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Dried Blood Spots in Toxicology – Towards New Insights and New Applications

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Dankwoord

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Table of contents

List of abbreviations

AAS	Atomic absorption spectroscopy
ALP	Allowable limit of performance
AMC	Academic Medical Center
AUC	Area under the curve
CDC	Center for disease control and prevention
CDT	Carbohydrate-deficient transferrin
CF	Cystic fibrosis
CI	Confidence interval
CLSI	Clinical and laboratory standards institute
CSF	Cerebrospinal fluid
CV	Coefficient of variation
CV_a	Analytical coefficient of variation
CYP	Cytochrome P
DBS	Dried blood spot
DMS	Dried matrix spot
DPS	Dried plasma spot
DRUID	Driving under the influence of drugs, alcohol and medicines
EBC	Exhaled breath condensate
EBF	European bioanalysis forum
EDTA	Ethylenediaminetetraacetic acid
EESI	Extractive electrospray ionization
EMA	European medicines agency
EQC	External quality control
EVF	Erythrocyte volume fraction
FA	Formic acid
FDA	Food and drug administration
GC-MS	Gas chromatography coupled to mass spectroscopy
GFAAS	Graphite furnace atomic absorption spectroscopy
GH	Growth hormone
Hb	Hemoglobin
HbF	Fetal hemoglobin
HC	Hemichrome
HT	Hematocrit
HI	Hemolytic index

HRMS	High resolution mass spectrometry
I(λ)	The light intensity reflected by a DBS
$I_{white}(\lambda)$	The light intensity reflected by the white reference
IATDMCT	International Association of Therapeutic Drug Monitoring and Toxicology
ICP-MS	Inductively coupled plasma coupled to mass spectrometry
IGF-1	Insulin-like growth factor 1
II	Icteric index
IS	Internal standard
ISE	Ion-selective electrode
ISF	Interstitial fluid
ISR	Incurred sample reanalysis
K_2EDTA	Ethylenediaminetetraacetic acid dipotassium
LA-ICP-MS	Laser ablation inductively coupled plasma mass spectrometry
LAKC	Laboratory of General Clinical Chemistry
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LI	Lipemic index
LLOQ	Lower limit of quantitation
LoAs	Limits of agreement
MAPE	Median absolute percentage predictive error
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
ME	Matrix effect
MeOH	Methanol
MetHb	Methemoglobin
MIC	Minimal inhibitory concentration
MilliQ water	Ultrapure water
MoM	Metal-on-metal
MPPE	Median percentage predictive error
MS	Mass spectrometry
NBS	Newborn screening
NIR	Near infrared
OF	Oral fluid
OxyHb	Oxyhemoglobin

PBS	Phosphate buffered saline
PCV	Packed cell volume
PEth	Phosphatidylethanol
PK	Pharmacokinetic
POC	Point of care
PT	Proficiency testing
QC	Quality control
R(λ)	Wavelength-dependent reflectance values
RBC	Red blood cell
RCPA	Royal College of Pathologists of Australasia
RE	Relative error
RE	Recovery
RM	Reference material
RSD	Relative standard deviation
RT	Room temperature
SD	Standard deviation
SF-ICP-MS	Sector field inductively coupled plasma mass spectrometry
SLS	Sodium lauryl sulphate
SoHT	Society of hair testing
SPE	Solid phase extraction
T4	Thyroxine
TB	Tuberculosis
TDM	Therapeutic drug monitoring
TLC	Thin layer chromatography
T_x	Time point at time x
ULOQ	Upper limit of quantitation
UV	Ultraviolet
VAMS	Volumetric absorptive microsampling
VAPD	Volumetric absorptive paper disc
VPA	Valproic acid

General background, structure and objectives

Based on:

Capiau S, Alffenaar J-WC, Stove CP. Alternative sampling strategies for therapeutic drug monitoring. Chapter in Clinical challenges in therapeutic drug monitoring: special populations, physiological conditions and pharmacogenomics. Edited by William Clarke and Amitava Dasgupta. Elsevier, The Netherlands.

Velghe S*, Capiau S*, Stove CP. Opening the toolbox of alternative sampling strategies in clinical routine: a key-role for (LC-)MS/MS. TrAC, 2016, 84: 61-73. (*Equally contributed)

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1. GENERAL BACKGROUND

Alternative sampling strategies include the collection of 'traditional' samples (blood, plasma, serum or urine) in an alternative way, as well as the collection of 'alternative' sample matrices[1]. A typical example of the former is the collection of dried blood spots (DBS) (i.e. the collection of blood in an unconventional manner), while examples of the latter include sampling of oral fluid (OF), hair, interstitial fluid (ISF), tears, exhaled breath condensate (EBC), sweat, nasal mucus, meconium, etc. Both the 'alternative sampling' and the 'alternative samples' offer interesting opportunities for clinical applications, as they do not only imply easier sample collection (particularly in special patient populations such as small children and neonates), but can also provide information that is impossible or hard to obtain using traditional sampling strategies such as venipuncture and urine collection. Moreover, the use of alternative sampling strategies is often coupled to matrix-specific advantages such as a broader detection window, increased analyte stability or the possibility of home sampling[1].

In this doctoral thesis the focus will be on blood-based sampling strategies. Indeed, blood remains the matrix of interest for most applications, as the analyte concentration in this compartment typically correlates with the (physiological) effect of the analyte.

1.1. Dried blood spots

DBS sampling is the collection of a drop of blood, derived from a finger or heel prick, onto a filter paper (card). The drop of blood can be collected either by allowing it to fall freely onto the card or by carefully letting the edge of the blood drop -when still attached to the finger- touch the filter paper (see Figure 1 and Chapter 2 section 2.2.1). Importantly, there should never be direct contact between the finger and the collection substrate[2].

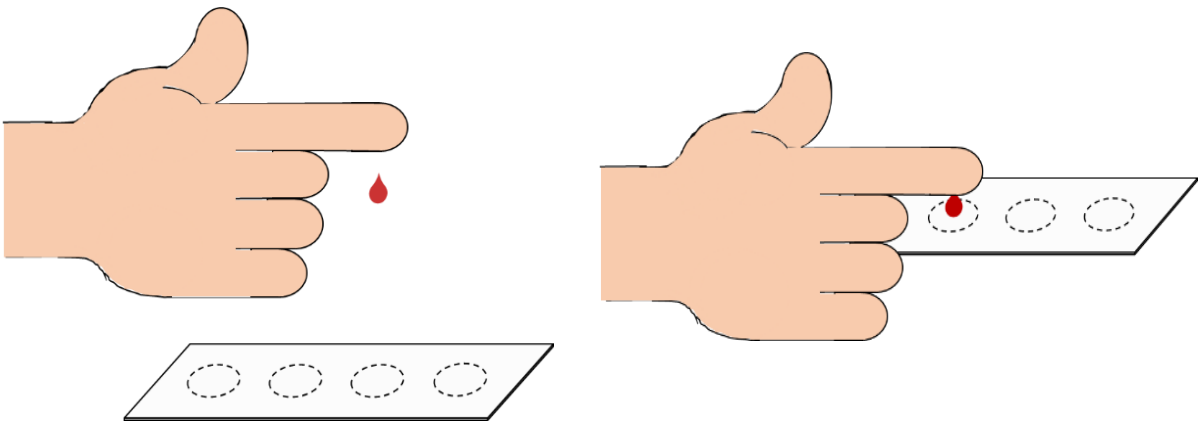

Figure 1: 'Falling drop' collection *vs*. 'hanging drop' collection

After collection, the samples are dried under ambient conditions and stored with silica in a zip-locked bag[3]. These samples can either be analyzed immediately (when collected at the site of analysis) or can be easily sent *via* regular mail to the laboratory for analysis. DBS analysis typically entails a punching step (i.e. the removal of a fixed-sized disk from the DBS), followed by extraction of the DBS punch. This extraction is generally performed with a mixture of organic and aqueous solvents (e.g. MeOH:H_20 80:20 v/v). Similar to traditional samples, additional sample preparation steps such as analyte derivatization, solid phase extraction (SPE), etc. may be used as well, if required[4,5]. Since generally only minute amounts of sample are available and/or low analyte levels may be present, sensitive analytical techniques are required. Although liquid chromatography tandem mass spectrometry (LC-MS/MS) is often the technique of choice when it comes to combining sufficient sensitivity with utmost selectivity, other techniques such as gas chromatography mass spectrometry (GC-MS), liquid chromatography with ultraviolet detection (LC-UV), inductively coupled plasma mass spectrometry (ICP-MS) or immuno-assays have been used for DBS analysis as well[4-8].

DBS sampling and analysis was introduced over a century ago by Ivar Bang for the determination of blood glucose levels[9]. However, its most successful application to date dates back to the 1960's when Dr. Robert Guthrie introduced DBS for the detection of phenylketonuria[10]. Since then, DBS sampling has played a pivotal role in newborn screening (NBS) programs for inborn errors of metabolism worldwide[11]. A major advantage of DBS sampling in this context is that it only requires a very small volume of blood, which can be obtained in a minimally invasive way (i.e. *via* a heel prick). With the arrival of more sensitive techniques the interest in this sampling strategy increased in other fields as well, where lower detection limits and quantitative rather than semi-quantitative analyses are required (see Figure 2).

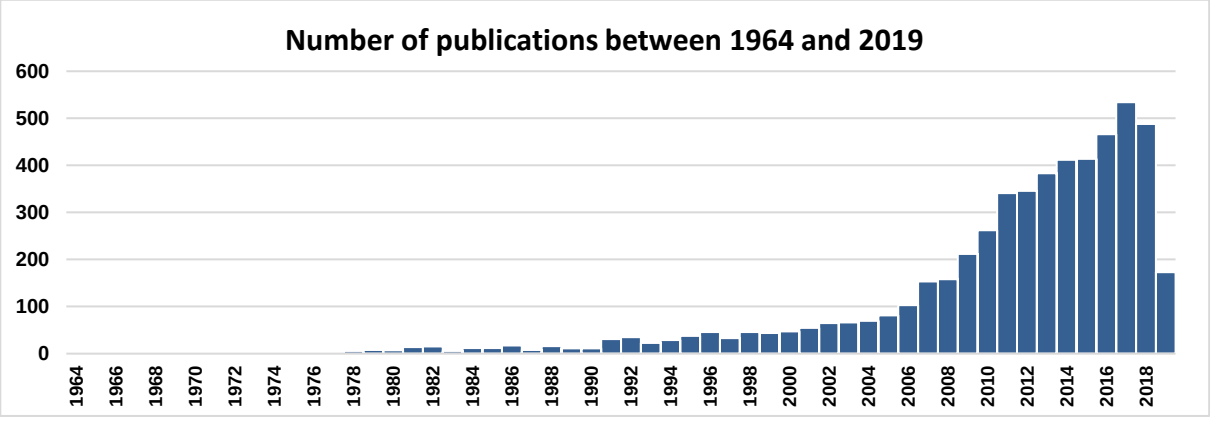

Figure 2: Number of publications regarding DBS between January 1964 and April 2019, as recorded by Web of Science.

This interest is the result of the many advantages that are associated with the sampling technique. As said before, it only requires a small sample volume and samples can be obtained in a minimally invasive way. Furthermore, the sampling procedure is relatively easy and can therefore be performed by the patient himself or by his caregiver, depending on the context[3]. This opens up a lot of opportunities, since samples can basically be collected at any time or place. This implies samples can be collected 'in the field' (e.g. in remote or resource-limited settings[12,13]) or at specific time points (e.g. after the occurrence of an epileptic episode[14]). Moreover, compared to liquid blood sampling, DBS display an increased *in vitro* stability for most analytes and they represent a reduced biohazard. In addition, sample transport and storage are convenient since there is no need for a cold chain.

DBS applications are very diverse[5] and include the analysis of (trace-)elements[15,16], small molecules[3,17], proteins[18,19], RNA[20] and DNA[21]. Fields which have shown interest in DBS sampling and analysis encompass NBS[11], screening programs for infectious diseases (e.g. HIV)[20], therapeutic drug monitoring (TDM)[3,22], toxicology[17,23], phenotyping[24,25], endocrinology[26,27], (pre-)clinical studies[28], proteomics[18] and metabolomics[29]. This diversity in applications can to some extent also be seen in Figure 3, which depicts the 20 research areas in which most DBS-related articles are published (i.a. pharmacology, endocrinology, immunology, tropical medicine, toxicology and environmental health). This map was generated by Web of Science using 'dried blood spots' as search term. Moreover, the versatility of DBS is also partly reflected in this doctoral thesis, as it includes methods for the determination of potassium, cobalt, hemoglobin (Hb), caffeine and paraxanthine using techniques such as LC-MS/MS, ICP-MS, indirect potentiometry and non-contact diffuse reflectance spectroscopy.

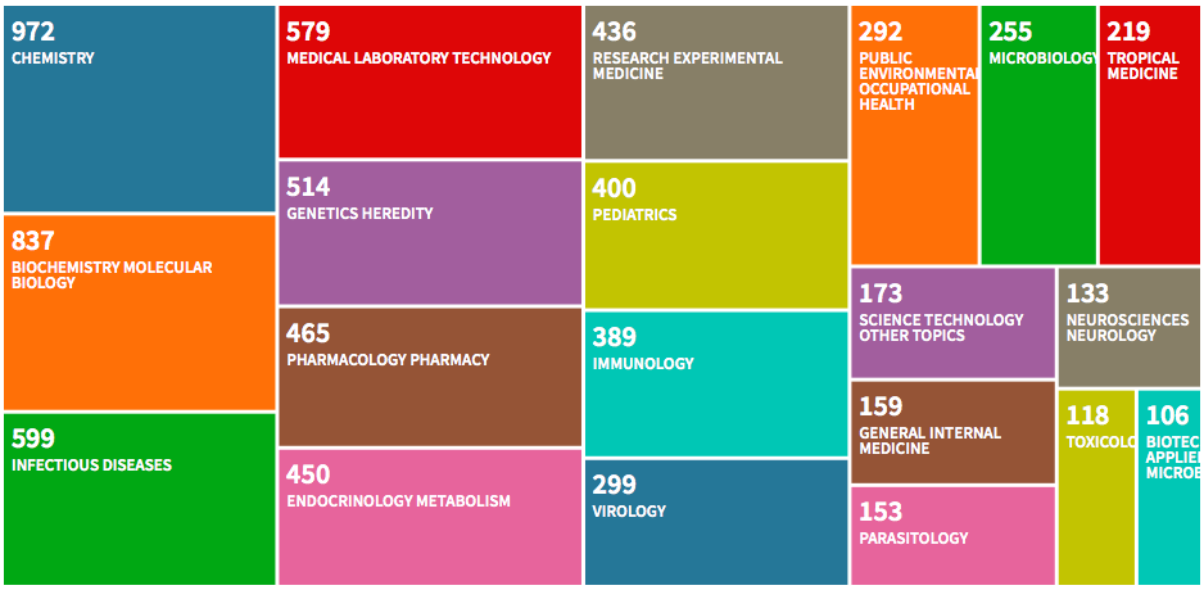

Figure 3: Overview of the 20 scientific areas in which the most DBS-related articles have been published, as generated *via* Web of Science.

1.1.1. The hematocrit effect

Although DBS sampling can be a valuable tool for both qualitative and quantitative purposes, it is also accompanied by some challenges[30-32]. The most widely discussed issue in this regard is the so-called hematocrit (HT) effect, which consists of multiple aspects, which can be subdivided in analytical and physiological aspects[30]. The analytical aspects refer to the fact that the analytical result may depend on a patient's HT, whereas the physiological aspect refers to the potential impact of the HT on the interpretation of the DBS-based result. The HT is the volume percentage of red blood cells (RBCs) in a blood sample and is sometimes also referred to as the erythrocyte volume fraction (EVF) or packed cell volume (PCV). A patient's HT depends on multiple factors such as gender, age, disease status, altitude at which the patient resides, smoking and medication use. A more detailed explanation on the HT effect as well as a guide on how to evaluate it can be found in Chapter 1, section 1.4.1 and Chapter 2, section 2.3.2.3, as well as in several recent reviews[30,33,34].

1.1.1.1. Analytical aspect

Since the HT affects the viscosity of blood, it also influences the spreading of the blood on/through the filter paper – with samples with a lower HT spreading out further compared to samples with a higher HT (see Figure 4)[30]. Consequently, whenever a fixed-size DBS punch is made, this will contain more blood when the sample has a higher HT compared to samples

with a lower HT. This phenomenon may lead to overestimation of drug levels in samples with a high HT, whilst samples with a low HT may be subject to underestimation of the drug levels (when compared to a calibration curve in a medium HT). Secondly, the HT may affect analyte recovery[35,36] and potentially also matrix effects[37].

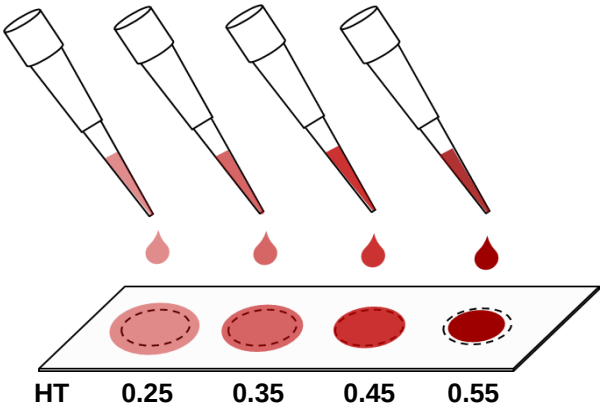

Figure 4: Schematic representation of the influence of the HT on the spreading of a blood drop.

1.1.1.2. Physiological aspect

Aside from these analytical aspects of the HT effect, there is also a physiological aspect to this issue. More specifically, the HT may influence a compound's blood to plasma ratio and hence, the correlation between DBS and plasma levels[38,39]. This is of great importance in DBS-based data interpretation as generally obtained results need to be converted to their corresponding plasma values[40,41]. Indeed, traditionally used therapeutic intervals are almost always established using plasma or serum. The impact of HT on the correlation between DBS and plasma levels can be easily demonstrated with a compound that is exclusively present in the plasma compartment (see Figure 5). Although the upper samples contain the same whole blood concentration of this analyte (represented by the green triangles), their plasma concentration is drastically different and depends on the sample's HT. In the bottom samples the plasma concentration of the analyte is the same for all samples, but their whole blood concentration is not and again depends on the sample's HT.

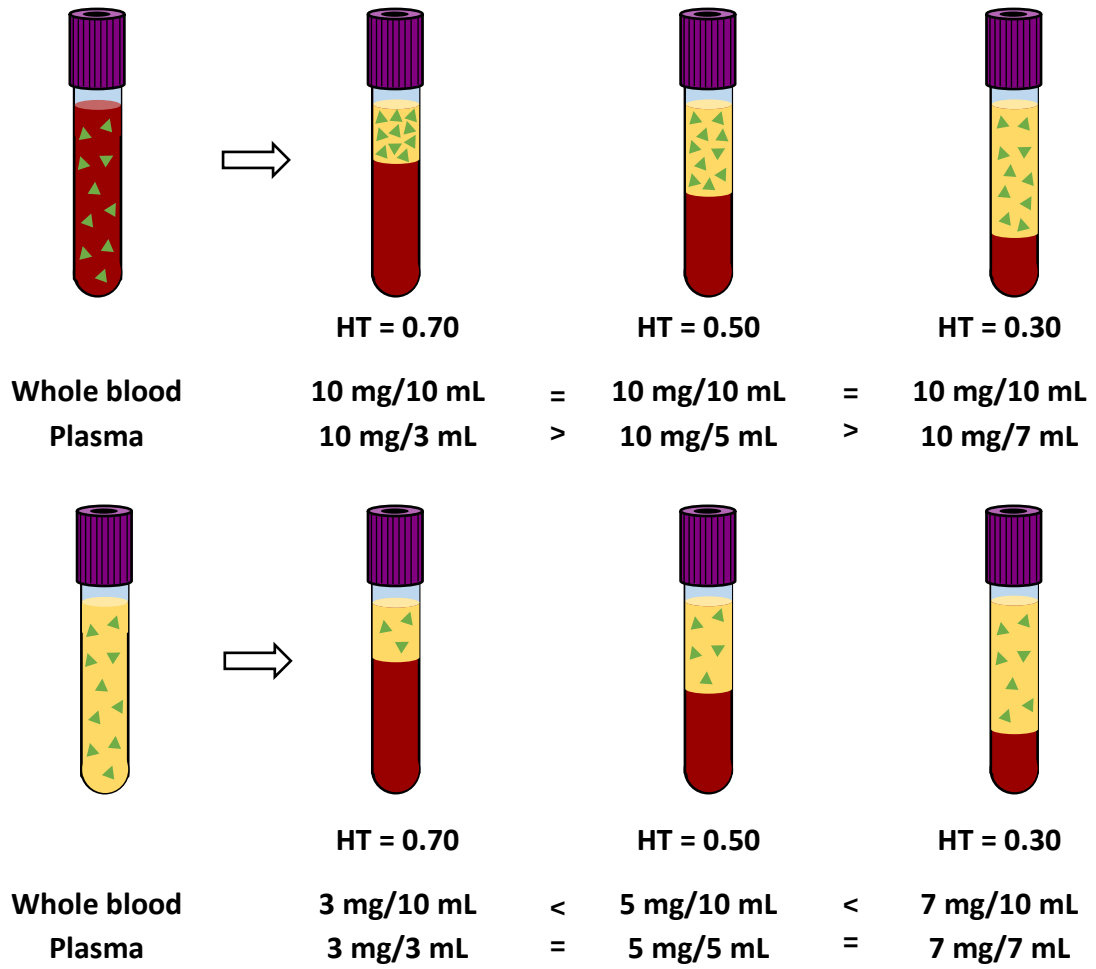

Figure 5: Schematic representation of the physiological aspect of the HT effect

1.1.1.3. Impact

In the scientific literature regarding DBS-based methods, the word HT is intrinsically linked to terms such as 'issue', 'hurdle' or 'barrier'[42-45]. The HT effect represents an additional source of variability which is not present in the analysis of traditional samples[30]. Therefore, although DBS may offer many opportunities, this additional variability prevents the more widespread use of DBS-based methods, both in a research context and a routine environment. Obviously, the HT effect mainly affects quantitative DBS methods, although it may also affect the interpretation of semi-quantitative analyses[46]. The relevance of the HT effect is illustrated in Figure 6 using quotes from literature.

Given the importance of the HT effect, many have researched potential ways to avoid or minimize the impact of this phenomenon on the end result[30,33,34]. Some of these suggested solutions were developed as part of this doctoral thesis (See Chapters 3-7). An overview of

other potential solutions along with their respective advantages and limitations is provided in Chapters 1-2 and in the section 'Broader international context, relevance and future perspectives'.

*"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." Timmerman et al., 2011*

*"Hematocrit 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." Fan & Lee, 2012*

*"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." Abu-Rabie et al., 2011*

*"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**" Blessborn et al., 2013*

*"Hematocrit-related issues **remain a major barrier for (regulatory) acceptance** of the classical dried blood spot (DBS) analysis **in the bioanalytical and clinical field**. Although many successful strategies have been put forward, **a combination of different technologies still provides the most complete solution**." Velghe et al., 2018*

Figure 6: quotes from literature illustrating the impact of the HT effect on the implementation of DBS sampling

1.1.2. Other issues

Aside from the HT effect, DBS analysis is accompanied by several other issues as well, which further hamper the more widespread use of DBS methods[1,6,30]. Analytical issues encompass the need for sensitive methods, the volume effect and the chromatographic effect. The latter two refer respectively to the fact that a DBS-based result may be affected by the volume of the blood drop that was applied and the area in which the DBS punch was made[32,47,48]. More practical issues on the other hand include the many manual steps required for DBS analysis (i.e. punching, extraction, etc.), the lack of DBS method validation guidelines and proficiency testing (PT) programs. An overview of these issues and the steps that have been taken during recent years to overcome these hurdles, is provided in Chapter 1 sections 1.4.1, 1.10 and 1.11.1, Chapter 2 section 2.3.2 and in 'The broader international context, relevance and future perspectives' section.

1.2. Volumetric absorptive microsampling

One of the more successful solutions that has been suggested to avoid the HT effect of DBS analysis, is to use volumetric absorptive microsampling (VAMS) instead of DBS sampling. VAMS is a sampling technique that was introduced in 2014 and uses a collection device that is able to collect a fixed volume of blood independent from a patient's HT[49]. The collection device is made of an absorbing polymeric tip attached to a plastic handle and allows even more convenient sample collection than DBS sampling[50]. After sample collection, the samples are typically dried under ambient conditions and the entire VAMS tip is extracted, intrinsically eliminating the possibility of a volume or chromatographic effect[51]. Unfortunately, recent evaluations of the VAMS approach (both in our laboratory and elsewhere) reveal that it does not entirely eliminate the HT effect[52]. Although there is no HT effect on the sample volume, VAMS appears to be quite susceptible to an HT effect on extraction efficiency. Obviously, the latter can be avoided by proper method development[53-57]. In addition, VAMS is still subject to the physiological aspect of the HT effect, which is inherent to each sampling strategy in which whole blood (dried or liquid) is analyzed. Therefore, HT remains an important variable.

2. STRUCTURE AND OBJECTIVES

In recent literature a multitude of potentially valuable dried blood sample-based applications has been described. Especially the possibility of home sampling and the opportunity to obtain information that is hard to procure using traditional sampling, renders dried blood micro-sample collection and analysis an appealing tool. Unfortunately, aside from (semi-quantitative) NBS programs, routine implementation is currently still scarce (see Chapter 1 and the 'Broader international context, relevance and future perspectives' section) due to several (practical) hurdles. Therefore, the **overall goal of this doctoral thesis is** to tackle these hurdles and **to render quantitative dried blood microsampling more compatible with implementation in (clinical) laboratories, so it can fulfill its full potential.**

An overview of alternative sampling strategies along with their specific advantages, challenges and limitations is provided in **Chapter 1**. Special attention is given to DBS and other dried blood microsampling strategies, since these are the common thread throughout this work. Indeed, for many applications blood remains the matrix of choice, since there is typically a correlation between an analyte's blood level and a certain effect one wants to measure. Selected applications of the abovementioned alternative sampling strategies are discussed as well and encompass the fields of TDM, NBS, endocrinology, toxicology, phenotyping, proteomics and metabolomics. TDM-related applications are discussed more elaborately, as TDM represents one of the main target domains of this thesis (i.e. it is the domain in which quantitative DBS-based methods are closest to routine implementation). In addition, the issues impeding routine implementation of alternative matrix analysis in general, and DBS analysis in particular, are also outlined in this chapter, along with the current and potential future developments that may help to overcome them.

One of these issues, i.e. the lack of a DBS method validation guideline, is tackled in **Chapter 2**. More specifically, a guideline was constructed in collaboration with the *Alternative Sampling Strategies Committee of the International Association of Therapeutic Drug Monitoring and Toxicology* (IATDMCT) to help ensure quantitative DBS-based methods provide reliable results. It not only covers the requirements for a thorough analytical method validation, but also encompasses points of attention during method development, as well as recommendations regarding clinical method validation. Furthermore, it also provides

suggestions on how to deal with DBS-related issues such as the HT, volume and chromatographic effect. Although this guideline was specifically constructed for the validation of quantitative DBS-based methods that use LC-MS/MS in the context of TDM, many of its aspects are also valid for other types of DBS methods as well.

A second issue which is tackled, and which represents the major focus point of this doctoral thesis, is the HT effect. Since this phenomenon is invariably linked to the word 'issue' (see above), it is a fundamental hurdle to overcome in our quest to render DBS-based results more reliable and hence, DBS-based methods closer to more widespread implementation. Therefore, several strategies were developed to predict the HT of the blood used to prepare a DBS (or VAMS sample). This predicted HT can be used to introduce a HT-dependent correction factor to a DBS (or VAMS)-based result and to compensate for the anticipated HT effect. The methods that were developed within the context of this doctoral thesis can be subdivided into two categories: those based on K^+ **(Chapters 3 and 4)**, and those based on Hb **(Chapter 5 – 7)**.

In **Chapter 3**, a method is described which allows to determine the HT of the blood used to generate a DBS based on its K^+ content. Following a simple and rapid extraction protocol, K^+ levels from a 3 mm DBS punch are measured *via* indirect potentiometry, using a routine chemistry analyzer. After successful analytical validation the method was applied to 111 venous patient DBS, yielding excellent results. Although VAMS can eliminate the analytical HT effect (see above), HT prediction may still be required to convert VAMS-based results to the corresponding plasma or serum values. Therefore, a HT prediction method was developed for VAMS samples as well, as outlined in **Chapter 4**. Both an aqueous and an organic extraction procedure were developed and applied to 95 venous patient VAMS samples. Special attention was paid to the optimization of the sample preparation procedure to ensure that both an LC-MS analysis and the K^+ determination could be performed on a single VAMS extract.

Despite the fact that the K^+-based method is straightforward, reliable and cheap and that the required instrumentation is available in every clinical laboratory, it does require the destruction of (part of) a DBS. As the sample volume that is available for analysis is already very limited with DBS analysis, a non-destructive alternative was developed. This method, which is presented in **Chapter 5,** predicts the HT of a DBS based on its total Hb content and

uses non-contact diffuse reflectance spectroscopy. The DBS are illuminated with halogen light, which is guided to the DBS surface *via* a fiber probe, and the light which is reflected by the DBS is transported *via* the same probe to a spectrometer. The reflectance spectra are fitted to a light transport model which takes into account the presence of oxyhemoglobin (OxyHb), methemoglobin (MetHb) and hemichrome (HC). The latter is essential since blood in DBS is originally present as OxyHb, which is then oxidized to MetHb and further denatured to HC upon storage. The fitting algorithm assigns a value with arbitrary units to each of the abovementioned Hb derivatives and the sum of those values is used as a surrogate measure of total Hb and HT. After an elaborate analytical validation, the method was successfully applied to 233 venous patient DBS. In **Chapter 6** a simplification of this non-destructive method is described. In this simplified method the HT is calculated using the reflectance at a single wavelength, located at a quasi-isosbestic point in the reflectance curve. At this wavelength, assuming 1-to-1 stoichiometry of the aging reaction, the reflectance is insensitive to the Hb degradation and only scales with the total amount of Hb and, hence, the HT. Furthermore, it is demonstrated in this chapter, using caffeine as a model compound, that this HT prediction method can be effectively used to implement a HT-dependent correction factor to DBS-based results to alleviate the HT bias.

Apart from developing strategies to cope with the HT effect, an alternative sample collection technique was evaluated which tries to avoid the HT effect altogether. More particularly, VAMS was employed to quantitate Co in whole blood using ICP-MS for the follow-up of metal-on-metal (MoM) prosthesis patients. The development, validation and application of this method is presented in **Chapter 7**. A good agreement could be observed between the venous VAMS results and those obtained on the corresponding liquid whole blood samples (n = 78).

Finally, an overall discussion is provided and the work is positioned within the broader international context.

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Chapter 1

Alternative sampling strategies: advantages, challenges and applications

Based on:

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1.1. INTRODUCTION

Alternative sampling strategies have proven to be of added-value for ample clinical applications, such as NBS programs[1,2], disease diagnosis and monitoring[3-8], vaccination status evaluation[6] and even therapy optimization and individualization[9-12]. Indeed, minimally or non-invasive sampling methods may facilitate home-based TDM[13], simplify TDM in e.g. a pediatric population[14] and promote TDM programs in remote settings and developing countries[15-17]. Furthermore, alternative sampling strategies facilitate pharmacokinetic (PK) studies in special patient populations[14], yielding important information that is currently still lacking for a lot of drugs. A better understanding of PK can in its turn help to reduce the need for TDM, as dosage regimens can be optimized for these special patient populations. Moreover, various easily obtainable matrices have been employed for cytochrome P (CYP) 450 genotyping[18] and phenotyping[19], further contributing to therapy optimization and individualization. Additionally, alternative sampling strategies have been employed in areas such as endocrinology[20], toxicology[21,22], proteomics[23] and metabolomics[24,25]. In conclusion, alternative matrices have the potential to contribute to better patient management and follow-up, and thus to help improve patient healthcare in general.

These so-called alternative sampling strategies refer to the patient-friendly sampling of non-conventional matrices as well as to the unconventional way of sampling traditional matrices such as whole blood, serum and plasma. Matrices that are discussed include DBS, VAMS samples, dried plasma spots (DPS), other dried matrix spots (DMS), OF, ISF, hair, tears, EBC, sweat, nasal mucus and meconium. In this chapter the benefits of the above-mentioned alternative sampling strategies will be discussed (predominantly in the context of TDM), with particular attention to special patient populations such as neonates, children, pregnant women and the elderly. Furthermore, some important limitations, pitfalls and challenges of these techniques will be highlighted, as well as the future perspectives they offer.

Because it is not our goal to provide an exhaustive overview of all published applications that have been developed using alternative matrices, selected examples will be discussed to highlight the potential added-value of alternative matrix sampling over traditional sampling. For more detailed information regarding existing applications and existing analytical methods we wish to refer to some published reviews[2,3,9-12,15,19-21,26-30].

1.2. THE IDEAL ALTERNATIVE MATRIX

In TDM systemic drug concentrations are measured to evaluate whether a suitable drug exposure has been achieved. This way, the risk for toxicity, inadequate efficacy or therapy resistance is minimized, whilst compliance problems as well as PK abnormalities or drug interactions can be detected. Unfortunately, traditionally used sampling strategies (i.e. venipuncture) are invasive and necessitate relatively large blood volumes which cannot always be justified in e.g. neonates or anemic patients[14,31]. Moreover, outpatients are obliged to frequent a clinic or a doctor's office for blood draws and samples often require a cold chain during transportation to the laboratory. Furthermore, for highly protein bound drugs, or in special situations such as pregnancy, hypoalbuminaemia, renal failure or liver insufficiency, it is advised to measure the free plasma concentration of a drug[32]. Indeed, under these circumstances the latter is better suited to predict drug toxicity and efficacy. However, these measurements are laborious and technically challenging, and are hence not easily incorporated in routine clinical laboratories.

Alternative matrices have gained a lot of attention in TDM since they are obtained in a minimally or non-invasive manner and may provide a more convenient way of assessing total or even free[32] systemic drug concentrations - depending on the type of alternative matrix. Ideally, a matrix for TDM fulfills the criteria summarized in Table 1.1. Briefly, the sample collection should be non-invasive and should require only a small sample volume to be applicable to vulnerable patient populations such as neonates. The collection procedure should also be universal (i.e. applicable to everyone) and straightforward enough to allow unsupervised patient self-sampling in a home setting. Preferably the sample collection is robust so that variables arising during sample collection have no impact on the analysis result. Importantly, sample collection should be inexpensive to be considered for routine use. The target analyte(s) should be stable in the alternative matrix for prolonged periods of time, both at room temperature (RT) and under transport conditions. Moreover, the sample matrix should represent no biohazard to facilitate transport of samples *via* regular mail services. The analysis of the sample should be as straightforward as possible (i.e. no or limited sample preparation), should not require expensive consumables and should be automatable. In addition, whenever other analytes are generally requested by clinicians concurrently with the therapeutic drug of interest, these should also be measurable in the alternative matrix, since

otherwise there would still be a need to draw a venous blood sample, limiting the usefulness of the alternative sampling strategy. Obtained results in the context of TDM should be quantitative, reproducible and should correlate with systemic levels - either free or total, depending on the type of alternative matrix. This correlation should show small intra- and inter-individual variability and should be independent of the analyte concentration, time after drug administration, as well as any other variable (either introduced by the sampling method or inherent to the patient). Most importantly, results obtained *via* alternative matrix analysis should not influence clinical decision making any differently than traditionally obtained results would.

Table 1.1: Overview of the criteria of the ideal alternative matrix for therapeutic drug monitoring.

SUITABILITY CRITERIA FOR AN ALTERNATIVE MATRIX
Sample collection
Non-invasive Requires little sample volume Straightforward Allows for self-sampling/home monitoring Applicable to everyone Robust Economic
Storage and shipment
Stable at ambient conditions No biohazard
Analysis
Limited pre-analytical phase Automatable Economic Allows co-analysis of other relevant analytes
Results
Reproducible Correlate with systemic (free) drug concentrations Cave: small intra- and inter-individual variability in this correlation is required! Should not be time-dependent Should not be concentration-dependent Should not be influenced by additional variables such as pH and flow rate Affect clinical decision making in the same way as traditional matrices

Unfortunately, the ideal alternative matrix does not exist, since in reality every matrix has its own limitations, challenges and inherent disadvantages (cfr. Table 1.2). The goal is to find those applications in which the benefits outweigh the drawbacks and to develop approaches that eliminate or minimize the impact of these matrix-specific issues on the analytical result and on the corresponding clinical decision.

Table 1.2: The main advantages and challenges of the different types of alternative sampling strategies

	Dried blood spots	Dried plasma spots	Capillary liquid sampling	Volumetric absorptive microsampling (VAMS)	Oral fluid	Hair	Meconium	Interstitial fluid (microneedles)	Sweat	Exhaled breath condensate	Sputum
Patient comfort (e.g. ease of sampling, invasiveness)	++	+	+	++	++	+	+++	++	++	+++	+
Small sample volume	++	++	++	+++	++	+	–	+++	NA	–	++
Analyte stability	+++	+++	+++	+++	+/–	+/–	++	+	++	–	–
Convenient storage and transport	+++	+++	–	+++	+	+++	–	–	+	–	–
Reduced infection risk (compared to blood samples)	+	+/–	+	++	+++	+++	+	+	+++	+++	+++
Resistance to contamination	–	–	+	–	–	–	++	++	–	+	++
Resistance to hematocrit effect	–*	+	++	++	NA	NA	NA	NA	NA	NA	NA

+++/++/ + /–: indication to what extent the statement on the left holds true; NA: not applicable.
* The hematocrit effect is a major issue, but to date, several attempts to cope with the issue have been explored.

1.3. THE CORRELATION BETWEEN ALTERNATIVE MATRIX AND SYSTEMIC LEVELS

As mentioned above, a prerequisite for the usefulness of alternative matrices for TDM is of course the existence of a correlation between the alternative matrix' drug level and the (free) systemic plasma or whole blood concentration. As can be seen from Figure 1.1., the free plasma concentration can diffuse into other tissues or biological fluids, indicating that alternative matrix' drug levels are -at least to some degree- determined by the corresponding free plasma concentration. However, the alternative matrix' drug levels are generally also affected by multiple other variables. First of all, the protein content of these matrices codetermines their total drug concentration. Only when drug-binding protein content is low, such as in tears, measured drug levels can be similar to plasma free drug concentrations. Since drugs generally require passive diffusion to enter an alternative matrix, drug levels are also defined by the molecular weight and lipophilicity of the compound. Moreover, the pH of the matrix and the pKa of the compound are of key importance for the equilibrium between both compartments. In acidic matrices such as sweat and OF, for example, basic compounds will become ionized and hence, be subject to ion-trapping. Another factor that is known to affect the measured concentration in e.g. OF, tear fluid and sweat is the flow rate (cfr. Sections 1.4.2 and 1.4.3). Taking the above-mentioned factors into account, drug concentrations in alternative matrices are rarely equal to (free) systemic concentrations, but can correlate with it. However, the latter has to be evaluated on a case-by-case basis during a clinical validation. To be truly useful in the context of TDM, it is crucial that this correlation is constant both intra- and inter-individually. Whenever only the intra-individual variability is small enough, the alternative matrix can only be used for patient follow-up after having established reference levels for that patient. As discussed above, the ratio between both matrices might be altered by differences in e.g. flow rate and pH and hence, also by the sampling procedure.

Therefore, sampling procedures often need to be standardized as much as possible. In addition, the observed correlation between alternative matrix' and systemic levels may be time-dependent or concentration-dependent, complicating result interpretation and limiting the practicability of the sampling strategy.

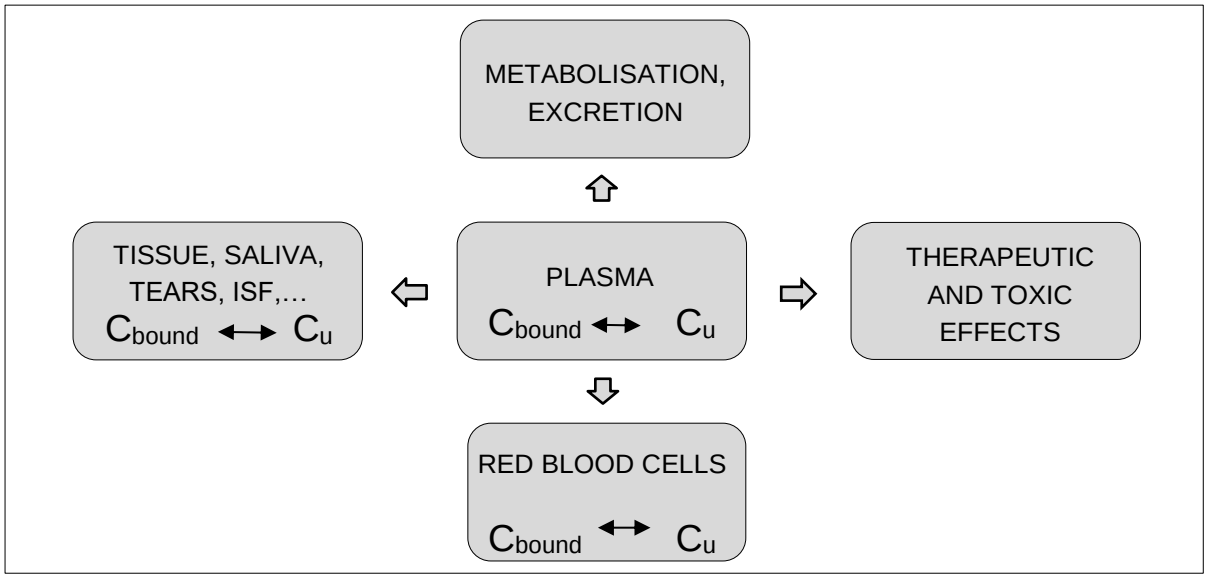

Figure 1.1: The equilibrium between the free plasma fraction and alternative matrices (with C_u, the unbound concentration and C_{bound}, the bound concentration of a compound)

Whether a sufficiently good correlation is present between the alternative matrix' and the systemic concentrations needs to be evaluated for every compound. Importantly, this correlation has to be strong enough to allow prediction of corresponding systemic levels for individual patients, since the latter will be employed to make therapeutic decisions (with the allowed deviation between true and back-calculated systemic concentrations depending on the specific target analyte). Therefore, a proper statistical evaluation of patient data obtained during a bridging study is essential. Although a high Pearson correlation coefficient can be a first indication of a good correlation between both matrices, as a stand-alone parameter this is still insufficient proof. Also other statistical tests such as Deming regression analysis, Passing-Bablok regression analysis and/or Bland-Altman analysis should be performed as well (also see chapter 2, section 2.4.3). In addition, an artificially high Pearson correlation coefficient can be obtained by including a broad range of analyte concentrations, even when the alternative matrix is actually not a good surrogate for the assessment of systemic levels. When the performance of matrices needs to be compared it is therefore better to employ other statistical methods such as Bland-Altman comparison, Deming regression and Passing-

Bablok analysis. Unfortunately, often only a small amount of patient samples is included in bridging studies, limiting the validity of the obtained results.

Additionally, the influence of different (matrix-specific) parameters on alternative matrix levels and hence, alternative matrix/systemic compartment correlation, needs to be evaluated. Whenever there is such an influence this should either be dealt with by standardizing the sample collection so that these variables are within acceptable limits (e.g. the use of printed concentric circles on DBS cards to control the volume effect[33]) or should be coped with in another appropriate fashion (e.g. HT correction to compensate for HT bias in DBS analysis[34]) (cfr. Section 1.4.1).

1.4. THERAPEUTIC DRUG MONITORING

1.4.1. Alternative specimen: dried blood spots

An alternative sampling technique that has gained a lot of attention for TDM purposes is DBS sampling[9,10]. Indeed, the collection of a drop of capillary blood from a finger or heel stick onto a filter paper offers a lot of advantages. First of all, it is a very simple, cheap and minimally invasive way of acquiring a representative sample, typically requiring only about 5-70 µL of blood (depending on the collection procedure, see Chapter 2). Furthermore, after proper drying, the analytes in DBS are generally stable at ambient conditions for prolonged periods of time and the dried blood only represents limited biohazard, facilitating transport under ambient conditions.

A major opportunity provided by DBS sampling is the possibility of home-based patient self-sampling with subsequent transport of the dried samples to the laboratory *via* regular mail services[35]. For outpatients this approach can reduce the frequency of clinic visits, which is undoubtedly more convenient, especially for active patients. Furthermore, this reduction may be beneficial to patients' health (e.g. in cystic fibrosis (CF) patients) and may contribute to an improved quality of life for chronically ill patients[36]. Moreover, self-sampling allows DBS to be collected at each moment of the day. This is convenient when e.g. trough levels need to be measured before the next dose, since this is often early in the morning or late in the evening (i.e. outside of clinic opening hours). Also when multiple samples need to be collected over a certain period of time this may be logistically challenging to organize in a clinic but feasible using DBS in a home setting. Home-based sample collection might also be preferable for

children and patients with mental, psychological or developmental disorders, since obtaining samples in a familiar environment might be less stressful[37,38]. Additionally, the convenience of home-based TDM offers the opportunity of more frequent patient follow-up, which in its turn may lead to improved therapy adherence and hence reduced health care costs[39]. Indeed, it has been proven multiple times that the lack of therapy adherence results in a lot of avoidable costs to the patient as well as to society due to i) the unnecessary changing to stronger and more expensive therapies, ii) increasing patient morbidity, invalidity and mortality, and iii) emergence of therapy resistant pathogens (in the case of treatment of infectious disease).

Another setting in which the use of DBS may contribute significantly to patients' health is the set-up of TDM programs in developing countries and other resource-limited areas[15-17], where limited accessibility is currently a big hurdle for the implementation of TDM. DBS samples can be collected in the field and sent to a central laboratory for analysis under ambient transport conditions. Consequently, the availability of a centrifuge, cooled storage or even electricity are not required at the place of sample collection. In addition, DBS present a reduced biohazard compared to traditional samples, which results in safer sample handling in areas where e.g. HIV is highly prevalent.

Aside from the clear advantages for outpatient TDM, DBS sampling also possesses several benefits for hospital-based TDM, albeit only in specific circumstances. First of all, DBS sampling can be an easy solution for compounds with stability problems. However, stability during drying and storage needs to be assessed on a case-by-case basis during the analytical method validation, as the use of DBS does not alleviate stability issues for all compounds. Furthermore, in particular patient populations such as neonates and severely anemic patients, DBS sampling may be preferred over classic venous sampling, as it is less invasive and requires a much smaller sample volume. Other inpatients for which DBS may be a worthy alternative are people with poor venous access (such as the elderly or people with phlebitis) and patients with fear of needles[5]. The advantage of DBS sampling is in the latter cases obviously limited to those patients for which no additional venous blood collection needs to be performed. Therefore, several analytical methods have been developed which can determine therapeutic drugs and/or other relevant parameters, such as creatinine, on the same DBS (extract)[5,40-42].

Additionally, DBS preparation can also be employed as an easy form of sample collection and/or sample pretreatment, either when preparing capillary or venous DBS. An example includes the on-card stabilization *via* derivatization of thiorphan, the active metabolite of the antidiarrheicum racecadotril[43]. By directly collecting a drop of blood onto filter paper, pretreated with the derivatizing agent 2-bromo-3'-methoxyacetophenone, sample collection can be greatly simplified compared to the traditional sample collection, which requires complex handling procedures at the clinical site. In addition, DBS can also be prepared from classic venous samples when samples need to be sent to another laboratory for analysis, as this facilitates more practical and economic sample transport.

DBS are mainly regarded as a suitable alternative for routine TDM checks of stable patients or for solving less urgent questions. When TDM results need to be reported urgently, the DBS format is of course less suitable, since a certain drying period is required (typically at least 2 hours). Although the drying process can be accelerated using for example microwave-assisted drying, which only takes minutes, liquid blood sampling at an emergency unit is more suited in this context[44].

Although DBS sampling can be valuable in the context of TDM, it is also accompanied by some challenges. The most widely discussed issue in this regard is the so-called HT effect, which consists of multiple aspects[45]. First of all, as the HT affects the viscosity of blood, it also influences the spreading of the blood on/through the filter paper – with samples with a lower HT spreading out further compared to samples with a higher HT. Consequently, whenever a fixed-size DBS punch is made, this will contain more blood when the sample has a higher HT compared to samples with a lower HT. This phenomenon may lead to overestimation of drug levels in samples with a high HT, whilst samples with a low HT may be subject to underestimation of the drug levels. Secondly, the HT may affect analyte recovery and potentially also matrix effects[45].

To cope with the HT effect on sample spreading multiple solutions have been postulated, which have been elaborately discussed by De Kesel *et al*.[45,46]. Briefly, the HT effect on spreading can be avoided by preparing volumetric DBS and analyzing the entire sample. However, the deposition of a fixed volume of blood requires the use of capillaries or pipets, which might be feasible in a laboratory setting, but is too complicated to allow for patient

self-sampling. To provide a solution for this issue, multiple formats have been developed recently to allow for the generation of volumetric dried blood samples starting from a non-volumetric drop of blood (cfr. Section 1.4.1.2)[47-49]. Another option (which is also applicable to non-volumetric DBS) is to estimate the HT of a DBS, based on its K^+ content, and to apply a K^+-content-dependent (i.e. a HT-dependent) correction factor to the obtained DBS-based result to compensate for the expected HT effect[33,34]. Recently, we developed a non-contact alternative to the K^+ method which allows to estimate the HT of a DBS by merely scanning its surface[50,51]. A third solution that has been suggested is the use of specially designed substrates on which sample spreading is not (or at least to a lesser extent) influenced by the HT[52]. Finally, it has been proposed to use *in situ* generated DPS instead of DBS (cfr. infra)[53-55]. Although some of these approaches are promising, more experience is required to build up sufficient confidence regarding the obtained results.

To avoid the HT effect on extraction recovery it is advisable to optimize the sample preparation procedure over a wide range of HT values (cfr. Chapter 2). To attain sufficient and reproducible extraction efficiencies, some have used heated extraction or even specially pretreated filter paper substrates[56-58]. Additionally, remaining variability in extraction efficiency can be accounted for by using an internal standard (IS) spray[59]. The latter can be applied to a DBS using an automated system. Unfortunately, only few laboratories have such resources to their disposal. Therefore, typically, the IS is included in the extraction solvent, which entails that differences in extraction efficiency cannot be accounted for. Alternatively, the filter paper can also be impregnated with IS prior to DBS preparation[59,60]. However, the latter approach is not practical to perform and requires homogeneous distribution of the IS throughout the filter paper. In addition, this distribution should not be affected by the deposition of the blood sample.

Aside from these analytical aspects of the HT effect, there is also a physiological aspect to this issue[45]. More specifically, the HT may influence a compound's blood to plasma ratio and hence, the correlation between DBS and plasma levels[61]. This is of great importance in DBS-based data interpretation as generally obtained results need to be converted to their corresponding plasma values. Indeed, traditionally used therapeutic intervals are almost always established using plasma or serum. Theoretically, new DBS-based therapeutic intervals could be established, but this is expensive and time-consuming. Furthermore,

physicians are familiarized with existing therapeutic intervals. To convert DBS-based obtained results into the corresponding plasma values, a conversion factor is often empirically determined. However, as this fixed correlation factor does not take the patient's HT into account, this approach might be an oversimplification[62]. Alternatively, the average HT of the target population (as well as the plasma protein binding of the compound) can be used to calculate the blood to plasma ratio[63]. This simplification is obviously only acceptable when the HT range of that population is quite narrow. However, when a DBS method has to be applied on both healthy subjects as well as on special patient populations such as anemic patients, neonates, children, pregnant women and elderly, the HT range will be much wider and the HT of each DBS sample has to be determined and needs to be taken into account in the DBS-plasma conversion. In addition, the blood to plasma ratio may also be concentration-dependent, as was observed for the anti-epileptic drug topiramate[64]. Obviously, establishing blood to plasma ratios should always be done using clinical samples, since *in vitro* experiments could sometimes yield deviating results[64].

Another essential issue for the analysis of capillary DBS (i.e. DBS collected directly from a heel or finger stick) is the possible difference between capillary and venous drug levels. The correlation between both needs to be assessed on a case-by-case basis during clinical validation. Therefore, to determine the usefulness of a developed DBS method, the latter should always be applied to true capillary patient samples. Several factors might influence the correlation between capillary and venous drug levels. On the one hand it needs to be taken into account that capillary blood contains both venous and arterial blood and that arteriovenous concentration differences have been observed for certain compounds[65,66]. These differences depend on drug characteristics (molecular size, lipid solubility and degree of protein binding) as well as on time after drug administration (after distribution an equilibrium should be attained)[65]. A time-dependent distribution between both matrices has for example been observed for the antiviral drug oseltamivir[67]. Moreover, differences between capillary and venous samples may be due to the contamination of the capillary blood sample with ISF. To minimize the admixture of ISF with capillary blood, the first drop of blood is discarded during DBS sample collection and "milking" the finger too extensively is dissuaded. In addition, discrepancies between capillary and venous HT have been observed previously and may contribute to differences in drug levels, especially when measured *via*

DBS[68]. Lastly, HT measurements performed *via* repeated capillary sampling are known to be more variable compared to HT measurements obtained *via* repeated venous sampling[68]. Yet again, this emphasizes the importance of knowing a patient's HT.

Other DBS specific variables that need to be evaluated during method development and validation include potential sources of contamination, the volume effect and sample homogeneity[45,69]. Contamination during sample preparation (e.g. during the punching step) should be eliminated during method optimization. However, contamination issues occurring during sample collection can never be excluded, especially when sampling is performed by the patient. The likelihood of the latter can however be minimized by properly educating patients *via* e.g. demonstration videos and by instructing the patients or their caregivers to thoroughly wash and disinfect the hands prior to the finger prick. To verify whether this type of contamination occurred, a second DBS or a blank DBS punch near the sampled DBS can be analyzed, whenever necessary[21,70]. The extent of the volume effect, which occurs due to differences in filter paper saturation, needs to be determined during analytical method validation. Importantly, an acceptable volume range should be established at low, medium and high HT. To evaluate whether the volume effect is within acceptable limits in patient samples, filter paper with concentric circles can be employed for DBS sampling[33]. In this format, the diameter of the inner circle corresponds to the smallest volume that is still acceptable for the lowest HT evaluated, whilst the diameter of the outer circle corresponds to the largest volume that is still acceptable for the highest HT. As long as samples are within these concentric circles the impact on target analyte quantitation should be acceptable. Also the evaluation of DBS homogeneity experiments should be performed at different volumes and different HT values. When a noticeable difference can be observed between quantitation on central and peripheral punches, only central punches can be employed for further analysis. Another option that can be evaluated in such a case is to use a larger punch size. The latter obviously does not influence DBS homogeneity *per se*, but might make the analysis less susceptible to location-dependent differences.

Since both the volume effect and DBS homogeneity depend on the type of filter paper used, results obtained with a different type of filter paper than the one used during validation may be unreliable[71]. Furthermore, a different type of filter paper may also affect blood spreading, sample stability and extraction efficiency[72]. In addition, extraction of each type of filter paper

may yield its own specific interferences[73], thereby potentially affecting matrix effect and method sensitivity. Therefore, the choice of filter paper should be made carefully during method development and should definitely remain constant throughout validation and method application. Intra- and inter-lot differences of several types of filter paper on the other hand, have proven to be acceptable even for quantitative applications[74].

Another challenge in DBS analysis is to obtain sufficient sensitivity when starting from such a small sample volume (a 3-mm punch corresponds to approximately 3 µL). Especially for compounds with low circulating concentrations this might be challenging at trough values. Generally, sufficient sensitivity can be obtained using GC-MS or LC-MS/MS, although LC coupled to UV or fluorescence detection, immunoassays and many other techniques have also been employed, depending on the intended application[75]. Importantly, the lower limit of quantitation (LLOQ) should be attainable at realistic volumes (i.e. starting from max. 25 – 30 µL). When this is not feasible, punch stacking might be a valuable option[76]. Also on the other end of the dynamic range DBS analysis may be challenging. Whenever a sample is above the upper limit (ULOQ) of quantitation, it needs to be diluted into the dynamic range. However, for DBS analysis this requires dilution with blank DBS extract, which is very cumbersome. To facilitate this step, IS-tracked dilution has been proposed[76,77]. Another option is to use the donut-punch approach, which is discussed in more detail in Chapter 2, section 2.3.1.3. In addition, alternatives which do not require the physical dilution of the DBS sample have been suggested and encompass mass spectrometer signal dilution (in which the signal that reaches the detector is artificially decreased by using suboptimal MS parameters), or the use of the signal of naturally occurring, less abundant isotopes[78].

Additionally, the evaluation of DBS sample stability might be challenging. In some cases this can be best evaluated on true clinical samples (i.e. *via* incurred sample stability). The stability of certain antiretroviral drugs, for example, which phosphorylate and accumulate in RBC *in vivo* and are released from RBC and dephosphorylated to generate additional parent compounds *in vitro*, cannot be mimicked in spiked samples[79]. Importantly, not only the stability under laboratory conditions should be evaluated. Whenever samples will have to endure more extreme temperatures (e.g. in a mail box in summertime), these conditions also need to be assessed during method validation. Concerning the application of DBS methods in developing countries, not only the effect of elevated temperatures should be evaluated, but

also the effect of humidity and sunlight exposure. Although DBS generally show enhanced analyte stability, this is not always sufficient and other approaches such as filter paper pretreatment or heat stabilization to stop enzymatic degradation need to be performed[80,81] . Even then, DBS may not alleviate all stability issues and they remain unsuitable for the analysis of volatile or air-sensitive compounds.

1.4.1.1. Applications of dried blood spot analysis in therapeutic drug monitoring

The first DBS-based method for TDM was published in 1978 by Albani *et al*. and evaluated the use of DBS for theophylline monitoring[82]. Since then, numerous other DBS-based TDM applications have been published, especially during the last decade[9,10]. Although DBS will never completely replace traditional matrices in TDM, they may provide valuable data that could not be (easily) obtained using traditional approaches, particularly in special patient populations or in certain challenging settings. To illustrate this, selected applications will be discussed below. Additionally, many different classes of therapeutic drugs have already been quantitated in DBS. These are summarized in Table 1.3, along with the most relevant advantages DBS sampling offers for each of these classes.

Pediatric patients

DBS sampling is minimally invasive and requires only a limited sample volume. Therefore, it is an interesting sampling technique for neonates and children[14]. Obviously, a lot of experience exists with DBS sampling in NBS programs[7]. However, DBS can also be employed for subsequent therapy monitoring. This has, for example, been done for dose optimization and monitoring of nitisinone, the treatment for tyrosinemia type I[83]. In this case also other parameters such as succinylacetone and tyrosine levels can be measured simultaneously to evaluate therapy effectiveness. Another use of DBS in children is the monitoring of busulfan, used in the myeloablative conditioning regimen prior to hematopoietic stem cell transplantation[84]. Moreover, home-based sample collection by a child's parents has also been performed. The feasibility of such an approach was first demonstrated by Rattenbury *et al*. in 1988 for the analysis of theophylline[85]. Since then, multiple other home-based applications have been evaluated such as the adherence assessment of children to anti-epileptic treatment[86] and the monitoring of immunosuppressant therapy in childhood solid organ transplant recipients[41].

Additionally, the use of DBS sampling in neonates, infants and children may provide an opportunity to obtain better age-specific dosing regimens, thereby optimizing therapy efficacy and safety and limiting the need for TDM[14]. Indeed, current dosing regimens are often derived from data obtained in an adult population, since obtaining age-specific PK data *via* traditional approaches would have required the collection of unethical amounts of blood. Moreover, the ease of sample handling, transport and storage facilitates multicenter pediatric PK studies, which are often required given the limited sampling pools available. In addition, pediatric nurses are receptive to the use of a method that they are familiar with and which can be fairly easily performed in the smallest babies with minimal training. Overall, DBS sampling fits well in a baby, parent and staff-friendly PK study design[87].

The elderly

In the elderly population venipuncture may be challenging to perform due to age-related changes in the skin (e.g. dermal thinning or reduced elasticity) and the blood vessels (e.g. atherosclerotic narrowing or the emergence of fragile subsurface blood vessels). Moreover, osteoarthritis may hinder straightening of the arm for venipuncture. Therefore, DBS collection may be more convenient in this population. Furthermore, reducing the number of hospital visits for elderly patients -by having DBS collected by the patient or his caregiver- may contribute to the patient's quality of life and could also be logistically easier to organize for a patient's family. In addition, many other parameters that are relevant to monitor in an elderly population can also be determined on DBS. DBS can for example be employed for the evaluation of glycemic control, renal function, cardiovascular risk, vitamin B12 status, thyroid function, iron status, and vitamin D status as well as for cancer screening [5]. An important point of attention in this population, however, is the finger prick depth, since many elderly patients take anti-clotting medication and prolonged bleeding may thus occur, if the finger prick is too deep.

Pregnant women

Strong changes in the level of anti-epileptic drugs such as lamotrigine are known to occur during pregnancy and the post-partum period, which can lead to more frequent seizures during pregnancy and potentially toxic levels post-partum. Since it is currently not possible to predict when these changes will occur exactly and since proper seizure control is essential for the mother's health as well as for the wellbeing of the fetus, these patients need to be closely

monitored to maintain a patient's pre-pregnancy drug level. Therefore, it has been suggested to collect DBS weekly at home to monitor lamotrigine levels in a more convenient way. Application of this approach in pregnant women demonstrated its applicability in a real life setting[13,88].

Anemic patients

A potentially valuable DBS-based application in anemic patients is the measurement of deferasirox, an iron chelating agent used in the treatment of transfusional iron overload in patients with chronic anemia. Deferasirox levels need to be closely monitored in these patients to assure therapeutic efficacy and DBS sampling may be an ideal format to do so, since it only requires very small volumes of blood, which will not aggravate the patient's anemia. Although the method developed by Nirogi *et al*. to measure deferasirox in DBS could be valuable in this context, it needs to be pointed out that the latter was developed to facilitate a PK study in rats and has not been evaluated in humans yet[89].

Psychiatric patients

As conventional sampling is often perceived as frightening by psychiatric patients, DBS sampling may provide a valuable alternative in this patient population[37]. Moreover, the use of DBS sampling may help improve patient co-operation during sample collection. In addition, since no syringes are required, this sampling procedure might contribute to the safety in a psychiatric facility. In addition, whenever a psychiatric facility does not have its own laboratory, samples need to be sent to a centralized laboratory for analysis, and in that case the stability and convenient transport provided by the DBS may be of added-value. The use of DBS sampling also facilitates (frequent) home-based adherence monitoring, which can be very relevant in this population, since adherence issues are highly prevalent. Importantly, as samples can be obtained in a familiar environment, this might be less stressful for the patient. The feasibility of DBS sampling in a psychiatric population was demonstrated by Patteet *et al*. for the analysis of a diverse panel of anti-psychotics[37].

Remote and resource-limited setting

A major hurdle in the set-up of TDM programs in remote settings is accessibility. A lot of patients live far away from a hospital and therefore samples should be obtainable 'in the field'. Other logistical issues regarding sample collection in resource-limited settings include

the unavailability of centrifuges or even electricity and the feasibility and cost of refrigerated transport. Here as well, DBS can provide a solution, since sampling can be performed conveniently at every location, no centrifuges are required and often transport to a centralized laboratory can be performed under ambient conditions. This is for example the case for TDM of the antiretroviral telaprevir. Whilst patient samples obtained *via* venipuncture typically require immediate centrifugation, acidification of the plasma fraction and freezing to stabilize the equilibrium between telaprevir's stereoisomers, DBS can be easily collected on a filter paper card pre-impregnated with 20% citric acid[81].

In addition, the reduced biohazard of DBS is a strong added-value in developing countries, since HIV is often highly prevalent. Therefore, numerous DBS-based methods have been developed for the monitoring of antiretrovirals, antituberculosis drugs and antimalarials[15,16,901]. Additionally, viral load monitoring, genotypic resistance testing and parasitic load determination can also be performed on DBS[16,91]. Another example for which DBS can be valuable is the set-up of a TDM program for tuberculosis (TB) treatment. In this context, DBS have been suggested to facilitate the set-up of a network of selected, quality-controlled reference laboratories which receive samples of difficult to treat patients from all over the world. Such an approach could help improve a patient's prognosis, prevent further development of multi-drug resistant TB (caused by underexposure due to PK variability or non-adherence) and reduce overall TB treatment costs[17,90,92]. In addition, DBS may be an ideal format for the set-up of specific PK studies that can only be performed in developing countries. Bartelink *et al*., for example, used DBS to facilitate a PK study in food insecure HIV-infected pregnant and breastfeeding women for multiple antiretroviral drugs[93].

Home monitoring

Since DBS sampling is fairly straightforward, it can even be performed at home by the patient himself or by his caregivers. Subsequently, samples can be sent to the laboratory for analysis and the results can be sent to the patient's attending physician. This way, results can already be available when the patient visits the doctor. Alternatively -when no visit is scheduled- the results can be reported to the patient *via* telephone, mail or an online system. The latter can be especially valuable for the frequent monitoring of outpatients on a long-time treatment. In this context, DBS sampling has for example been suggested for the follow-up of patients on an adjuvant breast cancer treatment with tamoxifen, since it was shown that DBS are able

to effectively identify underexposure of this drug[94]. Other possibilities for DBS sampling at home include more frequent home-based adherence monitoring, which may help to reduce healthcare costs[39].

Another important advantage of home-based sampling is the fact that samples can be obtained at every moment of the day. This is of particular relevance when the ideal sampling time is outside of regular clinic opening hours, as is for example the case for trough-level monitoring of prolonged-release tacrolimus[95]. Also when timing of the sampling needs to be accurate, DBS may be easier to collect than venous samples, since collection of the latter in a hospital setting might be difficult to organize logistically. Therefore, the collection of DBS has, for example, been evaluated for the C_2 (i.e. 2h post-dosing) monitoring of cyclosporine A[96]. When so-called abbreviated area under the curve (AUC) estimation using serial sampling is required, proper timing of venipuncture might be even more difficult[97,98]. In addition, patients have to wait in the medical center until the last sample is obtained. Therefore, Cheung *et al*. proposed the use of DBS for tacrolimus monitoring at C_0, C_2 and C_4, since the $AUC_{0\text{-}12}$ estimated from DBS collected by patients correlated well with the $AUC_{0\text{-}12}$ estimated from traditionally obtained venous samples ($r^2 = 0.96$)[97]. Home-based sample collection also provides the opportunity to collect samples when adverse effects occur or whenever the effect of the drug proves to be inadequate (e.g. immediately after an epileptic seizure or an asthma attack). This way valuable information can be obtained that a physician would not be able to acquire in any other way. This strategy was employed by Edelbroek *et al*. to pinpoint the reason for intermittent diplopia in a 63-year-old women who was taking multiple anti-epileptic drugs at the time[13].

Obviously, home-based DBS sampling is only helpful if the patient does not require venous sampling to assess other parameters. Therefore, it is important that also other relevant biomarkers can be determined on DBS, ideally even on the same DBS extract. In addition, the quality of the DBS samples has to be adequate to allow reliable quantitation. Fortunately, it has been shown multiple times that the majority of DBS samples have sufficient quality and that samples collected at home by the patient yield comparable results with DBS-based monitoring performed at specialized centers[96].

Home-based DBS sampling has a lot of potential. However, as with everything, it will never be suitable for all patients. Only when patients are properly instructed, motivated and willing to collect DBS themselves, these types of DBS programs can be successful (as has already been demonstrated for the routine DBS-based monitoring of anti-epileptic drugs in Dutch outpatient clinics[99]). Moreover, it is possible that a method is not applicable in certain patient subpopulations, for which the method was not validated. In addition, home-based TDM will not be applicable for all drugs or drug-related questions, since turn-around-time is of course higher when samples have to be sent *via* regular postal services.

Cystic fibrosis patients

A special population for which home monitoring may be particularly favorable are CF patients. Not only might the reduction of hospital visits contribute to the patients' quality of life, it may also reduce their risk to transmit their infection to other CF patients[36]. In addition, CF patients often have a difficult venous access, making venipuncture challenging[100]. Home-based DBS monitoring has already been performed in routine practice for the determination of the aminoglycoside tobramycin in CF patients[36]. Importantly, this strategy should only be performed when tobramycin is intravenously administered, since finger prick blood can be contaminated with tobramycin after nebulized administration, even after thorough handwashing[101]. Also for other types of antibiotics used in CF patients home-based monitoring has been proposed[102].

Table 1.3: Overview of the therapeutic drug classes determined in DBS and the advantages DBS has to offer.

DRUG CLASS	SPECIFIC ADVANTAGES OF DBS
Antibiotics [17,36,102]	CF patients [36]: Home monitoring of e.g. tobramycin Reduced number of hospital visits Increased quality of life Reduced risk of infection transmission to other CF patients TB: transport under ambient conditions to centralized reference laboratory (also in resource-limited settings) [17]
Antidepressants [37]	Psychiatric facility: transport under ambient conditions to laboratory Frequent home-based adherence monitoring
Antidiabetics [42]	Frequent home-based adherence monitoring HbA1c and creatinine can be evaluated on DBS as well [5,42]
Anti-epileptics [13,86,88]	Sampling immediately after seizure (in hard to control patients) [13] Facilitates more frequent monitoring e.g. during pregnancy [88] Neonates and children: small sample volume [86]
Anti-hypertensive drugs[103,104]	Frequent home-based adherence monitoring
Antimalarials [16]	Resource-limited settings (transport/stability) Reduced biohazard Parasitic load monitoring [16]

	Chloroquine: blood-based therapeutic interval [16]
Antimycotics [102,105]	Home-based TDM
	Children: small sample volume
Antipsychotics [37,106]	Frequent home-based adherence monitoring
	Psychiatric facility:
	Convenient sampling: patients might be less frightened [37] + safer
	Transport under ambient conditions to laboratory
Antiretrovirals [15,79,81]	Reduced biohazard
	Resource-limited settings (transport/stability)
	Recent and long term adherence can be evaluated for e.g. tenofovir [79]
	Viral load testing possible [91]
	Genotypic drug resistance testing possible [91]
	Anonymity [35]
Antivirals [107]	Reduced biohazard
	Ribavirin (hepatitis C treatment): anonymity [35]
Bronchodilators [108]	Sampling after asthma attack (in hard to control patients)
Immunosuppressants [40,41,56,96,97]	Abbreviated AUC e.g. tacrolimus: ease of timing, serial sampling [97]
	Cyclosporin A monitoring at C2: ease of timing [96]
	Blood-based therapeutic intervals for most immunosuppressants
Oncolytics [63,72,84,94]	Long term home-based monitoring e.g. tamoxifen [94]
	Anemic patients (common in cancer patients): low sample volume [31]
	Busulfan (myeloablation in children): low sample volume [84]
Pulmonary hypertension [109]	Treatment of pediatric patients: low sample volume [109]
	Frequent home-based adherence monitoring
Cholesterol control [104]	Caffeine (apnea of prematurity): limited sample volume [87]
Varia (selected examples)	Deferasirox (anemic patients): limited sample volume [89]
	Nitisinone (tyrosinemia I treatment) [83]:
	Neonates and children: limited sample volume, minimally invasive
	Simultaneous evaluation of tyrosine and succinylacetone
	Sodium oxybate: timing of sampling (late at night) [70]
	Thiorphan: more convenient sample collection [43]

1.4.1.2. Alternatives to classical dried blood spots

There are several alternatives to classical DBS. These are addressed in this section, as well as in Chapter 2, section 2.2.1 and the 'Broader international context, relevance and future perspectives' section.

Volumetric dried blood samples

The most convenient way to avoid the HT effect on DBS spreading is to analyze an entire fixed-volume DBS instead of a partial punch of a non-volumetric DBS. However, depositing a fixed volume of blood on a filter paper is not easily performed by patients, since this requires the use of pipettes or handheld volumetric capillaries. To resolve this issue, several approaches have been suggested that facilitate the preparation of volumetric DBS starting from a non-volumetric drop of blood. A first approach is the system developed by Leuthold *et al.*, in which a standard DBS card can be positioned and which utilizes integrated capillaries to transport a fixed volume of blood to the filter paper when a blood droplet is offered to the inlet of the

system[48]. Similarly, Lenk *et al.* used chip-based microfluidics to generate volumetric DBS on regular filter paper[47]. Alternatively, also VAMS devices can be employed to prepare volumetric dried blood samples[49]. These devices consist of a plastic handle with a hydrophilic polymer at the tip and absorb a fixed volume of blood independent of patient HT. However, experiments in our laboratory showed that although no HT effect could be discerned on the volume of blood absorbed, the VAMS devices seemed to be more prone to a HT effect on extraction recovery[110]. Although all these formats seem very promising, applications are absent or still limited, so further investigation is warranted. For a more elaborate discussion on these formats, see 'Broader internatonal context, relevance and future perspectives' section 1.2.1.

