag with dessicant

Figure A.3.1. Instruction folder on how to collect capillary DBS by finger prick, supplied to the CYP1A2 phenotyping study participants.

A.3.2.3. Chemicals

Caffeine, paraxanthine, theophylline, theobromine, the internal standards (IS) caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$, and formic acid were purchased from Sigma-Aldrich. Methanol of LC-MS quality was obtained from Biosolve (Valkenswaard, The Netherlands). A Synergy® Water Purification System (Merck Millipore, Overijse, Belgium) provided ultrapure water.

A.3.2.4. Sample preparation and analysis

For all DBS analyses, 3-mm discs were punched from the center of a DBS using a Harris micro-punch and cutting mat (Sigma-Aldrich). As capillary DBS were generated in a non-volumetric way, resulting in spots with varying sizes, we opted to punch 3-mm discs. These were extracted with 70 µL of a methanol/water (80/20, v/v) mixture, containing 0.01% formic acid and the internal standards. For plasma and whole blood, we used a protein precipitation procedure, starting from 50 µL sample, to which 10 µL of IS solution was added, prior to the addition of 100 µL methanol, containing 0.01% formic acid. After centrifugation, supernatants were diluted with water, containing 0.01% formic acid, until starting conditions were reached. Full details on sample preparation procedures are described in Chapter A.2., Section A.2.2.4. [24].

All samples were analyzed on a Waters Acquity UPLC® system (Waters, Milford, MA, USA) coupled to an AB SCIEX API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA), using the method described in Chapter A.2., Section A.2.2.2.2. [24]. Quantitative analyses were performed for caffeine and paraxanthine. Theophylline and theobromine, being metabolites of caffeine with the same molecular mass as paraxanthine, were qualitatively monitored in every analytical run to ensure selectivity. The methods used were fully validated based on U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [25, 26]. No interferences or carry-over were observed for any of the matrices. Linear calibration curves were obtained for both caffeine and paraxanthine (respectively 0.05-10.0 and 0.025-5.0 µg/ml). Intrabatch precision (%RSD; %relative standard deviation) was below 11.40% (DBS), 5.44 % (whole blood) and 5.47% (plasma), while interbatch precision (%RSD) was below 14.79% (DBS), 16.12% (whole blood) and 10.39% (plasma). Accuracy (%bias) was below 11.66% (DBS), 8.49% (whole blood) and 4.10% (plasma). All matrix effect and recovery data approximated 100% and were reproducible. DBS were stable for 324 days at room temperature and for 4 days at 50°C. Whole blood and plasma samples were stable for 172 days at -20°C. Full validation data can be found in Chapter A.2., Section A.2.3.3.

A.3.2.5. Impact of DBS-specific parameters on CYP1A2 phenotyping index

To evaluate the effect of varying hematocrit (Hct) on the concentration of caffeine and paraxanthine, we prepared blood samples with Hct values of 0.20, 0.25, 0.30, 0.43, 0.54 and 0.60 as described in Chapter A.2., Section A.2.2.5. [27]. These were used to prepare low and high quality control samples (QCs) (n=6; respectively 0.12 and 8.0 µg/mL for caffeine and 0.06 and 4.0 µg/mL for paraxanthine), from which 25-µL DBS were prepared. The influence of blood volume spotted and the impact of punch localization were investigated by preparing low and high QCs in blood with Hct at low (0.36 ± 0.007), intermediate

(0.43 ± 0.006) and high (0.50 ± 0.008) Hct. These Hct values were chosen based upon the reference ranges for women and men, which lie at approximately 0.36 – 0.44 and 0.41 – 0.50, respectively [11]. To evaluate the volume effect, different volumes of blood (7.5, 25 and 50 µL) were spotted onto the filter paper (n=6). For the effect of both Hct and volume, the results obtained for 25 µL DBS with a Hct of 0.43 were taken as a reference. The impact of punch localization was evaluated by comparing concentrations measured in central *versus* peripheral punches (n=6) using an independent samples t-test (α=0.05).

A.3.2.6. Data analysis

Peak areas were determined using AB SCIEX Analyst 1.5.2 software. Microsoft Excel 2010 and IBM SPSS Statistics 20 were used for statistical evaluation of the data. Bland-Altman plots and Passing-Bablok regression analyses were generated using MedCalc Version 12.7.5.

A.3.3. Results

A.3.3.1. CYP1A2 phenotyping study

Seventy-three healthy volunteers (age 21 – 48 years) participated in the study, including 50 women and 4 smokers. All participants received a 150 mg oral dose of caffeine. Caffeine and paraxanthine concentrations were determined in capillary DBS obtained 5 minutes before administration and in capillary DBS, venous DBS, whole blood and plasma obtained 6 hours post-administration. Table A.3.1. shows the median concentrations, along with the observed ranges at the 6-hour time point. For 12 volunteers, whole blood samples were not available. Since Perera *et al.* found that paraxanthine:caffeine ratios were not affected by pre-dose concentrations, whether or not a 24-hour abstinence period was applied [28], the pre-dose concentrations were not taken into account in this study.

Table A.3.1. Caffeine and paraxanthine concentrations in capillary DBS, venous DBS, whole blood and plasma (n=73, n=61 for venous whole blood) obtained 6 hours after administration of a 150 mg oral caffeine dose. The median concentrations together with the observed concentration ranges are listed.

Matrix	Caffeine concentration		Paraxanthine concentration	
	µg/mL	nmol/mL	µg/mL	nmol/mL
Capillary DBS				
Median	1.80	9.28	0.64	3.53
Range	0.73 – 3.92	3.76 – 20.20	0.27 – 0.96	1.51 – 5.34
Venous DBS				
Median	2.13	10.99	0.73	4.03
Range	0.90 – 3.95	4.62 – 20.32	0.28 – 1.10	1.53 – 6.09
Venous blood				
Median	2.15	11.06	0.73	4.05
Range	0.78 – 4.17	4.00 – 21.49	0.31 – 1.06	1.70 – 5.86
Venous plasma				
Median	2.56	13.17	0.90	5.00
Range	1.00 – 4.58	5.15 – 23.56	0.36 – 1.30	2.00 – 7.24

Bland-Altman comparisons were used to evaluate the data obtained for the different matrices. The mean differences and the limits of agreement (LoAs) are listed in Table A.3.2. Percentage plots were constructed since standard deviations increased with measured concentrations or concentration ratios for most comparisons [29]. Venous whole blood concentrations of caffeine and paraxanthine were on average respectively 15.2 and 16.6% lower than concentrations measured in corresponding plasma samples ($p < 0.001$). Surprisingly, also a difference between capillary and venous DBS was observed: concentrations of caffeine and paraxanthine in capillary DBS were on average respectively 12.7 and 13.8% lower than in venous DBS ($p < 0.001$). There was no significant difference between concentrations in venous DBS and venous whole blood samples ($p \geq 0.089$), demonstrating the validity of the DBS approach: the entire process of spotting, drying and storing of the samples had no influence on the analytical result. As a result, concentrations in capillary DBS were significantly ($p < 0.001$) and considerably lower than concentrations in venous plasma samples (on average -31.5 and -33.1% for caffeine and paraxanthine, respectively). However, the use of paraxanthine:caffeine molar concentration ratios alleviated both the blood-plasma difference and the capillary-venous difference: mean differences between the different matrices were only -1.6 to 0.6% (Table A.3.2.). In addition, paired samples t-tests showed no significant differences between ratios measured in the different matrices ($p \geq 0.053$).

Table A.3.2. Bland-Altman comparisons of concentrations of caffeine and paraxanthine and paraxanthine:caffeine molar concentration ratios measured in venous whole blood, plasma and DBS and capillary DBS (n=73, n=61 for comparisons with venous whole blood). The mean differences together with the limits of agreement (LoAs) are listed, with the respective 95% confidence intervals (values between square brackets).

Comparators	Statistics	Caffeine concentration	Paraxanthine concentration	Paraxanthine:caffeine molar ratio
Venous blood – venous plasma	Upper LoA (%)	-3.8 [-6.4 – -1.3]	-6.7 [-8.9 – -4.4]	9.4 [6.9 – 11.8]
	Mean difference (%)	**-15.2 [-16.7 – -13.7]**	**-16.6 [-17.8 –-15.3]**	**-1.4 [-2.8 – 0.012]**
	Lower LoA (%)	-26.5 [-29.1 – -24.0]	-26.5 [-28.7 – -24.2]	-12.1 [-14.6 – -9.7]
Venous DBS – venous blood	Upper LoA (%)	17.0 [12.8 – 21.3]	7.4 [5.5 – 9.3]	16.9 [13.2 – 20.5]
	Mean difference (%)	**-1.9 [-4.3 – 0.62]**	**-1.3 [-2.4 – -0.1]**	**0.6 [-1.5 – 2.7]**
	Lower LoA (%)	-20.7 [-25.0 – -16.5]	-9.9 [-11.8 – -8.0]	-15.7 [-19.3 – -12.0]
Capillary DBS – venous DBS	Upper LoA (%)	18.2 [11.9 – 24.5]	16.5 [10.3 – 22.7]	19.2 [15.1 – 23.3]
	Mean difference (%)	**-12.7 [-16.4 – -9.0]**	**-13.8 [-17.4 – -10.2]**	**-1.1 [-3.5 – 1.3]**
	Lower LoA (%)	-43.6 [-49.9 – -37.3]	-44.1 [-50.3 – -37.9]	-21.4 [-25.5 – -17.2]
Capillary DBS – venous plasma	Upper LoA (%)	-9.8 [-14.2 – -5.4]	-11.3 [-15.7 – -6.8]	13.0 [10.0 – 16.0]
	Mean difference (%)	**-31.5 [-34.1 – -28.9]**	**-33.1 [-35.7 – -30.5]**	**-1.6 [-3.3 – 0.1]**
	Lower LoA (%)	-53.2 [-57.7 – -48.8]	-54.9 [-59.3 – -50.4]	-16.2 [-19.2 – -13.2]

The differences between measured concentrations in capillary DBS and plasma on the one hand and the equivalence between the phenotyping indices obtained from these matrices on the other hand can readily be deduced from Figure A.3.2., depicting the Passing-Bablok regression analyses of caffeine concentrations (A), paraxanthine concentrations (B) and paraxanthine:caffeine molar concentration ratios (C), as well as the Bland-Altman comparison of these ratios (D) in capillary DBS versus those in the reference matrix for CYP1A2 phenotyping, i.e. venous plasma. Bland-Altman plots and Passing-Bablok regression analyses for all comparisons are shown in Figures A.3.3.-A.3.8.

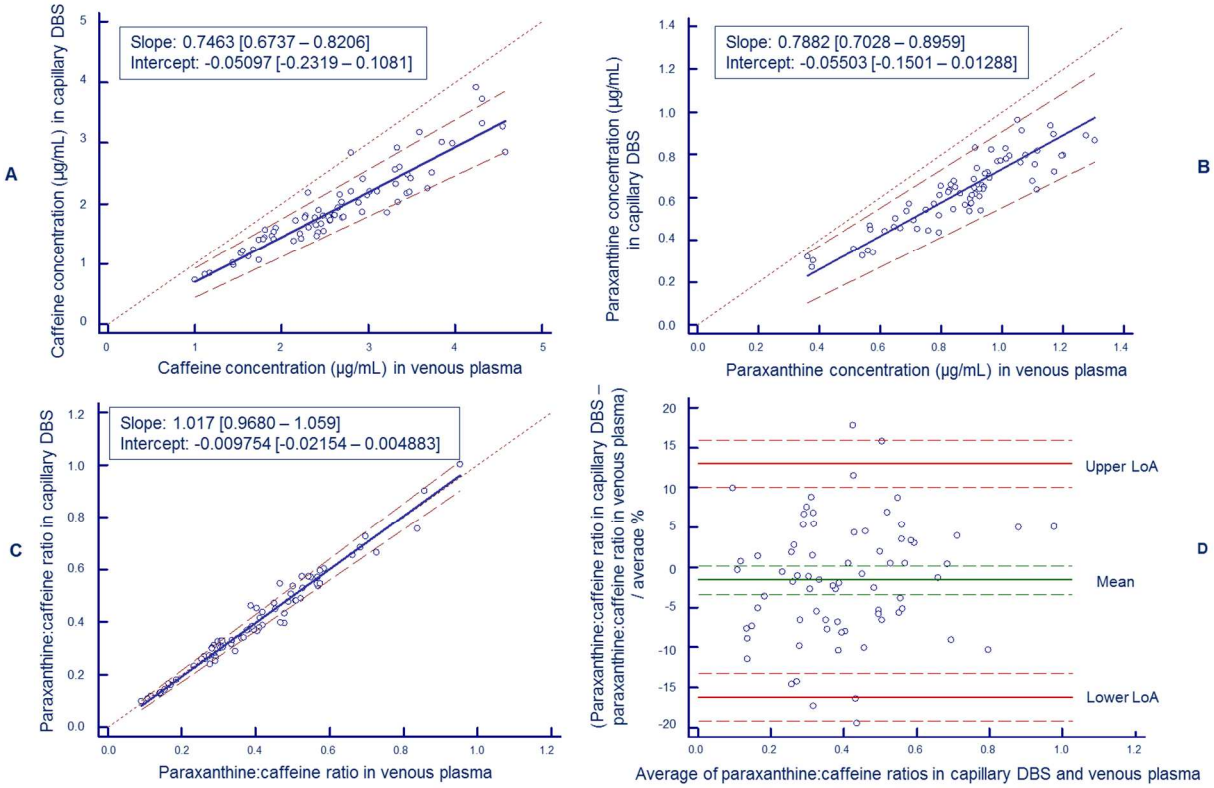


Figure A.3.2. Passing-Bablok regression analysis of caffeine concentrations (A), paraxanthine concentrations (B), paraxanthine:caffeine molar concentration ratios (C) and Bland-Altman comparison of the paraxanthine:caffeine molar concentration ratios (D) in capillary DBS and venous plasma samples (n=73). For the Passing-Bablok regression analyses, 95% confidence intervals of slope and intercept are shown between square brackets. In the Bland-Altman plot, the mean difference and the limits of agreement (LoA) are displayed, together with the 95% confidence limits (broken lines).

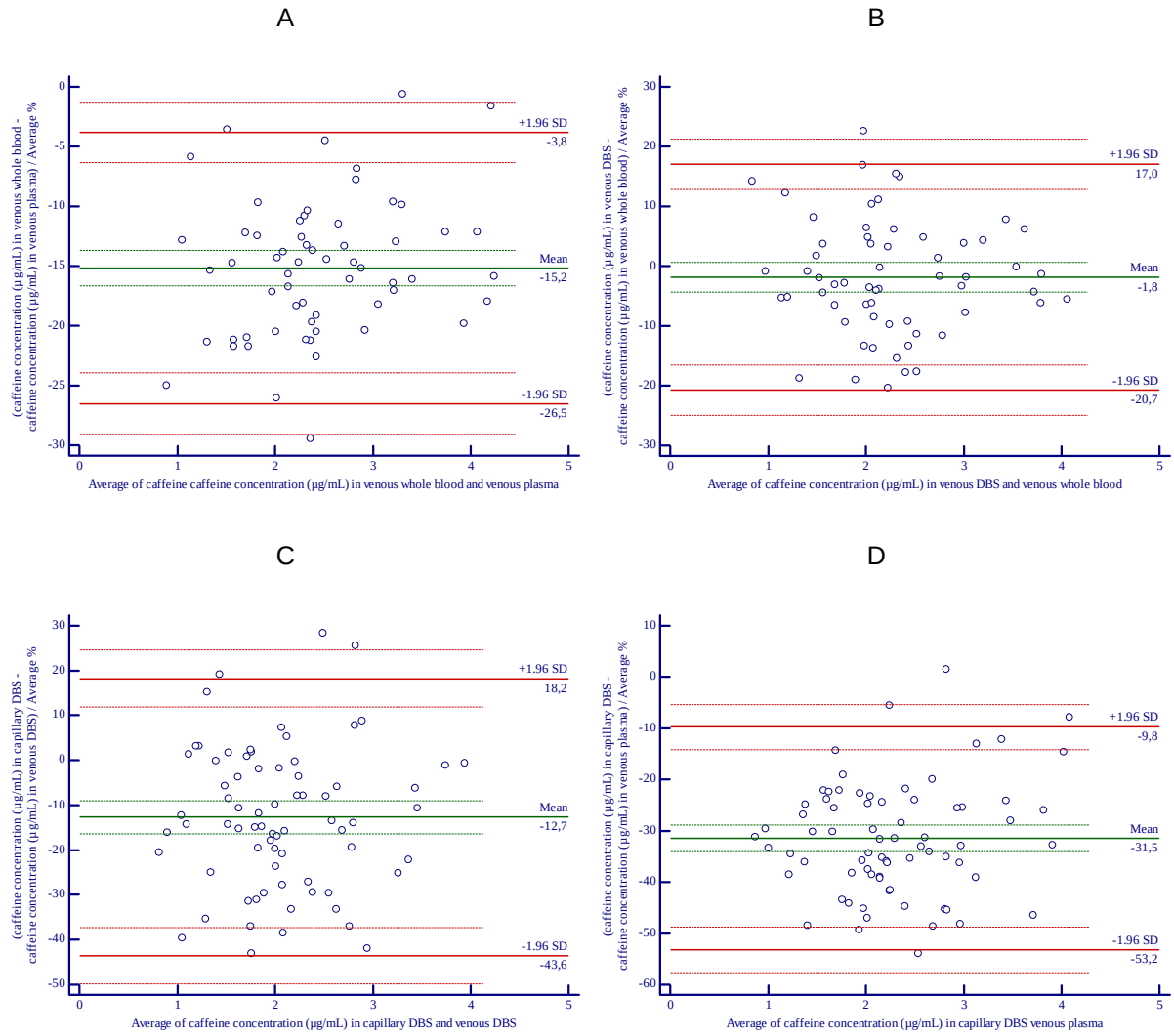


Figure A.3.3. Bland-Altman comparisons of caffeine concentrations (µg/ml) in venous whole blood and venous plasma (A), in venous DBS and venous whole blood (B), in capillary DBS and venous DBS (C) and in capillary DBS and venous plasma (D). The mean differences and the limits of agreement are displayed, together with the 95% confidence limits (dotted lines).

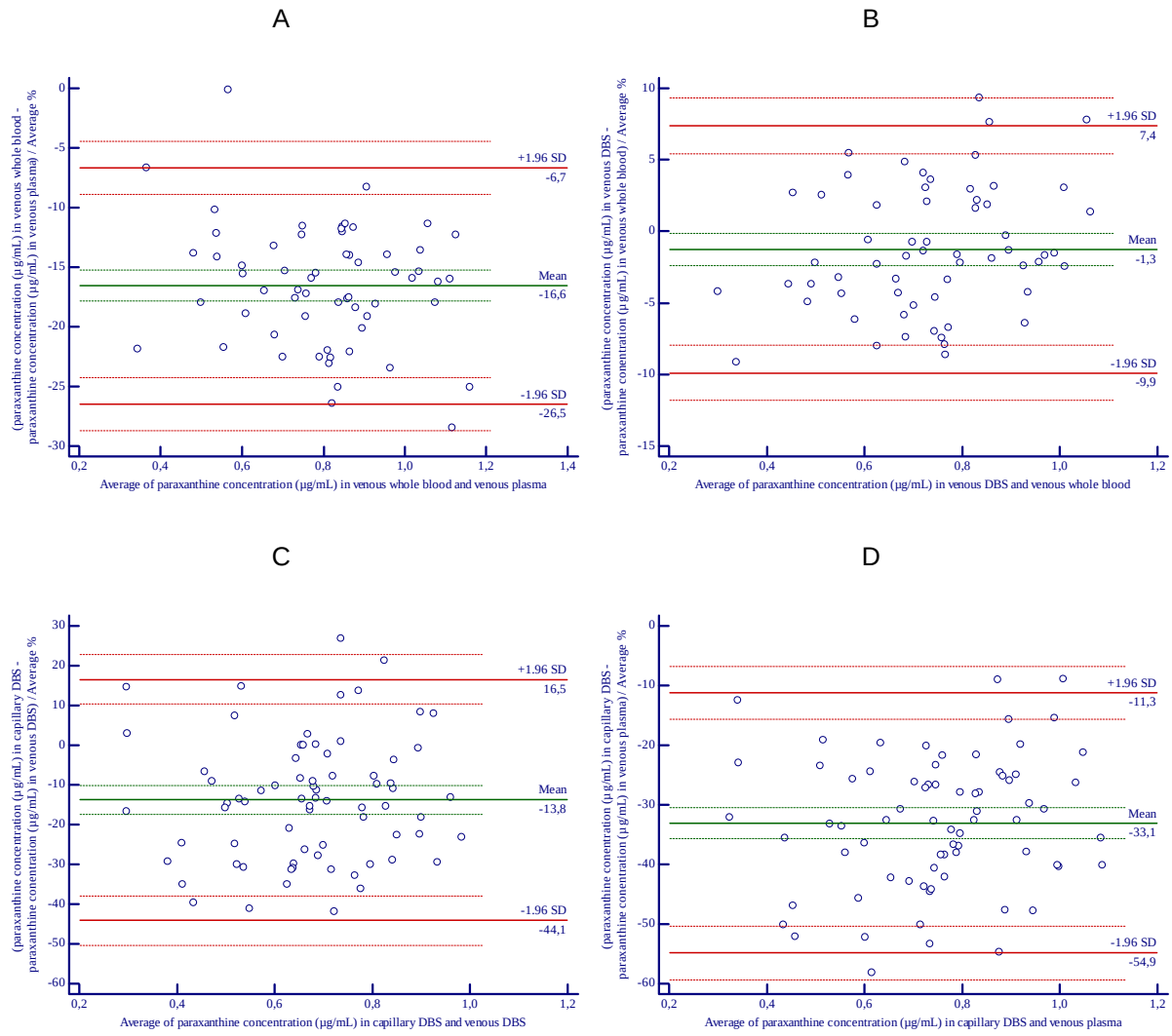


Figure A.3.4. Bland-Altman comparisons of paraxanthine concentrations (µg/ml) in venous whole blood and venous plasma (A), in venous DBS and venous whole blood (B), in capillary DBS and venous DBS (C) and in capillary DBS and venous plasma (D). The mean differences and the limits of agreement are displayed, together with the 95% confidence limits (dotted lines).

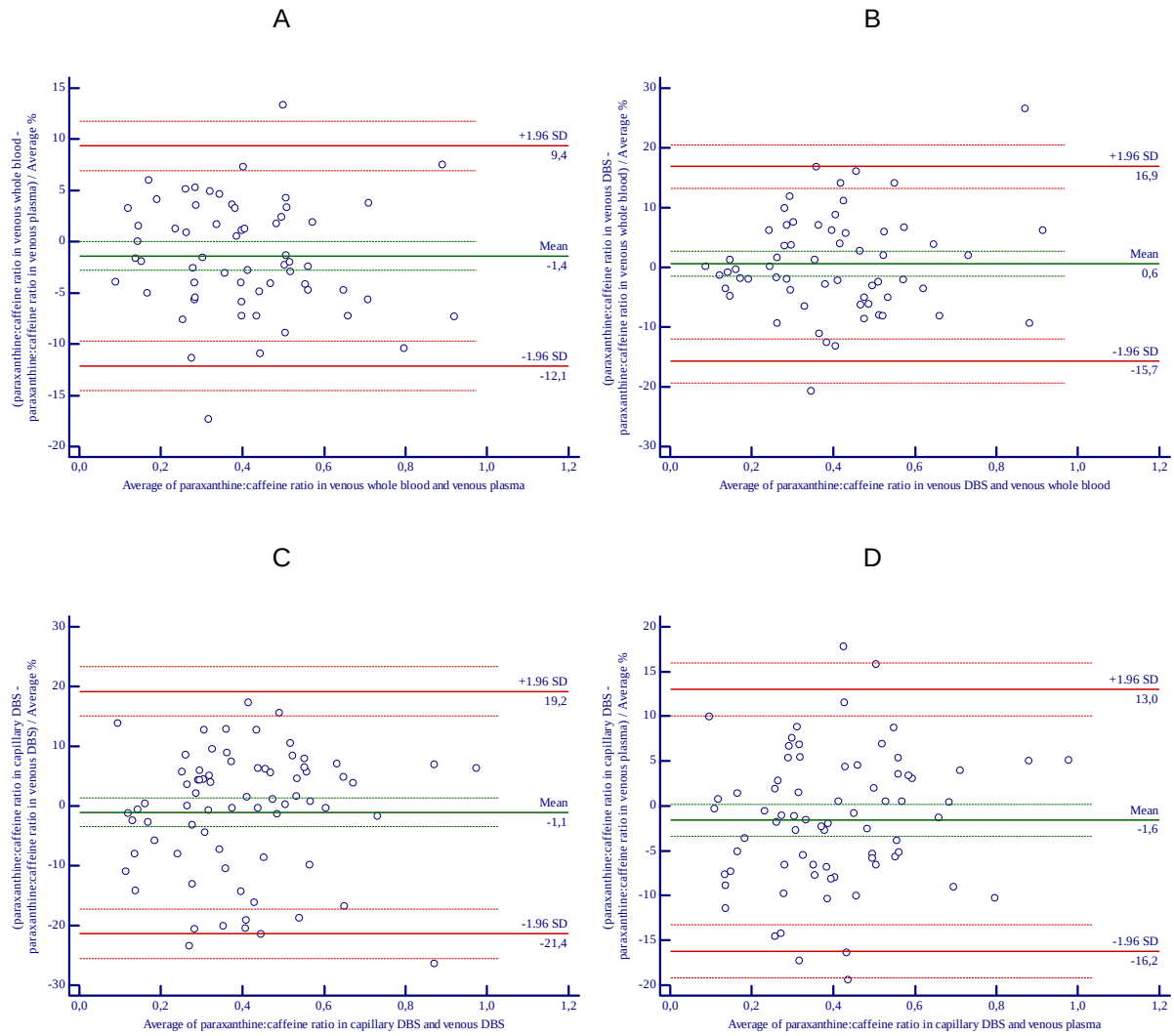


Figure A.3.5. Bland-Altman comparisons of paraxanthine:caffeine molar ratio in venous whole blood and venous plasma (A), in venous DBS and venous whole blood (B), in capillary DBS and venous DBS (C) and in capillary DBS and venous plasma (D). The mean differences and the limits of agreement are displayed, together with the 95% confidence limits (dotted lines).

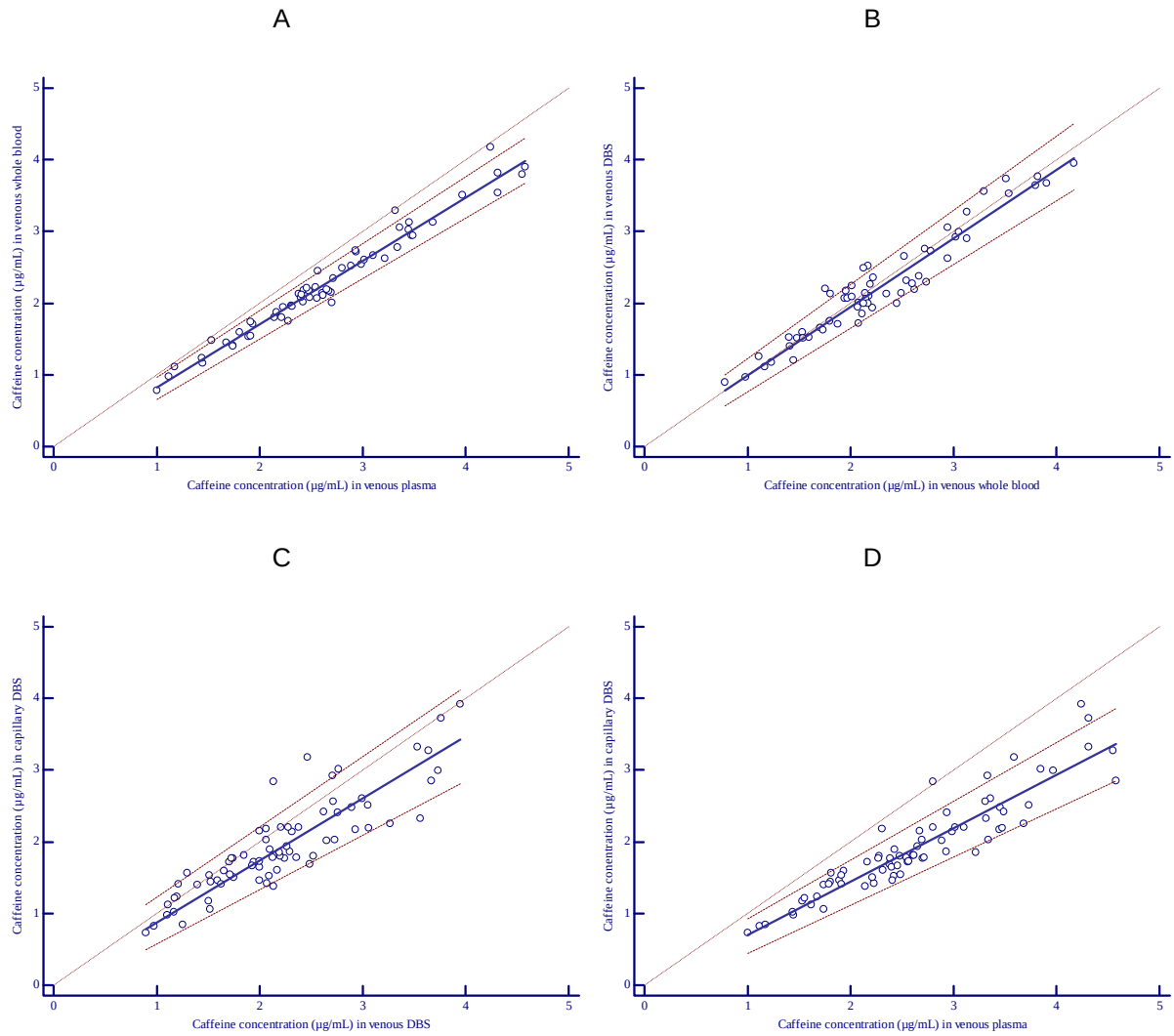


Figure A.3.6. Passing-Bablok regression analysis of caffeine concentrations (µg/ml) in venous whole blood and venous plasma (A), in venous DBS and venous whole blood (B), in capillary DBS and venous DBS (C) and in capillary DBS and venous plasma (D).

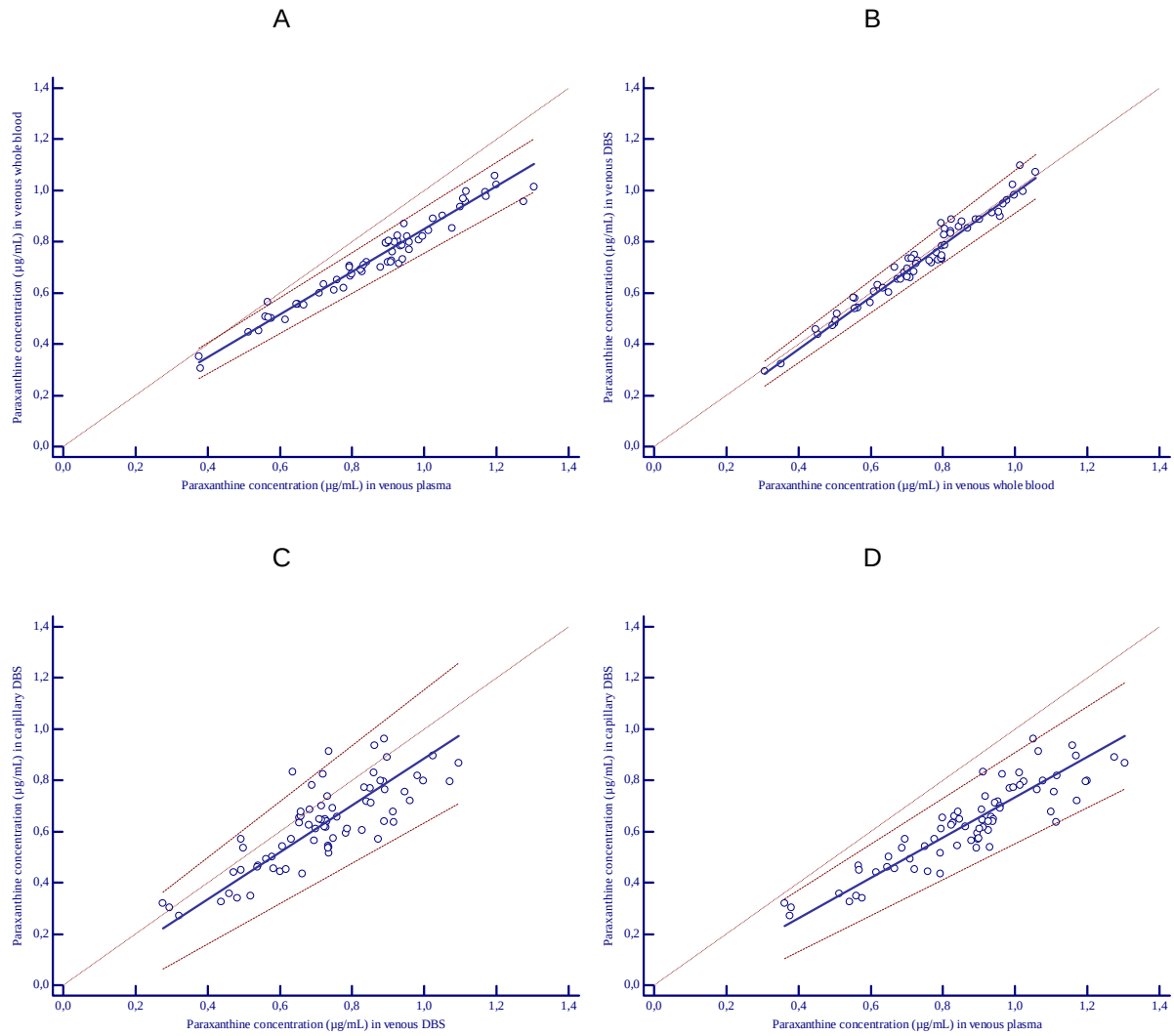


Figure A.3.7. Passing-Bablok regression analysis of paraxanthine concentrations (µg/ml) in venous whole blood and venous plasma (A), in venous DBS and venous whole blood (B), in capillary DBS and venous DBS (C) and in capillary DBS and venous plasma (D).

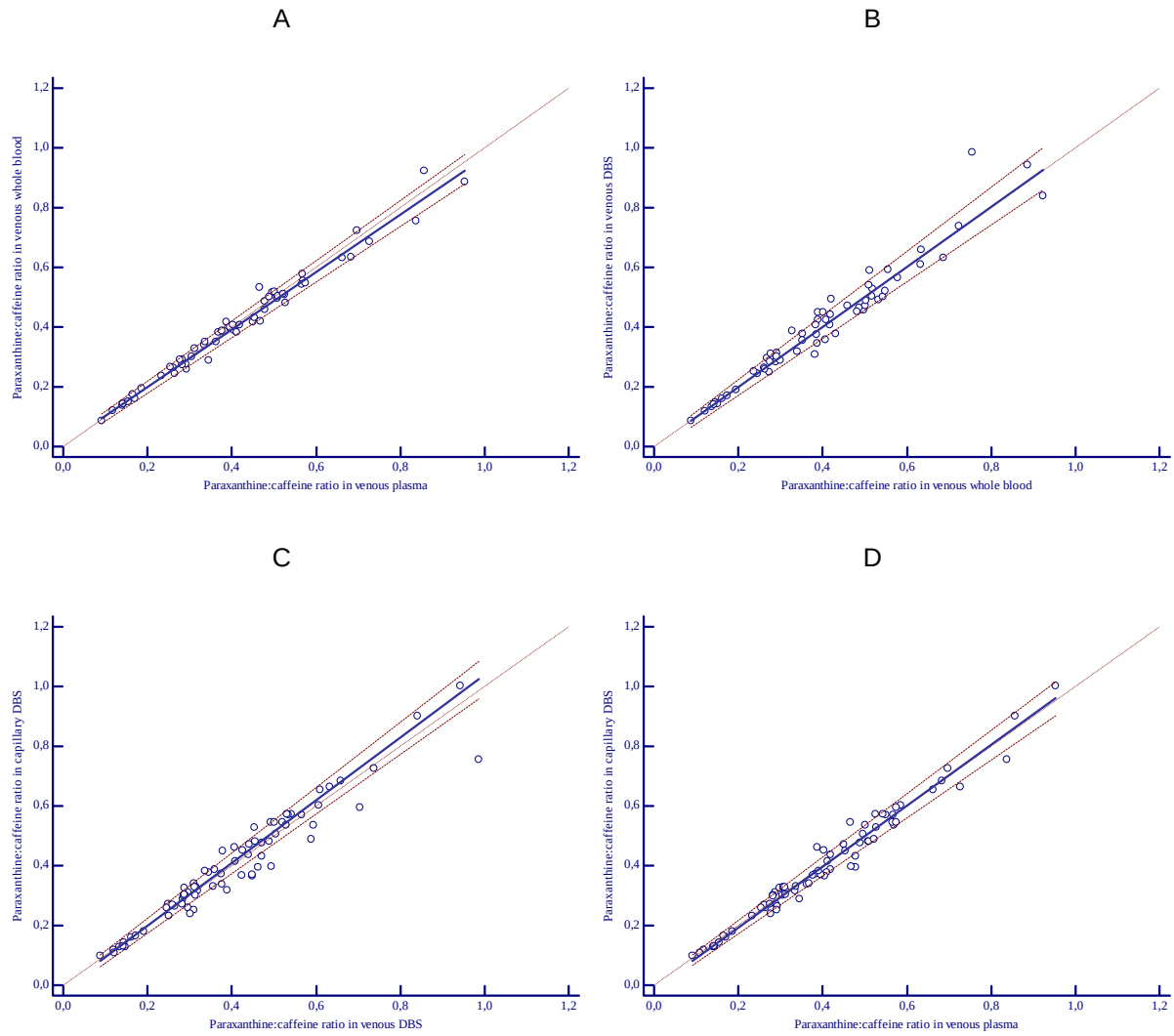


Figure A.3.8. Passing-Bablok regression analysis of paraxanthine:caffeine molar ratios in venous whole blood and venous plasma (A), in venous DBS and venous whole blood (B), in capillary DBS and venous DBS (C) and in capillary DBS and venous plasma (D).

A.3.3.2. DBS-specific parameters

To evaluate whether -besides capillary-venous differences- other DBS-specific parameters may have an impact on caffeine and paraxanthine quantitation, we varied the Hct, volume spotted and site of punching. Although we and others [20-24] demonstrated that DBS can be used for phenotyping CYP enzymes, no report has evaluated the impact of variation of these parameters on the phenotyping index. First, we did not observe significant differences between caffeine and paraxanthine concentrations measured in discs punched out peripherally versus centrally, irrespective of the Hct (Figure A.3.9.).

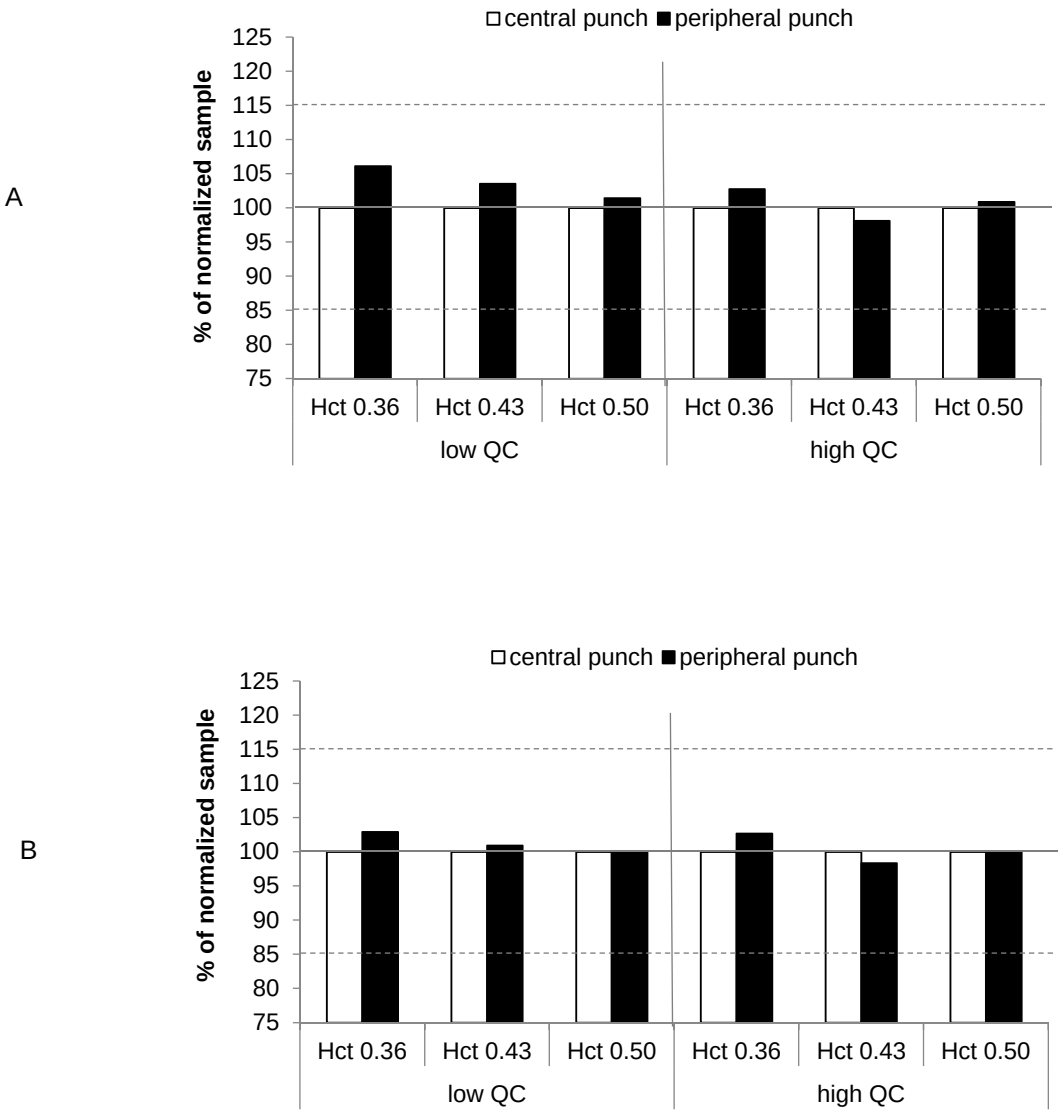
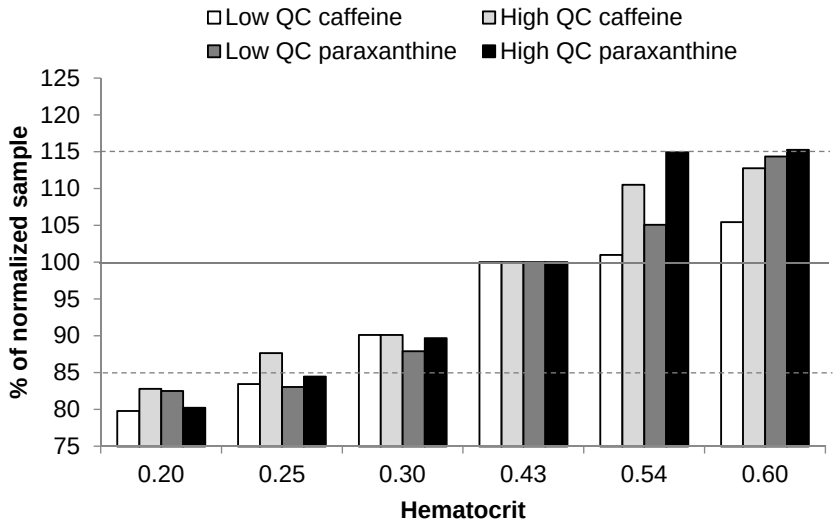


Figure A.3.9. Influence of the punch localization on the caffeine (A) and paraxanthine (B) concentrations measured in low and high QCs at three Hct levels. Data are presented as percentage of the normalized samples (central punch) (n=6). Dotted lines indicate the ± 15% deviation limits.