Dried plasma spots

To avoid the HT effect, others have suggested to use DPS instead of DBS. The former have the same advantages as DBS regarding biosafety, stability and ease of storage. Additionally, the obtained results are more easily compared to existing reference ranges and therapeutic intervals, which are generally established using liquid plasma or serum. Unfortunately, the preparation of DPS generally requires a centrifugation step (either when samples are obtained in the traditional manner or with special precision capillaries), which is not feasible in the real life home-based TDM context. Nonetheless, this approach might still be of added-value in specific cases due to the improved analyte stability and reduced transport and storage costs (e.g. when samples need to be transported to another lab for the required analysis). To circumvent the need for a centrifuge, several blood separation devices have been proposed for *in situ* DPS generation. The first one was a membrane-based device introduced by Li *et al*., which consists of a separation membrane that withholds the cellular material, letting the liquid part of the blood flow through, so it can be collected onto a filter[53,54]. A second device, commercialized as Noviplex™, is based on a similar principle, but yields volumetric DPS[55]. In addition, a novel blood separation device, which is composed of a spiral shaped filter paper, has been commercialized as HemaSpot-SE[111]. Although the above-mentioned approaches seem promising, published applications are still lacking. Importantly, it has also not yet been proven that the blood filtrate obtained is equivalent to plasma and at least some of these devices have shown a HT effect on the filtration process and on DPS-based quantitation[54].

Other dried matrix spots

The collection of biological fluids on filter paper has not been limited to whole blood and plasma, but has also been suggested for multiple other matrices. In the context of the analysis of therapeutic drugs the use of dried tear spots has been proposed for the quantitation of tobramycin[112], whilst dried OF spots have been employed for the analysis of lidocaine[113]. Dried OF spots could facilitate cost-effective sample transport and storage, as well as provide increased sample stability. Since for translucent matrices such as OF and tears, it can be hard to pinpoint the exact location of the spot on the filter paper, it has been suggested to impregnate the filter paper with a substance that changes color after contact with a biological sample, to facilitate correct analysis[114]. However, hitherto the use of these DMS in clinical practice seems limited.

1.4.2. Alternative specimen: oral fluid

OF consists of saliva (the aqueous secretion produced by the major salivary glands; the *glandula parotis, submandibularis* and *sublingualis*), the secrete of the accessory glands, gingival fluid, enzymes, other proteins, electrolytes, bacteria, epithelial cells, ora-naso-pharyngeal secretions, and food debris[115]. To enter the OF, drugs have to cross the capillary wall, the basement membrane as well as the cell membrane of the epithelial cells. This process is believed to be predominantly governed by passive diffusion, but also other transport mechanisms such as active transport, ultrafiltration and pinocytosis might play a role[115].

Since it is the free fraction of a therapeutic drug that can migrate into the OF, the latter has often been proposed as a convenient alternative to assess the free plasma concentration of a therapeutic drug (cfr. Section 1.3). This might be particularly relevant for TDM of highly protein bound drugs, as well as for the monitoring of drug levels in special patient populations such as pregnant women, hypoalbuminemic patients or patients with liver disease[32]. In addition, OF is readily available and easy to collect without the need for qualified personnel, even in difficult populations such as children, people with poor venous access or people with fear of needles.

OF can either be collected *via* drooling, expectoration, suction, swabbing or the use of commercial and non-commercial collectors[116-118]. Additionally, samples can be collected

without stimulation (*via* passive drooling), with mechanical stimulation (e.g. *via* movement of the tongue or by chewing of parafilm or paraffin wax), or with chemical stimulation (e.g. *via* the use of citric acid or a lemon juice drop). Importantly, although large differences exist in performance between OF collection devices, no particular one seems to consistently outperform the others[117]. Nonetheless, as will be discussed further, the choice of the collection method may be pivotal in the success of an OF-based TDM method[118].

A prerequisite for the usefulness of OF-based TDM is of course the existence of a (robust) correlation between the drug levels in OF and the (free) plasma levels. However, the amount of a therapeutic drug present in OF -and hence the correlation between OF and plasma levels- generally depends on a lot of variables such as compound molecular weight, size, lipophilicity, pKa and ionization status, as well as salivary flow rate and metabolism and plasma and salivary pH and protein binding[115,118]. Importantly, also the used collection method will affect the ratio between the OF and plasma drug levels, since stimulation (either mechanical or chemical) will change the flow rate and thereby the OF composition and pH[118]. The effect of the employed collection method on the observed ratio was for example demonstrated by Lomonaco *et al*. for the determination of warfarin in OF[119].

Furthermore, OF flow rate and/or composition are influenced by multiple factors such as circadian rhythm, diet, age, emotions, taste, smell, hormonal changes, the intake of medication with a parasympathetic or sympathetic (side) effect as well as the presence of certain disease states (e.g. infections increase the permeability of capillaries and kidney failure influences the salivary pH by increasing the amount of urea)[115,120]. In addition, the ratio between the OF and plasma drug levels may be concentration- or time-dependent. The former phenomenon was observed by Van Der Elst *et al*. for fluconazole. While at levels < 30 μg/mL OF and serum gave similar results, the OF/serum ratio was < 1 at higher levels, potentially indicating saturation of the amount of fluconazole in the OF[121]. A time-dependent ratio on the other hand was demonstrated by Wintergerst *et al*. for the quantitation of the antiretroviral indinavir, where a better correlation was observed at later time points, possibly due to a slow equilibrium between both matrices[122]. A similar time-dependent phenomenon was proposed by Berkovitch *et al*. to explain why a good correlation was found between OF and plasma levels of gentamicin after once daily administration, while no clear correlation could be observed when gentamicin was administered twice or thrice daily[123].

To minimize the influences of the parameters described above, standardization of the sample collection method and correct timing of the sampling are of utmost importance. However, standardization might be difficult in specific patient populations, such as neonates and children[124]. Another strategy that has been suggested to improve the correlation between the measured drug levels in both matrices is to correct for the salivary pH when the diffusion of the drug is pH-dependent[125].

Due to the variables described above, often a poor correlation between both matrices is observed or the obtained correlation displays an inter- and/or intra-individual variability that is too large to reliably derive the corresponding (free) plasma drug levels from the drug levels measured in OF[12,26,116]. Although, generally, unionizable drugs (or at least drugs that do not ionize within the salivary pH range) are regarded as optimal candidates for OF-based TDM, it always needs to be evaluated on a case-by-case basis during clinical validation whether the correlation between both matrices is adequate. However, even when no clear correlation can be observed between both matrices, it has been suggested that OF sampling can still be employed for short term adherence monitoring, as long as the therapeutic drug is consistently present in OF in quantifiable amounts (cfr. Section 1.4.2.1).

In addition, the Influence of the collection method (as well as other parameters) may explain the variability in reported OF/plasma ratios and correlation coefficients in different studies. Moreover, it needs to be remarked that sometimes OF drug levels are compared to total plasma drug levels, whilst other studies compare with free plasma drug levels, which may obviously yield different results.

The used collection method or collection device does not only affect the obtained correlation but also other parameters such as analyte stability and recovery[30,118,126]. Therefore, stability should always be evaluated in neat OF and on the collector, both over a relevant pH range and time period. Furthermore, recovery from collection devices can be concentration-dependent and should be determined over the entire concentration range[116]. Also the extent of analyte adsorption may be influenced by the used collector or stimulating aid (e.g. parafilm[127]). To improve stability and to reduce adsorption, some collection devices contain buffers, preservatives and surfactants. However, these may cause matrix effects, which should be taken into account during method development[28,116]. Also other materials used

during sample collection such as paraffin may yield interferences during analysis[116]. In addition, it has been postulated that the use of certain collection strategies may improve method reproducibility. For example, chewing of absorbent materials may help to standardize salivary flow rate and thereby reduce method variability[126].

As was stated previously, besides an optimal collection method, also correct timing is very important for OF sampling. Samples should be collected after steady state is reached and when an equilibrium between blood and OF is obtained. Since contamination from oral intake of medication may lead to erroneous results the first couple of hours after ingestion, it is advised to either collect samples before the next dose or after a sufficiently long 'wash-out' period[11,118]. Moreover, contamination issues may be aggravated if the patient has dental caries, since these may lead to the formation of so-called drug pockets[11]. To reduce the risk of contamination it has been suggested to ask donors to rinse their mouth before sample collection[11,128].

Although the collection of OF is easy to perform in most patients, sometimes it may be challenging to collect a sufficient sample volume due to xerostomia or dry mouth[30,115,120]. This issue might be age-related or can be caused by certain pathologies, medications, drugs of abuse or anxiety . Additionally, when using an OF collector, the volume of OF is unknown, which hampers OF-based quantitation[30,116]. Moreover, it is incorrect to assume that a fixed sample volume is collected, which is consistently recovered from the collection device[118]. To derive the collected OF volume, it has been proposed to weigh the OF collectors before and after sampling, which is of course tedious to perform[116]. Another strategy is to use a collection device such as the Greiner Bio-One Saliva Collection System (SCS), which contains a dye in the collection solution that allows to determine the collected OF volume photometrically[126].

Not only the sample volume is crucial, also sample quality is obviously of utmost importance. Samples can for example be contaminated with blood, which can drastically impact OF-based quantitation[11]. Especially for drugs with a very low OF/whole blood ratio, even a minor contamination with blood may yield extremely erroneous results. This can be a particularly important issue in people with gingivitis or gingival hyperplasia or severe mucositis[11]. To minimize the risk of blood contamination it is advised to collect samples before teeth brushing. Furthermore, OF samples may contain food debris or other contaminants[11,116].

Interestingly, it has been stated that unstimulated OF is more likely to contain debris[30], whilst OF samples collected after stimulation with citric acid yield cleaner samples and better chromatography[28]. Another practical issue is the fact that OF samples may be quite viscous, which leads to sample inhomogeneity, hampers pipetting and causes the formation of bubbles when samples are agitated[28,116,129]. To resolve this issue some have used sonication to break up the mucin[128], whilst others employed buffers to dilute the sample and to reduce sample viscosity[129]. Yet again, the choice of the collection method may impact sample quality. Gröschl *et al*., for example, noticed that neat OF samples remained sticky even after centrifugation, whilst samples collected with certain OF collectors yielded a cleaner matrix, potentially by removing mucopolysaccharides from the OF matrix[126]. Consequently, to warrant sample quality the collection method should be optimized during method development and colored or cloudy patient samples should always be discarded[11].

Another technical limitation for OF analysis might be the required method sensitivity[116]. Since OF drug levels (at least theoretically) correspond to the plasma free fraction, quite low drug levels can be anticipated. This is particularly true when trough levels are measured and/or when a dilution buffer is used during collection. For certain compounds which are subject to ion-trapping in OF, this issue might be less relevant.

1.4.2.1. Applications of oral fluid analysis in therapeutic drug monitoring

The non-invasive nature and the ease of OF collection may be favorable in certain patient populations and settings. This is illustrated below by discussing selected examples of OF-based TDM methods. Classes of therapeutic drugs that have been analyzed in OF include antibiotics[26,123,130], antidepressants[131], anti-epileptics[11,132,133], antimalarials[134,135], antimycotics[121,136], antipsychotics[137-140], antiretrovirals[122,141,142], immunosuppressants[128], and oncolytics[98,143].

Pediatric patients

Since OF collection is non-invasive and painless, it might represent a valuable alternative for TDM in neonates, infants and children. Furthermore, it may also provide a stress free alternative in children who are afraid of needles or who have poor venous access. In addition, OF collection is not only preferred over classic venous sampling by most pediatric patients, but also by their parents[144]. Since caffeine levels need to be monitored weekly during

treatment of apnea in prematures, it was evaluated whether OF would provide a good alternative to traditional TDM by comparing OF levels in infants with their corresponding plasma levels[145]. To facilitate OF collection a non-woven gauze attached to a cotton swab was employed, onto which the infants could suckle. Although unstimulated collection and stimulated collection using a citric acid impregnated gauze were more convenient to perform, the best correlation (r = 0.89) between OF and plasma levels was obtained when OF production was stimulated by depositing citric acid in the cheek pouch 5 to 10 min prior to OF collection. Another application of OF analysis in pediatric patients is the analysis of busulfan. Since conventional TDM requires serial blood sampling from a central (or peripheral) venous line -which is time-consuming and potentially distressing for both the children and their parents- OF collection could provide an interesting non-invasive alternative. The feasibility of such an approach was evaluated by Rauh *et al*. and a good correlation was observed between AUCs determined from OF and plasma busulfan levels. Interestingly, while a Salivette® collection device was generally used to collect OF, in patients under 2 years a modified medical pacifier was employed in which the teats had been perforated and a DBS filter paper had been inserted[98].

The elderly

Since albumin levels tend to be lower in the elderly, it is indicated to measure the free plasma levels of highly protein bound drugs in this population[32]. Therefore, OF analysis may be a good alternative, whenever OF levels correspond to the free plasma levels. This was for example demonstrated for the analysis of phenytoin in elderly patients[146]. In addition, the elderly are more likely to have difficult venous access, which further lends support to the use of OF in this population[5]. On the other hand, the elderly more frequently present with xerostomia, which may hamper OF collection. Indeed, the elderly typically take more chronic medication, amongst which anticholinergic medication or medication with anticholinergic side-effects, which is a major risk factor for dry mouth[120].

Pregnant women

As was discussed above, the levels of anti-epileptic drugs may change drastically during pregnancy and the post-partum period. In this context, Herkes *et al*. demonstrated that the frequent collection of OF samples at home may be a feasible approach to follow-up phenytoin plasma levels in pregnant women before and after delivery[132]. Although samples were

acquired more frequently during this study, the authors propose that pregnant women could collect OF at home once a week to minimize the risk of inadequate drug levels. In theory, similar results could be obtained *via* frequent plasma monitoring, however, this is generally hindered by logistic issues. In addition, OF phenytoin levels correspond well with free plasma levels[11], which is relevant for TDM in pregnant women, since it is known that pregnancy effects phenytoin protein binding[147].

Psychiatric patients

The fear and anxiety that accompany blood sampling in schizophrenic patients could potentially be avoided by using OF collection instead. In addition, a collection technique that does not require needles may be regarded as safer in a psychiatric facility. In some schizophrenic patients, however, OF collection might be challenging, since certain antipsychotics (or other co-medications) can have anticholinergic side effects, amongst which a reduction in salivary flow rate[148]. Recently, OF-based TDM of a diverse panel of antipsychotics was evaluated by comparing drug levels in OF and serum collected from psychiatric patients. Unfortunately, OF to serum concentration ratios showed a fairly large variability for all of the investigated antipsychotics, limiting the practical usefulness of the approach. Nonetheless, OF collection could potentially still be employed to screen for therapy non-adherence[137]. Another compound which has been evaluated in this context is lithium. Unfortunately, here as well, the correlation between OF and serum levels was not strong enough to make OF-based TDM a worthy alternative for classic Li TDM[138]. However, it has been postulated that although inter-individual variability in the OF to serum ratio is too large to allow OF-based TDM, intra-individual variability is much smaller and once an individual ratio is determined this could be safely used in clinically stable patients on prophylactic Li therapy[139]. In addition, El-Mallakh *et al*. found that when mucinous material was removed from the OF (*via* dialysis) the correlation with plasma levels increased drastically[140].

Patients with renal failure

Renal failure can have a pronounced effect on plasma protein binding. This was for example observed by Reynolds *et al*. who determined total and free phenytoin levels in 7 patients with chronic renal failure and found that these patients had low total plasma phenytoin levels while the free plasma levels were disproportionally high. Interestingly, drug levels determined

in OF correlated well with the plasma free drug concentrations, indicating that OF could be an adequate matrix to monitor phenytoin in patients with renal failure[133].

Remote and resource-limited settings

Considering no specialized personnel or equipment (e.g. centrifuges) are required to obtain OF samples, OF collection could potentially be of interest in resource-limited settings. In addition, OF samples may represent less of a biohazard than blood (e.g. for HIV), which is particularly important in areas where HIV is often endemic. However, to be applicable in resource-limited settings such as developing countries the analyte obviously needs to be stable in OF under field conditions. Unfortunately, that parameter -although crucial- is typically not evaluated.

To facilitate TDM of antiretroviral drugs in developing countries, multiple methods have been developed to analyze these drugs in OF. An example includes the semi-quantitative method developed by George *et al*. to detect nevirapine underexposure[142]. To render this method more field-compatible thin layer chromatography (TLC) with UV detection was employed due to its simplicity and low cost. Although this method originally proved to reliably identify those patients with subtherapeutic drug levels, remarkably, when Lamorde *et al*. tried to employ the same method, they could not visualize any spots on the TLC plates[141]. Since the reason for this problem could not be pinpointed, these authors employed LC-UV instead. However, the latter technique will probably only be available in specialist centers and therefore, requires samples to be sent over for analysis. Furthermore, also for the analysis of antimalarials several OF-based methods have been developed. While for some compounds, such as mefloquine, a good correlation (r = 0.88) was observed between OF and systemic levels[134], monitoring of other compounds, e.g chloroquine and proguanil, has been deemed unreliable[135]. Another disease which is endemic in developing countries and for which OF-based analysis could help in setting up TDM programs, is TB. The analysis of linezolid and clarithromycin in multidrug-resistant TB patients, for example, showed that the obtained $AUC_{0–12}$ values or $AUC_{0–24}$/MIC ratios (with MIC being the minimum inhibitory concentration) did not significantly differ from the ones calculated from simultaneously obtained serum samples[130]. Although this method was not specifically developed for use in developing countries, it might be valuable in this setting as well.

Home monitoring

As has been discussed previously, home-based TDM has multiple advantages for the patient, his caregiver and the clinician. The latter was nicely demonstrated by the questionnaire held by Baumann which revealed that clinicians who treat epilepsy patients would find it very valuable if a ‘‘real-time’’ concentration could be obtained when a seizure or adverse effect occurs at home .

Mycophenolic acid and fluconazole are two compounds for which home-based TDM using OF samples has been proposed. Mendonza *et al*. found a good relationship between mycophenolic acid concentrations in OF and the corresponding total (r = 0.909) and unbound (r = 0.910) plasma concentrations[128]. However, timing of sample collection seemed to be crucial as brushing of the teeth was suggested to have contaminated the majority of the early collected samples with blood. Van der Elst *et al*. observed a nice correlation (r = 0.96) between the OF and serum levels of fluconazole[121]. Moreover, fluconazole in OF was stable for at least 17 days when stored at RT and recovery from the collection device was not affected by storage at 20°C for up to at least 6 days. Therefore, fluconazole monitoring in OF might be feasible in patients receiving prolonged courses of antifungal treatment at home. Koks *et al*. also found a good correlation (r^2 = 0.80) between the levels of fluconazole in OF and plasma[136]. However, they concluded that it was still insufficient to derive the exact plasma fluconazole levels from the corresponding OF levels and that hence OF fluconazole levels could only be employed for compliance monitoring or for the semi-quantitative prediction of plasma fluconazole levels.

The feasibility of setting up a home-based TDM program using OF samples has been assessed by Tennison *et al*. for phenytoin, carbamazepine and phenobarbital[149]. Not only were these compounds stable during transport, patients were also able to collect the OF samples themselves. Similar stability results were obtained for the newer anti-epileptics as well[150]. In addition, it has been demonstrated that OF samples collected at home can be returned to the laboratory in a reasonable length of time[150]. However, it was also pointed out that in certain situations (e.g. to assess adverse effects) samples would have to be mailed via overnight carriers to allow a sufficiently short turn-around-time. To facilitate proper sample collection at home, the choice of the collection device might be crucial. The Quantisal™, for example, has a volume indicator which tells a patient whether a sufficient sample volume has been

collected, and may hence help to minimize the amount of samples with inadequate sample volume. The use of other collection devices such as the SCS on the other hand may be too complex and therefore less suitable for outpatient monitoring[126]. Additionally, dried sample formats (cfr. supra) might facilitate home-based TDM due to increased sample stability and ease of transport.

Cystic fibrosis patients

Although home-based OF analysis of antibiotics would be convenient for CF patients (cfr. supra), studies have suggested that this approach is not suitable for TDM of tobramycin and gentamycin[151,152]. In addition, CF patients tend to provide more viscous OF samples, which may complicate their analysis (cfr. supra)[115].

1.4.3. Alternative specimen: tears

Lacrimal fluid or tear fluid, which is composed of proteins, mucins, lipids, electrolytes, epithelial cells and ambient contaminants[153,154], has been proposed as a suitable alternative matrix for free plasma concentration monitoring[32,155]. This suggestion is based on the existing equilibrium between the free plasma fraction and the free fraction in tears, as well as on the low and constant protein content of lacrimal fluid[154,156]. Tear fluid offers the advantage over OF (which is also used for free fraction monitoring; cfr. supra) that it is more homogenous and has a more constant composition, particularly after stimulation[155]. Furthermore, contrary to OF, tears are only subject to relatively small changes in pH[154,156], implying a less pronounced, though still relevant, effect on transfer of ionizable drug into tear fluid[157].

Tear fluid can be obtained in multiple ways. First of all, direct tear collection can be performed by holding a capillary in the conjunctival sac[153]. This approach, however, requires a certain skill, as the capillary needs to be held in place, whilst avoiding injuries. Moreover, the eye has to be open for the entire collection procedure, which is unpleasant for the patient. Consequently, this technique cannot be utilized in non-cooperative patients and children, unless tears can be sampled from the cheek[158]. Alternatively, tears can be collected in an indirect fashion using Schirmer strips (i.e. a filter paper)[153]. To that end, a small part of a filter paper strip is folded at 90 degrees and positioned in the lower conjunctival sac for several minutes, during which the patient's eyes are closed. Afterwards the tear fluid can be recovered from the strip by centrifugation. The latter sampling method is not only more

feasible for non-ophthalmologists[153], but is also preferred by patients and yields more reproducible results[159]. Other indirect tear sampling methods include the use of absorbing materials such as sponges, swabs, filter paper discs and cotton thread[159,160].

Although 'basal tears' (i.e. unstimulated tears) can be sampled, it is also possible to stimulate tear production before sample collection[154]. The latter is often performed to obtain sufficient sample volume -especially when direct sampling techniques are employed- or to reduce collection times. These stimulated or 'reflex' tears can be triggered by i) non-contact methods such as looking into the sunlight, stimulation of the gag reflex or emotions ii) mechanical irritation caused by the use of e.g. the Schirmer strip itself or iii) chemical stimulation of the conjunctival or nasal mucosa with e.g. vix vapo rub, cigarette smoke, ammonia vapour, or formaldehyde vapour (although the use of the latter three has been abandoned in more recent times)[154,157,158,161,162]. As stimulation activates different lacrimal glands compared to standard physiological circumstances, the composition of the tear sample will be altered[154]. Furthermore, differences in flow rate between reflex tears and basal tears may contribute to discrepancies in measured drug concentrations[157,160]. Hence, the influence of tear stimulation on measured drug levels should always be evaluated. Especially the reproducibility of this phenomenon is of key importance, as dilution caused by stimulation may be time-dependent. To help improve sampling method reproducibility it has been advised to standardize both collection time and the volume withdrawn[159].

Although lacrimal fluid may provide an alternative way of assessing the free fraction of a drug, it suffers from certain drawbacks. First of all, tear sampling does not allow for patient self-sampling. Furthermore, the sampling itself requires a relatively long time and the stimulation of tear production can be noxious. Moreover, the composition of tear fluid and/or the volume produced can be influenced by age, flow rate, disease state, intake of (therapeutic) drugs, the use of contact lenses, the employed collection technique and the collection site[154,160]. Also differences in sample collection methods or lacrimal pH may yield contradictory results[157,160]. Additionally, as only a limited sample volume is available, tear analysis requires sensitive equipment.

1.4.3.1. Applications of tear analysis in therapeutic drug monitoring

Tear fluid analysis has mostly been discussed in the context of anti-epileptic drug monitoring. More specifically, the comparison between tear and OF-based therapeutic drug analysis has gained attention. Both for carbamazepine and phenobarbital, tear and plasma concentrations (r = 0.75 and 0.95, respectively) correlated better than OF and plasma concentrations (r = 0.38 and 0.60, respectively)[156]. Similar conclusions were obtained by Monaco *et al.*, although, the correlation coefficients found by the latter were less strong[161]. Furthermore, a better correlation was found between tear and cerebrospinal fluid (CSF) concentrations than between OF and CSF levels (r = 0.86 and 0.87 *vs*. r = 0.64 and 0.44), favoring the use of tears instead of OF for TDM purposes. Similarly, a good correlation was found between tears and plasma levels as well as tears and CSF levels for both diphenylhydantoin and primidone. However, only for diphenylhydantoine tear levels outperformed OF levels[163]. While for valproic acid (VPA) tear concentrations correlated better with plasma and CSF levels than OF levels did, it needs to be remarked that the correlation coefficients were still relatively low (r = 0.31 and 0.61, respectively)[164]. Nakajima *et al*. on the other hand found a correlation coefficient of r = 0.864 with total plasma concentrations and r = 0.936 with free plasma concentration[165]. Whilst the former group collected 100 µL tears after exposure to cigarette smoke or sniffing formaldehyde, the latter group employed Schirmer strips for tear collection, requiring only a very small sample volume (3.37 ± 0.41 µL), which may have contributed to the observed contradictory results. Furthermore, Nakajima *et al*. utilized on-card derivatization (i. e. direct derivatization to fluorobenzyl ester derivatives on the filter paper), followed by extraction and GC analysis, whilst the former employed an enzyme multiplied immunoassay technique. Lastly, for ethosuximide the correlation coefficients were similar for OF and tears *vs*. plasma levels (r = 0.73 and 0.74, respectively), whilst for both matrices no correlation could be found with CSF levels. The latter might have been due to the very limited sample number (n = 6)[166].

Furthermore, tears have been used for TDM of other drugs as well. Stoehr *et al.*, for example, employed tear sampling for the analysis of theophylline[158]. Although a good correlation was obtained between tear levels and either total (r = 0.94) or free plasma levels (r = 0.88), the method did not allow adequate free plasma level determination based on tear levels. Using the tear/free plasma concentration ratio as a conversion factor, only 50% of the patient

samples had a predicted unbound concentration within a 1 µg/mL deviation, limiting the clinical usefulness of this approach. Nakajima *et al*. also obtained similar results, with Pearson correlation coefficients of 0.8406 and 0.968 between tear and total, respectively free, plasma concentrations[167]. In addition, Steele *et al*. demonstrated that tear methotrexate concentrations correlated better with serum levels (r = 0.714) than OF levels did (r = 0.557)[168].

Although a correlation has been shown between tear and (free) plasma levels for multiple compounds, the clinical significance of tear sampling for TDM purposes is at best limited. Moreover, sampling is generally time-consuming, does not allow for self-sampling and is not easily applicable in children, a population for which alternative, non-invasive sampling would be particularly beneficial.

Recently, alternative ways of measuring tear concentrations have been proposed. Although not developed in view of TDM, a quantitative chromatographic method has been set up for the determination of tobramycin in 15 µL dried tear spots[112]. Furthermore, the use of contact lens sensors has been advocated for glucose monitoring in the lacrimal fluid of diabetics[169]. In the future this promising technique is likely to be expanded to the detection of other disease biomarkers. Moreover, the analysis of exogenous compounds, such as therapeutic drugs, present in lacrimal fluid may be envisaged, as TDM programs would greatly benefit from non-invasive, continuous drug monitoring.

1.4.4. Alternative specimen: interstitial fluid

Water and small, non-protein bound, solutes such as therapeutic drugs, are continuously exchanged between whole blood and the ISF under the influence of hydrostatic and osmotic pressure. Therefore, ISF forms an interesting alternative matrix for TDM, as its composition is closely related to plasma[170]. ISF can be sampled minimally invasively using reverse iontophoresis or microneedles[27,171]. Other methods of ISF sampling include clinical microdialysis[172,173], as well as the retrieval of fluid from induced skin blisters[155]. However, due to their less patient-friendly nature the latter sampling methods are not within the scope of this chapter.

The feasibility of using ISF as a valid alternative for blood-based TDM was demonstrated by Kiang *et al*.[170]. To this end, a diverse group of therapeutic drugs was administered to rabbits and the T_{max}, C_{max} and AUC were assessed in both blood and ISF. Although comparable results

were found for some compounds, ISF-based TDM did not seem feasible for others, indicating that the utility of ISF analysis needs to be evaluated on a case-by-case basis.

The most widely discussed technique regarding ISF-based TDM is reverse iontophoresis[27]. This sampling method extracts both charged and neutral drugs from the subdermal region in a non-invasive manner. To this end, a small electric current is sent through the skin after applying two electrodes, causing both electromigration as well as electroosmosis due to solvent drag. Eventually, positive and neutral molecules will accumulate at the cathode and negative components at the anode. As electroosmosis causes water migration from the anode towards the cathode, the extraction of positively charged molecules is facilitated, whilst the extraction of negatively charged molecules is hampered. In general, reverse iontophoresis is therefore most favorable for small hydrophilic or positively charged compounds[27].

In reverse iontophoresis the amount of drug excreted across the skin is assumed to correlate with the subdermal levels, which in turn need to correlate with the plasma concentrations. However, it needs to be taken into account that the measured ISF drug level can be dependent on the site of sampling[174]. Moreover, certain drugs may form a skin reservoir, which has to be depleted before a concentration can be measured that is proportional to systemic levels[175]. After collection, the sample can be analyzed in the lab or biosensors can be incorporated in the reverse iontophoretic system allowing ambulatory monitoring[27].

Despite the non-invasive nature of this sampling technique, it may cause some minor adverse effects such as erythema or edema. Furthermore, reverse iontophoresis is fairly costly and necessitates complicated technology. Moreover, it may require calibration with a whole blood sample and results can be affected by contamination from sweat[27].

1.4.4.1. Applications of interstitial fluid analysis in therapeutic drug monitoring

As the non-invasive nature of this technique offers the possibility of frequent sampling, even in vulnerable populations, Sekkat *et al*. evaluated the feasibility of monitoring caffeine and theophylline in neonates[176]. In this context they evaluated the effect of the functionality of the *stratum corneum* barrier on measured drug levels using tape-stripped porcine skin as a model. The authors concluded that at least for certain compounds, such as caffeine and

theophylline, which are transferred *via* electroosmosis and electromigration, the maturity of the skin drastically influences the obtained concentration.

Most research regarding the use of reverse iontophoresis for TDM purposes has been performed *in vitro* or *ex vivo*. However, a rare *in vivo* application was published by Leboulanger *et al*. regarding Li monitoring in patients with bipolar and schizo-affective disorders (n = 23)[175]. Interestingly, the correlation between extracted Li and serum concentrations was poor after 30 min of extraction, whilst it improved dramatically after 60, 90 and 120 min This observation indicated the presence of a drug reservoir in the skin and demonstrated the relevance of sufficient extraction time. Furthermore, the effect of using Na^+ as an IS was evaluated. Na was selected for this purpose as its extraction flux is constant. Using a training set of 10 patients, the ratio of Li_{ISF}/Li_{serum} and $(Li_{ISF}/Na_{ISF})/Li_{serum}$ was established and subsequently used to predict serum concentrations for the 13 remaining patients. This yielded excellent results, with the Na-normalized values leading to a smaller error on the serum level estimation. However, for applicability in routine practice miniaturization of the extraction system would be required as well as a shorter extraction time.

A more convenient alternative for ISF sampling is the use of microneedles[171]. Previously, these devices have been employed for drug or vaccine delivery, but recently their potential for ISF or blood sampling has been recognized. Microneedles have micron-scaled protrusions ranging from 50 to 150 µm for ISF sampling, and slightly larger needles (1000 – 1500 µm) for blood sampling. Biological fluid can be collected in hollow microneedles and either analyzed off-site or on-site using integrated detection systems. Alternatively, microneedle materials have been employed that undergo transition into a gel phase upon fluid collection[177]. Using the latter technique the risk for blockage or breakage of the needles and hence, non-biodegradable substances getting stuck in the skin, is avoided. Microneedles may not only be preferred over traditional blood sampling in a pediatric population[178], they also offer the potential for patient home sampling. However, when performing ISF sampling, attention must be paid to the fact that ISF levels may lag behind on systemic levels. Furthermore, as was discussed for reverse iontophoresis, skin reservoirs may exist for certain molecules, which must be depleted before microneedle sampling can be performed successfully.

1.4.5. Alternative specimen: hair

Hair analysis has been proposed as a convenient tool for long-term compliance monitoring, since it can provide retrospective information about a patient's drug exposure over the last weeks or months[179]. This could be particularly beneficial for the detection of so-called "white coat compliance". Indeed, patients who only adhere to therapy shortly before a hospital visit could be mistakenly considered as compliant based on their plasma levels, whilst their aberrant long-term adherence could be picked up using hair analysis[180]. However, contradictive opinions about the usefulness of hair analysis for TDM can be found in literature[181].

Although the exact incorporation mechanisms are not yet fully elucidated, therapeutic drugs are believed to enter the hair root *via* passive diffusion from the surrounding capillaries during the anagen phase. As the hair grows, they then become sequestered in the hair shaft during keratinization. Other routes by which drugs can access the hair are *via* sweat and sebum excretion, as well as *via* external contamination. Drug incorporation is favored for lipophilic and alkaline compounds and is influenced by many variables, as will be discussed further[179,182,]

Hair samples are preferably collected from the *vertex posterior* since the hairs in this region are more likely to be in the anagen phase, display a more stable growth rate and suffer less from age- and sex- related differences[179,182]. Furthermore, samples have to be cut as close as possible to the scalp and have to be stored at RT protected from sunlight. Although hair is generally an easily accessible matrix, the collection of hair samples may be challenging in certain populations due to cultural traditions[183], spiritual beliefs and superstition[184]. However, by properly informing the patient about the goal of the hair analysis and the fate of the hair sample, this issue can be overcome. The feasibility of hair collection in developing countries was confirmed by Gandhi *et al*.[185]. Moreover, acceptability of hair analysis may depend on the amount of hair that needs to be collected. The required sample size may vary from a single hair to about 200 mg[182,186]. Additionally, scalp hair is not always available. As an alternative, axial, pubic or other hairs could be collected. However, drug levels can be affected by the collection site, due to e.g. differences in growth rate. In this respect Liu *et al*. showed that pubic hair could not substitute for scalp hair in tenofovir monitoring[187].

As drug concentrations in hair are low (typically pg/mg to ng/mg), sensitive analytical techniques such as LC-MS/MS are required[179]. Moreover, hair analysis necessitates an elaborate pre-analytical phase, comprising multiple decontamination steps to remove external contamination, followed by hair homogenization, hair extraction, and additional sample clean-up procedures. These manual steps make hair analysis tedious and time-consuming[179].

Furthermore, the interpretation of quantitative and even qualitative hair analysis results is complicated by a multitude of factors. First of all, inadequate removal of external contamination leads to overestimation of hair levels or false positive results. Moreover, it has been postulated that traditionally used washing steps may actually promote the incorporation of external contaminants in hair instead of resulting in their removal[188,189]. In addition, hair treatments such as bleaching, coloring, perming and hair flattening may damage the hair matrix, making it more accessible for external contaminants[179]. On the other hand, an increased accessibility may also cause leakage of incorporated drugs, yielding lower hair levels and even false negative outcomes. In addition, high temperatures and noxious agents used during cosmetic hair treatment can have a direct deleterious effect on therapeutic drugs[179]. Other influences on the extent of drug incorporation are hair color (i.e. melanin content), ethnicity, gender, age and the amount of hairs in the anagen phase. To minimize the effect of hair color on the obtained quantitative results, it has been suggested to normalize drug levels to the hair melanin content[190].

As can be deduced from the above-mentioned issues, the correlation between systemic and hair drug levels is subject to ample variables which cause large inter-individual differences. Therefore, it is impossible to evaluate strict therapy adherence based on absolute hair levels[181]. However, as intra-individual variation is expected to be less pronounced, relative changes in compliance could potentially be monitored using segmental hair analysis, in which the patient acts as his own control[180]. To that end, consecutive 1-cm segments of hair are analyzed and drug levels compared. Each segment corresponds to approximately 1 month, with the most proximal segment corresponding to the most recent drug exposure. Although a brief interruption of therapy would not be noticeable using this approach, large variations in drug taking behavior (so-called drug holidays or white coat compliance) or complete non-compliance could be picked up.

Nonetheless, also segmental hair analysis is accompanied by challenges and limitations. First of all, the evaluation of recent drug intake is not feasible[179], since hair requires 7 to 10 days to emerge from the scalp[191]. Furthermore, segmental hair analysis is subject to a wash-out phenomenon, meaning that hair levels progressively decrease along the hair shaft. Attempts have been made to compensate for this phenomenon based on a correction factor established in adherent, healthy volunteers. However, this factor is tedious to determine and susceptible to inter-individual variability due to differences in e.g. hair structure and hair hygiene rituals. Moreover, although similarities have been observed in the temporal profile of hair concentrations and dose regimens, correlating drug levels in a certain segment to a specific moment in time remains challenging, as hair growth rates differ both inter- and intra-individually[192]. In addition, it has been observed that a single dose intake can yield detectable drug levels in multiple consecutive or even all hair segments, instead of only the segment encompassing the time of drug intake[193,194].

1.4.5.1. Applications of hair analysis in therapeutic drug monitoring

Williams *et al*. found that carbamazepine levels in the proximal 1-cm hair segment of adherent inpatients correlated with administered dose ($r = 0.581$)[180]. However, due to considerable inter-patient differences, absolute compliance could not be assessed based on hair levels. On the other hand, a relatively stable individual carbamazepine incorporation rate (an intra-patient coefficient of variation or CV of 15% ± 5.2%) was observed, indicating that segmental hair analysis could be utilized to detect compliance changes. Therefore, this approach was employed to reveal carbamazepine or lamotrigine therapy cessation during pregnancy, which is known to occur due to fear of teratogenic effects[195]. To that end, hair segments corresponding to periods before and during pregnancy were compared after correction for the wash-out phenomenon. Additionally, segmental hair analysis in post-mortem samples of epilepsy patients showed a larger intra-individual variability in anti-epileptic hair concentrations in sudden unexplained death victims, compared to the ones determined in epilepsy inpatients and outpatients[196]. These findings suggest that long-term compliance monitoring could help to prevent the occurrence of sudden unexplained death in epilepsy patients.

Furthermore, Müller *et al*. investigated the usefulness of hair analysis for cyclosporine A compliance monitoring[192]. Although a correlation (r^2 = 0.57) was observed between cyclosporine A hair levels and the average of blood trough levels measured within the corresponding timeframe, hair levels did not allow to determine drug intake. Yet, the observed hair concentration profile aligned with the dosage regimen, suggesting segmental hair analysis can be helpful in the detection of major non-compliance or drastic changes in drug taking behavior[192].

Moreover, hair analysis has received special attention in psychiatric patients and patients with behavioral problems, as compliance can be very challenging in these populations. In this context Uematsu *et al*. found a significant correlation between haloperidol hair and plasma levels (r = 0.772) as well as haloperidol daily dose (r = 0.555)[197]. Also here, relatively large inter-individual variations were observed, limiting the usefulness for TDM purposes. On the other hand, the same group demonstrated that haloperidol hair levels showed noticeable changes following (strong) dosage adjustments or therapy discontinuation[198,199], again indicating hair analysis could be valuable in detecting pronounced changes in therapy adherence. Furthermore, analytical methods have been developed to determine atomoxetine, methylphenidate and their respective metabolites in hair of children and adolescents treated for ADHD[200,201]. Although no correlation could be observed between daily dose and hair drug levels for either compound, the authors concluded that hair analysis could still be valuable to monitor long-term compliance.

Another domain in which hair analysis has been extensively investigated is TDM of (prophylactic) antiretroviral therapy. Because single plasma levels are known to show pronounced intra-individual variation, the analysis of multiple plasma samples over the course of several days is generally advised to assess average antiretroviral drug exposure. Since hair analysis is believed to provide an indication of average drug exposure, it has been proposed as a more convenient alternative[187].

To establish the usefulness of hair analysis for tenofovir compliance monitoring 2, 4 or 7 doses per week were administered to healthy volunteers to mimic different degrees of therapy adherence[187]. Although mean hair levels increased with the amount of doses received, the accompanying 95% concentration ranges clearly overlapped. Also, for 3 out of 23 patients

hair levels did not always increase with dosage frequency. Additionally, it needs to be noted that only individuals with dark hair were included in this study. Furthermore, Baxi *et al*. demonstrated that tenofovir and emtricitabine hair levels correlated significantly with Medication Event Monitoring System caps openings (r = 0.50 and 0.58, respectively), plasma (r = 0.41 and r = 0.51, respectively) and peripheral blood mononuclear cell levels (r = 0.43 and r = 0.50) and hence, concluded hair levels could be utilized for compliance monitoring[202]. Interestingly, these correlations were noticeably more pronounced after 16 weeks compared to 8 weeks (r = 0.61 – 0.86).

Besides the use of hair levels to monitor compliance to antiretroviral therapy, multiple papers have evaluated the correlation between these levels and therapeutic outcome. Bernard *et al*. demonstrated that mean indinavir hair levels were significantly higher in treatment responders (24.4 ± 16 µg/g) than in non-responders (12.9 ± 8.6 µg/g), further confirming their previous results[203]. Comparable data were obtained by Duval *et al*. who showed that, although a large overlap was observed between the hair levels of both groups, hair levels predicted viral response more adequately than a single plasma trough level[204]. Similarly, Huang *et al*. found higher mean lopinavir and efavirenz hair levels in responders than in non-responders (1.6 *vs*. 0.3 and 3.4 *vs*. 0.68 ng/mg)[205].

Others have taken it even one step further and have set up clinical decision trees based on antiretroviral hair levels. Van Zyl *et al*., for example, developed an algorithm based on lopinavir plasma and hair levels to verify the cause of therapy failure in second-line regimens in a cost-effective manner[206]. Only when both recent and average drug exposure levels were adequate (i.e. 1 µg/mL in plasma and 3.63 ng/mg in hair), the authors advise genotyping to confirm resistance. Furthermore, Gandhi *et al*. established a decision tree to prolong the durability of the first-line antiretroviral treatment with atazanavir, especially in settings where viral load monitoring is not available[207]. More particularly, the authors recommend to analyze a hair sample 4 to 6 weeks after therapy initiation. If the measured drug level is ≤ 1.78 ng/mg, this may be indicative of impeding treatment failure or resistance development and either additional compliance motivation or an appropriate PK intervention is required. Also in the case of disease progression or suspected PK changes hair level assessment is advocated.

1.4.6. Alternative specimen: exhaled breath

Not only volatile but also non-volatile exogenous compounds can be detected in exhaled breath. Indeed, after systemic uptake, drugs can enter the lungs *via* the bloodstream, after which they may become incorporated in aerosol particles, generated during exhalation from the fluid lining of the respiratory tract[208]. To this end, drugs need to pass the capillary wall, the interstitial space and the alveolar epithelial cells[209], a process that favors small, lipophilic compounds. Moreover, these lipophilic compounds are more likely to concentrate at the liquid surface and vaporize more easily, enhancing their concentration in aerosol particles[209].

Exhaled breath is an extremely accessible sample matrix that can be collected either in a bag, *via* adsorption to a sorbent material, collection on a filter or by breath condensation. Aside from these collection methods that are suitable for off-line analysis, direct breath analysis has also been performed. Concentrations of non-volatile compounds in exhaled breath are generally in the pg to ng/L range and hence, require very sensitive, mass spectrometry-based analytical systems. In this respect a collection method that allows for pre-concentration, as e.g. occurs with breath adsorption, may be advantageous[209].

1.4.6.1. Applications of exhaled breath analysis in therapeutic drug monitoring

Recently, Beck *et al*. evaluated the usefulness of D-amphetamine and methylphenidate measurements in exhaled breath for compliance monitoring in patients receiving oral therapy for ADHD[208,209]. Patients were sampled within 24 hours after last dose intake and the microparticles in their exhaled breath were captured on a polymer filter. The latter was attached to a bag to somewhat standardize the amount of breath that was collected. Subsequently, the filters were extracted and the extracts analyzed for their drug content. For D-amphetamine no correlation between breath level and dose could be observed (n = 9). On the other hand, a correlation coefficient of r = 0.84 was found between methylphenidate levels in exhaled breath and dose (n = 8).

Furthermore, direct breath analysis using extractive electrospray ionization (EESI) has been employed for the monitoring of VPA[210]. After VPA intake, extra peaks were observed at *m/z* 143 and 160, which the authors attributed to the presence of respectively 4-OH-VPA-γ-lactone (*m/z* 143) that was used as a biomarker of VPA exposure, and its non-covalent ammonium complex (*m/z* 160). When the EESI signal at *m/z* 143 was plotted against the free

VPA concentration in blood a r^2 of 0.89 was observed (n = 6). On the other hand, a different group reported on the use of 3-heptanone as a biomarker of VPA exposure in exhaled breath[211]. However, despite 3-heptanone levels being elevated in all VPA treated patients, these neither correlated with VPA serum concentrations nor with VPA dose. As 3-heptanone occurs naturally in exhaled breath, a possible explanation for this observation may be that other factors also affect the breath 3-heptanone level[210].

A completely different strategy to assess therapy adherence using a breath test was developed by Morey *et al.*[212]. More specifically, these authors proposed to co-formulate the active compound of a drug with a taggant, which after systemic uptake is metabolized into a volatile adherence marker that can be measured using a portable, miniature gas chromatograph. In this context it was demonstrated that either 2-butyl acetate or 2-pentyl acetate added to a vaginal gel leads to sufficient levels of the esters and corresponding alcohols and ketones in exhaled breath to assess vaginal gel adherence. Similarly, 2-butanol was used as a taggant to assess compliance to oral therapy[213]. The taggant 2-butanol was metabolized to 2-butanone and appeared in exhaled breath of all participants (n = 8) shortly after capsule intake. The concentration measured after 10, 15, 20 or 30 min yielded an area under the receiver operating-curve of 1 with a 95% confidence interval (CI) of 1.00 – 1.00, using a cut-off of 9.7, 5.4, 5.2 or 3.7 ppb, respectively.

1.4.7. Alternative specimen: sweat

The first report of therapeutic drugs being detected in sweat dates back to the nineteenth century, when quinine excretion *via* perspiration was observed[214]. However, a patch for more convenient sweat collection was not developed till 1980[215]. As these patches were occlusive and lead to skin irritation, local pH changes and alterations in colonizing bacteria, they have more recently been replaced by non-occlusive patches, which allow the diffusion of water vapor, oxygen and CO_2. These patches can be left on for more than a week, assessing cumulative drug exposure. Alternatively, swabs or wipes can be employed for sweat collection, providing information about more recent drug exposure (< 24h)[29].

Drugs are mainly incorporated into sweat *via* diffusion from the capillary system surrounding the sweat glands. Furthermore, transdermal migration of drugs and sebum excretion also contribute to the measured drug concentration in sweat. As these processes favor more

lipophilic components, mostly the parent compound is detected. In addition, alkaline drugs can accumulate in sweat due to its acidic pH (mean pH of 6.3)[29].

Since eccrine and apocrine sweat glands as well as sebum glands are unequally distributed throughout the body, sweat drug concentrations are dependent on sample location. Furthermore, these concentrations can be affected by sweat flow, which in turn depends on emotional status, ambient temperature and physical activity. This entails that the collected sweat volume is unknown, impeding quantitative analysis. Moreover, problems with drug stability in the sweat patch and the risk of accidental patch contamination upon patch application or removal further complicate interpretation of sweat-based results[29]. As drugs are also excreted *via* fingertips[216], proper precautions should be taken to avoid contamination during manual sweat patch manipulation. Due to the high inter- and intra-individual variability in sweat drug concentrations, sweat testing can only provide qualitative results[29]. This implies that only total non-compliance could be detected using this sampling method.

1.4.7.1. Applications of sweat analysis in therapeutic drug monitoring

The anti-epilieptic drugs phenytoin and phenobarbitone have been detected in sweat[217]. For phenytoin a good correlation with the free plasma concentration was reported, whilst phenobarbitone concentrations seemed to depend on sweat flow. Furthermore, clozapine quantitation in sweat patches has been investigated for compliance monitoring in schizophrenic patients[218]. However, although a correlation coefficient of 0.589 was found between clozapine sweat concentrations and administered dose, large inter-individual variations were noted, indicating sweat is not suitable to assess patient compliance.

Sweat-based non-invasive compliance monitoring in ADHD patients has been evaluated by Marchei *et al*. . Methylphenidate was found in 8h post-dose sweat patches (n = 3), whilst ritalinic acid could not be determined. However, a fairly large inter-individual variability in methylphenidate concentrations in patients receiving the same dose was reported[219]. Furthermore, it was estimated that only 0.08% of the administered methylphenidate dose was excreted *via* sweat over a 24h-period, indicating the necessity of a sensitive analytical technique[219]. In a second set of experiments, the determination of the norepinephrine reuptake inhibitor atomoxetine and its metabolites 4-OH-atomoxetine and N-desmethylatomoxetine in sweat was assessed[220]. Yet again, only the parent compound could

be quantified in sweat, whilst both metabolites could not be detected[220]. This method was applied to six pediatric patients in a follow-up paper and peak atomoxetine concentrations appeared not to correlate with dose nor with patient's body surface[221].

1.4.8. Alternative specimen: nasal mucus

An example was found in scientific literature where nasal mucus was used to monitor oral drug therapy[222]. This alternative approach was suggested to be of value for the assessment of drug efficacy and toxicity during hyposmia treatment with theophylline. To this end, nasal discharge was directly and non-invasively collected in a plastic tube and theophylline levels in nasal mucus and serum were compared. A Pearson correlation coefficient of 0.63 was found and nasal theophylline concentrations varied between 53 to 97% of the corresponding serum concentrations.

1.5. NEWBORN SCREENING

The use of DBS sampling as an alternative for conventional blood sampling in neonates has become a widespread technique in NBS programs, ignited by the demonstration of Guthrie and Susi in 1963 to use newborn DBS to determine phenylketonuria[1]. DBS-based NBS by (LC-)MS/MS has exponentially increased since the 1990s and has become an established procedure in developed countries[223]. Moreover, the experience gained from NBS has undoubtedly facilitated the development of DBS-based applications in other fields as well. NBS can be divided into primary screening tests and second-tier tests. The primary tests are designed to identify as many inborn errors as possible. Since diagnostic sensitivity is favored over specificity for disorder detection here, the number of false-positive tests increases. Therefore, second-tier tests have been implemented, enabled by the introduction of MS/MS methods, to improve the specificity of disorder detection. A second-tier test is performed using the same DBS, is characterized by a lower sample throughput and is extremely suitable to confirm or refute an initial positive result, due to the measurement of additional metabolites[224]. In addition, a distinction has to be made between direct and indirect screening. Direct screening examines endogenous substances, while indirect screening focusses on the conversion of substrates by specific enzymes[225]. Current NBS programs screen for up to over 50 disorders [2,226]. Of these, 20 to over 40 disorders can be screened for by LC-MS/MS[223]. However the exact number of disorders that is screened for varies strongly from

country to country[2,223]. A key advantage of the (LC-)MS/MS technology is that it is highly multiplexeable (e.g. a multiplex assay of lysosomal enzymes in DBS), making the procedure very attractive in routine NBS as a diagnostic platform for the early detection and confirmation of genetic disorders[227,228]. As it is beyond the scope of this chapter to provide a full overview of the metabolic diseases that can be screened for by tandem MS, we refer to a comprehensive review by Lehotay *et al*. on this topic[223]. Furthermore, DBS can also be used at a later age, for the follow up of (treatment) of inborn errors of metabolism, as is currently applied for patients with e.g. maple syrup urine disease, phenylketonuria or tyrosinemia type I[229].

1.6. ENDOCRINOLOGY

The measurement of sex steroids, testosterone and especially estradiol, serves as a key tool in the diagnosis or management of a wide range of disorders, such as hypogonadism, polycystic ovary syndrome, amenorrhea, disorders of puberty, male and female infertility and tumors of prostate, testes, breast and ovary[230]. Sex steroid testing has known a noticeable transition from colorimetric assays using urine, over manual radio-immunoassays and automated immunoassays using serum to LC-MS/MS methods[230]. Improved precision, sensitivity and selectivity compared to automated immunoassays and the capability of multiplexing methods has resulted in substitution of traditional sex steroid immunoassays by LC-MS/ MS methods in large reference clinical laboratories. OF serves, next to plasma and serum, as a matrix for sex steroid determination. Progress in LC-MS/MS has allowed to -at least partly- cope with the low hormone concentration, one of the challenges coupled to OF analysis. However, still, sensitivity remains the limiting factor when considering e.g. the assessment of testosterone in children and women, and, to a lesser extent, in hypogonadal men *via* alternative sampling strategies. Furthermore, cortisol and progesterone are also detectable in OF by LC-MS/MS[20]. Cortisol has been determined in OF for the diagnosis of Cushing's syndrome and numerous stress-related disorders, as it is considered a 'stress' biomarker. Importantly, OF can be used for home sampling, which can be of interest for example in the diagnosis of Cushing's syndrome, since late-night measuring of cortisol levels in OF is recommended as a first-line screening test[231]. Furthermore, additional stress -which could affect cortisol test results- due to venous sampling and/or hospital visits is avoided in this way. Progesterone, a hormone that plays a pivotal role in the regulation of the menstrual

cycle and in the maintenance of pregnancy, also has been measured in OF, to determine luteal and placental functions in non-pregnant and pregnant women, respectively[232]. Besides OF, also DBS can be used for the LC-MS/MS-based determination of steroid hormones, including corticosterone, deoxycorticosterone, progesterone, 17-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, testosterone, dihydrotestosterone and cortisol, although not in all cases the required sensitivity will be achievable[233]. An upcoming tool for the assessment of long-term cortisol secretion, as a biomarker of chronic stress in various settings, is the analysis of hair cortisol concentrations *via* LC-MS/MS[234]. Anti-Müllerian hormone is a predictor of the ovarian response in women undergoing ovarian stimulation for in vitro fertilization. Since it can be quantified in DBS, this minimally invasive sampling strategy could be another possible future LC-MS/MS-based application in women undergoing fertility treatment[235]. Other hormones that have been measured in OF and/or DBS *via* LC-MS/MS include 25-hydroxyvitamin D, melatonin and thyroxine (T4)[20,236,237]. T4 determination in OF may serve as a simple and cost-effective alternative to free T4 measurement in serum, used in the diagnosis of thyroid disorders. Clinical application of the method could be an interesting future prospect, since T4 measurement in OF turned out to be useful in the diagnosis of Graves disease[20]. Total T4 can also be measured in DBS *via* MS/MS, along with immunoassay-based determination of thyroid stimulating hormone and anti-thyroid antibodies[238-240].

Overall, OF- and DBS-based hormone tests are an upcoming tool allowing patient friendly evaluation of endocrine functions. While undoubtedly immunoassays will continue to be used for routine measurement of hormones, LC-MS/MS methods are increasingly being integrated in clinical routine due to the disadvantages (e.g. sometimes poor specificity and accuracy) associated with immunoassays. Clearly, for the accurate determination of hormones in OF and DBS, LC-MS/MS is the method of choice. For OF it needs to be remarked, though, that although the concentrations measured in this matrix may correlate with the serum/plasma free fraction, they are not necessarily equivalent. For example, Fiers *et al*. nicely demonstrated that salivary testosterone concentrations measured by LC-MS/MS are not identical to free testosterone concentrations in serum[241]. Moreover, as mentioned in section 1.4.2, pre-analytical issues, amongst which contamination of the OF with blood, as well as the choice of the collection method, may have an impact on the result[242]. Hence, the decision

whether OF may truly serve as a more convenient and inexpensive alternative to serum/plasma for free active hormone testing may actually depend on the clinical question.

1.7. TOXICOLOGY

Alternative samples like hair and OF have become an established part of toxicological investigations in many countries, with analyses being performed in both forensic and clinical labs. Although these samples can provide valuable information, their analysis is also accompanied by some challenges, as discussed in section 1.4.2. In several countries, OF has become -or will soon become- the matrix of choice for immunoassay-based on-site drug screening. Whereas blood is the classical matrix for unequivocal MS-based confirmation of a positive on-site screening test, also OF can serve this purpose. For this confirmation the toxicology section of clinical labs may play a role. Given the potentially high sample-throughput, focus has been put on the development of automated procedures, e.g. applying automated SPE (on-line or off-line) and on-line sample clean-up procedures, typically followed by LC-MS/MS[243]. Another matrix often considered in the context of drugs and driving -albeit covering another time frame- is hair. Indeed, hair analysis is increasingly being used for demonstrating drug abuse or for the determination of (bio)markers, such as the alcohol markers ethyl glucuronide and fatty acid ethyl esters. This approach is currently readily being applied on a routine basis in several countries (e.g. in Sweden, Switzerland and Germany) in driving license regranting programs, providing some labs with a throughput of several thousands of samples per year[244-246]. Such throughputs, combined with the need of ultimate sensitivity, are offered by LC-MS/MS.

Also the use of DBS for toxicology purposes has been advocated[6]. Samples can be obtained from adults or from newborns, e.g. to assess exposure to drugs, alcohol and other xenobiotics prior to birth. For an overview of analytes of particular forensic interest that have been measured in DBS the interested reader is referred to previous work of our group[21,247]. The ease of (rapid) sampling and the stabilizing effect are two significant advantages coupled to DBS sampling in (forensic) toxicology. In newborn DBS, benzoylecgonine and cotinine, respectively metabolites of cocaine and nicotine, have been determined to evaluate the use of cocaine and tobacco products among childbearing woman[6]. For most applications, the limited amount of material, combined with the required sensitivity, imposes the need for a dedicated LC-MS/MS configuration. Recent progress in this field includes the set-up of hands-

off on-line systems (see also section 1.10)[248]. When considering the cut-off for driving under the influence of drugs, alcohol and medicines (DRUID), for most analytes procedures have been described that are able to achieve the required sensitivity when starting from a ≤ 6.4-mm DBS punch or when starting from 10 µl or less dried blood[247]. Like OF, DBS may offer more convenient sample collection in the context of driving under the influence of drugs, as the usual urine sampling is coupled to privacy issues and the collection of a conventional blood sample by summoned medical staff is cumbersome and time-consuming[249]. Also in the context of driving license regranting programs, in which drivers with a history of alcohol abuse are followed up, DBS sampling may be beneficial. We recently demonstrated this for phosphatidylethanol (PEth), a direct alcohol marker that is used to monitor alcohol consumption during the past few weeks: capillary concentrations of PEth were equivalent to those found in venous blood, demonstrating that capillary DBS are a valid alternative for venous blood for this purpose[250]. Since the sampling procedure does not require dedicated staff and PEth outperforms indirect markers like carbohydrate-deficient transferrin (CDT), capillary DBS sampling offers a promising avenue for routine follow-up of drivers with a history of alcohol abuse. As is the case for CDT now, PEth determination might become a routine procedure integrated in the clinical lab. Again, LC-MS/MS is the method of choice, combining both high-throughput and sensitivity. In the toxicology lab, DBS can also be used as a sampling preparation strategy. We routinely use DBS (as well as other DMS) for quantitative determination of gamma- and beta-hydroxybutyric acid. Although for these particular analyses we use “on-spot derivatization” and GC-MS[251-253], the use of dried (blood, urine, . . .) spots as an analytical tool (also allowing automation – see section 1.10) prior to LC-MS/MS can be applied for other compounds as well[21,254,255]. When considering toxicology screening in an acute setting, liquid microsampling, coupled to e.g. on-line sample cleanup procedures like turbulent flow chromatography and MS/MS detection, are more likely to be used than DBS, since in most cases it would not make sense to wait for a sample to dry. Yet, it is conceivable that for screening purposes approaches like paper spray-MS/MS or -HRMS (see further in section 1.11.4) might be employed in future, to get an instant identification of an intoxicant. Again, a drop of blood might suffice.

Over the last years, meconium has proven to be a valuable matrix in the assessment of prenatal exposure to drugs of abuse and has gained a lot of interest due to the higher

sensitivity, the easier sample collection and the larger detection window than traditional matrices, such as neonatal hair and urine[256]. To date, effort has been put in the development of advanced broad-spectrum screening methods using LC-MS/MS, facilitating the use of meconium in clinical routine screening for drugs of abuse. Ristimaa *et al*. developed in this context an LC-MS/MS-based targeted analysis method for a wide range of drugs of abuse, amongst which MDMA, MDA and THC-COOH[257]. Another application of meconium analysis is the quantification by LC-MS/MS of meconium fatty acid ethyl esters, ethyl glucuronide and ethyl sulfate, three alcohol markers used for the identification of in utero alcohol exposure[258]. Furthermore, the use of other non-invasive matrices in toxicology, such as nails, sweat and breast milk, has been enabled by the introduction of sensitive analytical techniques. Although these matrices can be useful in some instances (e.g. doping control, determination of exposure to environmental contaminants), their more widespread implementation in clinical routine is less likely, given the specialized nature of these samples.

1.8. PHENOTYPING

Phenotyping aims at determining the exact actual enzymatic activity of CYP450 enzymes. In general, phenotyping for a drug-metabolizing enzyme consists of administering a selective probe drug of the enzyme, followed by determining a specific PK metric (e.g. systemic clearance of the probe drug, single-point concentrations or metabolite/parent drug concentration ratios)[19]. CYP450 enzymes mainly catalyze phase I metabolism reactions, the first step in the enzymatic biotransformation which is chiefly responsible for the elimination of drugs and other xenobiotics. Interindividual variability is seen in CYP450 enzyme expression and function, which is determined by genetic, epigenetic and non-genetic host factors (e.g. sex, age, pathophysiological conditions) and by environmental influences, such as tobacco smoke, drug intake and diet[19]. Therefore, every person has his own CYP450 enzyme activity profile, resulting in variability in drug metabolism and -consequently- variability in drug response. This variability is the prime reason why CYP450 phenotyping can be implemented in clinical routine for a selection of drugs, including tricyclic antidepressants (imipramine, nortriptyline), antipsychotics (haloperidol, risperidone, clozapine, olanzapine), opioid analgesics (codeine, tramadol), proton pump inhibitors (omeprazole, lansoprazole) and antithrombotic agents (clopidogrel, warfarine)[19]. The use of alternative sampling strategies for CYP450 phenotyping in combination with LC-MS/MS is currently confined to OF and DBS.

Although data obtained using EBC for CYP450 phenotyping are in some respect comparable with those obtained during OF-based phenotyping, the use of EBC in this context is less obvious due to the highly specialized equipment necessary for the measurement of $13CO_2/12CO_2$ ratios in breath. To date, reliable phenotyping methods for various clinically relevant CYP450 enzymes, including CYP1A2, CYP2C19 and CYP2D6 are available using alternative sampling strategies. For CYP3A4 on the other hand, additional studies are necessary[19]. For more details, the interested reader is referred to the recent comprehensive review by De Kesel *et al*.[19].

1.9. METABOLOMICS, PROTEOMICS AND PROTEIN ANALYSIS

The use of alternative sampling strategies in the 'omics' arena is to date limited to DBS, OF and EBC. In a clinical setting, metabolomics and proteomics are typically targeted approaches, following a discovery phase in which a selected set of biomarkers (proteins and small molecules, respectively) has been identified. Metabolomics, the global study of metabolites in human body fluids, is an emerging 'omics' science which intends, just as proteomics, to discover specific disease biomarkers. Clinical metabolome studies by LC-MS/MS have been performed for transplantation, cancer, diabetes, lipid profiling and coronary heart disease. LC-MS/MS is an indispensable partner for performing quantitative metabolomics[259]. Alternative sampling strategies in the metabolomics field include EBC as well as OF, with as an example the measurement of salivary biomarkers for the early diagnosis of various types of cancer[24,25,260]. EBC is an example of an alternative sampling strategy for which proteomic analysis has been performed for the detection of biomarkers related to asthma and COPD. Here, untargeted proteomics plays a distinct role in the search for the underlying pathobiology of these two most common chronic airway diseases. Analysis of EBC by an LC-MS/MS method revealed in this context the promising possibility of using a panel of proteins in the quest for the etiology of COPD[261].

Currently, clinical proteomics can be defined as the (large-scale) study of peptides/proteins in human biological matrices, aiming at validating and/or implementing biomarkers for the diagnosis, prognosis and/or therapeutic monitoring of diseases. Improvements in MS technology partly explain the increased focus on proteomics and protein analysis over the past decade, with applications in a variety of disciplines[262]. Recently, Chambers *et al*.

published a multiplexed approach for the (semi-)quantification of a panel of 97 proteins in DBS[23]. However, the performance of proteomics and protein analysis in clinical routine, both by the use of conventional and alternative biological matrices (e.g. DBS), has remained rather limited to date and mainly focusses on studying one or several proteins. DBS-based protein analysis by MS includes quantitative and qualitative Hb analysis, used in the diagnosis of sickle cell disease and other clinically relevant hemoglobinopathies[263]. Furthermore, Dewilde *et al*. developed a method for the determination of ceruloplasmin, a biomarker for Wilson's disease, in DBS using LC-MS/MS[264]. Also proteins used in doping can be determined in DBS *via* LC-MS/MS. Examples are insulin-like growth factor-1 (IGF-1), a biomarker of growth hormone (GH) abuse and Synacthen®, a synthetic human adrenocorticotropic hormone, causing increased plasma levels of cortisol[265,266]. Further, the rapid emergence of protein therapeutics will likely bring along the need in some clinical labs to measure these proteins in at least a subset of patients. This is typically done by targeted LC-MS/MS-based analysis of a representative set of peptides, generated by proteolytic digestion of a sample, such as a DBS.

Next to LC-MS/MS methods, several immunoassays were developed for the determination of relevant proteins in alternative samples, including DBS. Examples include thyroglobulin and prostate-specific antigen[267,268]. Although more challenging to set up and implement, LC-MS/MS assays offer the advantage over immunoassays that they do not suffer from false positive results, caused by autoantibodies (e.g. against thyroglobulin) and rely on unequivocal identification rather than on antibody-based recognition. Another disadvantage coupled to many immunoassays is the lack of reliable reference methodologies, sometimes causing incomparable results. In this context, LC-MS/MS methods could be developed as reference measurement procedures[269]. An example of such a reference method is the determination of HbA1c, a fundamental biomarker in the long-term follow-up of the glycemic state of diabetic patients, in whole blood. Several publications readily unveiled the advantages of HbA1c determination in DBS[270]. As blood lipids are also important risk determinants in patients with diabetes, a combined LC-MS/MS determination of HbA1c and lipids in DBS could be beneficial. Since LC-MS/MS-based quantification of cholesterol and related metabolites in DBS has already been performed in the screening of inborn errors, we believe that a combined method is certainly possible in the monitoring of diabetes patients[271].

1.10. TOWARDS ROUTINE IMPLEMENTATION

Although the use of alternative sampling strategies may be appealing for certain applications, routine implementation is still limited due to some practical hurdles and technical challenges[272]. First of all, method development typically takes longer when using alternative matrices, as additional variables need to be assessed[69]. Unfortunately, these variables are not included in standard validation guidelines. Therefore, white-papers covering matrix-specific best practice guidelines have been published for certain matrices (although not yet for all)[69]. Another potential limitation for the set-up of alternative TDM methods might be the availability of sufficient matrix[28]. However, if commutability is verified with spiked buffer solutions, the latter might be employed instead.

Furthermore, matrix-specific issues (e.g. the HT effect in DBS analysis and the influence of salivary pH in OF analysis) exist, which can lead to erroneous results and complicate data interpretation. However, as discussed above, multiple solutions have been suggested to cope with these issues. Another practical hurdle is the fact that existing therapeutic intervals, reference ranges or cut-off levels are generally established using serum or plasma. So either new intervals or cut-off levels need to be set up for the specific alternative matrix or bridging studies have to be conducted to correlate drug levels in both matrices.

Regrettably, thorough clinical validation is often lacking, as generally only a limited number of samples or even only spiked samples have been included in these studies. Moreover, the correlation between drug levels in the alternative and systemic matrix is often only evaluated in healthy volunteers, whilst this may be significantly different in the target population. In addition, the effect (and potential benefit) of the use of an alternative matrix on the clinical decision process is only rarely evaluated[63]. Also studies on patient outcome and economic impact are limited at best. In this context though, Gorodischer *et al*. calculated that OF-based TDM could be less expensive than traditional TDM since less nursing time, fewer visits to doctor's offices or hospitals, and fewer sampling materials are required[144]. Also important in a clinical setting is the fact that these alternative tests may not be included in nomenclature and hence may not be reimbursed by the public healthcare system.

An important step in incorporating the analysis of alternative samples in routine laboratories is automation; not only to increase throughput and safety, but also to decrease hands-on time

and to exclude human errors. Ideally, automation encompasses the pre-analytical, analytical and post-analytical phase. More specifically in the case of DBS or DPS, this means a lab technician would only have to introduce a patient's card into an analyzer after which the sample is automatically analyzed and the obtained result communicated into a laboratory management information system. Promising advancements have been made in this regard. The tedious punching step can be replaced by (semi-)automated punching devices, whilst sample preparation can be automated using e.g. readily available liquid handling systems[273]. Furthermore, completely automated DBS/DPS analyzers have become commercially available and can be directly coupled to standard LC-MS/MS configurations. These analyzers have accessories such as e.g. barcode readers that allow for sample registration and traceability. Every type of DBS/DPS automated analyzer uses solvents to elute a fixed area of matrix from a collection card, obviating the need for punching. The way the elution is performed depends on the type of analyzer: the DBS Autosampler™ and the Sample Card and Prep system™ both employ flow through desorption, whilst in the DBS-MS 500 system extraction solvent is guided horizontally through the DBS during a surface sealed extraction, after which the extract is guided into a sample loop[274-276]. After elution, the extract can be subjected to online sample clean-up and/or separation on an LC column or even direct injection into the MS, depending on the chosen configuration[59,248,274-276]. Importantly, using automated DBS analyzers, the entire DBS extract is introduced into the (LC-)MS/MS system, thereby increasing method sensitivity, since in offline approaches only part of the extract is injected into the analyzing system. The DBS-MS 500 system is currently the only analyzer in which IS can be sprayed onto the DBS before extraction[275]. In the two other types of analyzers the IS is automatically added to the elution solvent. To verify whether the correct portion of the card has been analyzed these analyzers can take a picture of the sample before and after analysis. In addition, it is also possible to use the DBS analyzers as automated sample preparation devices that are not coupled to an LC-MS/MS system. To reduce hands-on-time even further, also the preparation and spotting (in the case of DBS/DPS) of calibrators, quality controls (QCs) and blanks can be automated using a commercially available liquid handling system[243,277]. This procedure showed similar accuracy and precision as manual preparation, but was safer, more efficient and yielded samples of predictable quality.

Also for the analysis of other alternative matrices, automation is important to allow convenient implementation in a routine setting. Therefore, commercially available workstations can be employed to automate laborious sample pretreatment steps as much as possible. This was e.g. done for the analysis of drugs of abuse in preserved OF samples collected using Quantisal™ devices[243]. For this application the workstation was not only used for IS addition, but also for automated SPE. The only remaining manual step during sample pretreatment was the evaporation of the SPE eluate which was automatically collected in LC vials. However, for some matrices automation may prove challenging. In the case of hair analysis for example, the inability to handle the solid hair sample limits the degree of potential automation[22].

Importantly, when using LC-MS/MS (i.e. the analytical technique most frequently used in combination with alternative sampling strategies) in a routine setting, not only the sample handling and the online sample preparation need to be automated, also the LC-MS/MS modules and software programs should become more user-friendly. More specifically, to make LC-MS/MS technology as convenient as possible for lab technicians, ideally a sort of "black box" LC-MS/MS unit should be integrated in existing chemistry analyzers. One suggestion that has been made in this regard is the development of an analyzing module with hybrid technology combining the characteristics of immunoassays and tandem MS[278], potentially omitting the need for LC.

Another, complementary way of increasing throughput to which LC-MS/MS lends itself perfectly, is to multiplex different analytes in a single run. The latter also aids in retrieving as much information as possible from a limited sample. However, in hair analysis the development of such multi-analyte procedures might prove challenging, since authentic hair samples are required for e.g. extraction optimization during method development[22]. Additionally, multiple LC systems can be multiplexed on one MS, further allowing a more economic use of the MS. This can be *via* staggered analysis (in which the chromatographic eluate only enters the MS/MS system during the time window where the compounds of interest elute) and/or *via* a more convenient switching between different methods, since no hardware changes need to be performed. Even sample multiplexing (i.e. the simultaneous introduction of two samples that were differentially derivatized) may be considered an option to increase throughput[279].