Next, we measured caffeine and paraxanthine concentrations (low and high QCs) in DBS, prepared from blood with varying Hct. Figure A.3.10. A depicts the % of the normalized sample with Hct 0.43, being the Hct of the calibrators, for all tested Hct levels. A "Hct effect", i.e. increasingly deviating measured concentrations with increasingly deviating Hct levels, was observed for both compounds. While in the 0.30 - 0.60 Hct range, measured concentrations were overall within ± 15% of the concentration of the normalized sample, deviations overall exceeded 15% at the lowest Hct levels (0.20 and 0.25). Importantly, no suchlike influence of the Hct on the paraxanthine:caffeine molar ratio (i.e. the actual phenotyping index) was observed (Figure A.3.10. B), indicating that both compounds are subject to a similar Hct effect.

A



B

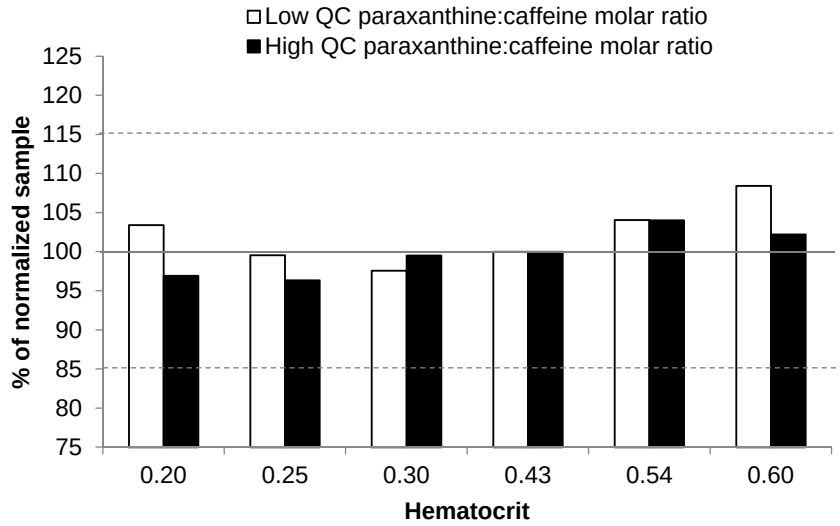
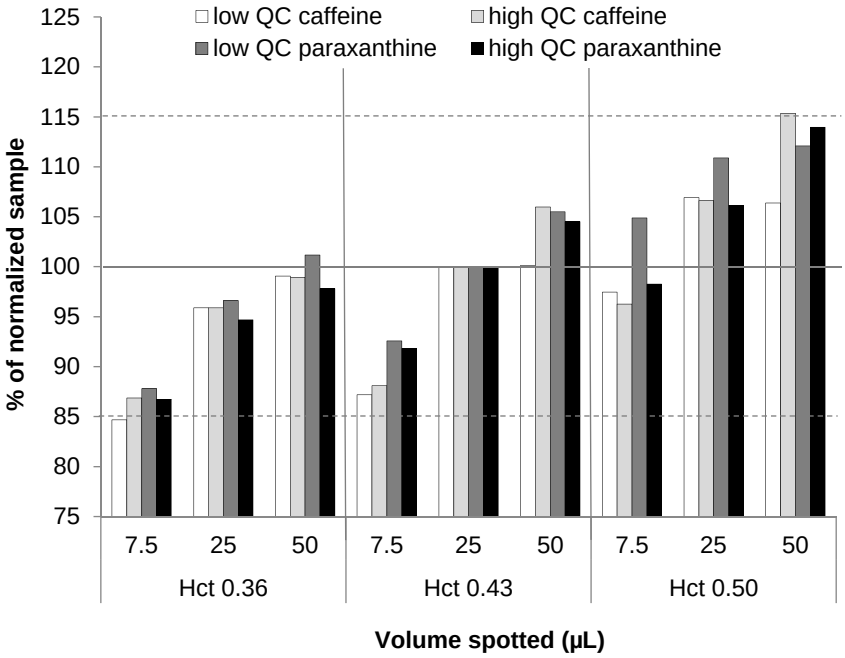


Figure A.3.10. Influence of hematocrit on caffeine and paraxanthine concentrations (A) and paraxanthine:caffeine molar ratios (B). Data are presented as percentage of normalized samples with Hct 0.43 and blood volume of 25 µL (n=6). Dotted lines indicate the ± 15% deviation limits.

The influence of the blood volume spotted was evaluated by analyzing DBS with three different blood volumes and Hct levels, taking a 25 µl DBS with Hct 0.43 as the reference. For both caffeine and paraxanthine, we observed a trend of increasing concentrations with increased blood volumes, although differences from the normalized sample were overall within ± 15% (Figure A.3.11. A). Similar to the Hct effect, the paraxanthine:caffeine molar ratio was unaffected by the blood volume (Figure A.3.11. B).

A



B

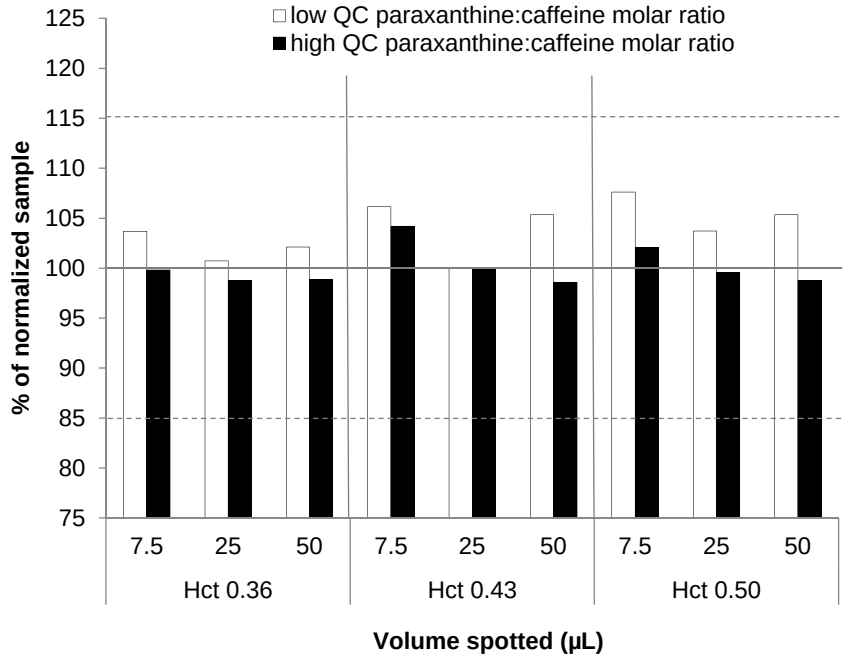


Figure A.3.11. Influence of blood volume spotted on caffeine and paraxanthine concentrations (A) and paraxanthine:caffeine molar ratios (B). Data are presented as percentage of normalized samples with Hct 0.43 and blood volume of 25 µL (n=6). Dotted lines indicate the ± 15% deviation limits.

A.3.4. Discussion

Several approaches have been proposed as convenient alternatives for classical plasma-based phenotyping. While for CYP1A2, Perera *et al.* readily demonstrated the utility of oral fluid for phenotyping [28], we investigated here in-depth the potential of DBS for this purpose. In this study, which is the largest published DBS-based phenotyping study to date, caffeine and its main metabolite paraxanthine were determined in capillary DBS, venous DBS, whole blood and plasma in the context of CYP1A2 phenotyping. Although a proof-of-principle of DBS-based CYP phenotyping is already available [20-24], all published studies had small data sets ($n \leq 16$) and did not include a systematic evaluation of DBS-specific parameters. Moreover, with a single exception [23], all studies started from volumetrically applied DBS, an approach that is difficult to sustain when envisaging patient self-sampling at home. In this study, by taking CYP1A2 as an example, the usefulness of a DBS-based phenotyping approach was demonstrated in 73 volunteers using spots prepared by non-experienced individuals in a non-volumetric way. By thoroughly investigating several known challenges associated with DBS analysis, we provide a better insight in the factors underlying the trustworthiness of DBS for phenotyping purposes.

First of all, significant differences were found between blood and plasma concentrations and between capillary and venous concentrations. As caffeine and most probably also paraxanthine (given their structural similarity) exhibit low binding to plasma proteins and freely enter blood cells without binding to cellular proteins, plasma and blood concentrations should be very similar. Taking into account the fraction of solid constituents in blood, the blood concentration could be expected to lie approximately 15% lower than the plasma concentration [9, 30]. The observed differences (15.2 and 16.6% for caffeine and paraxanthine, respectively) are in line with this expectation. Differences between capillary and venous concentrations have been found for several other small molecules (e.g. piperaquine, paracetamol) [7, 8]. However, lower capillary concentrations of caffeine and paraxanthine compared to venous concentrations, have, to the best of our knowledge, not been described before. Although we cannot fully exclude that anticoagulated blood spotted onto filter paper might exhibit a somewhat different behavior than blood without anticoagulant which is directly applied to the filter paper, we are not aware of evidence supporting this hypothesis. Instead, we consider it more likely that the observed differences between venous and capillary concentrations are caused by as yet incompletely understood physiological factors [31].

In addition to the factors described above, we also evaluated the influence of Hct, blood volume spotted and punch localization. Hct and blood volume had a considerable impact on the concentrations of caffeine and paraxanthine, similar to that described for several other small molecules [32-35]. The range in which the impact of Hct was evaluated, 0.20 – 0.60, was considered to be relevant and sufficient, as it covers approximately 99.5% of a hospital population (including both ‘normal’ and ‘critically ill’ patients) [10]. The 7.5 – 50 µL volume range was chosen to represent typical spot sizes resulting from a single drop of blood. Taken the above into account, it can be concluded that, apart from the plasma-blood and the capillary blood-venous blood difference, also the impact of Hct and volume spotted complicate the interpretation of DBS results. However, we demonstrated that the mean differences between ratios in

the different matrices were very limited (-1.6 to 0.6%) and that the paraxanthine:caffeine molar ratio was unaffected by both Hct and blood volume. The use of metabolite:substrate ratios may therefore offer a strategy to by-pass the impact of these DBS-related issues, demonstrating the usefulness of DBS-based phenotyping approaches.

Further evidence for the suitability of capillary DBS as a valid alternative for plasma, here considered the reference matrix, in the context of CYP1A2 phenotyping is given by the following: i) the 95% confidence interval of the average difference between paraxanthine:caffeine ratios in capillary DBS and plasma, obtained by Bland-Altman comparison, contained 0 (Figure A.3.2. D), indicating the absence of a consistent bias; ii) Passing-Bablok regression analysis yielded a good correlation between phenotyping indices from capillary DBS and plasma. As the 95% confidence intervals of the intercept and the slope contain 0 and 1, respectively (Figure A.3.2. C), there were no systematic and proportional differences between the two methods; iii) there was no significant difference between ratios in both matrices, as determined by a paired samples t-test; iv) although no general cut-off points concerning the CYP1A2 phenotype (poor versus rapid metabolizer) are available, we found that the 10% lowest and highest ratios measured in plasma and capillary DBS corresponded to the same subjects. In this study, the phenotyping index in plasma ranged from 0.090-0.95, which is in line with previous reports [36, 37].

A.3.5. Conclusion

In the largest published DBS-based phenotyping study to date, we demonstrated that quantitation of caffeine and paraxanthine in non-volumetrically applied capillary DBS is not only impacted by Hct and volume spotted, but also significantly differs from venous blood and plasma-based quantitation. However, as the use of the paraxanthine:caffeine molar ratio, i.e. the actual CYP1A2 phenotyping index, alleviates all these issues, we not only demonstrated here that capillary DBS-based CYP1A2 phenotyping is a valid and convenient alternative for the classical plasma-based approach, but, importantly, we also provided an objective basis as to why DBS are an ideal tool for CYP1A2 phenotyping. It needs to be evaluated whether these findings can be generalized for other CYP isoforms, using other CYP substrates.

A.3.6. References

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Chapter A.4.

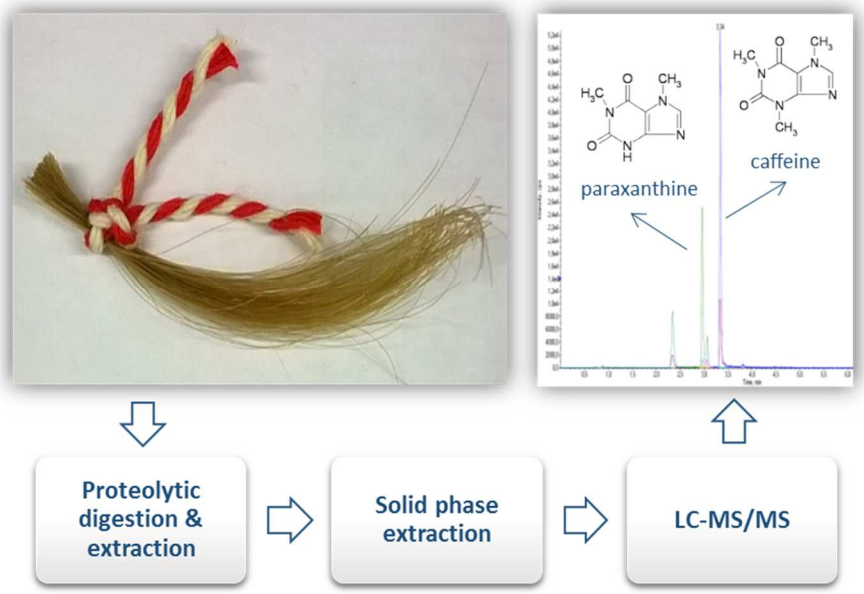
Development and validation of an SPE-LC-MS/MS method for the determination of caffeine and paraxanthine in hair

Based on

De Kesel PM, Lambert WE, Stove CP. An optimized and validated SPE-LC-MS/MS method for the determination of caffeine and paraxanthine in hair. Talanta. 2015;144:62-70.

Abstract

Caffeine is the probe drug of choice to assess the phenotype of the drug metabolizing enzyme CYP1A2. Typically, molar concentration ratios of paraxanthine, caffeine's major metabolite, to its precursor are determined in plasma following administration of a caffeine test dose. This chapter describes the development and validation an LC-MS/MS method for the determination of caffeine and paraxanthine in hair. The different steps of a hair extraction procedure were thoroughly optimized. Following a three-step decontamination procedure, caffeine and paraxanthine were extracted from 20 mg of ground hair using a solution of protease type VIII in Tris buffer (pH 7.5). Resulting hair extracts were cleaned up on Strata-X™ solid phase extraction (SPE) cartridges. All samples were analyzed on a Waters Acquity UPLC® system coupled to an AB SCIEX API 4000™ triple quadrupole mass spectrometer. The final method was fully validated based on international guidelines. Linear calibration lines for caffeine and paraxanthine ranged from 20 to 500 pg/mg. Precision (%RSD) and accuracy (%bias) were below 12 and 7 %, respectively. The isotopically labeled internal standards compensated for the ion suppression observed for both compounds. Relative matrix effects were below 15 %RSD. The recovery of the sample preparation procedure was high (> 85 %) and reproducible. Caffeine and paraxanthine were stable in hair for at least 644 days. The effect of the hair decontamination procedure was evaluated as well. Finally, the applicability of the developed procedure was demonstrated by determining caffeine and paraxanthine concentrations in hair samples of ten healthy volunteers. The optimized and validated method for determination of caffeine and paraxanthine in hair proved to be reliable and may serve to evaluate the potential of hair analysis for CYP1A2 phenotyping.



A.4.1. Introduction

Numerous approaches for qualitative and quantitative analysis of low molecular weight compounds in hair have been developed in the last decades. The majority of published methods deals with the determination of drugs of abuse and markers of alcohol use. Standard protocols are not available and several procedures to extract analytes from hair, such as extraction using organic solvents or aqueous buffers, digestion of the hair with NaOH or acid solutions and enzymatic digestion, have been combined with different separation and detection techniques, mainly GC- or LC- MS(/MS) [1.2]. Hair analysis is actively applied as an alternative matrix in different areas, such as clinical and forensic toxicology, abstinence monitoring for driving license regranting or child custody cases, workplace drug testing and doping control [3,4]. It provides some distinct advantages compared to traditional samples used in bioanalysis. Once drugs are trapped within the hair, they are no longer subject to biotransformation. Many drugs were shown to be stable in hair for prolonged periods of time, allowing retrospective assessment of drug use with a window of months up to years, depending on hair length. Furthermore, collecting hair strands represents a non-invasive sampling technique, eliminating the need for specialized personnel. On the other hand, hair analysis is characterized by certain inherent limitations, mainly in the interpretation of hair results. False positive results may result from external contamination of the hair [1-4]. Effective approaches to differentiate active ingestion from external contamination are subject of an ongoing debate in the hair analysis community. Certain drugs may also be lost from the hair due to cosmetic treatments, such as perming, bleaching or dying [5]. In many cases, hair concentrations cannot be correlated with plasma or serum concentrations.

Recently, a novel application for hair analysis has been proposed, in which specific metabolites are quantified along with their parent drugs. The corresponding metabolite-to-parent drug concentration ratios are then used to assess the metabolic phenotype of drug metabolizing enzymes [6]. For example, it was found that nortriptyline/amitriptyline ratios in hair correlated with the number of functional alleles of CYP2C19 [7]. In another study, different acetylisoniazide/isoniazide ratios were found in hair samples from individuals with different arylamine *N-acetyltransferase-2 (NAT-2)* genotypes [8]. Metabolite-to-parent drug ratios can also be used to (retrospectively) detect changes in drug metabolism. This approach has been applied to demonstrate altered metabolism of nicotine [9], citalopram [10] and methadone [11] during pregnancy.

This chapter describes the development and validation of an LC-MS/MS-based procedure for the determination of caffeine and its major metabolite paraxanthine in hair. Caffeine is a generally accepted and widely used probe drug to determine the phenotype of the drug metabolizing enzyme CYP1A2. Since N3-demethylation of caffeine to paraxanthine is entirely controlled by CYP1A2, the paraxanthine:caffeine ratio provides an ideal phenotyping index for this enzyme [12,13]. Typically, paraxanthine:caffeine ratios are determined in plasma or serum 5-7 h following the administration of a defined caffeine test dose [14]. Given the generalized consumption of caffeine, intake of a test dose would no longer be required in the case of a phenotyping procedure involving hair analysis. Caffeine has been determined in hair using a variety of extraction media and analytical techniques, such as GC-MS, LC-UV, ion mobility MS, LC-QTOF-MS and LC-MS/MS [15-20], although only one published

method was fully validated for caffeine [19]. To the best of our knowledge, this study is the first to provide an optimized and validated method for the combined determination of caffeine and its metabolite paraxanthine in hair. This method can be applied to evaluate the potential of hair analysis for CYP1A2 phenotyping purposes. The latter may be relevant in cases in which phenotyping is desired but not possible, e.g. in postmortem cases involving an intoxication with a suspected role for aberrant CYP1A2 activity. The kinetics of incorporation of caffeine and paraxanthine in hair has not been investigated in a systematic way. Incorporation of compounds in hair is a complex process in which various factors are involved. From a physicochemical point of view, the incorporation rate of a given compound is mainly determined by its lipophilicity and basicity [1,3,4]. Given the slightly higher lipophilic and basic characteristics of caffeine compared to its demethylated metabolite paraxanthine, it can be expected that caffeine may be better incorporated into hair. In general, lipophilic compounds accumulate more in hair as they easily penetrate cell membranes through passive diffusion. In addition, given the lower pH of keratinocytes and melanocytes compared to plasma, basic compounds will be trapped in hair matrix cells. The incorporation of basic compounds is also favored by a higher affinity for binding to melanin. On the other hand, the more polar compound paraxanthine may show a better retention in hair [3]. Apart from these factors, many other variables, such as melanin content or cosmetic treatment of hair, may affect the incorporation and stability of caffeine and paraxanthine in hair. Therefore, it is difficult to accurately estimate the kinetics of the incorporation of both compounds in hair or their potential to replace similar ratios in plasma. The latter is evaluated in the study described in Chapter A.5.

A.4.2. Material and methods

A.4.2.1. Chemicals and stock solutions

Methanol, acetonitrile and methylene chloride were obtained from Biosolve (Valkenswaard, The Netherlands). A Synergy® Water Purification System (Merck Millipore, Overijse, Belgium) provided ultrapure water. Analytical standards (caffeine, caffeine-$^{13}C_3$, paraxanthine, paraxanthine-$^{13}C_4$-$^{15}N_3$, theophylline, theobromine), proteases (proteinase K, protease type VIII, protease type XIV), dithiothreitol, trishydroxymethylaminomethane (Tris) and formic acid were purchased from Sigma-Aldrich (Diegem, Belgium). HCl (1N) was obtained from Merck KGaA (Darmstadt, Germany) and liquid N_2 from Air Liquide (Brussels, Belgium). Stock solutions of caffeine and paraxanthine (1 mg/mL) and the IS (100 µg/mL) were prepared as described in Chapter A.2., Section A.2.2.1 [21].

A.4.2.2. Optimization of the sample preparation procedure

Hair samples from two caffeine consuming healthy volunteers obtained after regular haircuts were used for optimizing hair extraction conditions. To remove external contamination, hair (± 2 g) was washed in methylene chloride (20 mL for 2 min), followed by two washes in water (20 mL for 2 min). Two homogenization methods -manual grinding in a mortar using liquid N_2 and mechanical grinding in a ball mill- were tested along with four different extraction solvents or conditions. The latter were methanol, an aqueous buffer (acetate buffer pH 5.0), digestion of the hair matrix with an aqueous NaOH solution (0.5 mol/L) and enzymatic digestion with protease type XIV (1.4 international units (IU)/mL) in acetate buffer

(pH 8.5). As enzymatic digestion was selected, solutions of three different proteases (proteinase K, protease type VIII and type XIV), corresponding to 30.0 IU/mL, were prepared in Tris buffer (50 mmol/L) at pH 7.5 and 8.5. For all conditions, 1 mL of extraction medium was added to 20 mg pulverized hair. All samples were gently shaken for 12 hours at 37 °C and 750 rpm on a Thermomixer® comfort (Eppendorf, Hamburg, Germany). Subsequently, several enzymatic digestion and extraction parameters were optimized. Evaluated extraction times ranged from 30 minutes to 12 hours and extractions at different temperatures (22, 37, 50 °C) were compared. Also the influence of the protease concentration (1.2, 6.0, 12.0, 24.0 IU/mL) and the Tris buffer concentration (25.0, 50.0, 100.0 mmol/L) was evaluated. Finally, the effect of the presence of different concentrations of dithiothreitol in the extraction medium was evaluated. To illustrate the performance of the optimized enzymatic procedure, a side-by-side comparison with extraction in methanol was performed. Therefore, aliquots of ground hair (20 mg) from a single volunteer were extracted using the enzymatic procedure and by adding 1 mL of methanol to the ground hair samples. All samples were shaken for 1 h at 750 rpm and 37 °C.

Prior to LC-MS/MS analysis, hair extracts were cleaned-up using solid phase extraction (SPE). Four different SPE cartridges (Bond Elut Phenyl, C8, C18 and Phenomenex Strata-X™) were tested using a general protocol. The cartridges were conditioned with 3 mL of methanol and 2 mL of water. Subsequently, 1 mL of hair extract was applied to the columns. For the wash step, 2 mL of a water/methanol mixture (85/15, v/v) was used. Finally, after drying for 2 minutes, the analytes were eluted from the cartridges with 4 × 250 µL of methanol. For all four cartridges, the recovery of caffeine and paraxanthine was determined by calculating the percent ratio of the peak areas of hair samples spiked at 100 pg/mg before extraction to those of samples spiked at the same concentration after extraction. Phenomenex Strata-X™ cartridges were selected (see Results section) and the SPE protocol was further optimized by evaluating different wash solvents, being mixtures of water with methanol (85/15, v/v) or acetonitrile (90/10, v/v). Also the influence of using incremental proportions of methanol in the wash solvent was investigated. Finally, methanol, acetonitrile, methanol/acetonitrile (50/50, v/v), methanol/ HCl 1 mol/L (95/5, v/v), acetonitrile/ HCl 1 mol/L (95/5, v/v) and methanol/ammonium aceteate 5 mmol/L (70/30, v/v) were evaluated as elution solvents.

A.4.2.3. Final sample preparation procedure

In the final procedure, a three-step decontamination procedure was used. Hair samples were first immersed in methylene chloride (20 mL) for 2 minutes and then twice in the same volume of water for 2 minutes. These large volumes of wash solvents were used to make sure there was a large excess of decontamination solvent, given the important issue of external contamination in hair analysis. Wash solvents were removed and samples were air-dried between the consecutive steps. Subsequently, samples were cut into small pieces (< 5 mm) using scissors and manually ground in a mortar with liquid N_2. Twenty mg of ground hair was weighed and 1 mL of a solution of protease type VIII (1.2 IU/mL) in Tris buffer (pH 7.5, 50 mmol/L) was added. The latter solution contained the internal standards (IS) caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$ at concentrations of 5 ng/mL. This results in signals that lie around the center of the calibration curve, in particular between the 150 pg/mg and 250 pg/mg calibrators. The samples were placed in a Thermomixer® comfort at 37 °C and 750 rpm for 1 hour and

subsequently centrifuged at 10000 x g for 10 minutes. An SPE procedure using Phenomenex Strata-X™ cartridges (200 mg, 3 mL) was used for clean-up of the supernatants. The cartridges were conditioned and equilibrated with 3 mL of methanol and 2 mL of water, respectively. Following application of the hair extracts, the cartridges were washed with 2 mL of a water/methanol mixture (65/35, v/v) and dried for 2 minutes. The analytes were eluted with 4 × 500 µL of a methanol/acetonitrile mixture (50/50, v/v). The resulting solutions were evaporated under a stream of N_2 and redissolved in 150 µL of the mobile phase.

A.4.2.4. LC-MS/MS method

A Waters Acquity UPLC® system (Waters, Milford, MA, USA) and an AB SCIEX API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) were used for all analyses. The LC-MS/MS configuration was controlled by AB SCIEX Analyst 1.5.2 and Waters Acquity console software. Identical chromatography parameters as described in Chapter A.2., Section A.2.2.2.2. for the analysis of caffeine and paraxanthine in dried blood spots, whole blood and plasma were used [21]. Mass spectrometer parameters were slightly adapted. The ion spray voltage was set at 4000 V, gas 1 at 40 psi, gas 2 at 80 psi, the curtain gas at 10 psi and collision-activated dissociation (CAD) vacuum at 9 (arbitrary setting). All compounds were detected in scheduled multiple reaction monitoring (sMRM) mode. Precursor to product ion transitions were identical as listed in Table A.2.1. (Chapter A.2.) [21].

A.4.2.5. Validation

Validation of the hair analysis procedure was based on European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA) guidelines for bioanalytical method validation [22,23]. Selectivity, carry-over, lower limit of quantification (LLOQ), linearity, precision, accuracy, matrix effect, stability and dilution integrity were evaluated. It should be noted that no blank, caffeine-free hair matrix was available. Hair samples from 4 young children (1 – 5 years), collected at different time points, were analyzed; however, caffeine and paraxanthine were detected in all samples. A pool of hair matrix to prepare calibrators and quality control (QC) samples was obtained by combining decontaminated and ground hair samples in which the lowest concentrations of both compounds were measured. In every analytical run, the resulting hair matrix pool (with IS added to it) was analyzed in triplicate. The mean ratio of caffeine and paraxanthine to their respective IS, obtained from this analysis of triplicates, was calculated. To correct for the background caffeine and paraxanthine, these mean ratios were subtracted from the ratios that were obtained for all calibrators and QCs. We did not consider applying standard addition, since this would require two analyses per sample. For hair analysis in general (and certainly for a volunteer study, as is the case here), the amount of hair to be sampled remains limited because of esthetic issues. In this respect, sampling the double amount of hair does not seem appropriate.

Given the lack of blank matrix, selectivity could not be assessed. Cross-interferences with caffeine or paraxanthine were evaluated by analyzing samples spiked with caffeine, paraxanthine, theophylline or theobromine at 500 pg/mg (n = 5). To evaluate the purity of the isotopically labeled IS, non-spiked and IS-spiked hair matrix pool samples were analyzed in every sample batch. Interferences were considered unacceptable if the increase in peak areas was higher than 20 % of the peak area of the LLOQ for the

analytes and 5 % for the IS [22]. Potential carry-over was assessed by applying these criteria on non-spiked samples injected after the highest calibrator as well.

Linearity was determined by constructing seven-point calibration lines (20, 40, 80, 150, 250, 350, 500 pg/mg) for caffeine and paraxanthine on four consecutive days. An F-test at the 99 % confidence level was performed and residuals were plotted against nominal concentrations to evaluate homoscedasticity of the data [24]. Unweighted and weighted ($1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$ and $1/\sqrt{y}$) linear regression were applied and slopes and intercepts of the corresponding calibration lines were calculated. The sum% relative error (RE) was calculated and %RE was plotted against nominal concentrations to select the best-fitted calibration model [24]. Mean back-calculated concentrations of the calibrators should be within ± 15 % of the nominal value in order to accept the chosen model [22]. The LLOQ was defined as the lowest concentration of caffeine and paraxanthine which could be measured with %bias and %RSD below 20 %.

On the same four consecutive days, QCs for caffeine and paraxanthine at four concentration levels were prepared and analyzed in duplicate to determine precision and accuracy. Assessment of accuracy ideally requires analysis of certified reference material (CRM); however, for caffeine and paraxanthine in hair, no CRM is available. Therefore, we opted to use spiked QCs for this purpose, as is commonly applied in hair analysis. Nominal QC concentrations were 20 (LLOQ), 60 (low), 200 (medium) and 400 (high) pg/mg. Intra- and interbatch precision (%RSD) were calculated using single factor ANOVA [25]. Accuracy (%bias) was calculated by dividing the difference between the obtained concentration and the nominal value by the nominal value and multiplying it by 100. %RSD and %bias should be within ± 15 % or within ± 20 % for the LLOQ [22].

Determination of matrix effects and recovery was based on the approach described by Matuszewski *et al.* [26]. Hair samples originating from five different individuals, including blonde, brown, dark and black hair, were spiked at low and high concentration levels (respectively 60 and 400 pg/mg) before (C) and after (B) extraction. Standard solutions of caffeine and paraxanthine in the injection solvent at the same concentration levels (A) were prepared as well. Absolute matrix effect values were obtained by calculating the percent ratio of peak areas of (B) to those of (A), absolute recovery values by calculating the percent ratio of peak areas of (C) to those of (B). The %RSD of absolute matrix effect and recovery values represented the relative matrix effect and recovery [26], the word “relative” referring to the comparison of the values for matrix effect and recovery between different sources of hair. Relative matrix effect should not exceed 15 % [22].

Long-term stability of caffeine and paraxanthine in hair was evaluated by analyzing ground hair samples in triplicate at time point zero and after various time points of storage (i.e. 7, 61, 369 and 644 days) at ambient temperature protected from light. Processed sample stability was examined by re-injecting extracts of low and high QCs after 4 days of storage at 4 °C and 7 days at -20 °C (n = 3). All stability samples were analyzed together with freshly prepared calibrators. The obtained concentrations should be within ± 15 % of the concentration at time point zero and the nominal concentration (for QCs).

In order to safely dilute sample extracts when caffeine or paraxanthine concentrations above the ULOQ (500 pg/mg) are measured in real hair samples, dilution integrity was evaluated. Extracts of hair samples spiked at a concentration of 1000 pg/mg (n = 6) were diluted (1:5) with the injection solvent and analyzed. Using this dilution, the signals of the IS were high enough to still allow trustworthy integration of the corresponding peaks, as they were still within the linear range (20-500 pg/mg). The peak areas of the IS in the diluted samples were multiplied by 5. These peak areas were used to calculate the analyte/IS peak area ratios, from which the analyte concentrations were derived using the calibration curve. Finally, these concentrations were multiplied by 5 to obtain the actual concentrations. Accuracy and precision of the back-calculated concentrations should be within ± 15 % [22].

A.4.2.6. Evaluation of the hair decontamination procedure

To evaluate the effect of the hair decontamination procedure, proximal 3-cm segments of hair samples collected from 6 volunteers were decontaminated using the procedure described in section A.4.2.3. All obtained wash solvents were analyzed for caffeine and paraxanthine. Therefore, methylene chloride washes were evaporated under a stream of N_2 and redissolved in 150 µL of the mobile phase. An identical SPE procedure as used for clean-up of hair extracts was applied to the water washes. To ensure that this procedure was reliable when a higher volume (20 mL) was applied to the SPE cartridges, recovery was determined using solutions in water (20 mL, n = 3) in which absolute amounts of caffeine and paraxanthine corresponded to those present in extracts of hair samples spiked at the LLOQ concentration level.

A.4.2.7. Application

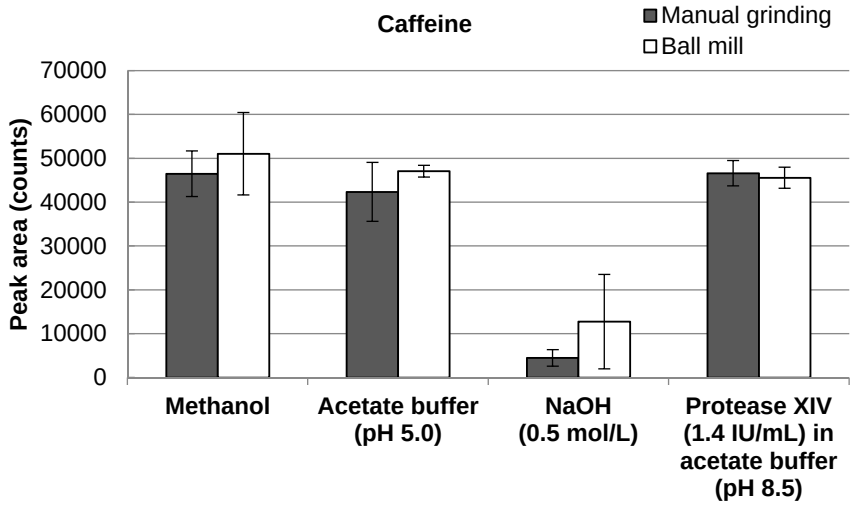
As a proof of concept, samples were collected from 10 healthy volunteers by cutting hair locks as close as possible to the scalp at the posterior vertex region. Caffeine and paraxanthine concentrations were determined in proximal 3-cm hair segments and corresponding paraxanthine:caffeine molar concentration ratios were calculated. This study was approved by the Ethics Committee of Ghent University Hospital (B670201111655). Written informed consent was obtained from all volunteers. No data on the participants' caffeine intake was available.

A.4.3. Results and discussion

A.4.3.1. Sample preparation

No significant differences were found between manual and mechanical grinding of hair (Figure A.4.1.). As the ball mill was not available for daily use, hair samples were manually ground in a mortar using liquid N_2. Extraction in methanol and enzymatic digestion with protease type XIV gave comparable results for both caffeine and paraxanthine (Figure A.4.1.). We opted to further optimize the enzymatic procedure as the resulting aqueous extracts were clearer compared to methanolic extracts and did not require an evaporation or dilution step prior to SPE.

A



B

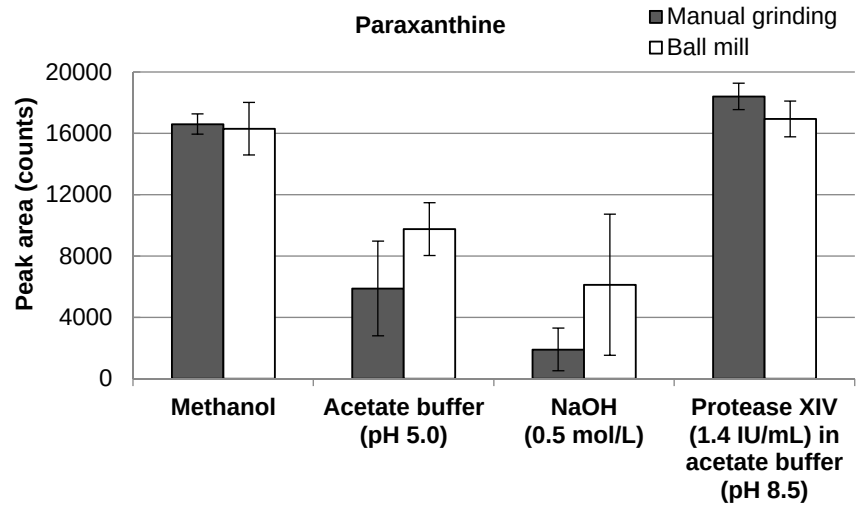


Figure A.4.1. Evaluation of different hair homogenization methods and extraction media to extract caffeine (A) and paraxanthine (B) from hair. Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

Solutions of three different proteases in Tris buffer at pH 7.5 and 8.5 were compared. No significant differences were found at pH 8.5 for caffeine, while slightly higher responses were seen for paraxanthine using protease type XIV. However, considerably higher responses for both compounds were obtained by using protease type VIII at pH 7.5 (Figure A.4.2.).

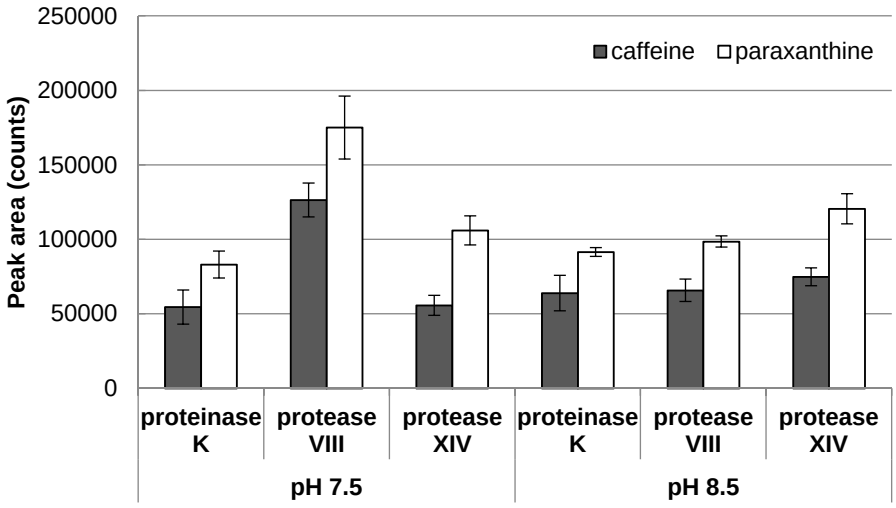


Figure A.4.2. Evaluation of solutions of three different proteases (30.0 IU/mL) in Tris buffer (50 mmol/mL, pH 7.5 and 8.5) for enzymatic digestion of hair. Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

Hitherto, hair samples had been incubated for 12 hours at 37 °C. Different extraction times were evaluated and decreasing responses for caffeine and paraxanthine were found when samples were extracted for 2 up to 12 hours (Figure A.4.3.).

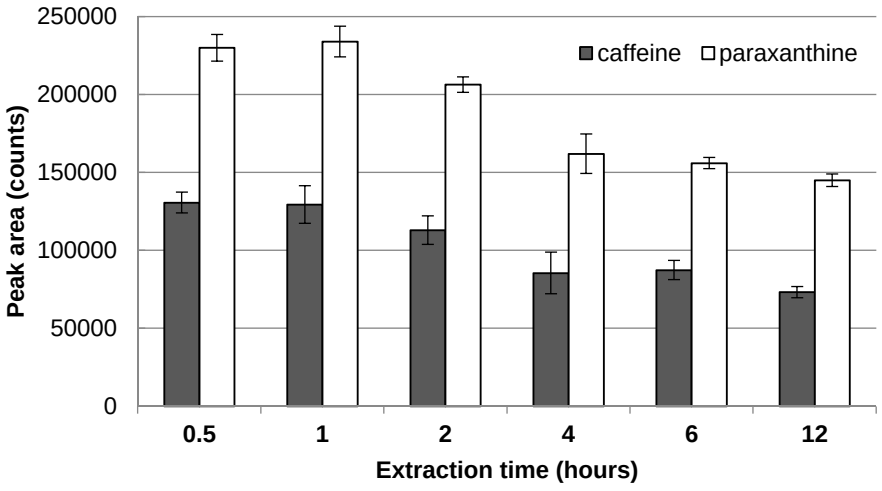


Figure A.4.3. Evaluation of different extraction times using 1 mL of protease type VIII (1.2 IU/mL) in Tris buffer (50 mmol/L, pH 7.5). Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

For caffeine, no influence of the extraction temperature was observed under the evaluated conditions, while slightly higher responses for paraxanthine were obtained at 37 °C (Figure A.4.4.).

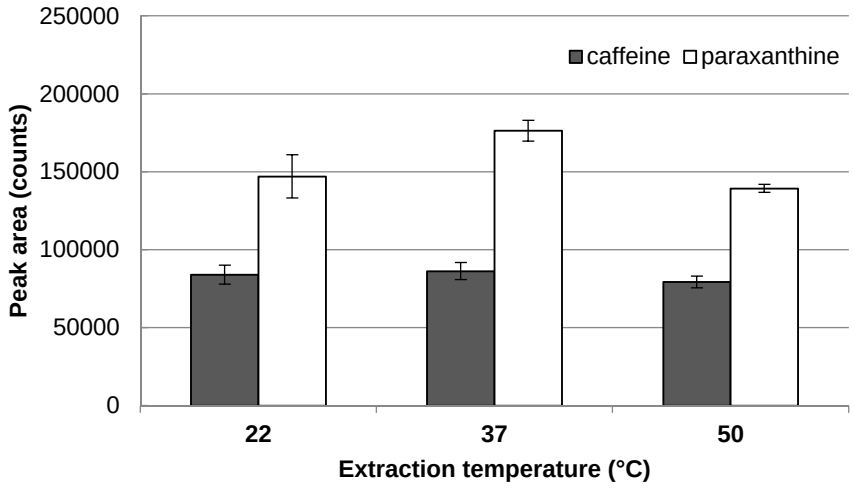


Figure A.4.4. Evaluation of different extraction temperatures using 1 mL of protease type VIII (1.2 IU/mL) in Tris buffer (50 mmol/L, pH 7.5). Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

Increasing the enzyme concentration or using Tris buffer solutions at different concentrations did not influence the extraction efficiency (Figure A.4.5. and A.4.6., respectively).

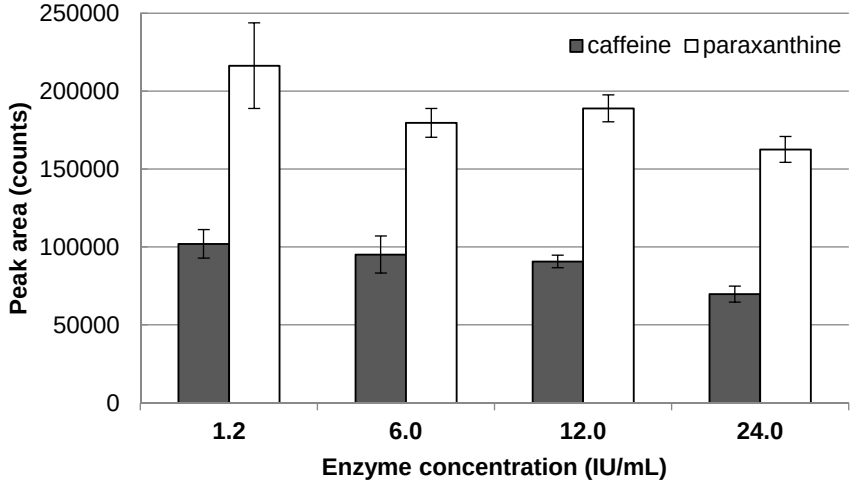


Figure A.4.5. Evaluation of different concentrations of protease type VIII in Tris buffer (50 mmol/L, pH 7.5). Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

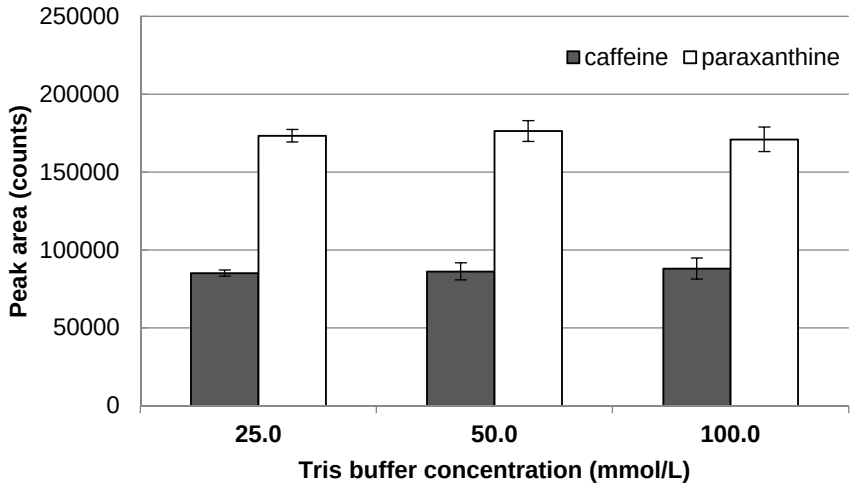


Figure A.4.6. Evaluation of different concentrations of Tris buffer (pH 7.5) as solvent for protease type VIII (1.2 IU/mL). Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

As dithiothreitol is able to reduce disulfide bonds of proteins, it is often applied to enhance enzymatic digestion [3]. In this study, however, adding dithiothreitol to the extraction medium had no positive effect, as analyte responses slightly decreased when higher dithiothreitol concentrations were used. Especially for paraxanthine, the highest peak areas were obtained when no dithiothreitol was present during extraction (Figure A.4.7.).

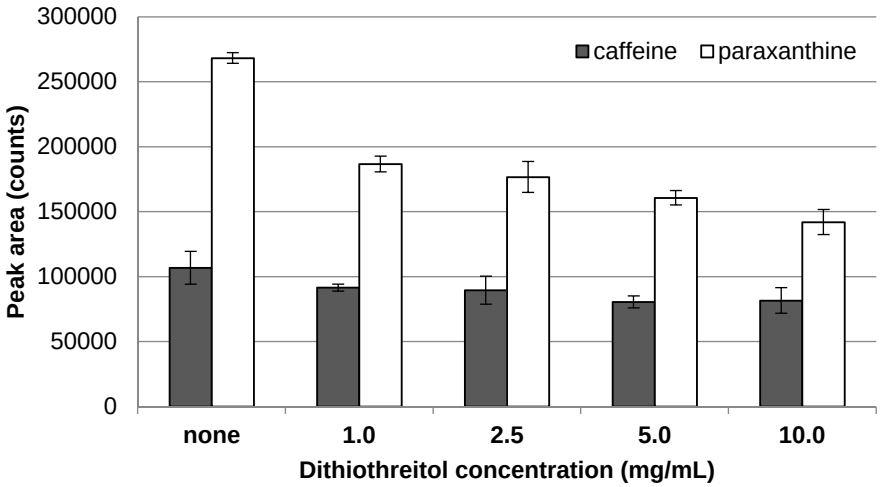


Figure A.4.7. Evaluation of different concentrations of dithiothreitol added to a solution of protease type VIII (1.2IU/mL) in Tris buffer (50 mmol/L, pH 7.5). Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

In general, peak areas of paraxanthine were higher than those of caffeine in the experiments described above. This was partly due the fact that hair from an individual with relatively high CYP1A2 activity was used. In addition, LC and MS parameters were specifically optimized to allow a sensitive determination

of paraxanthine, as it was expected that hair concentrations of this metabolite could be low in particular cases (e.g. in subjects with both low caffeine intake and reduced CYP1A2 activity). In the final procedure, 1 mL of a protease type VIII solution at 1.2 IU/mL in Tris buffer (50 mmol/L, pH 7.5) was added to 20 mg ground hair and samples were shaken for 1 hour at 37 °C and 750 rpm. To evaluate the impact of optimizing the extraction conditions, this procedure was compared with extraction in methanol. As mentioned below, hair extracts were cleaned-up using SPE. While the aqueous extracts resulting from the enzymatic procedure were directly applied to the cartridges, the methanol extracts were either evaporated under a stream of N_2 and redissolved in water or diluted 1:3 with water prior to SPE. The latter dilution step was feasible since a water/methanol mixture (65/35, v/v) is used in the SPE wash step. The results are shown in Figure A.4.8. Similar results were observed for both compounds when methanolic extracts were evaporated or diluted prior to SPE. The responses for caffeine and paraxanthine obtained after the enzymatic procedure were 30 %, respectively 43 – 47 % higher than the responses after extraction in methanol.

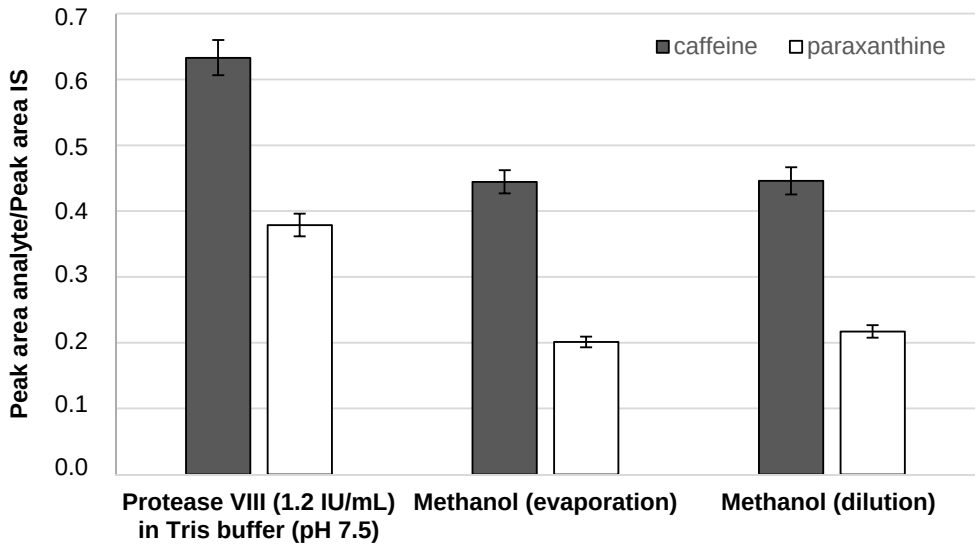


Figure A.4.8. Comparison of different conditions (enzymatic digestion and extraction in Tris buffer vs. extraction in methanol) to extract caffeine and paraxanthine from hair (n=4). Methanol extracts were either evaporated under a stream of N_2 and redissolved in water or diluted 1:3 with water prior to clean-up on Strata-X™ SPE cartridges.

Concerning the clean-up step using SPE, the highest recovery values for caffeine and paraxanthine were achieved with Phenomenex Strata-X™ cartridges (Table A.4.1.). Therefore, an optimized SPE protocol was developed for these columns.

Table A.4.1. Recovery data (n=3) for caffeine and paraxanthine spiked to hair samples at 100 pg/mg using four different SPE cartridges. An identical SPE protocol was used for all cartridges.

	Absolute recovery (mean ± SD, %)	
SPE cartridge	**Caffeine**	**Paraxanthine**
Bond Elut C18	59.17 ± 15.40	4.90 ± 1.89
Bond Elut Phenyl	83.26 ± 10.72	49.45 ± 6.90
Bond Elut C8	102.94 ± 18.29	60.52 ± 0.83
Phenomenex Strata-X™	98.32 ± 7.75	74.73 ± 4.69

The highest response for caffeine was obtained using water/acetonitrile (90/10, v/v) as SPE wash solvent, while water/methanol (85/15, v/v) gave the best results for paraxanthine (Figure A.4.9.). The water/methanol mixture was selected for further optimization because hair concentrations of paraxanthine, being a metabolite of caffeine, were expected to be lower.

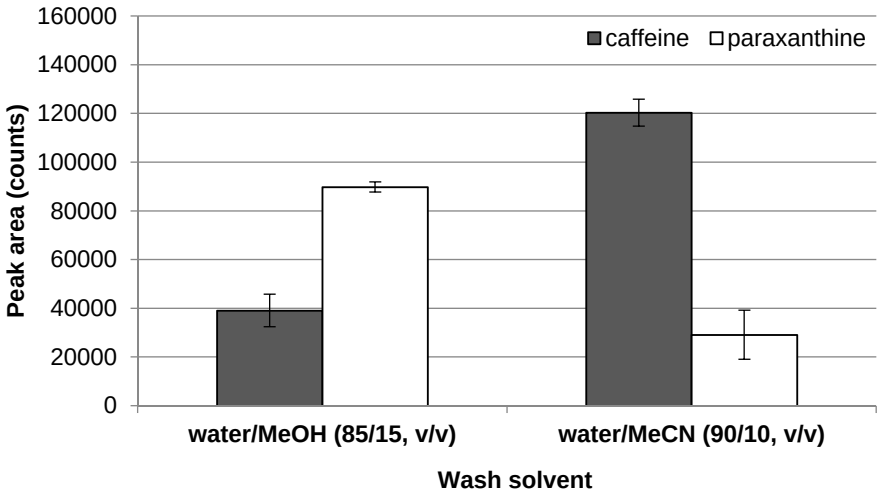


Figure A.4.9. Evaluation of different SPE wash solvents using Phenomenex Strata-X™ cartridges. Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

The response for caffeine decreased when 60 % of methanol in water was used in the wash step. Paraxanthine was increasingly lost from the cartridges when 40 % of methanol was used (Figure A.4.10.).

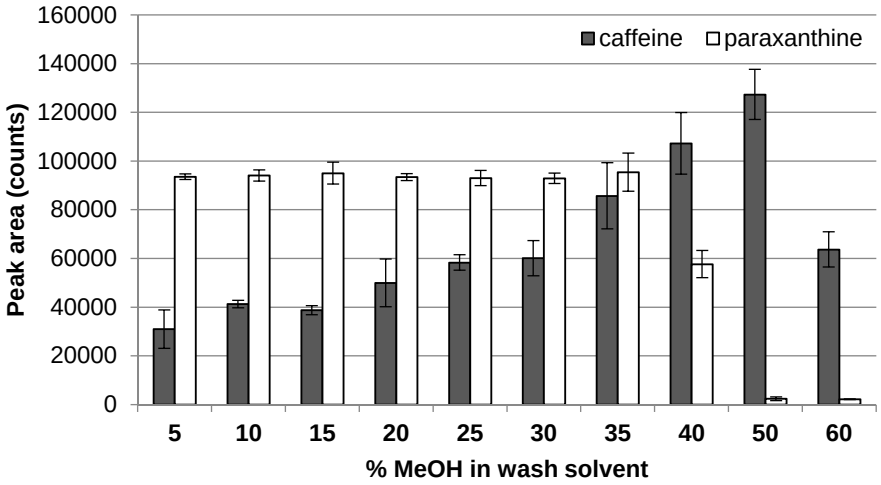


Figure A.4.10. Evaluation of the addition of incremental proportions of methanol to the SPE wash solvent (water) using Phenomenex Strata-X™ cartridges. Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

Finally, several elution solvents were tested. For paraxanthine, no differences between the evaluated solvents were found. For caffeine, the solvents containing acetonitrile gave the best results (Figure A.4.11.).

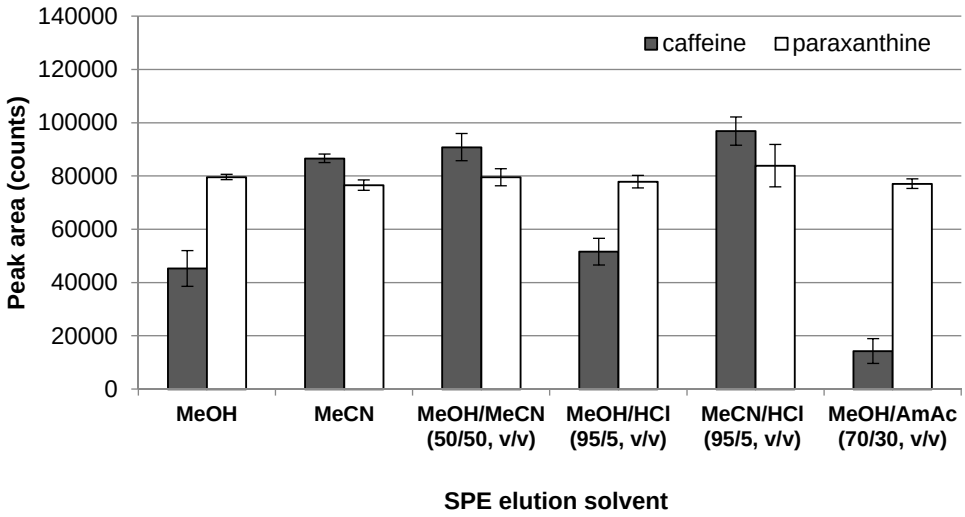


Figure A.4.11. Evaluation of different SPE elution solvents using Phenomenex Strata-X™ cartridges. Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

For the solvents containing acetonitrile, the effect of using higher elution volumes was investigated. For acetonitrile and methanol/acetonitrile (50/50, v/v), slightly higher responses were obtained when 4 x 500 µL was applied to the cartridges (Figure A.4.12.).

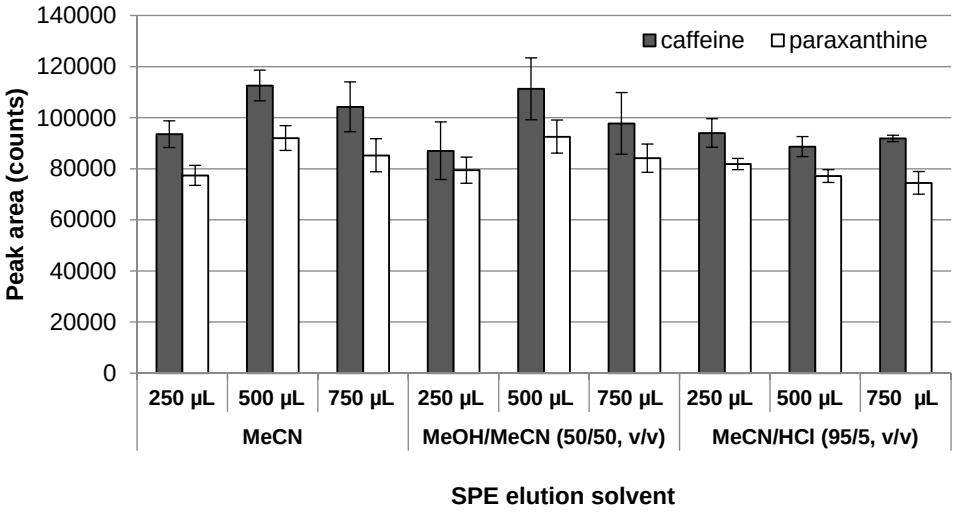


Figure A.4.12. Evaluation of different volumes of SPE elution solvents using Phenomenex Strata-X™ cartridges. Mean (n=3) peak areas of caffeine and paraxanthine in hair extracts are shown, with standard deviations.

In the final SPE protocol, 2 mL of a water/methanol mixture (65/35, v/v) was used in the wash step and 4 × 500 µL of a methanol/acetonitrile mixture (50/50, v/v) in the elution step.

A.4.3.2. Validation

No unacceptable interferences or carry-over were observed under the evaluated conditions. Calibrator data for both caffeine and paraxanthine were heteroscedastic. Sum% RE values as well as graphical representations of %RE plotted against nominal concentrations for the unweighted linear regression and after application of the different weighting factors were highly comparable. Therefore, no weighting was applied. Resulting calibration and sensitivity data are shown in Table A.4.2. Back-calculated concentrations of the calibrators were within 7 and 3 % of the nominal concentrations for caffeine and paraxanthine, respectively. Therefore, the selected regression models met the acceptance criteria and the calibration lines were linear.

Table A.4.2. Calibration and sensitivity data for the determination of caffeine and paraxanthine in hair using LC-MS/MS (n=4).

	Slope, mean ± SD [95% CI]	Intercept, mean ± SD [95% CI]	R^2	Standard deviation of residuals	Range (pg/mg)	LLOQ (pg/mg)
caffeine	3.376 ± 0.257 [3.124 – 3.627]	-0.004 ± 0.008 [-0.012 – 0.004]	0.999	0.028	20 – 500	20
paraxanthine	4.347 ± 0.311 [4.043 – 4.652]	-0.010 ± 0.018 [-0.027 – 0.008]	1.000	0.035	20 – 500	20

Intra- and interbatch precision and accuracy data for caffeine and paraxanthine QCs are summarized in Table A.4.3. As %RSD and %bias lay below 12 and 7 %, respectively, the predefined acceptance criteria were fulfilled.

Table A.4.3. Intra- and interbatch precision and accuracy (n = 4x2) for QCs of caffeine and paraxanthine prepared at four concentration levels in hair.

QC	Nominal concentration (pg/mg)	Intrabatch precision (%RSD)		Interbatch precision (%RSD)		Accuracy (%bias)	
		caffeine	paraxanthine	caffeine	paraxanthine	caffeine	paraxanthine
LLOQ	**20**	6.20	11.91	11.62	11.91	5.89	4.58
low	**60**	2.11	2.70	5.11	5.01	6.97	4.26
medium	**200**	1.72	2.16	4.75	2.55	1.02	-0.61
high	**400**	1.73	2.51	2.25	3.50	2.13	0.95

Ion suppression was observed for both compounds, as can be seen from the absolute matrix effect data shown in Table A.4.4. The signal of caffeine was suppressed to a larger extent compared to its metabolite paraxanthine. However, when compensation by the IS was taken into account, matrix effects approximated 100 %, although there was a slight overcompensation for caffeine at the low concentration level. Compensation by the IS also resulted in an improvement of relative matrix effects, which for both compounds fell within 15 % limits. As matrix effects were assessed using samples from 5 different subjects with varying hair color, the latter data indicate that hair color had no apparent effect on ion suppression, or, at least, the effect is compensated for by the labeled IS, given the lower values for relative matrix effects after IS compensation. In addition, the recovery of caffeine and paraxanthine was determined using the same 5 hair samples (Table A.4.4.). Also here, spiked samples were used and, therefore, it should be noted that these results reflect the recovery of the SPE procedure, rather than being a true measure of the actual extraction efficiency for caffeine and paraxanthine from human hair. Recovery was high (> 85 %) and found to be reproducible for both compounds, as all relative recovery values were below 15 %RSD. An additional experiment with repeated extraction [27,28] of non-spiked hair containing caffeine and paraxanthine revealed that the recovery following a single extraction was indeed high (> 73 %, data not shown), lying close to the values observed for spiked hair samples.

Table A.4.4. Absolute and relative matrix effect and recovery data (n=5) for caffeine and paraxanthine at two concentration levels in hair samples originating from five different individuals.

Caffeine								
	Absolute matrix effect (mean ± SD, %)		**Relative matrix effect (%RSD)**		**Absolute recovery (mean ± SD, %)**		**Relative recovery (%RSD)**	
QC	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS
low	66.24 ± 13.54	111.14 ± 13.36	20.43	11.98	88.43 ± 12.98	110.67 ± 9.31	14.68	8.42
high	56.58 ± 7.13	96.72 ± 4.41	12.60	4.56	89.14 ± 7.61	106.91 ± 6.66	8.54	6.23
Paraxanthine								
	Absolute matrix effect (mean ± SD, %)		**Relative matrix effect (%RSD)**		**Absolute recovery (mean ± SD, %)**		**Relative recovery (%RSD)**	
QC	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS
low	79.70 ± 4.98	95.53 ± 2.40	6.24	2.51	95.91 ± 5.46	120.54 ± 6.92	5.70	5.74
high	81.16 ± 4.88	99.49 ± 1.86	6.01	1.87	87.49 ± 4.10	106.93 ± 2.20	4.69	2.06

Hair samples proved to be stable at ambient temperature for at least 644 days, with measured concentrations being 102.61 ± 1.05 % and 102.03 ± 6.40 % of the concentrations measured at time point zero for caffeine and paraxanthine, respectively. Processed samples were stable after 4 days of storage at 4 °C (i.e. in the autosampler) and 7 days at -20°C. Stock solutions of the analytes and the IS were stable for at least 6 months when stored at -20 °C. Finally, the dilution integrity experiment showed that extracts of hair samples could be diluted 5-fold without affecting accuracy and precision. Following dilution, caffeine and paraxanthine concentrations were measured with an accuracy (%bias) of -0.99, respectively -3.45 % and precision (%RSD) of 1.23, respectively 1.02%.

A.4.3.3. Hair decontamination procedure

At the LLOQ concentration level, the recovery of the SPE procedure was 104.44 ± 5.11 % and 101.02 ± 5.96 % for caffeine and paraxanthine, respectively, when solutions with a volume of 20 mL were applied to the cartridges. Analysis of the wash solvents of 3-cm proximal hair segments from 6 volunteers revealed that paraxanthine was not present in any of the solvents, while caffeine was detected in all cases. Peak areas were determined and all responses for caffeine were below the LLOQ. Although strictly taken no quantitative conclusions can be deduced from these data, we compared the results from the different wash solvents by expressing peak areas as percentage of the peak area of the LLOQ. Peak areas ranged from 47.7 to 79.3 % of the LLOQ (mean ± SD; 63.8 ± 11.8 %) in the methylene chloride wash, from 56.9 to 72.82 % (mean ± SD; 64.0 ± 5.2 %) in the first water wash and from 28.7 to 50.2 % (mean ± SD; 37.1 ± 7.9 %) in the second water wash. Based on these data, it seems that the presence of caffeine in the wash solvents is more likely to result from limited external contamination of the hair, rather than from premature extraction from the hair matrix. First, caffeine was detected in the first wash solvent consisting of methylene chloride, a non-protic solvent that does not make the hair swell [1,3]. Second, responses for caffeine overall decreased from the first to the second wash step

using water, a protic solvent that causes swelling of the hair and may extract analytes [1,3]. Third, caffeine's metabolite paraxanthine was not detected in any of the wash solvents, supporting limited external contamination with caffeine rather than premature extraction of the analytes. Given the intensity of the caffeine responses in the last wash solvents, the effect of its presence as a presumed contaminant on final concentrations is considered to be limited, although it may play a role in cases with hair concentrations near the LLOQ.

A.4.3.4. Application

Caffeine and paraxanthine concentrations in proximal 3-cm hair segments from 10 healthy volunteers (age 21 – 36 years; 7 women) are listed in Table A.4.5., together with their corresponding paraxanthine:caffeine concentration ratios as potential CYP1A2 phenotyping metric. All measured concentrations fell into the linear concentration range of the developed method.

Table A.4.5. Caffeine and paraxanthine concentrations and corresponding paraxanthine:caffeine molar concentration ratios determined in proximal 3-cm hair segments of 10 healthy volunteers.

Volunteer	Caffeine (pg/mg)	Paraxanthine (pg/mg)	Paraxanthine:caffeine molar concentration ratio
1	173.5	87.5	0.54
2	343.3	94.5	0.30
3	384.0	74.0	0.21
4	365.6	220.8	0.65
5	253.9	143.9	0.61
6	284.6	33.3	0.13
7	409.9	134.6	0.35
8	204.9	70.9	0.37
9	311.1	91.5	0.32
10	389.0	268.9	0.75

A representative chromatogram of caffeine and its metabolites paraxanthine, theophylline and theobromine in a hair sample from one of the involved volunteers is shown in Figure A.4.13. To interpret paraxanthine:caffeine ratios in hair, they need to be compared with reference CYP1A2 phenotyping indices. Therefore, these results served as the basis for a larger study in which the usefulness of paraxanthine:caffeine ratios in hair for CYP1A2 phenotyping was evaluated by comparing hair ratios with similar ratios in the reference matrix plasma, following a classical CYP1A2 phenotyping protocol [29]. This study is described in Chapter A.5.

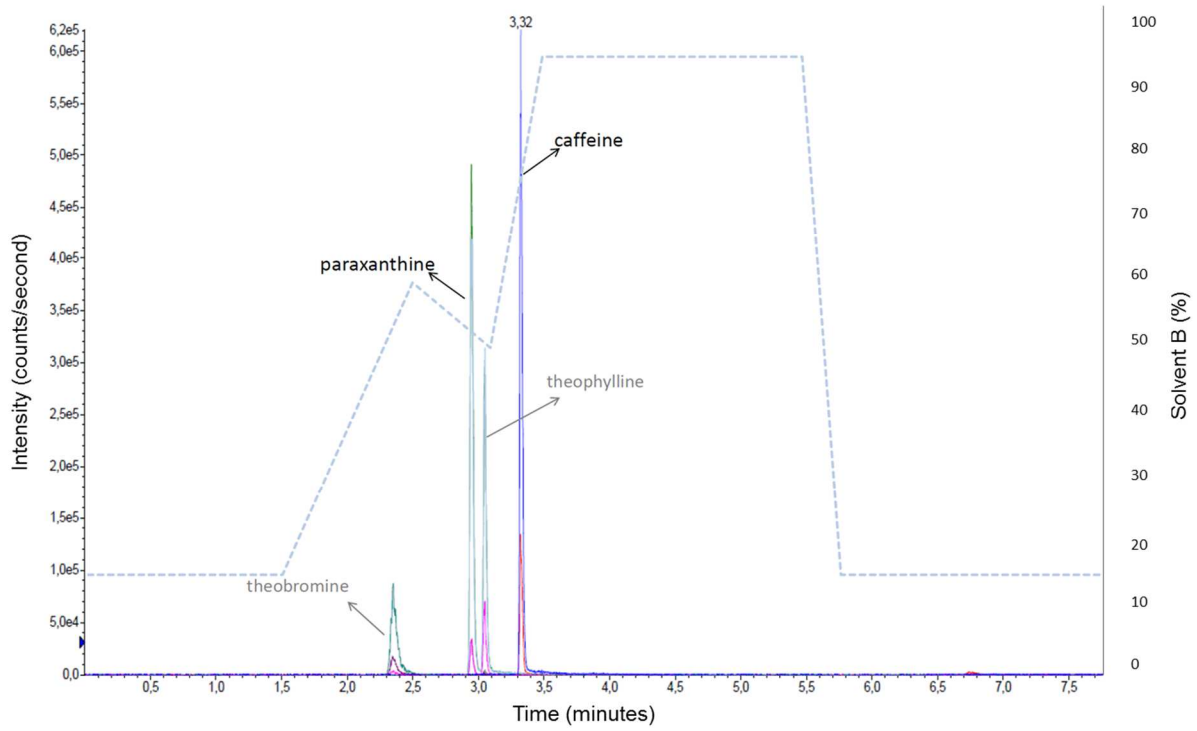


Figure A.4.13. Chromatogram of paraxanthine, caffeine, theobromine and theophylline in a hair sample from a healthy volunteer. The dashed line indicates the proportion of solvent B (methanol containing 0.01 % formic acid) in the mobile phase over the chromatographic run.

A.4.4. Conclusion

In this chapter, an SPE-LC-MS/MS-based procedure for the determination of caffeine and paraxanthine in hair was developed and validated. By thoroughly optimizing every step of the extraction and clean-up procedure, a reliable and sensitive method was obtained. The final method was fully validated based on international guidelines and all evaluated parameters met the pre-established criteria. Special attention was paid to the evaluation of the effect of the hair decontamination procedure. Finally, the suitability of the method was demonstrated by applying it to hair samples of 10 healthy volunteers.

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Chapter A.5.

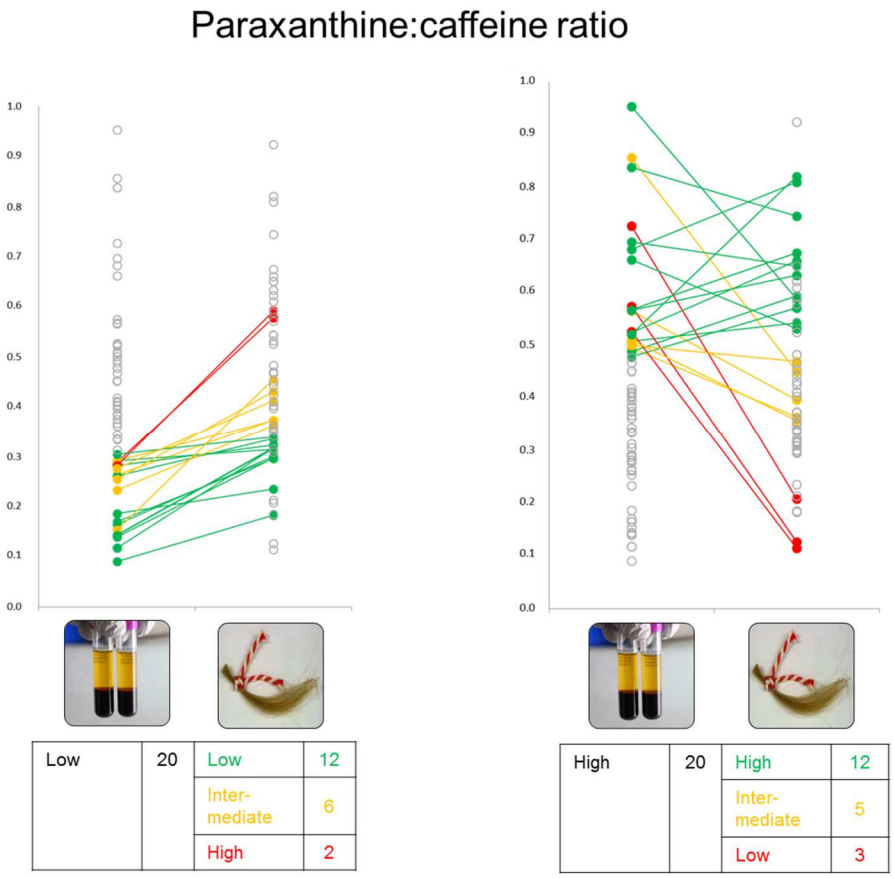
Evaluation of hair analysis for CYP1A2 phenotyping

Based on

De Kesel PM, Lambert WE, Stove CP. Paraxanthine/caffeine concentration ratios in hair: an alternative for plasma-based phenotyping of cytochrome P450 1A2? Clin Pharmacokinet. 2015; DOI 10.1007/s40262-015-0237-7.

Abstract

Although metabolite-to-parent drug concentration ratios in hair have been suggested as a possible tool to study the metabolism of drugs in a non-invasive way, no studies are available that evaluate this in a systematic way. CYP1A2 is a drug metabolizing enzyme characterized by large inter-individual differences in its activity. The standard approach for CYP1A2 phenotyping is to determine the paraxanthine:caffeine ratio in plasma, at a fixed time point after intake of a dose of the CYP1A2 substrate caffeine. The aim of the study described in this chapter was to evaluate whether paraxanthine:caffeine ratios measured in hair samples reflect the plasma-based CYP1A2 phenotype. Therefore, caffeine and paraxanthine concentrations were measured in proximal 3-cm segments of hair samples from 60 healthy volunteers and resulting paraxanthine:caffeine ratios were correlated with CYP1A2 phenotyping indices in plasma. Paraxanthine:caffeine ratios in hair ranged from 0.12 to 0.93 (median 0.41), corresponding ratios in plasma ranged from 0.09 to 0.95 (median 0.40). A statistically significant correlation was found between ratios in hair and plasma ($r = 0.41$, $p = 0.0011$). However, large deviations between ratios in both matrices were found in individual cases. Although the influence of several factors on paraxanthine/caffeine ratios and hair-plasma deviations was investigated, no evident factors explaining the observed variability could be identified. In conclusion, the variability between hair and plasma ratios complicates the interpretation of hair ratios on an individual basis and, therefore, compromises their practical usefulness as alternative CYP1A2 phenotyping metrics.



A.5.1. Introduction

Sampling and analysis of hair has earned an established place in bioanalysis, with applications in diverse fields, including, amongst others, postmortem toxicology, workplace drug testing, doping control, driving ability examination and detection of pre- and postnatal drug exposure. Owing to the ability to retrospectively determine drug use over a time window of several months (or even years), together with the long-term stability of many drugs in hair and the non-invasive sample collection, hair strands are collected as an alternative for, or in combination with, traditional blood or urine samples. On the other hand, important limitations associated with hair analysis are the lack of a clear-cut correlation between hair and plasma concentrations -the latter reflecting drug dose- for many substances and the risk of misinterpreting hair results due to external contamination [1,2].

In addition to the determination of parent drugs, highly sensitive analytical techniques, such as liquid chromatography (LC)- or gas chromatography (GC)-tandem mass spectrometry (MS/MS), also allow to quantity specific drug metabolites in hair, which are often present at low concentration levels. Resulting metabolite-to-parent drug concentration ratios have been applied to distinguish active ingestion of a drug from external contamination or passive exposure. For this purpose, cut-off values have been proposed for several drugs, such as cocaine, heroin and tramadol [3,4]. Apart from the latter application, the use of metabolite-to-parent drug concentration ratios in hair has recently been proposed as a possible means to assess the metabolic phenotype of drug metabolizing enzymes, as reviewed by De Kesel *et al*. [5]. Thieme *et al*. found that nortriptyline/amitriptyline concentration ratios measured in hair samples of children (n = 23) correlated significantly with the number of functional alleles of CYP2C19, the enzyme that is predominantly responsible for the demethylation of amitriptyline. Poor metabolizers showed significantly lower nortriptyline/amitriptyline ratios in hair compared to intermediate or rapid extensive metabolizers [6]. Eisenhut *et al*. determined concentrations of the anti-tuberculosis drug isoniazide and its metabolite acetylisoniazide in hair samples (n = 24) and correlated the corresponding metabolite-to-parent drug concentration ratios to the arylamine *N-acetyltransferase-2* (*NAT-2*) genotype. Acetylisoniazide/isoniazide ratios in hair samples of slow acetylators were significantly lower than ratios in subjects possessing rapid acetylator genotypes [7]. In a recent study, LeMasters *et al*. found higher cotinine levels in hair of secondhand smoke exposed children carrying the *NAT-1* minor allele compared to children with the major allele [8]. Several groups also reported on the use of metabolite-to-parent drug ratios to (retrospectively) detect changes in drug metabolism within individuals during pregnancy. Koren *et al*. found higher cotinine/nicotine ratios in hair segments corresponding to the third trimester of pregnancy than in segments corresponding to the first trimester, as determined in hair samples of 74 pregnant women. According to the authors, these results suggest an increased CYP2A6-mediated nicotine metabolism during pregnancy [9]. Similar findings for the antidepressant citalopram and its metabolite norcitalopram, determined in hair samples of 4 subjects, were described by O'Brien *et al*. [10]. Finally, by analyzing proximal hair segments from 29 women involved in a methadone-assisted therapy at different time points during pregnancy, Himes *et al*. found a positive correlation between 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)/methadone ratios and cumulative methadone dose. Also here, the authors suggested that these findings could indicate an altered metabolism during

gestation [11]. Although the above mentioned results seem very promising, it should be noted that in the latter 3 studies no control samples from non-pregnant women or reference phenotyping indices determined in non-hair matrices were included to support the validity of hair ratios in reflecting changes in drug metabolism.

In this chapter the usefulness of determining paraxanthine:caffeine concentration ratios in hair for CYP1A2 phenotyping is evaluated by comparing hair ratios of healthy volunteers with their reference, plasma-based CYP1A2 phenotyping indices. CYP1A2, a member of the CYP family of drug metabolizing enzymes, plays a role in approximately 9 % of drug metabolism pathways in which CYP isoforms are involved [12]. Several therapeutically used drugs are metabolized by CYP1A2, including olanzapine [13], clozapine [14], zolmitriptan [15] and warfarin [16]. CYP1A2 activity is affected by a combination of genetic, nongenetic and environmental factors [12] and, consequently, its activity displays a high inter-individual variability [17]. Therefore, a phenotyping approach involving controlled administration of a selective probe drug is preferred to assess CYP1A2 activity. As caffeine is predominantly metabolized by CYP1A2, it is the probe drug of choice [17,18]. In typical phenotyping approaches the paraxanthine:caffeine concentration ratio is determined 4-6 h post-administration in serum or plasma samples [19], although dried blood spots [20] and saliva [21] have proven to be appropriate matrices for this purpose as well. A CYP1A2 phenotyping procedure based on hair analysis would eliminate the need to administer a caffeine test dose, since it is expected that caffeine and paraxanthine will readily be detected in hair samples of a large part of the population given the widespread consumption of caffeine-containing beverages (e.g. coffee, tea, soft drinks) and food products (e.g. chocolate). This study is the first to compare hair ratios with plasma ratios for phenotyping purposes. The comparison between paraxanthine:caffeine ratios in hair and those obtained using a classical plasma-based approach may provide valuable insights into the usefulness and potential of hair analysis for this purpose. This could e.g. be relevant in fatal cases where it might be possible to deduce a phenotype postmortem by analyzing hair.

A.5.2. Material and methods

A.5.2.1. CYP1A2 phenotyping study

A CYP1A2 phenotyping study involving healthy volunteers was approved by the Ethics Committee of Ghent University Hospital (B670201111655). Scalp hair samples were collected at the posterior vertex region after obtaining written informed consent from each participant. Caffeine and paraxanthine concentrations were determined in proximal 3-cm segments and the corresponding paraxanthine:caffeine molar concentration ratios were calculated. For 4 volunteers, a segmental analysis of 6 consecutive 3-cm hair segments was performed as well. At the day of hair sampling, a standard CYP1A2 phenotyping procedure was conducted in the same study population, as described in Chapter A.3. [20]. Briefly, caffeine and paraxanthine concentrations were determined with a validated method in plasma samples from volunteers that had taken a 150 mg caffeine capsule 6 hours (± 5 minutes) before sample collection [20,22]. By means of a written questionnaire, data on sex, age,

general health status, smoking habits, caffeine intake, alcohol consumption, intake of drugs (including hormonal contraception) and cosmetic hair colouring were obtained. Caffeine intake was defined as the number of caffeine-containing drinks (including coffee, tea, soft and energy drinks) per day, while alcohol use was defined as the number of alcohol-containing drinks per week. Volunteers were asked to report chronic and occasional medication use at the time of inclusion and in the past without defining a time frame. A list of substrates, inhibitors or inducers of CYP1A2 was not available. Hair color was assessed by visual inspection of the collected samples in the laboratory.

A.5.2.2. Hair sample collection, pretreatment and analysis

Hair locks were cut as close as possible to the scalp at the posterior vertex region of the head and stored in aluminum foil envelopes at room temperature until analysis. Using adhesive tape, hair samples were either segmented into 3-cm sections or only the proximal 3-cm segment was used. As described in Chapter A.4, Section A.4.2.3., the resulting segments were decontaminated in methylene chloride (20 mL, 2 min), followed by two washes in water (20 mL, 2 min). Following each wash step, the wash solvents were removed and the hair was air-dried. Dried samples were cut into small snippets (< 5 mm) using scissors before being manually ground in a mortar with liquid N_2. One mL of a solution of protease type VIII (1.2 IU/mL) in Tris buffer (pH 7.5, 50 mmol/L), containing the internal standards caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$, was then added to 20 mg of ground hair. The samples were shaken for 1 hour at 37 °C and 750 rpm and centrifuged for 10 minutes at 10000 x *g.* Subsequently, the supernatants were cleaned-up by solid phase extraction (SPE) using Phenomenex Strata-X™ cartridges (200 mg, 3 mL). These were conditioned and equilibrated with 3 mL of methanol and 2 mL of water, respectively, prior to application of the hair extracts. The cartridges were washed with 2 mL of a water/methanol mixture (65/35, v/v) and dried for 2 minutes. The analytes were eluted from the columns with 4 × 500 µL of a methanol/acetonitrile mixture (50/50, v/v). The obtained solutions were evaporated under a stream of N_2 and redissolved in 150 µL of the mobile phase.

To evaluate the effect of the analytical wash procedure, the second (i.e. 3-6 cm) segments of 10 samples from the data set were decontaminated and all wash solvents were subsequently analyzed. The samples originated from 5 individuals with the largest positive differences between hair and plasma ratios and 5 individuals with the largest negative differences (see further).

All analyses were performed on an LC-MS/MS configuration consisting of a Waters Acquity UPLC® system (Waters, Milford, MA, USA) and an AB SCIEX API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA), controlled by AB SCIEX Analyst 1.5.2 and Waters Acquity console software. Chromatographic conditions for the determination of caffeine and paraxanthine in hair were identical to those described in Chapter A.2., Section A.2.2.2.2.for the analysis of dried blood spots, whole blood and plasma [22]. The same holds for the API 4000™ mass spectrometer parameters, except for some minor modifications (see Chapter A.4., Section A.4.2.4). All compounds were detected in scheduled multiple reaction monitoring (sMRM) mode. The monitored precursor to product ion transitions were identical as listed in Table A.2.1. (Chapter A.2.) [22].