Moreover, the set-up of a QC program might be particularly challenging for alternative matrices. QC materials in native matrix (or in an adequate surrogate matrix) are often not commercially available. Although standard kits exist for TDM purposes (e.g. for the determination of immunosuppressants *via* LC-MS/MS[280]), these are not necessarily suitable for methods using alternative matrices. For DBS methods, for example, the calibrator and QC materials should also have the same viscosity as true blood to have similar spreading properties. Therefore, calibrators and QCs are currently often prepared in-house from different (non-)certified starting materials. The development of more matrix-specific kits, encompassing calibrators and QCs in a suitable matrix as well as e.g. IS, extraction solvents and mobile phases, would hence be favorable. Although such a kit has, for example, already been developed for the analysis of amino acids and acylcarnitines in DBS, it needs to be pointed out that it was developed for MS/MS analysis and not specifically for LC-MS/MS analysis[281]. Importantly, some alternative matrices also have special QC requirements, which may complicate quality assurance programs. For DBS, for example, it is advisable to include different HT levels[69], whilst for hair analysis it is important to include different hair types[282]. Furthermore, to ensure appropriate accuracy, methods should ideally be traceable to a higher order reference method. However, these reference methods are often lacking. In addition, only few quality assurance schemes circulate specimens in alternative matrices. The Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology, for example, is setting up a pilot PT program for TDM of immunosuppressive drugs in DB[283]. An extra complicating factor that needs to be taken into account in PT programs for alternative matrices are the different types of substrates that are used to collect samples (e.g. different types of filter paper cards in DBS sampling and different collection devices in OF sampling). Therefore, to facilitate these types of programs, harmonization/standardization will be important in the future.

Not only the analytical method itself, but also the patient samples (even when collected at home) have to be of sufficient quality. A first step towards achieving this is by guaranteeing the quality of the substrate on which the sample is collected. For DBS sampling this is achieved *via* the Filter Paper Evaluation Project, set up by the center for disease control and prevention (CDC) which also offers its services to filter paper manufacturers[284]. The quality criteria postulated by the CDC resulted in the laboratory standard, Clinical and Laboratory Standards

Institute (CLSI) NBS01-A6, which has greatly contributed to the reduction of analytical imprecision due to batch- to-batch variability in filter paper. Furthermore, the quality of a DBS is also evaluated before analysis, either by experienced laboratory personnel or in an automated fashion using an optical scanning instrument[285]. Such instruments objectively evaluate spot area, circularity, convexity and consistency (i.e. DBS size, symmetry and uniformity). In this way, acceptable, marginally acceptable, and unacceptable DBS are distinguished and acceptable punch areas identified. To maximize the amount of acceptable spots in the event of home sampling, patient education *via* demonstration folders and movies has proven to be essential. To evaluate whether contamination occurred during sample collection, incurred sample reanalysis (ISR) or analysis of a blank filter paper area close to the DBS has been advocated[21,70]. Also for other matrices which are usable for home monitoring, evaluation of sample quality is essential. Therefore, it can for example be advised to perform OF home sampling using collection devices with volume indicators. To help harmonization and standardization of alternative matrix analysis, best practice guidelines have been proposed by several committees (e.g. the Society of Hair Testing (SoHT) or the European Bioanalysis Forum (EBF) consortium) highlighting important pitfalls and validation requirements[69,286]. Also harmonization of data interpretation is an essential goal of these committees. The SoHT and the DRUID project, for example, have played an important role in the determination of cut-offs for toxicological analysis of hair and OF, respectively[286,287].

1.11. FUTURE TRENDS

1.11.1. Development of new formats

New formats and approaches have been developed to improve the acceptance of dried blood (spot) analysis by tackling the crucial HT issue. Strikingly, most of these approaches have been designed to be compatible with automated analysis. First of all, volumetric approaches have been suggested which are able to produce fixed-volume DBS from a non-volumetric whole blood droplet. Whilst some of these approaches use formats which are compatible with existing DBS analyzers[47,48], others such as VAMS employ a different type of collection device[49,110]. However, also in this case, sample preparation can still be automated, more particularly *via* readily available liquid handling systems. Secondly, others have suggested the use of DPS instead of DBS. In such instance the former can be prepared *via* filtration of a blood drop over a size-exclusion membrane, which withholds blood cells[53,55]. The DPS cards

developed by Sturm *et al*. are compatible with commercially available automated analyzers[54]. Unfortunately, also DPS appear to be subject to a certain HT effect[54]. A third possibility is to estimate the HT of a DBS and to correct for the anticipated HT effect[34]. Recently, we developed a non-contact HT estimation method[50,51] which could potentially be more easily automated than the K^+-based HT estimation method we previously established[288]. Also for other alternative matrices innovation is ongoing. An example is the development of a new exhaled breath collection device for the analysis of drugs of abuse, which allows to standardize the volume of matrix collected (at least to some degree) and facilitates remote collection by non-trained personnel[289]. The device is composed of a filter which is located in a filter holder and a mouth piece which is attached to a plastic bag. When a person breathes into the device, aerosol particles will be collected onto the filter and the plastic bag will inflate. When the plastic bag is full, sufficient matrix has been collected onto the filter. Subsequently, the filter holder and the mouthpiece can be detached from one another and the filter holder can be closed with plugs and sent to a laboratory *via* regular mail.

1.11.2. Microfluidics

Microfluidics have been employed for alternative matrix collection and sample preparation in conjunction with tandem MS. One example includes the use of a lab-on-a-chip for sample preparation of either a DBS punch or a directly applied capillary blood droplet[290]. Multiplexed extraction of DBS using this type of chips was demonstrated by Lafrenière *et al*. using automated droplet control[291]. Due to its relative simplicity this technique has even been suggested to be able to bring clinical analysis closer to the patient. In this context, a proof of principle was published by Kirby *et al*. who used digital microfluidics to extract dried urine spots and to transport the extract to a nano-electrospray emitter to allow tandem MS-based detection of drugs of abuse using a portable mass spectrometer[292]. Evans *et al*. on the other hand, employed capillary-scale LC to analyze DBS extracts, since this increases assay sensitivity[293]. To avoid problems with column connections, a chip can be employed onto which column, connections and MS emitter and spray are co-located[294]. Although no published examples could be found by the authors, chip-based microfluidic extraction could potentially be made compatible with LC by integrating the required technology into a single chip format for a seamless workflow. Notwithstanding the promising nature of these new

developments, latest technological advancements are often only steadily implemented in a routine setting, as robustness first has to be well established.

1.11.3. High resolution mass spectrometry (HRMS)

Aside from its use in e.g. biomarker discovery research (possibly in OF or DBS), untargeted screening using HRMS is increasingly becoming a valuable tool for toxicological purposes, e.g. allowing detection of new psychoactive compounds and their metabolites[295]. Also in other cases where untargeted screening is advised, e.g. for the evaluation of the chemical and biological exposure of humans (which has been suggested to become more important in the future)[296], HRMS may be an ideal screening tool. In addition, for the detection of inborn errors of metabolism, the use of HRMS has been advocated as an alternative for the traditionally used electrospray ionization-MS/MS, since multivariate pattern recognition analysis would lead to better specificity and the identification of comorbidities and interferences caused by medical treatment or damaged DBS[297]. Last, although LC-MS/MS is likely to remain the workhorse for quantitative bio-analysis during the next couple of years, LC-HRMS/MS is increasingly advocated as a suitable alternative, further facilitating quantitative analysis of complex mixtures.

1.11.4. MS(/MS)-based point-of-care (POC) testing

Another important trend in clinical analysis is the development of MS(/MS)-based POC testing and/or near-patient analysis. The latter may be performed at a professional healthcare center, at the emergency unit or even in operating theatres. However, development of these decentralized analyses is most often -and ideally- still under the supervision of the clinical laboratory, which is responsible for e.g. quality assurance. In general, these POC techniques do not require sample preparation steps nor a separation step and employ ambient ionization techniques (although a multitude of ambient ionization techniques have been employed, selected examples will be discussed). In this context as well, alternative sampling strategies have been employed. Examples include the use of paper spray MS for the analysis of blood or OF collected on filter paper for e.g. TDM purposes and abstinence monitoring[298,299]. In paper spray MS, a drop of the biological sample is deposited on a triangle-shaped filter paper which is part of a disposable collection cartridge. After the cartridge has been positioned in front of the MS, a solvent is applied to the filter paper, as well as a high voltage. This causes

a spray to be formed at the tip of the triangle, which is then transmitted into the MS. Subsequently, the resulting signal is recorded for a fixed period of time, which results in a signal *vs*. time plot (called a chronogram). To quantify the amount of the target compound present, the ratio of the AUC of the target's and the IS's chronogram is employed[300,301]. To be workable in a POC-setting, the blood on the filter paper is either analyzed when it is still wet or after it has been quickly dried using either pre-spotted coagulants or heat application[298,299,302].

A first step towards automation has been accomplished by the development of a tray, which can hold multiple filter paper triangles which are consecutively analyzed[303]. In addition, to render POC-MS feasible, portable MS systems have been developed[304]. The mini 12, for example, has been employed for the analysis of the therapeutic drug amitriptyline using paper spray MS[305]. The goal of this instrument is to be able to offer a sample to a miniature MS after which analysis and data analysis are performed automatically and a result is directly generated on the screen.

Although PSI facilitates high-throughput analysis and potentially even POC-testing, the lack of sample preparation or a separation technique, may have a substantial effect on assay specificity and sensitivity. Despite this, the performance of e.g. PSI is still sufficient to cover the therapeutic ranges of most compounds, according to Manicke *et al.*[300]. Moreover, the efficiency of PSI can be improved in various ways, e.g. by optimizing the filter paper material [306], the wetting solution[306], as well as the sharpness of the filter paper tip[301]. Moreover, since the IS cannot be added to the sample before spotting, the IS either needs to be added to the wetting solvent or to the filter paper prior to the application of the blood droplet[300]. In this context a three-layered IS-containing cartridge has been developed by Siebenhaar *et al.*[307]. Although there is a growing literature on the use of PSI for TDM purposes, including e.g. the analysis of imatinib, citalopram, paclitaxel and tacrolimus, these generally only include a proof of concept and typically lack an appropriate analytical validation (e.g. the evaluation of volume and HT effect)[299,300,302]. In addition, in most cases the IS is added to the blood sample before spotting and only volumetric DBS are analyzed. Therefore, although promising results have been published, more research is necessary to demonstrate that PSI is able to deliver quantitative results that stand the comparison with traditional samples.

Similarly to the above-described applications, the concept of touch spray MS has been developed. This refers to the direct analysis of OF collected on a medical swab. In this case as well, a solvent and a voltage are applied to the collection device after which a spray is formed. Applications of the technique include the semi-quantification of various drugs and the detection of lipids which are specific to *S. pyogenes* to quickly diagnose strep throat[308,309]. In addition, even surgical smoke has been sampled *via* a surgical knife to help differentiate between cancerous and non-cancerous tissue during surgery[310,311].

1.12. CONCLUSION

Although ample valuable applications have been developed using alternative sampling strategies, their adoption in routine laboratories is still limited. Aside from the vast use of DBS in NBS programs, only few examples can be found which employ alternative sampling strategies for routine analyses. Examples include the determination of salivary cortisol levels, the determination of drugs of abuse in OF, and the use of DBS for TDM purposes. The use of alternative sampling strategies clearly provides valuable opportunities. Particularly in special patient populations such as neonates, children, CF patients, and patients with poor venous access, alternative sampling strategies can be of great added-value. Moreover, alternative sampling strategies may play a pivotal role in remote and resource-limited settings, where traditional sampling approaches are typically not applicable due to logistical and economic issues. In addition, the ease of collection and transport of certain alternative matrices facilitates home-based applications, in which samples are collected by the patient or his caregiver and transported to the laboratory *via* regular mail services. The latter is not only more convenient for patients or their caregivers, it also allows to collect samples when e.g. epileptic seizures or adverse effects occur, yielding information that could not be obtained in any other way. The growing automation of alternative matrix analysis will definitely contribute to the acceptance and introduction in routine practice. In addition, matrix-dependent issues are (successfully) being tackled and new, more robust formats are being developed, bridging the gap between research and routine. To guarantee the quality of alternative matrix-based assays, initiatives to set up guidelines for the development and validation of these assays (which take into account matrix-specific requirements) as well as matrix-specific PT programs are essential. Although alternative matrices will of course never completely replace classic blood samples, they may be extremely valuable whenever they can

offer (additional) information that could not be (conveniently) obtained using traditional approaches. Importantly, more studies are needed in which a proper clinical validation of alternative sampling strategies is performed to allow a more widespread implementation of these strategies in a routine context. Moreover, the effect on (clinical) decision making and overall costs needs to be thoroughly evaluated. Only when that results in a positive balance, may a given alternative sampling strategy become routinely implemented.

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Chapter 2

Guideline for DBS method set-up and validation

Based on:

Capiau S*, Veenhof H*, Koster R, Bergqvist Y, Boettcher M, Halmingh, O, Keevil B, Koch B, Linden, R, Pistos C, Stolk L, Touw D‡, Stove, CP‡, Alffenaar JW-C‡. Official International Association for Therapeutic Drug Monitoring and Toxicology guideline: Development and Validation of Dried Blood Spot-based Methods for Therapeutic Drug Monitoring. *Therapeutic Drug Monitoring*. 2019;41(4):409-430. *,‡Equally contributed

2.1. INTRODUCTION

DBS analysis has been introduced more and more into daily practice.[1] To assure the quality of bioanalytical methods and to assure that the results obtained with those methods are valid, it is of utmost importance that newly developed methods are fit for purpose. Those methods must have undergone adequate method validation and are monitored *via* a suitable QC program. Absence of DBS-specific method validation guidelines results in DBS-based methods lacking essential validation aspects with reduced credibility.[1-4] Validation requirements described in guidelines for the quantitative analysis of traditional matrices (i.e. liquid blood, plasma or serum) are not always easily translated to analysis of DBS.[5,6] Moreover, several additional parameters, like volume- and HT effects, which are not part of traditional guidelines, are often overlooked or not adequately assessed.[7]

Therefore, this guideline aims at defining the parameters necessary for the validation of quantitative DBS-based methods and to provide advice about how these can be assessed. In addition, guidance is given on the application of validated methods in a routine context. The recommendations in this guideline are based on existing guidelines for traditional matrix analysis -in particular the bioanalytical method validation guidelines issued by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA)-,[5,6] the guideline for measurement procedure comparison provided by the CLSI[8], several white papers on dried matrix analysis[9-11], as well as other published work and the personal experience of the authors.

The focus of this guideline is the analysis of DBS for the quantitative determination of small molecule drugs and drug metabolites using chromatographic techniques for TDM purposes. However, many elements of this guideline are also relevant for the analysis of samples obtained *via* VAMS and for DPS analysis, as well as for the analysis of DBS for other purposes than TDM. As the successful validation of a DBS-based analytical method starts with method development, this guideline commences by outlining the potential pitfalls encountered during that stage (see sections 2.2.1, 2.2.2 and 2.2.3). Furthermore, the importance of prevalidation stress testing is highlighted (2D). In a next section, the actual method validation is extensively discussed (see sections 2.3 and 2.4). This validation section encompasses both the analytical validation (comprising both the classical and the DBS-specific validation

parameters) and the clinical validation (i.e. demonstration of equivalence between DBS-based results and results obtained in the classical matrix). Finally, QC is briefly discussed (section 2.4.8.). A summary of this guideline can be found in Supplement S-1.

2.2. METHOD DEVELOPMENT: CONSIDERATIONS FOR SUCCESSFUL VALIDATION

Before embarking on the set-up of a DBS-based procedure, it is essential to carefully think about the purpose of the method. Already in this early stage, certain considerations need to be made to ensure the suitability of the method for a given application (i.e. to ensure the method is fit for purpose). These considerations are discussed below and the different options are schematically summarized in Figure 2.1. Furthermore, stress testing of the method during method development will allow potential issues to be detected at an early stage, which will eventually increase the chances of a successful method validation and application.

2.2.1. Considerations regarding sample collection

2.2.1.1. Collection procedure

Nowadays, the most frequently used dried blood sample collection method is the collection of a non-volumetric drop of blood (free falling or by touching) onto a filter paper (i.e. directly from a finger prick or heel stick). Alternatively, the blood sample may be deposited volumetrically using a capillary or a pipette. Furthermore, several blood collection strategies exist in which a volumetric dried blood sample can be directly generated from a non-volumetric drop of blood, without the use of pipettes or handheld capillaries. These strategies include i.e. HemaXIS[12], HemaPEN®[13] , Capitainer[14-16] and VAMS[17-19]. In addition, dried plasma spot (DPS) may be collected rather than DBS. These DPS may be generated either by centrifugation of a liquid blood sample and subsequent application of an amount of plasma onto a filter paper or by using a device which allows *in situ* DPS generation.[20-23]

While some of the above-mentioned collection strategies may allow patient self-sampling (e.g. non-volumetric DBS collection[24], VAMS™ [25], *in situ* generated DPS), other collection methods (e.g. volumetric DBS collection using exact volume capillaries, DPS generation following centrifugation) require trained professionals and/or laboratory equipment. Although the latter strategies are not suitable for home sampling, they may still be valuable

in another context. DPS generation *via* whole blood centrifugation and pipetting may for example be a suitable approach if DPS are prepared in a laboratory in a remote or resource-limited setting to allow more convenient transport to a centralized or reference laboratory.[26] Additionally, also other parameters such as the required sample volume, automation capabilities, commercial availability, the cost of a given microsampling device, as well as overall costs, may play an important role in the selection of the sample collection method.

2.2.1.2. Selection of the type of filter paper

If samples are to be collected on filter paper, the type of filter paper (card) that will be used needs to be carefully chosen. The type of filter paper may affect the occurrence of interferences, the blood's spreading behavior, sample homogeneity, as well as analyte stability and recovery.[27-29] Commercially available filter paper can either be untreated (e.g. Whatman® 903, Ahlström 226, DMPK-C), or pretreated with e.g. denaturing agents or enzyme inhibitors (e.g. DMPK-A or DMPK-B).[30] Furthermore, in certain DBS-based methods in-house pretreated filter paper has been used to increase analyte stability or recovery.[31-34] Moreover, some types of collection devices have been reported to be less affected by the HT effect and may help to overcome this issue.[35,36] Additionally, chitosan and alginate foams have been proposed as collection substrates to help increase analyte recovery, as they dissolve during sample extraction.[37] Although most DBS-based bioanalytical methods use regular, cellulose-based, untreated filter paper (cards), for certain applications it may be valuable to evaluate the use of pretreated or non-cellulose-based alternatives. However, it needs to be kept in mind that the use of non-commercially available substrates may hinder a generalized application of the method, and requires inhouse assessment of batch to batch quality.[38]

2.2.1.3. Interferences originating from the collection substrate

It is advised to analyze some blank collection cards during early method development to assess whether the collection material itself is blank and whether there are any interferences present that need to be separated chromatographically from the target compound(s).[28] If one of these issues occurs, it might also be valuable to evaluate different collection substrates.

2.2.1.4. Sample volume

The amount of sample that is required for a certain analysis will mainly depend on the envisaged LLOQ and is inherently linked to the available instrumentation. However, the minimally required volume should always relate to how the samples are collected. For the set-up and validation of the method a sample volume representative of the sample volume of the patient samples needs to be employed. Most people will typically generate DBS of 20 to 70 µL if free falling drops of blood are collected, whereas somewhat smaller DBS – typically 15 to 50 µL – will be obtained if a hanging blood drop is collected by bringing it into contact with the filter paper. With the latter approach it is essential that only the blood drop and not the fingertip touches the filter paper. If a DBS is smaller than what is typically expected, this may be an indication that the fingertip came into contact with the filter paper. On the other hand, if a DBS is larger than expected, multiple drops were likely collected. Obviously, whenever samples are collected volumetrically, the sample volume will be determined by the employed device. If a larger volume of blood is required to reach the LLOQ, sometimes punch stacking is used.[39] Nonetheless, the number of punches required for a single analysis should remain as small as possible, to limit the amount of good quality samples that needs to be collected and to allow ISR.

2.2.1.5. Drying and storage process

A parameter that is often neglected in DBS-based methods is the impact of drying time. If the sample is not completely dry before putting it in a zip-locked bag for storage, microbiological growth may occur and compromise sample quality.[40] Furthermore, improper drying might also affect analyte stability and recovery.[41,42] Therefore, it is advised to dry samples at least 3h under ambient conditions (preferably without direct sunlight) and to store them with a desiccant, which will remove an additional 5% of water from the dried samples.[40,43] In certain settings, however, the required drying time may be longer, as this depends on the ambient temperature and humidity, the sample volume and the type of filter paper.[42] In other settings, shorter drying times may suffice. Therefore, it is relevant to evaluate during early method development whether the drying time is adequate under the conditions likely to be encountered during the collection of the patient samples. This evaluation is preferably performed using DBS with a HT in the upper range of the HT of the target population and, if

applicable, a large sample volume (50 – 100 µL), as these will dry the slowest.[27] Furthermore, the ambient temperature and humidity during drying have been suggested to affect DBS homogeneity (although this effect also depends on the type of filter paper that is used).[44] Similarly, also the storage conditions should mimic the ambient conditions encountered during patient sample transport/storage.[45]

2.2.2. Considerations regarding sample preparation

2.2.2.1. Punch size

For volumetric DBS applications, the punch size needs to be large enough to punch out the entire DBS, independent of the HT of the sample. Hence, it is advised to select the required punch size based on samples with a HT of approximately 0.15, since this HT level will be lower than the lowest HT level of the patient population and will therefore yield DBS that are (slightly) larger than the largest expected patient DBS. The punches can either be made after application of the blood spot to the substrate or in advance.[46-48] For non-volumetric DBS applications, partial DBS punches are made that exclude the outer edge of the sample. If relatively small punches are made (≤ 4 mm or approximately 5.7 µL), most patients should be able to generate multiple DBS that are large enough to analyze. However, larger punch sizes may be required to obtain the desired LLOQ to increase method accuracy and precision or to exclude DBS homogeneity issues. Although generating larger DBS will be somewhat more difficult for a patient, when properly educated and trained, the vast majority of patients will be able to provide at least 1 or 2 samples that are large enough to make punches up to 8 mm (± 20 µL). The latter will also be easier if falling-drop-collection is used rather than hanging-drop-collection.

2.2.2.2. Internal standard incorporation

Ideally, an IS is mixed homogenously with the biological sample before sample preparation to compensate for any variability throughout the entire analytical process. Unfortunately, this is difficult to achieve with a DBS. For DBS analysis, the closest alternative is to spray the IS evenly onto the sample prior to extraction.[49] However, this requires the availability of a validated dedicated spraying system, which is not available in the majority of laboratories. Another option is to pre-coat the filter paper with the IS.[50] However, in that case the IS needs to be

applied to a larger surface, as it is not known where exactly the sample will be deposited. Furthermore, the IS should be stable for a sufficiently long period of time (i.e. during sample collection, transport, storage and analysis). In addition, the same batch of IS solution should be used for calibrators, QCs and patient sample collection cards, which is not feasible on a large scale. Another potential side-effect of pre-coating filter paper with IS (in the absence of matrix) is that the IS may show different recovery than the target analyte. To the best of the authors' knowledge, such strategies have not yet been evaluated for other dried blood samples nor has a successful application of IS-pre-coated microcapillaries been described. Again, such an approach would require the availability of tailor made devices, which will be at the expense of additional costs. In the majority of DBS-based methods, the IS is added to the extraction solution or directly to the DBS punch before extraction, and will hence not compensate for variability in analyte recovery.[9,51] Therefore, analyte recovery must be investigated extensively under different conditions (see below) during method development and validation.

2.2.3. Other important considerations

2.2.3.1. Type of blood used

For the set-up of calibration curves and internal QCs it is from a practical point of view impossible to use capillary blood samples derived from a finger prick. Instead, spiked samples generated from venous whole blood containing an anticoagulant, are used. Which type of blood is best suited for this purpose largely depends on how patient samples will be collected. If the DBS collection device that is used to generate the patient DBS contains a certain anticoagulant, the venous whole blood also needs to contain that same anticoagulant. On the other hand, if no anticoagulant is used during the collection of the patient samples, theoretically, the blood used to set up the calibration curves and QCs also has to be non-anticoagulated. Unfortunately, it is very impractical to prepare spiked samples from non-anticoagulated blood, as blood will start coagulating almost immediately after collection. Therefore, in most cases, a suitable anticoagulant will have to be selected. It is essential that the use of this anticoagulant does not impact the obtained results and that the stability of calibrators and QCs reflects that of real samples. Hence, we strongly advise to compare in an early stage results obtained from a non-anticoagulated sample with results from patient samples anticoagulated with different anticoagulants.[52] These blood samples should all be

obtained venously from the same volunteer or patient at (approximately) the same time and should be analyzed in quintuplicate. Based on the knowledge about the (lack of) impact of certain anticoagulants in liquid blood, some anticoagulants may readily be excluded. For example, if analytes are e.g. stabilized by oxalate/NaF, this type of blood should preferentially not be used to assess the analyte's stability in DBS (which in practice would not contain that stabilizing anticoagulant). On the other hand, if the anticoagulant stabilizes the analyte, and anticoagulant-containing DBS are commutable in any other way with DBS without anticoagulant, the former could be used for the set-up of calibrators and QCs as the prolonged analyte stability could help ensure consistent calibration.

2.2.3.2. Preparation of spiked samples

A first step in the preparation of spiked samples is to adjust the HT of the whole blood to the desired HT value. For most experiments the latter will correspond to the mean or median HT value of the target population.[53] Although there are several ways of preparing samples with a certain HT, the preferred procedure is to measure the HT of the original blood sample with a hematology analyzer and to calculate how much plasma needs to be added or removed to obtain the desired HT value.[54] After the addition or removal of the plasma, it is important to measure the HT again, to assure the sample was prepared correctly.

In a next step, the analyte needs to be spiked to the blood. It is important to only spike a limited volume of analyte solution to the blood (i.e. < 5% of the sample and preferably even less), to not change the nature of the sample.[5] Moreover, the addition of a larger volume of solvent would also change the sample's viscosity and/or cause cell lysis, thereby affecting its spreading behavior through the DBS filter paper. Furthermore, organic solvents may denature proteins. To further minimize the effect of the spiking volume on the sample's spreading behavior, stock solutions can be diluted with plasma, rather than with water or another solvent, if solubility allows for it. After spiking the blood with the target analyte, the samples should equilibrate for a sufficient amount of time at a suitable temperature, to mimic the analytes' in vivo RBC/plasma distribution.[55]

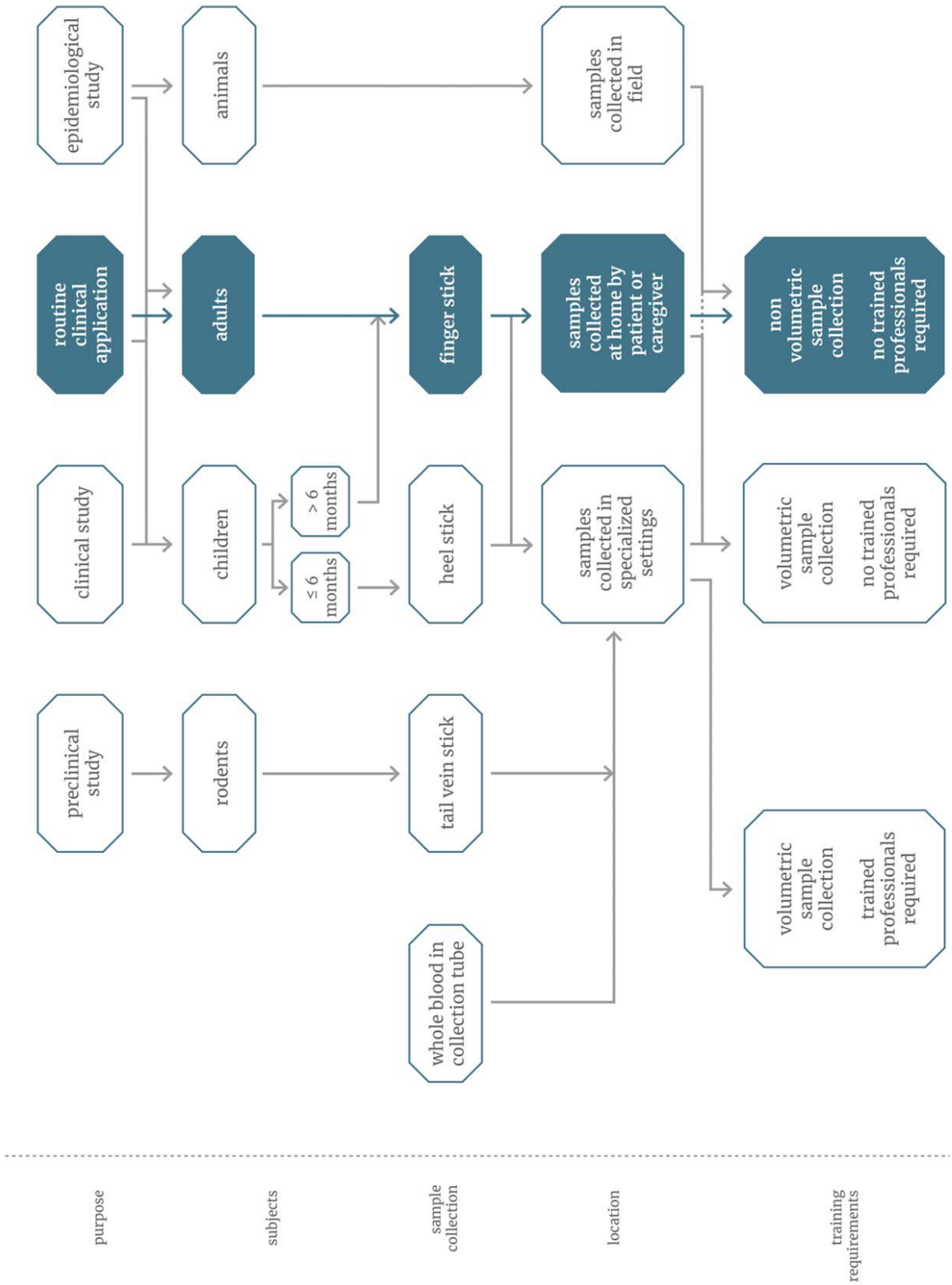
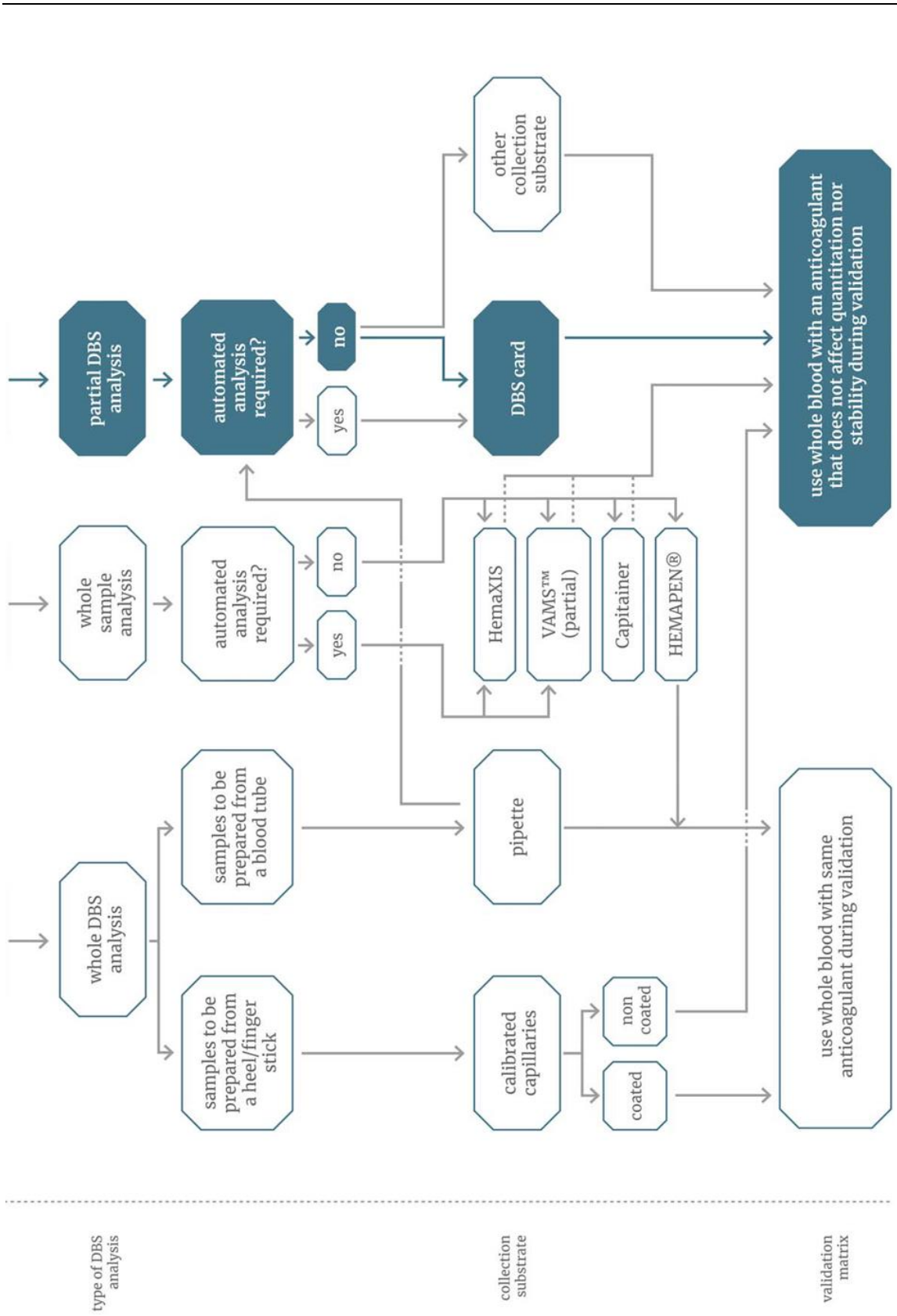


Figure 2.1: Flowchart depicting different options for the set-up of a DBS-based method which can be used before setting up a DBS-based procedure. The highlighted 'flow path' shows the procedure for TDM of immunosuppressants following home sampling by adult patients and partial spot analysis of DBS cards sent to the laboratory. Reprinted with permission from Anoek Houben. Copyright 2018


partial DBS analysis
automated analysis required?
no
yes
other collection substrate
DBS card
use whole blood with an anticoagulant that does not affect quantitation nor stability during validation
whole sample analysis
automated analysis required?
no
yes
HemaXIS
VAMS™ (partial)
Capitainer
HEMAPEN®
whole DBS analysis
samples to be prepared from a blood tube
pipette
samples to be prepared from a heel/finger stick
calibrated capillaries
non coated
coated
use whole blood with same anticoagulant during validation
type of DBS analysis
collection substrate
validation matrix

2.2.4. Prevalidation – stress testing

2.2.4.1. Exploratory tests

As with a traditional bioanalytical method, several exploratory tests need to be performed to assess whether a developed method is good enough to proceed towards validation. As with any chromatographic method, several technical aspects should be checked early on during method development, e.g. the absence of carryover and the influence of the sample matrix on the chromatographic method. Furthermore, the stability of the stock solutions used for spiking of calibrators and QCs should be guaranteed. Particular points of attention during prevalidation for DBS-based methods are short-term stability and extraction efficiency.

Although DBS generally tend to improve analyte stability, this is not always the case. Enzymatic analyte degradation may readily occur during the drying process.[56] Furthermore, oxidation sensitive analytes are likely to suffer from stability issues, since DBS are exposed to air during drying and/or storage.[30] If low signals are obtained from fresh samples (e.g. compared to a standard solution with the same concentration), this might be due to stability issues during the drying process. In addition, these low signals may also be caused by matrix effects, poor extraction efficiency or a combination of the above.

When using LC-MS/MS, the presence of matrix effects can be evaluated using post-column infusion. If present, these matrix effects may be eliminated by further optimization of the sample preparation and/or the chromatography. Poor extraction efficiency may be due to the analyte's interaction with the carrier or with endogenous matrix compounds.[29,57,58] However, the differentiation between extraction efficiency issues and actual analyte instability may not be so straightforward.[34] To get an idea about potential stability issues existing literature about the stability of the analyte in whole blood or about the chemical and physical properties of the analyte may be a good starting point. If degradation during sample drying is anticipated (e.g. for compounds with a very short in vitro half-life), flash heating may improve the analyte's stability (at least if the analyte is thermostable), as this inactivates the enzymes.[56] Unfortunately, this strategy is not suitable for home sampling. Nonetheless, it may help to figure out the cause of the poor method outcome. Other strategies to help improve the analyte stability may include preimpregnating the collection substrate with anti-oxidants or buffers.[34,59] However, these strategies may hamper generalized application of the method.

For some analytes instability issues remain unsolved, when taking into account a restrictive time frame for transportation of DBS. In those cases it should be decided that for that analyte dried blood sampling is not feasible. In specific situations, a volumetrically obtained sample could be brought into a stabilizing sampling buffer shortly after.[60] When poor extraction efficiency is suspected, further optimization of the extraction procedure may be required (i.e. the evaluation of different extraction solvents, additives and extraction temperatures, as well as more rigorous extraction techniques (like sonication). Furthermore, the use of different (pre-treated) collection cards/devices may also help to improve the extraction efficiency.

At this stage, it should also be evaluated whether the obtained results are affected by the time between sample collection and analysis. More particularly, the results from samples analyzed at T_0 (typically 30min – 3h after sample generation, depending on the required drying time) should be compared with results obtained at later time points, preferably up to 48h or 72h. This experiment is important since time-dependent extraction issues have been described.[61] More specifically, if the recovery decreases for the first (couple of) time points, but remains stable afterwards, it may still be possible to obtain good analytical results. This implies to only analyze samples older than a specified time point. Obviously, this strategy should not only be implemented for the patient samples, but also for the calibrators and QCs.

2.2.4.2. Evaluation of the robustness of the extraction procedure and short-term stability

In a next step, the robustness of the extraction procedure should be thoroughly investigated. This is a crucial experiment, since in most DBS applications the IS is not capable of correcting for variability in extraction efficiency. The extraction efficiency may be concentration, HT and time-dependent and, importantly, these parameters may also affect each other.[41,62-64] HT-dependent extraction efficiency may be present or more pronounced at one concentration level compared to another.[64] Similarly, time-dependent extraction efficiency issues may occur earlier at a more extreme HT level.

For non-thermolabile compounds, the occurrence of HT- and time-dependent extraction issues can be evaluated by comparing the results from fresh DBS at low, medium and high HT levels (with these HT levels encompassing the HT range of the target population; e.g. 0.20, 0.40, 0.60) with a second set of samples stored at 50-60°C for at least two days. This second set mimics thoroughly dried (aged) samples. This experiment should be performed at both

the low and high QC level (see Figure 2.2). Furthermore, to simultaneously determine the actual extraction efficiency at both QC levels, and to evaluate the presence of matrix effects, also samples spiked after extraction and standard solutions should be included in this experiment. Moreover, each of these samples should be analyzed in quintuplicate. Additionally, along with these samples, a calibration curve and QCs have to be analyzed. Importantly, in case of partial DBS analysis, these samples should be prepared by the accurate pipetting of a fixed amount of blood onto pre-punched filter paper disks to rule out any influence of the HT spreading effect on the amount of sample being analyzed.

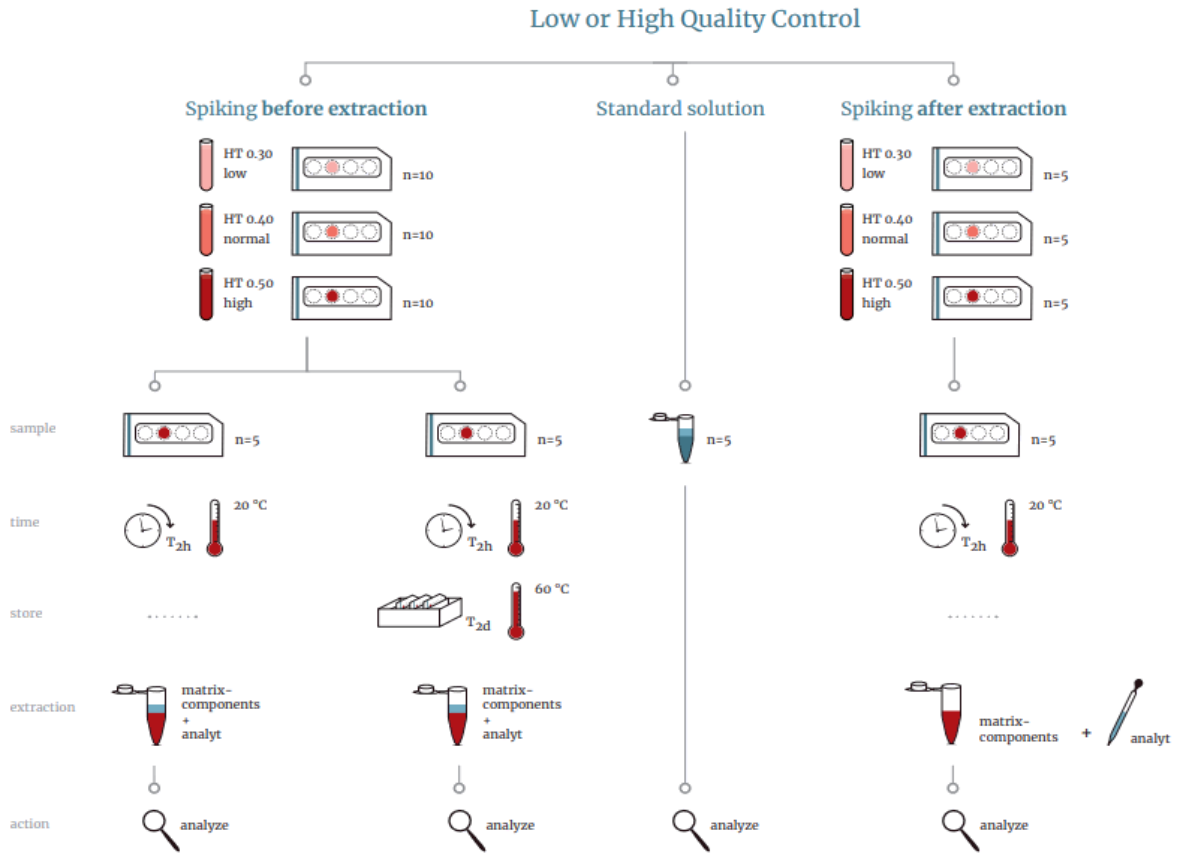


Figure 2.2: Schematic set-up of the experiments needed to assess the robustness of the extraction procedure and short-term stability. The total amount of samples to be analyzed for this experiment is 100 (plus calibrators and QC samples). Reprinted with permission from Anoek Houben. Copyright 2018

When no relevant differences (i.e. ≤ 15%) can be observed between the results obtained from fresh DBS and those stored at 50-60°C, it is unlikely that storage will have an impact on extraction efficiency. A good outcome in this set-up may also readily indicate good stability under ambient conditions, although this needs to be formally evaluated during method validation. However, it needs to be mentioned that the latter can also be affected by other parameters such as humidity and exposure to sunlight. Furthermore, by comparing the results

of the samples at the three different HT levels (both for the fresh and the stored samples) the occurrence of HT-dependent extraction efficiency issues can be evaluated. Moreover, using the Matuszewski approach, recovery and matrix effect can be evaluated at both concentration levels and at three HT levels.[65] While performing this experiment may seem fairly elaborate at first, it may prevent serious problems at a later stage, which may require a complete revalidation (e.g. if the extraction needs to be adapted). Moreover, if successful, the evaluation of matrix effect and recovery may not have to be repeated at different HT levels during the actual method validation, as long as the method remains unchanged. Also the evaluation of short-term stability at fairly extreme storage conditions (i.e. 50-60°C) is already incorporated in this experiment (cfr. section 3A).

For more thermo-labile compounds a similar experiment can be performed with samples stored at e.g RT for two weeks instead of at 60°C for two days. Although this is a less harsh experiment than the previously described one, it does cover a time span in which most clinical samples in a laboratory will have been analyzed. Alternatively, even lower storage temperatures may be used. However, if the analyte is not stable at RT for at least a couple of days, the method will not be suitable. Obviously, if satisfactory, these data can also be used as part of the stability data required for method validation.

To minimize the amount of samples that has to be analyzed at this stage, a simplified experimental set-up is suggested in Figure 2.3. More particularly, this set-up does not include 'spiked after extraction' samples or standard solutions, and all samples are only analyzed in triplicate. This simplified set-up offers the advantage that if the extraction procedure has to be adjusted (and consequently, this evaluation has to be repeated), the amount of samples that needs to be analyzed will not increase drastically. However, with this experiment, recovery and matrix effect will still need to be evaluated at different HT levels in a separate experiment during method validation.

If the results of the above mentioned experiments are non-satisfactory, this may be due to instability of the target analyte or to extraction efficiency issues. If the results for the different HT levels differ significantly and/or substantially (i.e. > 15%), this is due to a HT-dependent extraction efficiency issue and the extraction procedure needs further optimization. In this context, heated extraction, as well as the use of a mixture of organic solvents rather than a

single organic solvent may be helpful.[62,63,66,67] Furthermore, the use of a different collection card may also help to resolve this problem. Possibly, depending on the target population, the procedure can be repeated with less extreme low and high HT values, to evaluate whether acceptable results are obtained for a more limited HT span.

A difference between the fresh and the stored samples, on the other hand, might be due both to a time-dependent extraction efficiency issue and to actual instability of the target analyte.[68] However, if this difference between fresh and older samples is not observed at all HT levels, it is unlikely it is due to analyte instability. If the difference is observed at all HT levels, it may be worthwhile to repeat the experiment at a lower storage temperature, as this may indicate analyte instability.

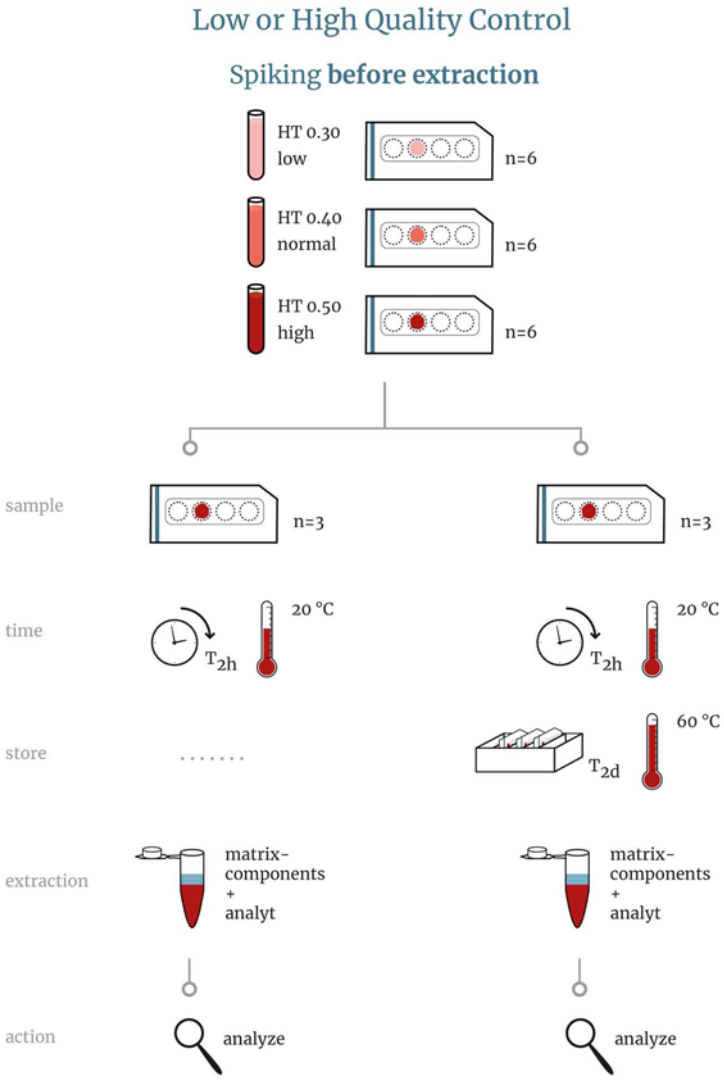


Figure 2.3: A simplified schematic set-up of an experiment to assess the robustness of the extraction procedure and short-term stability, requiring a minimum amount of samples. The total amount of samples to be analyzed for this experiment is 36 (plus calibrators and QC samples). Reprinted with permission from Anoek Houben. Copyright 2018

2.2.4.3. DBS homogeneity

In case of partial DBS analysis, it is essential to evaluate DBS homogeneity, i.e. to assess whether results from central punches are equivalent to peripheral (or decentral) ones.[69] By already evaluating this parameter during prevalidation, one knows whether during the next experiments it is required to make a central punch or whether a peripheral punch or multiple punches can be made from a single DBS.

This evaluation must be performed at two concentration levels (low QC and high QC), at different HT levels (low, medium and high) and at sample volumes representative of the anticipated patient sample volumes. Each of the evaluated conditions should be analyzed in quintuplicate. All samples should be compared to a calibration curve prepared with samples of medium HT level and average volume, of which a central punch was extracted. When both central and peripheral punches yield results within the standard bioanalytical acceptance criteria (typically, within 15% of their target value), the use of both types of DBS punches is considered acceptable.[69]

Obviously, this experiment only needs to be conducted if a central and a more peripheral punch can be made from a sample, which in turn will depend on the used punch size. When making peripheral punches, the very outer edge of the DBS should be excluded, as this has a different composition than the rest of the DBS (e.g. a higher amount of RBCs, when using conventional Whatman® 903 filter paper). In addition, the back of the filter paper should always be checked to assure that the peripheral punch is made in a part of the DBS in which the filter paper is saturated. Importantly, the samples should be prepared under similar conditions as the patient samples, as the drying process is known to influence DBS homogeneity.[27,70] Other parameters that may influence the equivalence between central and peripheral punches include the filter paper type, the position of the DBS card during drying, and the punch size (with larger punches being less affected by inhomogeneities within the DBS sample). The presence of an anticoagulant on the other hand, does not seem to influence DBS homogeneity.[27]

2.3. ANALYTICAL VALIDATION

None of the currently existing bioanalytical validation guidelines have been set up for dried blood sample-based methods. Certain experiments described in these guidelines may not be applicable (e.g. freeze-thaw stability, depending on the storage and transport conditions), whereas others may require some refinement (see section 2.3.1.). Moreover, some additional parameters will have to be evaluated (see section 2.3.2.).[9,71] An overview of the required additional investigations can be found in Table 2.1. These will result in a slightly larger amount of samples that will have to be analyzed during method validation (see Table 2.2.). Before starting any analytical validation, it is essential to contemplate what the desired quality of the method should be. Although the analytical performance requirements described in e.g. the FDA or EMA guidelines are widely applied and accepted, they may not always be suitable for DBS methodology. Depending on the analyte and the purpose of the method, these requirements can be set either more or less strict based on scientific evidence. In this context, some have suggested to use acceptance criteria based on biological variation, as is common practice in other areas of clinical chemistry.[72]

2.3.1. Classical validation parameters to be evaluated

Most of the validation parameters described in traditional bioanalytical method validation guidelines will have to be assessed for DBS-based methods as well.[5,6] Therefore, those documents will need to be consulted too when performing a DBS method validation. However, the particular points of attention when evaluating those classical validation parameters in the context of a DBS method, are given below. Furthermore, to assist the reader, a brief overview of these classical validation parameters is given in Table 2.3.

2.3.1.1. Selectivity

To assess the selectivity of the method, blank matrices of at least six different individuals should be analyzed without IS, as well as two zero samples (blank DBS extracted with extraction solvent containing IS). These blank samples should be obtained using the same sampling approach as the one that will be used to collect the patient samples. In addition, DBS prepared from blank blood spiked with common co-medications, metabolites, and other potential interferences could be tested. Furthermore, it is advisable to also test blank blood samples spiked with the same solvent(s) that is/are used to generate the stock solutions that

are employed to prepare calibrators and QCs. At this stage it may also be worthwhile to run a few authentic patient samples to ascertain there is no non-anticipated co-elution of a metabolite that may not be available as a standard

2.3.1.2. Calibration model, accuracy and imprecision, measurement range

For the evaluation of the calibration model, LLOQ and ULOQ, accuracy and imprecision, all experiments should be performed in accordance with existing guidelines.[5,6] The only difference is that all calibrators, blank, zero and QC samples should be prepared in blood with the median HT of the target population and should have a volume representative of the patient samples.[53] As with any bioanalytical method, the measurement range should be representative of the concentration range in patient samples. For the purpose of TDM, a calibration range minimally spanning from half of the lower end of the therapeutic interval to twice the upper end of the therapeutic interval should suffice. Furthermore, intra- and inter-card variability do not need to be evaluated separately, as these variables will be inherently included throughout the method validation.[9] For a method to be applied in a routine context, interbatch variability should be assessed. The latter can be done by including cards from multiple batches in the validation experiments. However, if non-certified filter paper is used, a more elaborate evaluation of the filter paper may be warranted.

2.3.1.3. Dilution integrity

Contrary to traditional liquid blood samples, DBS cannot be diluted directly. Hence, to analyze samples with a concentration above the measurement range, DBS extracts are typically diluted with blank DBS extracts or extraction solvent. Furthermore, IS-tracked dilution can be performed.[6,73] With this approach a higher concentration of IS is added to the extraction solvent, with the exact amount of IS depending on the envisaged dilution factor. This approach renders the dilution a volume-non-critical step. In addition, for DBS, the donut punch approach can be used.[74] With this approach, a small central punch (i.e. smaller than the regular punch size for a given DBS method) is made from a DBS sample and is extracted simultaneously with a donut punch prepared from a blank DBS sample. This donut punch is a regular sized DBS punch from which a small central punch (with the same punch size as used for the actual DBS sample) has been removed. However, to use the latter approach

successfully, DBS homogeneity should be adequate for the small punch size and the extraction efficiency should not depend on the punch size.

2.3.1.4. Carryover

Aside from classical carryover, in a DBS workflow, the punching step could be considered a potential source of contamination. Hence, we propose to include in the method validation the processing of one or more blanks following the processing of the highest calibrator.[9] To the authors' knowledge, however, no punch-mediated carryover has been described for (therapeutic) drugs, although it has been observed for PCR-based methods.[75] In addition, physical carryover between cards should be avoided by storing the cards separately. However, if multiple cards will be stored together, potential carryover between cards requires evaluation.[9] The same acceptance criteria as for classical carryover should be applied.[5,6]

2.3.1.5. Matrix effect, recovery and process efficiency

Matrix effect, recovery and process efficiency should be evaluated in line with the set-up proposed by Matuszewski *et al*. (also see section 2).[65] For this experiment, blood from at least six different donors should be used and two concentration levels should be evaluated (i.e. low and high QC level). In addition, since it is known that the HT may strongly impact the recovery -and possibly also the matrix effect- it is essential to evaluate recovery and matrix effect at different HT levels, prepared from the blood of at least one donor. These HT levels should encompass the anticipated HT range of the target population. Alternatively, this experiment could also be performed using five HT levels (0.20 – 0.30 – 0.40 – 0.50 – 0.60). The latter set-up has the advantage that whenever the most extreme HT values do not yield acceptable results, a narrower, acceptable HT range (regarding recovery and matrix effect) may still be determined, without having to repeat the experiment. This set-up is schematically depicted in Figure 2.4. As mentioned before, to accurately perform this experiment, a fixed volume of blank or spiked blood needs to be applied on pre-punched filter paper discs.

Although matrix effects are preferably as small as possible and recovery and process efficiency as high as possible, the exact values are not that relevant. It is essential, though, that they are reproducible (i.e. relative standard deviation or % RSD within 15% after IS normalization). It is relevant to note that observations by Abu-Rabie *et al*. suggest that extraction procedures with lower recoveries may be more subject to an impact of HT.[49]

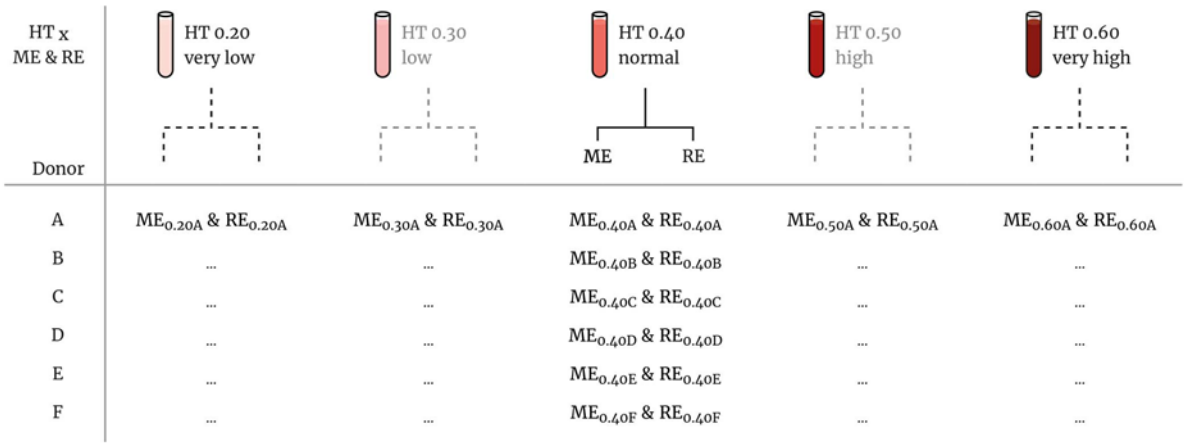


Figure 2.4: A schematic set-up for the evaluation of matrix effect (ME) and recovery (RE). The experiment can either be performed at five HT levels or at three (i.e. without the grey samples). This experiment allows to evaluate whether ME & RE are constant for different matrices and for different HT levels. Each condition is analyzed in quintuplicate. Reprinted with permission from Anoek Houben. Copyright 2018

2.3.1.6. Stability

The stability assessments performed during method validation should be representative of the ambient conditions encountered during sample transport, storage and processing. Therefore, stability should be evaluated at RT (the exact temperature depending on where the method will be applied) and the investigated time frame should cover the maximum expected time frame between sample collection, analysis and potential re-analysis. Furthermore, since temperatures may be significantly higher during transport (e.g. in a mail box in the sun during summer time) short-term stability at elevated temperatures (i.e. 2 or 3 days at 50°C – 60°C, or higher temperatures depending on the country) should also be tested.[45,76] If stability under ambient conditions is only sufficient for a couple of days (but long enough to allow transport to the laboratory), it may be evaluated if storage at lower temperatures in the lab may help stabilize the DBS until (re)analysis.

Importantly, stability may also be affected by other parameters such as humidity and exposure to (sun)light, conditions which are harder to replicate in the laboratory. To evaluate the effect of actual sample transport, samples which are generated in the laboratory can be analyzed immediately after drying, after storage for a certain time under controlled conditions and after sending them to the laboratory *via* mail service. Preferably the samples are deposited in a mail box which is relatively far from the laboratory. Furthermore, it may be relevant to repeat this experiment under different weather conditions, to rule out any seasonal effects on the stability of the samples. Although stability is typically evaluated using

spiked samples, it may be worthwhile to also evaluate the stability of incurred samples, as spiked samples may not always display the same stability profile as actual samples.[77] Additionally, autosamplerpost-preparative stability should be assessed.

2.3.2. Dried blood spot-specific validation parameters

The analytical validation of DBS methods requires the evaluation of several additional parameters (see Table 2.2): i.e. the volume effect, the volcano effect (i.e. DBS homogeneity) and the HT effect.[1,9,71] It is essential that these parameters are assessed simultaneously, as they may affect one another. These parameters can be evaluated in a single day experiment in which the obtained results are compared to those obtained from the reference condition (i.e. central DBS punches generated from DBS of average or median volume and HT). Alternatively, this evaluation can be combined with the accuracy and imprecision experiments (i.e. by measuring two series of DBS samples with different volumes, different HT levels etc. on each of three days). The latter approach has the advantage that accuracy profiles can be established.[78,79] Importantly, if a certain effect is observed (i.e. a relevant volume, HT or volcano effect) appropriate measures need to be taken to ensure patient samples are within the validated limits and patient results are reliable. Obviously, it should also be demonstrated that these measures are indeed adequate.

2.3.2.1. Volume effect

The volume range in which DBS-based results are still acceptable should be defined during method validation. Typical volume ranges to be evaluated are 10 – 50 µl for hanging-drop-collection and 20 - 70 µl for falling-drop-collection. The volume effect should also be evaluated at low (0.30), medium (0.40) and high (0.50) HT and at both low and high QC as shown in Figure 2.5. Whether a sufficient volume is collected from a patient should always be evaluated in the laboratory before DBS analysis. This evaluation should be performed based on the diameter of the DBS. More particularly, the diameter of the patient DBS should be between the diameter of the DBS prepared from the smallest validated volume at low HT and the diameter of the DBS prepared from the largest validated volume at high HT. To help patients to collect DBS of adequate volume, filter paper with two concentric circles may be used (see Figure 2.5).[80] These circles should correspond to the minimally required volume and the maximally allowed volume (also taking into account different HT levels, as described

above).[80] It should be noted, however, that this type of filter paper is not commercially available. Furthermore, although these circles may be printed onto commercially available filter paper, it should be considered that the printing itself may affect the analysis (interferences from ink or toner, potential effect on blood flow e.g. caused by paper compression or wax-like materials present in toner). Therefore, the printed filter paper should be used during the entire method validation. Alternatively, equivalence between the in-house printed filter paper and the filter paper used during validation should be demonstrated at both low and high QC levels, and at low, medium and high volume and HT. In addition, the volcano effect might have to be reevaluated, depending on the DBS punch size. Another option is to use, a phone app to assess whether the generated DBS are within the validated volume ranges.[81] Again, correct performance of the app should be verified during method validation using samples of known volume, covering the entire validated volume and HT range.

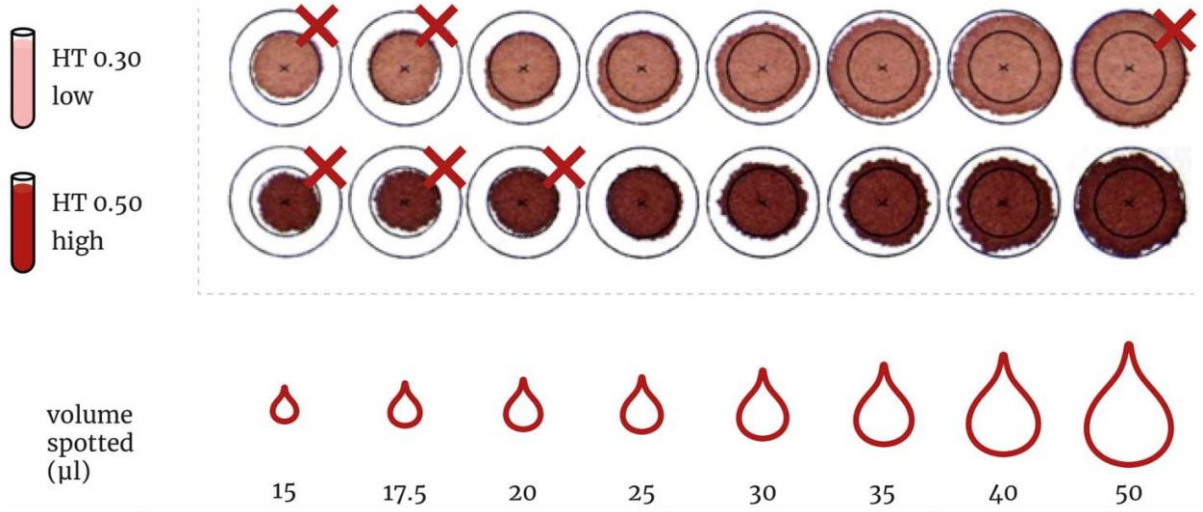


Figure 2.5: Example of filter paper with two concentric samples corresponding to the minimally required volume (e.g. 20 µL) and the maximally allowed volume (e.g. 50 µL), also taking into account different HT levels. Figure adapted from Capiau *et al.*[80] Reprinted with permission from Anoek Houben. Copyright 2018

2.3.2.2. Volcano effect

The homogeneity of the analyte distribution throughout a DBS should be evaluated when embarking upon partial-spot analysis (also see 2.2.4, prevalidation). If a relevant volcano effect is observed (e.g. punches from the central part of the spot yield different analytical results then punches from edges of the spot), only central punches should be analyzed.

2.3.2.3. Hematocrit effect

As mentioned before, it is important to actually determine the HT of the calibrators and the samples used during method validation. This will assure the exact HT value and, consequently, the validated HT range. At least three HT levels should be evaluated, more particularly, a QC generated with blood that has the same HT as the blood that was used to generate the calibrators, bracketed by HT values that encompass the expected patient HT range. At each HT level, two concentrations should be tested. The HT range that needs to be evaluated depends on the target population (see Figure 2.6). For a quasi-universal method, the range should span from 0.20 to 0.65, although a narrower range will suffice for most applications.[80] The exact range will depend on the target population and should encompass at least 95% of the target population.[53] Unless no relevant HT effect is observed over the entire HT range (both during analytical and clinical validation, cfr. part 2.4) or unless it is reasonable to assume that all patient HT values will be within the validated HT range, a method should be used to assess the HT of the patient samples. Besides confirming that the HT of the patient sample effectively lies within the validated HT range, this may also allow to perform a HT correction, to alleviate the HT bias.[82,83] Other options are to use volumetric dried blood samples (if there is no HT effect on recovery or matrix effect) or DPS (if there is no HT effect on DPS generation).[36]

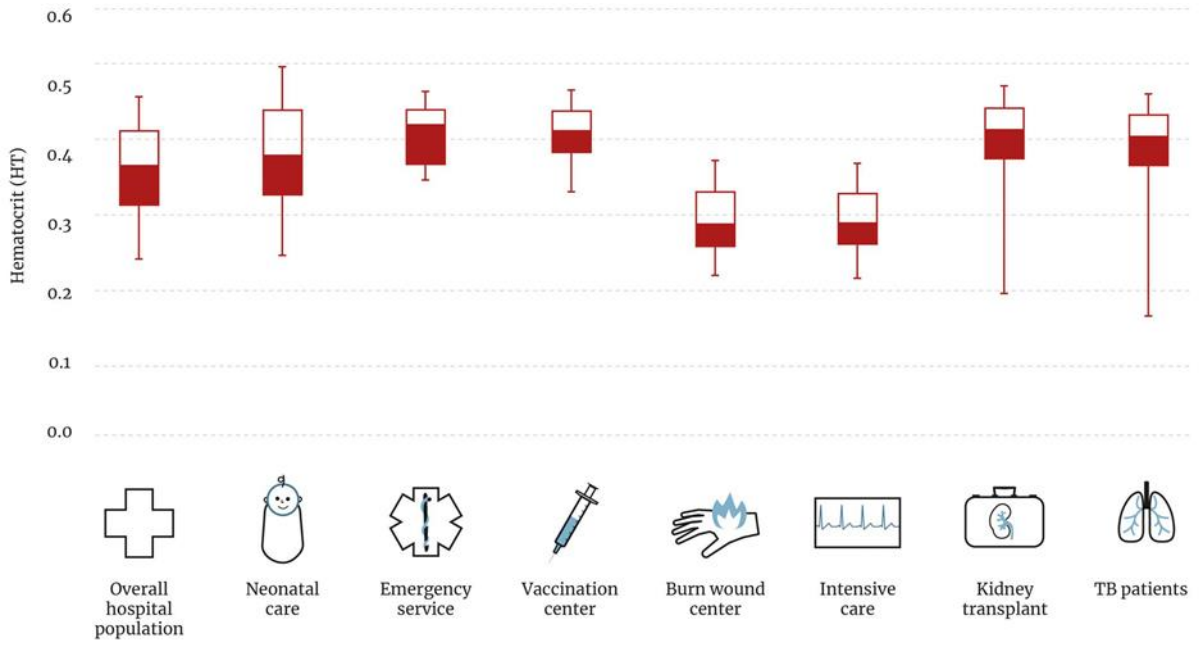


Figure 2.6: Overview of the expected HT range in different patient populations. The boxplots depict the distribution of HT values per patient population. The boxes show the HT values between the 25th and 75th percentile, as well as the median HT value. The flags show the 2.5% and 97.5% percentiles. Adapted from De Kesel *et al.*[53] Reprinted with permission from Anoek Houben. Copyright 2018

Table 2.1: Overview of the analytical validation parameters that require additional evaluation in DBS-based methods, and how to assess them.

Validation parameter	Evaluation	Statistical test/ Acceptance criterion
Recovery, matrix effect, process efficiency	Evaluate at both high and low QC levels using 6 different donors, (with one donor evaluated at minimally 3 HT levels), with each condition determined in quintuplicate*.	Should be reproducible, both between matrices and HT values (% RSD ≤ 15%).
Volume effect	Evaluate at both high and low QC levels and at least at 3 HT levels and 3 volumes*.	One-way ANOVA with bonferroni post-hoc analysis ($p \leq 0.05$). Back calculated values deviate ≤ 15% of medium volume.
Hematocrit effect	Evaluate at both high and low QC levels and at least at 3 HT levels*.	One-way ANOVA with bonferroni post-hoc analysis ($p \leq 0.05$). Back calculated values deviate ≤ 15% of medium HT values.
Volcano effect	Compare central and peripheral measurements. Evaluate at both high and low QC levels and at least at 3 HT levels and one volume (typically, the highest)*.	Paired t-test ($p \leq 0.05$) Back calculated 'peripheral' values deviate ≤ 15% of 'central' values

*HT levels should cover the entire HT range of the target population and the volumes should be representative of the sample volumes that will be generated by the patient.

Table 2.2.: An overview of the minimally required amount of analyses for the analytical validation of dried blood spots *vs.* whole blood.

Validation parameter	Amount of samples (DBS-based)	Amount of samples (liquid whole blood)
Selectivity	n = (6 + 6) x 1 x 1 = 12 6 blank matrices, 6 LLOQs, 1 day, in singulo	n = (6 + 6) x 1 x 1 = 12 6 blank matrices, 6 LLOQs, 1 day, in singulo
Calibration model	n = 6 x 5 x 1 = 30 6 calibrators, 5 days, in singulo	n = 6 x 5 x 1 = 30 6 calibrators, 5 days, in singulo
Accuracy & imprecision	n = 4 x 3 x 2 = 24 4 QC levels (LLOQ, low, mid, high), 3 days, in duplicate	n = 4 x 3 x 2 = 24 4 QC levels (LLOQ, low, mid, high), 3 days, in duplicate
Dilution integrity	n = 1 x 3 x 2 = 6 1 QC level (dilution QC), 3 days, in duplicate	n = 1 x 3 x 2 = 6 1 QC level (dilution QC), 3 days, in duplicate
Carry-over	n = (1 + 1) x 5 x 1 = 10 a blank and zero sample, 5 days, in singulo	n = (1 + 1) x 5 x 1 = 10 a blank and zero sample, 5 days, in singulo
Recovery, matrix effect, process efficiency	n = 2x (2 x 5 **x 1** x 1 x 5) + 2x (2 x 1 **x 3** x 1 x 5) + (2 x 1 x 5) = 170 2 QC levels, 6 donors, of which 1 donor at **3 HT levels**, 1 day, in quintuplicate (spiked before/after) 2 QC levels, 1 day, quintuplicate (standard solutions)	n = 2x (2 x 6 **x 1** x 1 x 5) + (2 x 1 x 5) = 130 2 QC levels, 6 donors, **1 HT level**, 1 day, in quintuplicate (spiked before/after) 2 QC levels, 1 day, quintuplicate (standard solutions)
Stability	n = 2 x 1 x 4 x 5 = 40 2 QC levels, 1 HT level, **4** points: T_0, T_{1w}, T_{2w} @ RT, T_{2d} @ 60°C, in quintuplicate	n = 2 x 1 **x 7** x 5 = **70** 2 QC levels, 1 HT level, **7** points, in quintuplicate: Bench-top stability: T_0 & T_{24h} @ RT Storage stability: T_{1w}, T_{2w} @ 4°C/-20°C **Freeze thaw stability: min. 3 cycles**
Volume effect, hematocrit effect, volcano effect	**n = 2 x 3 x 4 x 5 = 120** **2 QC levels, 3 HT levels, low, medium and high volume central punch + high volume peripheral punch, all in quintuplicate**	**N.A.**
TOTAL	**412**	**282**

RT = room temperature, T = time point, T_0 = starting point = at the minimum drying time (e.g. 2 hours), d = day, w = week.
*samples are prepared in blood of median HT, unless mentioned otherwise.

Table 2.3: An overview of the classical validation parameters and how to assess them

Validation parameter	Evaluation	Statistical test/ Acceptance criterion
Selectivity	6 individual blank matrices	≤ 20% of LLOQ (analyte) ≤ 5% (IS)
Calibration model	Use min. 6 calibrators + zero + blank. Zero and blank samples should not be included in the calibration curve.	Backcalculated concentrations ≤ 15% of nominal value (≤ 20% at LLOQ). ≥ 75% of all calibrators and ≥ 50% per calibration level should comply.
Accuracy & imprecision	Evaluate at 4 QC levels: - LLOQ - Low = ≤ 3 x LLOQ - Medium = 30 – 50% of range - High = ≥ 75% of highest calibrator	≤ 20% for LLOQ ≤ 15% for other QC levels
Dilution integrity	Evaluate a dilution factor (e.g. 1:9) applicable to the patient samples.	Accuracy and imprecision ≤ 15%
Carry-over	The analysis of (zero and) blank samples after the highest calibrator	≤ 20% of LLOQ (analyte) ≤ 5% (IS)
Recovery, matrix effect, process efficiency	Evaluate at both low and high QC, using 6 different blank matrices. - Recovery: spiked before/spiked after. - Matrix effect: spiked after/ standard solutions - Process efficiency: spiked before/ standard solutions	CV ≤ 15%
Stability	Evaluate at both low and high QC levels. Store stability QCs under representative conditions for a representative time frame and measure against fresh calibrators.	≤ 15% of nominal value (or ≤ 15% of value at T_0)

T_0 = starting point = when samples were fresh.

2.3.3. Validation of online DBS analysis

Whether the sample preparation and analysis are performed online or not, it does not affect the validation parameters that need to be evaluated. The way in which certain parameters (more particularly, recovery, matrix effect and process efficiency) are evaluated, however, will need to be adapted.[84-87]

Recovery is typically evaluated by comparing the peak areas from blank matrix samples spiked before extraction with the peak areas from blank matrix samples spiked after extraction. However, with an online sample preparation procedure, there is no option to spike the samples after extraction. Instead, the analytes are introduced to the system during the extraction step. Depending on the type of system used, this can be done *via* the IS loop or by spiking the extraction solvent. The results of the samples spiked during extraction are then compared to those of DBS samples containing the same absolute amount of analyte. This requires the entire DBS to be analyzed. When adding the analyte during extraction, the analyte passes through the filter paper and dried blank blood matrix, during which, theoretically, some analyte adsorption may occur. If such adsorption occurs, this will yield a falsely lowered '100% extracted' reference value, which in turn will result in an overestimation of the analyte's recovery. Alternatively, recovery may be evaluated by comparing the peak area resulting from a single extraction with the sum of peak areas resulting from e.g. 10 consecutive extractions. It needs to be considered that even after 10 extractions, not all the analyte may be extracted, again leading to an overestimation of the recovery. Moreover, these multiple extractions may technically not be possible because of filter paper deterioration (depending on the type of filter paper used). For the evaluation of the matrix effect, the peak areas resulting from the analysis of blank DBS samples and blank DBS cards can be compared. In both cases the analyte will be introduced during extraction.

2.4. CLINICAL VALIDATION

It is generally accepted that a DBS sampling method can only be implemented in routine care for the purpose of TDM–and thereby (partly) replacing the standard venous whole blood sampling with blood, serum or plasma analysis–after it has been successfully validated in a clinical validation study.[1,88-91] In a clinical validation study, paired DBS and venous blood, plasma and/or serum samples are obtained and analyzed. The analytical results are compared

and statistically evaluated. The purpose of a clinical validation is to demonstrate that results from DBS are interchangeable with those obtained with the standard method used for TDM, i.e. a blood, serum or plasma analysis. The aim of this part of the guideline is to provide recommendations on how to clinically validate a DBS assay for TDM in daily practice. Current recommendations regarding clinical validation are largely based on published clinical validation studies that used genuine finger prick blood-derived DBS, paired DBS and traditional matrix samples from at least 20 patients, and appropriate statistical analysis to compare both methods.[90-102]

2.4.1. Concentration range, number of clinical samples and patients

The concentration range that needs to be covered during clinical validation depends on the sampling time points of interest (i.e. trough, peak) and the shape of the PK time curve of a particular drug and the intra- and/or interindividual variability.[2] The CLSI guideline states that at least 40 patient samples should be analyzed for a clinical validation, ideally covering the entire measuring interval of the measurement procedures.[8] This sample size is based on linear regression described by Linnet *et al*.[103] The sample size that is necessary mostly depends on the CV of the method and the range ratio (maximum value divided by minimum value). Because most DBS methods have a CV > 5% and a range ratio > 25 the amount of samples needed following Linnet's calculation will always be 36 or 45. Therefore, using fewer than 40 samples is only possible if the CV of the method is < 5% and/or the range ratio < 25. Depending on the situation these 40 samples could either be paired capillary DBS-venous blood samples from at least 40 different patients collected at a single time point (i.e. trough or peak), or paired samples taken at 2-3 time points and from a smaller cohort, covering the whole concentration range of interest.[8,103] Ideally, in total 80 samples from at least 40 patients should be acquired for validation. This allows using one set of 40 randomly selected samples for fitting a line between DBS and blood (or serum or plasma) concentrations using appropiate statistical tests (see next paragraphs). If required, this allows to derive a conversion formula or factor to convert e.g. capillary DBS concentrations into venous plasma concentrations. The other set of 40 samples can be used to validate this conversion.[104] Despite the limitation of collecting multiple samples from the same patient this approach does not require a new cohort of 40 subjects. If the amount of patients is limited and multiple samples from the same patient (e.g. trough and peak) are acquired, it is our recommendation to have a minimum of

40 samples from at least 25 different patients to account for variation in matrix effects. In those cases where there is only a limited number of paired samples available, the conversion of a concentration in one matrix to that in another one can also be checked for by a jackknife method. In this approach the original set of n samples is resampled n times by systematically creating all possible subsets of n-1 samples. Each of these subsets is then used to set up a conversion equation, which is subsequently applied to the n^{th} sample (i.e. that sample which was not included in the subset that was used to set up the conversion equation).[105] To assess the predictive performance of the conversion equation the Median Percentage Predictive Error (MPPE) = median (corrected $[Analyte]_{Test\ matrix}$ - $[Analyte]^{Reference\ matrix}$)/ $[Analyte]^{Reference\ matrix}$) $*$ 100% and Median Absolute Percentage Predictive Error (MAPE) = median (|corrected $[Analyte]_{Test\ matrix}$ − $[Analyte]_{Reference\ matrix}$)|/ $[Analyte]_{Reference\ matrix}$) $*$ 100% can be calculated. These provide a measure of bias and imprecision, respectively.[106,107]

2.4.2. Comparing DBS concentrations to plasma or whole blood concentrations and effects of hematocrit

Peripherally collected blood consists of a mixture of venous and arterial blood and ISF. Therefore, the drug concentration in peripherally collected blood may differ from venously collected blood. This effect is mostly present during the distribution phase of the drug. Although drugs are usually rapidly distributed throughout the body, this process sometimes can take up to several hours, leading to unreliable results when samples are collected during the distribution phase.[2,108-110] To detect a potential capillary-venous difference (Figure 2.7), the results obtained from a DBS collected from a finger prick (sample A) can be compared with those from a DBS prepared from venously collected blood (sample B). This venous blood (sample C) can be used to generate plasma (sample D). Both sample C and D can be compared to blood collected by finger prick (sample A). Alternatively, another blood sample needs to be collected at the same time point if serum (sample E) is to be prepared. Serum or plasma is typically used for routine TDM. It is essential that samples B and C should give the same result. If they do not, this points to an effect of the DBS approach in se. In vivo, drugs can bind to components of plasma or accumulate in RBCs, leading to differences between observed concentrations in whole blood (and, hence DBS) and in plasma (or serum, depending on the matrix that is routinely used for an analyte).[98,108] The difference in drug concentration

between blood (DBS) and plasma can be explained by the fraction of drug in plasma relative to whole blood, the HT and the drug's affinity for RBCs. The study design may allow to derive this blood-plasma relationship. If a blood concentration has to be expressed as a plasma or serum concentration for easy interpretation by the clinician, HT values should ideally be measured, known or calculated for each blood (DBS) sample. Furthermore, when acceptance limits for the HT have been set based on the analytical validation, one should actually know whether the HT of a given sample effectively lies within these limits. When comparing capillary DBS values to reference whole blood values, correction factors (sometimes based on HT) can be necessary and should be derived from clinical validation studies comparing whole blood values to fingerprick (capillary) DBS values.[89,91,92,95,97,111-115]

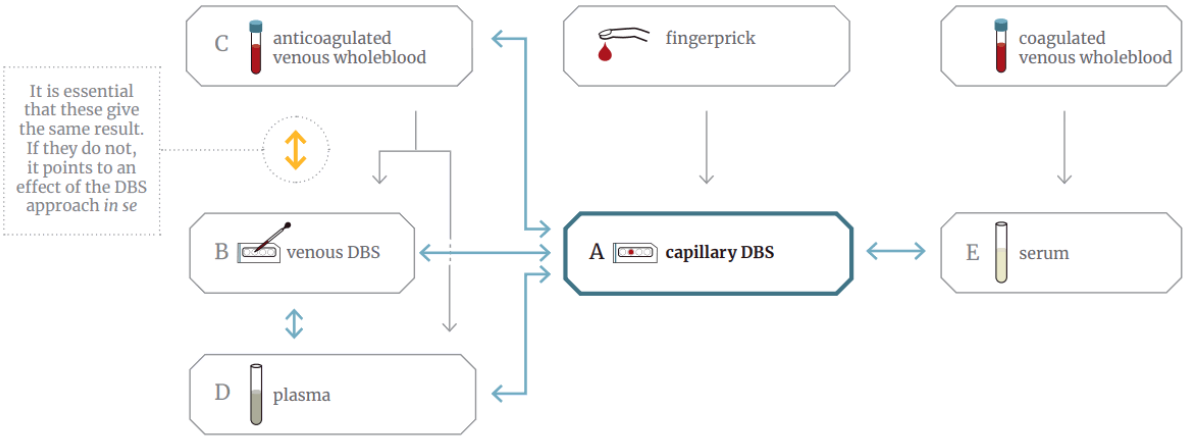


Figure 2.7: A schematic overview of the samples that could be collected during a clinical validation study. The bold blue lines depict which samples could be compared to one another. The grey lines show which samples can be generated from which sampling method. Reprinted with permission from Anoek Houben. Copyright 2018

If, for a specified HT range, the analytical validation has demonstrated that a DBS analytical method is independent of HT (or dependency is within acceptable analytical limits, see above), confirmation is required in a clinical validation study by plotting the differences between DBS results and reference method results *vs* the HT. The slope of the resulting curve should not be significantly different from zero.[80] When this has been confirmed, plasma or serum concentrations can be calculated based on the equation derived from the Passing-Bablok or weighted Deming regression line.[91,101,116-120] If an analytical method has proven to be dependent on HT values during analytical and clinical validation using appropriate statistical tests, a conversion formula should include a correction for HT.[121,122] An example is the estimation of plasma values from DBS concentrations using the formula 1-(HT/100).[122]

This will only be possible if there is a systematic effect from HT on estimated venous blood concentrations which is fixed within the relevant clinical range. [123] If this is not the case, the method might not be suitable for clinical application. If an HT-dependent method is to be used in routine care, the HT of the DBS should ideally be known. Procedures to derive HT from a DBS card include K^+ measurements[80], non-contact diffuse reflectance spectroscopy[52,83], near-infrared spectroscopy (NIR)[124] or the use of sulfolyser reagent.[125] If for a HT-dependent method it is – because of technical or other reasons – not possible to know the HT of a DBS, clinical validation can be performed for a specific patient population, provided the HT range in that specific population is narrow and lies within the method's acceptance limits (Figure 2.6).[94,98] In many instances the mean or median HT and range for a given patient population can be calculated from historical patient data.[53] For a different patient population it should be determined whether a new clinical validation should be performed.[10,98,122] Another approach to cope with the HT effect is whole blood spot analysis using a fixed spot volume. A volumetric capillary or pipet can be used to apply a fixed volume of finger prick blood to the filter paper.[14,126,127] In this situation, no conversion formula to correct for HT is needed. However, it should be clear from the analytical validation that the HT has no impact on recovery or matrix effects.[89,91,95,97,115] Moreover, this can be at the expense of the simplicity of sampling and/or bring along additional costs.

2.4.3. Statistical methods and interpretation

Technically, a DBS clinical validation is a cross validation study because a candidate method (DBS-based) is compared to a reference method (blood-, serum- or plasma-based). Although guidelines from the EMA, FDA and CLSI include cross validation and subsequent statistical analysis of results, this paragraph provides additional recommendations and guidance for the interpretation of results.[1,5,6,8]

As part of a clinical validation, the results obtained from DBS and the reference method should be compared using appropiate statistical tests. To compare two methods, regression analysis should be performed to measure the correlation, followed by an agreement and bias estimation test.[8] As both the reference and the DBS method have some inherent variability either Passing-Bablok or weighted Deming regression should be used instead of standard linear regression.[8,128-130] Both approaches have been used in various clinical validation studies.[91-102,131] Deming regression takes variability of both x and y into account, Passing-

Bablok regression makes no assumptions about the distribution of data points and is more resistant towards outliers.[8,129,132] Various clinical validation studies have shown that the absolute difference between results from a reference and a DBS method is propoportional to the concentration, at least at higher concentrations. However, in these studies, sometimes only a few high concentration samples were available.[91,96,120] Theoretically, an outlier in this region would impose an inflated or deflated estimate of proportional difference. In this case a Passing-Bablok regression analysis is the preferred statistical method.[8,133] Following regression analysis, a Bland-Altman difference plot should be made to assess the agreement between both methods and estimate the bias.[8] When using a (HT-dependent) conversion formula obtained from Passing-Bablok or weighted Deming regression, the Bland-Altman difference plot should be made using the (blood, plasma or serum) concentrations that were calculated from the DBS concentrations.[1,91]

Most clinical validation studies show some level of bias when performing a Bland-Altman test. While it may seem obvious that Bland-Altman graphs should be generated and interpreted in a correct manner, this is not always the case.[133] Several things can be deduced from a Bland-Altman difference plot. First, it can be observed whether there is an average bias between both methods and whether the 95% CI of this bias contains zero. Importantly, if the latter is not the case, it should have been formally decided beforehand what a clinically relevant or acceptable bias and corresponding limits of agreement (LoAs) should maximally be. For instance, for tacrolimus, where trough concentrations in blood are usually between 5-20 µg/L, a bias of 0.28 µg/L (LoA -0.45 µg/L – -0.12 µg/L), which is at most a bias of 5.6% (LoA 9.0% – 2.4%) would not impact clinical decision making, whereas a higher bias or LoA might.134 Second, the LoA's can be derived from the Bland-Altman plot. Here, the same holds true: pre-set criteria are needed to define what concentration or % difference span between the LoA's is still considered acceptable. This is a critical point that in many instances is lacking: e.g. whereas on average there may be no bias between a DBS- and blood-based procedure, the span of the LoA's may be too wide (implying there is too much variation) to be acceptable. What is considered acceptable in terms of bias or LoA will largely depend on the clinical setting, the lab's internal policy, the availability of guidelines (e.g. Royal College of Pathologists of Australasia or RCPA criteria)[135] and the drug of interest. Acceptance criteria should be decided by a multidisciplinary team of experts based on both clinical and analytical

acceptance criteria. In addition, during a clinical validation, it can be investigated for each measured pair of samples whether the clinical decision by the healthcare provider would differ, based on the DBS concentration *versus* the concentration in the reference sample.92,93,99,136 Again, acceptance criteria should be stated beforehand in the study protocol. The EMA guideline states for cross-validation study samples that 'the difference between the two values obtained should be within 20% of the mean for at least 67% of the repeats'.[5] It has been suggested that this guideline could also be aplied to assess agreement between DBS-based analytical results and reference results.[1] For
example, a study, in which for 30% of the samples a difference of more than 20% of the mean is observed, would theoretically fulfill the criteria put forward by the EMA guideline. However, this would likely be clinically unacceptable and in that case stricter LoAs would be preferred. It is also possible that, at lower concentrations, a maximum absolute deviation may be tolerated, while at higher concentrations a maximum allowable percentage deviation may be set.

2.4.4. Type of card/paper used

In a clinical validation study it should be stated which type of paper or DBS card is used. This type of paper should be the same as the one that was used during analytical validation.[29]

2.4.5. Sampling method and spot quality

A major problem during clinical validation is that the provided DBS may be of insufficient quality for analysis due to incorrect sampling.[42,137] Therefore, during clinical validation, the method of sampling and spot quality assessment by either an analyst or an automated quality assessment method should be mentioned in the study protocol.[138,139] As drug concentrations are dynamic, it is important to collect all paired samples within 5-10 minutes of each other.[91,116] Time-dependent changes in drug concentration are determined by PK and should be taken into account for the preparation of a sampling scheme. This is particularly relevant for drugs with a very short half-life or during the absorption and distribution phase of the drug. The sampling method that is used during clinical validation should be the same as the sampling method that will be used in daily practice. For example, if the method is intended for home sampling by patient finger prick, the DBS samples obtained for clinical validation should also be obtained by finger prick. Spotting of venous blood on a DBS card is only

appropriate if in clinical practice venous blood will be spotted on DBS cards. For instance, this may be the case when transport of tubes of whole blood is not possible due to instability of the compound or because of logistic difficulties (e.g. in remote areas or in resource-limited settings).58 This is highly relevant as for some analytes venous-capillary differences may -or are known to- be present.

If a method is designed for home sampling, patients should ideally perform a finger prick to collect a DBS sample themselves during clinical validation. However, in most clinical validation studies, a trained phlebotomist collects or helps to collect samples, to rule out variability due to inexperienced sampling by the patient.[91,95,97,99,116,123] Alternatively, both approaches can be used successively during clinical validation. Proper finger prick DBS sampling technique has been described earlier by the WHO, CLSI and in several studies[11,42,131,138,140,141] and is also shown in Supplementary Figure S-1. In short, sampling should be done after disinfecting the finger without excessive 'milking' or squeezing of the puncture site to avoid hemolysis or dilution by tissue fluid. When possible, finger prick blood should fall on the sampling paper instead of applying the droplet of blood to the sampling paper with the finger (without touching the sampling paper with the finger). Both patient and phlebotomist should be trained before samples can be obtained. This training should include practicing the whole sampling procedure under supervision of someone experienced in DBS sampling using either a test kit or a real finger prick aided by educational material such as a movie or a written instruction.[25,131,137,138,140] All spots provided in a clinical validation study should be checked for quality by an experienced analyst or *via* a validated automated quality assessment method. Some requirements for a good quality spot depend on the analytical method and should be stated on beforehand, such as minimum spot size imposed by punching size. Other requirements are independent of the analytical method. Criteria are stated in Supplementary Figure S-2. In short, all spots should be round, dried, consisting of one droplet of blood, and not touching other droplets.

2.4.6. Incurred sample reanalysis, duplicates and outliers

In their guideline, the FDA mentions ISR as a validation parameter for DBS methods.[6] In a clinical validation, ideally at least two replicate spots are available for analysis, to allow ISR and/or duplo analysis. However, reanalysis of the same spot (*via* a second punch) will not be

possible when the protocol involves the use of larger punching sizes (e.g. 6 or 8 mm).64 During clinical validation, it is recommended to analyze 2 different spots per sample, when possible, to evaluate within-card imprecision which can be calculated as the percentage difference (% difference = ((repeat value – initial value) / mean value) * 100).[5,24] The % difference between duplicates should not be greater than 20% of their mean for at least 67% of the samples.[5,6] In addition, ISR of the same spot is recommended when decentral punches may be used, provided spot homogeneity is supported by the analytical validation and small puch sizes (e.g. 3 mm) are used.[27]

The presence of an outlier may be explained by several reasons such as contamination of the sample, errors in sampling, extreme drying or storage conditions during transport or analytical errors.[42] In a clinical validation study most of the possible errors can be accounted for by, for instance, checking of spot quality of the sample upon arrival in the lab or checking and logging the drying time. When an outlier cannot be explained by such errors, the extreme studentized deviate technique[8] or a standardized score test can be used to exclude outliers.[121] However, outliers should be discussed in the context of clinical application of the DBS method. Therefore, outliers require an argumented discussion considering clinical setting and the aforementioned statistics tests.[8]

2.4.7. Clinical validation of automated analysis methods

Automation of a DBS assay could improve DBS sample- and workflow efficiency and reproducibility. Several examples exist of automated (on- or off-line) DBS assays using techniques like online extraction and SPE.[87,142,143] If an automated method is designed without a prior manual DBS method, the same recomendations for clinical validation apply. If a manual DBS assay used in clinical practice is replaced by an automated DBS method which is fully analytically validated, it is recommended to perform a cross validation including sample size of 40 samples from at least 25 different patients.[5,6,8] Due to the nature of DBS it will most likely be challenging in real practice to measure the same spot using both an on- and offline method. Therefore, if during the clinical validation the within-card imprecision is found to be acceptable and two spots per finger prick DBS sample are provided, it is recomended to analyze one spot using the automated method and one spot using the manual

method. Evaluation of agreement can again be performed by Passing-Bablok or Deming analysis and *via* a Bland-Altman plot, as described earlier.

2.4.8 Quality control

Laboratories should participate in external quality control (EQC) programs if a DBS assay is implented in routine care or provide objective evidence for determining the reliability of their results.[2,38] Apart from a prociency test pilot for the immunosuppresant tacrolimus no EQC programs are currently available for DBS assays for drugs.[144] There is an urgent need for DBS PT programs to facilitate uptake of DBS in routine care. Although EQC materials developed for the evaluation of liquid blood-based methods may be used to evaluate the quality of a DBS-based method, it should be taken into account that these materials typically have a different viscosity than true blood samples and will therefore yield DBS of deviating sizes. Therefore, when using these materials, they should always be analyzed using a full spot approach.[145] Furthermore, the extraction efficiency of an artifical matrix may always differ from the extraction efficiency of an actual sample. Since most EQC materials are only available for plasma analysis and not for whole blood analysis, anopther option might be to remove part of the plasma of a blank whole blood sample and to replace it with the EQC material. The resulting blood can then be used to generate DBS, as was successfully applied for e.g. conventional antiepileptics.[67]

2.5. CROSS-VALIDATION

Once a DBS assay has been successfully applied in clinical practice it is possible that changes have to be made to the sampling method, filter paper or analytical method. For some of these changes the standard guidelines for cross-validation are applicable.[5,6] This part will focus on additional recommendations when DBS assays or sampling methods are altered.

2.5.1. Different punch size

As stated before (see section 2), a punch size is preferably less than 4 mm because punching the sample in the lab will be easier and patients do not need to produce large blood spots. When the desired LLOQ, accuracy and imprecision can be met with a different punch (e.g. smaller or ‘donut’ punch)[74] than currently used in practice, a cross-validation study should be

performed. If during the clinical validation the within-card imprecision is within analytical limits and two spots per sample are provided, it is reccomended to analyze 1 spot with the new punch size and 1 with the old punch size. In total, 40 samples of at least 25 different patients should be analyzed. In addition, extraction efficiency and DBS homogeneity should be re-evaluated. The extraction volume used with smaller punches can be downscaled accordingly. Although theoretically possible, we do not recommend to use a surface-based formula to convert a result from a small (e.g. 3-mm) DBS punch to a theoretical bigger (e.g. 6-mm) DBS equivalent.

2.5.2. Different type of filter paper

In routine practice, several types of DBS filter paper are used such as the Whatman® 903, Whatman® FTA DMPK cards (type, A, B and C), and Perkin Elmer 226 cards.[29] Although performance of the FDA-approved Whatman® 903 and Perkin Elmer 226 paper is consistent and comparable in newborn screening,[146] the influence of drug concentration and HT can lead to a difference in recovery of up to 20% between cards.[29,147] This may be caused by the drugs' ability to form hydrogen bonds with the cellulose paper, leading to decreased recoveries[57], differences in spot homogeneity or differences in background signal.[27] Not only the recovery of the analyte may be altered, also matrix, volcano, volume and HT effects may have changed, as well as the analyte's stability. These parameters should all be re-evaluated as discussed before. Furthermore, QC samples for the new filter paper should be made using the same method as was done for the old filter paper.[54] Both old and new QC samples should be analyzed and the obtained mean accuracy should be within 15%.[5] The equivalence between both filter papers should be confirmed using a minimum of 40 samples obtained from at least 25 different patients. If not all parameters prove to be similar for both types of filter paper, a full analytical and clinical validation are required.

2.5.3. Different sampling method

Switching the sampling method will, most likely, be accompanied by some change in the method. For instance, it is likely that whole spot analysis rather than partial-punch analysis will be performed when a fixed volume of finger prick blood is deposited on a card instead of direct application of blood from the fingertip to the card. Moreover, it is possible that DBS-based assays are replaced by newer alternatives such as the earlier discussed VAMS

technique because of the convenience of sampling and/or automation possibilities.[25] Importantly, as stated earlier, volumetric sampling does not necessarily eliminate the effect of HT or ageing on recovery, so this remains an important parameter to be studied.[7,29,57,62,148] In addition, a new sampling technique might influence spot homogeneity, thereby introducing a possible unknown error in analytical results.[27] Therefore, when changing sampling technique, sample vehicle or changing to whole spot analysis it is recommended to perform a full clinical validation study, comparing the new method to the reference method, provided this change has been appropriately analytically validated.[25]

2.6. CONCLUSION

To successfully incorporate DBS-based methods in routine practice, good quality methods are a prerequisite. Since the quality of a method starts with its design, a sound method set-up not only ensures the method is suitable for a given application, it also increases the chances of a successful method validation. The quality of a method needs to be assessed both during analytical and clinical validation and should be compared with pre-set acceptance criteria. This is the first guidance document discussing how to evaluate the quality of a DBS-based method. This guideline outlines which traditional and non-traditional validation parameters should be assessed for this type of method, and provides suggestions on how to do this. Most importantly, each parameter should be evaluated in a way that reflects the real-life situation in which the method will eventually be applied. Furthermore, to ensure the method's quality on a day-to-day basis the first QC programs for quantitative DBS-based methods have been established recently. It is important to keep in mind that DBS for TDM applications only has a future if the quality of the result can be guaranteed. A proper analytical and clinical validation are essential to achieve this.

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Chapter 3

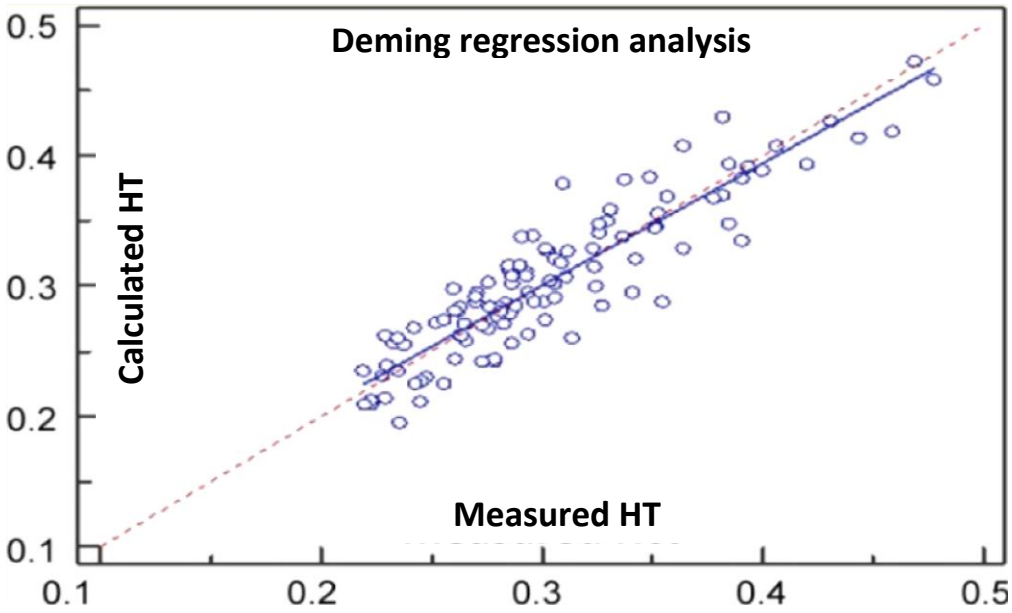
Hematocrit prediction of DBS *via* potassium measurement

Based on:

Capiau S, Stove VV, Lambert WE, Stove CP. Prediction of the hematocrit of dried blood spots via potassium measurement on a routine clinical chemistry analyzer. Anal Chem, 2013, 2;85(1):404-10.

Abstract

The potential of DBS sampling as an alternative for classical venous sampling is increasingly recognized, with multiple applications in, e.g., TDM and toxicology. Although DBS sampling has many advantages, it is associated with several issues, the HT issue being the most widely discussed challenge, given its possible strong impact on DBS-based quantitation. Hitherto, no approaches allow HT prediction from non-volumetrically applied DBS. Following a simple and rapid extraction protocol, K^+ levels from 3 mm DBS punches were measured *via* indirect potentiometry, using the Roche Cobas 8000 routine chemistry analyzer. The extracts' K^+ concentrations were used to calculate the approximate HT of the blood used to generate DBS. A linear calibration line was established, with a HT range of 0.19 to 0.63 (LLOQ to ULOQ). The procedure was fully validated; the bias and imprecision of QCs at three HT levels and at the LLOQ and ULOQ was less than 5 and 12%, respectively. In addition, the influence of storage (pre- and post-extraction), volume spotted, and punch homogeneity was evaluated. Application on DBS from patient samples (n = 111), followed by Bland-Altman, Passing-Bablok and Deming regression analysis, demonstrated a good correlation between the "predicted HT" and the "actual HT". After correcting for the observed bias, LoA's of ±0.049 were established. Incurred sample reanalysis demonstrated assay reproducibility. In conclusion, K^+ levels in extracts from 3 mm DBS punches can be used to get a good prediction of the HT, one of the most important "unknowns" in DBS analysis.



3.1. INTRODUCTION

DBS sampling is increasingly used as a minimally invasive tool to acquire a representative blood sample in the context of TDM and toxicology[1-4]. However, the analysis of DBS is associated with several issues, such as contamination risk, blood volume spotted, blood spot homogeneity, and HT[3-5], as elaborately discussed in Chapters 1 and 2. Of these, the HT is undoubtedly the most widely discussed challenge, as strongly deviating HT values may significantly impact DBS-based quantitation[3,5-15]. First of all, the HT strongly influences the spreading of a blood drop on filter paper, with higher HT values leading to smaller, more concentrated spots[3,5-9]. Second, the HT may influence parameters such as recovery and matrix effect[6,10]. Third, when DBS results are to be compared with those obtained from plasma, the distribution of an analyte between RBCs and plasma needs to be examined on a case-by-case basis[3,15]. These HT-associated issues, when compared to conventional plasma analysis, make DBS-based quantitation suffer from an additional unknown factor of uncertainty (see General Background).

Several strategies have been proposed to cope with the so-called "HT effect"[11]. The most easy approach is the analysis of complete, volumetrically applied DBS, obtained by pipetting or using precision capillaries or other microsampling devices, delivering a fixed amount of blood to filter paper[6,10]. Indeed, this approach copes with the most evident HT effect, i.e., the differential spreading of blood with varying HT. However, volumetric application requires some training and may be difficult to sustain when DBS are to be obtained by patients at home (e.g., in the context of TDM programs). In these cases, direct application from a cleaned fingertip may be the best feasible approach. As this typically implies, non-volumetric application, DBS punches rather than complete DBS should be evaluated, necessitating the definition of a HT range and a volume range in which the results for a given analyte still fulfill the acceptance criteria for imprecision and accuracy[5,8] (see Chapters 1 and 2).

Whereas some have considered the correlation between the DBS diameter and the HT of volumetrically applied blood[8,16], hitherto, there is no approach available that allows one to trace back the HT of DBS obtained by non-volumetric application of blood. A "marker" allowing one to trace back the HT should fulfill several criteria: first, it should correlate with the amount of RBCs; second, it should be universal (i.e., show minimal interindividual variation); third, it should be stable (i.e., being applicable to both freshly prepared and old

DBS, we found Hb to not fulfill this criterion); fourth, its determination should be possible on a minimal area of the DBS, while still being easy enough to allow universal implementation. Keeping these requirements in mind, K^+ was chosen as a candidate marker to predict HT. This electrolyte is primarily located intracellularly, its intracellular concentration (at approximately 140 mM) being about 35 times higher than its extracellular concentration[17]. In addition, K^+ levels are under tight physiological control, with normal serum or plasma levels ranging between 3.5 and 5 mM[17]. As RBCs are the predominant cells in the blood, roughly outnumbering white blood cells with a factor of 500–1000, these are the major contributors to the total blood K^+ concentration. Hence, the contribution by the serum or plasma and by other cells accounts for only a low % of the total blood K^+ concentration. As a consequence, physiological changes in serum or plasma concentration and/or in white blood cell count are only expected to have a minimal, if any, impact on total blood K^+ concentration. In this report, we describe that K^+ measurement in extracts from 3 mm DBS punches, using a routine clinical chemistry analyzer, allows one to trace back the HT of a DBS with acceptable accuracy and imprecision. Satisfactory results were obtained after evaluation of the developed procedure on DBS from patients.

3.2. MATERIALS AND METHODS

3.2.1. Preparation of DBS

Venous blood, used for preparation of DBS, was obtained from healthy volunteers and collected in tubes containing Li-heparin as anticoagulant (Venosafe 9 mL VF-109SHL, Terumo, Leuven, Belgium). DBS were generated the same day of blood collection by applying 25 µL of blood (unless mentioned otherwise) on Whatman® 903 filter paper (WHA10334885, GE Healthcare, Dassel, Germany), followed by air drying for a minimum of 2 h. Blood with different HT was prepared by centrifuging an aliquot of the blood in 2 mL safe-lock tubes in an Eppendorf 5804R centrifuge (Hamburg, Germany) for 5 min at 1000*g* and by removing or adding plasma. The obtained HT was measured at all instances in duplicate using a Sysmex XE-5000 hematology analyzer (Sysmex Corporation, Kobe, Japan); the mean of these duplicate measurements was considered as the actual HT. For comparing the HT of Li-heparin blood and ethylenediaminetetraacetic acid dipotassium (K_2EDTA) blood, coupled samples were obtained from both patients and healthy volunteers. Both groups received information and signed informed consent before entry into this study, which was approved by the Ethics

Committee of Ghent University Hospital (project number 2012/314). Harris cutting mats and micropunchers (Uni-Core, 3.00 mm diameter) were obtained from Sigma-Aldrich (Bornem, Belgium).

3.2.2. Extraction of potassium from DBS

Evaluation of the optimal conditions to extract K^+ from DBS was done using 3 mm punches from 2 days old and freshly prepared DBS. Different elution solvents were tested: ultrapure water (MilliQ water), generated using a Millipore device (Overijse, Belgium) with or without Triton X-100 (0.45%), phosphate buffered saline (PBS), and hypotonic (1:10) PBS with or without Tween 20 (0.05%). We opted to use solvents readily containing 2.5 mM KCl, as the final K^+ concentrations of the resulting solutions were within the validated range of the chemistry analyzer (see below), even at the extreme HT of 0.19 and 0.63. Two subsequent extractions were performed at RT in a 2 mL tube by adding 70 and 30 µL, respectively, to the DBS punch and shaking for 15 min at 1400 rpm on an Eppendorf Comfort Thermomixer. After spinning down the punch, the resulting supernatants were transferred to microcups (Sample Cup Micro 13/16, Roche Diagnostics, Mannheim, Germany). The results obtained for the DBS were corrected for those obtained for the extraction buffer. In line with the findings by Langer *et al*.[18], we did not find a measurable contribution from blank paper. Comparison of the different elution solvents (4–6 replicates for each solvent; 3 independent experiments) revealed that none outperformed elution with a 2.5 mM KCl solution in ultrapure water. Using the latter extraction solvent, the optimal extraction conditions (i.e. extraction volumes, time and temperature) were evaluated further (4–6 replicates for each condition).

3.2.3. Analyses

Routine HT and K^+ measurements were performed in the ISO 15189 accredited Laboratory of Clinical Biology at Ghent University Hospital. HT determinations were performed using a Sysmex XE-5000 hematology analyzer, having a measurement range of 0.17 (arbitrarily set, i.e., the lowest QC applied in the routine laboratory) to 0.75. K^+ measurements were performed by indirect potentiometry using an ion-selective electrode (ISE) using the ISE module of the Roche Cobas 8000 chemistry analyzer (Roche Diagnostics), disposing of two measurement units and having technical limits of 1.5 and 10 mM. HT determinations on the Sysmex XE-5000 hematology analyzer, as well as K^+ measurements using the ISE module of the Roche Cobas 8000 chemistry analyzer, are controlled by daily internal QC and trimestrial

EQC evaluations. For HT measurements, in-house validation revealed that none of the total errors (bias + 1.65 x inter-day CV) for QCs at a HT of 0.17, 0.36 and 0.45 exceeded 3.71%, meeting the desirable specification criterium of 4.1% total error, set by Ricos *et al*.[19] . For K^+ measurements, none of the between-day CVs and biases exceeded 1% and none of the total errors exceeded 2.02% for QCs at 3.48, 3.47, 6.15 and 6.17 mM (ISE 1 and 2), meeting the criterium set by Ricos *et al*. (maximum allowable total error of 5.8%)[19] and meeting the allowable limit of performance (ALP) set by RCPA Quality Assurance Programs (ALP of less than 0.2 mM for values up to 4 mM and of 5% for higher values)[20]. For the validation and application, K^+ and HT measurements were performed in duplicate (unless otherwise mentioned) and the average of these duplicates was used for the calculations.

3.2.4. Validation

Homoscedasticity (homogeneity of variances), linearity, and the choice of the calibration model were evaluated by generating five 7-point calibration curves from DBS, prepared from blood with a HT of ±0.25, 0.30, 0.39, 0.45, 0.50, 0.55, and 0.61 (prepared from a single donor). Homoscedasticity was examined by plotting the absolute residuals *versus* the HT and by performing the F-test at the lowest and highest calibrator levels, at the 99% CI[21,22]. Linearity was assessed by performing Fisher's test[23]. Calibration curves were generated by unweighted, $1/x$, $1/x^2$, $1/y$, and $1/y^2$ weighted linear regression. The choice of the calibration model was based on the sum % relative error (RE) and the % RE plot *versus* HT[21].

For the evaluation of accuracy (% bias) and imprecision (% RSD), we prepared on each of 4 different days, two 7-point calibration curves from blood with a HT of ±0.19, 0.26, 0.33, 0.42, 0.48, 0.55, and 0.63. In conjunction with every calibration line, three DBS QCs (with a HT of ±0.24, 0.41, and 0.58, further referred to as low, medium, and high QC) were prepared. For every calibrator and QC, two punches were analyzed. Eight calibration curves were obtained by plotting the mean of the K^+ measurements for the two punches *versus* the HT. The HT of the QCs (4 x 2 series of 2 punches per QC) was calculated from the respective regression lines. Accuracy, expressed as % bias, was calculated as the average % deviation of the calculated QC HT (mean of the 2 punches; n = 8) from the actual HT and needed to be less than 15%. Intra- and interbatch imprecision, calculated *via* one-way ANOVA, were expressed as % RSD and needed to be < 15%. The latter calculation started from the HT values obtained for the 8 series (batches) of QCs, every batch containing the results of 2 replicates (punches). To

confirm the validity of the calibration model, the same series of calibration curves (8 curves) and QCs (8 QCs at 3 levels; 2 punches analyzed for every QC) were prepared, starting from blood from another donor, followed by evaluation of accuracy and imprecision. In addition, the QCs from both donors were cross-tested using the regression equation obtained for the other donor. A schematic overview of the validation set-up can be found in Figure 3.1.

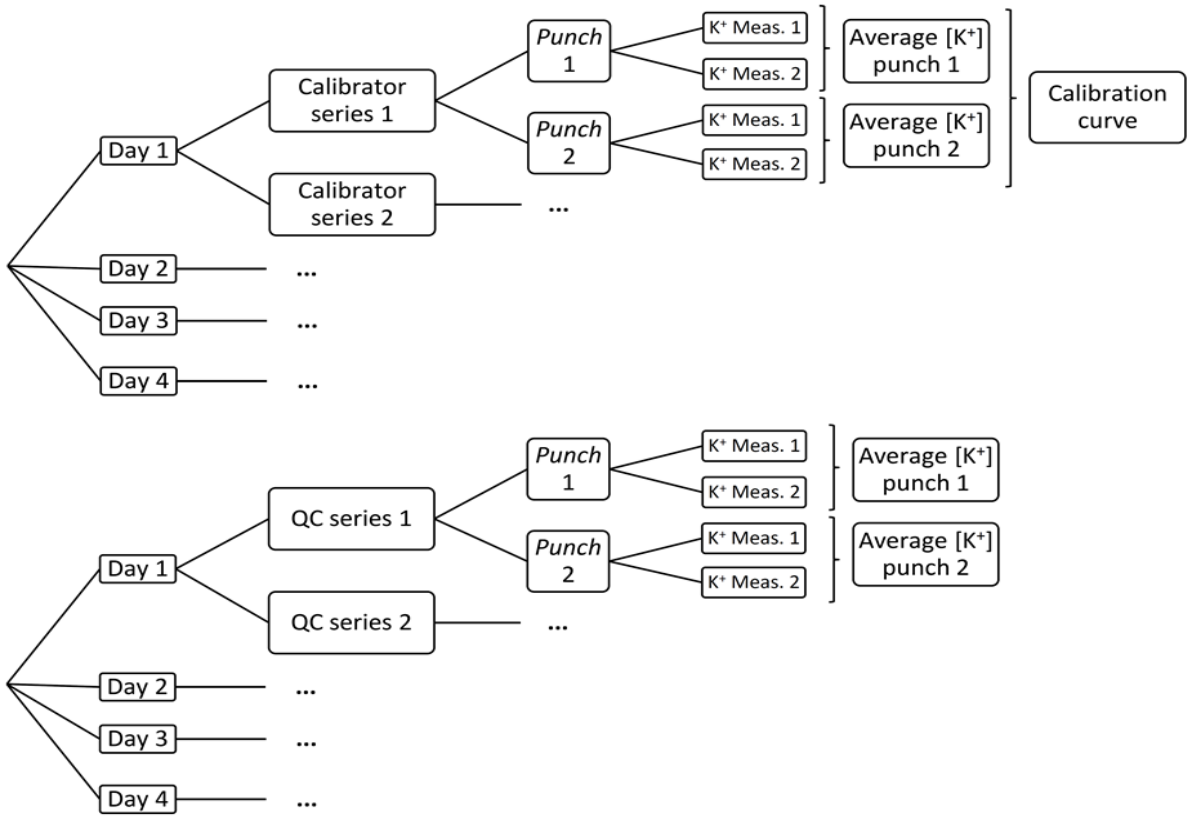


Figure 3.1: Schematic representation of how imprecision and accuracy were evaluated.

The LLOQ and ULOQ were arbitrarily set as the mean of the lowest and highest points of the calibration curves, respectively. The back- calculated values for these calibration points, using the final calibration curve, were used for determination of imprecision (% RSD) and accuracy (% bias) and needed to be < 15% (20% for LLOQ).

The impact of the punching site and of the applied volume was evaluated at three HT QC levels. K^+ concentrations obtained from central punches were compared with those obtained from peripheral punches, excluding the very edge (n = 6). The impact of the volume applied was evaluated using DBS of 15, 17.5, 20, 25, 30, 35, 40, or 50 µL of blood (n = 6).

The influence of storage was evaluated both before and after extraction of DBS. Post-extraction stability of the K^+ concentrations was evaluated by combining the extracts of 3 punches in 1 microcup (n = 6), followed by direct evaluation and after 1, 6, 17, 24, 42, and

72h. The influence of storage on K^+ in DBS was evaluated by comparing the K^+ concentrations from freshly prepared DBS (dried for 2h) with those from DBS, stored at RT for 1 or 55 days and stored at 60 °C for 20h.

Finally, we performed ISR (with 7 days in between both measurements) on a subset (n = 49) of patient DBS (see below). More than 2/3 of the repeated measurements should fulfill the acceptance criterion, i.e., lie within the limits of ± 20% of the mean of the original and the corresponding reanalysis result[224].

3.2.5. Application to patient samples

Evaluation of the procedure was performed using blood samples destined for routine clinical chemistry and hematology analysis, collected in Li-heparin tubes (Venosafe 2 mL VF-052SHL, 6 mL VF-106SAHL, or 9 mL VF-109SHL) and in 4 mL K_2EDTA tubes (Venosafe VF-054SDK) (all from Terumo, Leuven, Belgium). DBS were prepared upon arrival at the clinical laboratory by pipetting 25 µL of Li-heparin anticoagulated blood onto filter paper (n = 118). Relevant routine clinical parameters evaluated in the plasma obtained from these tubes included hemolytic index (HI) and plasma K^+ concentration. HT from corresponding K_2EDTA tubes was determined *via* a single measurement on the Sysmex XE-5000. Following extraction from one 3 mm DBS punch per patient, K^+ levels were measured in duplicate. Samples with evidence of (or no data on) hemolysis (HI > 88) (n = 2) and samples where no duplicate results of extracted K^+ were obtained (n = 5) were excluded, resulting in a final data set of 111 samples.

3.2.6. Data analysis

Statistical evaluation of the data was carried out using IBM SPSS Statistics 19 and Microsoft Excel 2010. Comparisons between the different extraction conditions were made using unifactorial ANOVA analysis. An unpaired *t* test ($\alpha = 0.05$; 95% CI) was used to evaluate the influence of storage pre- and post-extraction and to evaluate the effect of site of punching and applied volume.

Microsoft Excel 2010 was used to generate a Bland-Altman plot, while Medcalc software version 12.3.0.0 was used for Passing-Bablok and Deming linear regression analysis, for generating boxplots, and for generation of a mountain plot, depicting the distribution of the differences between the compared methods. The % RSDs, necessary for performing Deming linear regression analysis, corresponded to 0.81% for the Sysmex-measured HT (i.e., the %

RSD for the highest QC) and were derived from the duplicates (calculated from duplicate K^+ measurements) for the calculated HT.

3.3. RESULTS AND DISCUSSION

None of the evaluated elution solvents outperformed elution with a 2.5 mM KCl solution in ultrapure water (Figure 3.2). Further optimization of the extraction using 2.5 mM KCl in ultrapure water revealed that i) two subsequent elutions with 50 µL equaled elution with 70 and 30 µL respectively, and was better than a single elution with 100 µL; ii) maximal extraction was already obtained within 1 min of shaking at 1400 rpm; iii) extraction at 37 °C did not improve the extraction efficiency (Figure 3.3.). This resulted in the following extraction conditions: a 3 mm DBS punch is subjected to two subsequent 5 min extractions at RT, under continuous shaking (1400 rpm), using 50 µL of a 2.5 mM KCl solution in ultrapure water. After the first extraction, 40 µL was transferred to a microcup; after the second extraction, another 50 µL extract was added to the microcup. Although a 1 min extraction gave equivalent results, we opted in the final protocol for a 5 min extraction for practical reasons. The combined extracts can be stored in the microcups at 4 °C for at least 72 h (Figure 3.4A) before analysis by the routine clinical chemistry analyzer. The developed procedure is easy and straightforward and is in principle fully automatable, allowing high-throughput analyses.

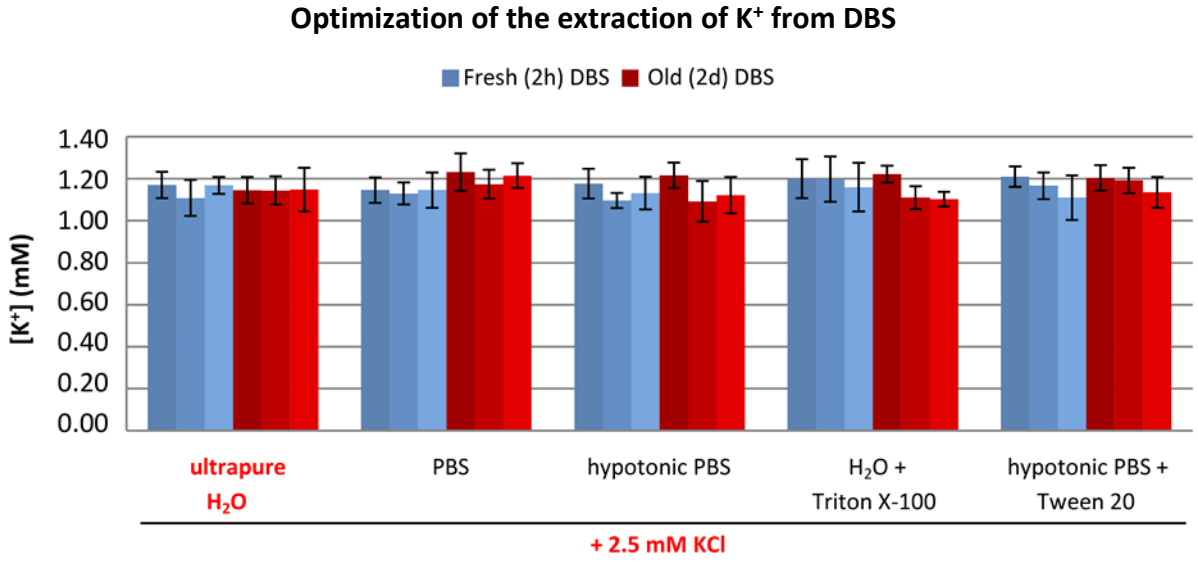


Figure 3.2: Evaluation of different extraction solvents to extract K^+ from DBS. K^+ concentration (mM) in the extract of 2h- and 2d-old DBS (approx. HT of 0.4) after correction for the blank. The extraction was performed using solvents containing 2.5 mM KCl: H_2O, H_2O with 0.45% Triton X-100, PBS, hypotonic (1:10) PBS and hypotonic PBS with 0.05% Tween 20. The different series of 3 blue and 3 red bars each indicate 3 independently performed experiments. In each bar, the average and standard deviation (SD) are shown (n = 4-6). The chosen condition is highlighted in bold & red.

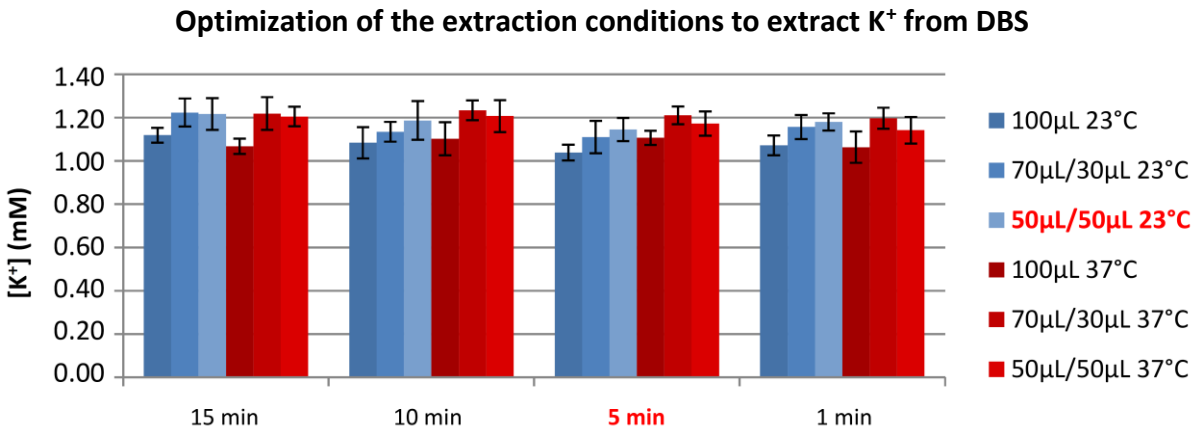


Figure 3.3: Evaluation of different extraction conditions to extract K^+ from DBS. Optimization of the volume of extraction solvent, extraction temperature and extraction (shaking) time. Shown are the average K^+ concentrations (mM), with standard deviations, in the extracts of DBS with an approximate HT of 0.4 (n = 6), after subtracting the K^+ concentration from the extraction solvent. Extraction was performed using 2.5 mM KCl in ultrapure H_2O. The chosen condition is highlighted in bold & red.

Several parameters have been shown to potentially influence the distribution of analytes in DBS. Therefore, it is important to control whether the K^+ concentration measured in the 3 mm DBS punches is affected by the punch location (peripheral *versus* central) or by the blood volume that was applied to prepare the DBS. Irrespective of the HT, no significant difference (95% CI) was seen between the K^+ concentrations in discs punched out from 25 µL DBS peripherally *versus* centrally (Figure 3.4B). Evaluation of the applied volume revealed that differences in K^+ concentrations never exceeded 15%, taking 25 µL DBS as the reference and as extreme volumes 15 and 50 µL, respectively, irrespective of the HT. However, although limited in extent, we did observe a trend of increasing K^+ concentrations upon increasing DBS volume (Figure 3.4C), as also others did, albeit for other analytes[5,9]. To minimize this volume effect, we propose that a volume criterion is set using filter paper with two preprinted concentric circles, in which a DBS should fill the inner circle (8 mm diameter) completely, while not exceeding the outer circle (13 mm diameter). Doing so, the volume of DBS will always lie between approximately 20–25 and 40–50 µL, whatever the HT (see Figure 2.5 in Chapter 2). In our experience, this volume range covers the typical volumes obtained from a single drop of blood following a fingerprick.

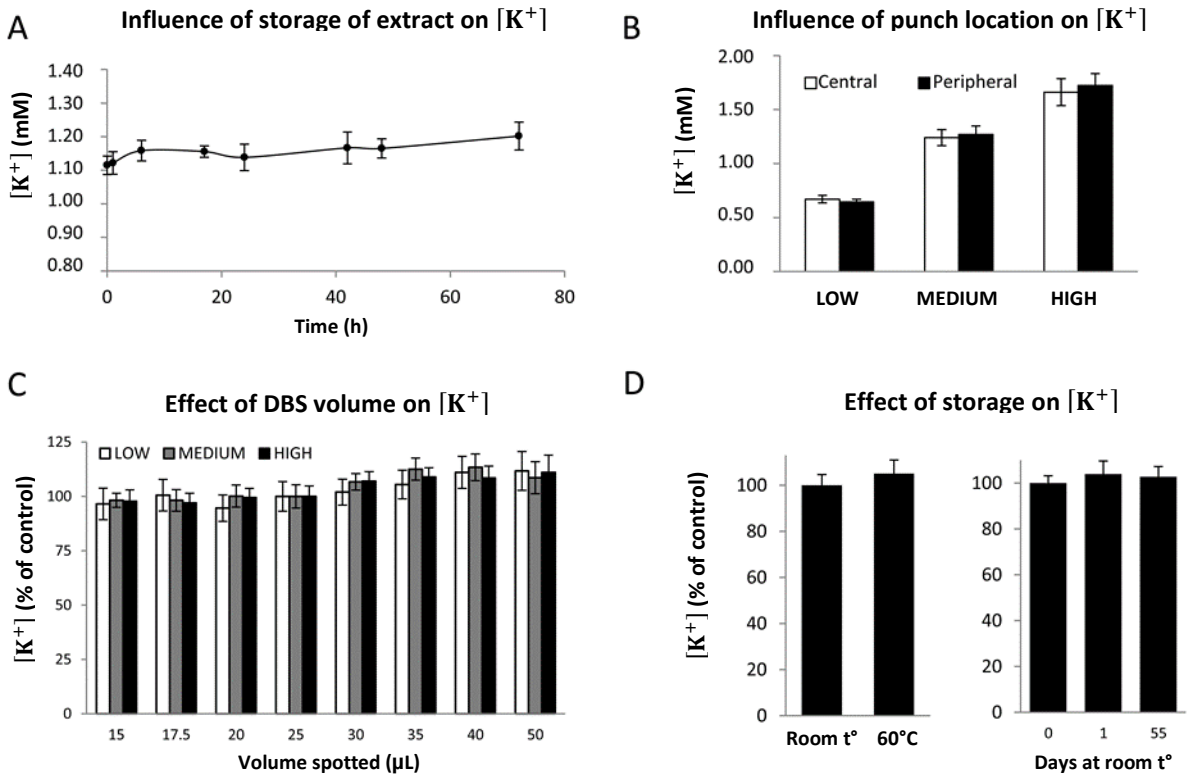


Figure 3.4. Influence of storage of extracts on the K^+ concentration in the extracts. The mean K^+ concentration at the different time points is shown, together with the standard deviation (n = 6) (A). Influence of the punch localization on K^+ concentration in the extracts, at three different HT levels. The bars show the mean K^+ concentration, together with the standard deviation (n = 6) (B). Influence of the volume used to generate DBS on the K^+ concentration in DBS extracts, at three HT levels. The bars show the mean of 3 independent experiments (each with n = 6 for every volume), with indication of the pooled standard deviation. A 25 µL DBS was taken as the reference (C). Influence of long-term storage (up to 55 days) at ambient temperature or short-term storage (20 h) at elevated temperature (60 °C) on K^+ concentrations in DBS. The bars show the mean and standard deviation (n = 6) (D).

Statistical evaluation of the calibration data revealed that the data were homoscedastic (i.e., had homogeneous variances) and that the calibration lines were linear, with no need for weighting. The slope and intercept of the calibration curve were 3.15 and −0.09, respectively, with respective 95% CI of 2.96–3.34 and −0.20 to −0.05. The LLOQ and ULOQ were arbitrarily set at the lowest (0.19) and highest (0.63) points of the mean calibration curve, respectively. Even in a hospital setting, with an obvious overrepresentation of critically ill patients, this HT range of 0.19–0.63 covers over 99.5% of the patients (1-year data, Ghent University Hospital). As shown in Table 3.1., imprecision and accuracy for the QCs (3 levels), LLOQ, and ULOQ fulfilled the predefined acceptance criteria at all instances (< 15% RSD and bias). To confirm the validity of our approach, this part of the validation was duplicated, using blood from another donor, giving similar results (see Table 3.2). Moreover, when the QCs from this second donor were fitted into the final regression equation, obtained using blood from the

first donor (and vice versa), imprecision and accuracy acceptance criteria were still met (Tables 3.1 and 3.2). Furthermore, evaluation of the influence of storage indicated that no significant change in K^+ concentration occurred upon prolonged storage of DBS at RT or after storage for 20h at 60 °C (Figure 3.4D).

Table 3.1. Overview of the data for accuracy and inter- and intrabatch (n = 8) imprecision for donor 1*

		Accuracy (% bias)	Intrabatch imprecision (% RSD)	Interbatch imprecision (% RSD)
A)	QC low	**-1.07**	**8.49**	**11.69**
	QC medium	**0.09**	**3.30**	**5.05**
	QC high	**-2.25**	**3.42**	**6.72**
B)	LLOQ (0.19)	**4.20**	**9.25**	**9.25**
	ULOQ (0.63)	**2.75**	**4.76**	**9.17**
C)	QC low (donor 2)	**-2.05**	**8.75**	**10.57**
	QC med (donor 2)	**0.57**	**3.37**	**6.26**
	QC high (donor 2)	**-1.31**	**3.54**	**6.61**

*A and B, respectively, give the data obtained for QCs (3 HT levels) and LOQs (LLOQ and ULOQ), prepared from blood from the same donor as the one in which the calibrators were prepared. C gives the data for QCs prepared from blood from another donor than the one in which the calibrators were prepared.

Table 3.2: Overview of the data for accuracy and inter- and intrabatch (n = 8) imprecision for donor 2*.

		Accuracy (%bias)	Intrabatch imprecision (% RSD)	Interbatch imprecision (%RSD)
A)	QC low	**1.06**	**6.77**	**7.44**
	QC medium	**-1.67**	**4.10**	**10.35**
	QC high	**0.12**	**7.00**	**12.84**
B)	LLOQ (0.19)	**2.76**	**7.34**	**16.37**
	ULOQ (0.63)	**2.70**	**5.80**	**9.75**
C)	QC low (donor 1)	**2.16**	**6.65**	**11.27**
	QC med (donor 1)	**-1.80**	**3.94**	**8.30**
	QC high (donor 1)	**-1.38**	**6.85**	**10.04**

*A and B respectively give the data obtained for QCs (3 HT levels) and LOQs (LLOQ and ULOQ), prepared from blood from the same donor as the one in which the calibrators were prepared. C gives the data for QCs prepared from blood from another donor than the one in which the calibrators were prepared.

The developed procedure was evaluated using paired K_2EDTA and Li-heparin blood samples, collected from patients (n = 111) for routine clinical purposes. These were used for direct HT determination and for generating DBS, respectively. For the latter, the developed procedure was applied to calculate the HT. Figure 3.6A shows the Bland-Altman comparison of the actually measured HT and the calculated HT, with indication of the mean difference and the

LoAs, all with their 95% CI. A first conclusion, which can be inferred from this comparison, is that there is a negative bias of 0.019. As suggested by Bland-Altman, when a consistent bias is observed between two methods (a reference method and a new method), this bias can be adjusted in the new method[25]. We opted to do this for three reasons. First, the slightly negative bias was consistently observed: when splitting the total data set of 111 patients into three subdata sets (each analyzed on a different day), a negative bias was observed for all subdata sets (Figure 3.5). Second, a trend line through all data points of the Bland-Altman comparison had a slope close to zero, demonstrating the absence of a proportional difference (i.e., the bias does not depend on the HT) (data not shown). Third, at least part of this bias can be explained by the fact that HT values obtained from Li-heparin blood, used for setting up the calibration lines and for generating the DBS, are significantly lower than the HT values obtained from the "gold standard" for HT measurement, i.e., K_2EDTA blood. We made this observation in paired blood samples of both patients and healthy volunteers and found it to be related to the mean corpuscular volume (MCV) of the RBCs, which is significantly lower in Li-heparin blood (data not shown).

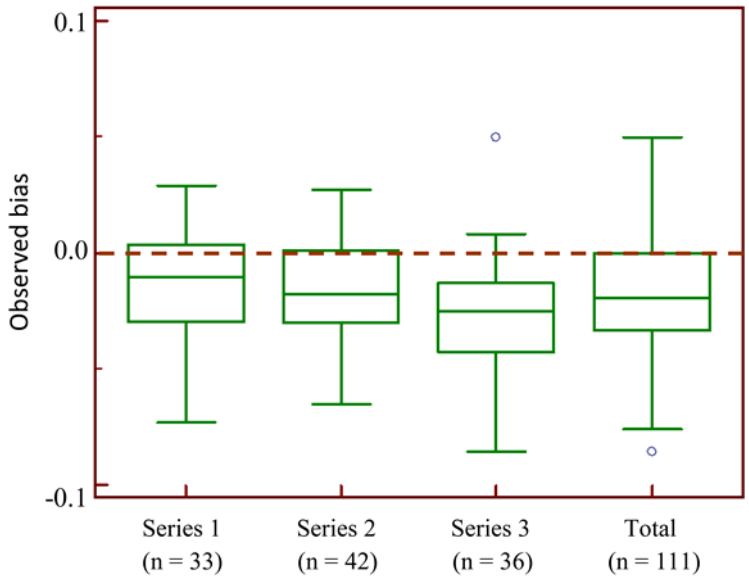


Figure 3.5: Boxplots indicating the distribution of the bias for the sub-datasets (analyzed on 3 different days, Series 1 to 3) and for the total dataset (Total), demonstrating a consistent slightly negative bias.

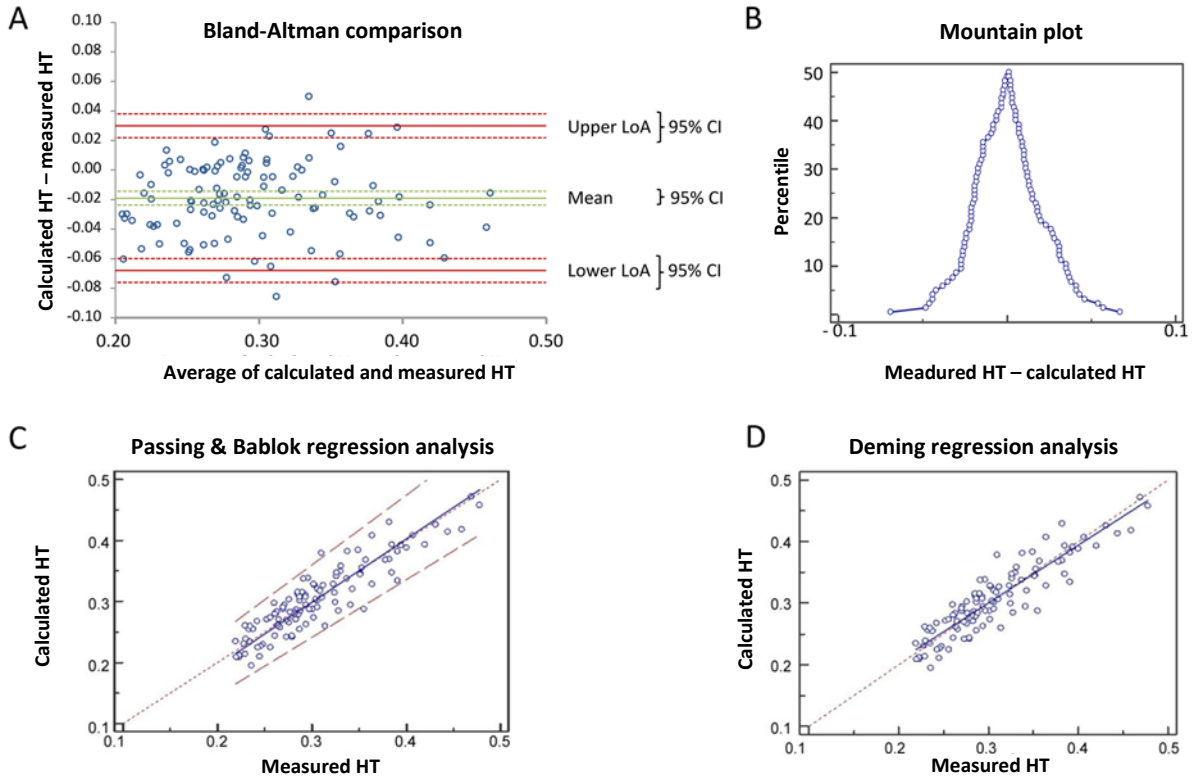


Figure 3.6. Bland-Altman comparison of calculated HT and measured HT. The mean difference, together with the upper and lower LoAs is indicated, with the respective 95% CI (A). Mountain plot depicting the distribution of the differences between the calculated and the measured HT, after correction for the bias (B). Passing-Bablok (C) and Deming (D) regression analysis of the calculated and measured HT, after correction for the bias.

Figure 3.6B shows a mountain plot, depicting the distribution of the differences between both methods for all data points, after bias correction. This plot nicely demonstrates that the differences are centered around zero. Passing-Bablok linear regression analysis was performed, also after correcting for the bias (Figure 3.6C, Table 3.3). A linear model fits the data, and the 95% CI of the intercept contains the zero value. Hence, after correction for the bias, there is no systematic difference between the two methods. In addition, as the 95% CI for the slope contains the value of 1, there is no proportional difference between the two methods. The same conclusions were obtained when performing Deming regression analysis (Figure 3.6D, Table 3.3).

Table 3.3. Results of the Passing-Bablok and Deming Linear Regression Analysis of the Comparison of the Calculated HT with the Measured HT*

	Passing-Bablok linear regression	Deming linear regression
Regression equation	1.036*x* – 0.012	0.938*x* + 0.019
95% CI slope	[0.943 - 1.141]	[0.861 - 1.015]
95% CI intercept	[–0.042 - 0.018]	[–0.004 – 0.042]

*The regression equations are given, together with the 95% CI for the slope and intercept.

A second conclusion that can be drawn from the Bland-Altman comparison is that the LoAs, after correcting for the bias, lie at ±0.049. These LoAs are acceptable, given the purpose of the method, i.e., getting an approximate estimation of the original HT. As such, the developed procedure allows one to make a statement whether results obtained with a certain analytical method are indeed valid (i.e., whether the HT of any given DBS lies within the predefined range of a validated method) or rather provide an under- or overestimation of the actual analyte concentration. The variation of the K^+-measurements themselves (i.e., technical variation) accounts for less than 0.01 of the observed LoAs. Although this is overall limited, it may be worthwhile to evaluate whether the use of alternative methodologies for K^+ measurement, such as flame photometry, atomic absorption spectroscopy (AAS), or ICP-MS-based methods, may further improve the LoAs. However, the possible benefit in increased sensitivity and/or precision of alternative configurations should be weighed against the advantage of high-throughput and simplicity offered by fully automated high-speed clinical analyzers (e.g., the Cobas 8000 configuration used here allows up to 600 samples/hour to be analyzed for Na^+, K^+ and Cl^-). The prime responsible factors for the LoAs are the variation induced by manipulation (manual punching, extraction, and transfer) and the biological variation. With respect to the former, it can be expected that the LoAs may be narrowed further by automation, as in this work all steps of the sample preparation procedure were performed manually. With respect to the biological variation, we evaluated whether there was a possible influence of the plasma K^+ concentration. To this end, we looked if a correlation could be observed between the deviation of the calculated HT from the expected HT and the deviation of the plasma K^+ concentration (range of 2.8–5.2 mM) from the median plasma K^+ concentration (4.0 mM). As expected, given the minor contribution of the plasma K^+ concentration, no such correlation was observed (Figure 3.7A).

ISR, performed on a subset of the patient samples (n = 49), demonstrated that, with one single exception, all repeated measurements lie within ± 10% of the mean of the repeated and the original measurement. Hence, the acceptance criterion (2/3 lying within ± 20%) was more than met, demonstrating good assay reproducibility (Figure 3.7B)[24].

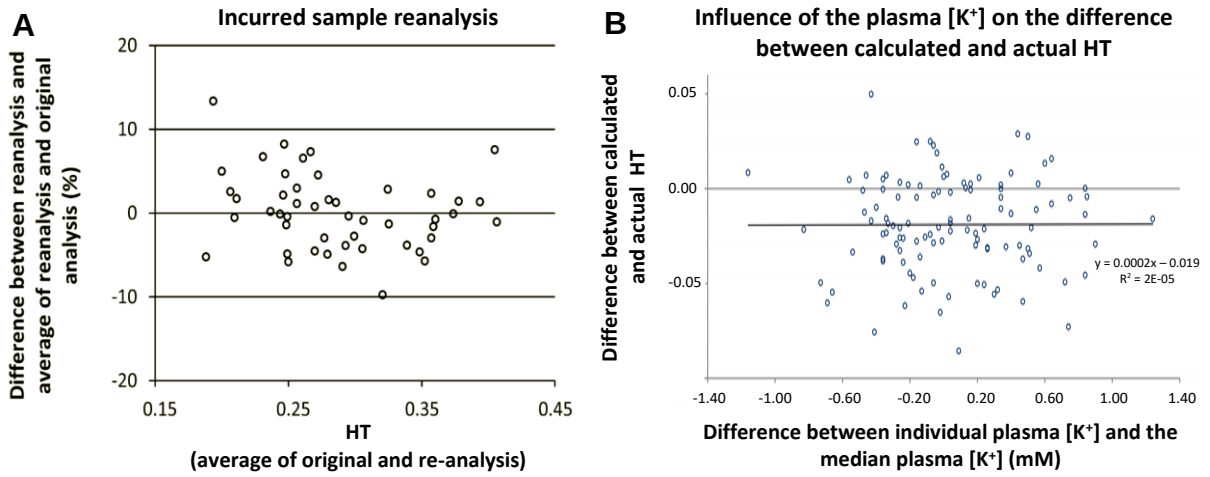


Figure 3.7A: Lack of correlation between the deviation of the individual plasma K+ concentrations (range 2.8 - 5.2 mM) from the median plasma concentration (4.0 mM) and the deviation of the calculated HT from the expected HT (n = 111) (non bias-corrected data). Figure 7B. ISR, performed on patient DBS samples (n = 49). The difference (in %) between the result of the reanalysis and the average of the reanalysis and the corresponding original analysis is plotted *versus* the HT (average of the original analysis and reanalysis).