Validation of the hair analysis procedure was based on U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [23,24]. For both compounds, no carry-over or interferences were seen in the chromatogram. Linear calibration curves for caffeine and paraxanthine ranged from 20 to 500 pg/mg, with 20 pg/mg being the lower limit of quantification (LLOQ) for both compounds. Intra- and interbatch precision was below 12% (% relative standard deviation, %RSD) and accuracy below 7% (%bias) for all QCs (20, 60, 200 and 400 pg/mg). Matrix effects were determined at two concentrations levels using hair samples from five different individuals. Ion suppression was observed for caffeine (56.58 – 66.24 % absolute matrix effect) and paraxanthine (79.70 – 81.16 % absolute matrix effect). When compensation by the IS was taken into account, absolute matrix effects approximated 100 % and relative matrix effects were within 15 % limits. The same five hair samples were used to determine the recovery of the sample preparation procedure by spiking samples before and after extraction and clean-up. Recovery was high (> 87 %) and reproducible (%RSD < 15 %). Incurred and processed samples were stable under the evaluated conditions. Finally, it was shown that extracts of hair samples could be safely diluted 5-fold. Full details on the validation of the hair method can be found in Chapter A.4., Section A.4.3.2.

A.5.2.3. Chemicals and stock solutions

Ultrapure water was obtained from a Synergy® Water Purification System (Merck Millipore, Overijse, Belgium). Methanol, acetonitrile and methylene chloride were purchased from Biosolve (Valkenswaard, The Netherlands). Caffeine, caffeine-$^{13}C_3$, paraxanthine, paraxanthine-$^{13}C_4$-$^{15}N_3$, theophylline, theobromine, formic acid, protease type VIII and trishydroxymethylaminomethane (Tris) were all purchased from Sigma-Aldrich (Diegem, Belgium). Liquid N_2 was obtained from Air Liquide (Brussels, Belgium). Stock solutions were prepared as described in Chapter A.2., Section A.2.2.1 [22].

A.5.2.4. Statistical analysis

Statistical evaluation and graphical representation of the data was performed using Microsoft Excel® 2010 (Microsoft, Redmond, WA, USA), IBM SPSS Statistics 20 (IBM Corp., Armonk, NY) and Medcalc 12.7.5 (MedCalc Software bvba, Ostend, Belgium). Normality of paraxanthine:caffeine ratios in plasma and hair was assessed by Kolmogorov-Smirnov test. Correlation was examined by Pearson coefficient of correlation. Differences between 2 groups were determined by independent samples t-tests, while one-way analysis of variances (ANOVA) and Tukey post-hoc tests were used when more than 2 groups were compared. Significance was defined as $p < 0.05$.

A.5.3. Results

Caffeine and paraxanthine concentrations in 6 consecutive 3-cm hair segments from 4 female, healthy volunteers overall decreased with increasing distance from the hair root (Figure A.5.1. A-D). Corresponding paraxanthine:caffeine molar concentration ratios showed a considerable variability between the consecutive hair segments (Figure A.5.1. E). Therefore, we decided to analyze proximal 3-cm hair segments in the actual comparative phenotyping study.

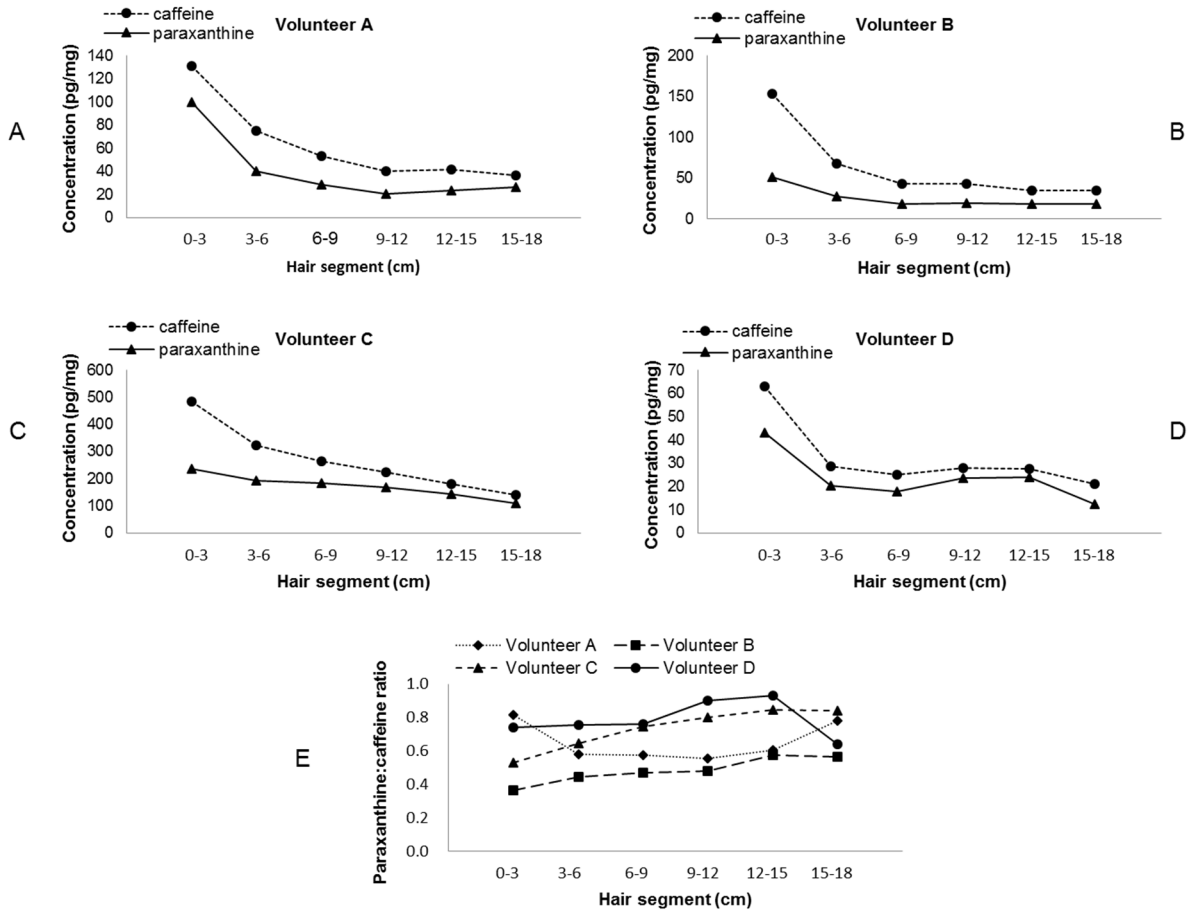


Figure A.5.1. Caffeine and paraxanthine concentrations (A-D) and paraxanthine:caffeine molar concentration ratios (E) in 6 consecutive 3-cm hair segments from 4 female, healthy volunteers.

Paraxanthine was not detected in any of the analyzed wash solvents. For caffeine, responses below the LLOQ were seen in all wash solutions from all 10 participants of the phenotyping study. Although no real quantitative conclusions can be drawn from these data, peak areas were compared with the peak area of the LLOQ. The average peak area of caffeine was 54.08 ± 10.16 % of the LLOQ for the methylene chloride wash, 39.80 ± 11.44 % for the first water wash and 21.46 ± 6.10 % for the second water wash.

Sixty healthy volunteers were involved in the CYP1A2 phenotyping study, including 43 women and 4 smokers. The age of the participants ranged from 24 to 48 years (mean ± standard deviation (SD); 28.8 ± 5.7). Paraxanthine:caffeine molar concentration ratios in plasma and hair samples were normally distributed (Kolmogorov-Smirnov test; p = 0.200 and p = 0.093, respectively). The median paraxanthine:caffeine ratios in both matrices, along with the measured ranges, are listed in Table A.5.1.

Table A.5.1. Paraxanthine:caffeine molar concentration ratios in plasma and hair samples. The median values together with the observed ranges are listed.

Variable		Paraxanthine:caffeine molar concentration ratio in plasma		Paraxanthine:caffeine molar concentration ratio in hair	
	n	median	range	median	range
All participants	60	0.40	0.09 – 0.95	0.41	0.12 – 0.93
Hair color					
Blond	13	0.41	0.14 – 0.95	0.37	0.12 – 0.81
Brown	16	0.38	0.14 – 0.73	0.41	0.21 – 0.82
Dark	15	0.42	0.12 – 0.86	0.40	0.21 – 0.93
Black	5	0.34	0.31 – 0.40	0.47	0.34 – 0.65
Cosmetic colouring					
Coloured	11	0.41	0.09 – 0.84	0.47	0.18 – 0.75
Non-treated	49	0.40	0.12 – 0.95	0.40	0.12 – 0.93
Sex					
Women	43	0.34 [a,b]	0.09 – 0.84	0.40 [a,b]	0.12 – 0.93
Men	17	0.51 [a,b]	0.31 – 0.95	0.59 [a,b]	0.13 – 0.82
Hormonal contraception					
Non-users	18	0.47 [a]	0.26 – 0.84	0.50 [a]	0.12 – 0.93
Users	25	0.28 [a]	0.09 – 0.66	0.37 [a]	0.18 – 0.68
Smoking					
Smoker	4	0.53	0.29 – 0.86	0.52	0.31 – 0.81
Non-smoker	56	0.40	0.09 – 0.95	0.41	0.12 – 0.93
Caffeine intake (consumptions/day)					
0	1	0.95		0.59	
1-3	23	0.45	0.09 – 0.84	0.48	0.12 – 0.75
4-6	32	0.37	0.12 – 0.70	0.37	0.13 – 0.93
> 7	4	0.43	0.28 – 0.86	0.43	0.19 – 0.82
Alcohol intake (consumptions/week)					
0	7	0.50 [a,b]	0.40 – 0.84	0.55	0.13 – 0.75
1-3	26	0.35 [a,b]	0.09 – 0.57	0.37	0.12 – 0.93
4-6	18	0.40	0.14 – 0.73	0.46	0.21 – 0.82
> 7	9	0.34	0.16 – 0.95	0.35	0.19 – 0.81
Age (years)					
20-29	43	0.39	0.09 – 0.95	0.40	0.12 – 0.82
30-39	13	0.41	0.19 – 0.84	0.41	0.13 – 0.93
40-49	4	0.41	0.26 – 0.66	0.50	0.34 – 0.57

a: significantly different , b: no longer significantly different when hormonal contraception users were excluded

Pearson correlation analysis revealed a correlation coefficient of 0.410 for ratios in hair and plasma (p = 0.001). Although the correlation was found to be statistically significant, a wide scatter of the data was observed, with large deviations between corresponding ratios in hair and plasma in individual cases (Figure A.5.2.).

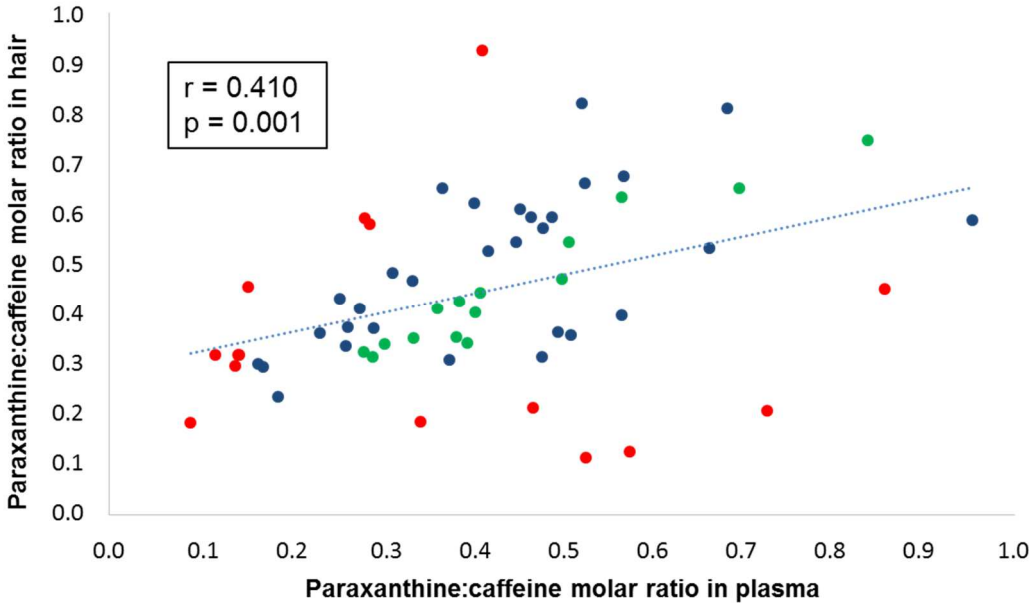


Figure A.5.2. Correlation between paraxanthine:caffeine molar concentration ratios determined in 3-cm proximal hair segments and in plasma samples from 60 healthy volunteers. Pearson correlation coefficient r is displayed, together with its p-value. Subgroups (n = 15) with a good and a poor correlation between ratios in hair and plasma are represented by green and red dots, respectively. The remaining subjects (n = 30) are represented by blue dots.

To evaluate further into depth to what extent ratios in hair correlate with corresponding plasma-based ratios, the datasets from both matrices (n = 60) were sorted from low to high and arbitrarily divided into 3 equally-sized groups (n = 20 for each group). From the 20 individuals showing the lowest ratios in plasma, 12 also showed the lowest ratios in hair, 6 were in the intermediate subgroup and 2 were in the subgroup with the highest ratios in hair (Figure A.5.3. A). A similar pattern was observed for the 20 individuals showing the highest ratios in plasma: 12 subjects were in the subgroup with the highest ratios in hair, while 5 were in the intermediate subgroup and 3 were in the subgroup with the lowest ratios in hair (Figure A.5.3. B).

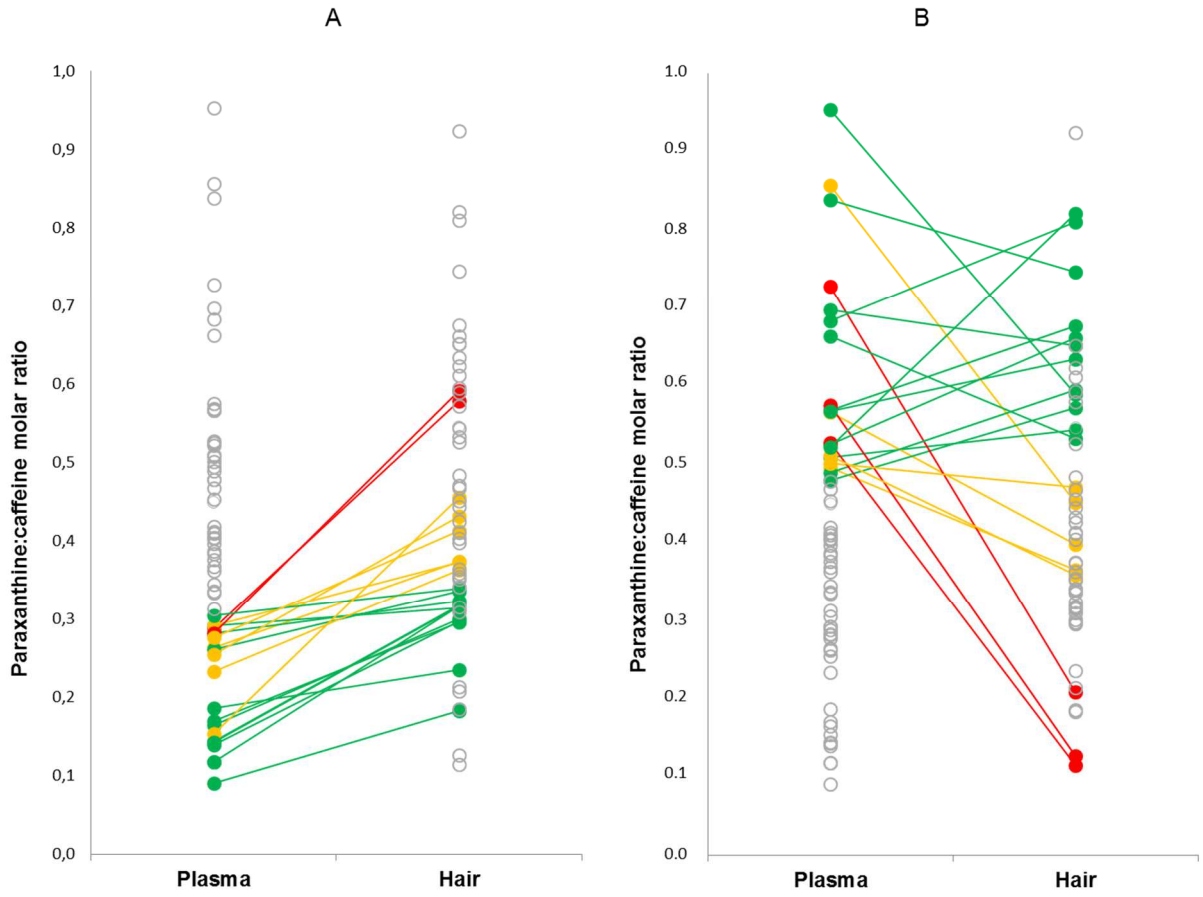


Figure A.5.3. Paraxanthine:caffeine molar concentration ratios in plasma and in hair from 60 healthy volunteers were arbitrarily divided into 3 subgroups containing the lowest, intermediate and highest ratios (n = 20 for each group). A: green lines between subjects showing the lowest ratios in plasma and in hair (n =12), orange lines between subjects showing the lowest ratios in plasma and intermediate ratios in hair (n = 6), red lines between subjects showing the lowest ratios in plasma and highest ratios in hair (n = 2). B: green lines between subjects showing the highest ratios in plasma and in hair (n = 12), orange lines between subjects showing the highest ratios in plasma and intermediate ratios in hair (n = 5), red lines between subjects showing the highest ratios in plasma and lowest ratios in hair (n = 3)

The influence of various factors, both related to the use of hair as a bioanalytical matrix and to a possible effect on CYP1A2 activity, was evaluated. The median values and corresponding ranges for paraxanthine:caffeine ratios in plasma and hair are listed in Table A.5.1. Hair color had no apparent effect on the paraxanthine:caffeine ratios. Ratios in plasma as well as in hair did not differ significantly between individuals with non-cosmetically-treated blond, brown, dark or black hair (p = 0.836 and 0.708, respectively). In addition, paraxanthine:caffeine ratios in subjects with cosmetically coloured hair were compared with ratios from subjects with non-cosmetically treated hair. Again, no significant differences were found for the ratios in both plasma and hair (p = 0.941 and 0.959, respectively). Concerning the effect of factors affecting CYP1A2 activity, we found that hormonal contraception users had 1.7-fold lower paraxanthine:caffeine ratios in plasma compared with women not taking hormonal contraception

($p < 0.0001$). Although also in hair the median ratio was lower in hormonal contraception users, this difference did not reach statistical significance when comparing hormonal contraception users and non-users ($p = 0.143$). Apart from hormonal contraceptives, none of the participants reported the use of any drugs that interfere with CYP1A2 activity. Both in plasma and hair, women showed lower ratios compared to men ($p = 0.00136$ and 0.0465, respectively); however, this sex effect disappeared when hormonal contraception users were excluded ($p = 0.317$ and 0.412 for plasma and hair, respectively). Paraxanthine:caffeine ratios in the small subgroup of smokers ($n = 4$) were not significantly different from ratios in non-smokers ($p = 0.119$ and 0.274 for plasma and hair, respectively). Also average caffeine intake had no apparent effect on measured ratios ($p = 0.154$ and 0.915 for different groups of caffeine intake in plasma and hair, respectively). Yet it is interesting to note that hair caffeine concentrations actually showed an increasing trend in subjects consuming higher amounts of caffeine, with significantly higher concentrations in hair from subjects reporting intake of > 7 caffeine consumptions/day ($p < 0.0001$) (Figure A.5.4.). Concerning the influence of alcohol intake, individuals not consuming alcohol showed higher paraxanthine:caffeine ratios in plasma compared to individuals consuming 1-3 consumptions/week ($p = 0.031$). However, again, the difference was no longer significant when excluding hormonal contraception users from the comparison ($p = 0.629$). Finally, we found no effect of age on paraxanthine:caffeine ratios ($p = 0.343$ and 0.880 for plasma and hair, respectively).

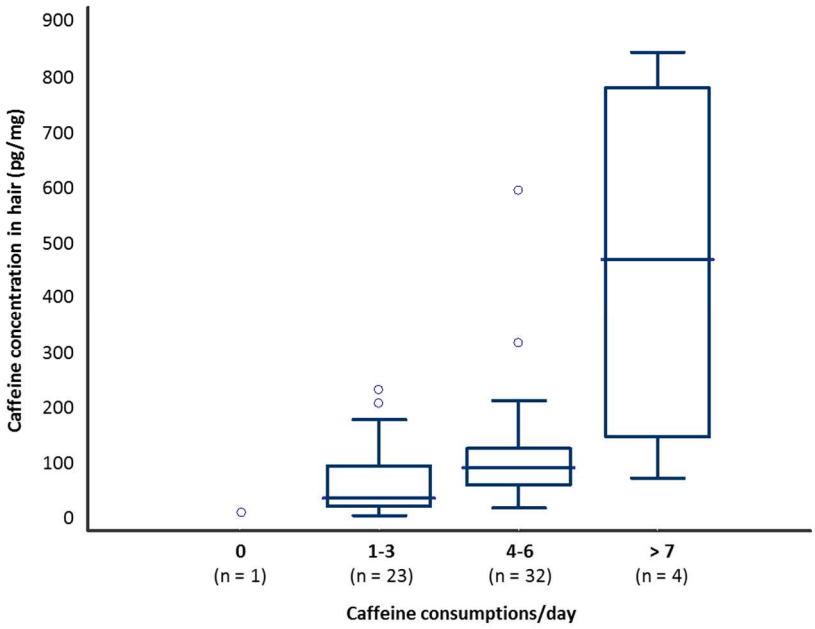


Figure A.5.4. Hair caffeine concentrations in groups of healthy volunteers consuming different amounts of caffeine-containing drinks per day. The central box represents the values from the lower to upper quartile. The middle line represents the median. The whiskers represent minimum and maximum values, excluding values larger than the upper quartile plus 1.5 times the interquartile range, which are represented by dots. In the case of a single data point, it is represented by a dot as well.

To further investigate the effect of these factors on the variability between ratios in hair and plasma, 2 subgroups (n = 15) were selected from the dataset, with either a good or a poor correlation between ratios in hair and plasma (Figure A.5.2.), with respective differences between corresponding ratios below 15 % and above 60 %. The distribution of all groups of participants in the poor and good correlation subgroup, as well as in the entire dataset, is displayed in Figure A.5.5. For none of the evaluated factors, a clear effect on the variability between hair and plasma ratios was found as the distribution in both subgroups was similar in all cases.

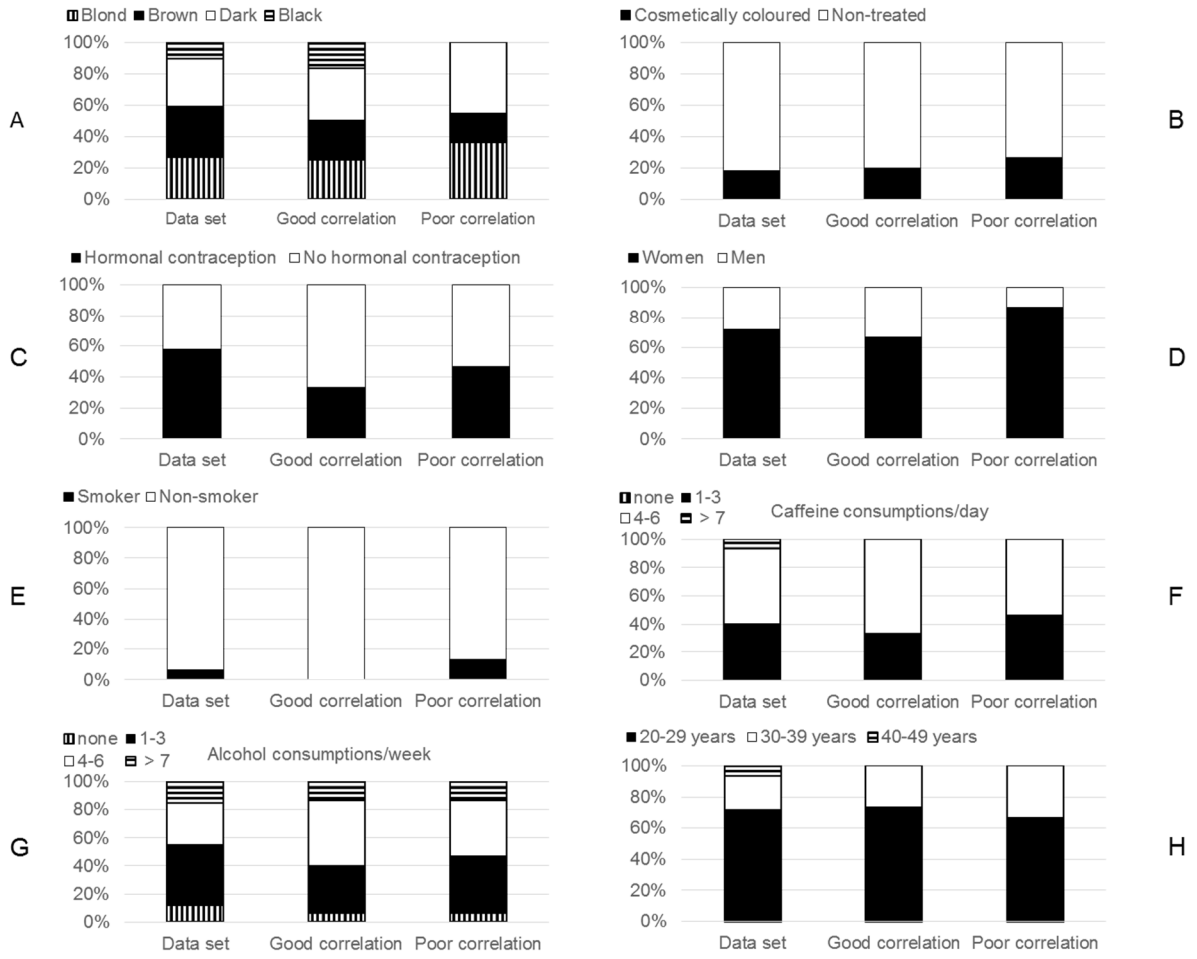


Figure A.5.5. Distribution of participants, sorted according to different factors (*), in the entire data set (n = 60) and in the subgroups of subjects showing a good (n= 15) or poor correlation (n = 15) between ratios in hair and plasma

(*) A: hair color (only non-cosmetically coloured samples), B: cosmetic hair colouring, C: hormonal contraception, D: sex, E: smoking, F: average number of caffeine consumptions per day, G: average number of alcohol consumptions per week, H: age

A.5.4. Discussion

Determining metabolite-to-parent drug concentration ratios in traditional matrices, such as plasma or urine [19], or alternative samples, such as dried blood spots [20] or saliva [21] is an accepted approach to assess the metabolic activity of enzymes. In the last few years, the use of hair analysis has been introduced in this field [5]. Already in 1996, Mizuno *et al.* suggested the use of the caffeine content in hair as an indicator of liver function, as they found higher caffeine concentrations in hair of liver cirrhosis patients compared to healthy volunteers [25]. In this study, using an optimized and validated LC-MS/MS method, paraxanthine:caffeine concentration ratios were determined in hair for the first time as a potential measure of CYP1A2 activity. This is, to the best of our knowledge, the first study in which metabolite-to-parent drug ratios in hair were compared with reference, plasma-based phenotyping metrics.

Segmental hair analysis showed decreasing caffeine and paraxanthine concentrations from the hair root to the distal end. As all 4 volunteers reported to generally have a constant caffeine intake over time and none of the hair samples had been cosmetically treated (i.e. coloured, bleached or permed), the observed decrease in analyte concentrations along the hair shaft is probably due to a wash-out effect caused by regular hair washing, mechanical stress and influence of UV-light over time [1]. Although the underlying reason for the observed variability between paraxanthine:caffeine ratios is not known, we cannot exclude the theoretical possibility that caffeine and paraxanthine might be unequally removed from the hair as a result of regular hair washing or other environmental factors to which hair is exposed over time, which could affect paraxanthine:caffeine ratios. Data on hair washing habits were not collected, as it would be very difficult to adequately assess the effect of these factors, given the large variability in regular hair washing procedures that can be expected. As a result, proximal 3-cm hair segments were used in this study, as is also commonly applied when determining markers of alcohol use in hair [26]. Furthermore, from a practical point of view, analyzing the first 3-cm segment was more convenient as it allowed to apply the procedure to individuals with short hair as well, thereby preventing that hair length would be a limiting factor for inclusion in the study. Although 3-cm segments could be obtained from all participants, hair length would be even less of a concern when shorter segments were used. However, this was not an option in this study for two reasons. First, we wished to avoid that samples needed to be excluded from the study due to limited sample amount (i.e. less than 20 mg of ground hair) or measured concentrations below the LLOQ, especially for the metabolite paraxanthine which was expected to be present in hair at lower concentrations. Second, while the aforementioned sensitivity issue might be coped with by sampling a shorter segment from a larger head area, this would undoubtedly have had a negative cosmetic impact, rendering it difficult to recruit sufficient volunteers.

Using data from 60 healthy volunteers, a statistically significant correlation between ratios in hair and plasma was found, albeit that large deviations were observed for some individuals. Given the lack of a clear link between CYP1A2 activity and known polymorphisms of the *CYP1A2* gene, a distinction between poor or extensive metabolizer status, as is the case for other CYP450 isoforms such as CYP2D6, cannot be made for CYP1A2 [12,27]. In addition, there are also no established reference ranges or cut-off values for CYP1A2 phenotyping metrics available. In this light, Figure A.5.3. illustrates

to what extent ratios in hair correlate with corresponding plasma-based ratios. This graphical representation shows that, in some cases, ratios in hair do not provide a reliable reflection of the CYP1A2 activity. For example, 3 individuals (2 women, 1 man) belonging to the subgroup of participants with the highest ratios in plasma, showed hair ratios that were among the 5 lowest measured in the entire study population (Figure A.5.3. B). As described below, the effect of several variables was evaluated for these individuals, as was done for the entire data set. They were non-smokers, had different hair color and did not report cosmetic hair colouring, use of CYP1A2-affecting medication, excessive caffeine or alcohol intake. Therefore, no clear explanation for their deviating results was available.

In general, many factors may contribute to the observed deviations between ratios in hair and plasma. We consider it most unlikely that analytical issues contributed to the observed variability. Validated LC-MS/MS methods were used for both matrices, with highly comparable data for precision (%RSD) and accuracy (%bias) in hair (Chapter A.4., Table A.4.3.) and plasma (Chapter A.2., Table A.2.3.) [22]. In addition, the differences between hair and plasma ratios cannot be explained by breakdown of the analytes during storage, as caffeine and paraxanthine were stable in hair stored at room temperature for at least 644 days (Chapter A.4., Section A.4.3.2.) as well as in plasma stored at -20 °C for at least 172 days (Chapter A.2., Table A.2.6.) [22]. Our evaluation of the analytical wash procedure only demonstrated the presence of caffeine in the wash solvents, with peak areas corresponding to less than 30 % of the LLOQ in the last wash step, consistent with our observations in the validation of this procedure (Chapter A.4., Section A.4.3.3.). In general, these amounts will have a limited impact on measured paraxanthine:caffeine ratios.

Another aspect that may have an effect on the observed variability is the methodological nature of the conducted comparative phenotyping study. Paraxanthine:caffeine ratios determined in plasma samples represent a measure of the CYP1A2 activity at the actual time of sampling. Ratios measured in 3-cm hair segments, on the other hand, rather may provide an image of the enzyme activity over a period of approximately 3-4 months prior to sampling, assuming that it takes approximately 2 weeks until a hair reaches the scalp and that hair grows with an average rate of 1.06 cm/month [28]. As a consequence, high intra-individual variability in CYP1A2 activity over time might be raised as an explanation for the differences between ratios in hair and plasma. However, using a similar CYP1A2 phenotyping approach as applied here, Simon *et al.* found that paraxanthine:caffeine ratios, determined in plasma 5 hours after administration of a 140 mg caffeine dose, did not differ significantly over a period of 12 weeks. The authors reported an intra-individual coefficient of variation (CV) of 17.6 ± 6 % and 16.2 ± 5.9 %, respectively in young and elderly subjects [29]. Based on the findings from this study, combined with the fact that none of the volunteers in our study reported to take (or have taken) medication of which an inhibiting or inducing effect on CYP1A2 has been described (apart from hormonal contraception), we consider it unlikely that intra-individual variability can explain the observed deviations.

In addition to the factors described above, the incorporation of caffeine and paraxanthine in hair may be a matter of concern. Incorporation of analytes into hair is a complex phenomenon in which multiple mechanisms are involved. Compounds can be incorporated via passive diffusion from blood capillaries

or other tissues surrounding the hair follicle, from sweat or sebum or through external contamination [30]. Many factors affect these processes, such as the physicochemical properties of the analyte and its affinity for binding to melanin in hair. As melanin determines hair pigmentation, a color-dependent incorporation has been described for several compounds [31,32]. Here, no influence of hair color on paraxanthine:caffeine ratios was found. Furthermore, it is known that cosmetic treatment of hair may decrease drug concentrations [33]. However, in our population, cosmetic hair colouring did not affect measured ratios in hair. In addition, both factors had no apparent effect on the observed hair-plasma deviations. Another aspect that may play a role is the site of hair sampling. Dussy *et al.* determined caffeine concentrations in hair locks from 3 individuals collected at 10 different areas of the scalp. Coefficients of variation of measured caffeine concentrations ranged from 12.6 to 61.9 %, while a CV of 4.5 % was found for a homogenized control sample analyzed in six-fold [34]. However, it should be noted that the areas of sampling were widely distributed over the scalp, which could have contributed to the observed variability in measured concentrations as the proportions of hair in the anagen or telogen phase may vary in function of the location. In our study, all hair samples were cut from the posterior vertex region of the head. It is generally recognized that the proportion of hair in the telogen phase is the lowest and that hair growth rate shows less variability in this region [1,2]. Furthermore, all samples were collected by the same operator to minimize variation in the actual sampling site.

As mentioned in Section A.5.1. (Introduction), CYP1A2 activity is characterized by a large inter-individual variability to which many factors have been reported to contribute. The influence of several factors on CYP1A2 activity was evaluated. The inhibiting effect of hormonal contraceptives found in our population is similar to that described in other studies [35,36]. When hormonal contraception users were excluded, no sex differences in paraxanthine:caffeine ratios were found. This is in agreement with other studies in which sex was found not to influence CYP1A2 activity [29,35]. Furthermore, an inducing effect of smoking [29,35,36] or caffeine consumption [35,36] was not observed in our study. However, as our study population contained only 4 smokers and 4 individuals reporting intake of > 7 caffeine consumptions/day, we might have been unable to detect an existing effect of these factors. In accordance with other studies [29,36], we also found no effect of age on paraxanthine:caffeine ratios. Finally, none of the above-mentioned factors could explain the variability between ratios in hair and plasma. However, it should be noted that subdividing the data set (n = 60) into different subgroups resulted in small (or, in some cases, very small) group sizes. Consequently, some statistical comparisons may be underpowered. Future studies with even more participants or with participants belonging to certain focus groups (e.g. more smokers) may be needed to make more conclusive statements.

A.5.5. Conclusion

In this chapter, the potential of using hair as an alternative matrix for CYP1A2 phenotyping was evaluated. Paraxanthine:caffeine molar concentration ratios were determined in proximal 3-cm hair segments from 60 healthy volunteers and compared with reference CYP1A2 phenotyping indices determined in plasma. Although paraxanthine:caffeine ratios in hair and plasma overall showed a

statistically significant correlation, large deviations in individual cases impede the interpretation of hair results on an individual basis. The influence of several factors on the variability between ratios in hair and plasma was evaluated, but, hitherto, none of these factors could explain the observed deviations. Therefore, hair sampling is not (yet) suitable for CYP1A2 phenotyping.

A.5.6. References

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Part B

The hematocrit effect in dried blood microsampling

Chapter B.1.

The hematocrit effect in dried blood spot analysis: challenges and solutions

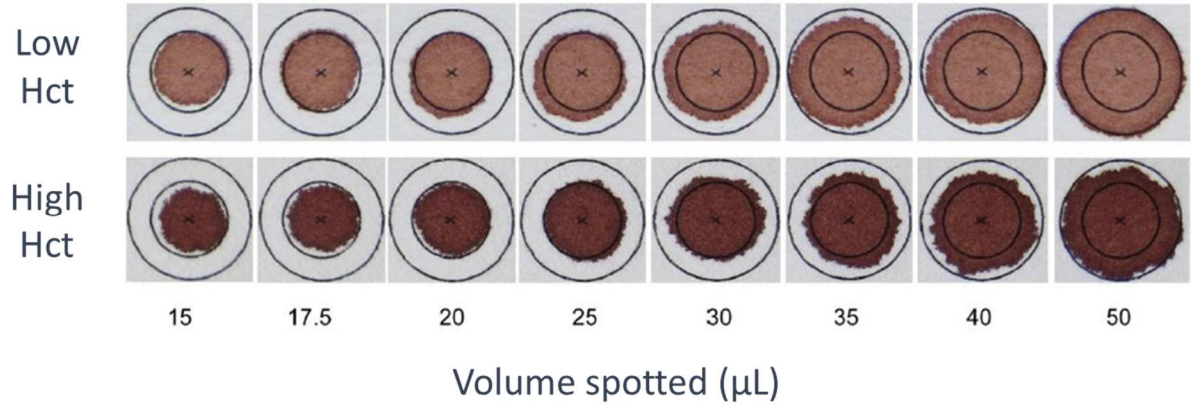
Based on

De Kesel PM, Sadones N, Capiau S, Lambert WE, Stove CP. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. Bioanalysis. 2013;5(16):2023-41.

De Kesel PM, Capiau S, Lambert WE, Stove CP. Current strategies to cope with the hematocrit problem in dried blood spot analysis. Bioanalysis. 2014;6(14):1871-4.

Abstract

Dried blood spot (DBS) sampling for quantitative determination of drugs in blood has entered the bio-analytical arena at fast pace during the last decade, primarily owing to progress in analytical instrumentation. Despite the many advantages associated with this new sampling strategy, several issues are remaining, of which the hematocrit issue is undoubtedly the most widely discussed challenge, as strongly deviating Hct values may significantly impact DBS-based quantitation. In this chapter, an overview of the different aspects of the 'hematocrit problem' in quantitative DBS analysis is given. The different strategies trying to cope with this problem are discussed, along with their potential and limitations. Implementation of some of these strategies in practice may help to overcome this important hurdle in DBS assays, further allowing DBS to become an established part of routine quantitative bioanalysis.



B.1.1. Introduction

As mentioned in the General Background section and Part A, dried blood spot (DBS) sampling is a microsampling technique, in which a drop of capillary blood, derived from a finger or heel stick, is collected on special filter paper. This approach has been used successfully in newborn screening since it was introduced in the 60's by Dr. Guthrie to determine phenylketonuria in neonates [1]. During the last decade, thanks to the development of more sensitive analytical techniques, DBS sampling has gained its place in a lot of other fields such as preclinical and clinical studies, epidemiological research, toxicology and therapeutic drug monitoring [2-5]. The increased use of DBS sampling is the logical consequence of the many advantages associated with this sampling technique, which are summarized in Box B.1.1. A. Despite the numerous applications using DBS and the many advantages associated with this sampling procedure, DBS sampling still faces some important challenges, as summarized in Box B.1.1. B. Among these challenges, the hematocrit (Hct) effect is undoubtedly the most widely discussed DBS-related problem, which is clearly reflected by the fact that in DBS publications the word "hematocrit" is almost invariably linked to "issue", "problem", etc. This is clearly exemplified by a selection of quotes that appeared the last few years in the literature [6-10] (Box B.1.2.). It needs to be remarked, though, as Xu *et al*. nicely pointed out [11], that the "hematocrit problem" is in fact a twofold problem. While the first problem is analytical in nature, affecting mainly the accuracy and possibly also the precision of the analytical methodology *in se*, the second is physiological in nature and relates to the blood/plasma ratio of an analyte, as detailed further.

Box B.1.1. Advantages and challenges associated with DBS sampling.

A. Advantages associated with DBS sampling ▪ Ease of sampling, enabling sampling at home ▪ Cost-effective sampling, transport and storage of samples ▪ Improved compound stability ▪ Small blood volumes ▪ Minimally invasive sampling ▪ Reduced risk of infection ▪ Simplification of sample preparation procedures ▪ Suitability for automation of sample processing and analysis
B. Challenges associated with DBS sampling ▪ Correlation between venous and capillary blood concentrations ▪ Adequate sampling ▪ Contamination risk ▪ Lack of sensitivity ▪ Chromatographic effect and influence of the site of punching ▪ Influence of spotted blood volume ▪ Hematocrit effect

Box B.1.2. Selected quotes illustrating the hematocrit problem in dried blood spot analysis.

"In summary we found that most metabolites used for newborn screening depend on hematocrit and on position of the disk. The ***effects of hematocrit and position of the disk were sometimes additive and sometimes even synergistic****."* [7]

"Current thinking is that this issue (hematocrit) *needs to be addressed* ***before practical application of DBS analysis can progress to the next level****, and any direct analysis technique needs to be compatible with this solution."* [6]

"Hematocrit is currently identified as the ***single most important parameter*** *influencing the spread of blood on DBS cards, which could impact the validity of the results generated by DBS methods, affecting the spot formation, spot size, drying time, homogeneity and, ultimately, the robustness and reproducibility of the assays."* [8]

"Hematocrit effect is clearly a ***major hurdle*** *to the success of any DBS method and attempting to ignore or avoid it is not an option in a regulated environment."* [9]

"The future of DBS in clinical bioanalysis is dependent on eliminating or limiting the so-called hematocrit effect, that is, inaccuracy caused by hematocrit variability." [10]

B.1.2. Hematocrit

The Hct is defined as the volume fraction of the blood which is taken in by red blood cells (RBC). It is determined by the amount and the size (volume) of these cells. Although reference ranges are available at the population level, important inter- as well as intra-individual differences exist. Among the factors determining the Hct are age, sex, health and nutritional status. Reference ranges for men and women lie at approximately 0.41-0.50 and 0.36-0.44, respectively [12]. Higher Hct values are typically observed in newborns, people living at high altitudes, as well as in persons suffering from e.g. polycythemia (primary) or chronic obstructive pulmonary disease (secondary). Patients with anemia have lower Hct values. Examples include patients receiving chemotherapy and immunocompromised patients, in which Hct values of 0.20-0.30 are not rare [13]. Figure B.1.1. shows the Hct distribution of a hospital population. This figure, representing over 200,000 Hct measurements can be considered as a worst-case scenario: patients with strongly deviating Hct values are likely to be followed up more closely, thus leading to an overrepresentation in this distribution profile. Still, 95% of the measurements lies in the Hct range 22.7 - 47.5, the range 0.19-0.63 covering over 99.5% of the measurements. Hence, when aiming at evaluating the impact of Hct (except when aiming at evaluating very specific populations), it is not useful to extend the range to even lower or higher Hct values.