3.4. CONCLUSION

In conclusion, the successful validation of the developed procedure and its application on real patient samples demonstrate its practical applicability, covering a HT range of 0.19–0.63. The developed methodology, which because of its simplicity and speed can be easily introduced into any automated clinical laboratory, allows one to make a good prediction of the HT, one of the most important "unknowns" in DBS sampling. Every analyst working with DBS is aware of the HT effect and, in our opinion, any validated method using DBS punches should define a HT range in which precision and accuracy for a given analyte are still acceptable. However, hitherto, there was no methodology available to actually confirm that the HT of blood used to generate a given DBS actually lies within the acceptable range. Being able to predict the HT of any given DBS may also render it possible in the future to cope with, and possibly even to adjust for, the "HT effect" in any given DBS-based analytical method. In addition, even though analysis of complete DBS, requiring volumetric application, has been advocated as one of the best solutions to overcome the HT effect[11], still, correct interpretation of the obtained results, and correlation with plasma data, requires knowledge of the HT of the DBS. It will be important to extend this study in the future to true capillary blood samples. Importantly, in these cases, capillary HT should be determined as a reference, as this may differ from venous HT[3,26,27]; a finding we also observed in our preliminary experiments.

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Chapter 4

Hematocrit prediction of VAMS *via* potassium measurement

Based on:

Capiau S, Stove CP. Hematocrit prediction in volumetric absorptive microsamples. *Journal of Pharmaceutical and Biomedical Analysis.* Submitted.

Abstract

Recently, VAMS has been suggested as an alternative to DBS sampling. With VAMS a fixed volume of blood (approximately 10 µL) is wicked up by the absorbent tip of a collection device, indepent of the HT of the blood sample. This way VAMS effectively avoids the HT bias which occurs in DBS analysis. Nonetheless, the HT remains an important variable in VAMS analysis, particularly if VAMS-based blood results need to be converted to serum or plasma values to allow comparison with e.g plasma-based therapeutic intervals. Indeed, an analyte's plasma to whole blood ratio may be HT-dependent. Therefore, we developed two straightforward methods to derive the HT value from a VAMS sample based on its K^+ content. One of these methods uses an aqueous extraction procedure, whereas the other one requires an organic extraction. Both methods have the potential to be seamlessly integrated with most existing VAMS analyses, allowing both target analyte quantitation and K^+ analysis on a single VAMS extract.

4.1. INTRODUCTION

Blood microsampling strategies allow minimally invasive collection of blood, without the need of a phlebotomist. Especially in the context of self-sampling at home, these strategies have received much interest over the last decades. Originally, microsampling applications focussed mainly on DBS collection, in which a drop of capillary blood is obtained *via* a heel- or finger prick and collected onto filter paper. Unfortunately, quantitative DBS-based results often depend on a patient's HT. Indeed, since the HT affects the blood's viscosity and thereby the spreading of the blood through the filter paper, the exact amount of blood (and thus the amount of a certain analyte) present in a fixed-size DBS punch depends on the HT[1,2] (see General Background, Chapter 1 sections 1.4.1 en 1.4.1.2, and Chapter 2 sections 2.3.2 and 2.4.2 for more information) . Therefore, we previously developed a method to estimate the HT of a DBS, based on the potassium content of an aqueous DBS extract[3] (see Chapter 3). Potassium is a suitable marker of HT as it is predominantly located intracellularly (at approximately 140 mM, a value which we also found during our experiments, see Supplementary section S-2). As RBCs are the most prevalent cells in blood, the potassium content of a dried blood sample, hence, correlates with the amount and volume of RBCs that were present in the liquid blood sample. Furthermore, potassium is a universal marker: gender, conditions such as thalassemia and treatments such as dialysis have proven to not significantly affect the intracellular potassium concentration[4-7]. This estimated HT has also been used to apply a HT-dependent correction factor to DBS-based results, successfully eliminating the HT effect[8].

Another microsampling strategy, i.e. VAMS, has recently gained more widespread use[9]. With VAMS, a fixed volume of blood (independent of a patient's HT) is wicked up by an absorbent tip attached to a plastic handle[10,11]. Although VAMS eliminates the influence of HT on the analyzed sample volume, it is still desirable to determine the HT of a VAMS sample, especially if VAMS-based blood results need to be converted to serum or plasma values. This conversion -e.g. for comparison with existing reference ranges or therapeutic intervals- also depends on the patient's HT[12]. Furthermore, in VAMS analysis, the HT tends to influence recovery. Therefore, it may be worthwhile to have an estimated value of HT, to verify whether this value is within the validated HT range or not (see General Background).

Bloem *et al*. recently demonstrated the potential of using a VAMS's potassium content as a HT marker[13]. However, the employed method required overnight extraction and the use of compounds such as tween, which should be avoided in case of LC-MS analysis. We therefore evaluated whether a shorter extraction procedure, which is more compatible with existing LC-MS workflows, would also yield acceptable results. Since both aqueous and organic solvents can be used for VAMS extraction, two extraction procedures were developed and evaluated. Special attention was paid to optimization of the sample preparation procedure to ensure that both the LC-MS analysis and K^+ determination could be performed on a single VAMS extract.

4.2. MATERIALS AND METHODS

4.2.1. VAMS preparation

VAMS samples were generated from Li-heparin anticoagulated whole blood by carefully touching the blood with the tip of the VAMS device (Mitra®, Neoteryx, USA). The VAMS devices that were used, were designed to wick up 10.6 µL of whole blood. After sample generation, the, samples were dried under ambient conditions for at least 2h before analysis. If samples needed to be stored, they were put in a clamshell in a zip-locked bag in the presence of desiccant (5g silica, MiniPax®, Sigma Aldrich, USA).

4.2.2. Extraction of potassium from VAMS

Solvents used for extraction were 1.6 mM KCl (UCB, Leuven, Belgium) in MilliQ water (aqueous extraction), generated using a Millipore device (Overijse, Belgium), 3.6 mM KCl in MilliQ water and MeOH/H_2O (80/20 v/v; organic extraction), a frequently used extraction mixture for VAMS/DBS analysis, the MeOH being LC-MS grade (Biosolve, Dieuze, France). All extractions were performed using an Eppendorf® Comfort Thermomixer (Eppendorf, Hamburg, Germany) at 1200 rpm and all K^+ measurements were performed with the ISE-module of the Cobas 8000 chemistry analyzer (Roche, Mannheim, Germany). The extraction conditions were optimized using samples with a high HT (approximately 0.65), as these samples pose most challenges regarding extraction efficiency reproducibility. Moreover, both fresh and artificially aged samples (stored at 60°C for 48h) were used, to ensure VAMS age would not affect K^+ extractability.

The evaluated extraction variables include extraction temperature, extraction solvent volume and extraction time. In a first experiment, a one-step extraction using 230 µL of 1.6 mM KCl was compared with two different two-step extractions. Of these two two-step extractions, the first one included an extraction with, respectively, 130 and 100 µL of 1.6 mM KCl and the second one an extraction with, respectively, 170 and 60 µL. For each of these extraction procedures the total extraction solvent volume was 230 µL. For the one-step extraction 180 µL of extract was transferred to a microcup (Roche) for potassium analysis. For the first two-step extraction procedure this was 100 µL of extract after the first extraction step and 80 µL after the second extraction step. For the second two-step extraction 140 and 40 µL were transferred after the respective extraction steps. The final extract volume was 180 µL for each of the evaluated extraction procedures. The extraction time was 30 min for the one-step extraction and 2x15 min for both of the two-step extraction procedures. Each of the abovementioned extraction procedures was evaluated at four different temperatures (21, 35, 50 and 60°C) and each condition was evaluated in triplicate. Based on the results of this experiment, both two-step extraction methods were repeated at 35°C and 50°C, for both fresh and artificially aged samples (n = 6). The different extraction conditions which were evaluated in the latter experiment were compared using a one-way ANOVA ($\alpha = 0.05$).

After the extraction volume and temperature were selected, the extraction time was optimized. To this end, the extraction was repeated at 2x15, 2x10 and 2x5 min (n = 6). The obtained results were compared using a one-way ANOVA ($\alpha = 0.05$). For the organic extraction procedure the same extraction volume and temperature were selected as those that were previously optimized for the aqueous extraction procedure. The extraction temperature, however, was optimized independently from the aqueous extraction. Here as well, the extraction was performed at 2x15, 2x10 and 2x5 min (n = 6) and the obtained results were compared using a one-way ANOVA ($\alpha = 0.05$).

During all experiments, all potassium measurements were corrected for the potassium concentration obtained after extraction of a double blank VAMS sample (i.e. a VAMS device containing no blood). Whenever a statistically significant result was detected using a one-way ANOVA, a post-hoc Bonferroni test was employed to assess between which results these significant differences occurred.

4.2.3. Analytical validation

To establish the calibration model, the accuracy (% bias) and intraday and total imprecision (% residual standard deviation), calibration curves and QC samples were prepared on three separate days, in duplicate. The calibrators and QCs were prepared separately from Li-heparin -anticoagulated whole blood from a healthy volunteer (BD Vacutainer®, USA). The HT of the calibrators (approximately 0.20, 0.27, 0.35, 0.42, 0.50, 0.57 and 0.65) and QCs (approximately 0.20, 0.42 and 0.65) was achieved by removing or adding a suitable amount of plasma, with verification of the actual HT using a Sysmex XE-5000 hematology analyzer (Kobe, Japan). Different calibration models (unweighted, 1/x, 1/y, $1/x^2$ and $1/y^2$) were evaluated based on the sum of the absolute values of the % RE, the distribution of the % RE values, and the backcalculated values of all calibrators. For all QC's it was assessed whether these fulfilled the preset acceptance criteria of % bias and % RSD ≤ 15% (≤ 20% for LLOQ). Since it is impossible to generate QC samples with exactly the same HT for each QC series, these small differences in actual HT were taken into account for the assessment of imprecision. To that end, the estimated HT values were normalized to the average true HT of a certain QC level.

4.2.4. Application to patient samples

Subsequently, the developed extraction methods were applied to patient samples. VAMS were prepared (in duplicate) from venous Li-heparin whole blood samples (n = 95) from patients for whom the HT (range 0.21 – 0.45) had been determined on the corresponding K_2EDTA sample using a Sysmex XE-5000. The local Ethics Committee granted permission to collect these samples, which arrived at the clinical laboratory of Ghent University Hospital for routine analysis (EC2015/0931). A subset of the available replicates (n = 56) was re-analyzed to assess method reproducibility. The remaining replicates (n = 39) were analyzed using the organic extraction procedure.

4.3. RESULTS AND DISCUSSION

4.3.1. Method development

The results obtained with the different extraction volumes and temperatures described in section 4.2.2 can be seen in Figure 4.1. The one step extraction with 230 µL of extraction solvent yielded systematically lower results at every extraction temperature when compared

to the other extraction conditions. For the extraction at 21°C an age-dependent result could be observed. Although not always statistically significant (p = 0.01, 0.03 and 0.13, respectively), this difference could be observed at all the evaluated extraction volumes (see Figure 4.1).

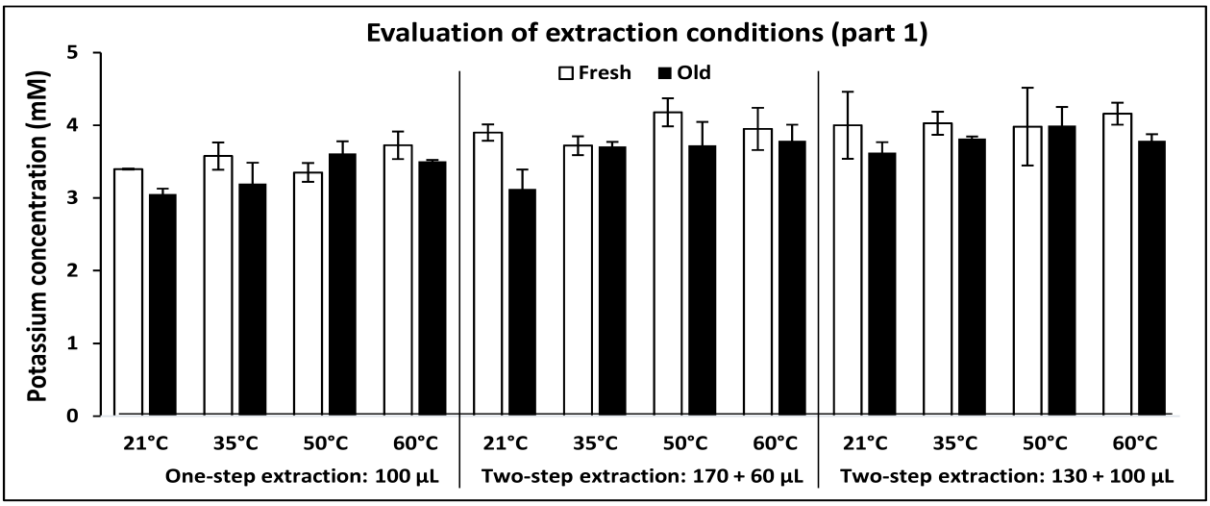

Figure 4.1: The evaluation of different extraction conditions: i.e. different extraction volumes (230 µL, 170 + 60 µL and 130 + 100 µL) at four different temperatures (21, 35, 50 and 60°C). The K^+-concentrations (mM) were corrected for the blank's K^+-content, and were obtained after extraction of 'fresh' and 'old' VAMS samples prepared from blood with a HT value of 0.65. The mean K^+-concentrations ± SD are plotted (n = 3).

When both two-fold extraction procedures were repeated at 35 and 50°C, all evaluated extraction conditions appeared to yield age-independent results and none of the evaluated extraction procedures outperformed the others (p = 0.18). However, as the two-step extraction with 170 + 60 µl at 35°C yielded results with somewhat lower variability (see Figure 4.2), this one was selected to further optimize the extraction time.

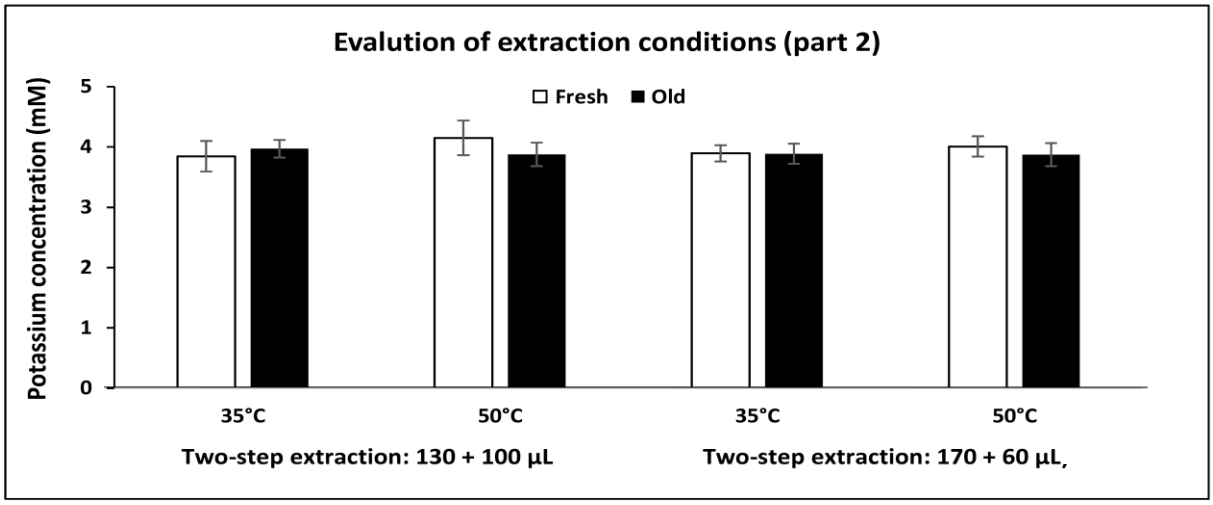

Figure 4.2: Further evaluation of the most promising extraction conditions: i.e. two-fold extractions with 170 + 60 µL and 130 + 100 µL at both 35 and 50°C. The K+-concentrations (mM) were corrected for the blank's K^+ content, and were obtained after extraction of 'fresh' and 'old' VAMS samples prepared from blood with a HT value of 0.65. The mean K+-concentrations ± SD are plotted (n = 6).

For the aqueous extraction, none of the evaluated extraction times outperformed one the other ones ($p = 0.027$). However, for practical reasons the extraction time of 2x10 min was selected. For the organic extraction on the other hand, the differences between the obtained results were statistically significant ($p = 0.001$). Bonferroni post-hoc analysis revealed there was indeed a significant difference between 2x5 and 2x15 minutes ($p < 0.001$). However, no significant difference could be observed between 2x15 and 2x10 minutes ($p = 0.364$) and 2x10 and 2x5 minutes ($p = 0.027$). Although not all differences were statistically significant, an inversely proportional trend could be discerned between extraction time and extraction efficiency. Therefore, it was deemed more prudent to select the 2x15 min extraction time for the organic extraction procedure.

Based on the results described above, a two-step extraction at 35°C with respectively 170 and 60 µL of extraction solvent was selected, yielding a final extract of 180 µL (140 + 40 µL). The extraction times for the aqueous and organic extraction procedures, were 2x10 min, and 2x15 min, respectively. For the organic extraction procedure, a realistic scenario was considered in which part (half) of the extract was left untouched (and could potentially be used for LC-MS analysis). The other half was transferred to a second tube and evaporated using a Zymark TurboVap LV evaporator (Hopkinton, USA) prior to reconstitution with 90 µL of a 3.6 mM KCl. Depending on the HT of the blood used to prepare the VAMS, potassium concentrations between 3.04 mM and 5.80 mM were obtained for the aqueous extraction method and between 4.66 mM and 5.81 mM for the organic extraction method..

4.3.2. Analytical validation

As there were only minimal differences between the best and the simplest calibration model, an unweighted linear calibration model was selected, yielding a bias of < 4.4%,an intraday imprecision of < 3.9% and a total imprecision of < 5.2% at each QC level, including the LLOQ.

4.3.3. Application

Application of the aqueous extraction procedure revealed a good agreement between the estimated and the actual HT ($r = 0.91$). Bland-Altman analysis (Figure 4.3A) showed LoA's of -0.043 and 0.051. These were both within the preset acceptance criterion of ± 0.06, which was selected based on our previous work on HT prediction of DBS samples[3]. Passing-Bablok

analysis revealed no proportional (95% CI of the slope $[0.91; 1.08]$) or systematic differences (95% CI of the intercept $[-0.02; 0.03]$). Analysis of a subset of the available replicates (n = 56) to assess method reproducibility showed that, with a single exception (24.4%), all repeat analyses fell within ± 15% of the original analysis, fulfilling the EBF criteria for ISR (i.e. ≥ 67% should fall within ± 20% of the original result).

The remaining replicates (n = 39) were analyzed using the organic extraction procedure. Also here, a good agreement could be observed between the estimated and true HT (r = 0.89). Bland-Altman analysis revealed LoA's of -0.059 and 0.054 (Figure 4.3B). Although these LoA's are somewhat broader than the ones observed for the aqueous extraction procedure, these still met the pre-set acceptance criterion. Passing-Bablok analysis revealed no proportional difference (95% CI of the slope $[0.98; 1.40]$) or systematic difference (95% CI of the intercept $[-0.12 - 0.00)$.

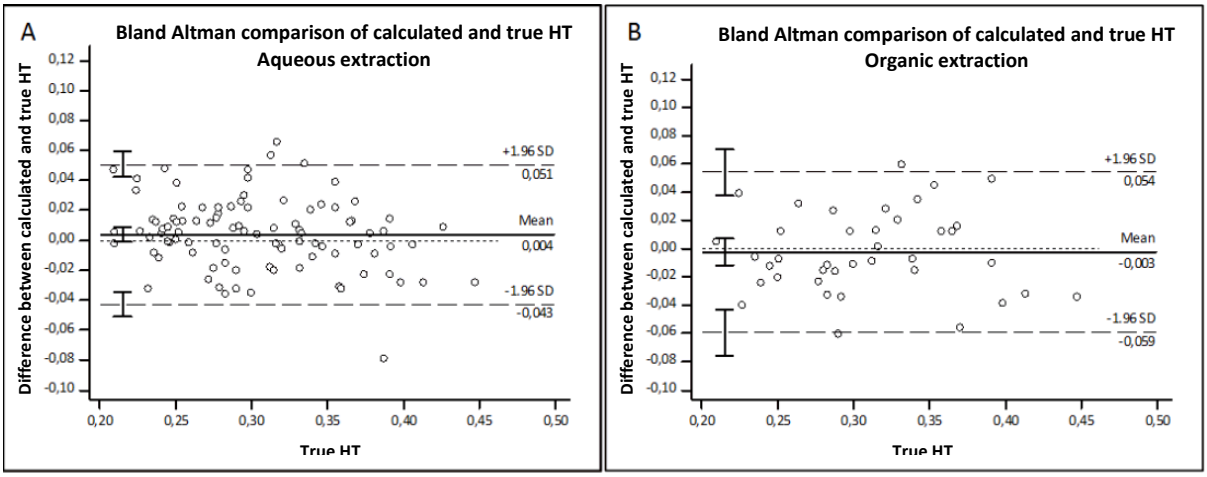

Figure 4.3: Bland-Altman plots depicting the difference between calculated and true HT *vs*. true HT. Panel A depicts the results obtained with the aqueous extraction (n == 95) and panel B depicts the results obtained with the organic extraction (n = 39). The dotted line represents the bias (with 95% CI) and the striped lines represent the LoA's (with their respective CI's).

4.4. CONCLUSION

In conclusion, two straightforward extraction procedures were developed which allow to estimate the HT of the blood used to generate a VAMS sample. These extraction procedures are simple, fast and straightforward and can seamlessly be integrated with most existing procedures[5], allowing both chromatographic and K^+ analysis on the same VAMS sample. In future studies, these methods should be applied to capillary VAMS samples.

4.5. REFERENCES

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Chapter 5

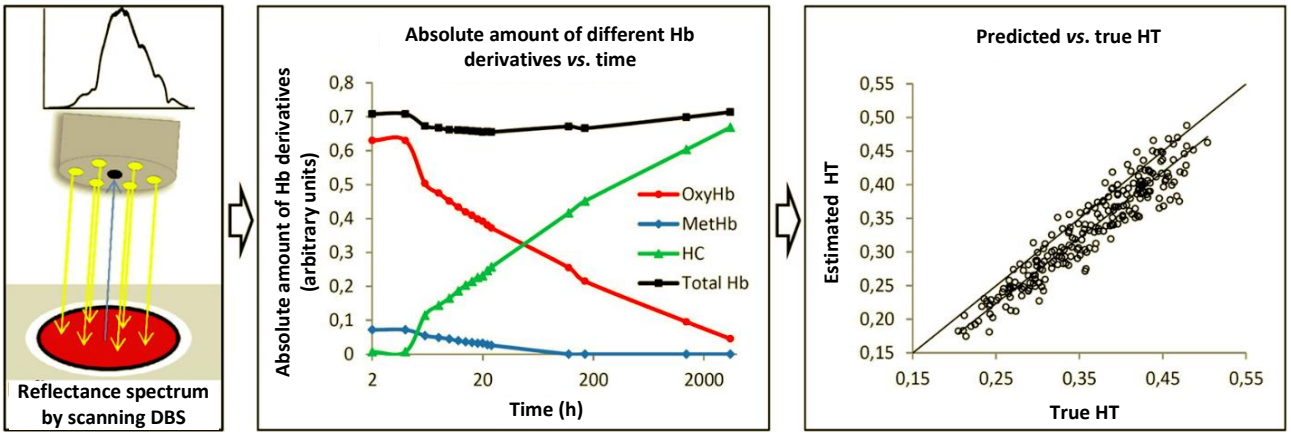
Hematocrit prediction of DBS based on hemoglobin content

Based on:

Capiau S, Wilk LS, Aalders MC, Stove CP. A Novel, Nondestructive, Dried Blood Spot-Based Hematocrit Prediction Method Using Noncontact Diffuse Reflectance Spectroscopy. *Analytical Chemistry,* 2016; 88(12):6538-46.

Abstract

DBS sampling is recognized as a valuable alternative sampling strategy both in research and in clinical routine. Although many advantages are associated with DBS sampling, its more widespread use is hampered by several issues, of which the HT effect on DBS-based quantitation remains undoubtedly the most widely discussed one. Previously, we developed a method to derive the approximate HT from a non-volumetrically applied DBS based on its K^+ content. Although this method yielded good results and was straightforward to perform, it was also destructive and required sample preparation. Therefore, we now developed a non-destructive method which allows to predict the HT of a DBS based on its Hb content, measured *via* non-contact diffuse reflectance spectroscopy. The developed method was thoroughly validated. A linear calibration curve was established after log/log transformation. The bias, intraday and interday imprecision of QCs at three HT levels and at the LLOQ and ULOQ (0.20 and 0.67, respectively) were less than 11%. In addition, the influence of storage and the volume spotted was evaluated, as well as DBS homogeneity. Application of the method to venous DBS prepared from whole blood patient samples (n = 233) revealed a good correlation between the actual and the predicted HT. Limits of agreement obtained after Bland-Altman analysis were -0.076 and +0.018. Incurred sample reanalysis demonstrated good method reproducibility. In conclusion, mere scanning of a DBS suffices to derive its approximate HT, one of the most important variables in DBS analysis.



5.1 INTRODUCTION

Over the last decades, DBS sampling has been increasingly recognized as a valuable alternative sampling strategy in ample domains[1], such as NBS[2], therapeutic drug monitoring[3,4], and toxicology[5,6], because of the various advantages that are associated with it (as was elaborately discussed in Chapter 1). Indeed, DBS sampling is minimally invasive, requires only very small volumes of blood and allows for home-based patient self-sampling[7]. Nonetheless, DBS still struggle to be implemented on a routine basis in quantitative bioanalysis. Among the prime causes for this is the so-called HT effect, which entails that the HT of the blood used to prepare a DBS may influence the obtained result[8] (see General background, Chapter 1 section 1.4.1 and Chapter 2 section 2.3.2.3). Indeed, with varying HT, and hence, varying blood viscosity, blood will spread differently throughout the filter paper, leading to a different volume of blood being analyzed when a fixed-size DBS punch is sampled. The latter is often preferred over whole DBS analysis after volumetric DBS application to enable simplicity of sample collection, especially when samples need to be collected by the patient himself in a home setting. In addition, the HT may also influence analyte recovery, matrix effect and DBS homogeneity[9]. Driven by the impact of this HT issue on the future of quantitative DBS analysis, ample solutions have already been proposed. These include the analysis of complete fixed-volume DBS[10-14] or other dried blood samples[15,16] HT prediction and subsequent HT effect correction[17,18] the use of new substrates on which the spreading of the blood is HT-independent[19], and the use of *in situ* generated DPS[20,21]. However, all of these solutions have certain limitations or drawbacks, as has been elaborately discussed elsewhere[8,22-24].

Previously, we developed a method which uses the K^+ content of a DBS extract to predict its HT (see Chapter 3)[17]. Although this method yielded excellent results when applied to real patient samples and was able to adequately correct for the HT effect using caffeine and paraxanthine as model compounds[18], it suffered from some practical drawbacks. Indeed, part of the DBS needed to be sacrificed for the K^+ analysis, which also required an additional sample preparation. Therefore, we set out to develop a non-contact method to predict the HT of DBS, since such a method would preserve the entire DBS sample and exclude the need for sample preparation, which in turn could facilitate integration in existing DBS analyzers. More particularly, it was our goal to develop a non-

contact method that allows to predict the HT of a DBS, based on its total Hb content.

Since previous experiments performed at our laboratory showed that Hb, upon aging of DBS, is converted from OxyHb to MetHb[8], which is also accompanied by spectral changes (cfr. the change in color of a DBS upon aging), both Hb forms need to be taken into account to allow spectrometry-based Hb quantitation. However, even when doing so, the Hb sum proved to be unstable in function of time. Interestingly, Bremmer *et al*. demonstrated that in aging blood stains Hb is originally present as OxyHb, which is then oxidized to MetHb and further denatured to HC. Therefore, we hypothesized that if a similar process would occur in DBS and no other major Hb derivatives or metabolites were formed upon aging, the sum of OxyHb, MetHb and HC (i.e., "total Hb") would remain constant and could be used as a marker of HT. As a basis for the development of the non-contact method we used the work of Bremmer *et al*. and Edelman *et al*., who used non-contact diffuse reflectance spectroscopy to determine the relative abundance of Oxy-Hb, MetHb and HC in dried blood stains at crime scenes for age estimation purposes[25,26]. For the HT prediction of DBS, however, these three Hb derivatives had to be determined quantitatively, rather than qualitatively, which required alteration and optimization of the method.

Total Hb has been previously determined colorimetrically in DBS extracts either "as is"[8] or after transformation of Hb into its cyano-methemoglobin derivative *via* extraction of the DBS in Drabkin's reagent[27-29]. However, in each case the age of the DBS influenced the obtained results. Others have used the total AUC of all Hb variants determined *via* HPLC-UV during hemoglobinopathy screening as a measure of total Hb and HT, albeit only semiquantitative results could be achieved[30]. Furthermore, LC-MS/MS has been successfully employed to determine total Hb by measuring a proteospecific peptide after tryptic digestion of a DBS extract[31]. Unfortunately, this method is not suitable for HT prediction of DBS, since in many cases the HT prediction would be more complicated than the analysis of the actual target analyte. Additionally, Miller *et al*. tried to utilize the Hb bands at 540 and/or 570 nm, measured with non-contact diffuse reflectance spectroscopy, as a measure of a DBS' HT[32]. Although these authors did not find a correlation between the HT and the reflectance at these Hb-specific wavelengths, they did find a correlation with the background scattering measured at 980 nm. The same optical technique has also been employed by Cecchi *et al*. to

measure total Hb after transformation of Hb into its isothiocyano-methemoglobin derivative[33]. To that end, derivatizing reagents were preimpregnated in a nylon-based filter paper used to collect DBS. Another non-contact method to determine total Hb was published by Yang *et al.*, which encompassed mixing Drabkin's reagent and blood before deposition of the sample onto the filter paper and using the color intensity determined on a digitized image of the DBS as a measure of total Hb[34]. However, such an approach would not be feasible in the context of home sampling.

In this Article, we describe a method that allows to predict the HT of a regular, non-volumetrically applied DBS, based on its Hb content using non-contact diffuse reflectance spectroscopy. In contrast to other published DBS-based total Hb analyses, this method is quantitative, non-destructive, applicable on both fresh and old DBS, does not require any sample preparation nor filter paper pretreatment, and allows to predict the HT of a DBS. This method was thoroughly validated, yielding acceptable accuracy and precision, and was applied to a diverse patient population, yielding satisfactory results.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of DBS

DBS were prepared as previously described in section 3.2.1: venous blood was collected from consenting healthy volunteers in blood collection tubes with Li-heparin as anticoagulant (Venosafe, VF-109SHL, Terumo, Leuven, Belgium) and DBS were prepared at the day of blood collection by depositing 25 µL of blood onto Whatman® 903 filter paper (GE Healthcare, Dassel, Germany), unless mentioned otherwise. Blood spots were always allowed to dry at ambient conditions for at least 2h. The obtained DBS were either analyzed immediately after drying or were stored in zip-locked plastic bags in the presence of a desiccant until analysis (Minipax, Sigma-Aldrich, Diegem, Belgium). Blood samples with different HT were prepared by centrifuging an aliquot of venous whole blood in 2 mL safe-lock tubes in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 5 min at 1000 g, followed by the removal or addition of a suitable amount of plasma. For evaluating the influence of the presence of different anticoagulants on the measured reflectance, venous blood from a healthy volunteer was collected in blood collection tubes with either Li-heparin, NaF/oxalate

(Venosafe, VF-052SFX, Terumo), K_2EDTA or citrate as anticoagulant and in a blood collection tube without any anticoagulant (all from BD Vacutainer, Becket Dickinson B.V., Breda, The Netherlands). The DBS without anticoagulant were prepared within 1 min after blood collection. Patient samples were prepared similarly to the ones from healthy volunteers and were collected in Li-heparin (Venosafe, VF-106SAHL and VF-054SAHL, Terumo) or K_2EDTA -containing blood collection tubes (Venosafe, VF-054SDK, Terumo).

5.2.2 Analysis

HT measurements on venous whole blood were performed using a Sysmex XE-5000 hematology analyzer (Sysmex Corporation, Kobe, Japan) and are expressed as L/L. All HT measurements during method development and validation were performed at the Laboratory of General Clinical Chemistry (LAKC) at the Academic Medical Center (AMC), Amsterdam, while the HT measurements of the patient samples were performed at the Laboratory of Clinical Biology at Ghent University Hospital. The analyzer at Ghent University Hospital was also used for the mean corpuscular hemoglobin concentration (MCHC) analysis of the patient samples. Additionally, the hemolytic, lipemic (LI) and icteric index (II) of the patient samples were determined using a Cobas 8000 analyzer at Ghent University Hospital.

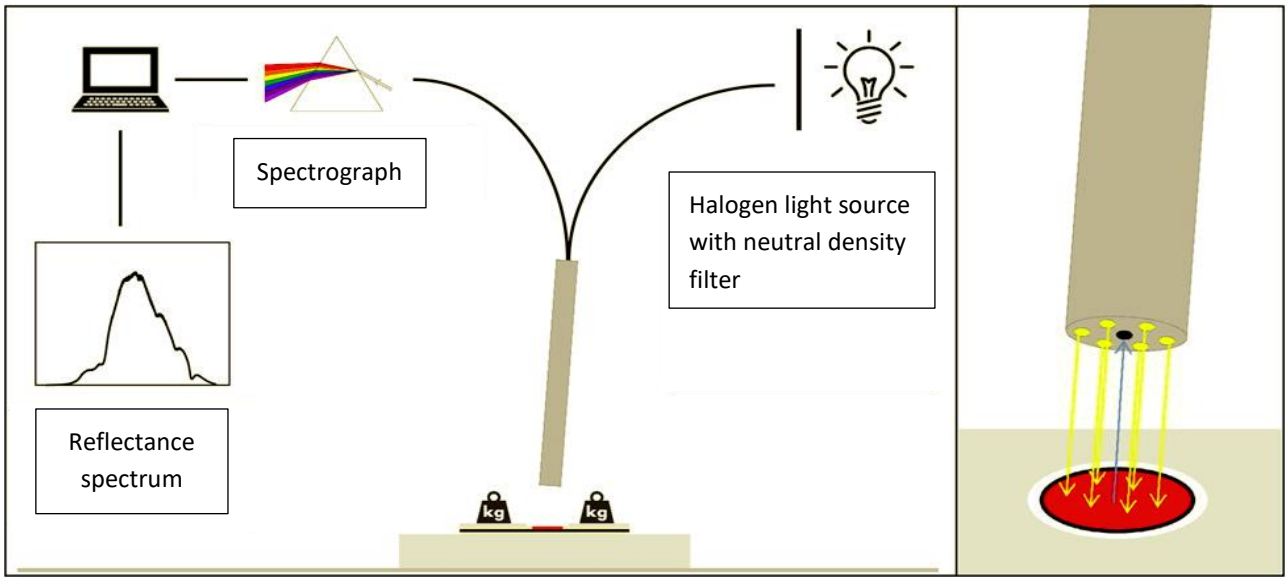


Figure 5.1.: Schematic representation of the used setup.

Non-contact diffuse reflectance spectroscopy was employed to determine the total Hb content and HT (L/L) of the DBS. The setup that was used to acquire the spectral data (depicted in Figures 5.1 and 5.2) comprised a 10W tungsten-halogen light source (AvaLight-HAL, Avantes, Appeldoorn, The Netherlands), a spectrometer (USB4000, Ocean Optics, Duiven, The Netherlands) and a non-contact fiber-probe (QR400-7-UV/BX, Ocean Optics, Duiven, The Netherlands). The fiber-probe consisted of a collection fiber encircled by six delivery fibers. All fibers had a core diameter of 400 µm. The probe tip was placed 1.3 cm above the sample and set at a slight angle to avoid specular reflection. Moreover, a neutral density filter with an optical density of 0.4 was inserted between the light source and the delivery fibers to prevent detector saturation. In this setup, the light source output was guided toward the sample surface *via* the six delivery fibers, illuminating a 5.9 mm-diameter spot, which corresponds approximately to a traditional 6 mm-punch. Light reflected by the DBS was guided to the spectrometer by the central collection fiber. The spectrometer recorded the wavelength dependence of the reflected light intensity (reflection spectrum) between 354 and 1042 nm using the SpectraSuite software (Ocean Optics, Duiven, The Netherlands). In addition to the recording of a reflection spectrum of each DBS, also the reflection spectrum of a white reference was recorded. The integration time was set at 20 ms and 20 spectra were averaged for one measurement. All recorded spectra were corrected for the electrical dark noise of the detector.

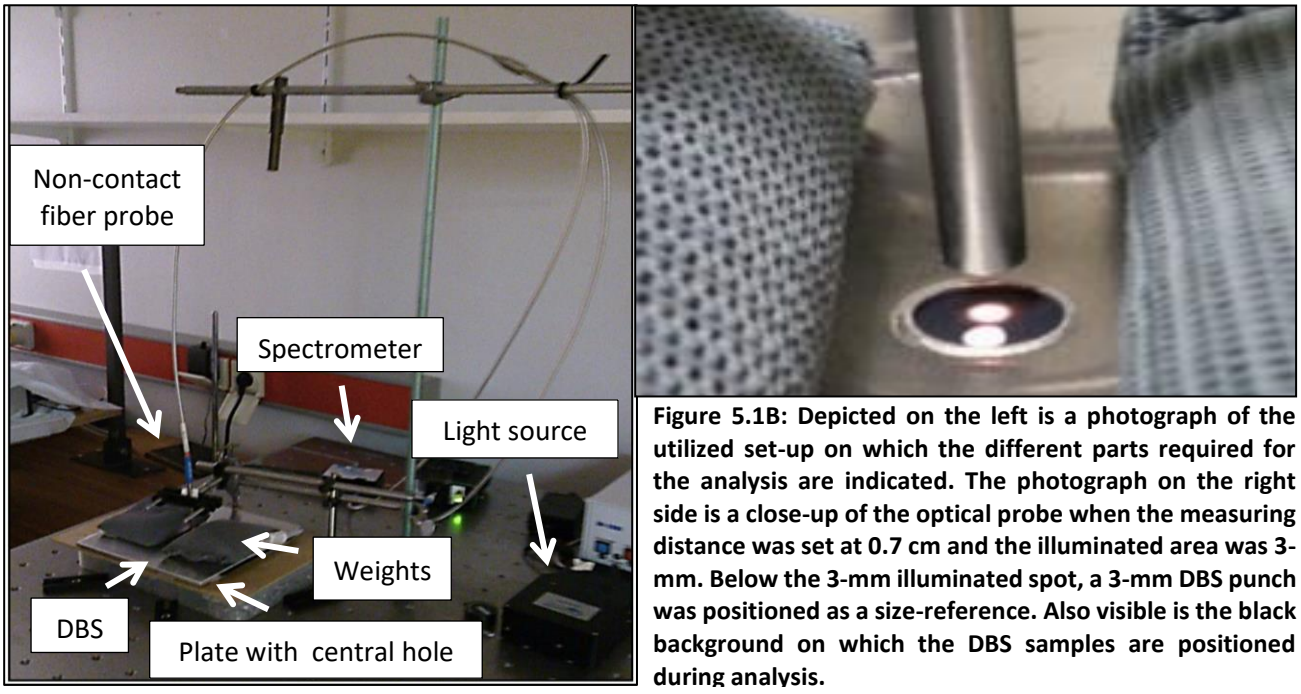


Figure 5.1B: Depicted on the left is a photograph of the utilized set-up on which the different parts required for the analysis are indicated. The photograph on the right side is a close-up of the optical probe when the measuring distance was set at 0.7 cm and the illuminated area was 3-mm. Below the 3-mm illuminated spot, a 3-mm DBS punch was positioned as a size-reference. Also visible is the black background on which the DBS samples are positioned during analysis.

5.2.3 Spectral data analysis

All data analysis was carried out using custom-made scripts written in MATLAB R2012a (The Mathworks Inc., Natick, MA, USA). Wavelength-dependent reflectance values R(λ) were obtained from the measured spectra using the following formula (Eq. 5.1):

$$\boldsymbol{R(\lambda)} = \frac{\boldsymbol{I(\lambda)}}{\boldsymbol{I_{white}(\lambda)}} \qquad \text{(Eq. 5.1)}$$

Here, λ corresponds to the wavelength of the light. Further, I(λ) refers to the light intensity reflected by a DBS, while $I_{white}(\lambda)$ denotes the light intensity reflected by the white reference. Applying equation 5.1 to the data served as a normalization step, normalizing the light intensity reflected by the DBS to the light intensity reflected by the non-light-absorbing white reflectance standard. R(λ) was then compared to a one-dimensional light-transport model for multilayered samples[35]. This light-transport model describes the wavelength-dependent reflectance of a DBS as a combination of light absorption and light scattering and takes into account the optical properties of both the blood and the blank filter paper. In this model the influence of the blank filter paper is described by its measured wavelength-dependent reflectance and the wavelength-dependent optical properties of the blood are described by an absorption coefficient and a scattering coefficient. Light absorption in the DBS is mainly attributed to three Hb derivatives which are assumed to be present, namely, OxyHb, MetHb, and HC. The overall absorption coefficient of the blood is therefore modeled as a linear combination of the individual absorption coefficients of the three Hb derivatives. Reference curves for these three absorption coefficients were taken from Bremmer *et al*.[25] For a given DBS, the present amounts of the Hb derivatives were calculated by fitting the output of the light-transport model to the measured R(λ) in the spectral range of 500–700 nm, employing a non-linear least-squares fitting algorithm. The range of 500–700 nm was used in the fitting procedure, since during method optimization this proved to yield the best fit between the measured spectra and the reference spectra. The least-squares fitting algorithm assigned a value with arbitrary units to each of the Hb derivatives and the sum of those values was then used as a measure of total Hb and HT. To further clarify the spectral data analysis process, a flowchart was included in Figure 5.3.

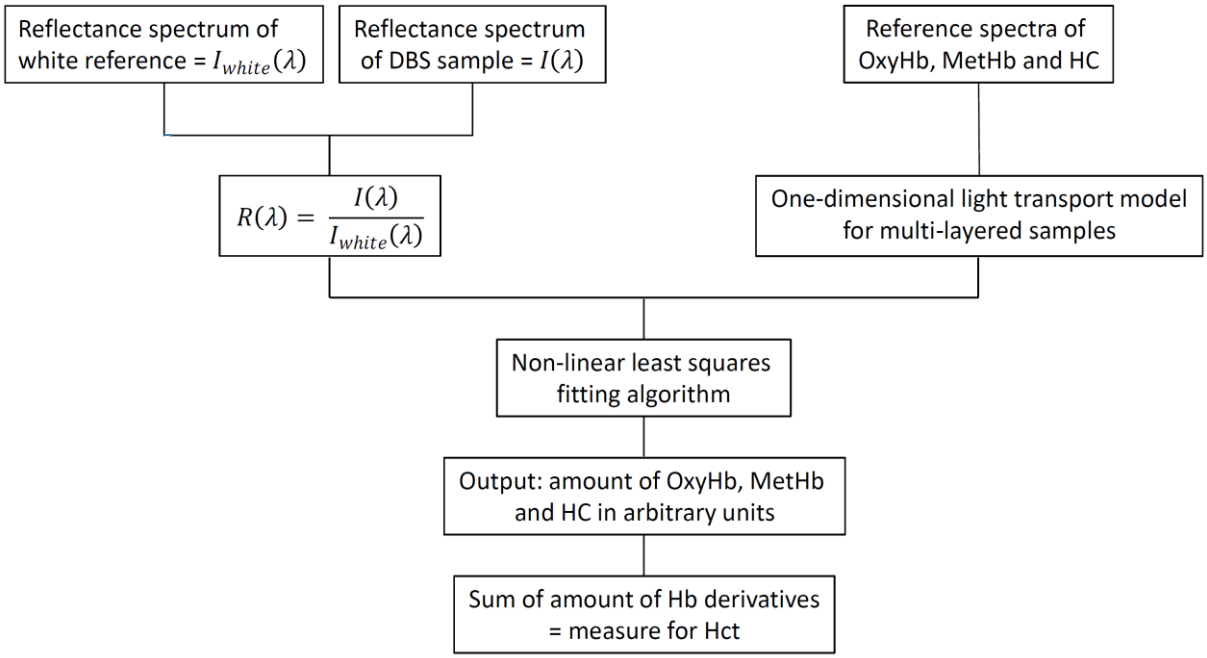


Figure 5.3: Flow chart of the spectral data analysis

5.2.4 Validation

To choose a calibration model, we set up six 7-point calibration curves in DBS, prepared from blood with a HT of 0.20, 0.27, 0.35, 0.41, 0.49, 0.57, and 0.66. All calibrators were generated from the blood of a single donor. Unweighted and weighted (1/x, 1/x2, 1/y, 1/y2) linear regression models, a second order regression model, a power regression model, as well as a linear regression model after transformation of the data (logaritmic or square root) were compared. The choice of the calibration model was based on the sum of the absolute % RE and the distribution of the RE in function of HT[36]. Linearity was evaluated using an F-test, acceptance of a linear model was based on the backcalculated values of all calibrators. A Levene's test for equality of variances was performed to assess homoscedasticity.

Accuracy (% bias) and imprecision (% RSD) were determined based on six 7-point calibration curves in DBS, prepared from blood with an approximate HT of 0.20, 0.27, 0.35, 0.42, 0.50, 0.57, and 0.65. On each of three different days two calibration curves were set up. Along with every calibration curve, three DBS QCs with an approximate HT of 0.20, 0.42, and 0.65 were prepared from blood of the same donor. Furthermore, additional QCs were prepared from five other blood sources. In addition, six 7-point calibration curves were also prepared from the blood of a second donor to exclude that the obtained results would be donor-dependent.

An overview of all calibrators and QCs is given in Table S-1. As it is not possible to prepare QCs with exactly the same HT for each measurement series, these slight differences in true HT had to be taken into account for the determination of imprecision. This was done by normalizing the calculated HT values to the average true HT value of a QC (Table S-2A and S2-B).

HT predictions based on measurements at the center of the DBS were compared with those obtained from measurements at a more peripheral location, avoiding the very edge of the sample (n = 6). This experiment was performed on 50 µL DBS to allow sufficient difference in location between a central and peripheral measurement. The impact of the blood volume spotted was evaluated in DBS prepared by applying 15, 20, 25, or 50 µL of blood on filter paper (n = 6). Both the impact of the measuring location and of the applied blood volume were evaluated at three HT levels (approximately 0.20, 0.42, and 0.65).

The effect of storage of the DBS on the HT prediction was evaluated by comparing the result from freshly prepared DBS (dried for 2h; three HT levels, six replicates) with results obtained after storage at RT for 1 day, 5 days, 1 week, 2 months, and 5 months. In addition, the influence of storage at 60 °C for 1, 2 or 3 days on the predicted HT was evaluated as well. Results were expressed as the average (n = 6) ± SD. Thanks to the non-destructive nature of the DBS analysis, the fresh spots could be reanalyzed at later time points. At every time point a fresh calibration curve (dried for 2h) was prepared.

5.2.5. Application

The developed method was applied on real-life clinical samples displaying a wide range of HT values (0.205–0.504). The samples had already undergone or were destined for routine clinical chemistry and/or hematology analysis and were collected in either Li-heparin tubes or in K_2EDTA tubes. Permission to use these samples was obtained from the Ethics Committee of Ghent University Hospital (project number 2015/0931). A first set of samples encompassed 57 Li-heparin whole blood samples that were used to prepare DBS upon arrival at the clinical laboratory by pipetting 25 µL of Li-heparin anticoagulated blood onto filter paper. We also prepared DBS of the corresponding K_2EDTA whole blood samples of these patients. A second set of samples encompassed 250 K_2EDTA left-over samples from which we prepared DBS on the same day of blood collection. Samples for which the routinely determined HT or MCHC value were not available were excluded from further data analysis, as will be discussed in

more detail below. This resulted in a final data set of 55 Li-heparin-containing samples with corresponding K_2EDTA samples and 233 "K_2EDTA -only" samples. The DBS were between 5 and 7 days old at the time of analysis.

Incurred sample reanalysis —with 3 days between the reanalysis and the original analysis— was performed on the second set of patient DBS (n = 233). At least two-thirds of the repeated measurements should meet the acceptance criterion, that is, lie within the limits of ± 20% of the mean of the original and the corresponding reanalysis result[37].

5.2.6 Data Analysis

Statistical analysis of the data was done using Medcalc Statistical Software, version 14.12.0 (MedCalc Software bvba, Ostend, Belgium, http://www.medcalc.org, 2014), SPSS statistics for Windows version 22.0 (IBM Corp, Armonk, NY, USA, 2013) and Microsoft Excel 2010. For the determination of intra- and interday imprecision, a model II ANOVA was used to allow analysis of the components of variation. Using this ANOVA approach, the value of the interday imprecision was equated to the intraday imprecision, whenever the latter would exceed the former. A paired t test (α = 0.05; 95% CI) was carried out to evaluate the effect of the measurement location on the predicted HT. To evaluate the influence of different volumes of blood spotted, a one-way ANOVA (α = 0.05) with Bonferroni post-hoc test was employed. Medcalc was used to perform a Levene's test for the evaluation of homoscedasticity. Furthermore, the same software was employed to calculate Pearson correlation coefficients, to generate Mountain plots and to perform Passing-Bablok regression analysis on the patient data. The corresponding Bland-Altman plots were generated in Excel 2010. Furthermore, a paired t-test was employed to evaluate whether a statistically significant difference existed between the predicted HT determined on Li-heparin-containing DBS and the predicted HT determined on K_2EDTA-containing DBS. The presence of outliers was always evaluated using a Grubbs test.

5.3 RESULTS AND DISCUSSION

5.3.1. Method Development - Optimization of Setup

To obtain reproducible reflectance spectra, it was of utmost importance to keep the distance between the probe and the sample constant. To achieve this, the setup needed to be fixed and the filter paper (in sheets, spotted with multiple DBS) needed to be kept flat. To that end, a plate was positioned on top of the filter paper and pressed down with weights. This plate had a 1 cm hole in the center (i.e., slightly larger than the DBS that needed to be analyzed) through which the measurement could be performed. Importantly, when using DBS cards instead of sheets of filter paper, the "waviness" of the substrate after DBS application and drying is much less of a problem, since the former are much more rigid in nature. Evidently, although the method could potentially be applied on either DBS cards or sheets of filter paper, it is important to prepare calibrators and patient samples on the same substrate and to record the respective spectra with exactly the same setup and hence, with the same fixed distance between DBS and probe.

To optimize the distance between the probe and the sample, three different distances (i.e., 0.7, 1.3, and 1.7 cm) were compared, yielding illuminated areas of respectively approximately 3, 6, and 8 mm. The distance of 0.7 cm resulted in a larger variability in the obtained spectra compared to the two larger distances (data not shown), likely because of some inhomogeneity in the DBS, which we could also observe *via* light microscopy (Figure 5.4). Since increasing the distance from 1.3 to 1.7 cm did not lead to a further improvement (i.e., a further reduction in variability), the distance of 1.3 cm was selected for the rest of the experiments. The resulting illumination diameter of 5.9 mm is acceptable, since DBS generated from a finger prick will typically have a larger diameter[7,38,39].

Additionally, the filter paper was positioned on a black matt surface to ensure that light going through the filter paper was absorbed by the background and not reflected toward the collection fiber. Indeed, with varying HT, and hence variable amounts of chromophores in a DBS, variable amounts of light may penetrate the sample and get reflected by, for example, a white background, which would lead to a variable, HT-dependent contribution of the background to the measured reflectance.

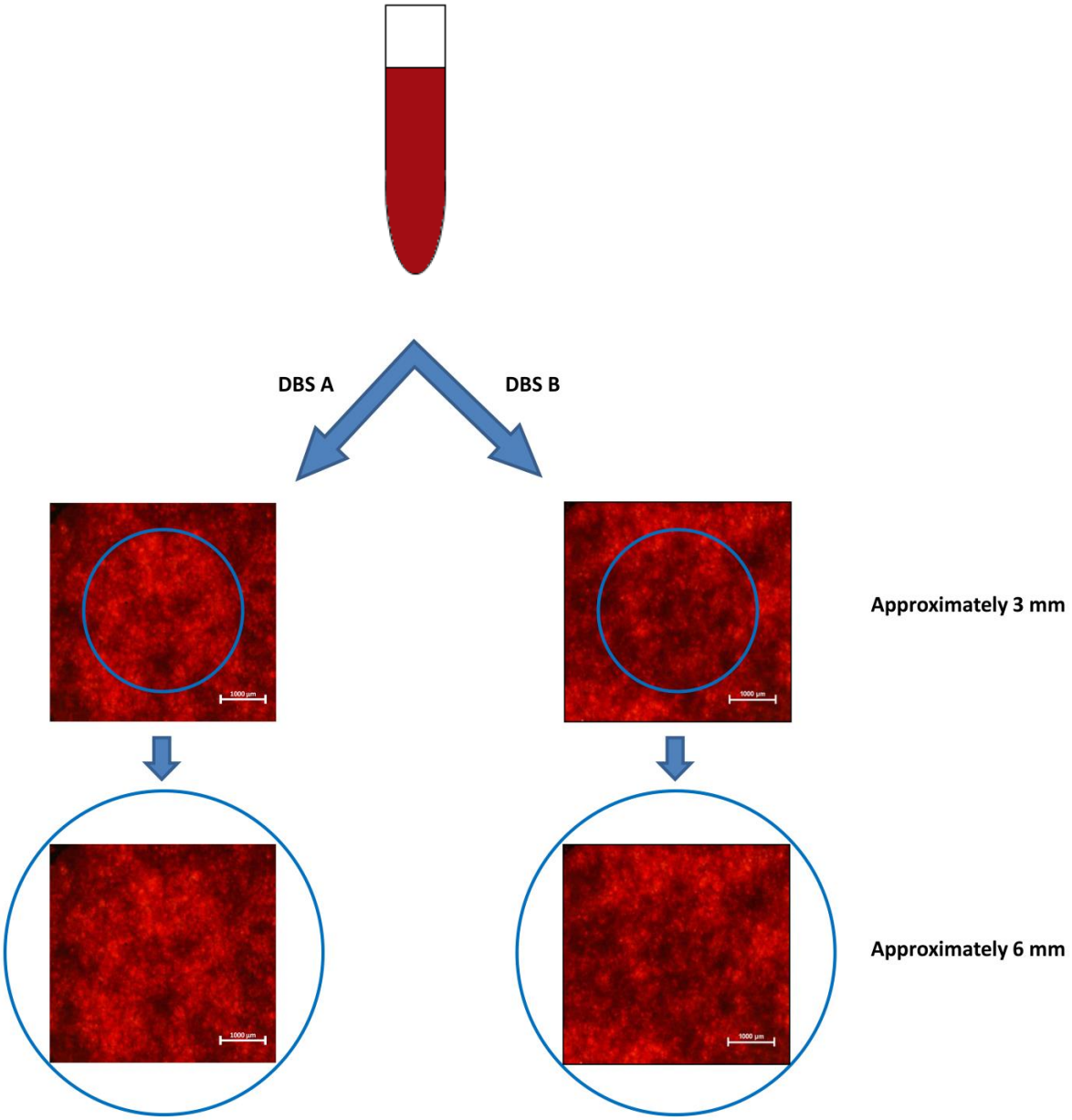


Figure 5.4: The influence of the illuminated spot diameter on method reproducibility. Depicted are images of two DBS prepared from the same blood source, recorded during light microscopy. A certain degree of inhomogeneity can be observed in the DBS. Whilst certain areas are darker, others are much lighter, indicating that light can penetrate more easily through the latter areas. These inhomogeneities may impact method reproducibility (at least in the case of a non-contact optical method), especially when the sampled area is relatively small. This can be clearly seen in the images below, as the smaller circles, which represent an illuminated spot with a diameter of 3 mm, clearly encompass almost only lighter areas in DBS A, and only darker areas in DBS B. By increasing the diameter of the sampled area to 6 mm (the larger circles), these inhomogeneities are leveled out (i.e. both 6-mm circles encompass both darker and lighter areas), and method reproducibility improves.

5.3.2 Selection of the used anticoagulant

A procedure aimed at measuring HT in DBS should be applicable to non-anticoagulated blood, since this is the type of blood that eventually will be analyzed (e.g., obtained from a finger prick in the context of home-based self-sampling). However, because of practical reasons, it is impossible to perform a validation or even to set up calibration curves in non-anticoagulated blood. We therefore compared at an early stage the reflectance spectra of DBS prepared from blood without anticoagulant with the reflectance spectra of DBS from blood that was anticoagulated with Li-heparin, K_2EDTA, citrate, or NaF/oxalate. The reflectance spectra of K_2EDTA and Li-heparin-containing DBS were nearly identical to the spectra of non-anticoagulated DBS (Figure 5.5). The spectra of the citrate-containing DBS on the other hand had a similar shape as the spectra from the non-anticoagulated DBS but showed a markedly higher reflectance. Furthermore, the spectra of the NaF/oxalate-containing DBS showed a slightly deviating shape. Therefore, only Li-heparin and K_2EDTA were deemed suitable anticoagulants to develop the non-contact HT prediction method. At this stage, we wished to preserve the possibility to also analyze the DBS samples with the existing K^+-based method for HT prediction. Therefore, we opted for Li-heparin anticoagulated blood to perform the validation.

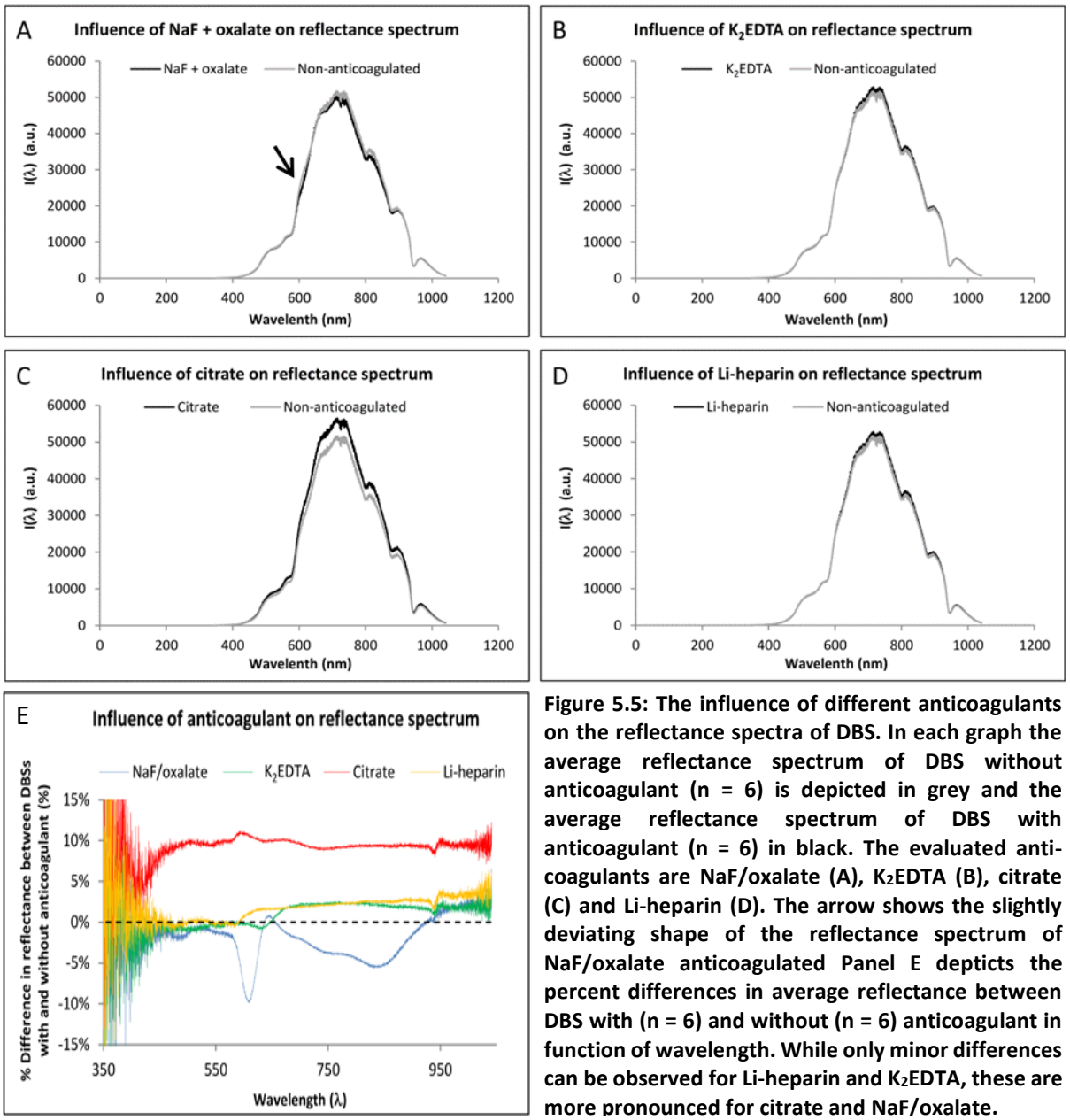


Figure 5.5: The influence of different anticoagulants on the reflectance spectra of DBS. In each graph the average reflectance spectrum of DBS without anticoagulant (n = 6) is depicted in grey and the average reflectance spectrum of DBS with anticoagulant (n = 6) in black. The evaluated anti-coagulants are NaF/oxalate (A), K_2EDTA (B), citrate (C) and Li-heparin (D). The arrow shows the slightly deviating shape of the reflectance spectrum of NaF/oxalate anticoagulated Panel E depicts the percent differences in average reflectance between DBS with (n = 6) and without (n = 6) anticoagulant in function of wavelength. While only minor differences can be observed for Li-heparin and K_2EDTA, these are more pronounced for citrate and NaF/oxalate.

5.3.3 Method Validation

A power regression model (and the derived linear regression model after log/log transformation) yielded the best calibration model. Furthermore, although data were originally heteroscedastic (p = 0.005), they became homoscedastic after log/log transformation (p = 0.429). Although an F-test performed on the logarithmically transformed data showed that the calibration model was statistically non-linear, a linear model could be accepted based on the backcalculated values of all calibrators, which never deviated more than 15% (range = −11.57% to 10.48%). The LLOQ and ULOQ were arbitrarily set at the average of the lowest, respectively, the highest calibrator. For donor 1 the LLOQ and ULOQ

corresponded to 0.20 and 0.67, while for donor 2 this was 0.20 and 0.65, respectively. The accuracy, intra- and interday imprecision for LLOQ and ULOQ were determined on the back-calculated values and always met the predetermined acceptance criteria (i.e., ≤ 20% bias and imprecision for LLOQ and ≤ 15% for ULOQ) (Table 5.1A). Also for the three QC levels % bias and % RSD did not exceed 11% (Table 5.1B). As described above, this experiment was also carried out using blood of a second donor, yielding very similar results (see Table 5.2A and 5.2B). Also when the QCs of donor 2 were inserted in the calibration curve of donor 1 (or *vice versa*) the results always complied with the acceptance criteria (Tables 5.1C and 5.2C). Moreover, QCs prepared from four additional blood sources (donors 3–6) were fitted into the calibration curves of donor 1 and donor 2, again leading to acceptable results (Tables 5.1C and 5.2C).

Table 5.1. Overview of the data for accuracy and imprecision (n = 6) for donor 1*

		Accuracy (% bias)	Intraday imprecision (% RSD)	Interday imprecision (% RSD)
A)	LLOQ	2.41	2.90	3.97
	ULOQ	–4.38	4.66	4.66
B)	QC LOW	0.896	10.7	10.7
	QC MID	–2.97	2.10	3.08
	QC HIGH	–2.50	4.63	4.63
C)	QC LOW (donor 2)	–1.81	2.30	5.65
	QC MID (donor 2)	0.630	5.56	6.91
	QC HIGH (donor 2)	0.857	7.38	7.38
	QC LOW (donor 3)	3.42	5.12	6.80
	QC MID (donor 3)	5.48	2.67	3.67
	QC HIGH (donor 3)	2.65	3.89	5.36
	QC LOW (donor 4)	1.62	6.42	6.42
	QC MID (donor 4)	0.629	7.68	8.38
	QC HIGH (donor 4)	–2.11	5.67	5.67
	QC LOW (donor 5)	4.01	7.77	7.77
	QC MID (donor 5)	–2.82	6.52	6.52
	QC HIGH (donor 5)	0.927	3.00	3.00
	QC LOW (donor 6)	5.13	5.45	6.15
	QC MID (donor 6)	4.92	11.8	11.8
	QC HIGH (donor 6)	4.34	10.8	10.8

***Sections A and B, respectively, give the data obtained for the LOQs (LLOQ and ULOQ) and QCs, prepared from blood of the same donor as the one in which the calibration curves were prepared. Section C gives the data for QCs prepared from blood of five other donors than the one in which the calibration curves were prepared.**

Table 5.2. Overview of the data for accuracy and imprecision (n = 6) for donor 2*

	Accuracy (% bias)	Intraday imprecision (% RSD)	Interday imprecision (% RSD)
A) LLOQ	0.386	1.73	3.80
ULOQ	-1.92	2.66	3.28
B) QC LOW	-1.29	4.66	4.66
QC MID	-1.03	6.29	6.75
QC HIGH	-2.25	5.19	5.19
C) QC LOW (donor 1)	1.36	6.14	7.81
QC MID (donor 1)	-4.55	4.09	4.78
QC HIGH (donor 1)	-2.50	3.00	3.00
QC LOW (donor 3)	3.60	3.43	3.43
QC MID (donor 3)	3.24	2.16	3.17
QC HIGH (donor 3)	-0.796	3.18	3.18
QC LOW (donor 4)	2.34	5.68	5.68
QC MID (donor 4)	-1.14	9.21	9.21
QC HIGH (donor 4)	-4.87	4.42	5.62
QC LOW (donor 5)	4.49	5.99	5.99
QC MID (donor 5)	-4.04	6.25	6.25
QC HIGH (donor 5)	-1.76	6.64	6.64
QC LOW (donor 6)	5.25	9.17	9.17
QC MID (donor 6)	2.19	11.2	11.2
QC HIGH (donor 6)	0.778	6.22	7.62

***Sections A and B, respectively, give the data obtained for the LOQs (LLOQ and ULOQ) and QCs, prepared from blood of the same donor as the one in which the calibration curves were prepared. Section C gives the data for QCs prepared from blood of five other donors than the one in which the calibration curves were prepared.**

As mentioned above, the reflectance spectrum of a DBS changes drastically upon aging because the relative amounts of the different Hb derivatives change throughout time (Figure 5.6A and 5.6B). It was therefore of utmost importance to evaluate the influence of storage time on the predicted HT. Long-term stability at RT did not seem to pose a problem for up to at least 5 months, as, with a single exception, the observed differences never exceeded 15% at all measured time points and at every HT level (Figure 5.6C). Also short-term storage (up to 3 days) at elevated temperatures (60 °C) did not seem to affect the obtained results, as can be seen in Figure 5.6D.

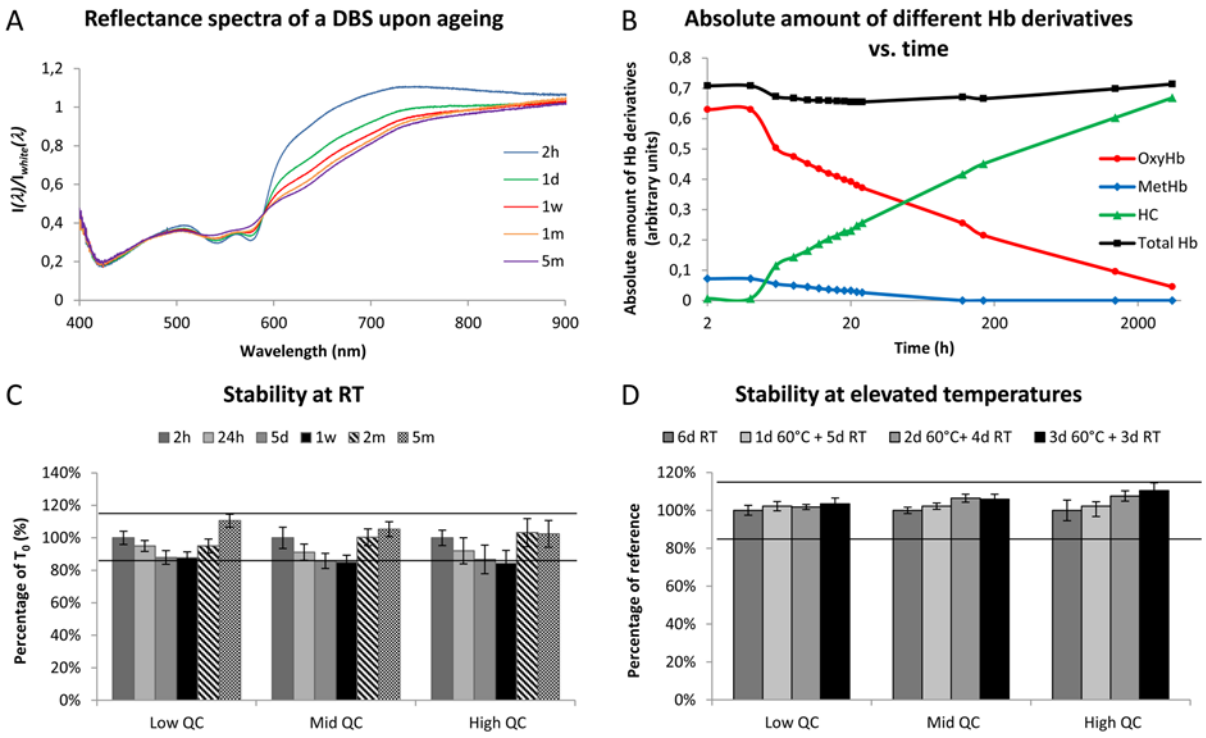


Figure 5.6. (A) The reflectance spectra of a DBS recorded after 2h, 1 day, 1 week, 1 month. and 5 months. The DBS which was analyzed had an approximate HT of 0.20. (B) The amounts of OxyHb, MetHb, and HC present in DBS up to five months. The DBS which were analyzed had an approximate HT of 0.20. Note the logaritmic scale of the x-axis. (C) The influence of storage at RT for up to five months on estimated HT, at three HT levels. At each HT level, the average estimated HT value at T0 was used as a reference value. (D) The influence of storage at elevated temperatures (60 °C) for up to 3 days on estimated HT, at three HT levels. At each HT level, the corresponding DBS stored under ambient conditions were used as a reference. The horizontal lines indicate the 15% acceptance levels. For panel B, C and D average values (n = 6) are depicted. In panels C and D, the error bars indicate SD.

Although one-way ANOVA showed a statistically significant difference in predicted HT between DBS prepared from 15, 20, 25, and 50 µL of blood at all the HT levels tested ($p < 0.05$), post-hoc analysis revealed that there was no statistical difference for any of the applied volumes when compared to the 25 µL reference volume. Moreover, these differences were sufficiently small to not pose a problem in practice (i.e., ≤ 8% compared to the reference of 25 µL) (Figure 5.7A). These differences are likely the result of a different degree of saturation of the filter paper, with higher sample volumes leading to a slightly higher saturation of the filter paper when compared to smaller volumes. This way, a smaller volume of blood will be present in a fixed-size DBS punch when a small volume of blood is deposited on the filter paper, whereas a larger volume of blood will be present in a fixed-size DBS punch when a larger volume of blood is deposited on the filter paper. This is a known phenomenon for DBS, as we described before[38,40].

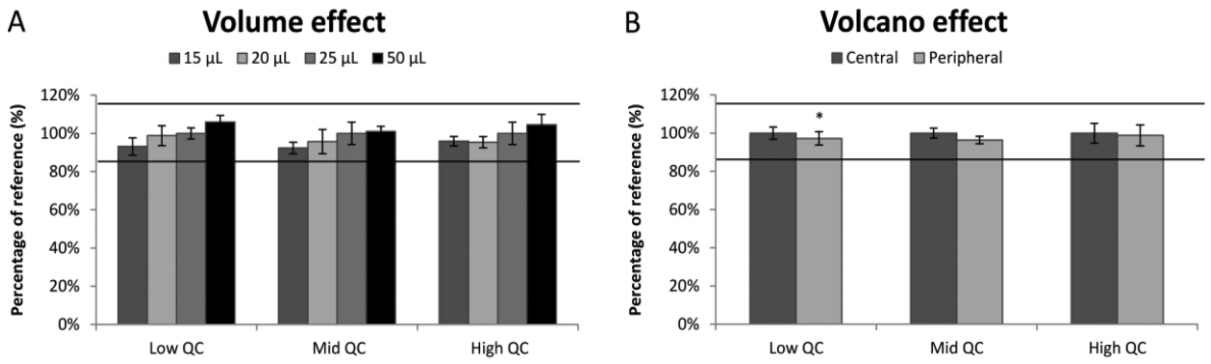


Figure 5.7. (A) Volume effect: Influence of the volume used to generate DBS on the estimated HT, at three HT levels. Per HT level the 25 µL DBS were taken as the reference. (B) Volcano effect: Influence of the measurement localization on the estimated HT, at three HT levels. Per HT level the measurements at a central localization were taken as a reference. Depicted in each graph are the averages (n = 6) ± SD. Results differing significantly ($p < 0.05$) from the reference have been indicated with an asterisk.

Although a marginally statistically significant difference ($p = 0.04$) could be observed between measurements performed in the center of DBS of low HT (HT = 0.19) compared to measurements performed at a more peripheral location of the same DBS, this difference is not of practical relevance (i.e., ≤ 4%), as can be clearly seen in Figure 5.7B. Furthermore, for the measurements at both mid (HT = 0.41) and high (HT = 0.65) HT neither a statistically significant ($p = 0.07$ and 0.15, respectively) nor a practically relevant difference was observed.

5.3.4 Method Application

The newly developed non-contact method was applied to patient samples with a wide HT range (0.205–0.504). The first set of samples included 55 Li-heparin DBS and their corresponding K_2EDTA DBS. The true HT of the patient samples was routinely determined on K_2EDTA anticoagulated whole blood, while the non-contact method was employed to predict the HT of the DBS. A good correlation ($r = 0.93$) was observed between the predicted HT of the Li-heparin DBS and the true HT. Nonetheless, Passing-Bablok regression analysis revealed a statistically significant difference. The 95% CIs of the slope and the intercept were 0.76 to 0.92 and −0.011 to 0.031, respectively, indicating the presence of a proportional error (1 is not included in the CI of the slope) but not of a systemic error (0 is included in the CI of the intercept). When the differences between the predicted and the true HT were plotted *versus* the true HT, an average bias of −0.031 could be observed (with a range of −0.069 to 0.014). However, importantly, aside from two exceptions which deviated −21.0% and −21.1%,

respectively, all predicted HT values were within ± 20% of the true HT (Figure 5.8), indicating that the non-contact method is fit for purpose (i.e., an approximate DBS HT prediction).

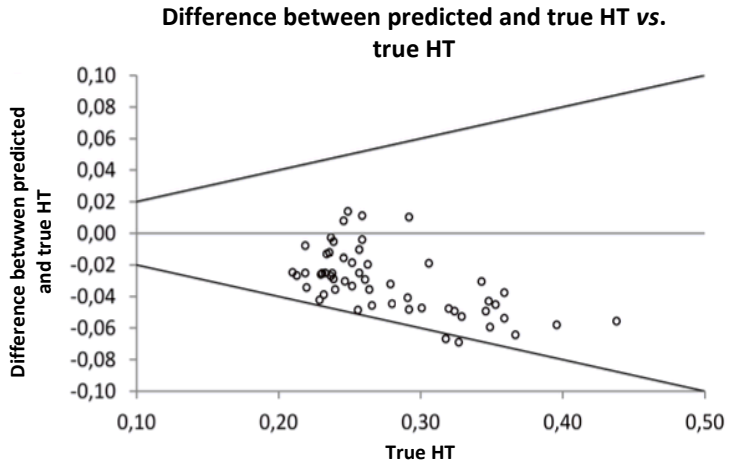


Figure 5.8: The application of the non-contact HT prediction method on Li-heparin anticoagulated DBS (n = 55). The absolute differences between the predicted and the true patient HT were plotted against the true patient HT. The latter HT values were determined on K_2EDTA anticoagulated whole blood.

Next, the predicted HT determined on the Li-heparin DBS was compared with the predicted HT determined on the corresponding K_2EDTA DBS. The Pearson correlation coefficient was 0.95, implying a good correlation between results obtained from Li-heparin and K_2EDTA DBS. In addition, no statistically significant difference was found between both matrices after applying a paired t-test (p = 0.19). Therefore, it was concluded that the non-contact method could be applied on both Li-heparin and K_2EDTA DBS.

In a next step, the method was applied to a second, independent set of 233 K_2EDTA-containing DBS. Again, a good correlation (r = 0.95) was observed between the predicted and true HT (Figure 5.9A). Yet, Passing-Bablok regression analysis demonstrated that a slight systemic error was present since the 95% CI of the intercept did not contain 0 (−0.056 to −0.022), whereas no proportional error could be detected as 1 was included in the 95% CI of the slope (0.98 to 1.07). Furthermore, a Mountain plot (Figure 5.8C) depicting the distribution of the differences between the predicted and true HT, showed a symmetrical distribution around a bias of approximately −0.029. This can also be observed in the Bland-Altman plot (Figure 5.9B), showing a mean difference of −0.029 (95% CI = −0.032 to −0.026) and LoAs of −0.076 (95% CI = −0.081 to −0.070) and 0.018 (95% CI = 0.012 to 0.023). These LoAs are similar to the ones that we obtained for our K^+-based method for HT prediction which has already proven to successfully alleviate HT bias in the quantitative DBS-based analysis of caffeine and

paraxanthine[17,18]. Hence, these LoAs (i.e., the degree of error) were considered acceptable for the intended purpose. Importantly, 95% of the predicted HT values were within 20% of the corresponding true HT.

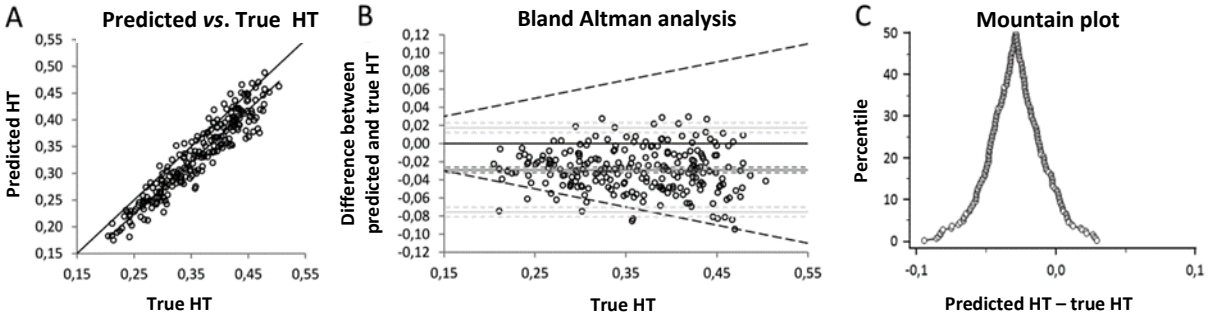


Figure 5.9. (A) Comparison of predicted and true patient HT values and corresponding regression line. Also depicted is the line of equality. (B) Bland-Altman comparison of predicted and true HT. Depicted are the mean difference, the upper and lower LoAs (all with their respective 95% CIs) and the 20% acceptance criteria. (C) Mountain plot depicting the distribution of the differences between the predicted and the true HT. In all graphs, the true HT refers to the HT determined on K_2EDTA anticoagulated whole blood.

We identified several factors that may have contributed to the observed slight negative bias. First, the "true" HT of the patient samples was determined on K_2EDTA anticoagulated whole blood, while the "true" HT of the calibrators was routinely determined on Li-heparin anticoagulated whole blood. The latter gives a slightly lower HT than the former, on average[17]. Second, because of logistical reasons, all HT measurements on patient whole blood were performed in Ghent University Hospital, whereas the HT measurements on the whole blood used to prepare the calibrators that were used to analyze the patient samples were performed in the AMC Amsterdam. Small differences may have been present between the results obtained in both laboratories. Furthermore, a clear influence of the patient's MCHC value on the predicted HT was observed. The MCHC is a measure of the concentration of Hb in a given number of packed RBCs and is calculated by dividing the Hb content of a sample by the HT. We observed that the lower the patients' MCHC values were, the more pronounced the underestimation of their HT values was on average (Figure 5.10). In addition, the influence of the HI, LI, and II on the predicted HT was evaluated. However, none of these parameters seemed to have a noticeable influence on the obtained results (Figure 5.11).

ISR performed on the second set of patient samples (n = 233) revealed good method reproducibility. After the removal of one outlier (54%; confirmed with a Grubbs test, $p < 0.05$) all data points were within the acceptance criterion of ± 20% (95% were within ± 8%).

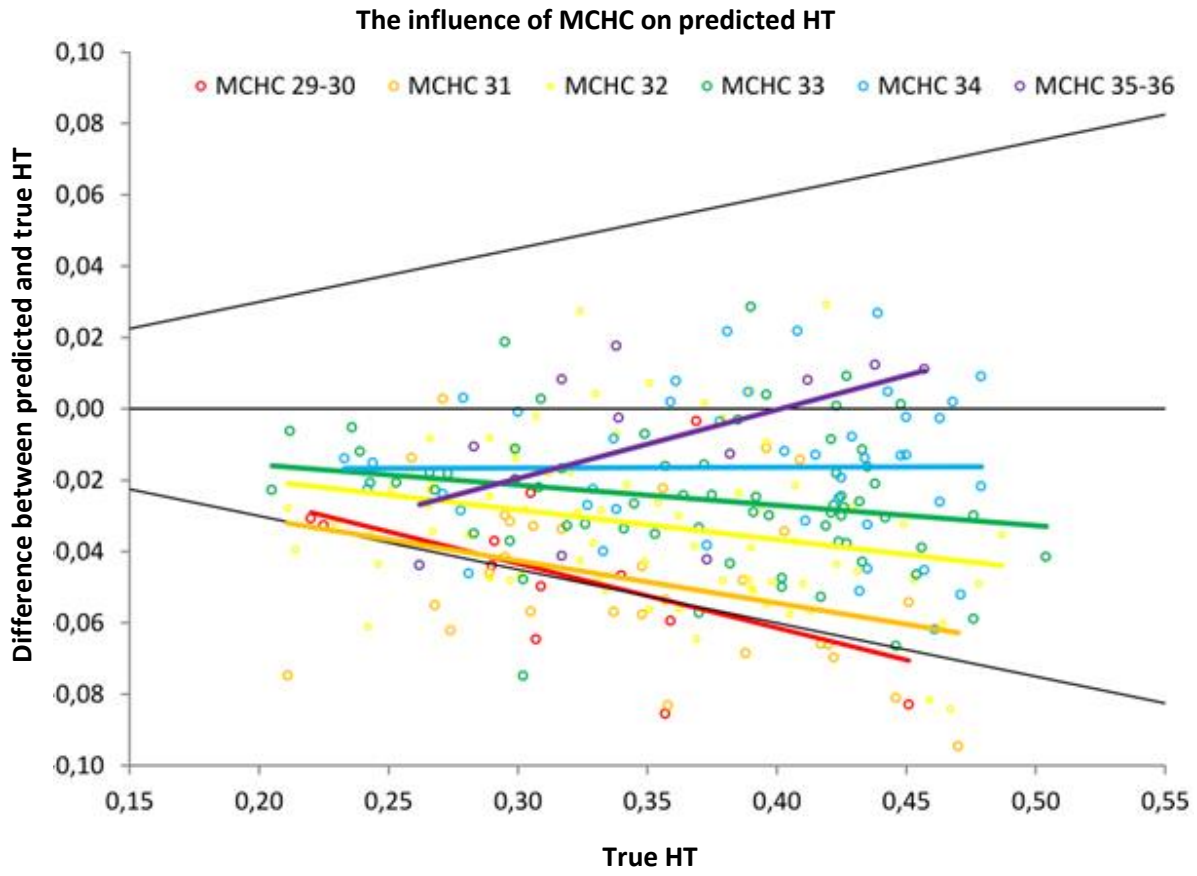


Figure 5.10: The influence of the MCHC value on the predicted HT. Depicted are the differences in predicted and true HT *versus* the true HT of the patient samples. The patient samples were divided into different groups based on their MCHC values and each group was assigned a different color. For each group, a trend line in the corresponding color was also incorporated in the figure to facilitate data interpretation. The samples with an MCHC value of 29 or 30 and 35 or 36, respectively, were combined into one group, because of the limited number of samples in these MCHC classes. Also included in the figure are the 15% acceptance criteria, which are depicted by the black lines. Based on the distribution of the patient samples, it can be concluded that the lower the patients' MCHC values were, the more pronounced the underestimation of their HT values was on average.

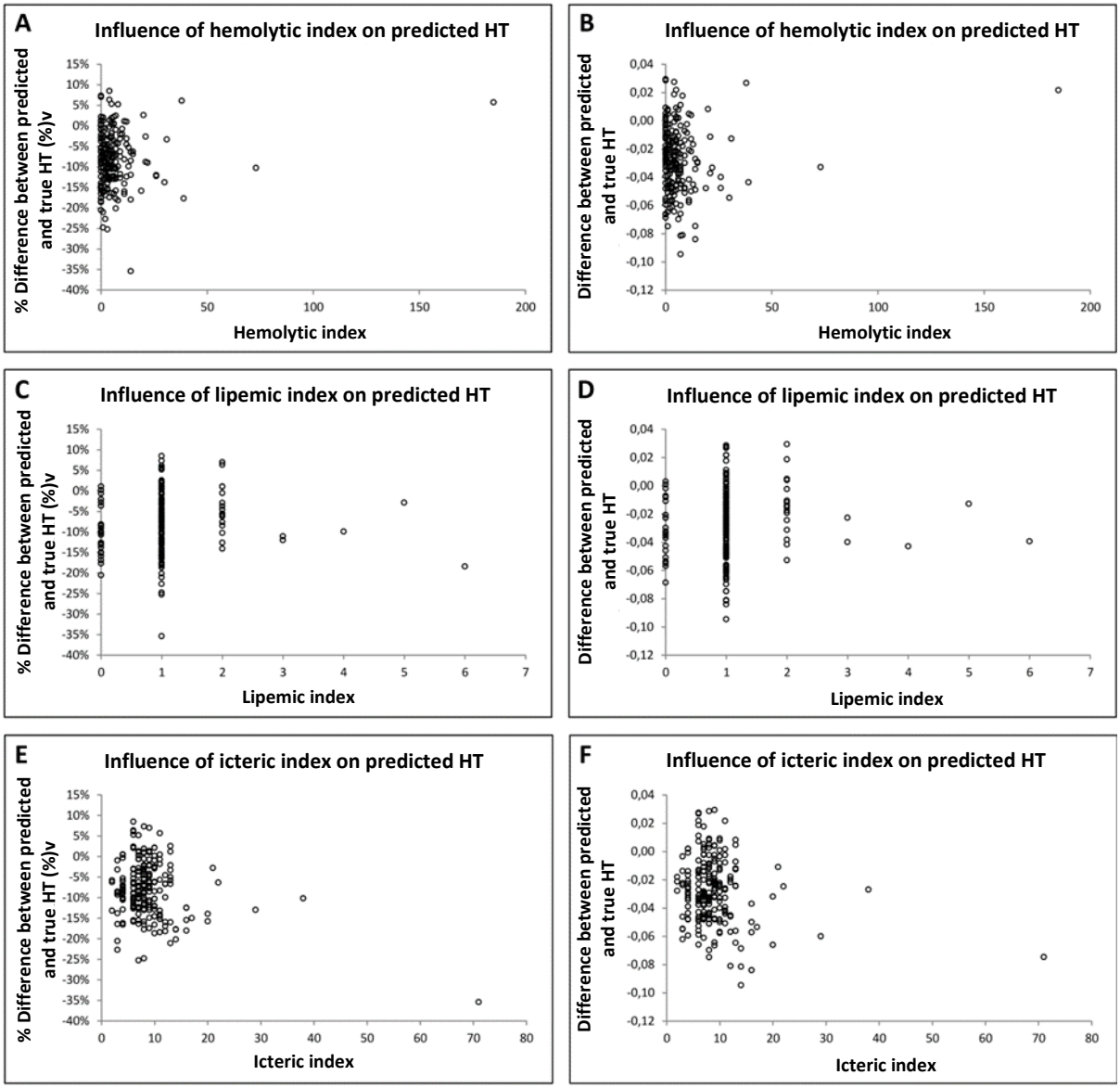


Figure 5.11: The influence of the HI (panel A & B), LI (panel C & D), and II (panel E & F) on the predicted HT. In panels A, C en E the percent difference between the predicted and true HT of the patient samples *versus* the HI, LI or II were plotted. In panels B, D and F the absolute differences between the predicted and true HT of the patient samples were plotted *versus* the different indexes.

5.4. CONCLUSION

We successfully developed a non-contact method to predict the HT of a DBS based on its total Hb content. In contrast to other published DBS-based total Hb analyses, this method is quantitative, non-destructive, does not require any sample preparation nor filter paper pretreatment, and most importantly, allows to predict the HT of both fresh and old DBS. This method was thoroughly validated and complied with all the predefined acceptance criteria generally used for bioanalytical procedures. Application on patient samples with a wide HT range showed the applicability of this method on real patient samples. Although a slight bias

could be observed, which could be attributed to (a combination of) several factors, this does not jeopardize the usefulness of the approach. This non-contact diffuse reflectance spectroscopy-based method overcomes the need for sample preparation, which in turn reduces the analysis time, minimizes the possibility of errors and, importantly, eliminates the need for sample destruction. Indeed, no part of the already limited sample volume of DBS needs to be sacrificed for the additional HT analysis, since mere scanning of a DBS suffices to derive the approximate HT of the sample, one of the most important variables in DBS analysis. The predicted HT could either be used to evaluate whether the HT of a sample lies within the valid HT range established during method validation or to correct the obtained result for the HT effect anticipated at that HT value. In both cases it is important to know the HT of a DBS, even if the HT value could also be determined on a corresponding liquid venous whole blood sample, as the venous and capillary HT may not be the same[41]. In addition, the capillary HT also shows more variation during consecutive measurements than the venous HT does[42], which may cause a larger variation in capillary analyte concentrations, as the latter are all subject to a (slightly) different HT effect. Yet again, knowing the DBS's HT may be beneficial in this context, as correcting the capillary DBS analyte concentration based on the predicted HT of that exact DBS may help to reduce the variability. In addition, the predicted HT may also be used to convert a DBS-based analyte concentration into the corresponding plasma-based concentration. Such a blood–plasma conversion is typically required to allow comparison of the DBS-based result with existing plasma-based reference values or therapeutic intervals and is often HT-dependent, since a compound's blood to plasma ratio is often influenced by the amount of RBCs present (i.e., a patient's HT). Importantly, all the solutions which have hitherto been suggested to cope with the HT effect on the spreading of the blood through the filter paper still yield a dried blood sample and therefore a dried blood sample-based result, necessitating a blood-plasma conversion and hence, a HT determination in those cases as well. The only exception to this is the use of *in situ* generated DPSs. However, also this approach has its (HT-related) issues[23]. In future, the newly developed non-contact method will have to be applied on true capillary samples and it will need to be evaluated whether the predicted HT will allow adequate HT effect correction. To ensure a correct evaluation, the true HT should be determined on a capillary blood sample, since the latter might differ from venous HT. Furthermore, since the employed equipment is simple and compact, automation of this analysis can be easily envisaged.

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Chapter 6

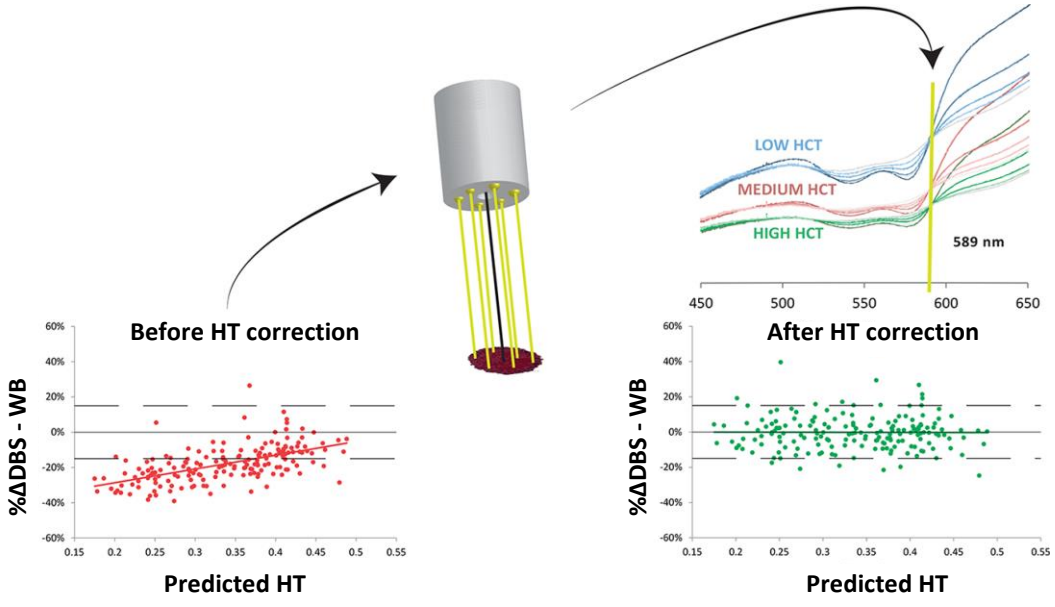
Hematocrit correction of DBS-based results using hemoglobin content

Based on:

Capiau S, Wilk LS, De Kesel PM, Aalders MCG, Stove CP. Correction for the Hematocrit Bias in Dried Blood Spot Analysis Using a Nondestructive, Single-Wavelength Reflectance-Based Hematocrit Prediction Method. *Analytical Chemistry*, 2018; 90(3):1795-1804.

Abstract

The HT may affect both the accuracy of DBS methods as well as the interpretation of DBS-based results. We previously developed a method to determine the HT of a DBS based on its Hb content using non-contact diffuse reflectance spectroscopy. Despite the ease with which the analysis can be performed and the good results that were obtained, the method did require a complicated algorithm to derive the total Hb content from the DBS's reflectance spectrum. As the total Hb was calculated as the sum of oxyHb, metHb, and HC, the three main Hb derivatives formed in DBS upon aging, the reflectance spectrum needed to be unmixed to determine the quantity of each of these derivatives. We now simplified the method by only using the reflectance at a single wavelength, located at a quasi-isosbestic point in the reflectance curve. At this wavelength, assuming 1-to-1 stoichiometry of the aging reaction, the reflectance is insensitive to the Hb degradation and only scales with the total amount of Hb and, hence, the HT. At each QC level as well as at the limits of quantitation (i.e., 0.20 and 0.67) bias, intra- and interday imprecision were within 10%. Method reproducibility was excellent based on ISR and surpassed the reproducibility of the original method. Furthermore, the influence of the volume spotted, the measurement location within the spot, as well as storage time and temperature were evaluated, showing no relevant impact of these parameters. Application to 233 patient samples revealed a good correlation between the HT determined on whole blood and the predicted HT determined on venous DBS. The bias obtained with Bland-Altman analysis was -0.015 and the LoA's were -0.061 and 0.031, indicating that the simplified, non-contact HT prediction method even outperforms the original method. In addition, using caffeine as a model compound, it was demonstrated that this simplified HT prediction method can effectively be used to implement a HT-dependent correction factor to DBS-based results to alleviate the HT bias.



6.1. INTRODUCTION

Since its introduction in the 1960s[1], DBS sampling has evolved into a valuable alternative for traditional venous blood sampling[2] (as was discussed in General Background and Chapter 1 section 1.4.1). This is illustrated by the countless semiquantitative and quantitative DBS applications that have since been developed in diverse domains, such as NBS[3], phenotyping[4, 5], therapeutic drug monitoring[6-8], and toxicology[9, 10]. The growing success of DBS can be attributed to the fact that they offer a minimally invasive way of collecting a representative sample, require only very small volumes of blood, tend to improve analyte stability as compared to whole blood, and, most importantly, allow for home-based patient self-sampling[11]. Unfortunately, despite these relevant benefits, routine implementation of quantitative DBS applications is still limited[2, 8, 12]. One of the root causes for this is the so-called HT effect[13] (see General Background). This effect refers to the impact of the HT on the quantitative result, obtained after DBS analysis. Indeed, by influencing the blood's viscosity, the HT affects the spreading of blood through the filter paper and consequently also the amount of blood in a fixed-size DBS punch. Typically, this phenomenon will lead to an overestimation of the analyte concentration in samples of high HT and to an underestimation in samples of low HT. Furthermore, the HT may also affect DBS homogeneity, analyte recovery, and potentially even the matrix effect (see Chapter 2). These factors render the HT one of the most important unknowns in DBS analysis and have led to the emergence of several strategies to cope with this problem. These strategies, which have been elaborately discussed elsewhere[13, 14], include the analysis of volumetric DBS[15-20] or other fixed-volume dried blood samples[21-23,] the use of HT-independent DBS collection substrates[24], the generation of DPS rather than DBS[25-28], and, finally, HT prediction and/or correction[29-36].

In this context, we recently developed the first non-contact method, able to predict the HT of a DBS based on its total Hb content[31] (see Chapter 5). This total Hb content was determined using non-contact diffuse reflectance spectroscopy, in which the DBS's wavelength-dependent reflectance is recorded. More particularly, broadband light from a halogen source is guided to the surface of a DBS *via* the delivery fibers of an optical probe, and the reflected light is transported to a spectrometer *via* the collection fiber of that same probe. The obtained reflectance spectrum is then compared to a one-dimensional light transport model for multilayered samples, which takes into account the presence of three Hb derivatives: OxyHb, MetHb, and HC. It is imperative that these three Hb derivatives are all taken into account to

determine the total Hb content of a DBS, as Hb is known to be present as OxyHb in fresh ex vivo blood, and is then gradually oxidized into MetHb and further transformed into denatured HC upon drying and aging of the blood[37-39]. To estimate the amount of each of these three Hb derivatives, a least-squares fit is performed between the light transport model and the measured reflectance spectrum, resulting in a value with arbitrary units being attributed to each Hb derivative. Finally, the sum of those three values is used as a measure of total Hb and, hence, HT. Although this approach offered a fast and convenient way of predicting the HT of a DBS, without the need for sample preparation or sample destruction, we set out to further simplify the approach in view of a more straightforward routine implementation in the future.

To that end, we evaluated whether the reflectance at a single wavelength rather than an entire reflectance spectrum could be employed as a measure of total Hb and HT, independently of the age of the DBS. More particularly, we reasoned that measuring the reflectance at a(n) (quasi-)isosbestic point of the three above-mentioned Hb derivatives might be a good alternative. This would not only be more straightforward, but it also requires almost no computing power nor specialized software programs (a patented MATLAB script is necessary to perform the reflectance spectrum analysis). Following successful validation and application, it was evaluated whether this simplified method can be used to implement an HT-dependent correction factor to DBS-based results to alleviate the HT bias. To that end, caffeine was used as a model compound, because DBS-based caffeine quantitation is known to be affected by the HT effect[40]. Furthermore, as most people, even hospitalized ones, regularly consume caffeinated food products, it is not necessary to set up a clinical study involving the administration of a probe drug, and left-over samples can be used instead.

6.2. MATERIALS AND METHODS

6.2.1. Chemicals

Caffeine, caffeine-$^{13}C_3$, and formic acid (FA) were obtained from Sigma-Aldrich (Diegem, Belgium), whereas LC–MS grade MeOH was purchased from Biosolve (Valkenswaard, The Netherlands). Ultrapure water was generated using a Synergy Water Purification System (Merck Millipore, Overijse, Belgium).

6.2.2. Preparation of DBS

The experiments conducted in this chapter were performed with the same DBS as those used in Chapter 5: to prepare calibration curves and to perform method validation, venous whole blood was collected from consenting healthy adult volunteers in Li-heparin containing blood collection tubes (Venosafe, Terumo, Leuven, Belgium). Within 24 h after blood collection, DBS were generated by applying 25 µL of whole blood (unless mentioned otherwise) onto Whatman® 903 filter paper (GE Healthcare, Dassel, Germany) and were left out to dry for at least 2h. This filter paper is among the most used filter papers in DBS studies. Obviously, if the method described here is to be applied on other filter papers, slightly different spectra may be obtained, due to characteristics intrinsically linked to the paper, as well as to potentially differential spreading of the blood, requiring a separate validation. Blood with different HT values was prepared by centrifuging an aliquot of venous whole blood and removing or adding a certain amount of plasma. Patient samples were collected in Li-heparin or K_2EDTA containing blood collection tubes (Venosafe, Terumo), followed by DBS generation within 24h, as mentioned above. DBS were stored at RT until analysis, while the liquid blood samples were stored at −20 °C (after the HT was determined). Permission was obtained from the Ethics Committee of Ghent University Hospital (project no. 2015/0931) to collect and analyze the above-mentioned samples.

6.2.3. Analysis

A Sysmex XE-5000 hematology analyzer (Sysmex Corp., Kobe, Japan) was used to determine the HT of venous whole blood samples and the MCHC. Other characteristics that were determined on the patient samples include the II, HI and LI, determined using a Cobas 8000 (Roche Diagnostics, Mannheim, Germany). These indices provide a semi-quantitative measure of the level of icterus, hemolysis or lipemia (turbidity) present in a patient sample. The HI is expressed in hemolysis units with a unit corresponding to approximately 1 mg/dL 0.62 µmol/L Hb, the II is reported in icterus units with an icteric unit corresponding to about 1 mg/dL or 17.1 µmol/L bilirubin, and the LI is reported in lipemia units, with each unit corresponding to the turbidity of a sample to which 1 mg/dL intralipid (an artificial lipid material) was added.

For the DBS-based HT determination, non-contact diffuse reflectance spectroscopy was employed. The setup that was used to collect the reflectance spectra has been elaborately

described elsewhere[31] (see sections 5.2.2 and 5.2.3). Briefly, light originating from a 10 W tungsten–halogen light source (AvaLight-HAL, Avantes, Apeldoorn, The Netherlands) is guided toward the DBS surface *via* the delivery fibers of a non-contact optical probe (QR400-7-UV/BX, Ocean Optics, Duiven, The Netherlands). This way, a 5.9 mm-diameter spot of the DBS is illuminated. The light reflected by the DBS is subsequently transported to a spectrometer (USB4000, Ocean Optics) *via* the collection fiber of that same optical probe, and a reflectance spectrum is recorded between 354 and 1042 nm using the SpectraSuite software (Ocean Optics). To normalize the measured reflectance to the light source output, the reflectance at each wavelength was divided by the reflectance of a white reference standard at that same wavelength. Additionally, both DBS and white reference spectra were corrected for the electrical dark noise of the detector. To be able to compare both the original and the simplified data processing approach side by side, the reflectance spectra that were recorded for the development, validation, and application of the original spectrum-based HT prediction method were also employed for the setup and evaluation of the new, simplified single wavelength-based HT prediction method[31]. Therefore, this Materials and Methods section only briefly outlines how the spectra were originally recorded and which samples were analyzed during method validation and application. For more details see section 5.2.

Caffeine was determined in all K_2EDTA anticoagulated whole blood samples and corresponding DBS samples, using previously validated LC–MS/MS methods[40]. Briefly, for whole blood samples, 100 µL of MeOH containing 0.01% FA is added to 50 µL of whole blood (after the addition of the IS) to perform protein precipitation. For DBS, a 3 mm punch is extracted using 70 µL of a MeOH/water mixture (80:20 v/v) containing the IS and 0.01% of FA. After 10 min of gentle shaking, the extracts of both whole blood and DBS samples are centrifuged. The resulting supernatant is further diluted with water containing 0.01% FA. Both whole blood and DBS extracts were analyzed using an Acquity UPLC System (Waters, Milford, MA) coupled to an API 4000 triple quadrupole mass spectrometer (Sciex, Framingham, MA). More details regarding these methods can be found elsewhere[40].

6.2.4. Method development

To elucidate which wavelength was most suited for HT prediction purposes, reflectance spectra of Li-heparin containing DBS with varying HT values (0.20–0.65) were recorded at multiple time points throughout a period of 5 months after DBS generation and were compared to one another. To evaluate the influence of different anticoagulants on the predicted HT, the reflectance at 589 nm of DBS from a healthy volunteer, prepared from blood that contained either Li-heparin, NaF/oxalate, K_2EDTA, or citrate as anticoagulant, was compared to the reflectance at 589 nm of DBS prepared from blood without anticoagulant.

6.2.5. Validation

The calibration model selection was based on the distribution of the % RE and the sum of the absolute % RE[41]. The evaluated models included unweighted and weighted ($1/x$, $1/x^2$, $1/y$, $1/y^2$) linear regression, a second-order regression, power regression, as well as linear regression after transformation (logarithmic or square root) of the data. For the determination of accuracy (% bias) and imprecision (% RSD), two seven-point calibration curves were set up on each of three different days and analyzed along with three DBS QCs with an approximate HT of 0.20, 0.42, and 0.65. To exclude donor-dependent results, this experiment was repeated with blood of a second donor. Furthermore, additional QCs were generated from blood of four other donors. For the calculation of the imprecision, a normalization step was implemented to take into account that each QC series had slightly different true HT values (as described in Capiau *et al*., 2016)[31]. For the evaluation of the effect of the blood volume applied, the predicted HT values obtained from 15, 20, 25, and 50 µL DBS were compared. The 50 µL DBS were also employed to compare the results obtained from measurements in the center of the DBS to those obtained at a more peripheral location. The stability of the reflectance at 589 nm (and, hence, of the predicted HT) was evaluated at RT (up to 5 months) and at 60 °C (up to 3 days). The effect of the blood volume spotted, the measurement location, and DBS storage conditions were all evaluated at three HT levels (approximately 0.20, 0.42, and 0.65, n = 6).

6.2.6. Method application

The newly developed methodology was applied to recorded reflectance spectra of patient DBS. These patient samples displayed a wide range of HT values (0.205–0.504) and were

collected in either Li-heparin or K_2EDTA containing blood collection tubes. As these samples were collected at Ghent University Hospital, they represent a heterogeneous population, encompassing both healthy and unhealthy individuals.

A first set of patient samples consisted of 55 Li-heparin whole blood samples and the corresponding K_2EDTA whole blood samples. A second set of samples consisted of 233 K_2EDTA whole blood samples for which the Li-heparin whole blood was not available. All DBS samples were 5–7 days old when their HT was determined. On the second set of patient DBS (n = 233), ISR was performed, 3 days after the original analysis. The results obtained with this simplified method were compared both to the true HT values determined on the whole blood samples and to the results obtained with the original, spectrum-based HT prediction method.

To assess whether the simplified, non-contact HT prediction method could be used to correct for the HT effect, caffeine was determined in all K_2EDTA containing whole blood samples and the corresponding DBS. From all sample sets with a caffeine concentration above the LLOQ, both in whole blood and in DBS, 50 were randomly selected to set up a reference set. This reference set was used to establish the HT correction algorithm. More particularly, the whole blood/DBS caffeine concentration ratios were plotted against the predicted HT values, and a linear regression line was fitted to the data. The HT correction algorithm was derived from that regression line *via* a simple transformation. The HT correction algorithm was applied to both the reference set and an independent test set (n = 178). To evaluate the effectiveness of the HT correction algorithm, the % difference between the DBS and whole blood caffeine concentrations was plotted against the predicted HT, both before and after implementation of the algorithm. To each data set was fitted a linear regression line, and the slope, intercept, corresponding 95% CIs, and the Pearson correlation coefficients were determined.

6.2.7. Statistical data analysis

For the statistical analysis, the AnalysisToolpak of Microsoft Excel 2010, SPSS statistics for Windows version 22.0 (IBM Corp, Armonk, NY; 2013) and Medcalc statistical software version 14.12.0 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014) were employed. The effect of different anticoagulants on the predicted HT was evaluated using a one-way ANOVA ($\alpha = 0.05$) with Bonferroni post-hoc test. Furthermore, a model II ANOVA for the analysis of the components of variation was utilized for calculating the intra- and interday imprecision. To assess the effect of the measurement location and the volume of blood

spotted, a paired *t* test and a one-way ANOVA (α = 0.05) with Bonferroni post-hoc test were performed, respectively. Pearson correlation coefficients were established using Medcalc, which was also employed to perform Passing-Bablok regression analysis and to set up a Mountain plot. The Bland-Altman plots were constructed in Excel 2010. To evaluate whether Li-heparin containing patient DBS and their corresponding K_2EDTA containing DBS yielded equal predicted HT values, a paired *t* test was carried out. To detect a single outlier, a Grubbs test (α = 0.05) was employed. Whenever multiple outliers were suspected, an ESD test was performed (α = 0.05). To calculate the 95% CIs of slope and intercept, the regression analysis in Excel was used.

6.3. RESULTS AND DISCUSSION

6.3.1. Method Development - Selection of the Optimal Wavelength

Several requirements need to be fulfilled, if the reflectance at a single wavelength is to be used for HT prediction of DBS. First, a unique relation must exist between the intensity of the reflection at this wavelength and the HT value of the DBS; second, it should allow one to accurately predict the HT of a DBS independently of its age; and third, it should show as little variation as possible. Ideally, an isosbestic point could be identified, that is, a wavelength at which the total reflectance does not change when OxyHb is transformed into MetHb and further into HC.

Although the shape of the DBS reflectance spectrum changes drastically upon aging of the DBS due to these transformations, several wavelengths were observed at which the reflectance of the DBS remained approximately constant during the investigated time period (Figure 6.1). Furthermore, because we wanted to use a wavelength that could be a surrogate for the total Hb content, wavelengths above 675 nm were excluded from further evaluation, as Hb shows minimal light absorption at these higher wavelengths[37]. In addition, wavelengths below 490 nm were discarded as an option, because of a lower signal-to-noise ratio in the reflectance spectra below this wavelength (Figure 6.1), which would negatively impact the imprecision of the final HT prediction method. The wavelength that fulfilled the above-mentioned criteria and that showed the least variable reflectance was 589 nm (Figure 6.1). As, for a given HT, the reflectance spectra did not completely overlap at 589 nm for all of the DBS ages, this wavelength is further referred to as a quasi-isosbestic point, rather than an isosbestic point. This quasi-isosbestic point at 589 nm can be clearly observed in Figure 6.1,

which depicts the reflectance spectra of DBS with HT values of 0.20, 0.42, and 0.65, recorded 2 h, 24 h, 1 week, 1 month, and 5 months after DBS generation. Consequently, we selected the reflectance at 589 nm as an alternative measure of the total Hb content and, hence, as a measure of HT for further evaluation during method validation and application.

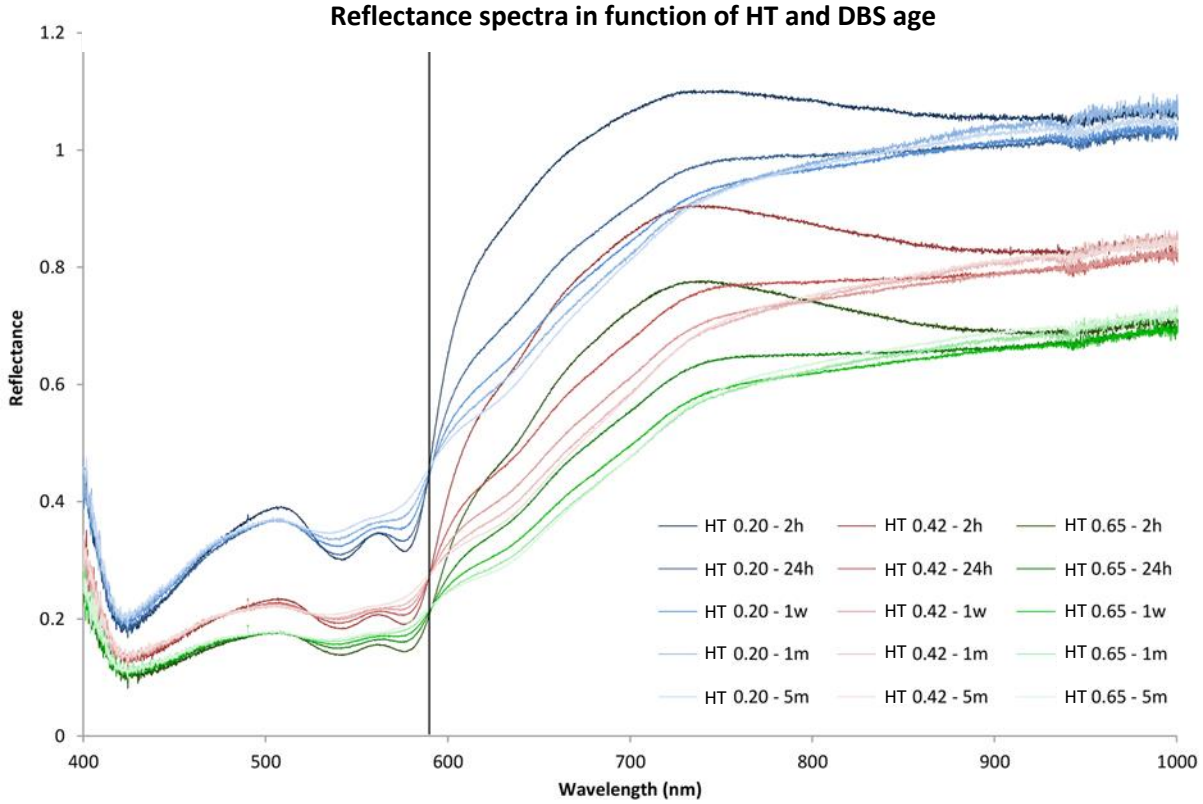


Figure 6.1. Depicted are the reflectance spectra of DBS with a HT of 0.20 (blue), 0.42 (red), and 0.65 (green), measured 2h, 4h, 1 week, 1 month, and 5 months after DBS generation. The black, vertical line represents the quasi-isosbestic point at 589 nm.

Contrary to the original spectrum-based HT prediction method, this simplified single-wavelength-based method only provides information on the total Hb content and no longer on the amount of OxyHb, MetHb, and HC present in a DBS. While knowledge of the amount of the different Hb derivatives, more particularly their fractions, could provide a DBS age estimate, lack of this knowledge has no repercussions on the HT prediction itself. Moreover, age estimation would only be valuable for some DBS applications, as in most cases the DBS age will obviously be known. Furthermore, age estimation of DBS requires knowledge of the exact DBS storage conditions, because the speed with which the transformation between the different Hb derivatives takes place is known to depend on both temperature and humidity[39]. Therefore, as DBS are typically transported and stored under “ambient” conditions and exact storage conditions are often unknown, reliable DBS age estimation may pose a challenge in many cases anyway.

6.3.2. Influence of anticoagulant

The reflectance at 589 nm of DBS samples prepared from blood without anticoagulant (the type of sample that eventually will need to be analyzed in the context of home sampling) did not differ from the reflectance observed in K_2EDTA- ($p = 1.00$) and Li-heparin- ($p = 1.00$) anticoagulated DBS samples (Figure 6.2). NaF/oxalate and citrate containing DBS, however, respectively showed a lower (although not statistically different $p = 0.21$) and a higher ($p < 0.001$) reflectance at 589 nm (Figure 6.2). This indicates that for this single wavelength-based HT prediction method, Li-heparin or K_2EDTA anticoagulated blood are the preferred sources to prepare DBS from during method development and to set up calibration curves for routine analysis. Indeed, the use of an anticoagulant is vital in these instances, because the required DBS samples can never be prepared from non-anticoagulated whole blood. Already in an early stage of this study, Li-heparin was selected as anticoagulant, as we wanted to keep the option open to also apply another HT prediction method, based on the K^+ content, on these same samples[28].

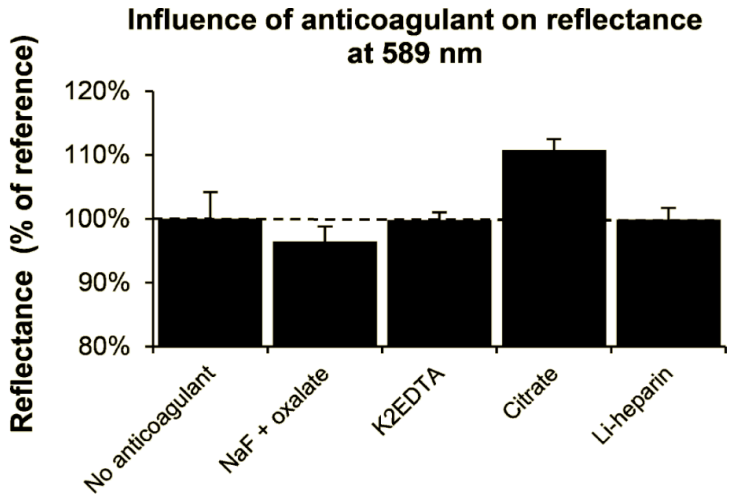


Figure 6.2. Depicted are the average (n = 6) relative intensities of the reflectance at 589 nm for DBS samples prepared from blood with different anticoagulants. All DBS were analyzed 2 h after DBS generation. The average reflectance of the DBS without anticoagulant was used as a reference. The error bars indicate the standard deviations.

6.3.3. Method Validation

On the basis of the sum of the absolute % RE and the distribution of the % RE, a power regression model (and the derived linear regression model after log/log transformation) was best suited to describe the correlation between the HT of the blood used to prepare the DBS and the reflectance measured at 589 nm (Table 6.1). The power regression model and the derived linear regression model yield the same results.

Table 6.1. Overview of the evaluation of the different calibration models.

Evaluated calibration model	% RE distribution	Sum |% RE|
Unweighted linear regression	Not random	N.A.
Weighted linear regression (1/x, $1/x^2$, 1/y, $1/y^2$)	Not random	N.A.
Linear model after log/log transformation	Random	119.38%
Power regression model	Random	119.38%
Linear model after square root transformation	Random	247.11%
2^{nd} order polynomial	Random	Not suitable at upper end of calibration range

For donor 1, the average calibration curve was $reflectance_{589nm}$ = 0.140 × HT–0.669, based on the power regression model, and $\log(reflectance_{589nm})$ = −0.669 × log(HT) – 0.855 when using the corresponding linear model. Similar calibration curves were obtained for donor 2: $reflectance_{589nm}$ = 0.144 × HT–0.651, respectively, $\log(reflectance_{589nm})$−0.651 × log(HT) – 0.843. The method was validated for HT values between 0.20 and 0.67. Using the above-mentioned calibration model, accuracy and imprecision were within 7% and, hence, well within the preset acceptance criteria (i.e., ≤ 15% and ≤ 20% at LLOQ) (Table 6.2, panels A and B). Equally good results were obtained when the calibration curves and QCs were prepared in the blood of a second donor (Table 6.3, panels A and B). Moreover, QCs prepared in the blood of five donors, different from the donor of the blood in which the calibration curves were prepared, yielded similar results when using the calibration curves of both donor 1 (Table 6.2, panel C) and donor 2 (Table 6.3, panel C), demonstrating that the obtained results were not donor-dependent.

Table 6.2. Overview of the data for accuracy and imprecision (n = 6) for donor 1*

	Accuracy (% bias)	Intraday imprecision (% RSD)	Interday imprecision (% RSD)
A) LLOQ	0.15	1.97	3.15
ULOQ	0.15	2.49	2.95
B) QC LOW	-1.46	4.44	4.61
QC MID	-0.59	5.96	6.55
QC HIGH	-0.37	5.52	5.52
C) QC LOW (donor 2)	1.09	4.37	8.00
QC MID (donor 2)	-4.69	4.01	4.82
QC HIGH (donor 2)	-3.18	5.06	5.06
QC LOW (donor 3)	4.89	3.44	3.44
QC MID (donor 3)	4.23	1.33	1.66

QC HIGH (donor 3)	1.60	3.98	4.32
QC LOW (donor 4)	3.06	5.60	5.60
QC MID (donor 4)	-0.07	9.13	9.13
QC HIGH (donor 4)	-4.00	6.74	7.03
QC LOW (donor 5)	5.16	6.08	6.08
QC MID (donor 5)	–2.38	5.24	5.24
QC HIGH (donor 5)	-0.27	9.03	9.03
QC LOW (donor 6)	6.16	8.62	8.62
QC MID (donor 6)	3.89	9.90	9.90
QC HIGH (donor 6)	2.05	7.56	7.88

*Sections A and B, respectively, give the data obtained for the LOQs (LLOQ and ULOQ) and QCs, prepared from blood of the same donor as the one in which the calibration curves were prepared. Section C gives the data for QCs prepared from blood of five other donors than the one in which the calibration curves were prepared.

Table 6.3. Overview of the data for accuracy and imprecision (n = 6) for donor 2*

		Accuracy (% bias)	Intraday imprecision (% RSD)	Interday imprecision (% RSD)
A)	LLOQ	2.12	2.63	3.68
	ULOQ	-3.42	4.66	4.66
B)	QC LOW	0.88	9.96	9.96
	QC MID	-2.90	2.24	2.85
	QC HIGH	0.13	6.31	6.31
C)	QC LOW (donor 1)	-1.70	5.36	6.67
	QC MID (donor 1)	1.39	4.33	6.63
	QC HIGH (donor 2)	3.28	8.41	8.41
	QC LOW (donor 3)	5.06	3.91	6.92
	QC MID (donor 3)	6.86	3.27	3.28
	QC HIGH (donor 3)	5.78	4.94	8.10
	QC LOW (donor 4)	2.61	5.40	6.39
	QC MID (donor 4)	2.04	8.38	8.95
	QC HIGH (donor 4)	-0.89	8.53	8.53
	QC LOW (donor 5)	4.99	6.72	6.72
	QC MID (donor 5)	-0.83	4.22	4.44
	QC HIGH (donor 5)	2.65	3.83	3.83
	QC LOW (donor 6)	6.42	4.58	5.52
	QC MID (donor 6)	7.07	11.73	11.73
	QC HIGH (donor 6)	6.46	14.53	14.53

*Sections A and B, respectively, give the data obtained for the LOQs (LLOQ and ULOQ) and QCs, prepared from blood of the same donor as the one in which the calibration curves were prepared. Section C gives the data for QCs prepared from blood of five other donors than the one in which the calibration curves were prepared.

As was already discussed above, Hb is not stable upon drying and storage of DBS, resulting in a drastical impact on the DBS' reflectance spectrum (Figure 6.1). It is therefore crucial to verify the effect of storage on the reflectance at the quasi-isosbestic point, and thereby on the

predicted HT. With the exception of a single time point at one HT level, the predicted HT was always within 15% of its value at T_0 when DBS samples were stored at RT for up to 5 months (Figure 6.3A). Furthermore, storage at elevated temperatures (60 °C) for up to 3 days did not affect the predicted HT value, as the latter was always within 6% of the reference value (i.e., the predicted HT of the corresponding DBS samples stored at RT) (Figure 6.3B).

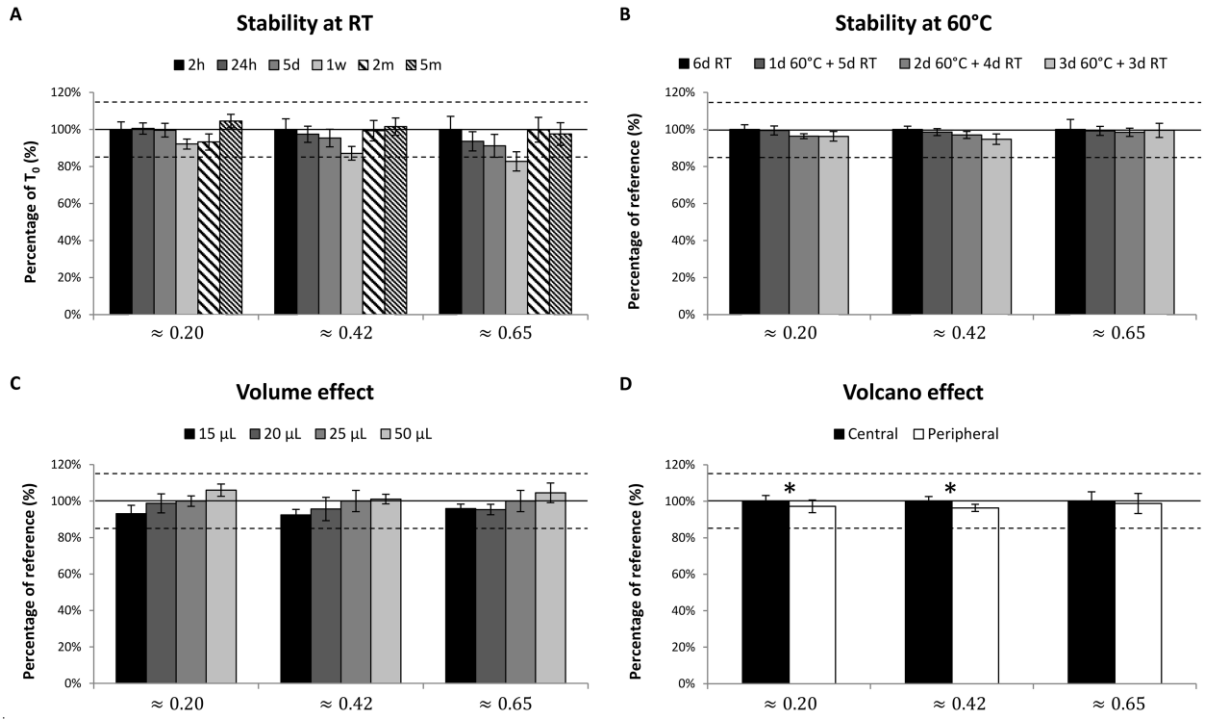


Figure 6.3. Influence of storage time, volume spotted, and measurement location on the predicted HT. (A) The influence of storage at RT for up to 5 months on the predicted HT. At each HT level, the average estimated HT value at *T_0* was used as a reference value. (B) The influence of storage at elevated temperatures (60 °C) for up to 3 days on the predicted HT. At each HT level, the corresponding DBS stored at RT were used as a reference. (C) The influence of the volume used to generate DBS on the predicted HT. At each HT level, the 25 µL DBS was used as a reference. (D) The influence of the measurement localization on the predicted HT in 50 µL DBS. Results of peripheral measurements were normalized to those of central measurements. All parameters were evaluated at three HT levels (0.20, 0.42, and 0.65). The dotted horizontal lines indicate the 15% acceptance levels, whereas the full line indicates 100% (i.e., the reference value). In each panel, the average results (n = 6) are depicted, and the error bars indicate the standard deviation. Asterisks in panel D indicate results that differ significantly ($p < 0.05$) from the reference.

Despite all HT levels (low, medium, and high) exhibiting a slight volume effect, this was neither of practical nor of statistical significance (Figure 6.3C). Indeed, all differences were within 8% of the predicted HT of the 25 µL DBS, which were used as a reference at each HT level. Although an ANOVA test showed a statistically significant difference between the different volumes tested at each HT, a bonferroni test ($\alpha = 0.05$) revealed no differences between the predicted HT at the reference volume of 25 µL and the predicted HT at any other evaluated volume at each HT level. The occurrence of a slight volume effect is inherent to DBS analysis,

as the deposition of a larger volume of blood will lead to a slightly higher degree of saturation of the filter paper, whereas the deposition of a smaller volume of blood will lead to a lower degree of filter paper saturation.

Furthermore, a small difference (< 4%) could be observed between the HT predicted from central and peripheral measurements (Figure 6.3D). This difference was marginally statistically significant at low and medium HT ($p = 0.041$ and $p = 0.038$) and of no practical relevance (i.e., ≤ 15%) at any HT level.

6.3.4. Method Application

The simplified, single wavelength-based data processing approach was first applied to a panel of Li-heparin containing DBS (n = 55). A good correlation (r = 0.93) could be discerned between the predicted HT and the true patient HT (routinely determined on K_2EDTA anticoagulated whole blood samples using a Sysmex XE-5000 hematology analyzer). For 49 out of 55 patients, the predicted HT was within 15% of the true patient HT, and all were within 20% (range −19.51% to 9.35%) (Figure 6.4). For these Li-heparin containing DBS samples, also the spectra of the corresponding K_2EDTA containing DBS samples were available. Data processing of the latter spectra yielded results similar to those of the Li-heparin spectra. Comparison of both results with a two-sided paired sample t test showed no statistically significant difference ($p = 0.920$), demonstrating that the simplified data processing method is applicable on either matrix.

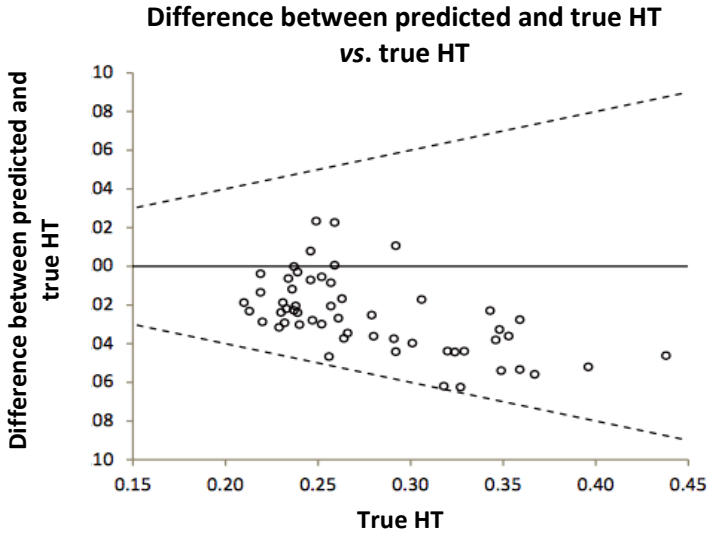


Figure 6.4: the application of the single wavelength-based HT prediction method on Li-heparin anticoagulated DBS (n = 55). Depicted are the absolute differences between the predicted and the true patient HT *versus* the true patient HT. The true HT values were determined on K_2EDTA anticoagulated whole blood. The dotted black lines represent the ± 20% limits.

In a second step, the simplified method was applied on an independent, more elaborate set of spectra of K_2EDTA containing DBS (n = 233). The patient samples were collected in a hospital setting. Therefore, at least part of the samples was from non-healthy individuals, implying that the test set is quite heterogeneous. Although Passing-Bablok regression analysis revealed that both a slight proportional (95% CI of slope = [1.02, 1.11]) and a constant error (95% CI of intercept = [−0.05, −0.02]) were present, a good correlation (r = 0.95) between the predicted and true HT could be observed in this set as well (Figure 6.5A). Furthermore, a Mountain plot (Figure 6.5C) showed a symmetrical distribution of the differences between the predicted and true HT around a bias of −0.015. This slight negative bias (−0.015; 95% CI = [−0.018, −0.012]) could also be discerned in the Bland-Altman plot (Figure 6.5B). Notably, this bias was less pronounced than the one that was observed with the spectrum-based HT prediction method (−0.029, 95% CI [−0.032, −0.026]). The difference in bias between both methods is statistically significant because their 95% CIs do not overlap. The LoAs that encompass 95% of the data were −0.061 (95% CI = [−0.065, −0.056]) and 0.031 (95% CI = [0.027, 0.036]). The span between these LoA is similar to that observed with the spectrum-based method (−0.076 and 0.018). Using the simplified single wavelength-based data processing approach, only 4.72% (respectively 1.72%) of the predicted HT values deviated more than 15% (respectively 20%) from the corresponding true HT values, whereas for the spectrum-based method this was 15.9% (respectively 3.9%).

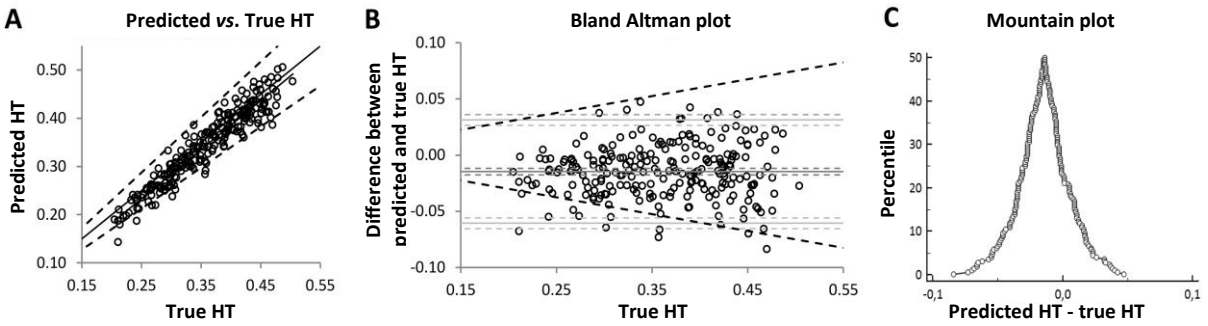


Figure 6.5. (A) Predicted HT plotted against the true patient HT and corresponding regression line. Also included are the line of equality (solid black line) and the 15% acceptance criteria (dotted black lines). (B) Bland-Altman plot of predicted and true HT. Depicted are the mean difference with CI (dark gray lines), and the upper and lower LoAs with CI (light gray lines), all with their respective 95% CIs. Also included are the 15% acceptance criteria (black dotted lines). (C) Mountain plot depicting the distribution of the differences between the predicted and true HT.

It was observed that the predicted HT was influenced by the patients' MCHC level (Figure 6.6, panels A and B). This MCHC level is defined as the ratio of a patient's Hb level and HT value. The lower this ratio is, the less Hb is present per HT unit and the higher the reflectance at 589 nm will be. This, in turn, results in a lower, Hb-based, predicted HT value and an underestimation of the true HT value. As the MCHC value may impact Hb-based HT prediction, the MCHC value of the calibrators should ideally correspond to the median of the target population to minimize method bias. This also implies that to reduce variability between calibration curves and to ensure reproducibility in obtained patient results, calibrators and QCs should always be prepared with blood of the same MCHC value. Although this is theoretically achievable using, for example, pooled blood, this is inconvenient and tedious. Therefore, it may be of added value to utilize artificial calibrators and QC materials instead. One option could be to use a series of colored materials, with each material containing a different amount of a given chromophore and thereby having a different reflectance intensity at 589 nm. These chromophore amounts would have to be carefully selected to ensure that the reflectance intensities at 589 nm correspond to the reflectance intensities of DBS-based calibrators and QCs with different HT values and of average MCHC. Moreover, the use of such artificial calibrator and QC materials would alleviate the need of preparing calibrators and QCs of different HT on a regular basis, thereby facilitating routine implementation of this HT prediction method. For other patient variables (i.e., HI, LI and II), no trend could be observed with the predicted HT variables (Figure 6.6, panels C–H). The ranges of the different serum indices measured in the patient samples were: HI: 0 – 185 (corresponding to 0 – 185 mg/dL or 0 – 114.7 µmol/L Hb, II: 0 – 6 (corresponding to 0 – 6 mg/dL or 0 - 102.6 µmol/L bilirubin, and LI: 2 – 71 (correspondng to 2 – 71 mg/dL intralipid).

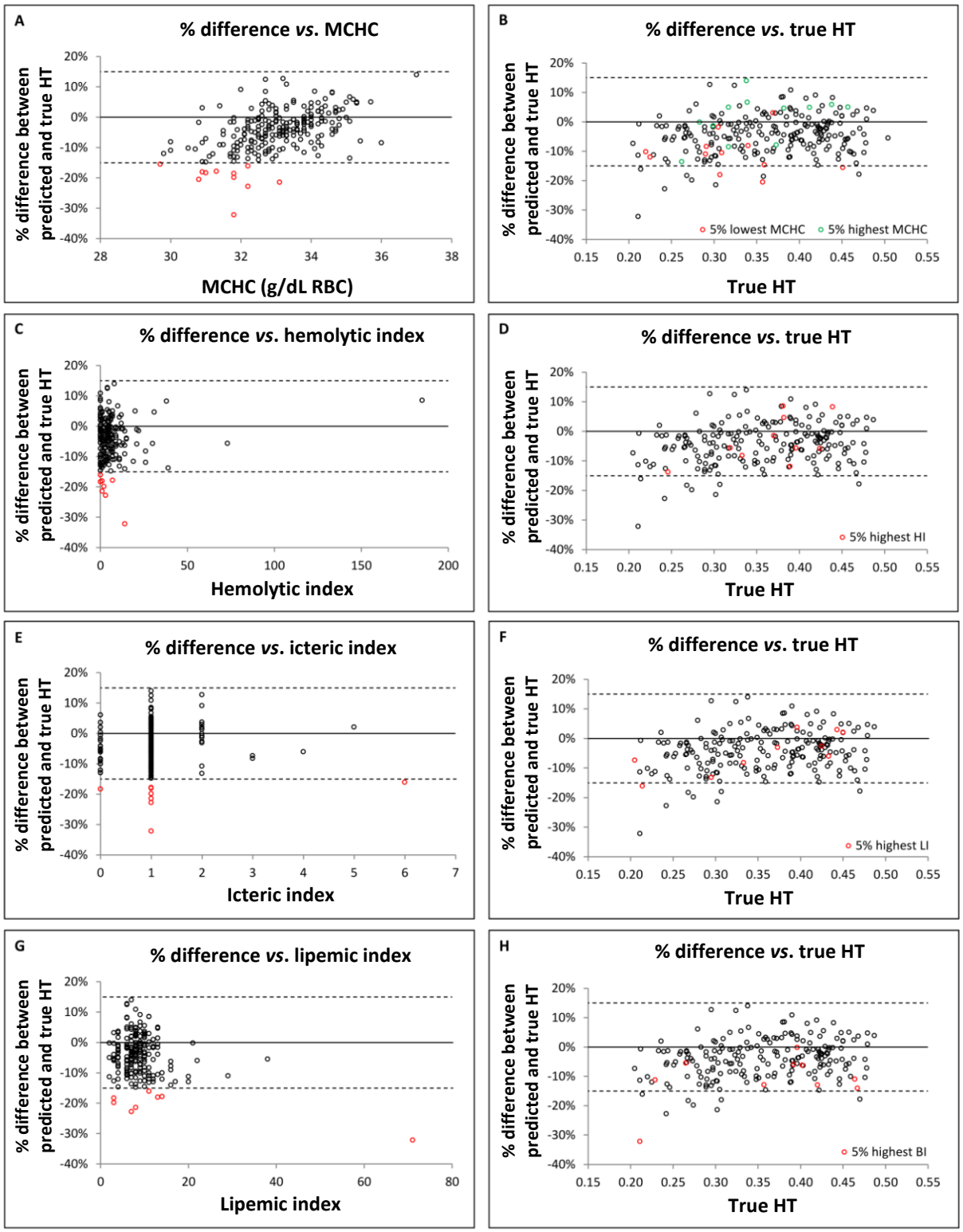


Figure 6.6: The influence of MCHC, HI, LI and II on the predicted HT. The MCHC value was available for all 233 patient samples, whereas the different indices were only available for 206 patient samples.

ISR of all 233 DBS samples of the second set of patient samples demonstrated excellent method reproducibility. After the removal of one outlier (51.8%; confirmed with a Grubbs test $p < 0.05$), the difference between the reanalysis result and the original result, divided by the mean of both analyses and multiplied by 100, was within 11% for all samples, thereby

easily fulfilling the acceptance criterion (i.e., two-thirds of the % differences should lie within ± 20%[42]) (Figure 6.7). The average and median % difference were, respectively, 1.99% and 2.04%, with 97% of the samples having a % difference of less than ± 5%. Moreover, the ISR results of the single wavelength-based HT prediction method outperformed those of the spectrum-based method, as for the latter 5% of the samples showed a difference of ≥ 8%.

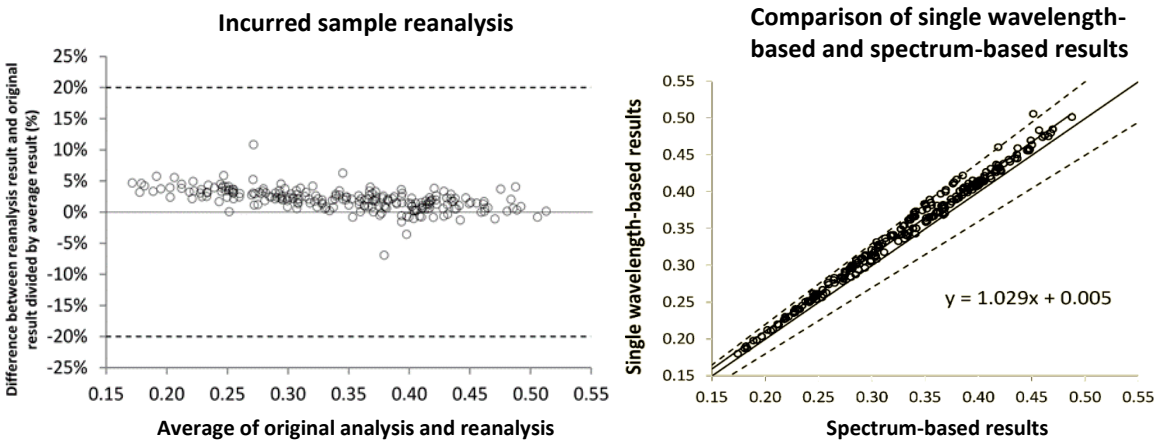


Figure 6.7: Plotted are the percentage differences between the reanalysis and the average results *versus* the average of the original and the reanalysis result (n = 233). The 20% acceptance criteria are depicted by a dotted line. Figure 6.8:. Comparison between the predicted HT obtained with the single-wavelength and the spectrum-based approach. Depicted by the dotted lines are the 10% limits. The longest solid line represents the line of equality.

6.3.5. Comparison between the original and the simplified hematocrit prediction method

A good correlation (r = 0.99) could be observed between the predicted HT values obtained on the basis of the reflectance at the quasi-isosbestic point of 589 nm (i.e., the single wavelength-based approach) and the predicted HT values obtained using the original spectrum-based approach (Figure 6.8). Moreover, with a single exception of 11.97%, all single wavelength-based results were within 10% of the spectrum-based results, demonstrating yet again that both methods yield similar results.

6.3.6. Hematocrit effect correction

Of all 288 patient sample sets, 231 had a caffeine concentration above the LLOQ in both DBS and whole blood samples. Of these caffeine-positive sample sets, 50 were selected to set up a reference set, and 181 were included in the test set. From each HT category (see Figure 6.9) a maximum of 8 samples was randomly selected for the reference set. Generally, no more than half of the samples from a category were selected. The only exception to this is the highest HT category, which only contained one sample. This sample was incorporated into the reference set as well. The HT distribution in the entire sample set, the reference set, and the test set is depicted in Figure 6.9.

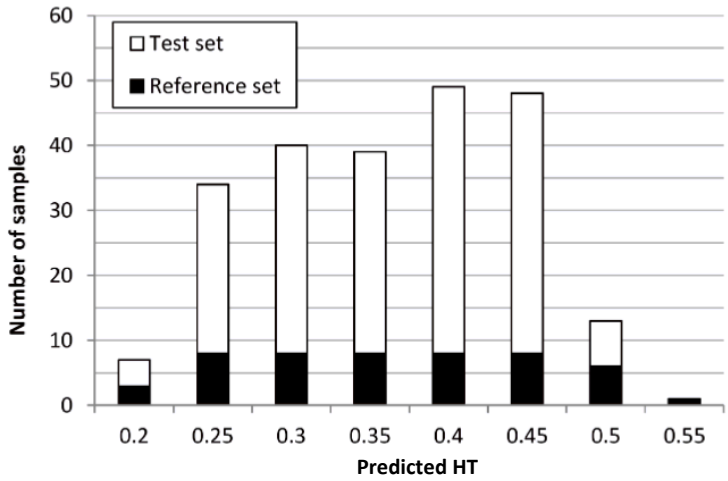


Figure 6.9: Depicted on the left is the HT distribution of the samples in the reference set and the test set. On the x- axis the upper limit of each HT category is displayed and on the y-axis the number of samples included in each of these HT categories. In total, 50 samples were incorporated in the reference set and 181 in the test set.

For the reference set, the linear regression line, which fitted the whole blood/DBS caffeine concentration ratios plotted against the predicted HT, had a slope of −1.196 and an intercept of 1.627 (Figure 6.10). The corresponding 95% CIs for slope and intercept were [-1.499, -0.893] and [1.522, 1.732], respectively. On the basis of this regression line (i.e. $[CAF]_{whole\ blood}/[CAF]_{DBS}$ = (-1.196 * predicted HT) + 1.627), the following correction algorithm could be derived: corrected DBS concentration = original DBS concentration × ((−1.196 × predicted HT) + 1.627).