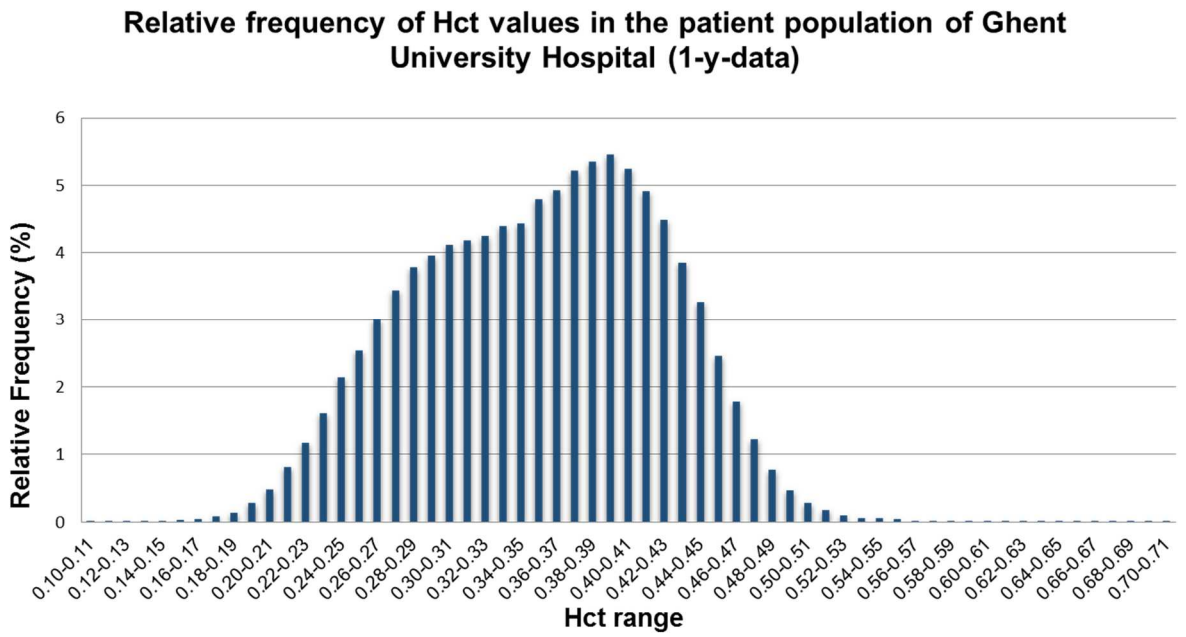


Figure B.1.1. Relative frequency of the Hct values measured over the course of 1 year at the clinical laboratory of Ghent University Hospital, measured via a Sysmex XE-5000 analyzer. From the data, representing over 200,000 data points, it is obvious that two populations are present, consistent with the nature of this population (i.e. a hospital population), with maxima at Hct values of approximately 0.31 and 0.40.

Nowadays, automated hematology analyzers determine the Hct of liquid blood (typically K_2-EDTA-anticoagulated) using electrical impedance. In this procedure, the cells in a liquid blood sample flow in a conductive fluid through a narrow channel between two electrodes. When a cell passes the electrodes, the conductivity of the fluid will lower. Since large cells displace more conductive fluid, the increase in electrical resistance will be dependent on the cellular volume. This way, RBC can be distinguished from the larger white blood cells and smaller platelets. In the end, the increase in electrical resistance will generate an electrical pulse. When all pulses derived from RBC are summed, the final Hct can be derived, based upon the number of RBC and their volume [14]. An alternative way to determine the Hct is to fill an anticoagulant-coated capillary with blood and subject it to centrifugation for an established period of time [15,16]. The results obtained by the impedance-based procedure will slightly differ from those obtained by microcentrifugation, since the former is not accompanied by plasma sequestration between the RBC [15]. Indirect measurement of the Hct is also possible by measuring the hemoglobin (Hb) content. More specifically, the “rule of 3” can be applied, by which the Hct (in % ± 3%) can be calculated by multiplying the Hb content (g/dL) with an arbitrary conversion factor of (approximately) three [17].

B.1.3. The Hct effect: Impact of Hct on quantitative DBS analysis

As observed by many authors, the Hct may have a large impact on the result in quantitative DBS analysis. As already mentioned, the impact of the Hct can be divided into an analytical and a physiological aspect.

B.1.3.1. The analytical impact of deviating Hct values

The most obvious impact of the Hct is that it determines the viscosity of the blood and will therefore have an impact on the spreading of the blood on filter paper. On 'classical' cellulose-based paper substrates used for DBS sample collection, such as Whatman 903™, FTA™ DMPK-C (GE Healthcare) or Ahlstrom 226 (PerkinElmer), blood with higher Hct will spread less than blood with lower Hct. In other words, following application of the same blood volume, DBS with a smaller diameter will be formed by blood with a higher Hct [12], as depicted in Figure B.1.2.

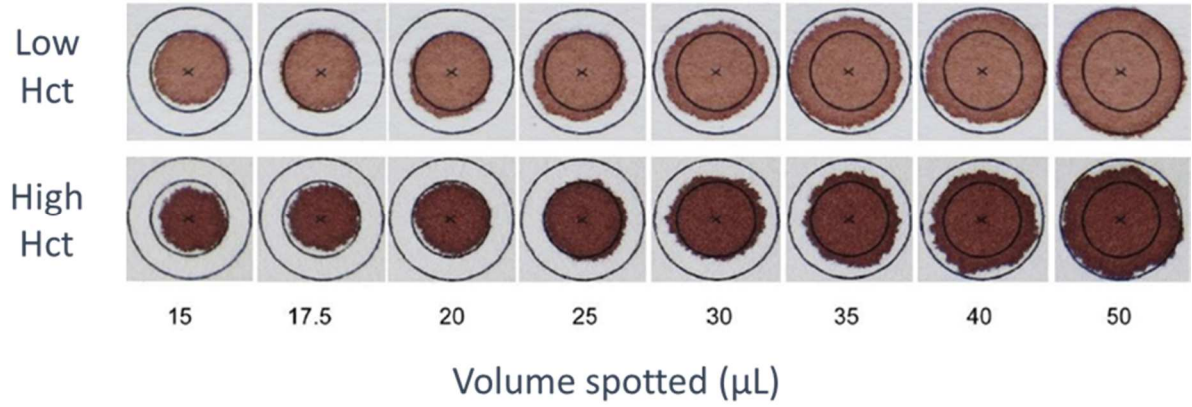


Figure B.1.2. Pictures of DBS, obtained by applying 15, 17.5, 20, 25, 30, 35, 40 or 50 µL of blood with low (0.25) or high (0.60) Hct on Whatman 903™ filter paper with two pre-printed circles of 8, respectively 13 mm diameter. (Figure taken from Capiau *et al.*, Supporting Information [74]).

Consequently, when a fixed-diameter partial-punch (e.g. 3 mm) is taken from these DBS, punches from $DBS^{high\ Hct}$ will contain larger volumes of blood than punches from $DBS_{low\ Hct}$. This positive correlation between the blood volume in a fixed punch taken from a DBS and the blood Hct has been described by several authors [12,18-20]. Denniff and Spooner, for example, found 35% difference in blood volume across the Hct range 0.20-0.80 [12]. Consequently, if a calibration line is established in blood with intermediate Hct, this may result in an underestimation in the case of $DBS_{low\ Hct}$ and an overestimation in the case of $DBS^{high\ Hct}$.

The impact that diverging Hct values may have on the quantitative result obtained from a DBS has been evaluated for small molecules, trace elements and proteins. The vast majority of studies evaluating the Hct impact on the analytical result deal with the determination of small molecules in DBS. Numerous authors reported on the effect of Hct on the assay accuracy when analyzing fixed-diameter punches from DBS. The influence of Hct on γ-hydroxybutyric acid (GHB) concentrations measured on Whatman 903 paper was examined by Ingels *et al.* [21]. These results are summarized in Figure B.1.3., the sample with a Hct of 0.44 being used for normalization. While little influence was observed in the Hct range of 0.39-0.51, inaccuracy became more pronounced when the Hct range was extended, with biases up to -15% and 11.1% at Hct values of 0.34 and 0.56, respectively [21].

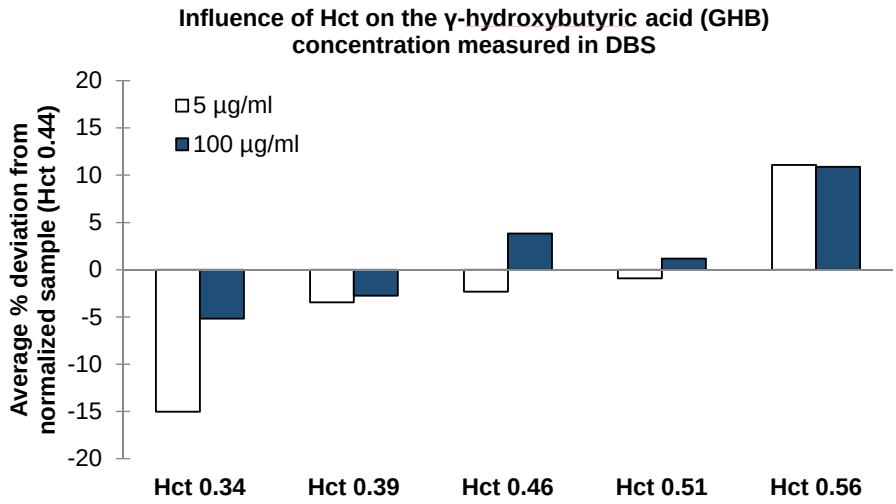


Figure B.1.3. Influence of Hct on the γ-hydroxybutyric acid (GHB) concentration measured in DBS samples (n = 5), using gas chromatography coupled to mass spectrometry in selected ion monitoring mode. For QCs at 2 concentration levels (5 and 100 µg/mL), the average % deviation from the GHB concentration obtained for the sample with a Hct of 0.44 is shown. (Data taken from Ingels *et al.* [21]).

Holub *et al*. investigated the effect of Hct on the analysis of amino acids, acylcarnitines and guanidinoacetate on S&S 2992 paper [7]. Most compounds showed a significant increase in measured concentration with increasing Hct over a range of 0.20-0.60. For tyrosine, aspartate and some acylcarnitines, however, no significant correlation with Hct was found [7]. Wilhelm *et al.*, quantifying immunosuppressants in DBS on Whatman 903 paper, demonstrated biases of -12% and 14% for cyclosporine A, at Hct levels 0.20 and 0.72, when normalized to the sample with 0.35 Hct, and of -11 to 19% for mycophenolic acid in the Hct range 0.22-0.45 (normalization to 0.36 Hct) [22]. Vu *et al*. found high assay biases for the determination of moxifloxacin in samples with varying Hct values on Whatman 31 ET CHR paper; more specifically, a difference of approximately 40% between concentrations measured in samples with Hct of 0.20 and 0.50 was seen. After correction for Hct (see further), the biases were below 15% [23]. For the analysis of thiorphan on DMPK-A cards, Mess *et al*. also observed increasing assay biases with increasing Hct values, ranging from -4.8 to 11.2% in the 0.25-0.65 Hct interval, when normalized to the 0.45 Hct sample [24]. A similar effect of Hct in the range 0.20-0.50 was described for losartan and its active metabolite losartan carboxylic acid on Whatman FTA cards by Rao *et al*. [25]. Peng *et al*. found large biases for the determination of succinylacetone on 903 paper over the Hct range 0.30-0.60. For samples with a Hct of 0.30 the bias was -45%, while for samples with a Hct of 0.60 a bias of 24% was observed [26]. Shah *et al*. examined the influence of Hct on the determination of antiepileptic drugs (levetiracetam, lamotrigine, phenobarbital, carbamazepine and its active metabolite carbamazepine-10,11 epoxide) on Whatman 903 paper. For all five compounds, the bias of measured concentrations was below ± 5% at Hct levels of 0.30 and 0.55, when normalized to the samples with 0.425 Hct [27]. The influence of the paper substrate on the Hct-dependent bias was evaluated by Denniff *et al.* These authors quantified acetaminophen and sitamaquine in DBS on three different paper substrates (Whatman 903, FTA and FTA Elute) over a Hct range of 0.20-0.80 and observed considerable variation in assay bias [12]. On FTA paper, the bias went from negative to

positive for both compounds with increasing Hct. On 903 paper, as well as on FTA Elute paper, a similar trend was observed for acetaminophen with a Hct between 0.20 and 0.60; however, for Hct values between 0.60 and 0.80 the bias decreased again. A possible explanation for the latter might be a negative effect on recovery of the analyte at high Hct levels, as has been demonstrated for other compounds (see further). Remarkably, the bias for sitamaquine on 903 paper was not significantly affected over the entire Hct range, while on FTA Elute, the bias had a rather complex course [12]. Hence, the impact of Hct on the bias depended both on the paper substrate and on the analyte. In order to explore the role of various analyte parameters, O'Mara *et al*. selected a set of five compounds with varying physicochemical and binding characteristics and analyzed this set on four different paper types (Whatman FTA, FTA Elute, FTA DMPK-C and Ahlstrom 226) over a Hct range of 0.25-0.75 [28]. Measured concentrations were compared to those at the reference Hct level of 0.45. All compounds displayed a negative bias at Hct levels below 0.45 and a positive bias above this reference Hct value. Again, the degree of bias was influenced by both compound characteristics and paper type [28].

Considering the analysis of trace elements in DBS, most attention has been given to the determination of lead in DBS [4]. There seems to be no real consensus regarding the effect of deviating Hct on the measured lead concentrations. While Carter already reported in 1978 on increased blood lead values with increasing Hct [29], more recent studies found little or no influence of different Hct levels on lead concentrations in DBS [30,31].

In addition to small molecules and trace elements, DBS have also been used in analytical methods for the determination of peptides and proteins, large molecules predominantly residing in the plasma [20]. Here, fixed-size punches originating from $DBS^{high\ Hct}$ will contain less plasma than punches from $DBS_{low\ Hct}$. This effect may counteract the above-described Hct effect (i.e. punches from $DBS^{high\ Hct}$ contain more blood than punches from $DBS_{low\ Hct}$). Hence, the net result will be determined by two opposing factors: on the one hand the spreading of the DBS (with more concentrated spots in the case of $DBS^{high\ Hct}$); on the other hand the presence of plasma in the DBS punch (with less plasma in the case of $DBS^{high\ Hct}$). Hoffman *et al*. investigated the influence of varying Hct on the concentration of prostate-specific antigen (PSA) over the Hct range 0.24-0.61. As PSA concentrations decreased with increasing Hct, it can be concluded that the decreased presence of plasma in $DBS^{high\ Hct}$ prevailed in this case. More specifically, the bias ranged from 18 to -10% over the tested Hct interval, when normalized to the sample with a Hct of 0.45 [32].

The above-mentioned data clearly demonstrate that the impact of Hct on assay bias differs from case to case and may depend on e.g. paper type, differential spreading of blood, compound characteristics (blood cell affinity, plasma binding, molecular weight, pKa, hydrophobicity,…), extraction solvents, etc. Thus, there is a clear need to define a Hct interval in which the impact of the Hct on the accuracy of the analytical result is still acceptable. Consequently, evaluation of the impact of the Hct on the accuracy should be part of the validation procedure for any DBS-based bio-analytical method. In general, the acceptance criteria are set at ± 15% (% bias); at the LLOQ at ± 20%. Important to note as well is that a distinction should be made between "absence of a Hct effect", "no significant impact" and "the Hct effect fell within the acceptance limits of 15%". Whereas all of these statements have been used in literature,

they do not always imply the same. In many cases where no Hct effect was described this actually refers to the fact that the impact of deviating Hct on the accuracy did not exceed 15 or 20%. However, even when during the validation a Hct interval has been described in which the bias is still acceptable, still, one does not know whether the Hct of the blood used to prepare a given DBS actually does lie within this interval. Strategies to cope with this problem are outlined further in this chapter.

Apart from the differential spreading of blood with low and high Hct on filter paper, the Hct may also have an impact on the extraction efficiency and, consequently, on the recovery of an analyte. $DBS^{high\ Hct}$ may, upon drying, form some kind of barrier from which it may be more difficult to extract the analyte of interest, when compared to extraction from DBS samples with a normal or low Hct, resulting in an underestimation of the analyte concentration. A Hct-dependent effect on the recovery of naproxen from DBS was observed by Youhnovski *et al*. [33]. Since these authors extracted the entire spotted blood volume, at least theoretically, no significant bias from varying Hct levels was expected. However, measured naproxen concentrations decreased with increasing Hct values. Biases of -9.2 and -11.1% at 0.75 Hct were observed for FTA and DMPK-C cards, respectively, when compared to the 0.45 Hct sample. Post-column infusion profiles, as well as a stable response for the internal standard, revealed no differences in ion suppression or enhancement between 0.45 and 0.75 Hct samples. The hypothesis that the observed negative bias could be owing to a lower recovery of the analyte at high Hct levels was confirmed by the fact that optimization of the extraction procedure eliminated this effect. Möller *et al*. described a similar effect on the determination of peginesatide, a peptide of approximately 45 kDa, in whole DBS. While the assay bias showed little variation between 0.45 and 0.55 Hct, a steep decline in measured concentration was observed at 0.60 Hct, when compared to the 0.40 Hct sample. Again, as the internal standard response showed no Hct dependency, it is likely that this effect is also caused by decreased extraction efficiency [34]. Likewise, Cox *et al.*, quantifying insulin-like growth factor-1 in DBS, observed a negative bias at 0.60 Hct, when compared to a 0.40 Hct reference [35]. This bias was most pronounced at lower concentrations. Although the above-mentioned examples did not establish any apparent effect of the sample Hct on ion-suppresion or enhancement, each DBS sample with a different Hct can be considered as having a different matrix, with the potential of giving rise to a Hct-dependent matrix effect. This matrix effect can significantly impact the accuracy of an analytical result, leading to an under- or overestimation, depending on the Hct of the sample. Furthermore, varying recoveries and matrix effects in function of the sample Hct may affect the precision of an analytical method as well. Therefore, during method validation, it could be valuable to evaluate recovery and matrix effects using blood samples covering a broad range of Hct values. Very recently, Abu-Rabie *et al.* presented an approach to eliminate the Hct-induced recovery bias by spraying the internal standard (IS) solution onto the DBS prior to extraction, or alternatively by spraying the IS solution on the filter substrate before whole blood is spotted. Combined with analysis of whole, volumetrically applied DBS (see further), this approach allowed to overcome the overall Hct effect, caused by differential spreading of blood and altered recovery at deviating Hct values, on DBS concentrations of paracetamol and sitamaquine [36].

To conclude, the above-described phenomena explain how Hct levels may have a large impact on the accuracy of an analytical result. This in turn may influence the diagnostic decision based on those results. In particular, when a person has a strongly deviating Hct, this may lead to seemingly elevated or lowered

levels of the determined compound, resulting in a false positive or false negative diagnosis [26]. Important to note, however, is that the effect of Hct is not an isolated factor in the analysis of fixed-diameter punches taken from DBS. Other factors, such as spotted blood volume and/or punch location, could interact with the Hct effect. E.g., the impact of taking a peripheral versus a central punch, in some cases, appears to be more pronounced at lower Hct levels [7,28]. Overall, the bias originating from different punch location may counteract, as well enhance the bias caused by deviating Hct [28]. In conclusion, also the combined effect of Hct, volume spotted and site of punching should be evaluated on a case-by-case basis when using partial-cut DBS.

B.1.3.2. The physiological impact of deviating Hct values

Besides the effect of the Hct on the accuracy of an analytical result, it may also have a significant influence on the interpretation of a DBS result that in itself may be correct. Indeed, currently applied reference intervals that are used to evaluate whether a result is normal are mostly based upon plasma or serum concentrations. When comparing DBS results with these reference intervals, one has to be aware that no straightforward conversion of one value to another is possible. Concerning the correlation between DBS and plasma concentrations, an important factor is the blood-to-plasma concentration ratio (B/P ratio), which is, according to Emmons & Rowland, expressed by the following equation: $C_b/C_p = (1-Hct) + Hct*\rho*f_u$, where C_b is the total blood concentration, C_p the total plasma concentration, ρ the blood cell-to-unbound plasma concentration ratio, f_u the unbound fraction in plasma and Hct the hematocrit [37,38]. From this equation it can be deduced that Hct is one of the prime factors determining the B/P ratio. Compounds that do not, or only to a limited extent, enter erythrocytes, will display low B/P ratios and consequently the presence of erythrocytes may be seen as a ‘dilution’ of the plasma fraction of whole blood. In this situation, Hct is an important parameter influencing the B/P ratio. Typical examples of such analytes, exclusively residing in the plasma, are proteins or peptides. When not considering the impact of Hct on the spreading of a DBS, theoretically, for a given plasma concentration, the B/P ratio of these molecules will lower with increasing Hct values. For molecules that do partition into erythrocytes and bind to both cellular and plasma proteins, both ρ and f_u are of importance. When blood cell affinity is more important than plasma binding, the B/P ratio will be higher and Hct is a factor influencing that ratio. In this case, higher Hct levels will lead to higher B/P ratios. Other compounds freely enter erythrocytes but do not bind to cellular proteins, neither to plasma proteins. In this case the B/P ratio will approximate one and almost no impact of Hct on B/P ratio is expected [11,37-39]. It needs to be remarked that in all scenarios, irrespective of the B/P ratio, the impact of Hct on spreading of the DBS plays a role.

These data indicate that Hct, amongst other factors, needs to be taken into account when a correlation or conversion is to be made between DBS and plasma concentrations, especially in populations with deviating Hct levels, such as newborns or patients suffering from anemia. Currently, such conversion is often based on the assumption that all patient’s Hct correspond to what is considered normal for the population they belong to. However, for clinical validation of the correlation between DBS and plasma measurements, *in vivo* bridging studies are essential [11]. In these studies, DBS and corresponding plasma samples are collected, and the correlation between both is evaluated statistically.

B.1.4. Strategies to cope with the Hct effect

It needs to be remarked that the following strategies only overcome -or assist in overcoming- the issue of differential spreading of blood with different Hct. Although the impact of diverging Hct values on other parameters, e.g. matrix effects and recovery, may be alleviated by optimizing the extraction and chromatographic conditions [33], these validation parameters still need to be investigated, as outlined above. Furthermore, if a comparison is to be made with plasma or serum concentrations, one ideally still needs to know the actual Hct of the sample.

B.1.4.1 Avoid the Hct effect

B.1.4.1.1 Generate volumetrically applied DBS, followed by whole DBS analysis

Without a shadow of a doubt, the easiest way of overcoming the Hct problem is to avoid it by analyzing complete, volumetrically applied spots, instead of using discs generated by punching DBS. Roughly, two strategies have been followed in literature for whole-spot analysis; in the first approach, the complete DBS is punched after volumetric application of the blood [3,9,40-43]; in the second approach the blood is volumetrically applied on pre-punched discs [33,44-46]. The latter approach exists in different formats. In the concept of perforated DBS (PDBS), paper discs with a diameter of several millimeters are punched out from the filter paper and placed back into that filter paper before DBS sampling [44,45]. Then, a limited amount of blood can be applied volumetrically on these discs. In the case of pre-cut DBS (PCDBS) the perforated filter paper discs are attached on a support system instead of being placed back into the sheet of filter paper [33]. A variant of the latter is 'Dried Matrix on Paper Discs' (DMPD), a whole-cut DBS microsampling technique with a dedicated support system, described by Meesters *et al*. [46]. Although these approaches may seem very convenient, we recommend to always reflect on the context in which sampling will need to be done eventually. Additionally, the choice between a partial- and whole-cut DBS method should ideally be made before setting up the method, as validation of both methods differs in several aspects, as outlined above.

When the aim is to establish a method for analysis of DBS, obtained from e.g. animals, patients in a hospital environment or from post mortem matrices, volumetric application by dedicated staff (e.g. an experienced animal technician, a dedicated nurse or laboratory staff) may be feasible. In these cases, sample application can be done by accurate pipetting or by using anticoagulant-coated microcapillaries [47]. Such capillaries can be used for direct spotting or can be used in conjunction with devices allowing repeated volumetric dispensing. A prototype of an "incremental dispenser" device that facilitates the process of volumetric spotting has been developed by Drummond Scientific Company. This device allows the precise and accurate generation of multiple volumetric spots from a single blood-filled microcapillary [9,45,48]. Li *et al.* also found Microsafe® pipettes (Safe-Tec, PA) suitable for accurate microsampling [45]. These are one-time-use plastic pipettes that draw blood via capillary forces and from which an accurate volume (e.g. 5 µL) can be delivered on filter paper by squeezing an attached bulb. In the context of forensic toxicology, when dealing with post mortem matrices, volumetric application should be the rule, as blood from a post mortem source is often lysed and spreads differently

on filter paper than fresh blood. Here, the process of spotting a matrix (e.g. blood) on paper can actually be considered as an alternative sample preparation technique [49].

When sampling is to be performed by non-experienced individuals (e.g. patients at home), all of the above-mentioned whole-DBS approaches may be difficult to sustain. In this scenario, the requirement for accurate volumetric application of the blood constitutes a non-negligible disadvantage and direct application of a blood drop from the fingertip onto the paper may be the most realistic and feasible option. Although this non-volumetric application may be relatively easy and straightforward, careful instruction of the individual is still needed as incorrect sampling will inevitably lead to erroneous results. Some instructions to be given to self-sampling patients are indicated in Box B.1.3. [2]. These can be given via e.g. a hands-on demonstration, a flyer (see Chapter A.3., Figure A.3.1.), a video, a dedicated website or a combination of these.

Box B.1.3. Instructions to be given to self-sampling patients in a DBS study (not limiting) [2]

- Required size of the DBS (this can be indicated by using custom pre-printed circles)
- Contact of the fingertip with the DBS card should be avoided (to avoid contamination and 'smearing' of the DBS)
- Only 1 drop of blood should be applied per DBS
- The first blood drop should be wiped off (may contain interstitial fluid)
- Too much 'milking' of the fingertip should be avoided
- There should be minimal manipulation of the DBS card (to avoid contamination)
- The disinfectant used to clean the skin, should be completely dry before puncture (may interfere with the blood spreading)

Recently, two interesting microfluidic devices have been developed, allowing to collect a fixed volume of blood from a non-volumetrically deposited sample. The first is a 'disposable metering device' prototype developed by Lenk *et al.* [50]. The second is a microfluidic device consisting of a foldable support system that holds a DBS card on one side and a microfluidic plate with sized capillaries on the other side. A drop of blood is applied to the inlet of a capillary, which is subsequently filled by capillary force. By closing and turning the support system, a blood spot is formed on the DBS card as a result of the capillary outlet and the filter paper making contact. As traditional DBS cards are inserted in the support system, the resulting spots can be processed using previously developed methods. The reproducibility of the deposited blood volume obtained with this device was comparable with that of a volumetric micropipette and no effect of Hct on the accuracy and precision for the test compound Mavoglurant was observed in the 0.26 – 0.62 Hct interval [51]. Another promising approach for volumetric collection of blood to generate dried samples is the use of a very recently developed alternative collection device (VAMS - Volumetric Absorptive Microsampling), which wicks up a fixed volume of blood, independent of the Hct [52]. We evaluated the potential of this new sampling technique to overcome the hematocrit bias in real incurred human samples, using caffeine and paraxanthine as model compounds. This study is described in Chapter B.3.

B.1.4.1.2 Use dried plasma spots instead of DBS

Another way of avoiding the Hct problem may be to use dried plasma spots (DPS) instead of DBS, as this matrix does not comprise RBC. This way, the convenience of sample transport, handling, storage and analysis may be maintained. Several publications have described the use of DPS, demonstrating a close correlation between the results obtained from DPS and those obtained from plasma [53-59]. However, in all but two of the DPS reports, plasma was prepared by centrifugation of whole blood, which is not feasible for sampling at home. A good to excellent correlation between concentrations measured in DPS and plasma has been reported for paroxetine [53], triazole antibiotics [56] and nevirapine [57]. Likewise, Ruhwald *et al*., evaluating the chemokine interferon-γ inducible protein 10 (IP-10) [55,58] and Flowers *et al.*, measuring ferritin and transferrin receptor for iron status assessment [59], found an excellent agreement between DPS and plasma measurements. While the previous authors prepared DPS from plasma obtained from venous blood, recently, several reports described the preparation of plasma, starting from capillary microsamples [60-62]. For pharmaco- and toxicokinetic studies in plasma from rats and mice, Jonsson *et al.* applied capillary microsampling of blood [60-62]. Plasma was prepared from the blood by sealing the microcapillary at one end and centrifuging it. Next, after cutting it with a ceramic cutter above the blood cell phase, an exact volume of plasma was collected by holding an exact volume capillary (4 or 8 µL) end-to-end to the first capillary. Two centrifugation-independent methods to prepare DPS have been described, both using interesting plasma separator devices [63,64]. With these devices, depicted in Figure B.1.4., DPS are generated by applying drops of blood onto multi-layered extraction cards, in essence consisting of a separation and a collection membrane. While the plasma can flow through the separation membrane to the bottom collection membrane, the cellular fraction is trapped on top. After waiting for a few minutes, this top layer can be peeled off and plasma spots (a fixed volume or not) are formed on the collection membrane. Although more studies involving different analytes and evaluating the correlation between plasma and DPS concentrations are needed to support the general usefulness of this approach, the application of these devices seems a promising strategy to overcome the Hct problem.

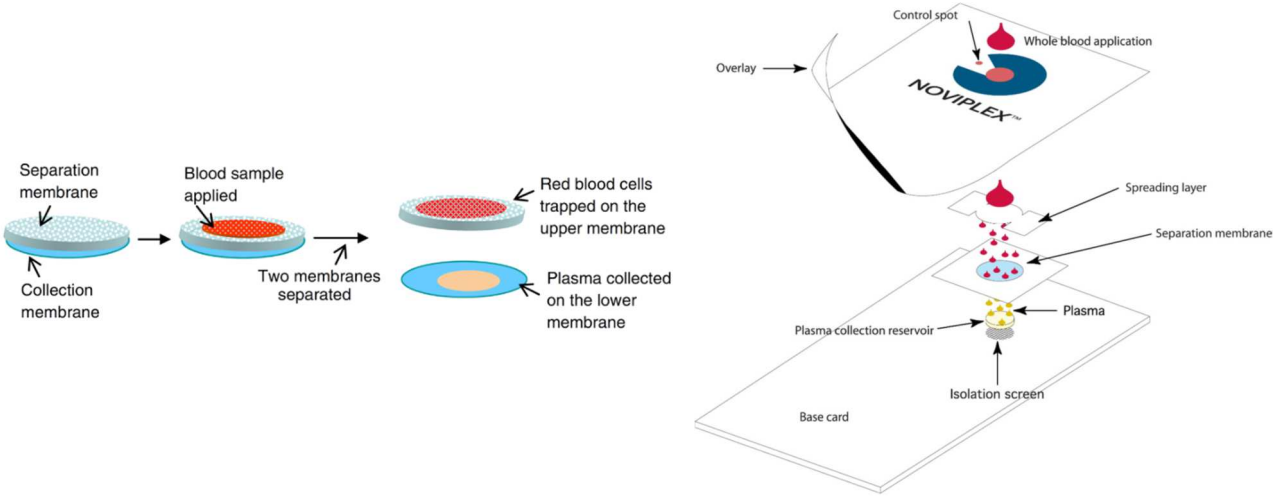


Figure B.1.4. Schematic drawing of multilayered membrane filtration devices for the generation of DPS, utilized by Li *et al.* (left panel) [63] and Kim *et al.* (right panel) [64]. (Figures taken from [63] and [64]).

B.1.4.2 Minimize the Hct effect

B.1.4.2.1 Spot onto a special type of filter substrate

Several companies have tried to reduce the Hct effect by developing special filter material or special filter paper formats. Agilent Technologies for instance has developed a special type of non-cellulose filter paper, Bond Elut Dried Matrix Spotting (DMS), which is acclaimed to be less influenced by the Hct, i.e. different Hct values lead to less variation in DBS diameters compared to the classically used cellulose filter paper [65]. In our hands, however, the reduced rigidity of this paper, as compared to cellulose paper, makes it less suitable for reproducibly punching out discs (at least, via manual punching). Moreover, we found that blood did not always spread out evenly on this type of paper, also compromising reproducible punching. HemaForm™, a fan-shaped filter paper with eight blades protruding from a center (Figure B.1.5.), distributed by Spot On Sciences, is also claimed to have a reduced Hct effect, although no data have been published [66].

Figure B.1.5. HemaForm™ (left) compared to a traditional DBS (right). (Picture taken from [66]).

Likely, other developments will follow in the near future, as also exemplified by a possibly promising, supposedly Hct-independent, material developed by Mengerink and colleagues [67]. However, more data are needed, as also here, no publications are available, yet. Other recently developed non-paper substrates are glass paper filters [68] and alginate and chitosan foams [69]. The latter are porous, swollen hydrophilic matrices that can absorb water-containing matrices, such as blood, and can be dissolved in dilute acidic solutions. The impact of Hct on the spreading of blood on these matrices remains to be evaluated.

B.1.4.2.2 Use calibrators with a Hct close to the range of the target population

As outlined above, it is necessary to evaluate the impact of the Hct on the accuracy, as well as on the precision of any DBS-based bio-analytical method and to establish an interval in which this impact remains acceptable. A good starting point before setting up a validation procedure for quantitative DBS analysis is to first reflect on the population onto which the procedure will be applied: newborns, elderly people, healthy individuals, patients with a specific disease,… This way, the impact of the Hct can be minimized for a large part of the study population. The Box Plots in Figure B.1.6. depict the median Hct and central 50% range of Hct values measured in distinct hospital populations, the flags indicating the range containing 95% of the Hct values. From these data, it is obvious that, depending on the population under investigation, one may opt to prepare calibration lines in blood with Hct close to the range of the target population [2,3,9,20,28,70-72]. E.g., when aiming at quantitative monitoring of biomarkers in

healthy newborns (in which Hct values may exceed 0.50), a different (higher) calibration line should be used than when aiming at monitoring these same biomarkers in immunosuppressed patients (often having Hct values around or even lower than 0.30).

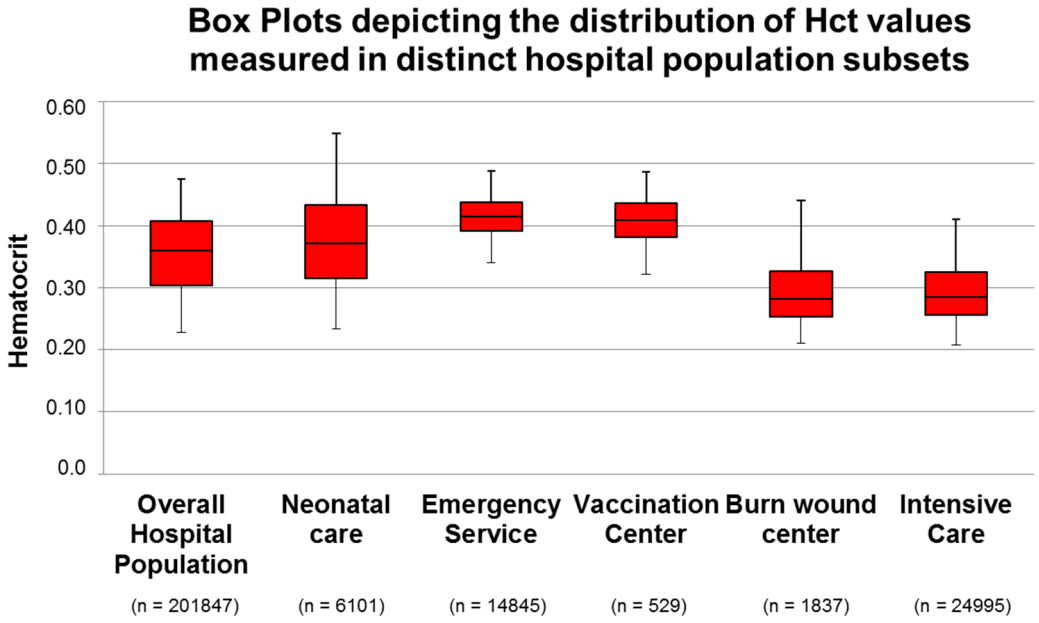


Figure B.1.6. Box plots depicting the distribution of Hct values measured in distinct hospital population subsets. Indicated are the median, the central 50% interval (boxed) and the 2.5% and 97.5% percentiles (flags). The number of measurements underlying these data are indicated. It should be noted that this number is distinct from the number of patients and that patients with (strongly) deviating Hct and/or residing for a long time in the hospital will provide a bias to the result. A clear distinction can be seen between on the one hand the 'normal' population, represented here by the patients at the emergency service and those having attended the vaccination center, and on the other hand, the 'ill' patients, represented here by patients at the burn wound center and at the intensive care unit. Whereas a mixed picture is obtained in the overall hospital population and in patients from neonatal care, it is clear that the latter population contains more patients with higher Hct values, in line with higher Hct values in healthy newborns.

Such an approach was e.g. followed by Sadilkova *et al*., who prepared calibration and QC material in blood with a Hct corresponding to the median level of Hct (0.30-0.35) of the target population, transplant patients receiving immunosuppressants [73]. Likewise, Ingels *et al*., monitoring GHB in -predominantly male- drug abusers, opted to prepare calibrators in blood with a Hct of 0.44 [21]. When the population is (expected to be) heterogeneous, an intermediate calibration curve (prepared in blood with Hct of approximately 0.40) may be a good option. Alternatively, two calibration curves may be set up, one at the higher end and one at the lower end, both with intervals in which the impact of Hct on accuracy is still acceptable. Obviously, this further extends the amount of work.

Even when during the validation procedure a Hct interval has been defined, still, one does not know whether the Hct of the blood to prepare a given DBS actually does lie within this interval. Also, in the

case where two calibration lines would be set up, knowledge of the Hct of the blood that was used to prepare the DBS is necessary, in order to assign a given DBS to one of both calibration curves. As outlined further in this chapter, recently a strategy has been developed that does allow approximate prediction of the Hct from a DBS [74].

B.1.4.3 Measure or estimate the Hct of a DBS

Rather than focusing on strategies that avoid or minimize the Hct effect, another possible strategy that may be followed to cope with the Hct problem is to get an idea of the Hct of a given DBS sample. This may allow two things: first, it can be confirmed that the Hct of a given DBS does lie within the range of a given validated bio-analytical procedure; second, knowledge of the Hct may allow to introduce a correction factor compensating for the impact of the Hct, as described in Chapter B.2.. The approaches followed in literature, as well as potential future approaches, are discussed below.

B.1.4.3.1 Acquire an extra blood sample to measure the Hct

Taking an extra blood sample in conjunction with the capillary blood sample has been suggested as a logical way to know the Hct [9]. However, when this extra sample is a venous sample, again dedicated staff is necessary and one may wonder what the added-value of the capillary sample still is. Indeed, except in cases where because of stability issues direct application onto (pre-treated) filter paper is required, the most straightforward way may be to analyze the venous blood right away or prepare venous DBS from it. DBS prepared from venous blood may be useful in the scenario where classical Hct determination is possible in e.g. the treating hospital, but analytical measurements are to be done elsewhere and thus require transport. Moreover, it needs to be reminded that differences may exist between capillary and venous blood, also with respect to the Hct [75,76]. Hence, if an analyte is to be measured in capillary DBS, one ideally should know the Hct of the capillary DBS. Therefore, as an alternative for an extra venous blood sample, a small volume of capillary blood can be sampled using a calibrated anticoagulant-coated capillary, of which the Hct can be read out after centrifugation [16]. However, also here one of the main advantages associated with DBS sampling - the relative ease of the procedure - is lost. Indeed, both of the above-mentioned scenarios to acquire an extra blood sample just to know the Hct are not only impossible in the case of patient self-sampling at home, but will undoubtedly also complicate the sampling procedure in other circumstances. A more feasible and convenient alternative may be to use point-of-care-tests, such as those developed by Hemocue® [77,78]. These are relatively simple to handle, require only 10 µL of blood and allow fast (within a minute) determination of Hb, from which the Hct can be calculated, as outlined above. However, cost is likely to be a limiting factor here, when considering the context of patient self-sampling (it is unlikely that every patient would have a device). However, in remote areas with centralized DBS sampling and/or in centres specializing in DBS sampling from patients, these devices may represent a simple means to know a patient's Hct.

B.1.4.3.2 Calculate the Hct using physical characteristics of DBS

Several authors have proposed to estimate the Hct based upon some physical characteristics of the DBS, such as diameter, surface, color and/or frustum volume [12,23,79]. Denniff and Spooner for example took photographs of DBS to measure the spot area on different types of paper [12]. However, as the blood did not spread homogenously through all types of filter paper, it was not always easy to define the edges of the spot. The frustum volume, which is calculated from the front diameter, the back diameter and the thickness of the filter paper, would correlate linearly with the Hct [79]. Vu *et al.* also introduced a correction factor, based on the diameter of the punch, the blood volume and the DBS area [23]. These authors, however, observed that despite the correction for Hct, the bias may not be totally eliminated. In addition, all characteristics based upon the dimensions of the DBS primarily depend on the amount of blood spotted. Ergo, this approach is only feasible when using volumetric application, which is, as previously pointed out, not an ideal situation. However, it needs to be remarked that it is possible to know the approximate volume of blood spotted on filter paper by weighing the DBS [23,80]. Theoretically, the volume deduced from this weight could be combined with the surface of the DBS to read the approximate Hct from a calibration curve. However, this approach may be both impractical and imprecise. Lastly, while the color of a DBS darkens as the Hct of blood increases [12], also this aspect is not suited for the purpose of estimating Hct as the DBS color changes upon ageing of the DBS, as described by Bremmer *et al.* for blood stains (cfr infra) [81,82].

B.1.4.3.3 Predict the Hct by measuring endogenous compounds

As readily suggested by Denniff and Spooner, an "*approach could be to normalize the measured concentrations in the punched disc to another endogenous component of blood that also varies with Hct*" [12]. In order for such an endogenous compound to be a suitable candidate it has to fulfill certain criteria (see Box B.1.4.).

Box B.1.4. Criteria to be fulfilled by a potential hematocrit marker.

1. Correlation with Hct: a good Hct marker should correlate with the number and size of RBC
2. Universal applicability: a good Hct marker should be measurable in every member of a population, independent of e.g. age, race or sex
3. Minimal inter-individual non-Hct-related variation: a good Hct marker should correlate with the Hct, independent of a variety of disease conditions
4. Stability: a good Hct marker should be measurable in both freshly prepared and old DBS
5. Easy to determine: ideally, a good Hct marker is easy to determine (simple and inexpensive sample preparation), starting from small (e.g. 3 mm) punches, leaving enough of the DBS to determine the actual analyte of interest.

Potassium

At a first glance, potassium as a correlate of Hct may seem somewhat surprising. However, the choice for this electrolyte is less of a surprise when considering the following:

1. Erythrocytes are the predominant cells in the blood, outnumbering white blood cells with a factor of about 500-1000. When also considering points 2 and 3 below, erythrocytes can be seen as the major contributors to the total blood potassium concentration.
2. Potassium is primarily located intracellularly in all cells of the body: its intracellular concentration (at roughly 140 mM) is approximately 35 times higher than its extracellular concentration [83].
3. Potassium levels are under tight physiological control, with normal serum or plasma levels ranging between 3.5 and 5 mM and representing only a low % of total blood potassium [83]. As a consequence, physiological variations in plasma- or serum potassium levels are expected to have only a minimal, if any, impact on the total blood potassium concentration. In addition, even in anemic or inflamed patients erythrocytes still greatly outnumber other cell types in the blood.
4. Potassium can reasonably be indicated as a stable analyte.
5. High-throughput, low-cost measurement of physiological potassium concentrations in serum is routinely performed by clinical laboratories worldwide.

As, theoretically, potassium appeared to fulfill all criteria we had put forward, it was investigated in our group whether potassium concentrations could indeed be used as a correlate for the Hct of a DBS, starting from a 3 mm DBS punch [74]. Potassium could be efficiently extracted from 3 mm DBS punches by soaking the discs in ultrapure water for 5 min in a thermoshaker at room temperature. Since it was opted to determine the potassium concentration via a routine clinical chemistry analyzer, 2.50 mM KCl was readily added to the extraction buffer so that the final potassium concentration of all extracts (from DBS from blood with low and high Hct) would lie within the validated range of the chemistry analyzer. The value of this ‘blank’ was then subtracted from the measured value to know the contribution of the DBS-derived potassium. Addition of twice 50 µL to the DBS allowed to recover 90 µL of extract in total, which was transferred to a microcup and analyzed with a Cobas 8000 routine clinical chemistry analyzer. This approach turned out to be successful: the developed procedure was validated and its application on real patient samples demonstrated its practical applicability and potential to provide a good Hct prediction (Figure B.1.7.) [74]. Moreover, the applied procedure is very simple, fast, comes at almost no cost and may be universally implementable, as virtually every clinical laboratory -even remote ones- measure potassium on a routine basis. The limited sensitivity of the clinical chemistry analyzer necessitates the addition of potassium to the extraction solvent, which inextricably results in an additional error and, hence, additional uncertainty in the calculated Hct. Consequently, there may be room for improvement of the current procedure, e.g. by using more sensitive instrumentation, overcoming the need to add potassium to the extraction solvent. However, this may be at the expense of the simplicity and the low cost. In Chapter B.2., we evaluated whether Hct prediction by itself may

effectively assist in overcoming the Hct problem for a given DBS-based analytical procedure in real practice.

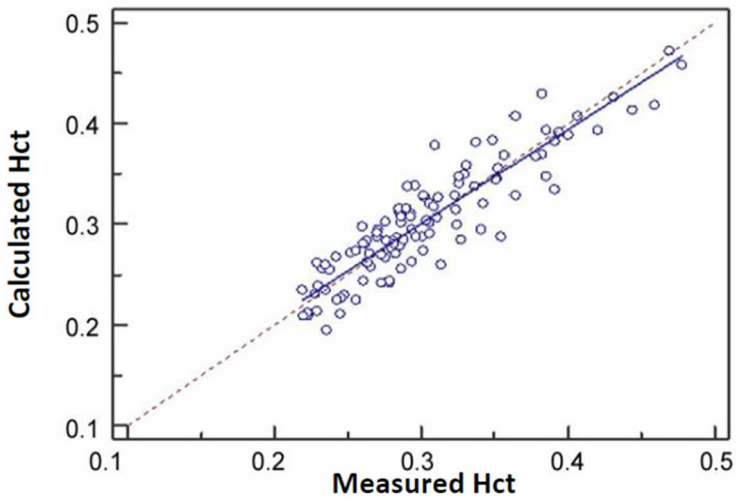


Figure B.1.7. Deming regression analysis of the calculated and measured Hct. (Figure taken from [74]).

Hemoglobin

Another obvious candidate for Hct prediction is hemoglobin (Hb), an iron-binding protein that transports oxygen through the body and which is exclusively present in RBC. As readily outlined above, Hb is already used to calculate the Hct of fresh blood. However, whether Hb determination can effectively be used for accurate Hct prediction of the blood used to prepare DBS remains to be demonstrated. Orsini *et al.* have suggested that a HPLC-UV method, originally aimed at qualitative detection of Hb variants in a newborn screening for hemoglobinopathies, may allow semi-quantitative Hct prediction [84]. These authors reasoned that, since each peak in the chromatogram corresponds to a Hb variant, the total area of all peaks should be an indication of the total Hb concentration. While a calibration line could be set up successfully, these authors did not include a comparison with direct Hct measurement. Instead, a comparison was made with Hct prediction, based upon another in-house developed Hb-based colorimetric method. Hence, it is not surprising that a promising trend could be observed. In addition, it needs to be remarked that a very large part of the evaluated population -newborns- had Hct values of over 0.65. Even in newborns, however, which indeed have higher Hct values than adults, values of over 0.65 are the exception rather than the rule. This work provides an interesting, but very preliminary proof-of-concept of using Hb as Hct-predictor.

An important factor that needs to be kept in mind when studying Hb is that the form in which it is present changes in time, which is already evident by the changing color of DBS upon ageing. This has important implications when Hb is measured using e.g. routine clinical chemistry analyzers, which typically only measure absorption at certain wavelengths. The consequence is that a higher signal is obtained for Hb eluted from fresh, as compared to older DBS (Figure B.1.8. A). Whether this truly represents a lower total Hb concentration or may be the result of a conversion to another Hb form, which absorbs less at

the utilized wavelength(s), has not been established, yet. Anyway, this implies that the Hb concentration from fresh DBS cannot be calculated straightforwardly using calibration curves generated by Hb measurements from older DBS (and vice versa). Interesting in this context are the findings by Bremmer *et al.* [81,82,85], working on dried blood stains rather than DBS. Using spectrometric techniques, these authors demonstrated that in dried blood stains the original form present in blood, oxy-Hb, is oxidized to met-Hb, which is further converted into the denatured form, hemichrome, which is the most prominent Hb form after longer storage [81,82,85]. We also observed a conversion in DBS, as measured by CO-oximetry performed on DBS extracts, albeit met-Hb was the main species we observed in extracts from older DBS (Figure B.1.8. B). It needs to be remarked, though, that no definitive conclusions can be drawn from our results, as it is not clear if or to what extent hemichrome contributes to the signal of oxy- and met-Hb in the CO-oximeter, or, alternatively, may renature to met-Hb upon dissolution. In conclusion, if total Hb would remain constant in DBS and if it would be possible to quantitatively determine the different Hb species in DBS, Hb-based Hct determination from DBS may remain possible. As a side note it needs to be remarked that the correlation between Hb and Hct is not absolute: a near-normal Hct may be present, with below-normal Hb values, as may occur in hypochromic RBC, associated with iron-deficient anemia [13].

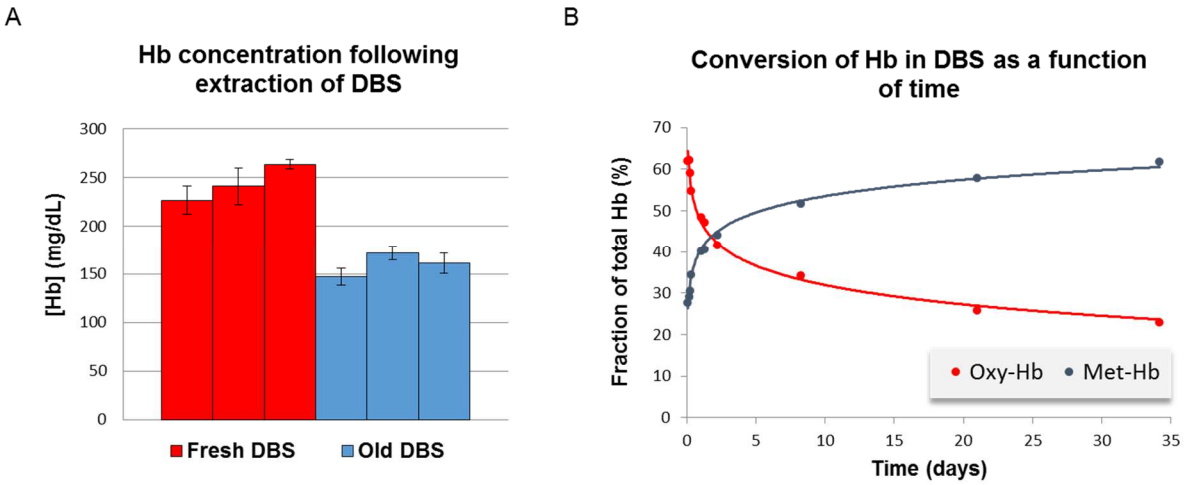


Figure B.1.8. A: Hb concentration (mg/dl) in the extracts of "fresh DBS" and "old DBS", resp. processed 2h and 2d post-spotting. Ultrapure H_2O was used for extraction. The two series of bars each represent 3 experiments (n = 6). Indicated are mean ± standard deviation. B: Fraction (%) of oxy- and met-Hb, measured in function of time in extracts from DBS. Measurements were done using a GEM OPL CO-oxymeter (Instrumentation Laboratory, Lexington, MA) (Capiau *et al*., upublished data).

Other candidates

Rather than focusing on an electrolyte *in* RBC, such as potassium, Erhardt *et al*. focused on another tightly regulated electrolyte, sodium, for normalization purposes [80]. These authors determined retinol in DBS, which -like sodium- primarily resides in the plasma. The sodium content in a DBS extract, measured via flame photometry, was used to calculate the plasma content of the examined specimen, assuming a value of 140 mM sodium in plasma. A similar strategy could be followed, determining

chloride, rather than sodium. A prerequisite for the usefulness of other physiological elements (e.g. calcium, magnesium, iron, copper, zinc, …) is that these show minimal non-Hct-related variation and are concentrated either in the RBC or in the plasma, allowing estimation of the Hct or the plasma content, respectively. Iron, being complexed with Hb in RBC, but also zinc, occurs at higher levels in RBC. However, since the levels of these elements may fluctuate more than e.g. those of potassium or sodium, a higher intra- and interindividual non-Hct-related variation may be expected. Also calcium and magnesium concentrations in blood, which both are under tight physiological control, have been suggested to have potential for normalization [86]. However, also here, experimental evidence is currently lacking.

Apart from trace elements, also amino acids may represent candidate markers to predict the Hct of DBS. Using a metabolomics approach, Wang *et al.* demonstrated that the plasma concentrations of five branched-chain and aromatic amino acids in fasting subjects may be predictive of the risk of developing future diabetes [87]. While these authors primarily focused upon amino acids differentially present in subjects, their findings also imply that the levels of various amino acids remain fairly constant between individuals. Focusing on these amino acids may help to trace back the Hct of the blood to prepare DBS. This hypothesis has been put forward by Hill *et al.*, who also demonstrated a good correlation between plasma and RBC concentrations of amino acids [66]. Also here, the absolute amount of certain amino acids may correlate with the plasma content, rather than with the RBC content of the blood. Additionally, a difficulty not to be underestimated may be the influence that food consumption, physical activity or pre-analytical variables may have on the levels of certain amino acids, e.g. necessitating DBS to be taken from fasting individuals [88].

Theoretically, also the erythrocyte proteome could be exploited to search for RBC-specific markers [89,90]. These may constitute both membrane-associated markers (e.g. surface markers) as cytoplasmic proteins. Not surprisingly, globins constitute the most abundant group of erythrocyte-specific proteins, although RBC markers such as glycophorins (major sialoglycoproteins present at the RBC surface), as well as other proteins, may be valuable candidates as well [90]. However, the use of such protein markers would require that expression is subject to minimal inter-individual variation. Moreover, determination of proteins may be more cumbersome, because of possible issues of stability. If suitable antibodies are available, determination of the RBC markers may be feasible via immuno-assays (e.g. ELISA), which are, however, typically associated with a significant cost. Theoretically, quantitative determination via proteomics may be an alternative option; however, application of such sophisticated methodology may represent overshooting, considering the aim (i.e. Hct prediction).

DNA-based procedures (e.g. PCR) are not suited for Hct prediction as erythrocytes anucleate during maturation, with as a consequence that mature erythrocytes do not possess nuclear DNA. As mature erythrocytes have also lost organelles such as mitochondria, they also lack mitochondrial DNA. Consequently, the major DNA contributors of the blood are the nucleated, mitochondria-containing non-RBC. As for RNA, Kabanova *et al.* reported the presence of transcripts of over 1000 different genes in a >99.9999% pure erythrocyte preparation [91]. Although it remains to be established whether there are true RBC-specific “housekeeping” transcripts amongst these, mRNA’s encoding Hb might be a

candidate, as these are the most abundant mRNA's in whole blood extracts, with reticulo-derived globin mRNA constituting up to 70% of total mRNA from whole blood [92]. However, even if there would be a correlation between globin mRNA and Hct of a DBS, a limiting problem may still be the sensitivity of mRNA to decay, which may give rise to differences between DBS with the same Hct, depending on the length and conditions of storage [93,94]. In addition, mRNA determination requires conversion to cDNA, again lengthening and complicating the procedure.

B.1.5. Conclusion

As generally recognized, DBS sampling offers several advantages over classical wet sampling. On the other hand, despite the progress made during the last decade, DBS analysis still faces considerable challenges. Among these, the effect of Hct on the measured concentration is unquestionably the most addressed problem. From an analytical point of view, although analyte-dependent, differential spreading of blood with different Hct mainly affects the accuracy of an analytical result, possibly leading to unacceptable assay biases at extreme Hct levels. At elevated Hct levels, this effect may in some cases be partially counteracted by reduced extraction efficiency of the analyte. Hence, a Hct interval in which the impact of the Hct on the analytical result falls within acceptable limits needs to be defined for any DBS-based bio-analytical method. From a physiological point of view, the Hct is an important factor to be taken into account when DBS results are to be compared with reference plasma values.

Different strategies to cope with the Hct-dependent spreading of blood on paper substrates were discussed. The Hct effect may simply be avoided by analyzing whole, volumetrically applied DBS instead of fixed-size partial DBS punches. The volumetric approach, however, excludes direct application of a drop of blood from the fingertip onto the paper card and may be difficult to sustain when envisaging home-sampling, although new devices have been developed very recently that should allow accurate disposition of a defined volume of blood by non-experienced users. The use of DPS instead of DBS may offer another way of avoiding the Hct problem. On the other hand, the effect of Hct may be minimized by using special types of substrates which are less influenced by the Hct, or by preparing the calibration line in blood with Hct close to the expected Hct range of the target population. Apart from avoiding or trying to minimize the Hct effect, another possible strategy to cope with this effect may be to measure or estimate the Hct of the blood used to prepare DBS. Also here, several strategies are possible, amongst which direct Hct measurement, Hct estimation based upon the physical characteristics of DBS and Hct prediction based upon endogenous compounds. With respect to the latter, potassium measurement in extracts from DBS punches has been demonstrated to be a promising strategy.

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Chapter B.2.

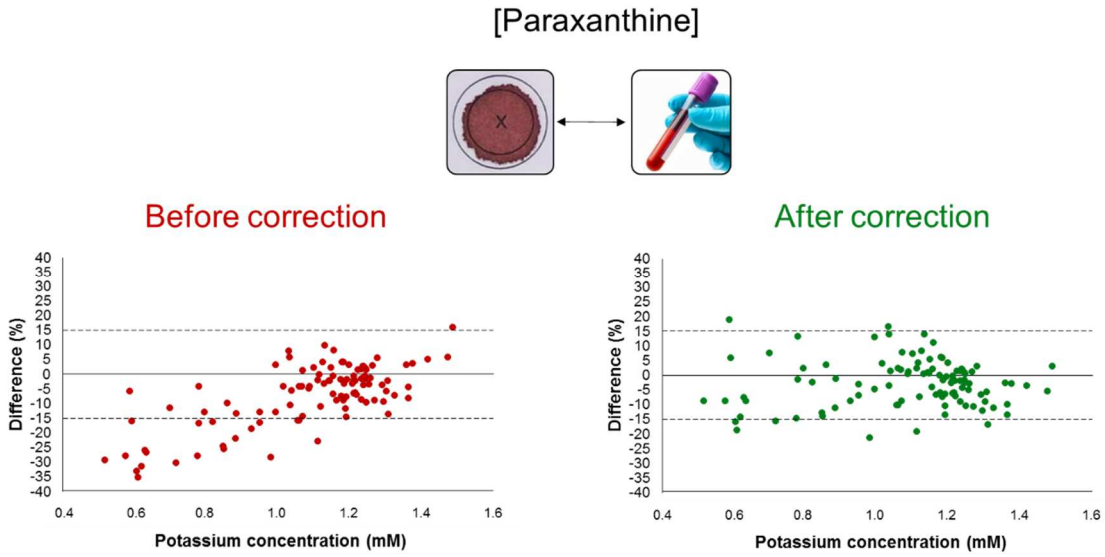
Potassium-based algorithm allows correction for the hematocrit bias

Based on

De Kesel PM, Capiau S, Stove VV, Lambert WE, Stove CP. Potassium-based algorithm allows correction for the hematocrit bias in quantitative analysis of caffeine and its major metabolite in dried blood spots. Anal Bioanal Chem. 2014;406(26):6749-55.

Abstract

Although dried blood spot (DBS) sampling is increasingly receiving interest as a potential alternative to traditional blood sampling, the impact of hematocrit (Hct) on DBS results is limiting its final breakthrough in routine bioanalysis. To predict the Hct of a given DBS, potassium (K^+) proved to be a reliable marker. The aim of the study described in this chapter was to evaluate whether application of an algorithm, based upon predicted Hct or K^+ concentrations as such, allowed to correct for the Hct bias. Using validated LC-MS/MS methods, caffeine, chosen as a model compound, was determined in whole blood and corresponding DBS samples with a broad Hct range (0.18 – 0.47). A reference subset (n = 50) was used to generate an algorithm based on K^+ concentrations in DBS. Application of the developed algorithm on an independent test set (n = 50) alleviated the assay bias, especially at lower Hct values. Before correction, differences between DBS and whole blood concentrations ranged from -29.1 to 21.1 %. The mean difference, as obtained by Bland-Altman comparison, was -6.6 % (95 % CI; [-9.7 – -3.4 %]). After application of the algorithm, differences between corrected and whole blood concentrations lay between -19.9 and 13.9 % with a mean difference of -2.1 % (95 % CI; [-4.5 – 0.3 %]). The same algorithm was applied to a separate compound, paraxanthine, which was determined in 103 samples (Hct range 0.17 – 0.47), yielding similar results. In conclusion, a K^+-based algorithm allows to correct for the Hct bias in the quantitative analysis of caffeine and its metabolite paraxanthine.



B.2.1. Introduction

In recent years, dried blood spot (DBS) sampling has increasingly received interest as an alternative sampling strategy. However, despite the numerous DBS-based methods that have been developed and the many advantages associated with this sampling technique [1,2], its implementation in routine quantitative bioanalysis has to a great extent been limited by the hematocrit (Hct) issue [3]. As outlined in Chapter B.1., this Hct issue is a problem that is both analytical and physiological in nature. The analytical facet comprises the impact of Hct on blood viscosity and, hence, the spreading of the blood on filter paper. Consequently, fixed size punches, taken from DBS with varying Hct values, will contain different amounts of blood. This facet also encompasses the influence of Hct on sample homogeneity, extraction efficiency and matrix effect [4]. The physiological facet of the Hct issue, on the other hand, relates to the influence of the Hct on the blood-to-plasma concentration ratio of an analyte. The latter is of key importance when DBS results are to be compared with established plasma- or serum-based reference intervals or therapeutic ranges [5].

As strongly deviating Hct values may have a significant impact on method accuracy and precision, as well as on the interpretation of DBS results, multiple strategies have been suggested to overcome this obstacle (see Chapter B.1.). These include analysis of whole, volumetrically applied DBS, the use of dried plasma spots instead of DBS or spotting on special filter substrates that are less prone to differential spreading of blood [3,6]. On the other hand, when DBS are prepared in a non-volumetric way, followed by analysis of partial punches, it is vital to define a Hct interval for every analyte in which method accuracy and precision are still adequate. Additionally, to minimize the Hct impact, calibration curves should be centered around a Hct value close to the median of the population of interest. However, even when such a Hct interval has been established, one still needs to confirm whether or not the Hct of a given DBS actually lies within this interval. To this end, a reliable marker is required that allows to predict the Hct of the blood used to prepare a given DBS. Our group previously demonstrated that potassium (K^+), an endogenous compound that correlates with the amount of red blood cells, allows to predict the Hct over a wide range with acceptable accuracy and precision [7]. This method was thoroughly validated and application on real patient samples showed a good correlation between the estimated Hct (based on the K^+ content) and the real Hct. However, hitherto, it has not been demonstrated if –and how– this Hct prediction may effectively assist in overcoming the Hct problem for a given DBS-based analytical method in real practice. Therefore, the aim of the study described in this chapter was to evaluate whether application of an algorithm, based upon the predicted Hct or the K^+ concentrations as such, could adjust for the Hct-induced bias in the quantitative analysis of caffeine. Caffeine was selected as a model drug, since we previously observed a Hct effect on the accuracy of measured caffeine concentrations in DBS by analyzing quality control samples prepared from spiked blood with a wide Hct range (0.20 – 0.60) (see Chapter A.3., Section A.3.3.2.) [8]. In this context, we also found that strongly deviating Hct values had no impact on matrix effect and recovery, using a similar Hct range (0.19 – 0.63) (see Chapter A.2., Section A.2.3.3.) [9]. Furthermore, given the widespread consumption of caffeine-containing food products, a sufficient number of positive samples could be obtained without the need to set up a clinical study involving actual administration of a probe drug.

B.2.2. Material and methods

B.2.2.1. Chemicals

Caffeine, caffeine-$^{13}C_3$, paraxanthine, paraxanthine-$^{13}C_4$-$^{15}N_3$ and formic acid were purchased from Sigma-Aldrich (Sigma-Aldrich, Diegem, Belgium), while KCl was obtained from UCB (UCB, Leuven, Belgium). LC-MS grade methanol was purchased from Biosolve (Biosolve, Valkenswaard, The Netherlands) and a Synergy® Water Purification System (Merck Millipore, Overijse, Belgium) provided ultrapure water.

B.2.2.2. Sample collection

Venous whole blood samples were obtained from both healthy volunteers (n = 61) and hospital patients (n = 117) and collected in Venosafe® 4 mL VF-054SHL or 9 mL VF-109SHL Li-heparin tubes (Terumo, Leuven, Belgium). Prior to blood collection, all healthy volunteers (age 24 – 48 years, 44 women and 17 men) were informed and signed informed consent. This study was approved by the Ethics Committee of Ghent University Hospital (Belgian registration number B670201111655 and B670201214201), which also approved the use of aliquots of routinely collected Li-heparin patient blood samples, admitted to the Laboratory of Clinical Biology of Ghent University Hospital (B670201319311). Because of an opting-out procedure, foreseeing the possibility of comparative analysis on left-over samples, no individualized informed consent was needed for the hospital patients. The samples were included in this study to obtain blood samples with deviating Hct values. As these were anonymized left-over samples, no additional data concerning age or sex of the donors could be traced back. On the day of venous blood sampling, corresponding DBS were prepared by spotting 25 µL whole blood on WHA10334885 Whatman 903 filter paper (GE Healthcare, Dassel, Germany). The resulting DBS were air-dried for 2 hours and stored at ambient temperature in zip-closure plastic bags, containing two 5-g Minipax® absorbent packets as desiccant (Sigma-Aldrich, Diegem, Belgium). Liquid blood samples were stored at -20 °C until analysis.

B.2.2.3. Analyses

Concentrations of caffeine and its major metabolite paraxanthine were determined in whole blood and DBS using validated methods on a Waters Acquity UPLC® system (Waters, Milford, MA, USA) coupled to an AB SCIEX API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA), described in Chapter A.2., section A.2.2.2.2. [9]. For whole blood samples, a protein precipitation was performed by adding 100 µL of methanol, containing 0.01 % formic acid, to 50 µL of blood after the addition of 10 µL of an internal standard (IS) solution. DBS punches (3 mm) were extracted in 70 µL of a methanol/water (80/20, v/v) mixture, containing 0.01 % formic acid and the IS. After gentle shaking for 10 minutes, whole blood and DBS samples were centrifuged and the resulting supernatants were diluted with water, containing 0.01 % formic acid [9]. Further details of these methods can be found in Chapter A.2., Section A.2.2.4.

K^+ concentrations in DBS extracts were measured by indirect potentiometry, using the ion-selective electrode of a Roche Cobas 8000 routine chemistry analyzer (Roche Diagnostics, Mannheim, Germany) [7]. K^+ was extracted from 3-mm DBS punches by two-fold addition of 50 µL of a 2.5 mM KCl solution in

ultrapure water and shaking for 5 minutes. Actual DBS K^+ concentrations were obtained by subtracting the value in the extraction solution from the measured value. These K^+ concentrations allowed to predict the approximate Hct of the blood used to prepare the DBS. Direct Hct measurement in whole blood was performed on a Sysmex XE-5000 hematology analyzer (Sysmex, Kobe, Japan).

B.2.2.4. Data analysis

Samples in which caffeine was measured were randomly assigned to a reference and test set of samples, thereby ensuring that Hct values were similarly distributed in both groups. For the reference set, the whole blood/DBS caffeine concentration ratios were plotted against K^+-based calculated Hct. A linear regression line was fitted to these data by applying the least squares method. The slope and intercept, together with their 95 % confidence intervals (CIs), were obtained using the Analysis Toolpack of MS Excel® 2013 (Microsoft, Redmond, WA, USA). From the resulting linear model, a Hct-based correction algorithm was derived by a simple transformation. As the calculated Hct used to generate the algorithm was based on K^+ concentrations measured in DBS, a similar algorithm could be generated using the K^+ concentrations as such. The resulting algorithm was applied to the independent test set. To further evaluate the usefulness of this approach, the same algorithm that was constructed for caffeine was applied to another compound, paraxanthine, which was also determined in samples from healthy volunteers and hospital patients.

To assess the extent of the Hct effect before and after implementation of a correction algorithm, the differences (%) between DBS concentrations (uncorrected or corrected) and whole blood concentrations were plotted against measured and calculated Hct values for the caffeine reference set and against K^+ concentrations for the caffeine and paraxanthine test sets. Percentage differences were calculated by dividing the difference between DBS and whole blood concentrations by the whole blood concentration and multiplying the result by 100. To the data of the test sets, linear regression lines were fitted by applying the least squares method. Slope, intercept and corresponding 95 % CIs were obtained as described above. Additionally, uncorrected and corrected concentrations were compared with corresponding whole blood concentrations using Bland-Altman plots, which were generated with Medcalc version 12.7.5 (Medcalc Software bvba, Ostend, Belgium).

B.2.3. Results and discussion

Caffeine concentrations above the lower limit of quantification (LLOQ) (50 ng/mL) were measured in whole blood and DBS samples from 100 subjects, both healthy volunteers and hospital patients, displaying a wide Hct range (0.18 – 0.47). Figure B.2.1. shows the Hct distribution in the entire sample set.

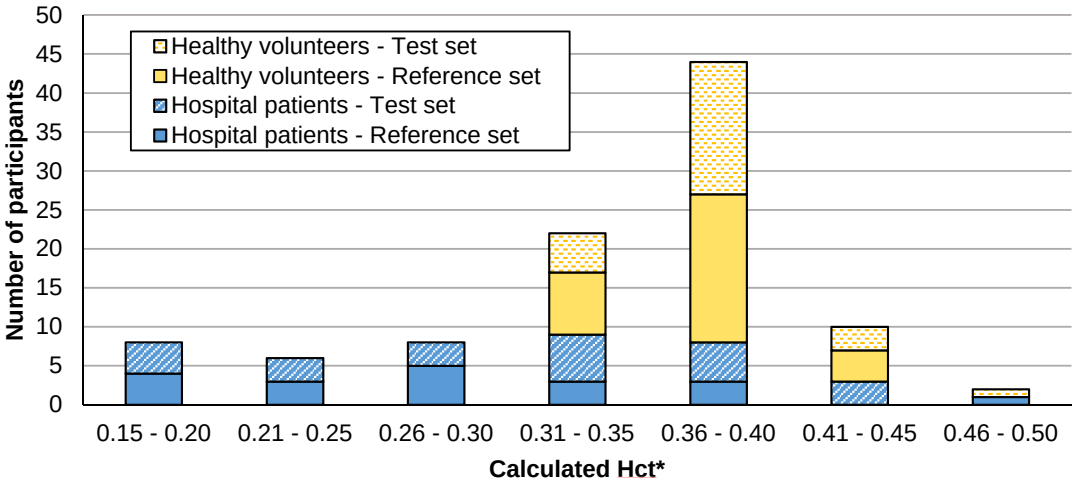


Figure B.2.1. Hct distribution of blood samples collected from healthy volunteers and hospital patients in which caffeine concentrations were measured.
*Calculated Hct: Hct of the blood used to prepare the DBS, as calculated using the K^+ concentration measured in the DBS.

The differences (%) between uncorrected caffeine concentrations in DBS and whole blood, plotted against Hct values measured in whole blood for the reference set (n = 50), are shown in Figure B.2.2. A. Lower DBS concentrations were found at low Hct levels, while higher concentrations were measured at high Hct levels, with differences ranging from -29.3 to 25.4 %. A similar pattern of decreasing uncorrected DBS concentrations with decreasing Hct was observed when differences (%) were plotted against calculated Hct values (Figure B.2.2. B), illustrating the validity of K^+-based Hct prediction [7]. The linear regression line fitted to the whole blood/DBS concentration ratios versus the calculated Hct for the reference set had a slope of -1.33 (95 % CI; [-1.74 – -0.93]) and an intercept of 1.51 (95 % CI; [1.37 – 1.65]). From this linear relationship, an algorithm to correct the caffeine DBS concentrations, taking into account the calculated Hct of the DBS, was derived: *corrected DBS concentration = uncorrected DBS concentration * ((-1.33 * calculated Hct) + 1.51).* The impact of applying the developed algorithm can be deduced from Figure B.2.2. C, showing the differences between corrected DBS concentrations and whole blood concentrations plotted against the calculated Hct.

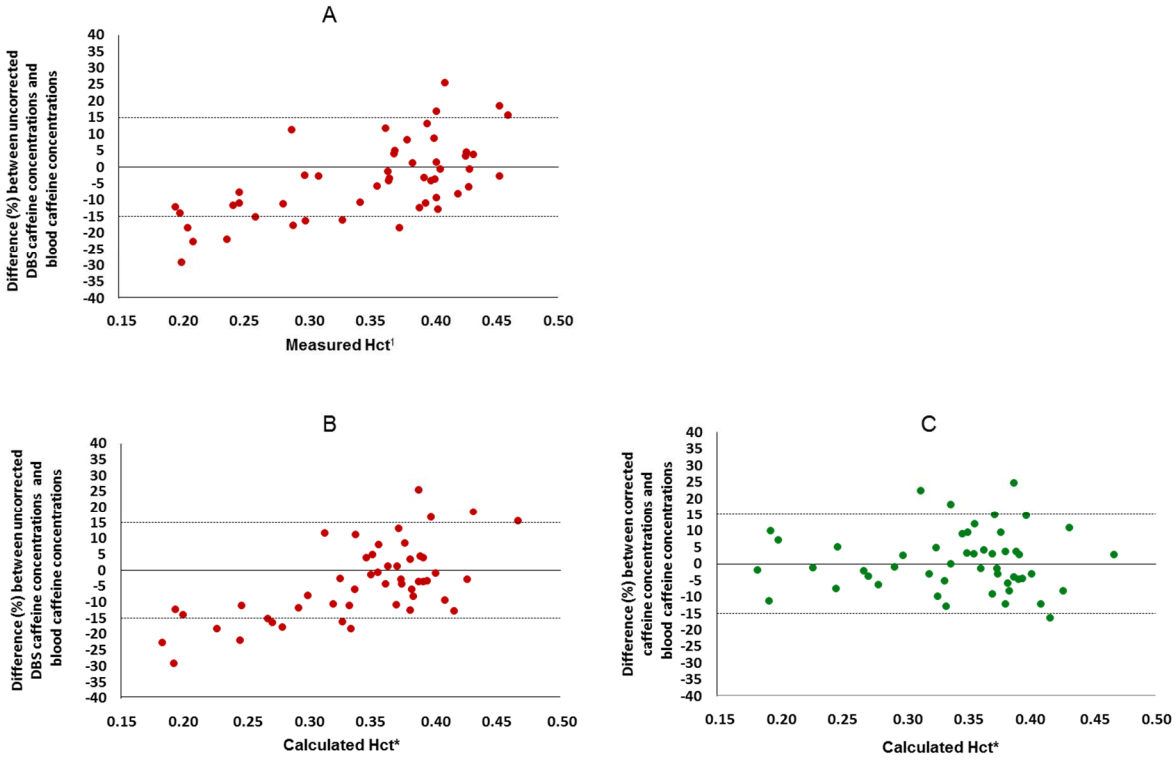


Figure B.2.2. Percentage differences (%) between uncorrected caffeine concentrations in DBS and whole blood plotted against hematocrit (Hct) measured in whole blood (A), against calculated Hct (B) and percentage differences (%) between corrected and whole blood caffeine concentrations plotted against calculated Hct (C). Dashed lines indicate ± 15 % difference limits, the full line indicates 0 % difference. All data were obtained from a reference set of whole blood and DBS samples (n = 50).
*Calculated Hct: Hct of the blood used to prepare the DBS, as calculated using the K^+ concentration measured in the DBS; [1]Measured Hct: Hct directly measured in whole blood using a Sysmex XE-5000 hematology analyzer.

When whole blood/DBS concentration ratios were directly plotted against K^+ concentrations, as the latter were used to calculate the Hct, the resulting linear regression line had a slope of -0.41 (95 % CI; [-0.54 – -0.29]) and an intercept of 1.51 (95 % CI; [1.37 – 1.64]). From these data, the following algorithm was derived: *corrected concentration = uncorrected DBS concentration * ((-0.41 * K^+ concentration) + 1.51).* However, as this algorithm was based on the reference sample set, it is likely to work for this given set of samples. Therefore, the algorithm needed to be applied to the remaining samples (n = 50), representing an independent test set. The differences (%) between uncorrected DBS caffeine concentrations and whole blood caffeine concentrations versus measured K^+ concentrations in this test set are shown in Figure B.2.3. A. Before application of the algorithm, a Hct effect similar to that in the reference set was observed, as the regression line of the plotted variables, with K^+ concentrations representing a measure of Hct, had a slope of 32.03 (95 % CI; [21.38 – 42.68]) and an intercept of -41.05 (95 % CI; [-53.00 – -29.10]). Differences between uncorrected caffeine concentrations in DBS and

whole blood ranged from -29.1 to 21.1 %. For 14 samples (i.e. 28 % of the data set), the difference was beyond ± 15 % limits. After application of the developed algorithm (Figure B.2.3. B), the regression line had a slope of -3.81 and an intercept of 2.44. The 95 % CIs of slope and intercept, respectively [-14.59 – 6.97] and [-9.65 – 14.53], included the zero-value. The differences between corrected and whole blood concentrations ranged from -19.9 to 13.9 %. Now, for only 4 samples (i.e. 8 % of the data set) the differences exceeded ± 15 % limits and none of the calculated concentrations deviated more than 20 % from the measured whole blood concentration. These findings were supported by the results of Bland-Altman comparisons, which clearly demonstrate the effect of the correction algorithm. The mean difference between uncorrected DBS caffeine concentrations and whole blood caffeine concentrations was -6.6 % (95 % CI; [-9.7 – -3.4 %]) (Figure B.2.3. C), while the mean difference between corrected concentrations and whole blood concentrations was -2.1 % (95 % CI; [-4.5 – 0.3 %]) (Figure B.2.3. D). As the latter 95 % CI contained 0, a consistent negative bias was no longer observed after correction.

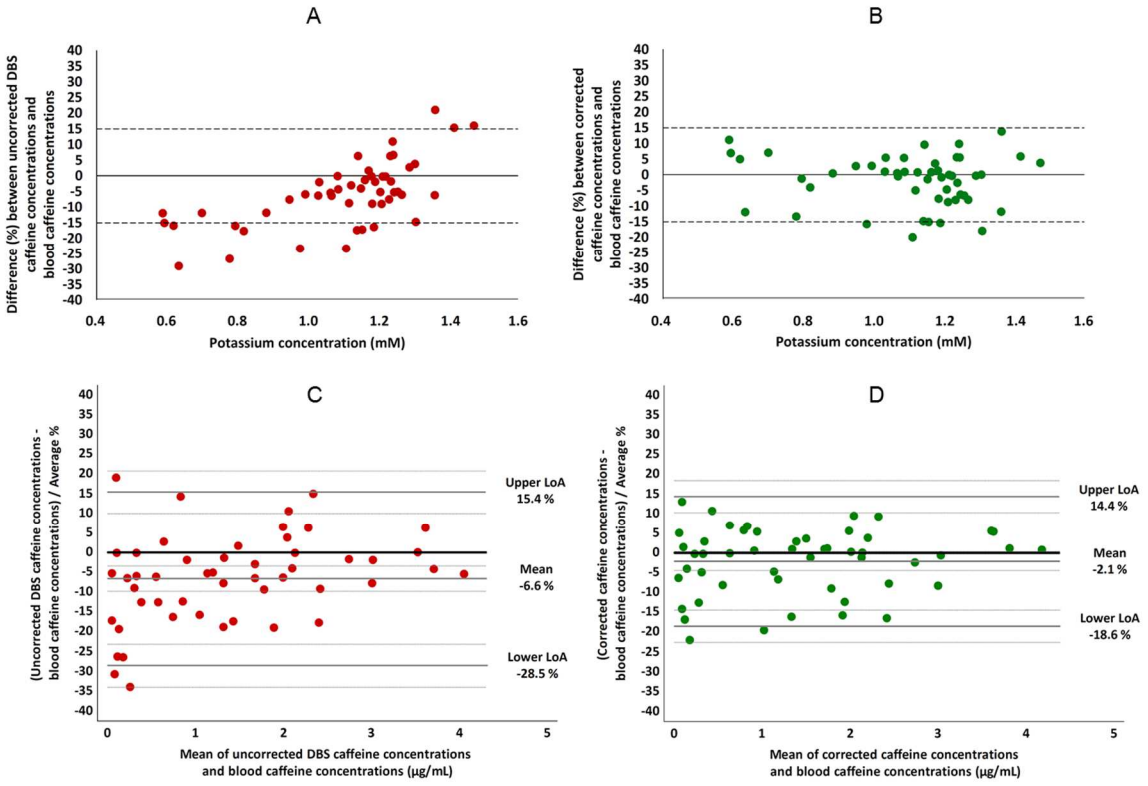


Figure B.2.3. Percentage differences (%) between uncorrected caffeine concentrations in DBS and whole blood (A) and between corrected and whole blood caffeine concentrations (B). Differences are plotted against potassium concentrations in DBS, dashed lines indicate ± 15 % difference limits and the full line indicates 0 % difference. Bland-Altman plot for the comparison between uncorrected DBS caffeine concentrations and whole blood caffeine concentrations (C) and between corrected and whole blood caffeine concentrations (D). The mean differences and the limits of agreement (LoAs) are displayed (full lines), together with their 95 % confidence limits (dashed lines). All data were obtained from a test set of whole blood and DBS samples (n = 50).

Application of the same algorithm to paraxanthine, caffeine's major metabolite, further demonstrated the usefulness of the presented approach. Paraxanthine concentrations above the LLOQ could be measured in samples from 103 participants (Hct range 0.17 – 0.47). The Hct distribution in this sample set is shown in Figure B.2.4.

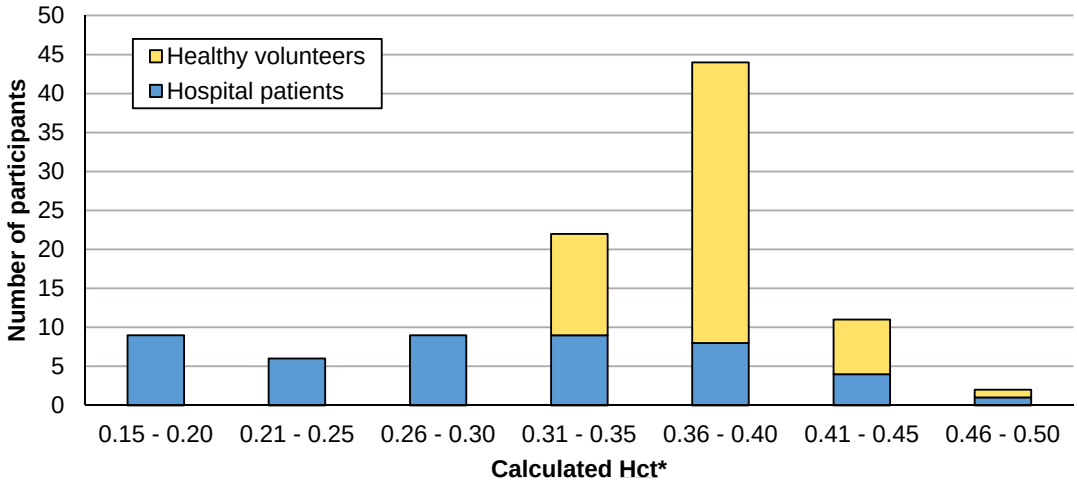


Figure B.2.4. Hct distribution of blood samples collected from healthy volunteers and hospital patients in which paraxanthine concentrations were measured.
*Calculated Hct: Hct of the blood used to prepare the DBS, as calculated using the K^+ concentration measured in the DBS.

The differences (%) between uncorrected DBS paraxanthine and whole blood paraxanthine concentrations versus measured K^+ concentrations are shown in Figure B.2.5. A. Also for this compound, uncorrected DBS concentrations were considerably affected by Hct, as the slope of the regression line of the plotted variables was 34.83 (95 % CI; [28.33 – 41.33]) and the intercept -45.12 (95 % CI; [-52.32 – -37.92]). The differences between uncorrected DBS and whole blood paraxanthine concentrations ranged from -35.2 to 15.8 %. Application of the algorithm yielded similar results as obtained for caffeine (Figure B.2.5. B), with the slope of the regression line being 1.58 and the intercept -4.56. Also here, the 95 % CIs of slope and intercept, respectively [-5.60 – 8.76] and [-12.51 – 3.39], included the zero-value. Differences between corrected and whole blood paraxanthine concentrations lay between -21.3 and 18.8 %. Before correction, the difference was beyond the ± 15 % limits for 22 samples (i.e. 21 % of the data set), while this was the case for only 8 samples (i.e. 8 % of the data set) after correction. With a single exception (-21.3 %), all corrected paraxanthine concentrations were within ± 20 % of the concentrations measured in whole blood. Also here, Bland-Altman comparisons supported the obtained results. Before correction, the mean difference was -8.2 % (95 % CI; [-10.6 – -5.9 %]) (Figure B.2.5. C). Again, the observed negative bias decreased considerably after correction, as the mean difference between corrected and whole blood paraxanthine concentrations was -3.2 % (95 % CI; [-4.8 – -1.6 %]) (Figure B.2.5. D).