When this correction algorithm was applied to the reference set, the HT bias was alleviated (Figure 6.11). Before implementation of the HT correction algorithm, the % difference between the DBS and the whole blood caffeine concentrations ranged from -35.5 to 8.4%. Only 40% of the samples had a % difference ≤ 15% and 62% of the samples had a difference

≤ 20%. The linear regression curve which best described the relationship between the % difference and the predicted HT (according to the least squares fitting method), had a slope of 0.790 (95% CI: [0.587, 0.992]) and an intercept of -0.437 (95% CI: [-0.508, -0.367]). The squared Pearson correlation coefficient was 0.562. After implementation of the correction algorithm, the % difference ranged from -14.1 to 22.7% with 96% of the samples having a % difference within 15% and all samples (with a single exception of 22.7%) having a % difference within 20%. Furthermore, the slope of the regression line was -0.013 (95% CI: [- 0.261, 0.236]) and the intercept 0.011 (95% CI: [-0.076, 0.097]), with both CIs including 0. The squared Pearson correlation coefficient was 0.000

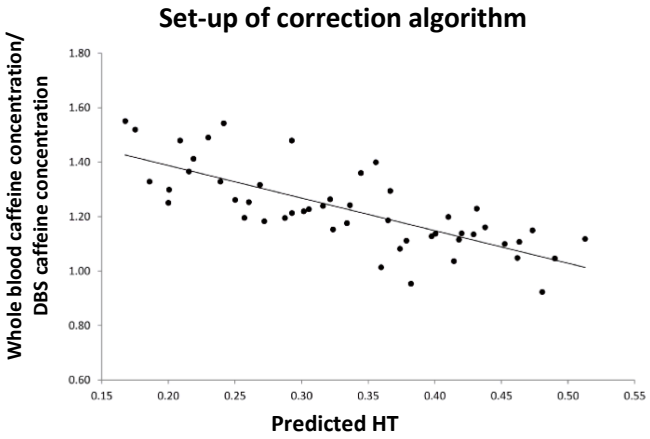


Figure 6.10: For all samples in the reference set the whole blood/DBS caffeine concentration ratios were plotted against the predicted HT values. The resulting linear regression line was used to derive the correction algorithm.

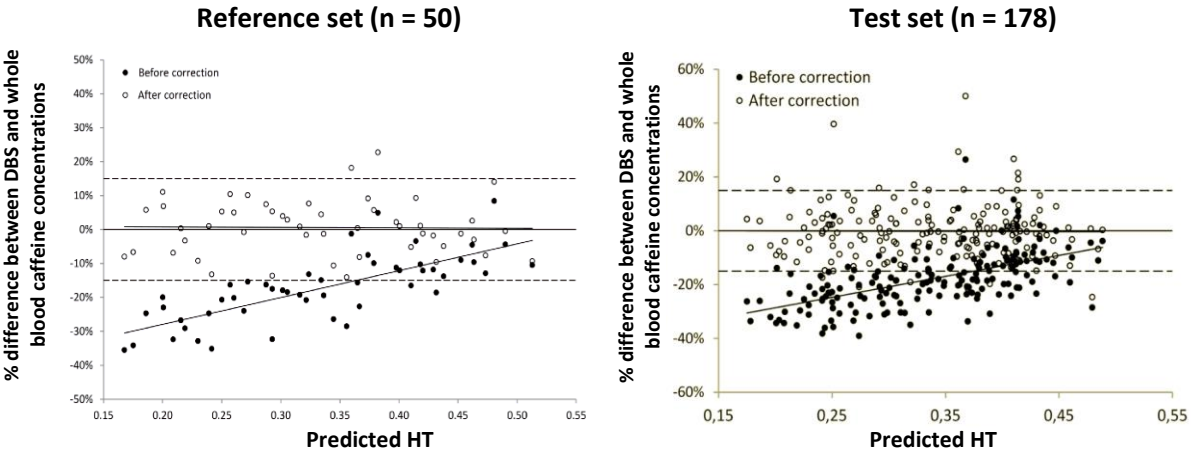


Figure 6.11: Displayed are the differences (%) between the DBS and the whole blood caffeine concentrations, determined on the reference set, both before and after HT correction. The % difference was plotted *versus* the DBS-based predicted HT. Figure 6.12: Plotted are the % difference between DBS and whole blood caffeine concentrations (n = 178), both before and after HT correction. The dotted lines depict the 15% limits, the long solid line at 0% represents 0% difference, and the other solid lines represent the regression lines of the test set before (●) and after (○) HT correction.

Subsequently, this correction algorithm was also applied to the separate test set of caffeine-positive samples (Figure 6.12). Of the 181 sample sets, 3 were statistically found to be outliers, with % deviations between whole blood and DBS caffeine concentrations exceeding 77%. As these large % differences could impossibly be explained by a HT effect, these samples were left out to evaluate the HT correction algorithm. This eventually resulted in a test set of n = 178. Before implementation of the HT correction algorithm, the 2.5% and 97.5% percentiles of the difference between the DBS and whole blood caffeine concentrations were −35.8%, respectively 5.3% (Figure 6.13). Furthermore, only 34.8% of the samples had a % difference within 15%, and only 54.5% had a % difference within 20%. The slope of the regression line fitted to the data set was 0.779 (95% CI [0.612, 0.945]), the intercept −0.442 (95% CI [-0.500, −0.385]), and the squared Pearson correlation coefficient 0.326. After implementation of the HT correction algorithm, the 2.5% and 97.5% percentiles of the difference between the DBS and whole blood caffeine concentrations were −19.8%, respectively 19.2% (Figure 13). Moreover, for 89.9% of the samples, the difference was within 15%, while for 95.5% of the samples, the difference was within 20%. Additionally, the slope of the regression line became −0.010 (95% CI [−0.212, 0.193]), the intercept 0.001 (95% CI [−0.069, 0.071]), and the squared Pearson correlation coefficient 0.000. As both the CI of the slope and the intercept included 0 after implementation of the correction algorithm, this further indicates that the correction algorithm based on the predicted HT is able to alleviate the HT bias in DBS-based caffeine quantitation.

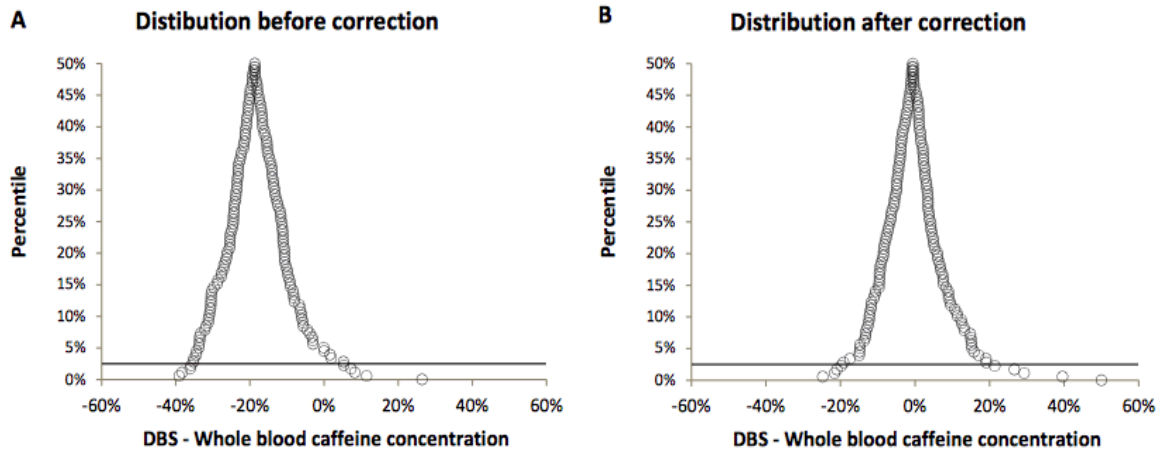


Figure 6.13: Depicted is the distribution of the % difference in DBS and whole blood caffeine concentrations before and after the implementation of the HT correction algorithm to the test set (n = 178). Both distributions are depicted as a mountain plot (with panel A depicting the distribution before HT correction and panel B the distribution after HT correction). In each mountain plot a horizontal solid black line is included which intersects the mountain plot at the 2.5% and 97.5% percentiles.

6.4 CONCLUSION

We simplified a previously developed non-contact, Hb-based HT prediction method. In this simplified method, the reflectance at the quasi-isosbestic point of 589 nm was measured as a surrogate of the total Hb content (and thereby the HT). The latter was previously determined as the sum of OxyHb, MetHb, and HC, three Hb derivatives formed upon DBS aging. Although the DBS reflectance spectra change as a function of DBS age due to these Hb-transformations, the reflectance at the quasi-isosbestic point remains approximately constant throughout time, allowing DBS age-independent HT prediction. This single wavelength-based method was thoroughly validated and fulfilled all of the preset acceptance criteria. Furthermore, application on patient samples yielded similar, but slightly better, results when compared to the original, more complicated spectrum-based HT prediction method. Moreover, the method reproducibility (based on ISR) was excellent, even exceeding that of the original method. The simplification of the HT prediction method allows more straightforward data processing as no complicated algorithms and MATLAB scripts are required to obtain the predicted HT value. In addition, it was demonstrated that the HT predicted by this simplified non-contact method can be used to implement a HT-dependent correction factor to DBS-based results to alleviate the HT bias. Implementation of such a method in routine DBS analysis, either as a stand-alone approach or potentially integrated in an automated DBS analyzer, could help to improve the quality of DBS-based quantitation in a straightforward manner, while preserving the entire DBS sample. However, to consolidate general applicability of this approach, further performance evaluation on a wider range of analytes is needed.

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Chapter 7

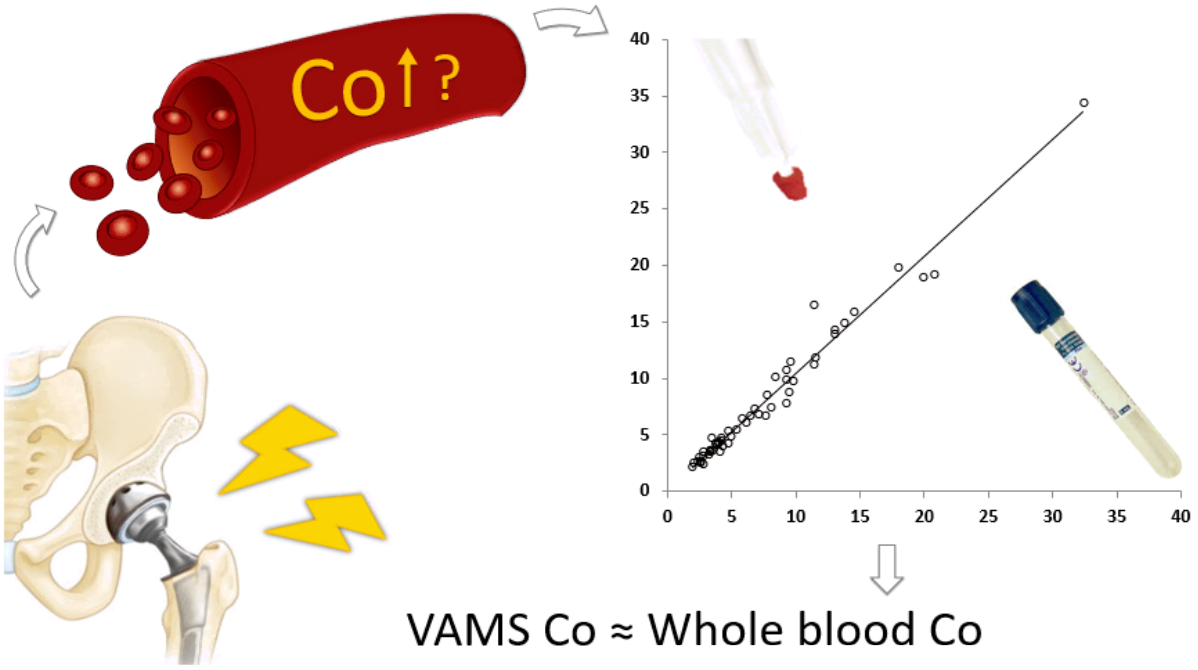
The determination of cobalt in VAMS

Based on:

Capiau S, Bolea-Fernandez E, Balcaen L, Van Der Straeten C, Verstraete AG, Vanhaecke F, Stove CP. Development, validation and application of an inductively coupled plasma – mass spectrometry method to determine cobalt in metal-on-metal prosthesis patients using volumetric absorptive microsampling. *Talanta*, 2019; *In Press*.

Abstract

MoM prostheses, in which the bearing surfaces are made of a metal alloy, may release metal ions upon wear and corrosion, potentially inducing both local and systemic toxicity. As the systemic Co concentration increases with the degree of implant wear, this concentration needs to be monitored as a means of assessing implant function and the risk of adverse effects. Here, we report on the development, validation and application of a method to quantitatively assess these Co concentrations in whole blood, based on the combination of VAMS and ICP-MS. This method could allow patients to collect the required samples at home, as VAMS samples are easy to collect and can be transported to the laboratory *via* mail. The extraction procedure utilized an alkaline extraction mixture with Y as IS and proved to be independent of the HT and age of the VAMS samples. The Co concentrations in the VAMS extracts were measured using quadrupole-based ICP-MS. The analytical method covers a range of 2 – 300 µg/L and displays excellent accuracy (bias ≤4%) and imprecision (RSD ≤5% and ≤15% at LLOQ). The method was applied to venous VAMS samples of MoM prosthesis patients (n = 78), yielding promising results. The comparison of these results with those obtained on the corresponding liquid whole blood samples, showed a correlation coefficient of 0.99 and 87% of the data fulfilled the criteria proposed by the RCPA.



7.1. INTRODUCTION

Approximately 1.5 million patients worldwide have undergone total hip replacement or hip resurfacing with metal-on-metal (MoM) hip prostheses[1]. These prostheses, in which the bearing surfaces are made of a metal alloy, may release excess metal ions upon wear and corrosion. This can potentially cause both adverse local tissue reactions and systemic toxicity, including neurological, cardiovascular and endocrine toxicity[2].Although the use of MoM has drastically decreased over the last few years[3], specialist centers continue to advise MoM hip resurfacing in young and active male patients, if performed by an experienced orthopedic surgeon[4-7]. Furthermore, at least 80% of the previously used MoM implants are still *in situ* and the prevalence of MoM revision surgery due to adverse reactions is increasing[8]. Moreover, new recommendations advocate more intensive follow-up of MoM prosthesis patients, with annual investigations during the entire life time of the prosthesis, as it can take a long time for metallosis to occur and it is impossible to predict which patients will be at risk for adverse events at which time after prosthesis implantation[9,10]. In addition, with other types of MoM prostheses, such as knee and trapeziometacarpal prostheses, increased metal ion concentrations have been observed as well[11-14].

As the systemic Co concentration increases with the degree of implant wear, blood Co concentrations are monitored as a means of assessing implant function, the risk of adverse effects and the need for implant revision[2,15-17]. Although the concentrations of other metal ions (e.g. Cr) also increase with metal wear, experts state it is sufficient to only test for one metal, preferably Co, and preferably in whole blood[5,17-18]. Patient assessment should be done based on a multi-parametric evaluation model including metal ion concentrations, clinical symptoms and imaging results, as normal whole blood Co concentrations do not exclude the occurrence of adverse local events (e.g. due to metal hypersensitivity)[16,19]. Currently, there is no consensus on the Co concentration that can be regarded as a safe upper limit for local adverse events and thresholds ranging between 2 and 7 µg/L have been suggested[1,15,16]. Systemic adverse effects have been reported to occur at a median Co concentration of 35 (14–288) µg/L [20].

Since patients' Co concentrations are measured regularly, it is worthwhile to evaluate whether this can be done using dried blood samples, such as DBS or using samples obtained *via* VAMS.

Rather than using conventional filter paper to which a drop of blood is applied, VAMS makes use of an absorbent tip wicking up a fixed volume of blood[21-23]. Such dried blood samples can be easily obtained at home by the patient himself; this procedure only requires a simple finger prick and the samples can be sent by mail to the laboratory for subsequent analysis. That way, patients do not need to visit a phlebotomist and the results can readily be available when they visit the orthopedic surgeon for a check-up. Additionally, as adequate measurement of such Co concentrations requires specialized instrumentation, the use of dried blood samples could also facilitate sample transport to laboratories thus equipped. Several toxic metals, such as Pb and Hg, have already been determined in dried blood samples, although mainly using DBS[24-33]. However, also other elements, including Al, As, Ba, Be, Bi, Ca, Cd, Co, Cs, Cu, Fe, I, K, Li, Mg, Mn, Mo, Na, Ni, P, Rb, S, Sb, Se, Sr, Ti, Tl, V, Zn and Zr , have been determined in DBS and other dried matrices[25,34-39]. Different techniques have been used for this purpose, including ISE[40], AAS[41], graphite furnace atomic absorption spectroscopy (GFAAS)[42,43], ICP-MS[44], and laser ablation ICP-MS (LA-ICP-MS)[35,36,45].

Previous methods for the quantification of Co in dried blood samples mainly focused on DBS. In addition, the equipment used in previous works is often not available in routine laboratories and/or sample volumes were relatively high, and/or methods were deemed unsuitable for toxicological screening due to the combined effect of background contamination and sample dilution[34,37,44-46].

Since VAMS has the advantage over conventional DBS sampling that it i) samples a fixed volume of whole blood and is hence less affected by the HT effect, and ii) facilitates home sampling[47], VAMS samples represent an interesting matrix for (ultra-)trace element determination in the context of metal monitoring for clinical purposes. However, there are only few reports on the use of VAMS for elemental analysis[37,38,48]. We previously reported on the potential of VAMS for determining a variety of trace elements, including Co, using tandem ICP-MS (ICP-MS/MS) and chemical resolution to overcome spectral overlap[37]. However, in that study, only reference materials (RM) and spiked samples were analyzed, and no clinical validation study was undertaken. Moreover, ICP-MS/MS technology is still not easily accessible for routine clinical laboratories.

The goal of this work was the development of a simplified sample preparation procedure and analytical method for the follow-up of Co concentrations in patients with MoM prostheses, focusing on the needs and capabilities of most clinical laboratories equipped with ICP-MS analysis. Special attention has been devoted to both analytical and clinical validation, aiming at bridging the gap between future potential and present-day applicability of this technique in clinical routine.

7.2. MATERIALS AND METHODS

7.2.1. Chemicals and materials

1 g/L standard solutions of Co, Rh, Y and Ga were obtained from Instrument Solutions (The Netherlands). Ultrapure water (resistivity > 18.2 MΩ cm) was generated with a Milli-Q system (Millipore, France). Depending on the VAMS extraction procedure, pro-analysis 12M HCl (Chemlab, Belgium), further purified by in house subboiling distillation, Triton X-100 Ultra grade (Sigma-Aldrich, Belgium), NH_4OH solution 32%, 1-butanol EMPROVE® ESSENTIAL NF and $Na_2EDTA\ 2H_2O$ Titriplex® III for analysis (all from Merck, NJ, USA) were used. The extraction was carried out in 15 mL metal-free conical tubes (VWR, Belgium). The sample preparation was performed using a vortex (VWR), a Comfort thermomixer and/or a centrifuge (Eppendorf, Hamburg, Germany). Acid digestion was accomplished in Teflon® beakers (Savillex, MN, USA) using pro-analysis 14M HNO_3 (ChemLab), further purified by in house subboiling distillation, and 9.8M H_2O_2 (Fluka, Belgium). Se ronorm™ Trace Elements Whole blood level 3 (Sero, Norway) was used for quality assessment.

7.2.2. VAMS generation

For method development and validation, blank blood from healthy volunteers (B670201627300 EC UZG 2016/0119) was collected in K_2EDTA sample collection tubes for trace element analysis (BD Vacutainer™, USA). The HT of the blood was determined using a Sysmex XE-5000 (Sysmex Corporation, Kobe, Japan) and adjusted to the required HT level, if necessary, by adding or removing a suitable amount of plasma. For the majority of the experiments, the HT of calibrators and QCs was approximately 0.40, whereas QCs with a HT of 0.20 and 0.60 were used for certain experiments as well. Aliquots of the “blank blood” were spiked with an appropriate amount of Co stock solution to prepare calibrators with Co concentrations of 2, 3, 4, 10, 20, 30 and 40 µg/L, and QCs with Co concentrations of 2, 4, 20,

30 and 300 µg/L. The intermediate solutions of Co with concentrations of 0.1 and 1 mg/L, respectively, were prepared by diluting the standard solution with Milli-Q water. The spiked volume was never more than 5% of the total sample volume. Samples were homogenized by brief vortexing (2s) and gently inverting the samples 10 times. A fixed volume of approximately 10 µL of whole blood was wicked up using a VAMS device (Phenomenex, CA, USA), with the exact volume depending on the lot of VAMS devices which was used. Careful attention was paid to only touch the blood samples with the outer part of the absorbent VAMS tip, to avoid overfilling of the VAMS device. The VAMS samples were left out to dry for at least 2h under ambient conditions and were either processed immediately after drying or stored in zip locked bags with desiccant until analysis (Minipax, Sigma-Aldrich). Samples stored at 60°C could not be stored in zip locked bags and were positioned upright in a sample rack and covered with a pipet tip to avoid contamination during storage (see Figure 7.1). This pipet tip did not touch the VAMS absorbent tip.

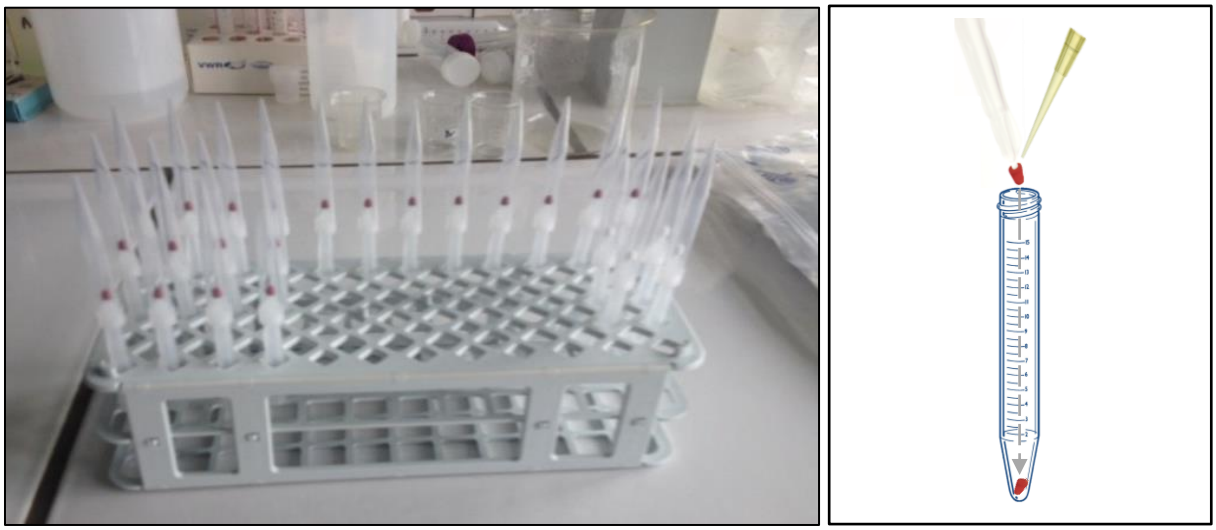


Figure 7.1: Set-up used to store VAMS samples in the oven (left panel) and schematic representation of the first step in the sample preparation.

7.2.3. Sample preparation

The first step of the sample preparation consisted of removing the absorbent VAMS tip manually from the plastic holder using a 200 µL pipet tip to avoid contamination (see Figure 7.1). The VAMS tip was directly collected into a 15 mL metal free plastic tube. Different extraction approaches were evaluated in this work to identify the optimal procedure, which allows a high and robust recovery, independent of VAMS age, HT level and Co concentration. The latter are three variables which are known to affect analyte recovery in VAMS analysis. It

is now well-established that, when insufficient attention is paid to developing a robust extraction procedure, VAMS samples prepared from blood with a high HT and/or samples which have been stored for a while tend to display a lower extraction efficiency, when compared to other VAMS samples[23,49,50].

The first sample extraction procedure (extraction 1) was based on the work of Bolea-Fernandez *et al*.[37] and consisted of extracting the VAMS with 1960 µL of Mili-Q water using a vortex mixer, whereafter 20 µL of 12M HCl and 20 µL of IS solution (i.e. 10 µg/L of ^{103}Rh) were added. This mixture was then centrifuged at 3000g for 3 min. To semi-automate this procedure, a thermomixer was used instead of a vortex to facilitate the extraction. In contrast to the previous procedure, in this procedure the IS was added to the extraction solvent, rather than at the end. Furthermore, the 12M HCl was only added after transfer of the VAMS extract to a second tube. This resulted in the following extraction procedure: VAMS tips were extracted with 1990 µL of Milli-Q water (containing IS), 1900 µL of the VAMS extract was transferred to a second tube and 20 µL of 12M HCl was added (extraction 2). In this procedure, analyte extraction was further optimized by comparing the results obtained at different extraction temperatures (21°C and 60°C) and after various extraction times (5, 15 and 30 min). All extraction procedures were performed at the maximum mixing speed of 1000 rpm. The best extraction procedure was selected based on the highest and most reproducible Co/Rh ratio. These experiments were conducted using VAMS samples prepared from venous whole blood with a high HT level (approximately 0.60) and a Co concentration of 4 µg/L, which were stored overnight in an oven at 60°C to simulate sample ageing, as this type of samples is known to display the most extraction-related issues[23,49].

In addition, we attempted to obtain cleaner extracts (i.e. lower turbidity as a result of partial removal of the heavy matrix) in order to facilitate the ICP-MS measurement and to increase the sample throughput. For this purpose, an acidic extraction procedure (extraction 3) and an aqueous extraction followed by protein precipitation (extraction 4) were evaluated as well. Briefly, the acidic extraction procedure (extraction 3) consisted of extraction of a VAMS sample with 450 µL of 0.6M HCl and a 1:4 dilution of the extract with Milli-Q water. The aqueous extraction procedure (extraction 4) consisted of VAMS extraction with 300 µL of Milli-Q water, the addition of 40 µL of HCl to 250 µL of the obtained extract and centrifugation at 3000g for 3 min. After protein precipitation, 270 µL of the supernatant was diluted with 1400

µL of Milli-Q water. To evaluate the robustness of these extraction procedures, we verified whether they were independent of VAMS sample age and HT level. In addition, the results were compared with i) those obtained after several other extraction procedures; ii) those obtained after traditional sampling; and iii) those of acid digestion. In addition, the suitability of Rh as an IS was assessed and compared to that of other IS, such as Ga and Y. For the acid digestion, the procedure was as follows: 100 µL of whole blood or 100 µL of a Co stock solution were added to a pre-cleaned Teflon® beaker. The stock solutions contained 0, 50, 75, 100 or 125 µg/L of Co, respectively. Next, 1 mL of HNO_3 and 250 µL of H_2O_2 were added to each beaker and all samples were left on the hot plate at 110°C until digestion was complete. For the whole blood samples the lids of the Teflon beakers were a little bit loosened, to allow the generated fumes to escape. After digestion was complete, 100 µL of the digest was diluted with 1900 µL of Milli-Q water containing a mixture of IS (i.e. Ga, Rh and Y at a final concentration of approximately 0.2 µg/L for Ga and 0.1 µg/L for Rh and Y).

Aside from the different acidic extraction procedures described above, an alkaline extraction procedure (extraction 5) was also assessed. Analyte extraction was accomplished by addition of 1990 µL of a mixture containing 3mM EDTA, 7.5% v/v butanol, 0.14 mM NH_4OH, 1% w/v Triton X-100 and 0.1 µg/L Y in Milli-Q water. The samples were shaken for 15 min at 1000 rpm and at 60°C using a thermomixer. Subsequently, 1.5 mL of the VAMS sample extracts was transferred to a second tube prior to analysis. The results obtained with this extraction procedure were compared with those obtained *via* whole blood acidic digestion, as well as with the routinely used method at Ghent University Hospital. The latter consists of diluting 400 µL of whole blood with 5.6 mL of an alkaline extraction mixture (containing $Na_2EDTA.2H_2O$ (3mM), 1-butanol (7.5% V/V), NH_4OH 25%, Triton X-100 (1.0% w/V), Nb (1000 µg/L), Tm (1000 µg/L), Sc (1000 µg/L), Y (1000 µg/L) and H_2O) followed by ICP-MS analysis. In addition, to assess whether the alkaline extraction procedure yielded results which were independent of VAMS sample age and HT level across the envisaged measurement range, samples (n = 6) were generated at three HT levels (approximately 0.20, 0.40 and 0.60) and at two concentration levels (4 and 30 µg/L). Half of the samples (n = 3) were analyzed immediately after sample drying, whereas the other half of the samples (n = 3) were analyzed after storage at 60°C for 2 days, the latter yielding very dry, brownish samples and reflecting 'accelerated ageing'. This short evaluation, performed before the actual method validation, is referred to as 'stress

testing', as it evaluates how the method performs with samples subjected to extremes of certain variables (i.e. HT and 'sample age'), which are known to potentially affect the extraction efficiency.

It needs to be pointed out that a slight Co contamination (increased background level) was found when using 1 mL pipet tips compared to 200 µL ones (both from VWR). Although this issue could be resolved by pre-cleaning the 1 mL pipet tips (see Figure 7.2), for practical reasons, it was opted to only use 200 µL pipet tips instead.

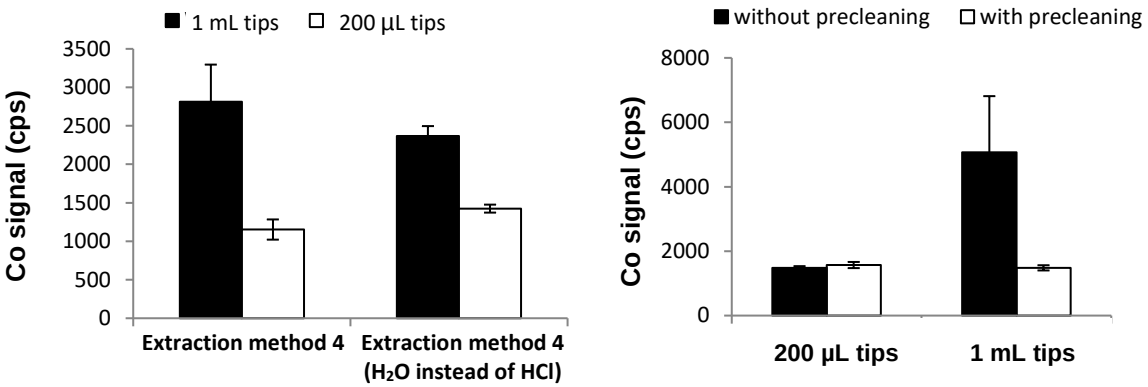


Figure 7.2: The effect of using different pipet tips on the Co background signal. Whenever an extraction of a blank VAMS sample was performed using exclusively non-1mL pipet tips, the detected Co levels were systematically lower than when 1 mL tips were used during sample preparation (see left panel). This phenomenon was both observed with extraction method 4 (left side of left panel) and with an adapted version of extraction method 4 in which HCl was replaced by water (right side of left panel). The abovementioned effect disappeared whenever the 1 mL tips were pretreated overnight with 2.5% HCl (see right panel). For the 200 µL tips this pretreatment did not reduce the background level of Co any further (n = 3).

7.2.4. Analytical method

All measurements were performed using an Agilent 7900 quadrupole-based ICP-MS unit equipped with an Agilent SPS 4 autosampler (Agilent Technologies, Japan). The instrument was operated in no gas or "vented" mode, without the requirement of any collision and/or reaction gas to avoid spectral overlap. Depending on the experiment, the nuclides ^{59}Co, ^{71}Ga, ^{89}Y and/or ^{103}Rh were monitored. The wash solutions used for cleaning between samples were adapted depending on the extract composition to avoid precipitation and blockages in the nebulizer. During the analysis of acidic sample extracts the cleaning solution consisted of 0.3M HCl, while the analysis of alkaline sample extracts required Milli-Q water instead. An overview of the ICP-MS instrument settings can be found in Table 7.1. To verify the analytical quality of the developed ICP-MS method, "blank blood" samples spiked with different Co concentrations and real patient samples were also analyzed using a sector field ICP-MS (SF-ICP-MS)

instrument (Element XR, Thermo Scientific, Germany). In addition, the results were compared to those obtained for the same samples at Ghent University Hospital, where an Elan DRC-e quadrupole-based ICP-MS instrument (Perkin Elmer, USA) was used.

Table 7.1. Overview of the instrument settings for the final method.

Parameter	Agilent 7900
Sample introduction system	MicroMist nebulizer (400 µL/min) and Peltier-cooled Scott-type spray chamber (2°C)
Nebulizer pump speed	0.30 rps
Collision/Reaction gas	---
Carrier gas flow rate	1.05 L/min
Uptake time	55s
Stabilization time	30s
Nuclides monitored	^{59}Co and ^{89}Y
Measurement time/Mass	1s
Replicates	10
Sweeps/Replicate	100
Rinse fluid	H_2O
Probe rinse time	10 s
Probe rinse time solution 1	30 s
Probe rinse time solution 2	40 s

7.2.5. Analytical validation

The validity of a linear calibration model was evaluated by generating two 7-point calibration curves and corresponding zero samples from VAMS samples, on each of three different days. The Co/Y ratio of the zero sample was subtracted from all other results to compensate for any endogenous Co present in the "blank blood". The calibration model was accepted if the back-calculated values are within 15% for at least two thirds of the data points. In order to (i) determine the LLOQ, (ii) evaluate dilution integrity and (iii) assess accuracy (% bias) and imprecision (% RSD), 5 QC samples were prepared along with every calibration curve (i.e. QCs with a Co concentration of 2, 4, 20, 30 and 300 µg/L, which are further referred to as LLOQ, LOW, MID, HIGH and DIL QC). Dilution integrity was assessed in two different ways: by diluting the VAMS extract 1:9 with either the extract of blank VAMS samples, or with the extraction solvent mixture. Carry-over/memory effect was evaluated by measuring the Co and Y level of

Milli-Q water after the analysis of the highest calibrator and comparing those results with the original background level. The extraction efficiency, i.e. the percentage of a known amount of analyte contained in the final extract after that known amount of analyte is carried through the sample extraction and processing steps of the method, was determined as suggested by Matuszewski *et al*.: by comparing results of extracts from VAMS samples generated from spiked blood with results from extracts from VAMS samples generated with "blank blood", which were spiked after extraction[51]. The influence of VAMS sample age and HT level on extraction efficiency was evaluated by comparing the extraction efficiency of fresh VAMS samples at both LOW and HIGH QC levels at three different HT levels (approximately 0.20, 0.40 and 0.60) with the extraction efficiency of corresponding samples that underwent 'accelerated ageing' by storage in an oven for 2 days at 60°C. Stability was evaluated for up to 48 days at RT and up to 48 hours at 60°C. The stability of the sample extract at RT was also evaluated by re-analyzing VAMS extracts which had been left in the autosampler overnight.

Several calibrators and samples were also analyzed using the Element XR SF-ICP-MS to ensure the analytical validity of the ICP-MS method. In addition, Seronorm™ Trace Elements Whole blood level 3 RM, was analyzed for quality assessment purposes. This RM, which is delivered as a lyophilized material, was reconstituted in Milli-Q water according to the manufacturer's instructions and was used to generate VAMS samples. Since the viscosity of this reconstituted RM is drastically different from that of actual whole blood, which may result in a different volume to be wicked up, an exact volume of 10 µL was pipetted onto a VAMS tip instead of wicking up the RM.

7.2.6. Clinical validation

Venous left-over samples collected from patients with MoM prosthesis (n = 78) were used to generate VAMS samples (two replicates per patient). Permission to do so was granted by the ethics committee of Ghent University Hospital (EC UZG 2016/0119). The venous blood samples were collected in trace-element K_2EDTA-coated blood collection tubes. As for practical reasons it was not possible to generate VAMS samples from the venous blood samples right away, the venous blood samples were stored at -20°C after routine analysis. Since the whole blood samples were hence hemolyzed before VAMS generation, it was evaluated whether the patient VAMS samples should be generated by pipetting an exact

amount (i.e. 10.6 µL) on the absorbent VAMS tip or whether patient VAMS samples could be generated by directly wicking up the hemolyzed whole blood (n = 7). As no significant differences in measured Co concentration could be observed between both VAMS generation methods, patient VAMS samples were generated by directly wicking up a patient's venous whole blood, after thawing the samples for at least 1h at RT and homogenizing the samples. These patient VAMS samples were processed and analyzed as described above. For the traditional liquid whole blood analysis, the sample preparation was essentially the same as the one used for VAMS, except starting from 10 µL of whole blood instead of from a VAMS tip.

To compare the results obtained from liquid whole blood with those from the VAMS samples, the criteria postulated by the RCPA were applied to the obtained data set. These criteria state that the differences between both methods should be ≤ 1 µg/L for Co concentrations below 10 µg/L and ≤ 10% at higher Co concentrations. Furthermore, a squared Pearson correlation coefficient was calculated. In addition, the effect of using VAMS samples instead of liquid whole blood on clinical decision making was assessed. To that end, the four categories which are used at Ghent University Hospital for Co concentration interpretation in patients with a single hip prosthesis were employed: category 1: < 4 µg/L (normal concentrations, annual follow-up suffices), category 2: 4 to 10 µg/L (additional investigations and closer follow-up required), category 3: 10 to 20 µg/L (even more thorough investigations and strict follow-up required) and category 4: > 20 µg/L (implant revision should be considered). Moreover, to assess the reproducibility of the VAMS-based method, ISR was performed. A replicate VAMS sample was measured 5 days after the initial analysis. Over two thirds of the repeated measurements should fulfill the acceptance criterion, i.e., lie within ± 20% of the original result[52].

7.2.7. Data analysis

Data analysis was performed using Microsoft Excel® 2016 and MedCalc version 18.10. For the evaluation of the different extraction conditions, a one-way ANOVA ($\alpha = 0.05$) was performed, whereas a two-sided paired-sample t-test was employed for the assessment of potential differences between VAMS samples generated by wicking up the blood and VAMS samples generated by pipetting the blood directly onto the VAMS tip. To assess whether results obtained after 48 days of storage at RT were lower than those at T_0, a one-sided paired sample

t-test was used. To compare whole blood and VAMS-based patient results, a Bland-Altman and Passing-Bablok plot were generated and a squared Pearson correlation coefficient was calculated.

7.3. RESULTS AND DISCUSSION

7.3.1. Sample preparation

Although there were no statistical differences between the different extraction (extraction 2) temperatures and times evaluated (p = 0.23), the 15 min extraction at 60°C tended to show the least variation (see Figure 7.3). In literature as well, heated VAMS extraction has proven to be advantageous[23,53]. Both the acidic extraction procedure (extraction 3) and the aqueous extraction procedure followed by protein precipitation (extraction 4) yielded much cleaner extracts than the original procedures (extraction 1 and 2). However, both procedures displayed issues. The former resulted in an age-dependent extraction efficiency for Co (see Figure 7.4), whereas the latter displayed commutability issues (see Figure 7.5).

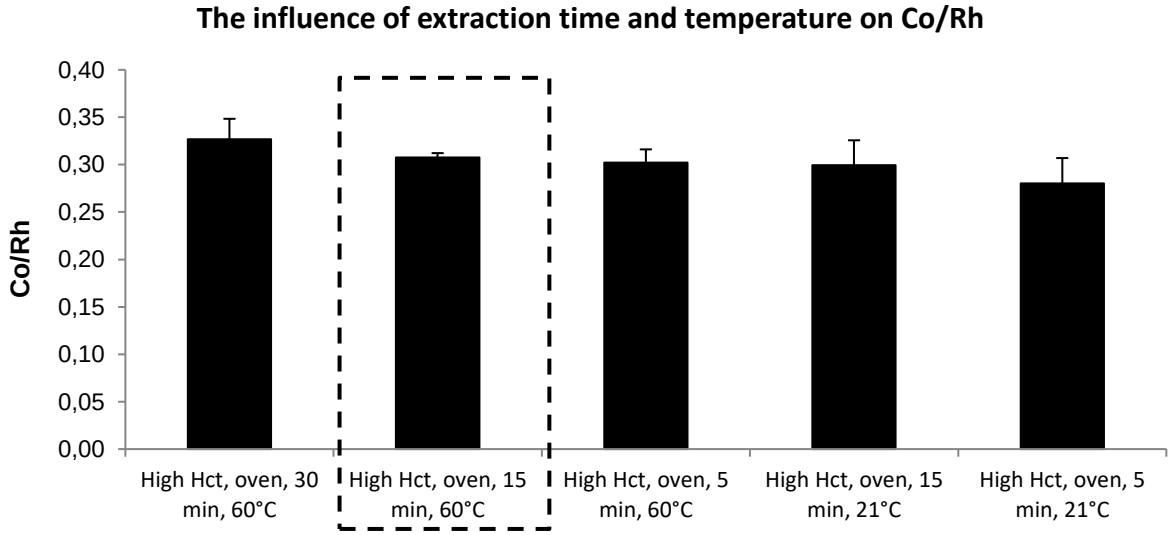


Figure 7.3: The effect of different extraction temperatures and times. A one-way ANOVA analysis showed no significant difference between the evaluated extraction conditions (p = 0.23). However, the 15 min extraction procedure at 60°C yielded the most reproducible results and was therefore selected as optimal extraction procedure (highlighted with dotted lines). Shown are mean and standard deviation (n = 3).

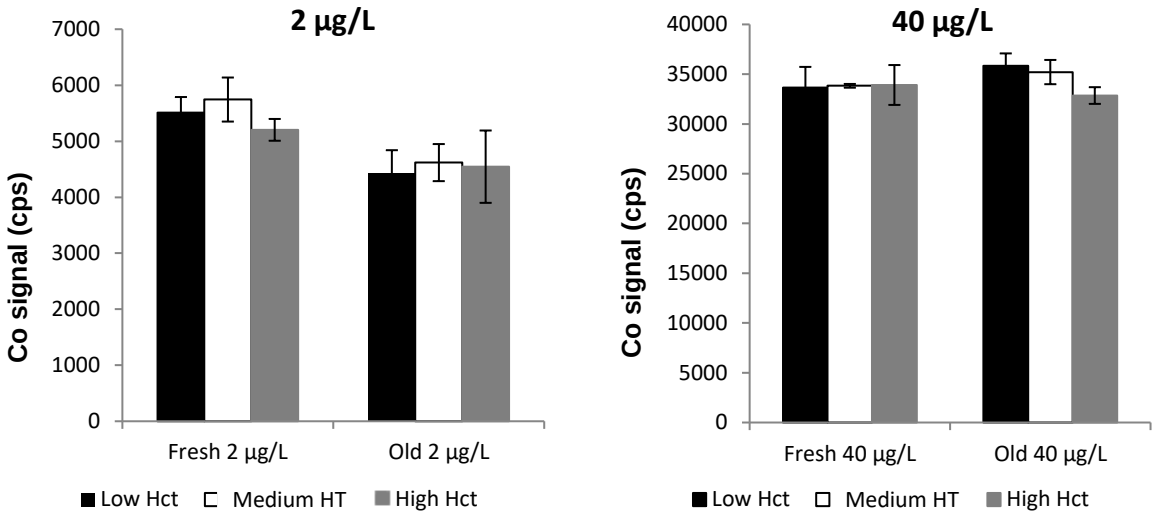


Figure 7.4: Ageing-dependent extraction efficiency with acidic extraction procedure. When VAMS samples (n = 3, at each HT level) were extracted with a 5% HCl solution, this resulted in an ageing-dependent result for Co. At a Co level of 2 µg/L, samples that were stored for 2 days at 60°C only yielded about 80% of the Co-concentration obtained for fresh VAMS samples. This phenomenon was observed at a low, medium and high HT level. For the Co level of 40 µg/L the result appeared to be HT-dependent in case of aged samples (p = 0.04), whereas no such effect was observed for fresh VAMS samples (p = 0.97).

The discrepancies observed between spiked and real samples (extraction 4) could not be attributed to the measurement procedure itself, since Co measurements with the SF-ICP-MS gave similar results as those obtained with the Agilent 7900 (see Table 7.2A). Co concentrations measured with the routine method of Ghent University Hospital using a Perkin Elmer Elan DRC-e quadrupole-based ICP-MS instrument on the other hand did yield discrepant results. For the measurements at Ghent University Hospital their in house sample preparation method was used (i.e. dilution of whole blood with an alkaline solvent mixture), whereas for the measurements with the Agilent 7900 and Element XR, the same sample preparation method (i.e. aqueous dilution of 10 µL of liquid whole blood followed by protein precipitation) was used. These results led us to conclude that the observed discrepancies were not due to the measurement procedure, but were rather due to differences in the sample preparation procedure. When whole blood extracts prepared at Ghent University Hospital were analyzed with the Elan DRC-e and the Agilent 7900, similar results were obtained with both analytical methods, confirming the previous hypothesis (see Table 7.2B). To analyze the latter samples on the Agilent 7900, these were first diluted 1:3 with Milli-Q water and Rh was added as an IS in a final concentration of 1 µg/L.

Table 7.2A: Co concentrations measured in a spiked sample and several patient samples with Agilent 7900, Element XR and Elan DRC-e.

		Co concentration (µg/L)		
		Ghent University Hospital	Agilent 7900	Element XR
A.	Spiked sample	20.0 ± 1.4	20.1 ± 0.1	20.3 ± 0.6
	Patient 1	14.5 ± 0.9	5.9 ± 0.2	6.0 ± 0.1
	Patient 2	10.6 ± 0.8	2.2 ± 0.2	< LLOQ
	Patient 3	32.7 ± 2.8	8.8 ± 0.2	9.1 ± 0.3
	Patient 4	3.1 ± 0.3	< LLOQ	< LLOQ
	Patient 5	4.1 ± 0.4	< LLOQ	< LLOQ
	Patient 6	7.5 ± 0.4	< LLOQ	< LLOQ
	Patient 7	0.5 ± 0.2	< LLOQ	< LLOQ
	Patient 8	5.0 ± 0.5	< LLOQ	< LLOQ
	Patient 9	9.1 ± 0.8	< LLOQ	< LLOQ
	Patient 10	2.0 ± 0.2	< LLOQ	< LLOQ

* At Ghent University Hospital an in house sample preparation method was used, whereas an aqueous extraction followed by protein precipitation was used in conjunction with the two other methods. All results are displayed with their respective SD. These SD values are calculated based on the replicate measurements performed with each measurement (n = 4 for Ghent University Hospital, n = 10 for Agilent 7900, n = 5 for Element XR).

Table 7.2B: Co concentrations measured in spiked samples and several patient samples with Agilent 7900 and Elan DRC-e.

		Co concentration (µg/L)		
		Ghent University Hospital	Agilent 7900	Element XR
B.	Spiked sample 1	2.4 ± 0.2	< LLOQ	n.a.
	Spiked sample 2	15.2 ± 1.3	13.2 ± 0.1	n.a.
	Patient 1	4.1 ± 0.4	2.6 ± 0.03	n.a.
	Patient 2	4.7 ± 0.5	3.1 ± 0.1	n.a.
	Patient 3	7.1 ± 0.4	5.2 ± 0.04	n.a.
	Patient 4	9.5 ± 0.9	7.6 ± 0.1	n.a.
	Patient 5	13.2 ± 0.8	11.4 ± 0.1	n.a.
	Patient 6	18.5 ± 1.4	15.8 ± 0.1	n.a.
	Patient 7	23.7 ± 2.0	20.5 ± 0.1	n.a.
	Patient 8	35.6 ± 3.0	31.7 ± 0.3	n.a.
	Patient 9	52.8 ± 3.8	46.4 ± 0.3	n.a.
	Patient 10	71.5 ± 5.0	62.5 ± 0.4	n.a.

* All samples were extracted using the in house sample preparation method of Ghent University Hospital. All results are displayed with their respective SD. These SD values are calculated based on the replicate measurements performed with each measurement (n = 4 for Ghent University Hospital, n = 10 for Agilent 7900, n = 5 for Element XR).

Therefore, this issue was considered to be related to a sample preparation issue. This was confirmed in a subsequent experiment in which different sample preparation methods were applied to both a spiked and a real sample (see Figure 7.5). As can be seen in Figure 7.5, a considerable part of the Co content was lost during protein precipitation in case of patient sample analysis, while this did not occur in case of spiked sample analysis. Indeed, for the spiked samples, results obtained using protein precipitation were comparable to those obtained after e.g. simple dilution of 10 µL of whole blood with MilliQ water. This underscores the importance of including real patient samples even at the method development stage.

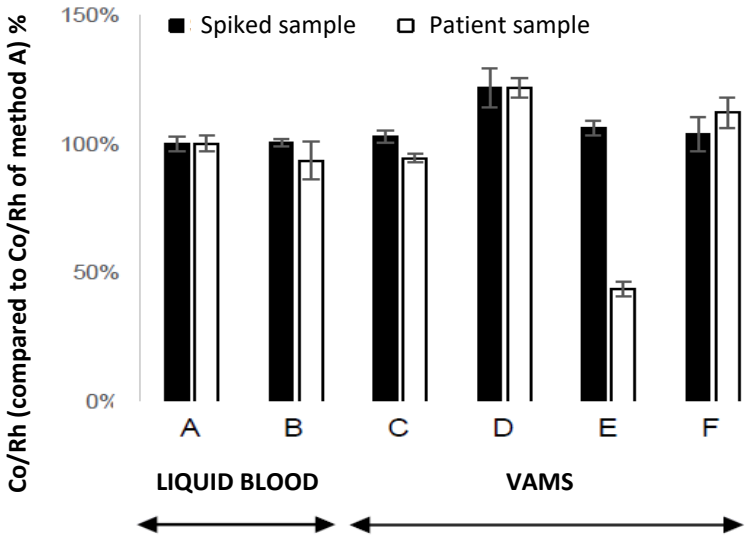


Figure 7.5: The effect of different sample preparation methods on the Co/Rh ratio for both a spiked sample (20 µg/L Co) and a real patient sample (16 µg/L). Method A = 10 µL of whole blood + 1990 µL Milli-Q water; method B = method A + 3 min centrifugation at 3000g; method C = VAMS sample extraction with 1990 µL of Milli-Q water; method D = method C with extraction at 60°C; method E: aqueous VAMS sample extraction + protein precipitation; Method F: VAMS sample extraction with 1980 µL of Milli-Q water + addition of 20 µL of HCl + 3 min centrifugation at 3000g. Depicted are the average Co/Rh with the corresponding standard deviations (n = 3). The average Co/Rh ratio obtained with method A was used as a reference.

The alkaline extraction method on the other hand did not display such commutability issues. This approach yielded similar results for both spiked and patient samples. Moreover, these results compared well to those obtained after acid digestion and the method routinely used at Ghent University Hospital (see Table 7.3). The results were quite similar between the three methods, indicating that the alkaline extraction method is fit for purpose. The results obtained *via* acid digestion, however, tended to be somewhat higher, which might be due to some evaporation occurring during the digestion on the hot plate, resulting in preconcentration of the samples.

Table 7.3: Comparison between different sample preparation methods: alkaline extraction, acid digestion and the sample preparation method routinely used at Ghent University Hospital*

	Co concentration (µg/L)		
	Ghent University Hospital	Acid digestion Agilent 7900	Alkaline dilution Agilent 7900
Patient 1	54.0 ± 0.7	65.2 ± 0.2	53.8 ± 0.4
Patient 2	74.4 ± 0.9	89.4 ± 0.2	64.3 ± 0.7
Patient 3	78.0 ± 1.5	75.8 ± 0.3	67.9 ± 0.5
Patient 4	81.2 ± 1.1	98.3 ± 0.2	83.4 ± 0.9
Patient 5	102.0 ± 1.0	119.4 ± 0.3	90.6 ± 0.7
Patient 6	125.5 ± 1.4	153.1 ± 0.3	125.3 ± 1.3

*All results are displayed with their respective SD. These SD values are calculated based on the replicate measurements performed with each measurement (n = 4 for Ghent University Hospital, n = 10 for Agilent 7900).

Furthermore, when subjected to stress testing, the alkaline sample preparation method proved to be concentration-independent, HT-independent and VAMS sample ageing-independent (see Figure 7.6). In addition, the alkaline extraction procedure was also the most straightforward one (i.e. everything is added in one step), and would hence be more convenient in routine use. Therefore, the alkaline extraction method was considered most suitable for the determination of (ultra-)trace concentration of Co using VAMS. Further analytical and clinical validations were thus carried out using this extraction method. It needs to be noted that different IS (Ga, Rh and Y) were also evaluated when using the alkaline extraction method and, in this case, Y was selected owing to the lower variability compared to Ga and Rh (data not shown).

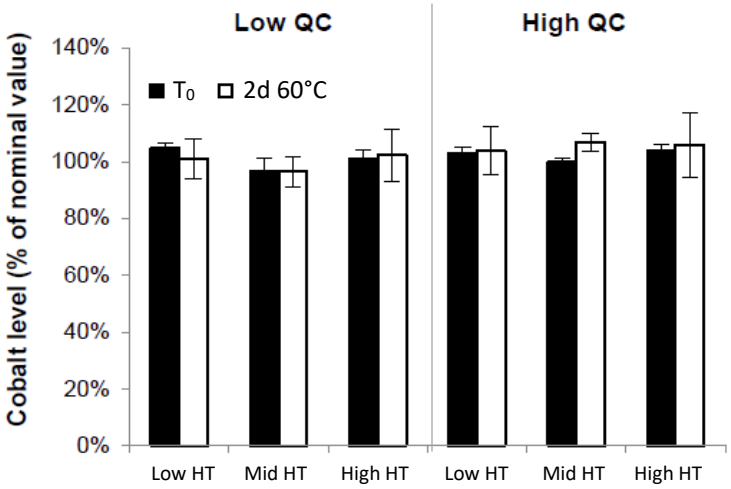


Figure 7.6: The effect of stress testing on measured Co concentrations (extraction 5). The column graph shows the average Co concentrations (expressed as the % of the nominal value). The error bars represent the standard deviations (n = 3).

7.3.2. Analytical validation

Since all back-calculated values were between -11 and 9%, the pre-set acceptance criterion was easily fulfilled and a linear unweighted calibration model could be accepted. Moreover, the differences were randomly distributed around zero at all calibrator levels, further indicating the validity of a linear calibration model (data not shown). An overview of the results for accuracy, and intra- and inter-day imprecision can be found in Table 7.4A. Since the % bias and % RSD should be within 15% at all QC levels (except at LLOQ where it should be within 20%), these data more than fulfill the acceptance criteria. The LLOQ was set at the lowest evaluated QC level (i.e. 2 µg/L), since this fulfilled the acceptance criteria for % bias and % RSD for LLOQ. The bias ranged from -1 to 1% (3% at LLOQ). Intra-day imprecision varied from 1 to 5% (7% at LLOQ) and inter-day imprecision from 3 to 5% (15% at LLOQ).

Table 7.4: Overview of accuracy (% bias), imprecision (% RSD) and dilution integrity (n = 6).

		Accuracy (% bias)	Intra-day imprecision (% RSD)	Inter-day imprecision (% RSD)
A	LLOQ (2 µg/L)	3	7	15
	LOW QC (4 µg/L)	0	5	5
	MID QC (20 µg/L)	-1	1	3
	HIGH QC (30 µg/L)	1	4	4
B	DIL QC (300 µg/L) blank extract	3	7	7
	DIL QC (300 µg/L) solvent	4	2	2

The dilution integrity experiment also yielded excellent results for both dilution strategies (see Table 7.4B). The dilution with extraction solvent yielded even slightly better results than the matrix-matched dilution, with a % bias of 4% and a % RSD of 2%. Neither for Co, nor for Y carry-over/memory effect could be observed (data not shown).

Although the HT level of the whole blood used to generate a VAMS sample should not impact the amount of blood that is wicked up, VAMS analysis may still be subject to a HT effect, more particularly at the level of extraction efficiency[23,49]. However, for the extraction method selected, no such HT effect on the extraction efficiency of Co could be observed. More specifically, the extraction efficiency proved to be high (approximately 90%) and reproducible at all HT levels and at both low and high Co concentrations (see Table 7.5).

Table 7.5: Overview of the extraction efficiency at different HT and QC levels (n = 6).

	Low HT	Mid HT	High HT
Low QC	89 ± 1%	98 ± 2%	88 ± 3%
High QC	89 ± 2%	91 ± 5%	90 ± 3%

The influence of storage at RT was acceptable up to at least 48 days, and at 60°C up to at least 2 days (see Figure 7.7). Although there was some effect discernable after 48 days, the results were not significantly lower compared to T_0 ($p \geq 0.36$) and were still within 85 – 115% of the nominal value, both at low and high QC. This effect is most likely not due to element instability -since an element such as ^{59}Co is not expected to precipitate or volatilize- but rather due to a possible effect of VAMS sample age on extraction efficiency. However, this will not pose a problem in real life, as most samples would be analyzed well within this time frame. In addition, the extract proved to be stable when stored overnight in the autosampler (data not shown).

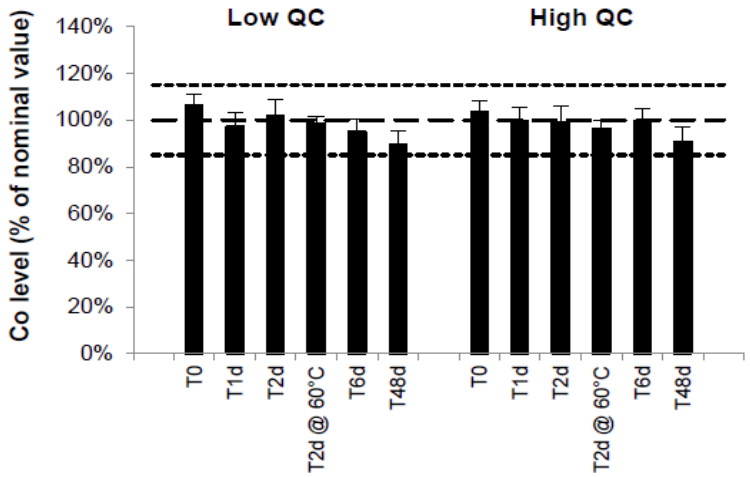


Figure 7.7: The influence of VAMS sample storage on measured Co concentrations. Shown are the mean obtained Co concentrations (expressed as the % of the nominal value) and the corresponding standard deviations (n = 6).

In addition, the analysis of the RM Seronorm™ whole blood level 3 yielded Co concentrations (9.1 and 9.2 µg/L) which were within the acceptable range (i.e. within 8.3 – 12.4 µg/L), further confirming the validity of the developed method for the quantitative determination of Co in VAMS samples.

7.3.3. Clinical validation

Since no significant differences ($p = 0.59$) could be observed between Co concentrations of extracts from VAMS samples generated by depositing a fixed volume of whole blood (i.e. 10.6 µL) onto the VAMS tip and those from VAMS samples generated by directly wicking up the

whole blood, all patient VAMS samples were generated by directly wicking up the whole blood. Of the 78 sample pairs that were analyzed, 61 samples showed a Co concentration above the method LLOQ, both in whole blood and VAMS. The measured Co concentrations ranged from 2 to 158 µg/L and were all within the validated range of 2 – 300 µg/L. When the VAMS results were compared with the corresponding whole blood results, an excellent squared Pearson correlation coefficient of 0.99 was obtained (see Figure 7.9). In the narrower actual calibration range of 2 – 40 µg/L, where most quantifiable (54/61) results lie, the squared Pearson correlation coefficient was still 0.99, (see Figure 7.8B and 7.9B). When a Bland-Altman plot was generated by plotting the absolute differences between the Co concentrations in both matrices against the average of those concentrations, an average bias of 0.39 µg/L could be observed in the range of 2 – 40 µg/L. Doing the same but plotting the % difference instead of the absolute differences, yielded an average bias of 5.3% (5.0% for the complete dataset, see Figure 7.9C). When the absolute differences were compared to the RCPA criteria, 87% of the samples (53/61) fulfilled these criteria (see Figure 7.8A and 7.9A). Passing-Bablok regression analysis revealed neither a proportional nor systematic error when taking into account the samples between 2 and 40 µg/L, since the 95% CI of the slope [1.00 – 1.10] and the intercept [-0.14 – 0.30] contained 1 and 0, respectively (See Figure 7.8B). When looking at the entire data set, Passing-Bablok regression analysis showed a slight proportional error (slope = 1.06 with 95% CI of [1.02 – 1.09]), but no systematic error (intercept = 0.09 with 95% CI of [-0.13 – 0.24]), see Figure 7.9B. In addition, ISR showed that 80% of the reanalysis results were within 20% of the original result, thereby fulfilling the pre-set acceptance criteria for ISR (see Figure 7.10).

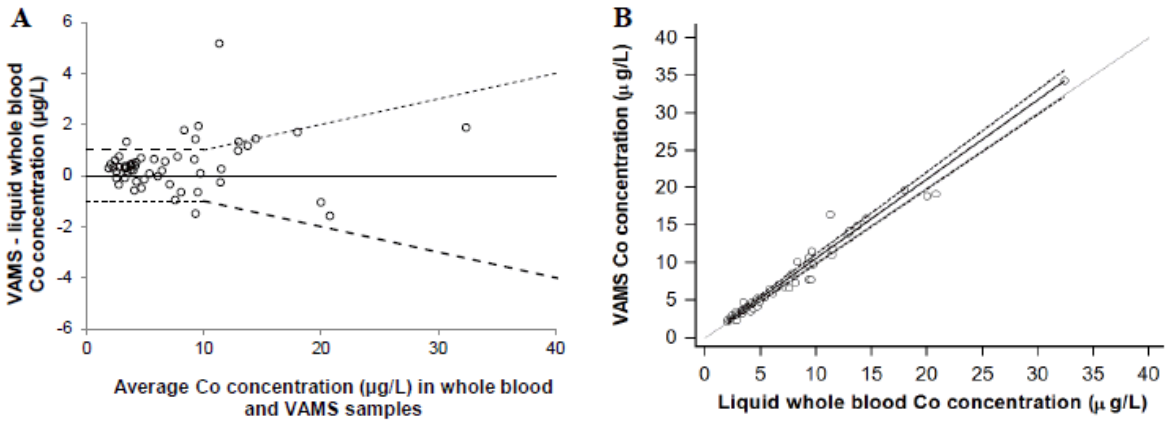


Figure 7.8. Comparison between VAMS and liquid whole blood sample Co concentrations, for the sample set with Co concentrations between 2 and 40 µg/L. In the panel A the absolute differences (i.e. [VAMS] – [whole blood]) are plotted against the average of both measurements. The differences are compared to the RCPA criteria, depicted by the dotted lines. In panel B, a Passing-Bablok plot is depicted, comparing the VAMS sample Co concentrations with the whole blood Co concentrations.

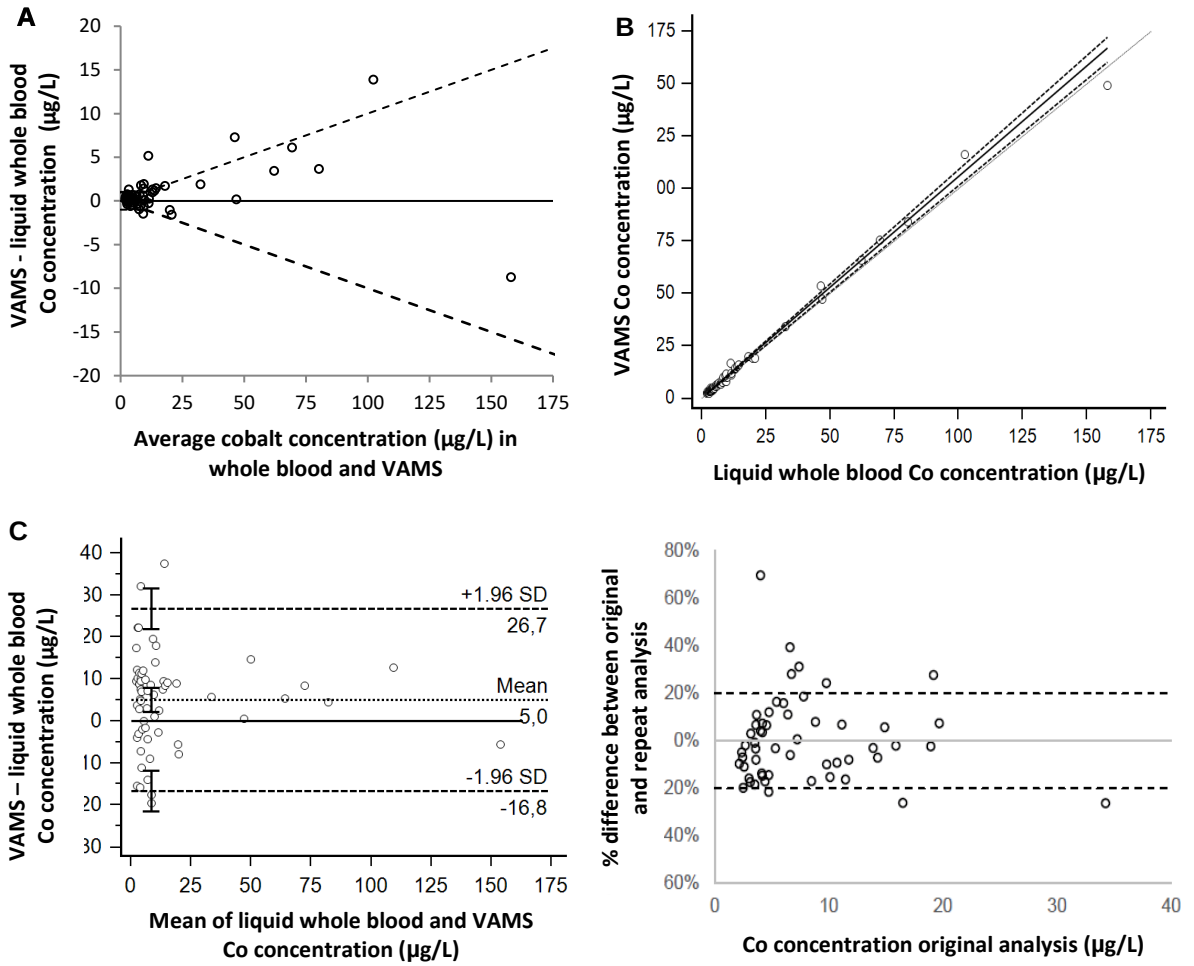


Figure 7.9: Method comparison including all measurements. Depicted are three plots that compare the Co levels determined on liquid whole blood and on VAMS samples, for all samples with measurable Co concentrations (n = 61). In panel A the absolute differences between both measurements (i.e. VAMS – whole blood) are plotted against the average of both measurements. These differences are compared to the RCPA criteria. A figure focusing on the samples below 40 µg/L can be found in figure 7.8. Panel B depicts a Passing-Bablok plot in which the VAMS Co concentrations are plotted against the whole blood Co concentrations. An excellent correlation can be observed between both matrices (r^2 = 0.99). Panel C represents a Bland-Altman plot in which the % difference between both measurements is plotted against the average of both measurements. Figure 7.10 (right bottom): ISR. The difference (%) between the original and repeat VAMS analysis result is plotted *versus* the original result (Co concentration in µg/L). For clarity, only the samples with a Co concentration lower than 40 µg/L are depicted.

Moreover, using VAMS instead of liquid whole blood proved to have only a minimal effect on the assessment of a patient's Co level, as can be observed in Figure 7.11. Inevitably, there are some samples which were classified in another category. However, this only affected samples with a Co concentration relatively close to the limits of a certain category, and for none of the samples the clinical interpretation was drastically different (i.e. a difference of two categories or more). Furthermore, this slight misclassification would not drastically impact patient management, as Co levels are always interpreted along with the results of the clinical examination, medical imaging and other laboratory tests (see Supplementary Figure S-3 for an example of a decision tree used for interpreting Co levels in MoM prosthesis patients[55]).

In addition, when taking into account the method uncertainty of the method by establishing a 95% CI around each cut-off (i.e. cut-off ± (bias +1.96*CV_a), with both the bias and analytical CV or CV_a being 5%) for none of the patient samples a truly different result could be observed between the whole blood and VAMS result.

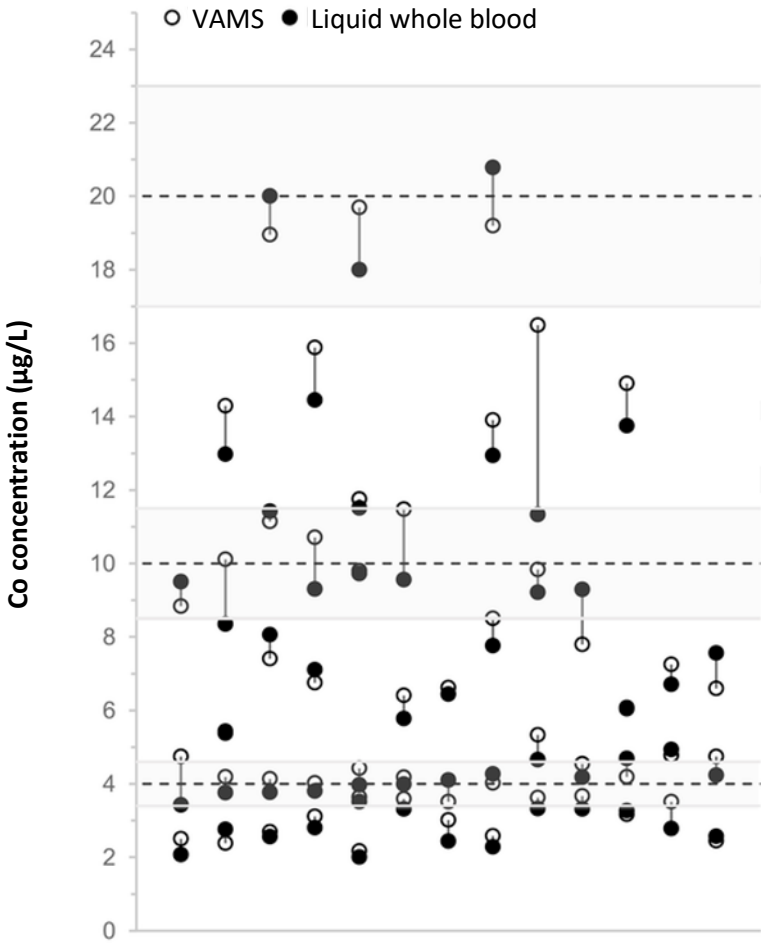


Figure 7.11. Depicted are the liquid whole blood and VAMS Co levels for each patient. Both results are connected with a line to visualize the effect the use of a different matrix had on the interpretation of the Co concentration. For clarity, the graph only represents all data points between 0 and 25 µg/L. Cut-off levels are indicated by dashed lines. The grey rectangles represent the area corresponding to cut-off ± (bias + 1.96*CV_a), with both the bias and the CV_a being 5% (cfr. analytical method validation and method comparison). The respective CIs correspond to 3.4 – 4.6 µg/L, 8.5 – 11.5 µg/L and 17.0 – 23.0 µg/L

7.4. CONCLUSION

A procedure to quantitatively determine Co in VAMS samples using ICP-MS was developed. This procedure is characterized by an easy sampling method (patients can easily collect a VAMS sample themselves at home) and an analysis method that allows for routine use in clinical laboratories, since it does not require high performance ICP-MS instrumentation. If patients could collect a VAMS sample at home and send it to the laboratory for analysis in

advance to their appointment with the orthopedic surgeon, results could already be discussed during this appointment, rather than a couple of weeks later, resulting in a more efficient patient follow-up. A robust extraction method, which was simple, yet independent of a sample's HT, Co concentration and/or VAMS age, was developed and extensively and successfully validated, with special attention to these VAMS-specific issues. Despite strict clinical acceptance criteria for Co quantitation, the application to patient samples proved promising. This study provides a solid and promising basis to proceed towards direct patient sampling, where true capillary blood samples are to be derived from a finger prick. An important aspect to evaluate in such study is whether there are any issues related to the sampling and/or related to contamination, e.g. by the lancet used to perform the finger prick.

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Broader international context, relevance and future perspectives

In this section an overview is given of other strategies that have been developed during recent years to deal with the HT effect in dried blood sample analysis. The real life applicability of these strategies is evaluated, and the specific factors that may facilitate or hinder this are outlined. Obviously, special attention was paid to the added value and future perspectives of the methods that were developed in the context of this PhD thesis. Aside from the recent trends in dealing with the HT effect, also other advances that may improve routine implementation of dried blood sample-based methods are reviewed, such as automation and the set-up of QC programs. Furthermore, an update is provided on the current status of routine DBS analysis in various settings. In addition, to demonstrate the current general interest in minimally or non-invasive sampling strategies for home sampling or self-sampling purposes, several other sampling formats that may facilitate this, are briefly discussed.