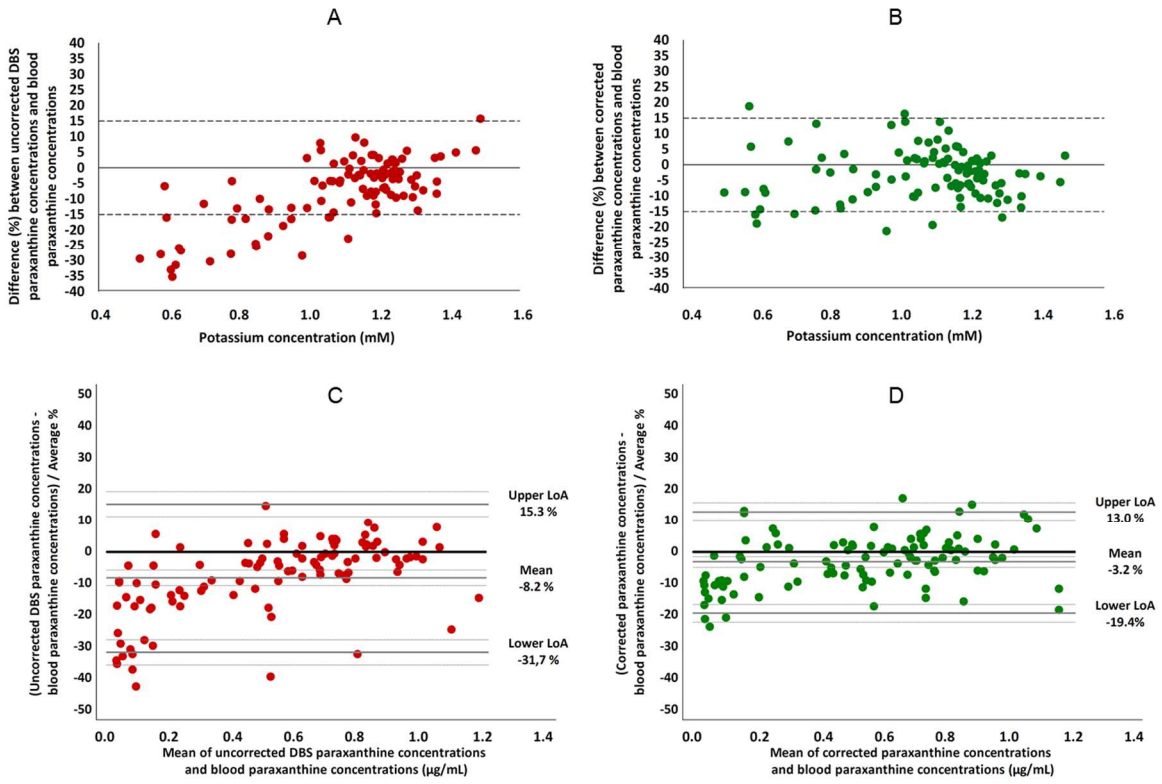


Figure B.2.5. Percentage differences (%) between uncorrected paraxanthine concentrations in DBS and whole blood (A) and between corrected and whole blood paraxanthine concentrations (B). Differences are plotted against potassium concentrations in DBS, dashed lines indicate ± 15 % difference limits and the full line indicates 0 % difference. Bland-Altman plot for the comparison between uncorrected DBS paraxanthine concentrations and whole blood paraxanthine concentrations (C) and between corrected and whole blood paraxanthine concentrations (D). The mean differences and the limits of agreement (LoAs) are displayed (full lines), together with their 95 % confidence limits (dashed lines). All data were obtained from a test set of whole blood and DBS samples (n = 103).

B.2.4. Conclusion

Based on the findings described above, we conclude that we are able to correct for the Hct bias in the quantitative analysis of caffeine in DBS, using a K^+-based algorithm. The same algorithm, initially designed for caffeine, was applied to a separate compound, paraxanthine, yielding similar results. Caffeine and paraxanthine are weakly basic compounds that show low binding to plasma or red blood cell proteins [5]. To further support the broader applicability of the presented approach, its usefulness should be evaluated for more analytes, displaying varying physicochemical properties and binding characteristics. The latter should include, amongst others, neutral, acidic and basic compounds, hydrophilic and hydrophobic substances and analytes with different plasma protein binding and blood cell association profiles.

B.2.5. References

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Chapter B.3.

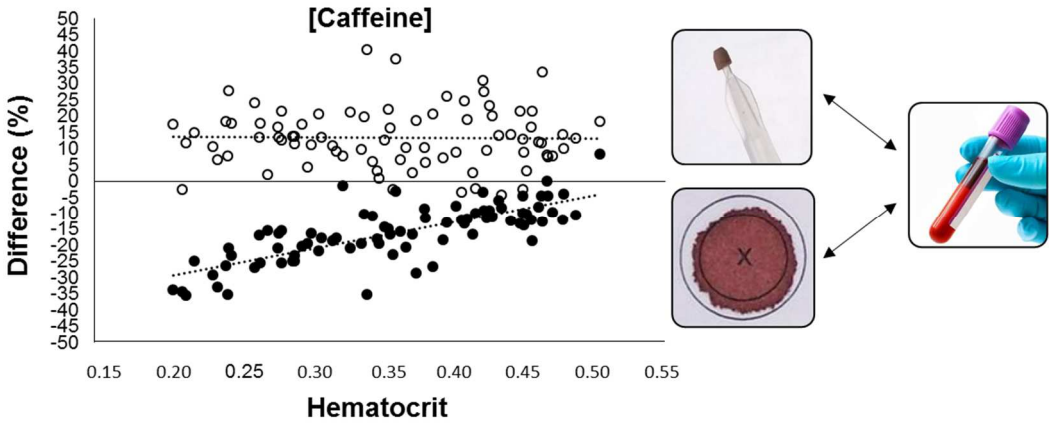
The hematocrit effect in volumetric absorptive microsampling

Based on

De Kesel PM, Lambert WE, Stove CP. Does volumetric absorptive microsampling eliminate the hematocrit bias for caffeine and paraxanthine in dried blood samples? A comparative study. Anal Chim Acta. 2015;881:65-73.

Abstract

Volumetric absorptive microsampling (VAMS) is a novel sampling technique that allows the straightforward collection of an accurate volume of blood (approximately 10 µL) from a drop or pool of blood by dipping an absorbent polymeric tip into it. The resulting blood microsample is dried and analyzed as a whole. The aim of the study described in this chapter was to evaluate the potential of VAMS to overcome the hematocrit bias, an important issue in the analysis of dried blood microsamples. An LC-MS/MS method for analysis of the model compounds caffeine and paraxanthine in VAMS samples was fully validated and fulfilled all pre-established criteria. In conjunction with previously validated procedures for dried blood spots (DBS) and blood, this allowed us to set up a comparative study in which both compounds were determined in over 80 corresponding VAMS, DBS and liquid whole blood samples. These originated from authentic human patient samples, covering a wide hematocrit range (0.21 – 0.50). By calculating the differences with reference whole blood concentrations, we found that analyte concentrations in VAMS samples were not affected by a bias that changed over the evaluated hematocrit range, in contrast to DBS results. However, VAMS concentrations tend to overestimate whole blood concentrations, as a consistent positive bias was observed. A different behavior of VAMS samples prepared from incurred and spiked blood, combined with a somewhat reduced recovery of caffeine and paraxanthine from VAMS tips at high hematocrit values, an effect that was not observed for DBS using a very similar extraction procedure, was found to be at the basis of the observed VAMS-whole blood deviations. Based on this study, being the first in which the validity and robustness of VAMS is evaluated by analyzing incurred human samples, it can be concluded that VAMS effectively assists in eliminating the effect of hematocrit.



B.3.1. Introduction

Owing to distinct advantages over traditional liquid blood samples (whole blood, serum, plasma), dried blood spot (DBS) sampling has been increasingly applied over the last years in various bioanalytical areas including preclinical animal studies [1-3] and clinical trials [4] in drug development programs, therapeutic drug monitoring [5,6] and toxicology [7,8]. Implementing dried blood microsamples in an analytical workflow may simplify sample collection, transport, storage and processing. Furthermore, it enables collection of representative samples in the patient's home setting or in resource-limited areas. Nevertheless, several issues are still limiting the generalized use of DBS in routine bioanalysis, amongst which the influence of hematocrit (Hct) is one of the most addressed topics, along with the volume of blood spotted onto the filter paper cards and the spot homogeneity. As Hct is directly related with blood viscosity, a drop of blood with high Hct will spread less on filter paper, resulting in spots with a smaller diameter. Consequently, a fixed-sized sub-punch taken from a high Hct DBS will contain a higher amount of blood (and analyte) than a punch taken from a low Hct DBS, giving rise to a Hct-dependent assay bias [9-11]. Apart from the latter phenomenon, high Hct levels may negatively impact the recovery of an analyte from DBS, an effect that can be minimized by optimizing the extraction conditions [12,13].

As outlined in Chapter B.1., several strategies to cope with the Hct effect caused by differential spreading of blood on filter paper have been proposed in the past few years [10,14]. In general, for every sub-punch DBS method, the impact of Hct can be minimized by preparing the calibration line using blood with a Hct value close to the expected median Hct of the study population. Furthermore, by analyzing quality control (QC) samples prepared from blood with varying Hct values during method validation, a Hct interval can be established in which assay bias is within acceptable limits. To verify whether the Hct of a given DBS actually lies within this interval, potassium (K^+) concentrations can be measured in extracts of DBS as they allow to predict the Hct of the blood used to prepare the DBS [15]. The study described in Chapter B.2., as well as work from others, showed that these predicted Hct values, or the K^+ concentrations as such, can actually be used to correct for the Hct-induced bias [16,17]. An alternative approach is to use devices, consisting of a multilayered membrane filtration system, that generate dried plasma spots (DPS) upon application of blood [18,19]. Although also these systems hold promise to overcoming the Hct issue, their validity and practical applicability needs to be supported by more data.

Analyzing complete volumetrically applied DBS is perhaps the simplest way to avoid the Hct effect [20]. Here, the blood can be applied on pre-punched paper discs [12,21,22] or, alternatively, the entire DBS can be punched [23]. However, these approaches require the accurate and precise application of a fixed volume of blood onto the filter paper substrate using pipettes or microcapillaries. Whereas this is a feasible strategy when sampling is to be carried out by trained personnel, it precludes e.g. home-sampling by non-experienced individuals.

Recently, two interesting new approaches that allow to collect a fixed volume of blood from a non-volumetrically deposited sample have been developed. The first is a microfluidic device consisting of a foldable support system that holds a DBS card on one side and a microfluidic plate with sized capillaries on the other [24]. The second, termed volumetric absorptive microsampling (VAMS), allows

straightforward collection of an accurate volume of blood without the need for specialized devices, such as pipettes or capillaries. The device, shown in Figure B.3.1., consists of a plastic handler with attached to it an absorbent polymeric tip, which, when dipped into blood, wicks up an accurate volume of blood (~ 10 µL) [25].

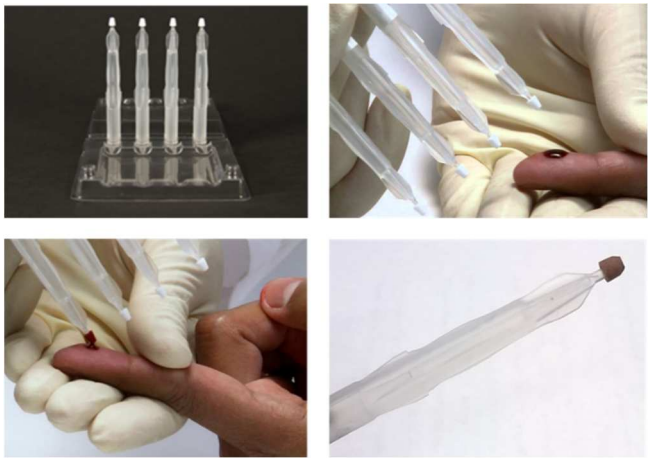


Figure B.3.1. Volumetric absorptive microsampling (VAMS) (Pictures taken from [35] and [36]).

By sampling blood with Hct values ranging from 0.20 – 0.70, it has been shown that the absorbed blood volume was independent of Hct [25]. In addition, analysis of VAMS samples with a similar Hct range (0.20 – 0.65) in six different laboratories revealed an acceptable variability in absorbed blood volume (8.7 %CV) [26]. Very recently, VAMS was used in a toxicokinetic (TK) study in rats. Although controlling the blood flow rate from the tail vein appeared to be an issue, leading to overfilling of the VAMS tips, the authors concluded that TK parameters for paracetamol obtained by VAMS were comparable to those measured in conventional diluted whole blood microsamples [27]. Based on these studies, VAMS shows promise to overcome the Hct and volume bias in the analysis of dried blood microsamples, while maintaining the benefits of collecting dried samples. Furthermore, in contrast to sub-punch DBS methods, potential sample inhomogeneity is no longer an issue as the entire tip is extracted. However, up to now, only artificial human samples (obtained via spiking blood that was prepared to have a certain Hct) have been used to demonstrate the ability of VAMS to overcome the Hct effect [25-27]; the impact of Hct on the analysis of real life human samples remained to be established. Therefore, the aim of the study described in this chapter was to evaluate the potential of VAMS to eliminate the Hct effect by analyzing over 80 incurred human patient samples with a wide Hct range (0.21 – 0.50). This is the first in-human study in which analyte concentrations obtained by VAMS are compared to concentrations measured in corresponding whole blood and DBS samples. The analyzed samples originated from patients admitted to 18 different hospital departments, including critical units, such as surgery or burn wound center, and consultation departments of diverse disciplines, such as radiology, cardiology, nephrology or oncology. Therefore, in addition to deviating Hct values, it was expected that this study batch includes a wide variety of sample characteristics, representing a highly relevant study batch to evaluate the practical applicability and robustness of VAMS. Caffeine and paraxanthine, determined using a validated LC-MS/MS method, were selected as model compounds since we previously observed

that DBS sub-punch concentrations of both compounds are subject to a Hct-induced bias (see Chapters A.3. and B.2.) [16,28].

B.3.2. Material and methods

B.3.2.1. Chemicals and stock solutions

Caffeine, paraxanthine, their internal standards (IS) caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$ and formic acid were purchased from Sigma-Aldrich (Diegem, Belgium). LC-MS grade methanol was obtained from Biosolve (Valkenswaard, The Netherlands). Ultrapure water was provided by a Synergy® Water Purification System (Merck Millipore, Overijse, Belgium). Stock solutions of caffeine and paraxanthine in water (1 mg/mL) and the IS in methanol (100 µg/mL) were prepared as described in Chapter A.2., A.2.2.1. [29].

B.3.2.2. Sample collection

Caffeine- and paraxanthine-free venous whole blood from a caffeine abstinent healthy volunteer was collected in ethylenediaminetetraacetic acid (EDTA) tubes (Venosafe® 9 mL VF-109SDK, Terumo, Leuven, Belgium) and used to prepare calibrators and QC samples. Patient whole blood samples (n = 96) with a wide Hct range (0.21 – 0.50) were obtained by taking aliquots (400 µL) from EDTA blood samples (Venosafe® 4 mL VF-054SDK, Terumo) admitted to the Laboratory of Clinical Biology of Ghent University Hospital for routine analysis. All samples were anonymized and, apart from Hct values and hospital departments, no clinical parameters or patient data were available. Hct was measured using a Sysmex XE-5000 hematology analyzer (Sysmex, Kobe, Japan). This procedure was approved by the Ethics Committee of Ghent University Hospital.

VAMS devices (brand name Mitra™) were purchased from Phenomenex (Utrecht, The Netherlands). VAMS samples were generated by dipping the upper part of the tip into a volume of whole blood contained in 2 mL tubes, thereby ensuring that the tips were not completely immersed into the blood to prevent overfilling. Upon turning completely red, the tips were held in place for an additional 2 s. Subsequently, the devices were positioned in a dedicated rack to prevent samples from touching each other while being air-dried for 2 h at room temperature. DBS samples were prepared by spotting 25 µL whole blood on WHA10334885 Whatman 903 filter paper (GE Healthcare, Dassel, Germany) using a calibrated pipette and dried under the same conditions as VAMS samples. Whole blood samples were obtained by transferring 50 µL blood to 2 mL tubes. Until analysis, dried samples were stored at room temperature in the presence of desiccant (two 5 g Minipax® absorbent packets, Sigma-Aldrich) in a closed plastic box or a zip-closure plastic bag for VAMS and DBS samples, respectively. Liquid blood samples were stored at -20 °C for 41 days.

B.3.2.3. Sample preparation and LC-MS/MS procedures

VAMS tips were separated from the handler and placed in 2 mL cups before 140 µL of a methanol/water (80/20, v/v) mixture, containing 0.01 % formic acid and the isotopically labeled IS of caffeine and

paraxanthine, was added. The tips were extracted for 10 minutes in a Thermomixer® comfort (Eppendorf, Hamburg, Germany) set at 1000 rpm and 22 °C. Following a centrifugation step at room temperature (10 minutes; 10000 x g), 90 µL of the supernatant was isolated and mixed with 390 µL of water, containing 0.01 % formic acid. The resulting mixture was transferred to an LC vial and 10 µL was injected onto the UPLC® column. DBS and whole blood samples were processed using the methods described in Chapter A.2., Section A.2.2.4. [29].

All samples were analyzed by LC-MS/MS using an Acquity UPLC® (Waters, Milford, MA, USA) - API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) configuration. Chromatography and mass spectrometry parameters for DBS and whole blood analysis are described in Chapter A.2., Section A.2.2.2.2. [29]. Instrument settings for analysis of VAMS samples were identical as those for DBS.

B.3.2.4. Validation of the VAMS method

Validation of the VAMS method was based on European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA) guidelines for bioanalytical method validation [30,31] and included evaluation of selectivity, carry-over, lower limit of quantification (LLOQ), linearity, precision, accuracy, matrix effect, recovery and stability.

Selectivity was assessed by analyzing VAMS samples that were prepared using blank whole blood from six different healthy volunteers. Blank samples, along with zero (IS-spiked blank matrix) samples, were also analyzed at the start of every analytical run to verify whether the IS potentially contributed to the responses of the analytes as a result of impurity. All resulting chromatograms were inspected for interfering peaks, which were considered acceptable if responses were less than 20 % of the LLOQ for the analytes and 5 % for the IS [30]. Blank samples injected after the highest calibrators were used to determine carry-over, thereby applying identical criteria as for assessment of selectivity.

On four non-consecutive days, eight-point calibration lines were constructed using blank whole blood and analyzed to evaluate linearity. The concentrations of the calibrators were 0.050, 0.075, 0.25, 0.50, 1.0, 2.5, 5.0, 10.0 µg/mL for caffeine and 0.025, 0.050, 0.100, 0.25, 0.50, 1.0, 2.5, 5.0 µg/mL for paraxanthine. The LLOQ was the lowest calibrator and was defined as the lowest concentration of caffeine and paraxanthine which could be measured with %RSD and %bias below 20 %. To evaluate whether the obtained calibration data were homoscedastic, an F-test was performed at the 99 % confidence level and residual vs. nominal concentration plots were constructed [32]. Both unweighted and weighted linear regression were applied to find the best fitted-model. The following weighting factors were tested: $1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$ and $1/\sqrt{y}$. The obtained models were compared by calculating the sum% relative error (RE) and plotting %RE against nominal concentrations [32]. In order to accept the selected model, mean back-calculated concentrations of the calibrators should be within ± 15 % of the nominal value or within ± 20 % for the LLOQ [30].

QCs at four concentration levels were prepared and analyzed in duplicate on four non-consecutive days to determine precision and accuracy. The nominal concentrations were 0.05 and 0.025 (LLOQ), 0.12 and 0.06 (low), 4.0 and 2.0 (medium), 8.0 and 4.0 (high) µg/mL for caffeine and paraxanthine,

respectively. Independent stock solutions were used for QCs and calibrators. For all spiked samples, the volume of non-matrix solvents never exceeded 5 % of the total sample volume. A single factor ANOVA was used to calculate intra- and interbatch precision values (%RSD) [33]. Accuracy (%bias) was calculated by dividing the difference between the obtained concentration and the nominal value by the nominal value and multiplying it by 100. Precision and accuracy should be within ± 15 % (± 20 % for the LLOQ) [30].

Blank whole blood originating from six different sources with Hct values ranging from 0.40 - 0.46 (0.43 ± 0.02; mean ± SD) was used to determine matrix effect and recovery. Blood samples were spiked at low and high concentration level before (C) or after (B) extraction. Solutions of caffeine and paraxanthine in the starting eluent at the same concentrations (A) were prepared as well. To prepare solutions A and B, a nominal blood volume of 10 µL, absorbed by the VAMS tips when dipped into the blood samples, was taken into account. The ratios of peak areas of (B) to those of (A) were multiplied by 100 to obtain absolute matrix effect values, while absolute recovery values were calculated by multiplying the ratios of peak areas of (C) to those of (B) by 100. Relative matrix effect and recovery were obtained by the %RSD of absolute matrix effect and recovery values [34]. Relative matrix effect should not exceed 15 % [30]. To evaluate the impact of Hct on recovery and matrix effect, blood samples with different Hct values (0.21, 0.42, 0.62) were prepared starting from blood with Hct 0.48, as described in Chapter A.2., Section A.2.2.5. [15]. For all four Hct levels, recovery and matrix effect were determined in triplicate on low and high concentration level. Here, 10 µL of blank or spiked blood was spotted onto the upper surface of the VAMS tips using a calibrated pipette.

Stability of VAMS samples was evaluated by analyzing low and high QCs in triplicate at time point zero and after 4 days of storage at 60 °C and 82 days at room temperature. VAMS samples were placed in dedicated clamshells and stored in zip-closure plastic bags with dessicant until analysis. Processed sample stability was assessed by re-injecting extracts of low and high QCs (n = 3) after 4 days of storage in the autosampler (4 °C) and 30 days at -20 °C. All stability samples were analyzed against a freshly prepared calibration curve. The obtained mean concentrations at a given time point should be within ± 15 % of the concentration measured at time point zero and of the nominal concentration [30].

B.3.2.5. Comparative study involving VAMS, DBS and whole blood sampling

Caffeine and paraxanthine concentrations were determined in VAMS, DBS and whole blood samples originating from 96 hospital patient blood samples. All samples were analyzed against freshly prepared matrix-matched calibration curves, prepared from blank blood from a single donor with a Hct of 0.48. To examine the impact of Hct on VAMS and DBS results, differences (%) between VAMS or DBS concentrations and whole blood concentrations were plotted against Hct values. Percentage differences were calculated by dividing the difference between VAMS or DBS and whole blood concentrations by the whole blood concentrations and multiplying the result by 100. Using least squares regression analysis, linear regression lines were fitted to the resulting data. Slopes, intercepts and their 95 % confidence intervals (CI) were calculated by the Analysis Toolpack of MS Excel® 2013 (Microsoft, Redmond, WA, USA). VAMS and whole blood concentrations were also compared by constructing

Bland-Altman plots using Medcalc statistical software version 12.7.5 (Medcalc Software bvba, Ostend, Belgium).

Furthermore, caffeine and paraxanthine concentrations were determined in VAMS and whole blood samples (n = 3) originating from spiked and incurred blood. Therefore, blank EDTA blood from a healthy volunteer was spiked with caffeine and paraxanthine to obtain nominal concentrations of 1.0 and 0.8 µg/mL, respectively. These target concentrations were based on the results of a previously conducted CYP1A2 phenotyping study (see Chapter A.3.) and should approximate whole blood caffeine and paraxanthine concentrations 6 h after the intake of a 150 mg caffeine test dose [28]. Hence, on the same day, a second blood sample was collected from the same volunteer 6 h after oral ingestion of a capsule containing 150 mg caffeine. Spiked and incurred samples were analyzed in the same analytical run. Calibration curves for VAMS and whole blood samples were prepared using a single pool of blank blood.

B.3.3. Results & discussion

B.3.3.1. Validation of the VAMS method

No unacceptable interfering peaks were observed in VAMS samples prepared from blank blood from 6 individual sources and the IS did not contribute to the responses of caffeine and paraxanthine. Furthermore, no carry-over was found in blank samples injected after the highest calibrators. Calibration data for caffeine and paraxanthine were found to be heteroscedastic. Weighted linear regression considerably improved %RE values compared to unweighted regression. The selected weighting factors for both compounds are shown in Table B.3.1., together with calibration and sensitivity data. Using these regression parameters, mean back-calculated concentrations of the calibrators deviated less than 5 and 7 % from the nominal concentrations for caffeine and paraxanthine, respectively. The chosen models fulfilled the acceptance criteria and the calibration lines were linear.

Table B.3.1. Calibration and sensitivity data for the determination of caffeine and paraxanthine in VAMS samples using LC-MS/MS (n = 4).

	Weighting	Slope, mean ± SD [95% CI]	Intercept, mean ± SD [95% CI]	R^2	Standard deviation of residuals	Range (µg/mL)
caffeine	$1/y^2$	2.021 ± 0.215 [1.833 – 2.209]	0.036 ± 0.010 [0.028 – 0.045]	0.996	0.250	0.050 – 10.0
paraxanthine	$1/x^2$	4.456 ± 0.480 [4.035 – 4.877]	0.025 ± 0.014 [0.012 – 0.037]	0.995	0.284	0.025 – 5.0

Intra- and interbatch precision (%RSD) and accuracy (%bias) values for caffeine and paraxanthine, displayed in Table B.3.2., were below 15 % and, therefore, also met the acceptance criteria.

Table B.3.2. Intra- and interbatch precision and accuracy (n = 4 x 2) for QCs of caffeine and paraxanthine at four concentration levels in VAMS samples.

QC	Intrabatch precision (%RSD)		Interbatch precision (%RSD)		Accuracy (%bias)	
	caffeine	paraxanthine	caffeine	paraxanthine	caffeine	paraxanthine
LLOQ	8.08	6.65	14.31	13.36	8.96	1.77
low	7.82	5.68	9.03	7.73	3.48	4.21
medium	4.84	3.22	5.70	5.38	-0.64	1.07
high	3.12	4.30	5.42	5.85	1.63	2.17

Table B.3.3. shows matrix effect and recovery data for caffeine and paraxanthine, determined in VAMS samples prepared from 6 individual lots of blood with an average Hct of 0.43 ± 0.02. The absolute matrix effect for both compounds was close to 100 %, with or without compensation by the IS. Relative matrix effect, being lower than 4 and 6 %RSD for caffeine and paraxanthine respectively, was well within the predefined acceptance limits (15 %RSD). High recovery values (> 87 %) were obtained for both compounds. As recovery was somewhat lower for the IS than for the analytes, a slight overcompensation for caffeine was observed when the IS was taken into account. However, it should be noted that, in order to calculate the amount of analyte that was spiked after extraction, a nominal absorbed blood volume of 10 µL was assumed. According to the manufacturer, the average absorbed volume of the VAMS tips used in this study was actually 10.7 µL. Therefore, the reported recovery values in Table B.3.3. represent a limited overestimation of the real recovery. Importantly, recovery for both compounds was reproducible (< 6 %RSD).

Table B.3.3. Absolute and relative matrix effect and recovery data (n = 6) for caffeine and paraxanthine at two concentration levels in VAMS samples prepared using whole blood from six different individuals with Hct values ranging from 0.40 – 0.46 (0.43 ± 0.02; mean ± SD).

Caffeine

	Absolute matrix effect (mean ± SD, %)		Relative matrix effect (%RSD)		Absolute recovery (mean ± SD, %)		Relative recovery (%RSD)	
QC	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS
low	101.71 ± 3.23	98.27 ± 3.87	3.17	3.94	99.10 ± 4.33	116.42 ± 6.10	4.37	5.24
high	102.50 ± 1.23	100.08 ± 1.18	1.20	1.18	94.04 ± 2.60	108.73 ± 2.77	2.77	2.55

Paraxanthine

	Absolute matrix effect (mean ± SD, %)		Relative matrix effect (%RSD)		Absolute recovery (mean ± SD, %)		Relative recovery (%RSD)	
QC	without IS	with IS	without IS	with IS	without IS	with IS	without IS	with IS
low	99.79 ± 5.09	99.81 ± 5.68	5.10	5.69	90.16 ± 3.71	100.20 ± 2.66	4.11	2.66
high	101.09 ± 1.08	99.44 ± 1.71	1.07	1.72	87.74 ± 2.34	95.74 ± 2.01	2.66	2.10

As the blood samples used in the latter experiment had a narrow Hct range (0.40 - 0.46), the effect of Hct on recovery and matrix effect was further evaluated using blood samples with deviating Hct values (0.21 – 0.62). Absolute recovery and matrix effect values, determined in triplicate at low and high concentration levels, are presented in Table B.3.4. For these data, compensation by the IS was already taken into account. While matrix effect was not affected by Hct, recovery for both compounds was lower at higher Hct values (0.48 and 0.62). A similar trend was observed by Denniff *et al.*, who found a reduced recovery for low paracetamol concentrations in VAMS samples, with recovery values ranging from 92.9 to 70.6 % over a comparable Hct range (0.20 – 0.69) [27]. It seems that as Hct increases, the higher relative amount of erythrocytes trapped in the VAMS tips renders it more difficult for compounds to be desorbed from the tips. Since 10 µL of blood was directly spotted onto the VAMS tips in this experiment, it can be excluded that lower volumes of blood absorbed by the tips were at the basis of the reduced recovery at high Hct. Although beyond the scope of this study, this recovery issue can most probably be resolved by optimizing the extraction conditions, for example by using sonication [12] or elevated extraction temperatures [13], as demonstrated in DBS analysis. Here, we opted to apply a very similar protocol as previously developed and validated for the extraction of caffeine and paraxanthine from DBS and for which a suchlike influence of Hct on recovery was not observed (see Chapter A.2., Section A.2.3.3.) [29].

Table B.3.4. Absolute recovery and matrix effect data (n = 3) for caffeine and paraxanthine at two concentration levels in VAMS samples prepared using whole blood with varying Hct values.

	Hct	Caffeine		Paraxanthine	
		Low QC	High QC	Low QC	High QC
Absolute recovery (mean ± SD, %)	**0.21**	101.45 ± 2.26	101.79 ± 0.67	86.98 ± 1.52	87.14 ± 1.75
	0.42	101.30 ± 1.28	100.53 ± 2.67	84.70 ± 3.53	84.33 ± 1.38
	0.48	93.86 ± 0.89	91.08 ± 2.03	75.48 ± 0.60	75.93 ± 1.09
	0.62	92.01 ± 4.80	92.91 ± 4.74	73.51 ± 1.60	77.72 ± 5.69
Absolute matrix effect (mean ± SD, %)	**0.21**	104.29 ± 3.37	100.49 ± 1.54	101.46 ± 2.49	99.69 ± 2.01
	0.42	101.16 ± 0.53	99.52 ± 1.71	98.28 ± 3.28	99.48 ± 0.34
	0.48	97.46 ± 0.50	97.48 ± 0.99	99.03 ± 2.73	99.10 ± 1.31
	0.62	103.07 ± 2.09	98.36 ± 0.31	100.58 ± 4.26	99.48 ± 1.78

Stability data for low and high QCs, analyzed in triplicate, are presented in Table B.3.5. and demonstrate that caffeine and paraxanthine were stable in VAMS samples for at least 82 days when stored at room temperature and for at least 4 days when stored at 60 °C, the latter representing potential shipping conditions. Furthermore, processed samples were stable when stored for at least 4 days in the autosampler set at 4 °C and for at least 30 days at -20 °C. Stock solutions of both compounds and the IS were stable for at least 6 months at -20 °C (Chapter A.2., Section A.2.3.3.) [29].

Table B.3.5. Stability data for caffeine and paraxanthine in VAMS samples at two concentration levels (n = 3). Data are presented as the percentage of the concentrations measured at time point zero.

QC	Room temperature 82 days (mean ± SD, %)		60 °C 4 days (mean ± SD, %)	
	caffeine	paraxanthine	caffeine	paraxanthine
Low	100.10 ± 11.24	97.44 ± 10.38	97.58 ± 8.64	90.94 ± 8 .28
High	99.11 ± 3.50	99.41 ± 4.92	94.09 ± 7.08	95.50 ± 5.09

B.3.3.2. Comparative study involving VAMS, DBS and whole blood sampling

In 81 out of 96 corresponding VAMS, DBS and whole blood samples, caffeine concentrations above the LLOQ (0.050 µg/mL) could be measured, while paraxanthine concentrations above the LLOQ (0.025 µg/mL) were measured in 83 samples. As we relied on daily consumption of caffeine containing beverages or food to determine caffeine and paraxanthine concentrations in the collected samples, no data on caffeine dose or time since the last intake were available. The latter, combined with the availability of a single blood sample per subject, did not allow to calculate pharmacokinetic parameters. Hct values in the analyzed samples ranged from 0.21 to 0.50, with a median of 0.36. It should be noted that all samples were analyzed against calibration curves prepared using blood with a Hct of 0.48. We specifically opted to use a calibration curve generated from blood with a relatively high Hct to clearly visualize the Hct effect on DBS concentrations and create a worst case scenario, both for DBS and VAMS samples. This allows a challenging assessment of the Hct effect. The differences (%) between VAMS or DBS concentrations and whole blood concentrations, plotted against Hct values, are shown in Figure B.3.2. for caffeine (A) and paraxanthine (B). A similar Hct-induced bias was observed for DBS concentrations of both compounds, as regression lines fitted to the differences between DBS and whole blood concentrations had a slope of 83.67 (95 % CI; [67.80 – 99.54]) and an intercept of -46.30 (95 % CI; [-52.17 – -40.44]) for caffeine and a slope of 82.25 (95 % CI; [67.61 – 96.90]) and an intercept of -52.16 (95 % CI; [-57.57 – -46.75]) for paraxanthine. Measured DBS concentrations markedly decreased with decreasing Hct values. These results are in accordance with previous findings for both compounds, described in Chapter B.2., Section B.2.3. [16]. With differences between DBS and whole blood concentrations ranging from -35.52 to 8.39 % for caffeine and from -41.44 to 4.16 % for paraxanthine over the evaluated Hct range, the assay bias was unacceptable at low Hct levels. However, as we demonstrated in Chapter B.2. [16], DBS concentrations of both compounds can be corrected for the Hct bias in a convenient way by means of an algorithm based on K^+ concentrations measured in DBS extracts. VAMS concentrations, on the other hand, were not affected by a suchlike Hct-effect. The regression lines fitted to the differences between VAMS and whole blood concentrations had a slope of -1.70 (95 % CI; [-26.96 – 23.56]) and an intercept of 14.09 (95 % CI; [4.76 – 23.43]) for caffeine and a slope of -5.98 (95 % CI; [-28.42 – 16.47]) and an intercept of 19.13 (95 % CI; [10.84 – 27.42]) for paraxanthine. As, for both compounds, the 95 % CIs of the slopes included the 0 value, it can be

concluded that the differences between VAMS and whole blood concentrations did not change in function of Hct (in the range between 0.21 and 0.50). These findings on real patient samples lend support to other studies using artificial samples, demonstrating that VAMS devices are able to collect an accurate volume of blood over a wide Hct range [25,26].

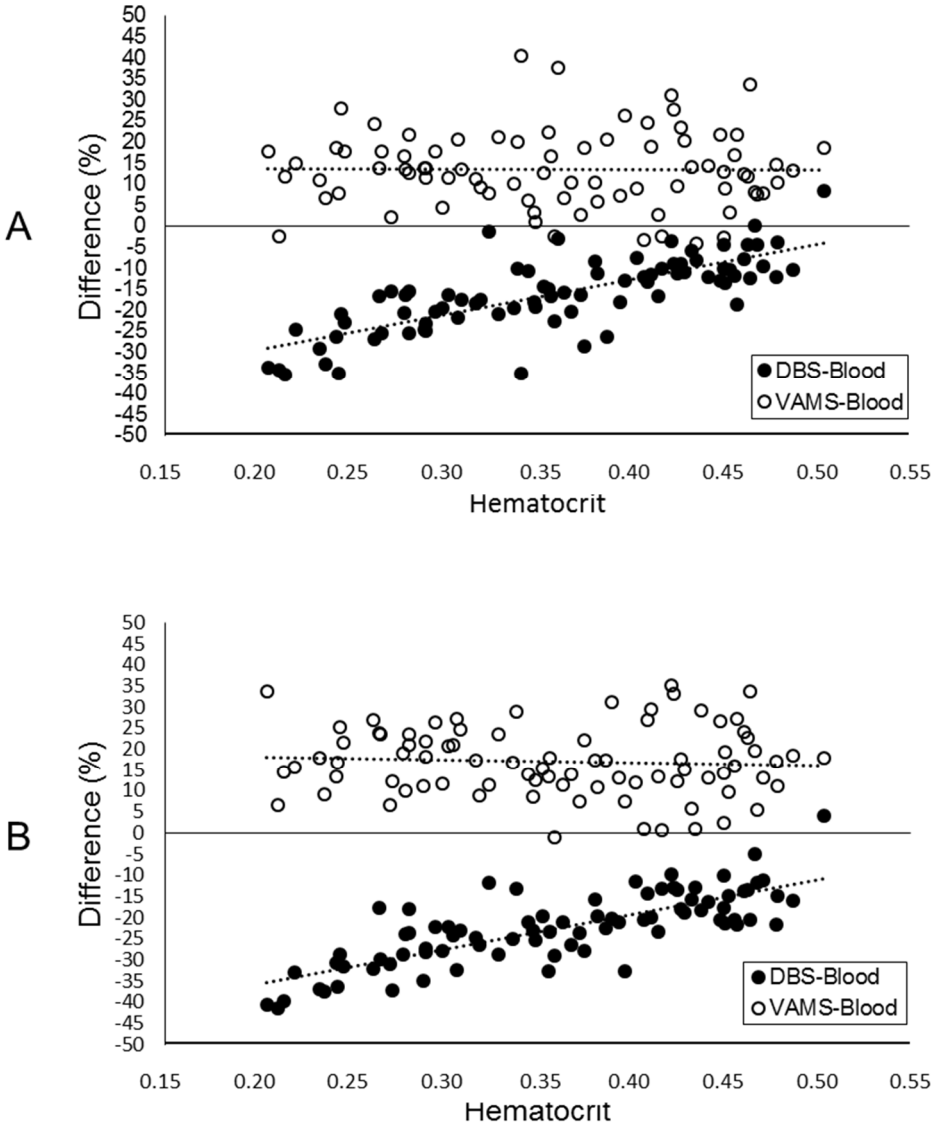


Figure B.3.2. Differences (%) between VAMS (white dots) or DBS (black dots) concentrations and whole blood concentrations plotted against hematocrit for (A) caffeine (n = 81) and (B) paraxanthine (n = 83). Broken lines represent linear regression lines.

Remarkably, VAMS concentrations were subject to a consistent positive bias, irrespective of Hct. Bland-Altman plots for the differences between VAMS and whole blood concentrations (Figure B.3.3.) revealed a mean positive difference of 12.3 % (95 % CI; [10.5 – 14.1 %]) and 15.4 % (95 % CI; [13.9 – 16.9 %]) for caffeine and paraxanthine, respectively.

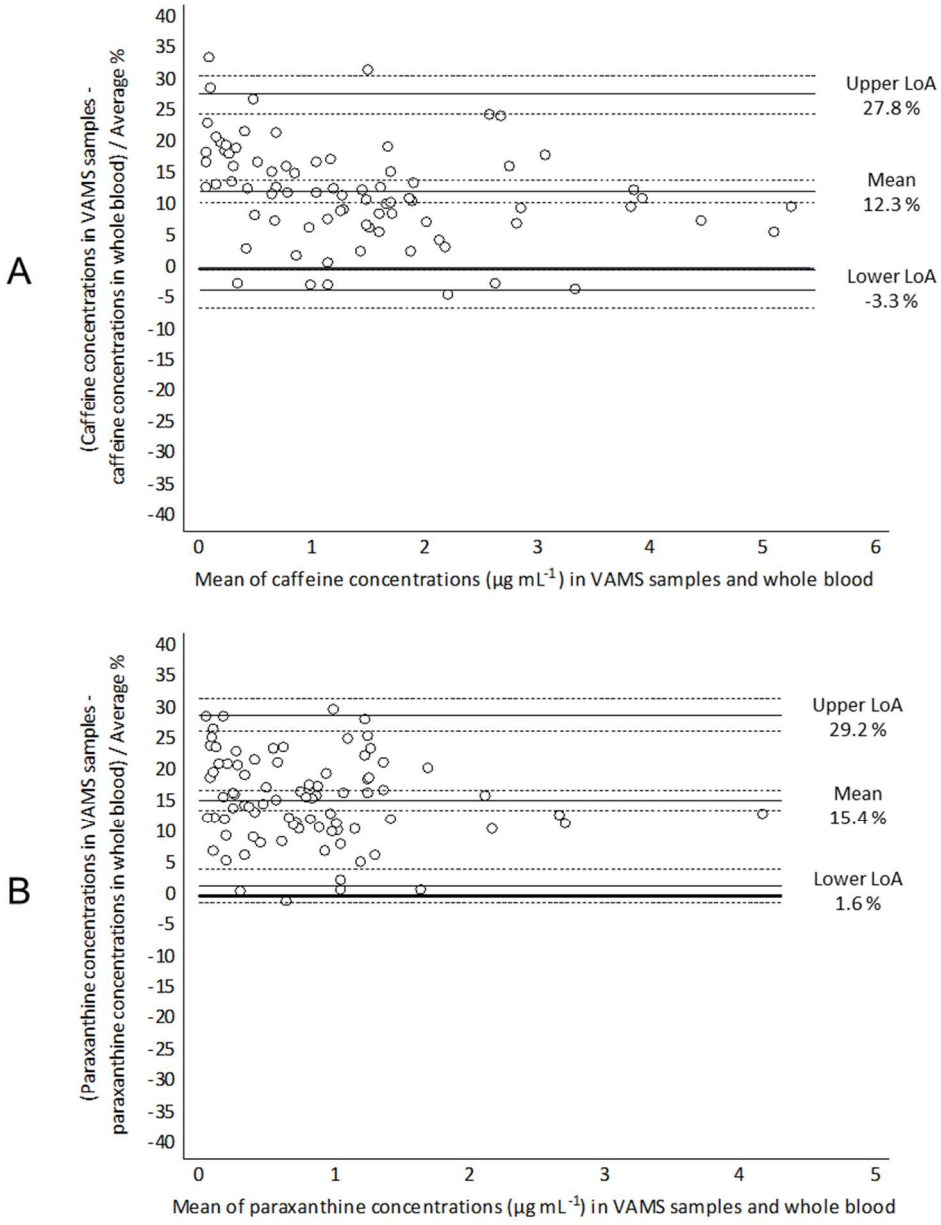


Figure B.3.3. Bland-Altman plots for the comparison between VAMS and whole blood concentrations for (A) caffeine (n = 81) and (B) paraxanthine (n = 83). Mean differences and limits of agreement (LoAs) are represented by full lines, 95 % confidence limits by broken lines.

Thus, analyte concentrations determined in VAMS samples tend to overestimate whole blood concentrations, although deviations were relatively limited, taking into account that results from different matrices (i.e. dried VAMS tips vs. liquid whole blood), obtained by different extraction procedures and analyzed in separate analytical runs, were compared. For example, for 86 and 74 % of the samples for

caffeine and paraxanthine, respectively, differences between VAMS and whole blood concentrations were below 20 %, thereby fulfilling the acceptance criteria of incurred sample reanalysis, intended for reanalysis of a single matrix [30]. Interestingly, a positive bias for VAMS results was also described by Denniff *et al.* [27]. In a paracetamol TK study in rats, mean C_{max} was up to 40 % higher when measured in VAMS samples compared to diluted whole blood samples. The authors concluded that this bias was caused by a combination of overfilled VAMS tips, as a result of the rate of blood flow from the tail vein being higher than the rate at which tips could absorb the blood, and the fact that VAMS and whole blood samples were collected from different groups of animals. In another study, the authors found a significant positive bias for midazolam when VAMS tips were intentionally submerged into blood past the shoulder [25]. In our study, we paid special attention to avoid overfilling of the tips. All VAMS samples were prepared by dipping the tip into a small volume of blood (± 400 µL) contained in 2 mL plastic tubes, enabling good visibility of the blood surface, by operators experienced in handling blood (micro)samples. Care was taken to prevent that VAMS tips were dipped too deeply into the blood samples.

The differences observed in our study may be attributed to a combination of factors. First, from an analytical point of view, the recovery of caffeine and paraxanthine from VAMS samples was reduced at higher Hct values, as discussed in section B.3.3.1. Data in Table B.3.4. show that the recovery of both compounds was lower starting from Hct 0.48, with highly comparable values at Hct 0.48 and 0.62. The latter also applies for the recovery data at the lower evaluated Hct levels (0.21 and 0.42). Importantly, the patient samples in our study were analyzed against calibrators prepared from blood with Hct 0.48. However, all but two patients samples had Hct values below 0.48, with 0.21 being the lowest Hct. Consequently, analyte concentrations in patient VAMS samples will have been consistently overestimated, albeit to a limited extent, due to the difference in recovery between calibrators and study samples. Hence, although VAMS results were not subject to a “traditional Hct effect”, with lower concentrations measured at low Hct levels and higher concentrations at high Hct values, as seen in DBS analysis, Hct did have an impact on the analysis of VAMS samples, by affecting the recovery. We consider it unlikely that other analytical factors contributed to the disparity between VAMS and whole blood concentrations. Hct had no effect on ion suppression or enhancement for VAMS samples. The different types of samples were analyzed against calibration curves prepared in the respective matrices. Accuracy values (%bias) for QCs of caffeine and paraxanthine in liquid whole blood (Chapter A.2., Table A.2.3.) and VAMS samples (Table B.3.2.) were within ± 5% (± 9% at LLOQ level). Furthermore, under the storage conditions used here, both compounds were stable in whole blood (Chapter A.2., Table A.2.6.) and VAMS samples (Table B.3.5.) for prolonged periods of time, exceeding the storage times the actual study samples were subject to.

From a methodological point of view, several additional factors may have played a role. First of all, the observed mean differences between VAMS and whole blood concentrations (12.3 and 15.4 % for caffeine and paraxanthine, respectively) are highly comparable to the mean differences found between plasma and whole blood concentrations (15.2 and 16.6 % for caffeine and paraxanthine, respectively (see Chapter A.3., Table A.3.2.)) [28]. This might lead one to hypothesize that VAMS tips might preferentially absorb the plasma component of blood and that the resulting VAMS concentrations would reflect plasma rather than whole blood concentrations. However, by comparing the hemoglobin content

of VAMS tips prepared using the standard dipping technique to that of tips to which 10 µL of blood was spotted, Denniff *et al.* demonstrated that there is no indication for such an effect [25]. Furthermore, the positive bias of VAMS vs. whole blood may result from the fact that measured whole blood concentrations underestimated the actual values, instead of VAMS concentrations overestimating whole blood concentrations. In this light, we verified whether whole blood samples were completely hemolyzed by the applied sample preparation procedure [29]. During validation of the whole blood method, QCs of caffeine and paraxanthine at four concentration levels, stored at -80 °C for 90 minutes and thawed at room temperature, were analyzed in duplicate on three different days along with freshly prepared QCs. As for dried VAMS samples, hemolysis was considered to be complete in frozen and thawed blood samples. The resulting caffeine and paraxanthine concentrations are summarized in Table B.3.6. No significant differences between caffeine and paraxanthine concentrations in fresh and hemolyzed blood were found; all differences were within ± 3%. These results exclude that potential incomplete hemolysis of blood samples may have contributed to the observed deviations.

Table B.3.6. Caffeine and paraxanthine concentrations in fresh and hemolyzed whole blood quality control (QC) samples at four concentration levels determined in duplicate on three non-consecutive days. Hemolyzed samples were stored at -80 °C for 90 minutes and thawed at room temperature. Percentage differences were calculated by dividing the difference between concentrations in fresh and hemolyzed blood by the concentration in fresh blood and multiplying the result by 100.

	QC	Fresh blood (µg/mL) mean ± SD [%RSD]	Hemolyzed blood (µg/mL) mean ± SD [%RSD]	Difference fresh-hemolyzed blood (%)
Caffeine	**LLOQ**	0.048 ± 0.007 [14.53]	0.049 ± 0.006 [12.80]	-2.28
	Low	0.12 ± 0.008 [6.87]	0.12 ± 0.007 [5.64]	-1.17
	Medium	3.96 ± 0.16 [4.14]	4.06 ± 0.20 [4.92]	-2.53
	High	7.68 ± 0.36 [4.67]	7.86 ± 0.63 [8.03]	-2.22
Paraxanthine	**LLOQ**	0.023 ± 0.003 [12.11]	0.024 ± 0.003 [12.65]	-2.86
	Low	0.057 ± 0.004 [7.69]	0.057 ± 0.004 [7.72]	0.66
	Medium	1.94 ± 0.11 [5.39]	1.94 ± 0.12 [6.22]	-0.15
	High	3.90 ± 0.30 [7.64]	3.86 ± 0.35 [9.12]	1.07

Interestingly, we observed an apparently differential behavior of VAMS samples prepared from incurred vs. spiked blood. Table B.3.7. shows caffeine and paraxanthine concentrations measured in VAMS and whole blood samples (n = 3) originating from either spiked blank blood or blood collected 6 h after the administration of a 150 mg caffeine dose. For both compounds, no significant differences were found between spiked whole blood and corresponding VAMS samples. Analyte concentrations in incurred blood, however, were higher when measured in VAMS compared to whole blood samples. Although differences were limited (6.86 and 6.35 % for caffeine and paraxanthine, respectively), these were statistically significant, as determined by independent samples t-tests ($p = 0.005$ and 0.008 for caffeine and paraxanthine, respectively). Therefore, we concluded that the latter effect, for which the underlying

reason is unknown, combined with the somewhat reduced recovery of caffeine and paraxanthine from VAMS samples at the Hct of the calibration line used for analysis of the study samples, was at the basis of the observed positive difference between VAMS and whole blood results.

Table B.3.7. Caffeine and paraxanthine concentrations in VAMS and whole blood samples (n = 3) originating from spiked and incurred blood. Blank EDTA blood from a healthy volunteer was spiked with caffeine and paraxanthine to obtain nominal concentrations of 1.0 and 0.8 µg/mL, respectively. Incurred blood was collected on the same day from the same volunteer 6 h after oral ingestion of a capsule containing 150 mg caffeine.

		VAMS (µg/mL) mean ± SD [%RSD]	Whole blood (µg/mL) mean ± SD; [%RSD]	Difference VAMS-whole blood (%)
Caffeine	**Spiked samples**	1.03 ± 0.01 [1.36]	1.01 ± 0.01 [1.31]	1.49
	Incurred samples	1.12 ± 0.01 [1.07]	1.04 ± 0.02 [1.74]	6.86*
Paraxanthine	**Spiked samples**	0.81 ± 0.01 [1.45]	0.81 ± 0.01 [0.62]	0.35
	Incurred samples	0.82 ± 0.02 [1.95]	0.77 ± 0.01 [0.88]	6.35*

B.3.4. Conclusion

VAMS, a novel sampling technique that allows to accurately collect a fixed volume of blood, represents a promising approach to overcome the effect of deviating Hct values in the analysis of dried blood microsamples. In this study, the potential of VAMS to effectively eliminate the Hct bias was evaluated by analyzing over 80 VAMS samples prepared from incurred whole blood collected from hospital patients displaying a wide Hct range (0.21 - 0.50). Therefore, an LC-MS/MS method for the determination of caffeine and paraxanthine in VAMS tips was fully validated. All evaluated parameters met the pre-established criteria. Analyte concentrations in VAMS samples were compared to corresponding DBS and whole blood concentrations. VAMS results were not affected by a bias that changed in function of Hct, as differences between caffeine and paraxanthine concentrations in VAMS and whole blood samples did not vary over the evaluated Hct range, in contrast to the observed DBS-blood differences. However, on the other hand, it should be noted that measured VAMS concentrations consistently overestimated whole blood concentrations. It was found that this effect was caused by a combination of a different behavior of VAMS samples prepared from incurred or spiked blood and a reduced recovery of the analytes from VAMS tips at high Hct values. Interestingly, in a previous study, the latter effect was not observed for DBS, although very similar procedures were used to extract caffeine and paraxanthine from DBS and VAMS samples. On the basis of this study, it can be concluded that VAMS indeed allows to overcome the Hct bias for caffeine and paraxanthine, although care should be taken when protocols developed for DBS analysis are transferred to VAMS samples. As this study is the first in which the usefulness of VAMS is evaluated based on results obtained from real incurred human samples, our findings should be compared to those for other compounds with varying characteristics, determined using validated procedures, in order to further accept VAMS as a reliable sampling technique in bioanalysis.

B.3.5. References

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Conclusions and future perspectives

In this work, several aspects of dried blood spots (DBS) and hair as alternative sampling strategies have been addressed, using caffeine and its major metabolite paraxanthine as model compounds.

In **Part A**, alternative procedures for CYP1A2 phenotyping were developed, validated and applied. **First**, it was investigated whether DBS sampling could provide an alternative for the traditional plasma-based approach. Therefore, a simple and fast procedure was developed to extract caffeine and paraxanthine from 3-mm DBS punches prior to analysis on an LC-MS/MS system. To allow comparison between DBS and classical samples, similar methods were developed to determine both compounds in 50 µL whole blood and plasma. Validation of these methods showed excellent method performance, as all evaluated parameters met the acceptance criteria defined in international guidelines. To examine the influence of hematocrit (Hct) on the quantitation of caffeine and paraxanthine in DBS, quality control (QC) samples prepared from blood with different hematocrit (Hct) levels were included in several validation experiments. While recovery, matrix effects and precision were unaffected, an effect of Hct on the accuracy was observed. Deviations were within acceptable limits (i.e. ± 15 %) for the applied 0.36 – 0.50 Hct range, which encloses the reference intervals for healthy, adult women and men. The actual value of DBS sampling for CYP1A2 phenotyping was assessed in a study involving 73 healthy volunteers. Caffeine and paraxanthine concentrations 6 h after the intake of a caffeine test dose were significantly lower in capillary DBS, prepared in a non-volumetric way, compared to corresponding concentrations in venous DBS and plasma, the latter being the reference matrix for CYP1A2 phenotyping. Additionally, concentrations of both compounds varied in function of the volume of blood spotted on the DBS cards and were influenced by strongly deviating Hct values. However, as the actual CYP1A2 phenotyping metric, the 6 h paraxanthine:caffeine ratio, in the different matrices showed an excellent agreement and was unaffected by blood volume or Hct, capillary DBS proved to be a valid alternative for plasma for CYP1A2 phenotyping. These findings, as well as those of studies evaluating other CYP450 isoforms, indicate that DBS sampling provides a reliable tool for phenotyping purposes. The characteristics of this technique, such as the minimal invasiveness, ease of sampling, limited sample volumes, improved compound stability and convenient transport, combined with the possibility of collecting samples in a cost-effective way by informed individuals themselves without the need for a phlebotomist, may aid to expand the application of CYP450 phenotyping to areas other than controlled research settings, such as routine clinical practice or even phenotyping in the patient's home environment. Therefore, our results for CYP1A2, and those for other enzymes, need to be confirmed in large population studies, allowing to define reference ranges for phenotyping metrics in DBS.

Second, the potential of hair analysis for CYP1A2 phenotyping was evaluated. A method for the determination of caffeine and paraxanthine in hair was developed by thoroughly optimizing every step of the extraction protocol and the subsequent clean-up procedure. The resulting method, involving a three-step decontamination procedure, proteolytic digestion of the hair matrix, analyte extraction in an aqueous buffer, clean-up of the hair extracts on Strata-X™ solid phase extraction (SPE) cartridges and, finally, LC-MS/MS analysis was fully validated. Also here, all parameters fulfilled the acceptance criteria. Analysis of wash solvents of 3-cm proximal hair segments collected from 6 volunteers suggested a limited external contamination of the hair samples with caffeine, rather than premature extraction of the analyte during the decontamination procedure. Using this procedure, we examined whether

paraxanthine:caffeine ratios in 3-cm proximal hair segments of 60 healthy volunteers reflected reference, plasma-based CYP1A2 phenotyping indices. Although ratios in hair and plasma showed a similar range and a statistically significant correlation, large deviations between ratios in both matrices were observed in some cases. The latter complicated the interpretation of hair ratios on an individual basis. The influence of various factors on paraxanthine:caffeine hair ratios and hair-plasma deviations was investigated, but none of these could explain the observed variability. Although metabolic ratios in hair have been proposed to study drug metabolism or assess the phenotype of drug metabolizing enzymes in a non-invasive way, our study is the first to evaluate this approach in a systematic way. Based on the results obtained here for CYP1A2 and the fact that incorporation of compounds into hair is a complex process influenced by many factors, the usefulness of metabolic ratios in hair for phenotyping purposes needs to be further evaluated on a case-by-case basis.

In **Part B**, two strategies to cope with the Hct effect in dried blood microsampling were presented. **First**, an algorithm was developed that allowed to correct for the Hct effect encountered in the analysis of caffeine and paraxanthine in DBS. This algorithm was based on caffeine concentrations in 50 corresponding DBS and whole blood samples with a wide Hct range (0.18 – 0.47) and on potassium DBS concentrations, as the latter proved to be a reliable Hct marker. Application of the algorithm to an independent set of samples with an identical Hct range alleviated the Hct bias for caffeine. The usefulness was further supported by the observation that similar results were obtained for paraxanthine, following application of the same, caffeine-based algorithm. Being a promising strategy to correct for the Hct bias in DBS analysis, this approach should be applied for more analytes with varying physicochemical characteristics and different plasma protein or blood cell binding profiles. Recently, also another group successfully used a modification of this strategy to correct DBS creatinine concentrations for Hct bias.

Second, the potential of a recently developed sampling technique, volumetric absorptive microsampling (VAMS), was evaluated. An LC-MS/MS method for the determination of caffeine and paraxanthine was successfully validated. The analytes were extracted from VAMS tips using a very similar procedure as developed for DBS analysis. By comparing caffeine and paraxanthine concentrations in over 80 corresponding VAMS and whole blood samples with Hct values ranging from 0.21 to 0.50, it was found that analyte concentrations in VAMS were not subject to a bias that changed over the evaluated Hct range. On the other hand, VAMS concentrations consistently –though slightly– overestimated whole blood concentrations. The latter could be attributed to the combined effect of a different behavior of VAMS samples prepared from spiked and incurred blood and a reduced recovery of caffeine and paraxanthine from VAMS devices at higher Hct values. Overall, VAMS effectively assisted in eliminating the effect of Hct for caffeine and paraxanthine. As it retains all benefits of DBS sampling and analysis, VAMS appears to be a promising new sampling technique. However, its applicability and ability to overcome the Hct effect needs to be further investigated for other analytes. In addition, it may be interesting to evaluate whether Hct has an effect on the recovery of these analytes, as observed here for caffeine and paraxanthine.

Summary

The aim of this work was twofold; first, we evaluated whether dried blood spots (DBS) and hair could be implemented as alternative sampling strategies in phenotyping procedures of the drug metabolizing enzyme CYP1A2; second, we explored two strategies to cope with a major issue in the analysis of dried blood microsamples, being the effect of hematocrit (Hct). Throughout this thesis, caffeine and its major metabolite paraxanthine were used as model compounds.

A general introduction to DBS and hair sampling and analysis is given in the **General Background** section. DBS are prepared by applying a drop of capillary blood on a filter paper card following a finger or heel prick. The resulting spots are dried to the air. This sampling technique offers several advantages over traditional blood sampling by venipuncture, such as minimal invasiveness, limited sample volume, improved compound stability and ease of sample collection, transport, storage and processing. Although DBS sampling and analysis has been increasingly applied in various fields in the last decade, this sampling techniques still faces several challenges. For example, being a capillary sampling technique, one has to be aware of the correlation between capillary and venous concentrations of the analyte of interest. In addition, specific DBS-related parameters, such as the influence of hematocrit, the volume of blood spotted onto the filter paper and the punch location, should be evaluated for every partial punch DBS method. Hair samples are obtained by cutting a strand of hair, typically with a diameter of a few mm, as close as possible to the scalp at the posterior vertex region of the head. As hair analysis provides an extended window of detection (up to several months) compared to traditional blood or urine sampling, this approach has been mainly applied for drugs of abuse testing in diverse contexts. The major challenge of hair analysis lies in the interpretation of hair concentrations, given the risk of false positive results due to external contamination or passive drug exposure. On the other hand, analytes may be lost from the hair by cosmetic hair treatment, giving rise to false negative results.

In vivo phenotyping of a drug metabolizing enzyme involves administration of a selective enzyme substrate, followed by the determination of a specific phenotyping metric in traditional matrices, such as plasma, serum or urine. In the first chapter of **Part A**, **Chapter A.1.**, an overview of CYP450 phenotyping procedures involving alternative sampling strategies is given. We discussed the role of DBS, hair, oral fluid, exhaled breath and sweat in this context, with a focus on the correlation between phenotyping metrics in alternative and standard matrices. Based on the available literature, we concluded that oral fluid, breath and DBS provide reliable matrices for phenotyping of several clinically relevant CYP450 enzymes. The utility of hair analysis for this purpose needs to be supported by more data.

In the following chapters, we evaluated the potential of DBS and hair for CYP1A2 phenotyping. **Chapter A.2.** describes the development and validation of LC-MS/MS-based methods for the determination of caffeine and paraxanthine in DBS, whole blood and plasma. Caffeine is the most widely applied probe drug for CYP1A2 phenotyping. As the biotransformation of caffeine to paraxanthine is solely mediated by CYP1A2, the paraxanthine:caffeine ratio in plasma, determined at a defined time point following intake of a caffeine test dose, is generally accepted as a valid measure of CYP1A2 activity. For all three

methods, validation parameters met the predefined acceptance criteria. By including quality control (QC) samples prepared from blood with different Hct values in the validation protocol of the DBS method, we found that Hct had an effect on the accuracy of caffeine and paraxanthine, although deviations stayed within acceptable limits over the 0.36 – 0.50 Hct interval. Other parameters, such as precision, recovery, matrix effect and stability, were not influenced by Hct. A small-scale CYP1A2 phenotyping study revealed a good agreement between paraxanthine:caffeine ratios in capillary DBS, venous DBS, whole blood and plasma. The developed methods and the preliminary CYP1A2 phenotyping results allowed to set up a larger phenotyping study in healthy volunteers in which the actual utility of DBS sampling for CYP1A2 phenotyping was evaluated. This study is described in **Chapter A.3.** Caffeine and paraxanthine concentrations were determined in non-volumetrically applied capillary DBS, venous DBS, whole blood and plasma -the latter being the reference matrix for CYP1A2 phenotyping- of 73 healthy volunteers. Samples were collected 6 h after the intake of a capsule containing 150 mg caffeine. As 3-mm DBS punches were analyzed, the influence of punch location, blood volume spotted on the filter paper and Hct was examined as well. Remarkably, capillary DBS concentrations of both compounds were significantly lower than those in venous DBS. Furthermore, DBS concentrations were affected by blood volume and strongly deviating Hct values. However, no significant differences were observed between the paraxanthine:caffeine ratio, i.e. the actual CYP1A2 phenotyping index, in the distinct evaluated matrices. This ratio also alleviated the impact of Hct and blood volume. Based on these results, we concluded that CYP1A2 phenotyping in capillary DBS provides a valid and convenient alternative for the plasma-based approach.

In **Chapter A.4.**, we describe the optimization of a procedure for the determination of caffeine and paraxanthine in hair. The final procedure consisted of a decontamination step, proteolytic digestion of the hair matrix, analyte extraction in an aqueous buffer and clean-up of the hair extracts on Strata-X™ solid phase extraction (SPE) cartridges. The resulting hair extracts were analyzed using LC-MS/MS. This procedure was fully validated, thereby fulfilling all acceptance criteria. Using this procedure, we evaluated the potential of hair analysis for CYP1A2 phenotyping. Given the ubiquity of caffeinated drinks and food products, a large part of the population was expected to be positive for caffeine in hair, eliminating the need to administer a caffeine test dose. To investigate whether hair analysis could be useful for CYP1A2 phenotyping, paraxanthine:caffeine ratios determined in 3-cm proximal hair segments of 60 healthy volunteers were compared with reference ratios in plasma. As described in **Chapter A.5.**, ratios in hair and plasma were significantly correlated. However, considerable deviations between ratios in both matrices in some cases impeded the interpretation of hair results on an individual basis. Investigation of the influence of several factors on paraxanthine:caffeine ratios and hair-plasma differences did not provided an explanation for the observed variability. Although we concluded that hair analysis is not (yet) suited for CYP1A2 phenotyping, this study, being the first to evaluate the usefulness of hair analysis for phenotyping purposes in a systematic way, may provide valuable information concerning the role of hair analysis in this context.

In **Part B**, we addressed the Hct effect, which is considered as the most important factor limiting a more widespread use of quantitative DBS analysis. An overview of the different aspects of this Hct effect is given in **Chapter B.1.** As Hct is directly related with blood viscosity, it determines the spreading of blood on filter paper. A drop of blood with high Hct will spread to a lesser extent, resulting in spots with a smaller diameter. Consequently, a fixed-size sub-punch taken from a high Hct DBS will contain a higher amount of blood (and analyte) than a punch taken from a low Hct DBS. This may result in an overestimation of analyte concentrations at high Hct levels and an underestimation at low Hct levels. This effect may in some cases be counteracted by a reduced analyte recovery from high Hct DBS. In addition, Hct is one of the main factors determining the blood-to-plasma ratio of a given compound. In Chapter B.1., we also discussed available strategies to overcome the effect of Hct on differential spreading of blood.

Two new strategies to cope with the Hct effect were examined in the following chapters. Caffeine and paraxanthine were used as model compounds, as we previously observed (Chapters A.2. and A.3.) that caffeine and paraxanthine were subject to a Hct-dependent bias. In **Chapter B.2.**, we present an approach to correct for the Hct bias, using an algorithm based on the potassium concentration in DBS, serving as Hct marker. This algorithm was constructed using 50 corresponding DBS and whole blood samples from healthy volunteers and hospital patients with a wide Hct range (0.18 – 0.47). Application of the algorithm on caffeine concentrations measured in an independent set of samples (n = 50) alleviated the Hct bias, especially at lower Hct values. Similar results were obtained when the same algorithm, designed for caffeine, was applied to paraxanthine concentrations determined in 103 samples with a similar Hct range (0.17 – 0.47). In the last chapter, **Chapter B.3.**, we describe a study in which a recently developed sampling technique, volumetric absorptive microsampling (VAMS), was applied Using this technique, an accurate volume of blood is collected by dipping an absorbent polymeric tip, attached to a plastic handle, into a pool or drop of blood. Subsequently, the obtained blood microsample is dried and analyzed as a whole. To evaluate the potential of VAMS to overcome the Hct effect, caffeine and paraxanthine concentrations were determined in over 80 corresponding VAMS, DBS and whole blood samples originating from hospital patients with a wide Hct range (0.21 – 0.50). Therefore, an LC-MS/MS method was fully validated and similar conditions as for DBS were used to extract the analytes from VAMS tips. In contrast to DBS results, VAMS concentrations were not affected by a bias that changed over the Hct range. However, VAMS concentrations tended to slightly overestimate reference whole blood concentrations, as a consistent positive bias was observed. The latter was caused by a combination of a reduced recovery of caffeine and paraxanthine from VAMS tips at high Hct levels and a different behavior of VAMS samples prepared from spiked and incurred blood. Based on these results, we concluded that VAMS represents a promising new sampling technique, as it assisted in eliminating the effect of Hct and holds the same benefits associated with DBS sampling and analysis.

Samenvatting

Het doel van dit werk was tweeledig; enerzijds evalueerden we of gedroogde bloedspots (*dried blood spots*, DBS) en haar kunnen geïmplementeerd worden als alternatieve staalnametechnieken in procedures voor de fenotypering van het enzym CYP1A2; anderzijds onderzochten we twee strategieën die een oplossing kunnen bieden voor een belangrijk probleem in de analyse van DBS, nl. het effect van hematocriet (Hct). Doorheen deze thesis werden cafeïne en zijn belangrijkste afbraakproduct paraxanthine gebruikt als modelcomponenten.

Algemene informatie rond het verzamelen en analyseren van DBS en haarstalen wordt gegeven in de ***General Background*** sectie. DBS worden bereid door een druppel capillair bloed aan te brengen op een filterpapier na een vinger- of hielprik. De verkregen spots worden gedroogd aan de lucht. Deze staalnametechniek biedt verschillende voordelen t.o.v. traditionele bloedafname, zoals minimale invasiviteit, een beperkt staalvolume, verbeterde stabiliteit van bepaalde verbindingen en eenvoudige transport, opslag en verwerking van de stalen. Hoewel DBS analyse gedurende de voorbije jaren in toenemende mate werd toegepast in verscheidene disciplines, bestaan er nog enkele belangrijke uitdagingen. Aangezien dit een capillaire techniek betreft, is het bv. belangrijk om de correlatie tussen capillaire en veneuze concentraties van een bepaalde component te kennen. Daarnaast is het aangewezen om, voor elke methode die gebruik maakt van schijfjes genomen uit een DBS, de invloed van specifieke parameters, zoals de invloed van Hct, het bloed volume dat aangebracht wordt op het filterpapier en de plaats waar het schijfje genomen wordt, te evalueren.

Een haarstaal wordt verkregen door een haarbundel met een diameter van enkele mm zo dicht mogelijk bij de hoofdhuid af te knippen ter hoogte van de posterior vertex regio achteraan het hoofd. Een belangrijk voordeel van haaranalyse is het feit dat de techniek een veel breder detectievenster biedt in vergelijking met traditionele stalen, zoals bloed of urine. Om deze reden wordt haaranalyse voornamelijk toegepast voor het retrospectief opsporen van (illegale) drugs. De interpretatie van de verkregen concentraties vormt de belangrijkste uitdaging van haaranalyse. Zo is er altijd een risico op vals positieve resultaten als gevolg van externe contaminatie met of passieve blootstelling aan de drug die opgespoord wordt. Anderzijds kunnen bepaalde verbindingen ook verwijderd worden uit de haarmatrix na cosmetische behandeling van het haar, hetgeen aanleiding kan geven tot vals negatieve resultaten.

Voor de *in vivo* fenotypering van een enzym wordt een selectief substraat van dit enzym toegediend, waarna een specifieke parameter bepaald wordt in een traditionele matrix, zoals plasma, serum of urine. In het eerste hoofdstuk van ***Part A, Chapter A.1.***, wordt een overzicht gegeven van CYP450 fenotyperingsprocedures waarbij gebruik wordt gemaakt van alternatieve staalnametechnieken. De rol van DBS, haar, speeksel, adem en zweet wordt besproken, met nadruk op de correlatie tussen fenotyperingsparameters in alternatieve en klassieke stalen. Op basis van de beschikbare literatuur besloten we dat speeksel, adem en DBS betrouwbare matrices zijn voor fenotypering van verschillende klinisch relevante CYP450 enzymen. Het nut van haaranalyse in deze context moet verder onderzocht worden in bijkomende studies.

In de volgende hoofstukken evalueerden we het potentieel van DBS en haar voor de fenotypering van CYP1A2. ***Chapter A.2.*** beschrijft de ontwikkeling en validatie van LC-MS/MS methoden voor de bepaling van cafeïne en paraxanthine in DBS, vol bloed en plasma. Cafeïne is de meest gebruikte testcomponent voor CYP1A2 fenotypering. Aangezien de omzetting van cafeïne naar paraxanthine volledig gecontroleerd wordt door CYP1A2, wordt de paraxanthine:cafeïne ratio in plasma, gemeten op een welbepaald tijdspunt na de inname van een cafeïne dosis, algemeen aanvaard als een maat voor de activiteit van CYP1A2. Voor elk van de drie methoden voldeden de validatieparameters aan de vooropgestelde aanvaardingscriteria. Door het includeren van kwaliteitscontrolestalen in het validatieprotocol van de DBS methode stelden we vast dat de accuraatheid van de bepaling van cafeïne en paraxanthine beïnvloed wordt door het Hct. De afwijkingen bleven echter binnen aanvaardbare grenzen in het toegepaste Hct-interval (0.36 – 0.50). Andere parameters, zoals precisie, terugvinding, matrix effect en stabiliteit, werden niet beïnvloed door het Hct. Op basis van een kleinschalige CYP1A2 fenotyperingsstudie vonden we een goede overeenkomst tussen paraxanthine:cafeïne ratio's in capillaire DBS, veneuze DBS, vol bloed en plasma. De ontwikkelde methoden en de preliminaire resultaten voor CYP1A2 fenotypering dienden als basis om een grotere fenotyperingsstudie op te zetten. Aan de hand van deze studie, die beschreven is in ***Chapter A.3.***, werd de effectieve bruikbaarheid van DBS voor CYP1A2 fenotypering geëvalueerd. Cafeïne en paraxanthine concentraties werden bepaald in capillaire DBS, veneuze DBS, vol bloed en plasma -deze laatste als referentiematrix voor CYP1A2 fenotypering- van 73 gezonde vrijwilligers. De stalen werden verzameld 6 u na de inname van een capsule die 150 mg cafeïne bevat. Aangezien DBS schijfjes met een diameter van 3 mm geanalyseerd werden, werd de invloed van de plaats waar deze schijfjes genomen worden, het bloed volume en het Hct geëvalueerd. Het was opmerkelijk dat de capillaire DBS concentraties van beide componenten lager bleken te zijn dan de concentraties in veneuze DBS en plasma. Bovendien werden de DBS concentraties beïnvloed door het bloedvolume en sterk afwijkende Hct waarden. De paraxanthine:cafeïne ratio's, de eigenlijke CYP1A2 fenotyperingsparameter, in de verschillende matrices waren echter niet verschillend. Verder werden deze ratio's ook niet beïnvloed door Hct of bloedvolume. Op basis van deze resultaten besloten we dat CYP1A2 fenotypering in capillaire DBS een geschikt alternatief biedt voor de klassieke procedure in plasma.

In ***Chapter A.4.*** beschrijven we de optimalisatie van een procedure voor de bepaling van cafeïne en paraxanthine in haar. De finale procedure omvat een decontaminatiestap, enzymatische afbraak van de haarmatrix, extractie van de componenten in een waterige buffer en opzuivering van de haarextracten m.b.v. vaste fase extractie op Strata-X™ kolommen. De verkregen haarextracten worden geanalyseerd met LC-MS/MS. De procedure werd volledig gevalideerd, waarbij voldaan werd aan alle aanvaardingscriteria. Met behulp van deze procedure evalueerden we het potentieel van haaranalyse voor CYP1A2 fenotypering. Rekening houdend met de alomtegenwoordigheid van cafeïne-bevattende dranken en voedingsproducten, verwachtten we dat haarstalen van een overgroot deel van de populatie cafeïne bevatten. Hierdoor is het niet langer nodig om een cafeïne dosis toe te dienen. Om na te gaan of haaranalyse kan gebruikt worden voor CYP1A2 fenotypering werden paraxanthine:caffeine ratio's bepaald in proximale haarsegmenten (3 cm) van 60 gezonde vrijwilligers. Deze ratio's werden vervolgens vergeleken met referentieratio's in plasma. Deze studie is beschreven in ***Chapter A.5.***

Hoewel de ratio's in haar en plasma een statistisch significante correlatie vertoonden, werd de interpretatie van de ratio's in haar in bepaalde gevallen bemoeilijkt door sterke afwijkingen tussen ratio's in beide matrices. De invloed van verschillende factoren op de paraxanthine:cafeïne ratio's en op de afwijkingen tussen haar en plasma werd onderzocht. Dit leverde echter geen evidente verklaring op voor de variabiliteit. Hoewel we besloten dat haaranalyse (voorlopig) niet geschikt blijkt te zijn voor CYP1A2 fenotypering, kan deze studie nuttige informatie opleveren betreffende de rol van haaranalyse in deze context, aangezien het de eerste studie is waarin de bruikbaarheid van haaranalyse voor fenotypering op een systematische wijze werd geëvalueerd.

Aangezien het Hct effect beschouwd wordt als de belangrijkste factor die een bredere toepassing van kwantitatieve DBS analyse vertraagt, wordt dit verder behandeld in ***Part B***. In ***Chapter B.1.*** wordt een overzicht gegeven van de verschillende aspecten van dit Hct effect. Aangezien Hct de viscositeit van bloed bepaalt, beïnvloedt het ook de spreiding van bloed op filterpapier. Een bloeddruppel met een hoog Hct zal bijgevolg in beperktere mate uitspreiden, hetgeen aanleiding geeft tot de vorming van spots met een kleinere diameter. Een schijfje dat genomen wordt van een DBS met hoog Hct zal dus meer bloed bevatten dan een gelijkaardig schijfje genomen uit een DBS met laag Hct. Dit kan resulteren in een overschatting van concentraties bij hoog Hct en een onderschatting bij laag Hct. Dit effect kan in bepaalde gevallen tegengewerkt worden door een verminderde terugvinding bij hoog Hct. Daarnaast is Hct ook een van de belangrijkste factoren die de bloed-plasma ratio van een bepaalde component bepaalt. In ***Chapter B.1.*** bespreken we eveneens beschikbare strategieën die een oplossing kunnen bieden voor het Hct effect.

In de volgende hoofdstukken werden twee nieuwe strategieën onderzocht. Aangezien we voorheen vaststelden dat cafeïne en paraxanthine onderhevig zijn aan een Hct effect, werden deze componenten gebruikt als modelcomponenten. In ***Chapter B.2.*** stellen we een methode voor die toelaat om te corrigeren voor het Hct effect. Deze methode maakt gebruik van een algoritme dat gebaseerd is op een merker voor Hct, nl. het kalium gehalte in DBS. Dit algoritme werd opgebouwd a.d.h.v. cafeïne concentraties gemeten in 50 DBS en bloedstalen afkomstig van gezonde vrijwilligers en ziekenhuispatiënten met een breed Hct interval (0.18 – 0.47). Toepassing van het ontwikkelde algoritme op cafeïne concentraties in 50 onafhankelijke stalen bevestigde de bruikbaarheid van deze aanpak om te corrigeren voor het Hct effect. Gelijkaardige resultaten werden verkregen wanneer hetzelfde algoritme toegepast werd op paraxanthine, bepaald in 103 stalen met vergelijkbare Hct waarden (0.17 – 0.47). In het laatste hoofdstuk, ***Chapter B.3.***, beschrijven we een studie waarin een recent ontwikkelde staalnametechniek, nl. *volumetric absorptive microsampling* (VAMS), werd onderzocht. Met deze techniek wordt een welbepaald bloedvolume opgenomen door een absorberende tip, bestaande uit een polymeer, die in contact gebracht wordt met een bloeddruppel. Het verkregen staal wordt vervolgens gedroogd en in zijn geheel geanalyseerd. On na te gaan of VAMS het Hct effect kan elimineren, werden cafeïne en paraxanthine concentraties bepaald in meer dan 80 overeenkomstige VAMS, DBS en volbloed stalen met een breed Hct interval (0.20 – 0.50). Deze stalen waren afkomstig van ziekenhuispatiënten. Hiervoor werd eerst een LC-MS/MS methode gevalideerd. Om de componenten

te extraheren uit VAMS tips werden gelijkaardige condities gebruikt als voor DBS. VAMS concentraties waren niet onderhevig aan een bias die veranderde over het geëvalueerde Hct interval, dit in tegenstelling tot de resultaten voor DBS. Anderzijds bleken de VAMS concentraties de concentraties in vol bloed, hier gebruikt als referentie, in beperkte mate te overschatten. Dit laatste effect kon toegewezen worden aan een combinatie van een gedaalde terugvinding van cafeïne en paraxanthine bij hoge Hct waarden en een schijnbaar verschillend gedrag van VAMS stalen die bereid werden uit aangerijkt bloed en bloed die de componenten bevatte als gevolg van een inname. Op basis van deze resultaten besloten we dat VAMS een veelbelovende nieuwe staalnametechniek is, aangezien deze aanpak effectief bijdraagt tot het elimineren van het Hct effect terwijl alle voordelen van DBS analyse behouden blijven.

Curriculum Vitae

Personalia

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2009-2010: Master in Industrial Pharmacy, Ghent University (with distinction).

2007-2009: Master in Drug Development/Pharmacist, Ghent University (with distinction).

2004-2007: Bachelor in Pharmaceutical Sciences, Ghent University (with distinction).

1998-2004: Mathematics-Sciences, College O.-L.-V.-ten-Doorn, Eeklo

Work experience

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2010-2015: PhD-student in the Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University (Prof. Christophe Stove & Prof. Willy Lambert).

Educational experience

2010-2015: - Support of practical courses Toxicology, Master in Pharmaceutical Care and Drug Development.

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2013-2014: Laura De Meester. CYP1A2 fenotypering in haarstalen en aantonen van coagulatie in gedroogde bloedspots.

2012-2013: Jeroen Lievens. Ontwikkeling en validatie van een UHPLC-MS/MS methode voor de bepaling van cafeïne en paraxanthine in bloed en plasma.

2011-2012: Tom Devriendt. Ontwikkeling en validatie van een UHPLC-MS/MS methode voor de bepaling van cafeïne en paraxanthine in haar.

2010-2011: Nariman Esteki. Ontwikkeling van een UHPLC-MS/MS methode en optimalisatie van de staalvoorbereiding voor de bepaling van cafeïne en paraxanthine in haar.

2013-2014: Labinstructor and tutor, Pharmaceutical Bachelor dissertation.

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Scientific curriculum

A1-publications (status 22/06/2015 – ISI Web of Science™)

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1. **De Kesel PM**, Lambert WE, Stove CP. Alternative sampling strategies for cytochrome P450 phenotyping. Clin Pharmacokinet (in revision).
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Book chapters (B2)

1. Ingels AS, Sadones N, **De Kesel PM**, Lambert WE, Stove CP. Derivatization techniques in dried blood spot analysis. In: Li W & Lee M, editors. Dried Blood Spots: Applications and Techniques. John Wiley & Sons, 2014.

2. **De Kesel PM**, Lambert WE, Stove CP. The role of (bio)markers in hair analysis. In: Stove CP, editor. New sampling strategies in toxicology and therapeutic drug monitoring. Future Science, in press.

Oral presentations

1. 16th Forum of Pharmaceutical Sciences of the Belgian Society of Pharmaceutical Sciences (BGFW), Blankenberge, Belgium, May 7-8, 2012. Development and validation of an LC-MS/MS determination of caffeine and paraxanthine in human hair to assess CYP1A2 activity (De Kesel PM, Lambert WE, Stove CP).

2. AB SCIEX Clinical Research and Toxicology Workshop, Ghent, Belgium, January 25, 2013. Alternative approaches for CYP1A2 phenotyping (De Kesel PM, Lambert WE, Stove CP).

3. Seminar on Forensic Toxicology, Leuven, Belgium, May 24, 2013. Alternative approaches for CYP1A2 phenotyping (De Kesel PM, Lambert WE, Stove CP).

4. 13th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology (IATDMCT), Salt Lake City, USA, September 22-26, 2013. Dried blood spots as an alternative sampling strategy for CYP1A2 phenotyping (De Kesel PM, Lambert WE, Stove CP).

5. Analytical, Clinical and Forensic Toxicology Meeting, Bordeaux, France, June 10-13, 2014. CYP1A2 penotyping by measuring paraxanthine/caffeine concentration ratios in hair and comparison with the plasma-based phenotype (De Kesel PM, Lambert WE, Stove CP).

6. 1st European Congress of Mass Spectrometry: Applications to the Clinical Lab (MSACL), Salzburg, Austria, September 2-5, 2014. CYP1A2 penotyping by measuring paraxanthine/caffeine concentration ratios in hair and comparison with the plasma-based phenotype (De Kesel PM, Lambert WE, Stove CP).

7. Alternative Sampling Strategies in Toxicology and Therapeutic Drug Monitoring, Ghent, Belgium, September 18-19, 2014. CYP1A2 penotyping by measuring paraxanthine/caffeine concentration ratios in hair and comparison with the plasma-based phenotype (De Kesel PM, Lambert WE, Stove CP).

8. 7th Open Meeting of the European Bioanalysis Forum, Barcelona, Spain, November 18-21, 2014. Potassium-based algorithm allows correction for the hematocrit bias in quantitative analysis of caffeine and its major metabolite in dried blood spots **(**De Kesel PM, Capiau S, Stove VV, Lambert WE, Stove CP).

Poster presentations

1. 50th Annual Meeting of the International Association of Forensic Toxicologists (TIAFT), Hamamatsu, Japan, June 3–8, 2012. Caffeine and paraxanthine in human hair to asses CYP1A2 activity: development and validation of an LC-MS/MS-based procedure (De Kesel PM, Lambert WE, Stove CP. Presenting author: Stove CP).

2. 17th Meeting of the Society of Hair Testing (SOHT), Toronto, Canada, June 25–28, 2012. Caffeine and paraxanthine in human hair to asses CYP1A2 activity: development and validation of an LC-MS/MS-based procedure (De Kesel PM, Lambert WE, Stove CP).

3. Annual Meeting of the Belgische Vereniging voor Klinische Biologie (BVKB), Brussels, Belgium, October 5, 2013. Overcoming all issues associated with dried blood spot sampling? The case of CYP1A2 phenotyping (De Kesel PM, Lambert WE, Stove CP).

4. 6th Open Meeting of the European Bioanalysis Forum, Barcelona, Spain, November 20–22, 2013. Overcoming all issues associated with dried blood spot sampling? The case of CYP1A2 phenotyping (De Kesel PM, Lambert WE, Stove CP. Presenting author: Stove CP).

5. Knowledge for Growth, Ghent, Belgium, May 8, 2014. Challenges & promises of dried blood spots in bioanalysis: the case of CYP1A2 phenotyping. (De Kesel PM, Lambert WE, Stove CP. Presenting author: Stove CP).

Scientific prizes

2nd Poster prize at the 6th Open Meeting of the European Bioanalysis Forum, Barcelona, Spain, 20–22 November, 2013.

Best Oral Presentation at the Analytical, Clinical and Forensic Toxicology Meeting, Bordeaux, France, June 10-13, 2014.

Memberships of scientific organizations

International Association for Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT)
Society of Hair Testing (SoHT)
Toxicological Society of Belgium and Luxemburg (BLT)
Belgisch Genootschap voor Farmaceutische Wetenschappen (BGFW)