1. PROPOSED SOLUTIONS FOR THE HEMATOCRIT EFFECT

The HT effect remains a relevant issue in dried blood sample analysis[1]. It hinders more widespread implementation of dried blood sample-based methods and represents a major barrier for regulatory acceptance of DBS-based methods. Many solutions have been proposed to tackle the HT issue, with each approach having its specific advantages and limitations[1-3]. There are three main options: i) minimize the HT effect, ii) avoid the HT effect and iii) predict the HT and compensate for the anticipated HT effect.

1.1. Minimize the HT effect

This 'solution' could actually be referred to as good DBS practices. First of all, set up a calibration curve using blood with a HT close to the median HT of the target population. This way, the HT differences between the patient samples and the calibrators will be as small as possible. Furthermore, to minimize the HT effect on recovery and matrix effect, the sample preparation and analytical method, respectively, have to be optimized. The HT dependency of recovery and matrix effect should therefore be evaluated during method development and the (pre-)analytical method(s) adjusted, if necessary (see Chapter 2).

Another option to minimize the HT effect is to use substrates which are less prone to the HT effect (see Figure 1). Mengerynck *et al.*, for example, developed a substrate consisting of hyfrophilic-coated woven polyester fibers, called Qyntest, on which spot sizes are not affected by the sample's HT[4]. Another substrate for which the spreading of the blood is claimed to be independent of HT is the HemaSpot™ HF[5]. Unfortunately, data to prove this

claim have not been published yet. Despite the fact that both substrates have been developed multiple years ago, no applications have been published yet, indicating that this strategy has probably little future.

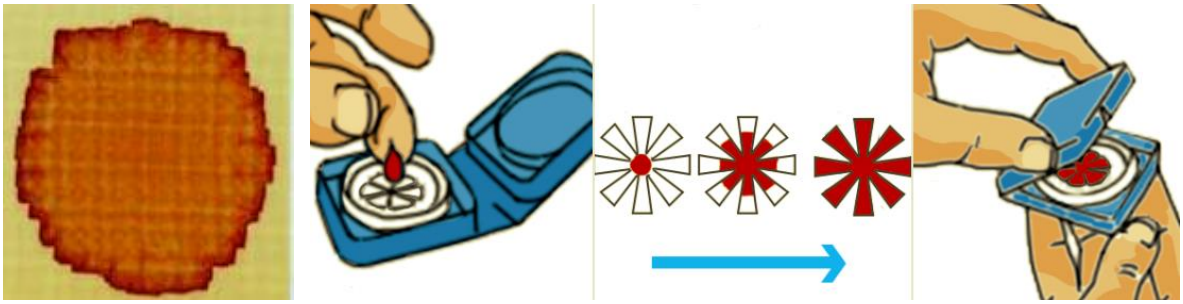

Figure 1: The Qyntest[4] (left) and the HemaSpot™ HF[5] (right) collection substrates.

1.2. Avoid the HT effect

A second solution is to avoid the HT effect. This may be achieved by using devices that can collect a volumetric dried blood sample from a non-volumetric drop of blood or by using devices that allow *in situ* DPS generation. At least in theory, both should yield quantitative results that are not influenced by a patient's HT.

1.2.1. Volumetric dried blood sample collection

Volumetric deposition of a drop of blood onto filter paper followed by whole DBS analysis alleviates the HT bias. Previously, however, this required the use of pipettes or microcapillaries, which was only feasible in a specialized setting and not at all compatible with self-sampling at home[6-8]. Over recent years several new formats have been developed that allow the generation of volumetric dried blood samples starting from a non-volumetric drop of blood (see Figure 2). These include VAMS™, HemaPEN®, Hemaxis DB, Capitainer-B, volumetric absorptive paper discs (VAPD) and VAPD-mini[9-17].

In 2014 VAMS™ devices, also called Mitra® microsamplers, were launched. These sample collection devices consist of an absorbent tip attached to a plastic handle that is able to wick up a fixed volume of blood (i.e. 10, 20 or 30 µL, depending on the type of device). Originally, only the 10 µL device was available. Recently, the larger volumes have been commercialized as well. Although the larger volumes may help to attain the desired LLOQ for certain applications, they may also render extraction efficiency optimization even more precarious.

Indeed, although VAMS alleviates the HT-bias on the analyzed sample volume, this sampling technique is sensitive to HT-and/or time-dependent extraction issues[10,18].

Contrary to VAMS™, several other devices have been developed that use a DBS format. One of them is the HemaXis DB, a book type collection device that allows to collect four volumetric DBS of 5.5 µL or 10 µL. each. Interestingly, the DBS are collected on a DBS card, rendering this sampling technique suitable for automation. A second microfluidics-based collection device is the Capitainer-B. With this collection device four spots of 13.5 µL are generated on pre-punched paper discs. Another sample collection system that uses pre-punched paper discs is the HemaPEN®. This handheld device contains four microcapillaries that each transfer 2.74 µL of blood to the pre-punched discs. Although methods with pre-punched paper discs can be semi-automated, they are not suitable for full automation.

Recently, a new sample collection approach was presented which combines the principles of DBS and VAMS™ and which is called VAPD or VAPD-mini, depending on the sample volume that is collected. These devices consist of a filter paper disc that is fixed within a filter paper sheet that contains holes that have a slightly larger diameter than the discs themselves. These paper discs should be able to collect a fixed volume of blood, independent of HT. Although further evaluations are still required, a proof of principle paper showed promising results[17].

Each of the collection devices described in this section has its own advantages and limitations. Interestingly, most of them have become commercially available (except for the VAPD and VAPD mini), indicating there is a clear need to deal with the HT effect in an elegant way. Which collection device is best suitable depends a.o. on the LLOQ, the need for automation and the price of the devices. Unfortunately, the use of each of these collection devices is more expensive than the use of classical DBS cards, which hampers their routine use. Importantly, these devices only deal with the HT effect on the volume of blood in a DBS punch. Therefore, when one of these ‘solutions’ is selected, it remains valuable to also determine the HT value of the sample, as the HT may still affect recovery, matrix effect and/or the conversion of dried blood sample-based results to the corresponding plasma or serum levels.

Figure 2: Different devices to collect volumetric dried blood samples: Capitainer, VAMS™, HemaPEN® and HemaXis DB[19-22].

1.2.2. DPS collection

The first device that was used to generate DPS *in situ*, i.e. without the need for centrifugation, was the Yorktest plasma separator[23]. An improved version of this collection system, called the autoDPS card, was designed by Stürm *et al*.[24] This card-based collection system has the advantage of being compatible with commercially available DBS analyzers. Unfortunately, results obtained with the autoDPS Card appeared to be HT-dependent, which might be due to the fact that varying volumes of plasma are generated from blood with a different HT. NoviPlex cards on the other hand are able to collect a fixed volume of plasma (i.e. 2.5 or 3.8 µL, depending on the card type) and yield adequate quantitative results which are independent of HT, as long as at least 50 µL of blood is applied to the cards[24-25]. These Noviplex cards, however, use pre-punched filter paper discs instead of actual DBS cards and are hence not compatible with commercially available DBS analyzers. Another DPS collection system that has been commercialized is the HemaSpot™-SE device, which consists of a spiral-shaped filter paper[26]. Regrettably, there are also no publications regarding this HemaSpot™ device. A potentially interesting new format is the HemaXis DX system, which appears to be able to generate fixed volume DPS on a DBS card[27]. Hitherto, no proof of principle paper has been published. If a system would be designed that is able to generate volumetric DPS, yields quantitative results independent of HT, is compatible with commercially available DBS analyzers and is affordable of course, this might potentially become the matrix of choice for most applications, instead of DBS. Although plasma might not be inherently superior to whole blood, it remains the matrix that was used to establish most therapeutic intervals or reference ranges. An overview of several of the above-mentioned devices can be found in Figure 3.

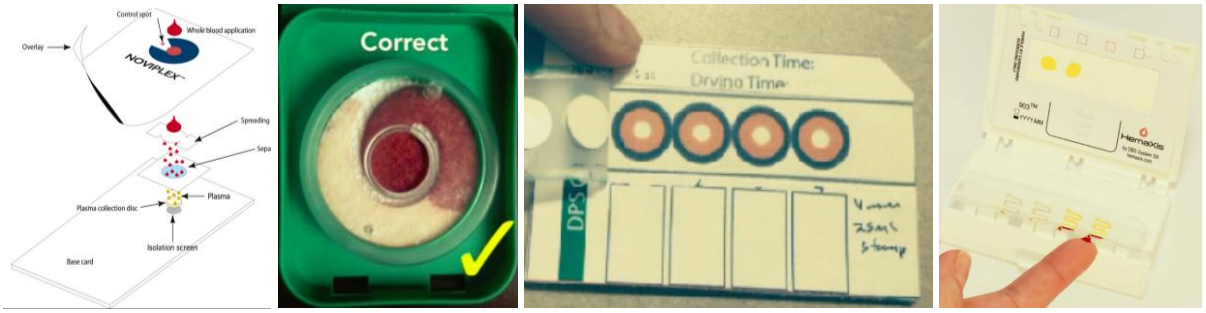

Figure 3: Different devices to collect DPS: Noviplex card, HemaSpot™ HF, autoDPS card and HemaXis DS[24-27].

1.3. Hematocrit prediction and correction

Although several of the above-mentioned sampling strategies yield promising results, the low cost of classical DBS, as well as the experience people have with this sampling format, make classical DBS still the most frequently used dried blood matrix. Therefore, a multitude of methods have been developed to determine the HT of DBS and/or correct for the anticipated HT impact. Whenever the HT is determined, the obtained value can be used to i) evaluate whether the HT of the sample is acceptable (i.e. falls within the validated HT range), ii) to implement a HT-dependent correction factor to a DBS-based result and/or iii) to convert DBS-based results to the corresponding plasma or serum levels.

1.3.1. Measuring the hematocrit on a liquid sample

One option to know a patient's HT is to collect an additional, liquid sample. However, this would negate many of the advantages of DBS sampling. Furthermore, differences between venous and capillary HT have been documented[28] and the HT of capillary blood also displays variability from drop to drop, even when the first drop is discarded[29]. Therefore, it is relevant to know the HT of the exact DBS that will be used for the analysis of the target analyte.

1.3.2. Estimate the HT based on physical DBS properties

Several attempts to cope with the HT effect have tried to deduce the HT of a DBS sample based on its physical properties[30]. Unfortunately, the diameter, surface, weight and frustum volume all depend on both the HT and the sample volume. The color of a DBS on the other hand is determined by the dried blood sample's age (cfr. Chapter 5). Therefore, these approaches are not applicable to classical, non-volumetric DBS.

1.3.3. Endogenous compounds as a marker of HT

1.3.3.1. Potassium

As was elaborately discussed in Chapters 3 and 4, K^+ is an excellent marker of HT[31-32]. The obvious need for a HT correction method, the simplicity of the method and the fact K^+ can be measured in any laboratory, has resulted in this HT prediction method being adopted by other laboratories as well (either as is, or with some changes to render the method compatible with the target analyte analysis). Den Burger *et al.* for example used 8-mm DBS in combination with a 30 min extraction, to allow for the combined analysis of K^+ and creatinine[33]. Rufail *et al.* on the other hand has measured K^+ in perimeter ring samples instead of in DBS punches[34]. This has the advantage that a part of the DBS sample is used that would typically not be used for the target analyte analysis. However, the latter method is only applicable to volumetric DBS. Additionally, it has been demonstrated that K^+ can also be used to measure the HT of a VAMS sample. In this context, Bloem *et al*. designed a method that was compatible with the immune-assays they used for the quantitation of therapeutic monoclonal antibodies[35], whereas we set-up K^+-based methods that are more compatible with LC-MS/MS analysis.

1.3.3.2. Hemoglobin

Since Hb is a marker of HT in clinical routine, this seems to be a logical choice for the set-up of a HT prediction method. As was mentioned in Chapter 5, multiple methods have indeed been used for total Hb determination in DBS, but unfortunately, most yielded DBS age-dependent results, semi-quantitative results or required mixing of the blood sample with a derivatizing agent before or upon DBS collection[36-37]. Such approaches are thus not suitable for a Hb-based HT prediction method.

One Hb-based HT prediction method that did yield promising results employs a commercially available, sodium lauryl sulphate (SLS) containing reagent to transform all the Hb into SLS-Hb, which is subsequently measured at 550 nm[38]. Importantly, the obtained results were not affected by DBS age (up to 6 months at least) or the sample volume that was applied. This method is certainly applicable in routine laboratories, but requires the extraction of a 6 mm DBS punch. This entails a separate DBS is required for HT prediction.

Another potentially valuable method has been published by Yu *et al*. and uses LC-MS/MS to determine a proteospecific peptide, obtained after tryptic digestion of a DBS extract[39]. Although this method is too complex to be universally applicable to all DBS analyses, it can be

of value if a similar sample preparation procedure and analytical method are to be used for the determination of the target analyte as well.

As was elaborately described in Chapters 5 and 6, we developed non-destructive methods to determine the HT of a DBS based on its total Hb content using non-contact diffuse reflectance spectroscopy. These approaches basically require mere scanning of a DBS to derive the sample's HT[40-41]. Oostendorp *et al.* has also published a non-destructive Hb-based HT prediction method, for which NIR was employed[42]. However, their method appears to be less extensively validated and has not yet been used for actual HT correction purposes. For these non-contact HT prediction methods in particular, automation and even integration in a commercially available DBS analyzer can be envisaged.

1.3.3.3. Other options

Aside from K^+ and Hb, sphingomyelins have been suggested as a marker of HT[43]. As these compounds are constituents of the RBC membrane, they appear to be a logical choice for HT prediction purposes. As these lipophilic compounds are determined via LC-MS, it is unlikely they will become a universal method to determine HT from DBS. In addition, this method was only evaluated using volumetric DBS with a HT between 0.20 and 0.40, so it still needs to be demonstrated that application to non-volumetric DBS with a wide HT-range yields acceptable results.

Another LC-MS-based solution that has been suggested to estimate the volume in a DBS, employs post-column infusion[44]. More particularly, the sample volume is derived from the extent of ion suppression caused by early eluting non-volatile salts present in the blood sample. Unfortunately, it was not evaluated whether this method could adequately determine the volume of blood in a DBS sub-punch, which could potentially be used to normalize DBS-based results.

When looking at non-destructive (and non-Hb-related) options, two methods have been published. The first one derives the volume that was present in a DBS punch from the electrical conductivity of a DBS extract, which is determined using a custom made ring disk electrode[45]. Although this method requires an extract, it does not use part of the extract, so the latter can be completely used for the actual target analyte analysis. A second non-destructive method, uses non-contact reflectance spectroscopy, but rather than looking at a

Hb-specific wavelength, the reflectance at 980 nm is measured[46]. The reflectance at this wavelength was attributed to background scattering but appeared to correlate well with the sample volume in a 3 mm DBS punch. As the sample volume may also affect the background scattering, the authors advise to only employ this method for DBS of similar size, which limits its real life applicability. Regrettably, the authors did not evaluate the effect of DBS storage on the measured reflectance at 980 nm, an essential element for any DBS-based method, and for this HT-prediction method in particular, as the reflectance spectrum of a DBS is known to change drastically during storage (see Chapter 5 and 6).

Yet again, each HT prediction method has its own strengths and weaknesses. The ideal method to predict the HT depends on the context (e.g. the target analyte, the type of collection substrate that is used, the amount of sample that is available, whether the analysis needs to be compatible with automation, the available instrumentation in the laboratory, the analytical technique used to quantitate the target analyte, specific patient characteristics, etc.). In addition, for most HT prediction methods it has not been evaluated whether the predicted HT can be used for HT correction. This will probably also factor in to which method will be selected.

Regarding the above-mentioned patient-specific characteristics it is noteworthy that the effect of e.g. hyperleucocytosis on the K^+ level in DBS extracts has not been evaluated. However even in these cases the amount of RBCs will still outweigh the amount of WBCs. Furthermore, hyperleucocytosis might also affect a blood sample's viscosity, so it might actually be better to also take into account the K^+ content of these cells. The Hb-based approaches on the other hand have not been applied to DBS from neonates (which have fetal hemoglobin or HbF) or patients with hemoglobinopathies. Depending on the influence the presence of another type of Hb has on the reflectance spectrum, an impact on the HT estimation might be anticipated. These are elements that could be evaluated in future experiments

2. ROUTINE DBS-BASED APPLICATIONS

Aside from NBS programs and applications in resource limited settings, DBS analysis is still only scarcely adopted in routine laboratories. Although DBS analysis fits within the general trend of personalized medicine and definitely has added value for certain applications, no routine DBS-based methods could be found when consulting Belgian laboratory guides. In Dutch clinical laboratories on the other hand, DBS-based applications are slowly being introduced in the context of TDM. Analytes that are monitored *via* DBS include antidepressant, anti-epileptic drugs, immunosuppressants and antibiotics. Some hospitals even offer the simultaneous determination of the target drug, creatinine and HT[47].

In our toxicological laboratory whole DBS analysis is used for the determination of GHB in post-mortem samples. Volumetric application is feasible in this context and DBS is basically used as an easy sample preparation method. Furthermore, dried blood microsamples are also used to assess recent alcohol consumption by analyzing PEth. Although theoretically DBS sampling may also facilitate blood sample collection in the context of road side drug testing, this would be difficult from a legislative perspective, as police officers are not allowed to collect blood samples.

Originally, there was also major interest from pharmaceutical companies to use DBS during (pre-)clinical studies. Unfortunately, there interest has diminished as regulatory institutions still require bridging studies to demonstrate the validity of DBS-based data. Therefore, for the time being, their interest has mainly shifted towards liquid microsampling. Only when samples cannot be collected in any other way, DBS are still used (e.g. if the participants of a clinical study need to collect the samples themselves at home).

On the other hand, a lot of commercial laboratories, located mainly in the US, but also in other countries such as Australia and the UK, offer DBS analysis for a vast array of analytes. These laboratories use slogans such as ‘home health testing made easy’ and tap into the general trend of ‘people taking control of their own health’[48]. For some tests samples are collected at home and sent to the laboratory, other tests are performed at home (using liquid capillary blood samples). It is of course true that it is convenient to collect samples at home at any time, however, quality should remain the primary concern. If these tests are of adequate quality and/or reach people which would otherwise not get tested, home sampling should be applauded. Unfortunately, the above-mentioned phenomenon should be treated

with great care, as it is unlikely that while methods take a long time to be introduced in a scientific environment, that a thorough analytical and clinical validation was performed for all the parameters offered by these laboratories. Despite the large appeal of home sampling or self-sampling, the quality of the tests remains essential. Therefore, a thorough method validation, representative of how the tests are used in 'real-life', is indispensable.

3. OTHER ADVANCES

3.1. Automation

Regarding the automation of DBS-analysis no fundamental changes have occurred during recent years. Two types of DBS analyzers are available that allow full automation of a DBS workflow, from sample preparation to extract analysis using LC-MS/MS. Experience with these instruments is slowly being built. In the near future the non-contact HT prediction method might be integrated into one of these analyzers, as evaluations in this context are ongoing. For VAMS analysis, no full automation systems are currently available. However, thanks to the shape of the VAMS devices and the relative small size of the absorbing tips, these samples are compatible with the use of liquid handling systems available in routine laboratories[49]. These facilitate easy tip transfer to and from extraction solvents contained in e.g. a 96 deep well plate. However, leaving the VAMS tips on the plastic bodies impacts the liquid flow during extraction and may therefore yield a lower extraction efficiency[50]. Furthermore, a dedicated system for semi-automated VAMS analysis has been commercialized recently[51].

3.2. Quality assurance

To help ensure the quality of DBS methods, a matrix specific method validation guideline has been written (see Chapter 2). A similar guideline regarding VAMS analysis is in the process of being written. The amount of EQC programs has also increased, although they remain scarce, aside from those for NBS programs[52]. Furthermore, to help improve the quality of the DBS received by the laboratory, a phone app has been developed to help patients evaluate the quality of the spot based on physical properties. This way, a new sample can be collected if required[53].

3.3. Other sampling formats to facilitate home-based sample collection

As was discussed in section 2 also liquid capillary microsampling is used in home sampling applications. To facilitate sampling in this context, several options have been suggested (see Figure 4). Although no longer available, The Nanotainer® was easier to manipulate than a single, relatively fragile microcapillary[54]. Another device that is designed to render liquid microsampling easier is the Innovac®, which is designed to promote capillary blood flow and thereby eliminate the need for milking[55].

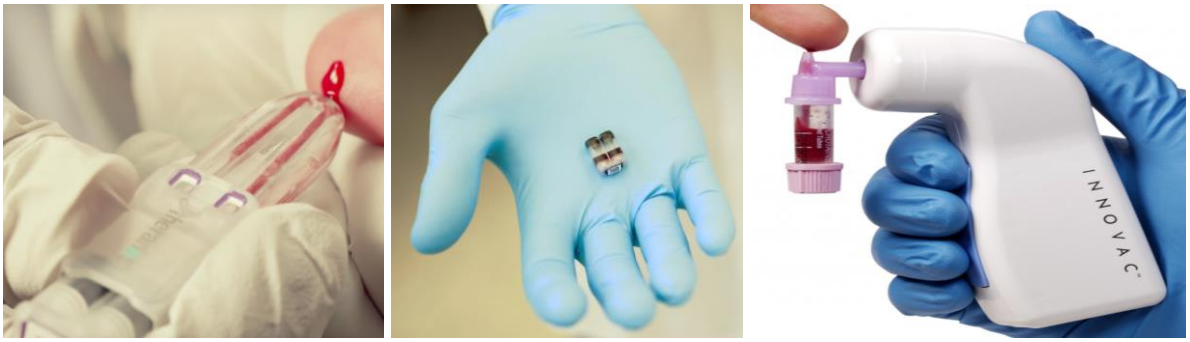

Figure 4: different formats to facilitate liquid microsampling. Left and center panel: the Nanotainer®. Right panel: the Innovac® system[54-55].

Another commercially available way of collecting capillary blood samples is the TAP device[56] (see Figure 5). This does not require a finger prick, but uses an array of 30 microneedles to create skin punctures on the upper arm. A vacuum helps draw blood from the puncture sites and the blood is mixed with Li-heparin before being collected in a designated reservoir. An indicator window turns red when the reservoir is full. The device is easy to operate as it only requires a single push on a button. The current device collects 100 µL of blood and another device that should be able to collect 250 µL of blood, is being developed. Although this device probably does not collect pure blood, but rather a mixture of capillary blood and ISF, HbA1c measured from these samples appeared to be equivalent to the corresponding venous HbA1c values (n = 144).

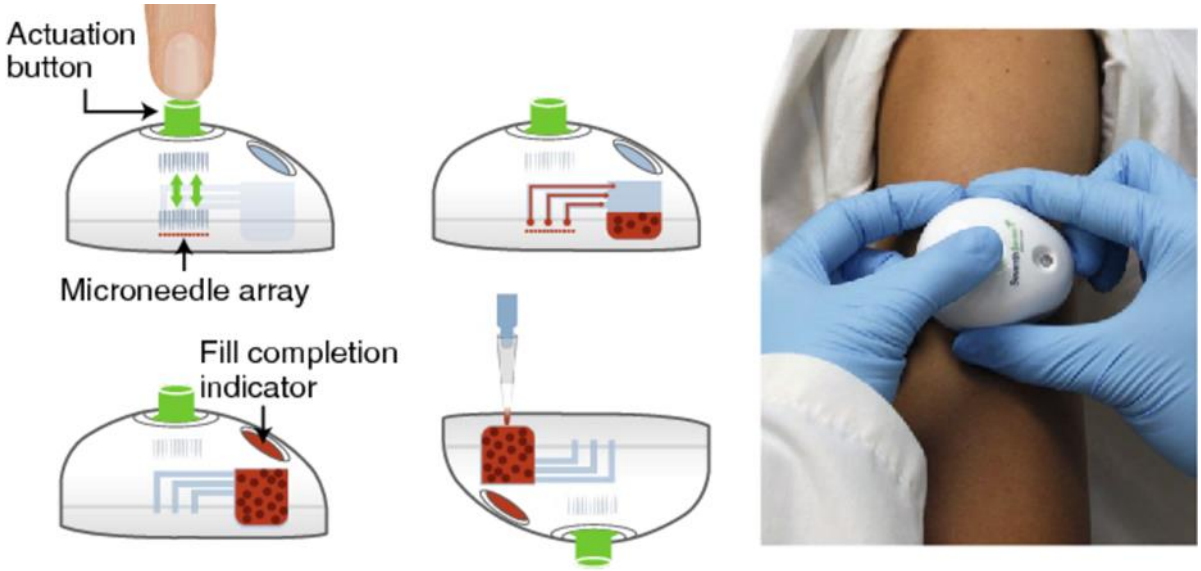

Figure 5: The TAP device, a microneedle-based device used to collect liquid capillary blood[56].

To facilitate serial DBS sampling, which is relevant during pharmacokinetic studies or for certain TDM applications, the Fluispotter® has been developed[57] (see Figure 6). This is a wearable microfluidic DBS sampling device that is able to collect and store up to 20 DBS over a time frame of 20 minutes to 20h. The sample collection device does not only contain the DBS collection substrate but also a IS reservoir. Interestingly, the IS can be mixed with the blood sample before being volumetrically spotted onto the DBS collection substrate. Currently, this device is cleared for preclinical applications and trials are ongoing to approve human use as well.

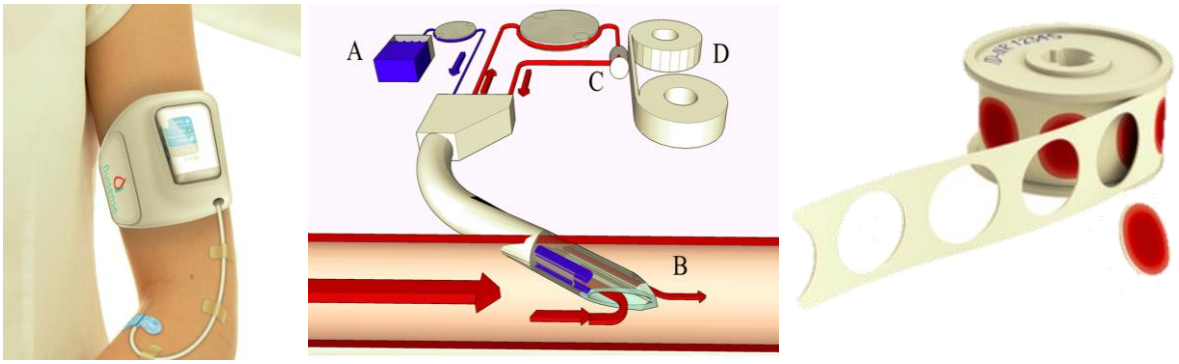

Figure 6: The Fluispotter®, a wearable microfluidic sampling device. The blue color in the right panel represents the IS[57-58].

Also the collection of other alternative matrices has been subject to changes during recent years. Many of the applications focus on glucose monitoring as this is a parameter that numerous people need to monitor and diabetes is a pathology that results in a great cost for healthcare systems. One example is the Freestyle Libre depicted in the left panel of Figure 7, a device that was designed for continuous glucose monitoring in ISF[59-60]. By scanning the

sensor the glucose level can be examined at any time. This device has been used in Belgium since 2016 for the monitoring of glucose in patients with type 1 diabetes and has already proven to decrease the risk of severe hypoglycemic events. Another innovative device that was developed for the purpose of continuous glucose monitoring is the Smart Contact Lens, which measured glucose in tear fluid[61] (see Figure 7). Unfortunately, although the preliminary results were satisfactory, the development of the product has since been cancelled, since the quality of the obtained results in further studies proved to be inadequate[62]. Once again, this underlines the importance of a thorough analytical and clinical validation of all analytical methods. Recently, the trend of minimally or non-invasive sampling has even been moving towards optical monitoring of blood glucose levels, eliminating the sampling step altogether[63]. However, these applications are still within a research context and require further evaluation.

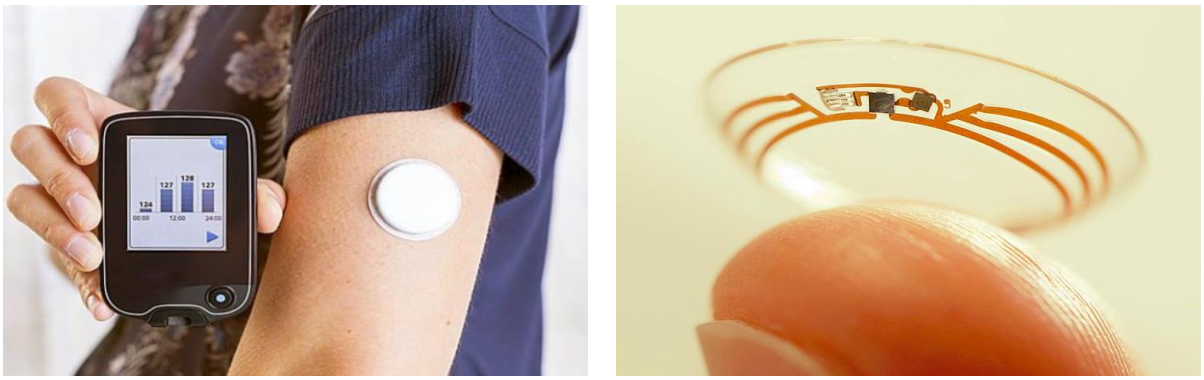

Figure 7: Left panel: the FreeStyle Libre, a sensor that continuously measures glucose in ISF. Right panel: Smart Contact Lens developed by Google for glucose monitoring[60-61].

4. CONCLUSION

Overall, a definite trend towards minimally invasive or non-invasive sampling can be observed, as well as towards personalized medicine. Dried blood sample analysis can be of added value in this context. Obviously, it will never completely replace traditional sampling, but it may aid in obtaining information that would otherwise be difficult or inconvenient to obtain. During the last 5 to 10 years great steps forward have been made to implement dried blood sample analysis in a routine environment. When compared to a couple of years ago, scientists now have multiple tools to their disposal to evaluate the extent of the HT effect and to cope with it in an adequate way. Unfortunately, two important hurdles remain: i) the lack of applications with an in depth clinical validation, and ii) studies regarding the cost/benefit ratio of implementing dried blood sample analysis for certain applications. Only when this ratio is favorable will more widespread implementation of dried blood sample methods occur.

5. REFERENCES

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Supplementary information

Supplementary section S-1: DBS guideline summary

SCOPE

This guideline aims to define the elements necessary for the validation of quantitative DBS-based methods. The main focus of this guideline is the analysis of dried blood spots (DBS) for the quantitative determination of small molecules drugs and drug metabolites using chromatographic techniques for TDM purposes.

METHOD DEVELOPMENT

Before embarking on the set-up of a DBS-based procedure, it is essential to carefully think about the purpose of the method. Sampling method: which sample collection method is best suited for a certain application largely depends on the context in which the method will be applied (e.g. home sampling). Also the required sample volume, automation capabilities, commercial availability and cost may play an important role in the selection of the sample collection method. Filter paper: the type of filter paper may affect the occurrence of interferences, the blood's spreading behavior, sample homogeneity, as well as analyte stability and recovery. Analyze some blank collection substrates to assess whether the collection material itself is blank and whether there are any interferences present. Sample volume: the required sample volume will mainly depend on the envisaged LLOQ. However, the minimally required volume should always relate to how the samples are collected. For the set-up and validation of the method a sample volume representative of the sample volume of the patient samples needs to be employed. The number of punches required for a single analysis should remain as small as possible (preferably one).

Drying and storage process: dry samples at least 3h under ambient conditions outside of direct sunlight and store them with desiccant. Evaluate whether the drying time is adequate under the conditions likely to be encountered during the collection of the patient samples using dried blood samples with a high HT (0.50 – 0.65, depending on the target population) and, if applicable, a large sample volume, as these will dry the slowest. The ambient conditions during drying may also affect DBS homogeneity.

Punch size: for volumetric DBS applications, the punch size needs to be large enough to punch out the entire DBS, independently of the HT of the sample. Hence, select the required punch

size based on samples with a HT of approximately 0.15. For non-volumetric DBS applications, partial DBS punches are made that exclude the outer edge of the sample. The punch size largely depends on the desired LLOQ and DBS homogeneity. Typical punch sizes are between 2 and 8 mm.

Internal standard incorporation: the IS is typically added to the extraction solution or directly to the DBS punch before extraction, and will not compensate for variability in analyte recovery. Therefore, analyte recovery has to be investigated extensively.

Type of blood used: for the set-up of calibration curves and QCs it is impossible to use finger prick samples. Instead, spiked samples generated from venous whole blood containing an anticoagulant, are used. If the DBS collection device that is used to generate the patient DBS contains a certain anticoagulant, the venous whole blood also needs to contain that same anticoagulant. If no anticoagulant is used during the collection of the patient samples, the anticoagulant should not impact the obtained results nor the analyte's stability. Compare results obtained from a non-anticoagulated sample with results from samples anticoagulated with different anticoagulants. These samples should be obtained venously from the same person at the same time.

Preparation of spiked samples: first adjust the HT of the whole blood to the desired HT value. For most experiments the latter will correspond to the median HT value of the target population (e.g. for the calibration curve). Measure the HT, to assure the sample was prepared correctly. Spike a limited volume of analyte solution to the blood (i.e. < 5% of the sample and preferably even less), to not change the nature of the sample. Equilibrate for a sufficient amount of time at a suitable temperature, to mimic the analytes' in vivo RBC/plasma distribution.

PREVALIDATION

Proper stress testing of the method during method development will allow potential issues to be detected at an early stage, which will eventually increase the chances of a successful method validation and application.

Extraction efficiency and short-term stability

Since time-dependent extraction issues shortly after sample generation have been described, results from samples analyzed at T0 (typically 30min – 3h after sample generation) should be

compared with results obtained at later time points, preferably up to 48h or 72h. If the measured analyte concentration only decreases for the first time points, but remains stable for the later ones, it is possible to obtain good analytical results if only samples older than a certain age (depending on the time point from which stable results were obtained) are measured. The extraction efficiency may be concentration, HT and time-dependent and, importantly, these parameters may also affect one another. For non-thermolabile compounds, this can be evaluated by comparing the results from fresh dried blood samples at low, medium and high HT levels (with these HT levels encompassing the HT range of the target population; e.g. 0.20, 0.40, 0.60) with a second set of samples stored at 50-60°C for at least two days. This second set mimics thoroughly dried (aged) samples. This experiment should be performed at both the low and high QC level. Importantly, in case of partial DBS analysis, these samples should be prepared by pipetting a fixed amount of blood onto prepunched filter paper disks to rule out any influence of the HT effect on the amount of sample being analyzed. For more thermolabile compounds a similar experiment can be performed with samples stored at e.g. RT for two weeks instead of at 60°C for two days

ANALYTICAL VALIDATION

Selectivity: blank samples should be obtained using the same sampling approach as the one that will be used to collect the patient samples.

Calibration model, accuracy and imprecision, measurement range: all experiments should be performed in accordance with existing guidelines. All calibrators, blank, zero and QC samples should be prepared in blood with the median HT of the target population and should have a volume representative of the patient samples. For the purpose of TDM, a calibration range spanning from half of the lower end of the therapeutic interval to twice the upper end of the therapeutic interval suffices.

Dilution integrity: to analyze samples above the measurement range, dried blood sample extracts are typically diluted with blank dried blood sample extracts or extraction solvent. Furthermore, IS-tracked dilution or the donut punch approach can be used.

Carry over: aside from classical carryover, in a DBS workflow, the punching step could be considered a potential source of contamination. Hence, we propose to include the processing of one or more blanks following the processing of the highest calibrator.

Matrix effect, recovery and process efficiency: these should be evaluated in line with the set-up proposed by Matuszewski *et al*. Blood from at least six different donors should be used and two concentration levels should be evaluated. In addition, since it is known that the HT may strongly impact the recovery -and possibly also the matrix effect- it is essential to evaluate recovery and matrix effect at different HT levels, prepared from the blood of at least one donor. These HT levels should encompass the anticipated HT range of the target population. To accurately perform this experiment, a fixed volume of blank or spiked blood needs to be applied on prepunched filter paper discs or VAMS tips.

Stability: the stability assessments should be representative of the ambient conditions encountered during sample transport, storage and processing and the investigated time frame should cover the maximum expected time frame between sample collection, analysis and potential re-analysis. Furthermore, since temperatures may be significantly higher during transport (e.g. in a mail box in the sun during summer time) short-term stability at elevated temperatures (i.e. 2 or 3 days at 50°C – 60°C) should also be tested. Additionally, autosampler post-preparative stability should be assessed.

DBS-specific validation parameters: the analytical validation of DBS methods requires the evaluation of the volume effect, the volcano effect and the HT effect . It is essential that these parameters are assessed simultaneously, as they may affect one another. If a relevant volume, HT or volcano effect is observed, appropriate measures need to be taken to ensure patient samples are within the validated limits and are reliable. Obviously, it should also be demonstrated that these measures are indeed adequate.

- Volume effect: the volume range in which DBS-based results are still acceptable should be defined during method validation. Typical volume ranges to be evaluated are 10 – 50 µl for hanging-drop-collection and 20 - 70 µl for falling drop collection. The volume effect should also be evaluated at low, medium and high HT and at both low and high QC.

- Volcano effect (DBS homogeneity): in case of partial DBS analysis, it is essential to assess whether results from central punches are equivalent to peripheral ones. This evaluation must be performed at low and high QC, at different HT levels (low, medium and high) and at sample volumes representative of the patient sample

volumes (n = 5). All samples should be compared to a calibration curve prepared with samples of medium HT and volume, of which a central punch was extracted. If a relevant volcano effect is observed, only central punches should be analyzed.

- Hematocrit effect: the HT range that needs to be evaluated depends on the target population (see Figure 6). For a quasi-universal method, the range should span from 0.20 to 0.65, although a narrower range will suffice for most applications. At each HT level, two concentrations should be tested.

Table 1: Overview of the minimally required analyses for the analytical validation of DBS.

Validation parameter	Amount of samples
Selectivity	n = 6 x 1 x 2 x 1 = 12 6 blank matrices, 6 LLOQs, 1 day, in singulo
Calibration model	n = 6 x 5 x 1 = 30 6 calibrators, 5 days, in singulo
Accuracy & imprecision	n = 4 x 3 x 2 = 24 4 QC levels (LLOQ, low, mid, high), 3 days, in duplicate
Dilution integrity	n = 1 x 3 x 2 = 6 1 QC level (dilution QC), 3 days, in duplicate
Carry-over	n = 2 x 5 x 1 = 10 a blank and zero sample, 5 days, in singulo
Recovery, matrix effect, process efficiency	n = 2x (2 x 5 x 1 x 1 x 3) + 2x (2 x 1 x 3 x 1 x 3) + (2 x 1 x 3) = 102 2 QC levels, 6 donors, of which 1 donor at 3 HT levels, 1 day, in triplicate (spiked before/after) 2 QC levels, 1 day, triplicate (standard solutions)
Stability	n = 2 x 1 x 4 x 5 = 40 2 QC levels, 1 HT level, 4 points: T0, T1w, T2w @ RT, T2d @ 60°C, in quintuplicate
Volume effect, hematocrit effect, volcano effect	n = 2 x 3 x 4 x 5 = 120 2 QC levels, 3 HT levels, low, medium and high volume central punch + high volume peripheral punch, in quintuplicate
TOTAL	**344**

*samples are prepared in blood of average HT, unless mentioned otherwise.

CLINICAL VALIDATION

Comparison between DBS and traditional matrix

- Samples should be collected after the distribution phase and within 5 to 10 minutes from each other. They should be collected as will be done during routine use and should be processed and analyzed as was done during analytical validation.
- Samples should be collected after disinfecting the finger without excessive milking or squeezing of the puncture site. The drop of blood should either freely fall onto the DBS card or should be deposited onto it by gently bringing the drop of blood into contact with the filter paper without the finger touching the filter paper.
- Ideally, collect at least 80 samples from at least 40 patients (if samples are difficult to obtain, collect at least 40 paired samples from at least 25 patients).
- Collect finger prick DBS (at least in duplicate) and venous whole blood
- Analyze capillary DBS, venous DBS, venous liquid whole blood and, depending on the traditional matrix used, also venous plasma or serum.
- Compare venous whole blood and venous DBS: a difference between both methods indicates a problem with the DBS approach per se. By plotting the differences between the DBS results and whole blood results *vs*. HT, the presence of a HT effect can be evaluated. If there is no HT effect, the slope of the resulting curve should not be significantly different from zero.
- To find out a potential capillary-venous difference, the results obtained from fingerprick DBS can be compared with the corresponding venous DBS.
- The differences in analyte concentration between liquid whole blood and plasma depend on the distribution of an analyte between RBCs and plasma and may be HT-dependent (the latter can be evaluated as described earlier).
- Eventually capillary DBS results should be compared with reference method results. Conversion between both may be HT-dependent or not (which can be evaluated as described earlier) and will be the result of the of the differences observed during the above-mentioned comparisons.
- Whenever two matrices are compared, a conversion factor, if required, can be derived from the Passing-Bablok or weighted Deming regression analysis (if the conversion is not HT-dependent). If the conversion is HT-dependent the conversion should also include the patient's HT value. A conversion factor should be determined

on the first 40 randomly selected samples and should be validated by applying it to the other 40 samples.

- If fewer samples are obtained, conversion factors may be validated using a jackknife approach.

Required statistics

- Passing-Bablok or weighted Deming regression analysis to assess the correlation between two matrices and the presence of proportional and/or systemic errors.
- Bland-Altman plot to assess both bias and agreement between both matrices.
- Acceptance criteria for bias and LoA should be agreed upon beforehand by a multidisciplinary team of experts based on clinically and analytically relevant parameters.
- These statistics should be ran before and after conversion calculations have been performed.
- ISR (to assess method reproducibility, as described in FDA guideline draft). The % difference between duplicates should not be greater than 20% of their mean for at least 67% of the samples. These duplicates may be two punches from the same DBS or two punches from two different DBS on the same card (also depends on punch size).

How to ensure good spot quality?

- Patient education
- Cards with two concentric circles corresponding to the minimally and maximally required sample volume that should be collected (taking into account the Ht-range of the population).
- Checking the spot quality in the laboratory, either by lab technicians or by an automated system.
- The same method to ensure/check spot quality should be used during clinical validation and routine use.

How to deal with volume, volcano and hematocrit effect?

a) Volume effect

- Train patient to collect samples of adequate volume
- To ensure the spot volume is within the validation volume range a card with two concentric circles (corresponding to the minimally and maximally required volume may be used).

b) Volcano effect

- If a relevant volcano effect is present, only use central punches (both during validation and routine use).

c) Hematocrit effect

- Unless no relevant HT effect is observed over the entire HT range (both during analytical and clinical validation), a method should be used to assess the HT of the patient samples.
- Besides confirming that the HT of the patient sample effectively lies within the validated HT range, this may also allow to perform a HT correction, to alleviate the HT bias.
- If the target population has a narrow HT-range, the median HT value of the target population may be used instead of the actual patient HT (e.g. for DBS – plasma concentration conversion).

Quality control

- Join a dried blood sample-based EQC program if available
- EQC materials for liquid blood-based methods can be used if whole sample analysis is performed (although differences in extraction efficiency between the EQC and a genuine dried blood sample cannot be ruled out).
- EQC materials for plasma-based methods can be used to replace (part of) the plasma of a blank donor sample. The spiked sample can then be used to generate a dried blood sample (again whole sample analysis is required and differences in extraction efficiency cannot be ruled out).

CROSS VALIDATION

Different punch size

- Compare 40 samples of at least 25 different patients with both punch sizes.
- Thoroughly re-evaluate extraction efficiency for potential concentration, time and HT-dependent issues.
- Re-evaluate DBS-homogeneity (although the DBS homogeneity will not change per se, smaller punch sizes may be more sensitive to this phenomenon).

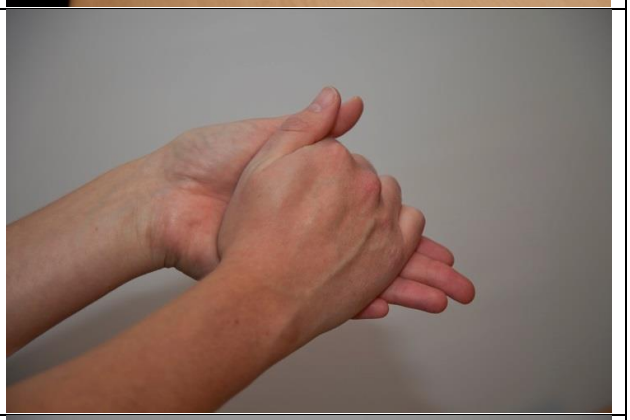
Different type of filter paper

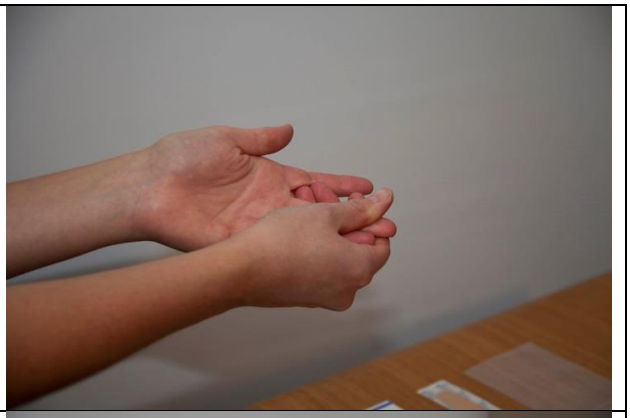
- Compare 40 samples of at least 25 different patients with both types of filter paper.
- Thoroughly re-evaluate extraction efficiency and matrix effect for potential concentration, time and HT-dependent issues.
- Re-evaluate DBS-homogeneity, stability as well as volume and HT effect.

Different sampling method

- Requires a completely new analytical and clinical validation

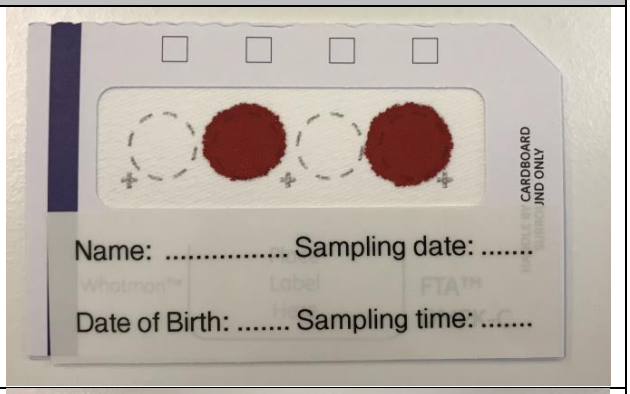
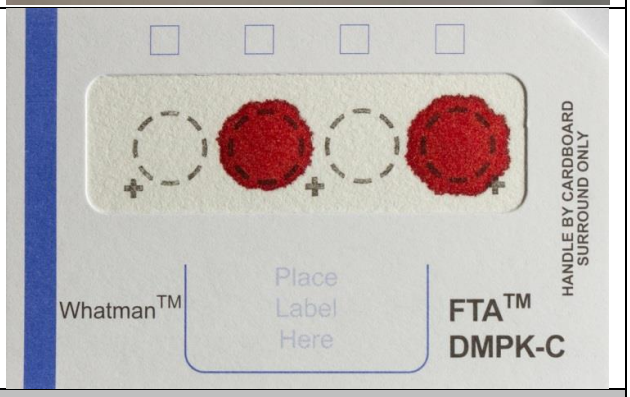
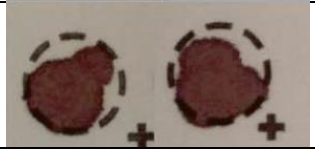
Supplementary Figure S-1: DBS sampling procedure for sampling at home

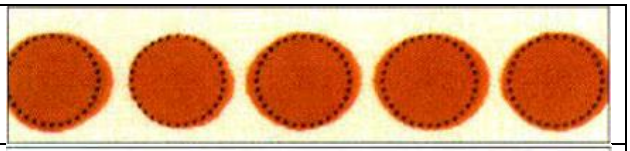
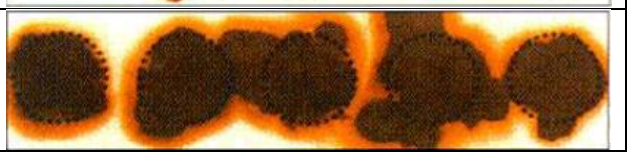
1. Place all sample accessories (blood collection card, lancets, sealable plastic bag, desiccant pack, band-aid, alcohol prep) on a smooth and clean surface. Do not touch the filter paper.	
2. Wash hands with soap and warm water for at least 30 seconds. Thoroughly dry hands.	
3. Warm hands by rubbing the hands together to increase blood flow. Afterwards, disinfect the top of the middle- or ring finger with the alcohol prep and let dry for at least 40 seconds.	
4. Prepare the lancet by removing the plastic cover.	

5. Gently massage the fingertip in a downward motion to increase blood flow.	
6. Place lancet on the finger and push to prick the finger.	
7. Allow a blood drop to form and wipe away the first drop with a tissue or cotton ball.	
8. Allow a large drop to form. If a drop doesn't form, gently massage the finger downwards without excessive 'milking' of the finger. Ideally, a single drop falls on the sampling card filling the circle completely. If the drop doesn't fall within 30-60 seconds, lightly touch the circle with the blood drop without touching the filter paper with the finger and without layering successive blood drops.	

9. Fill at least 2 circles completely, without the droplets touching each other. Puncture another finger (repeat steps 4-8) if the blood flow from the first puncture is insufficient.	
10. Clean the finger(s) and, if needed, place a band-aid on the finger(s). Dispose of all lancets appropriately.	
11. Record time and date of sampling and patient details or study number on the sampling card. Dry for a least 3 hours in a non-humid environment at ambient temperature on a clean and flat surface. Keep away from direct sunlight.	
12. After drying, place each individual sampling card inside a plastic (biohazard) sealbag with a desiccant pack and transport as soon as possible to the laboratory using regular mail or other transportation methods.	

Supplementary Figure S-2: DBS quality assessment

Correctly collected samples	
General ▪ Patient details / study number provided. ▪ Date and time of sampling provided.	
Package and shipping ▪ DBS is sealed in a plastic bag with desiccant sachet. ▪ DBS and plastic bag are intact and blood is only applied to filter paper. ▪ Time between sampling and analysis is within validated stability range.	
Valid samples ▪ Circles should be filled with enough blood to meet criteria set by the lab. ▪ Both sides of the filter paper should be equally soaked with blood. ▪ Blood drops should not touch each other. ▪ Blood drops should be round and consisting of one droplet.	
Invalid samples	
Lighter circles around the darker blood spots due to applying heat, contamination or excessive squeezing of the puncture site.	
The blood spots are crusted on the card, causing the DBS card to be contaminated. Also, fingersmearing is visible on the right spot.	
The premarked circle contains multiple overlapping blood drops.	

Sample not dried before mailing.	
Clotted or layered spots.	
Serum rings, possible due to contact with alcohol or excessive squeezing of the puncture site.	

Supplementary section S-2: calculation of erythrocyte potassium concentration

A. Aqueous VAMS extraction LOW HT

HT = 0.20 (=> 2.1 µL RBC + 8.5 µL of plasma)

Extract potassium concentration ≈ 3.05 mM

VAMS ≈ 10.6 µL of whole blood

Total extraction volume = 230 µL

Final extract volume = 180 µL

Extraction solvent = 1.6 mM KCl

Plasma potassium concentration arbitrarily set at 4 mM

Amount of potassium in final extract:

$$3.04\ \text{mM} * 180\ \mu\text{L} = \frac{3.04\ \text{nmol}}{\mu\text{L}} * 180\ \mu\text{L} = 547.2\ \text{nmol}$$

Contribution of VAMS potassium content to final amount of potassium

$$547.2\ \text{nmol} - \left(180\ \mu\text{L} * \frac{1.6\ \text{nmol}}{\mu\text{L}}\right) = 547.2\ \text{nmol} - 288\ \text{nmol} = 259.2\ \text{nmol}$$

Amount of potassium extracted from VAMS in total

$$259.2\ \text{nmol} * \frac{230\ \mu\text{L}}{180\ \mu\text{L}} = 331.2\ \text{nmol}$$

Intracellular potassium concentration

$$331.2\ \text{nmol} = 2.1\ \mu\text{L} * \text{intracellular } [K^+] + 8.5\ \mu\text{L} * 4\ \text{mM}$$

$$331.2\ \text{nmol} = 2.1\ \mu\text{L} * \text{intracellular } [K^+] + 8.5\ \mu\text{L} * \frac{4\ \text{nmol}}{\mu\text{L}}$$

$$331.2\ \text{nmol} = 2.1\ \mu\text{L} * \text{intracellular } [K^+] + 33.9\ \text{nmol}$$

$$297.3\ \text{nmol} = 2.1\ \mu\text{L} * \text{intracellular } [K^+]$$

$141.6\ \text{mM} = \text{intracellular } [K^+]$

B. Aqueous VAMS extraction HIGH HT

HT = 0.65 (=> 6.9 µL RBC + 3.7 µL of plasma)

Extract potassium concentration ≈ 5.80 mM

VAMS ≈ 10.6 µL of whole blood

Total extraction volume = 230 µL

Final extract volume = 180 µL

Extraction solvent = 1.6 mM KCl

Plasma potassium concentration arbitrarily set at 4 mM

Amount of potassium in final extract:

$$5.80\ \text{mM} * 180\ \mu\text{L} = \frac{5.80\ \text{nmol}}{\mu\text{L}} * 180\ \mu\text{L} = 1044\ \text{nmol}$$

Contribution of VAMS potassium content to final amount of potassium

$$1044\ \text{nmol} - \left(180\ \mu\text{L} * \frac{1.6\ \text{nmol}}{\mu\text{L}}\right) = 1044\ \text{nmol} - 288\ \text{nmol} = 756\ \text{nmol}$$

Amount of potassium extracted from VAMS in total

$$756\ \text{nmol} * \frac{230\ \mu\text{L}}{180\ \mu\text{L}} = 966\ \text{nmol}$$

Intracellular potassium concentration

$$966.0\ \text{nmol} = 6.9\ \mu\text{L} * \text{intracellular } [K^+] + 3.7\ \mu\text{L} * 4\ \text{mM}$$

$$966.0\ \text{nmol} = 6.9\ \mu\text{L} * \text{intracellular } [K^+] + 3.7\ \mu\text{L} * \frac{4\ \text{nmol}}{\mu\text{L}}$$

$$966.0\ \text{nmol} = 6.9\ \mu\text{L} * \text{intracellular } [K^+] + 14.8\ \text{nmol}$$

$$951.2\ \text{nmol} = 6.9\ \mu\text{L} * \text{intracellular } [K^+]$$

137.9 mM = intracellular $[K^+]$

Supplementary Table S-1: Overview of the HT values (measured with a Sysmex XE-5000 hematology analyzer) of all calibrators and QCs (in Li-heparin-anticoagulated blood) prepared for the evaluation of accuracy and imprecision.

	Day 1	Day 2	Day 3
Donor 1			
Calibration curve 1	0.20, 0.27, 0.35, 0.42, 0.50, 0.57, 0.66	0.21, 0.28, 0.36, 0.43, 0.52, 0.59, 0.67	0.20, 0.28, 0.35, 0.41, 0.52, 0.59, 0.68
QCs 1	0.20, 0.43, 0.66	0.21, 0.43, 0.67	0.20, 0.43, 0.69
Calibration curve 2	0.20, 0.27, 0.35, 0.42, 0.50, 0.57, 0.65	0.21, 0.28, 0.36, 0.43, 0.52, 0.59, 0.68	0.20, 0.28, 0.36, 0.43, 0.52, 0.59, 0.69
QCs 2	0.20, 0.42, 0.65	0.20, 0.44, 0.68	0.20, 0.43, 0.67
Donor 2			
Calibration curve 1	0.20, 0.27, 0.35, 0.42, 0.50, 0.57, 0.65	0.20, 0.27, 0.34, 0.42, 0.50, 0.58, 0.66	0.19, 0.26, 0.34, 0.41, 0.48, 0.54, 0.63
QCs 1	0.20, 0.42, 0.65	0.20, 0.42, 0.66	0.19, 0.40, 0.64
Calibration curve 2	0.20, 0.27, 0.35, 0.42, 0.50, 0.57, 0.65	0.20, 0.27, 0.35, 0.42, 0.50, 0.57, 0.65	0.19, 0.26, 0.33, 0.40, 0.49, 0.56, 0.65
QCs 2	0.20, 0.41, 0.65	0.20, 0.42, 0.65	0.20, 0.40, 0.64
Donor 3			
QCs 1	0.20, 0.44, 0.68	0.20, 0.42, 0.66	0.20, 0.42, 0.66
QCs 2	0.21, 0.44, 0.70	0.20, 0.42, 0.65	0.20, 0.42, 0.67
Donor 4			
QCs 1	0.18, 0.39, 0.61	0.20, 0.42, 0.66	0.19, 0.40, 0.65
QCs 2	0.18, 0.39, 0.62	0.20, 0.43, 0.66	0.19, 0.41, 0.66
Donor 5			
QCs 1	0.19, 0.41, 0.63	0.19, 0.41, 0.64	0.19, 0.42, 0.64
QCs 2	0.19, 0.40, 0.63	0.19, 0.41, 0.64	0.20, 0.42, 0.65
Donor 6			
QCs 1	0.19, 0.40, 0.62	0.19, 0.40, 0.62	0.20, 0.42, 0.68
QCs 2	0.19, 0.40, 0.63	0.18, 0.39, 0.60	0.20, 0.43, 0.67

Supplementary Tables S-2A and S-2B: Normalization procedure.

The equation used to calculate the normalized predicted HT is depicted below (Eq. S-1). An example of its implementation can be found in tables S-2A and S-2B.

$$Normalized\ predicted\ HT = \frac{Predicted\ Hct\ x\ Average\ true\ Hct}{True\ HT} \quad \text{(Eq. S-1)}$$

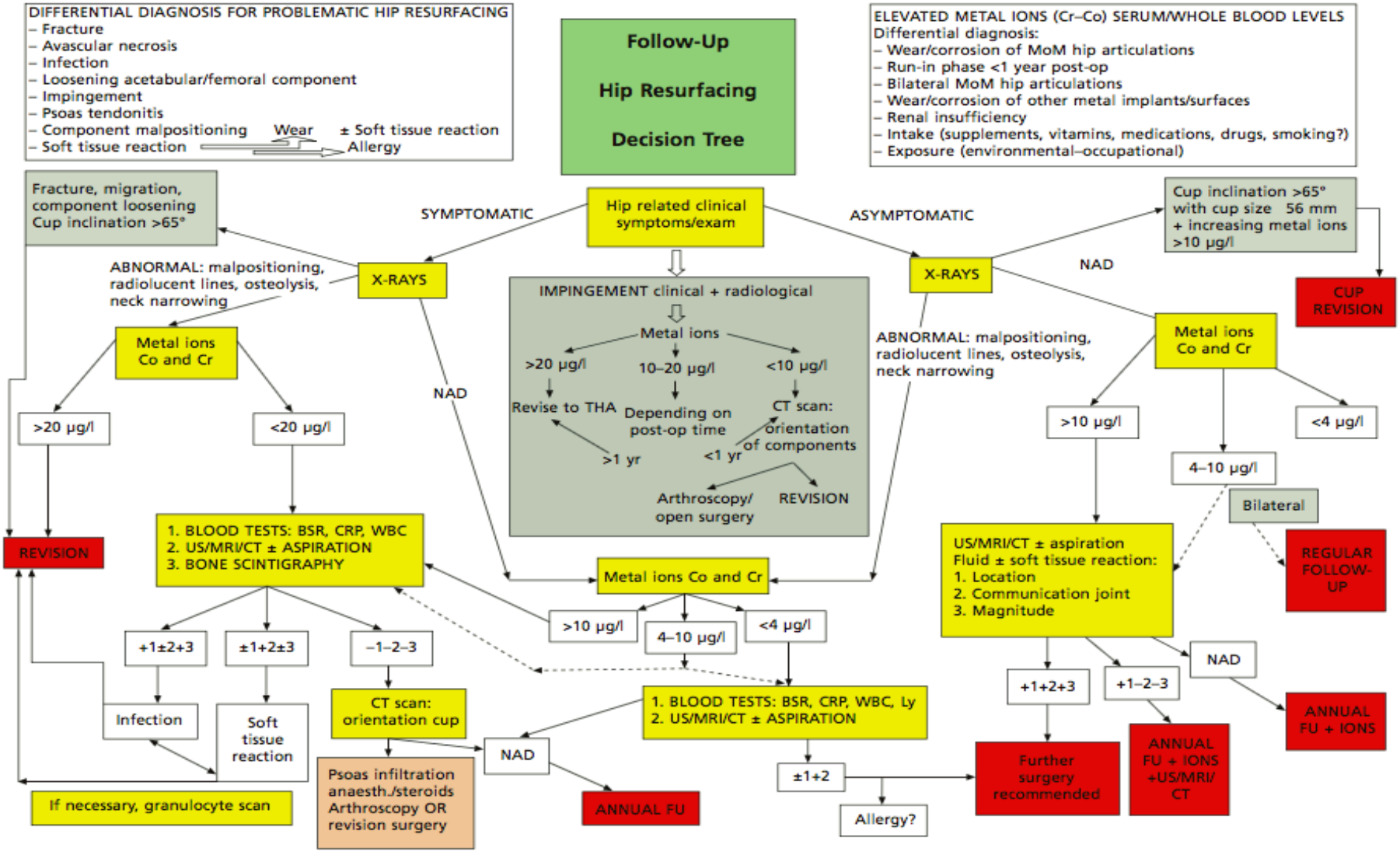
Table S-2A: Original true and predicted HT values.

	True HT	Predicted HT
Day 1 QC1	0.20	0.223
Day 1 QC2	0.20	0.175
Day 2 QC1	0.21	0.205
Day 2 QC2	0.20	0.208
Day 3 QC1	0.20	0.195
Day 3 QC2	0.20	0.214
Average true HT	0.202	
SD	0.41	
% bias	0.896%	
% RSD (intraday)	N.A.	
%RSD (interday)	N.A.	

Table S-2B: Normalized true and predicted HT values.

	True HT	Predicted Ht
Day 1 QC1	0.202	0.224
Day 1 QC2	0.202	0.177
Day 2 QC1	0.202	0.197
Day 2 QC2	0.202	0.210
Day 3 QC1	0.202	0.196
Day 3 QC2	0.202	0.216
Average true HT	0.202	
SD	0.00	
% bias	0.896%	
% RSD (intraday)	10.7%	
% RSD (interday)	10.7%	

Supplementary Figure S-3: Follow-up hip resurfacing decision tree



NAD = no abnormalities discerned, BSR = blood sedimentation rate, WBC = white blood cells, CRP = C-reactive protein, Ly = lymphocytes, US = ultrasound, FU = follow-up. At any moment, the surgeon can decide to take a 'wait and see' decision depending on the severity of the symptoms or the results of the exams.

Summary

The overall goal of this doctoral thesis was to help bridge the gap between research and routine in quantitative dried blood spot (DBS) analysis by tackling some of the hurdles that hinder(ed) more widespread implementation of this technique. These hurdles included the lack of a guideline for DBS method validation and the lack of adequate ways to cope with the hematocrit (HT) effect.

A general introduction to DBS sampling and analysis is provided in **General background, structure and objectives.** DBS are prepared by collecting a (typically non-volumetric) drop of blood onto a filter paper. This drop of blood can be obtained *via* a finger or heel stick, depending on the patient's age. DBS sampling provides several advantages over traditional venipuncture such as minimal invasiveness, the very small sample volume that is required, increased analyte stability as well as the ease of sample collection, transport and storage. DBS analysis has been used in many fields for the analysis of a wide range of compounds. However, this sampling technique also faces several challenges and issues, of which the HT effect is considered to be an essential one. The HT effect can be subdivided in analytical and physiological aspects. The analytical aspects refers to the fact that the analytical result may depend on a patient's HT, whereas the physiological aspect refers to the potential impact of the HT on the interpretation of the DBS-based result. In this section volumetric absorptive microsampling (VAMS) is also discussed. Using this sampling technique, a fixed volume of blood is wicked up from a non-volumetric drop of blood using an absorbent tip connected to a plastic handle. Therefore, VAMS-based results should be unaffected by a sample's HT.

A more in-depth overview of dried blood sample techniques, as well as other alternative sampling strategies is provided in **Chapter 1**. These alternative sampling strategies refer to the patient-friendly sampling of non-conventional matrices as well as to the unconventional sampling of traditional matrices such as whole blood, serum and plasma. Matrices that are discussed include DBS, VAMS samples, dried plasma spots, dried matrix spots, oral fluid, interstitial fluid, hair, tears, exhaled breath, sweat and nasal mucus. For each matrix specific advantages, challenges and limitations are discussed as well as some relevant applications. These applications encompass the fields of therapeutic drug monitoring (TDM), newborn screening, endocrinology, toxicology, phenotyping, proteomics and metabolomics. Special attention is given to dried blood sampling strategies, as this is the main topic of this work. Blood remains the matrix of choice, since there is typically a correlation between an analyte's blood level and its (therapeutic) effect. TDM-related applications are discussed more

elaborately, as TDM is the domain in which quantitative DBS-based methods are closest to routine implementation.

To help ensure the quality of DBS-based assays, a DBS method validation guideline was set up in collaboration with the Alternative Sampling Strategies Committee of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT). This guideline is the subject of **Chapter 2.** To successfully incorporate DBS-based methods in routine practice, good quality methods are a prerequisite. Since the quality of a method starts with its design, a sound method set-up not only ensures the method is suitable for a given application, it also increases the chances of a successful method validation. The quality of a method needs to be assessed both during analytical and clinical validation and should be compared with pre-set acceptance criteria. Most importantly, each aspect of a method should be evaluated in a way that reflects the situation in which it will eventually be applied. Throughout this guideline special attention is given to matrix-specific issues such as the HT, volume and chromatographic effect, and suggestions are provided on how to deal with these. Although this guideline was specifically constructed for the validation of quantitative DBS-based methods that use LC-MS/MS in the context of TDM, many of its aspects are also valid for dried blood microsample analysis in general.

In the following chapters the HT effect was tackled. This was done on the one hand by developing several strategies to determine (predict) the HT of dried blood microsamples. On the other hand, a 'HT-independent' alternative sampling strategy, i.e. VAMS, was evaluated. The first method we developed to derive the HT of a DBS, which is described in **Chapter 3**, estimated the HT based on a DBS's K^+ content. This electrolyte was chosen as a HT marker as its intracellular concentration is about 35 times higher than its extracellular concentration and its levels are under tight physiological control. As red blood cells are the predominant cells in blood, these are the major contributors to the total blood K^+ concentration. The method requires a two-step extraction of a 3-mm DBS punch with MilliQ water containing 2.5 mM KCl and the K^+ levels were measured *via* indirect potentiometry, using a routine chemistry analyzer. After successful analytical validation the method was applied to 111 venous patient DBS with a wide HT range (0.19 – 0.63), yielding excellent results. The developed methodology can be easily introduced into any automated clinical laboratory, due to its simplicity, speed and the fact a routine analyzer can be employed. The predicted HT can be used to assess whether the HT of the sample is within the validated HT range or to compensate for the

anticipated HT effect by introducing a HT-dependent correction factor. Furthermore, it may allow conversion of DBS-based results to the corresponding plasma or serum values.

This K^+-based HT prediction method was adapted to be applicable to VAMS samples as well. Although a VAMS-based result should be HT-independent, this is not always the case, as this matrix is susceptible to HT-dependent extraction efficiency issues. Therefore, it is valuable to check whether the HT of a VAMS sample is within the validated HT range. Alternatively, the HT may be required to convert VAMS-based results to the corresponding plasma or serum concentrations. In **Chapter 4,** two straightforward methods are described that allow to derive the HT from a VAMS sample based on its K^+ content. One of these methods uses an aqueous extraction procedure, whereas the other one requires an organic extraction. Both methods have the potential to be seamlessly integrated with most existing VAMS analyses, allowing both target analyte quantitation and K^+ analysis on a single VAMS extract. These methods were successfully validated and applied to 95 venous patient VAMS samples with a broad HT range (0.21 – 0.45).

Although the K^+-based HT prediction method is straightforward, reliable, cheap and requires instrumentation that is available in every clinical laboratory, it does necessitate the destruction of (part of) a dried blood sample. As the sample volume that is available for analysis is already very limited with DBS analysis, a non-destructive alternative was developed. This method, which is described in **Chapter 5,** predicts the HT of a DBS based on its total Hb content and uses non-contact diffuse reflectance spectroscopy. The DBS are illuminated with halogen light, which is guided to the DBS surface *via* a fiber probe, and the light which is reflected by the DBS is transported *via* the same probe to a spectrometer. The reflectance spectra are fitted to a light transport model which takes into account the presence of oxyhemoglobin (OxyHb), methemoglobin (MetHb) and hemichrome (HC). This is essential, since Hb is originally present as OxyHb, which is then oxidized to MetHb and further denatured to HC upon storage (cfr. the change in color of a DBS upon aging). The fitting algorithm assigns a value with arbitrary units to each of the above-mentioned Hb derivatives and the sum of those values is used as a surrogate measure of total Hb and HT. After an elaborate analytical validation, the method was successfully applied to 233 venous patient DBS. This non-contact diffuse reflectance spectroscopy-based method overcomes the need for sample preparation, which in turn reduces the analysis time, minimizes the possibility of

errors and, importantly, eliminates the need for sample destruction. Basically, mere scanning of a DBS suffices to derive the HT of a DBS.

In **Chapter 6** a simplification of this non-destructive method is described. In this simplified method the HT is calculated using the reflectance at a single wavelength, located at the quasi-isosbestic point of 589 nm. At this wavelength the reflectance is insensitive to the Hb degradation and only scales with the total amount of Hb and, therefore, the HT. This simplification eliminates the need for a complicated algorithm to derive the total Hb content from the DBS's reflectance spectrum. Interestingly, this simplified method even slightly outperforms the original, spectrum-based one. Furthermore, it is demonstrated, using caffeine as a model compound, that this HT prediction method can be effectively used to implement a HT-dependent correction factor to DBS-based results to alleviate the HT bias.

As mentioned above, apart from developing strategies to compensate for the HT effect, an alternative sample collection technique was evaluated which tries to avoid the HT effect altogether. More particularly, **in Chapter 7**, a quantitative VAMS-based method is described to determine Co using inductively coupled plasma coupled to mass spectrometry (ICP-MS) for the follow-up of metal-on-metal (MoM) prosthesis patients. Co is monitored in this patient population as a marker of the degree of implant wear. The development of this method revealed that VAMS-based results may still be subjected to a HT effect, more particularly to HT-dependent extraction efficiency issues. This observation stresses the importance of evaluating the HT effect in VAMS methods as well, using both fresh and aged samples. After adequate optimization of the sample preparation procedure, a robust, HT- and VAMS age-independent method was obtained. Furthermore, application to patient samples (n = 78), revealed a good agreement between the venous VAMS results and those obtained on the corresponding liquid whole blood samples. Yet again, special attention was paid to develop a method which is simple, applicable to realistic sample volumes and compatible with the capabilities of most clinical laboratories equipped with ICP-MS analysis.

In the **'Broader international context, relevance and future perspectives'** section an overview is given of other strategies that have been developed during recent years to deal with the HT effect in DBS analysis. In general there are three main trends: i) the development of several devices that allow to generate fixed volume DBS (or other dried blood samples) starting from a non-volumetric drop of blood; ii) the development of devices that allow the *in*

situ generation of DPS; iii) the development of other methods to estimate and/or correct for the HT of dried blood samples. Importantly, there does not seem to be a 'best' way to deal with HT effect. Multiple strategies have proven to be valuable, and a combination of different approaches still provides the most complete solution. Which (combination of) approach(es) is best suited, depends on the context in which the dried blood microsample analysis will be employed. Aside from the recent developments with regard to dealing with the HT effect, also other advances that may facilitate routine implementation of dried blood sample-based methods are briefly discussed. Furthermore, a non-exhaustive overview is provided of real life DBS applications in various settings. In addition, to demonstrate the current general interest in home-based sampling, several other sampling formats that may facilitate this, are reviewed.

Samenvatting

Het doel van deze doctoraatsthesis was om kwantitatieve gedroogde bloedspot (dried blood spot - DBS) methoden dichter bij de routine te brengen door een aantal problemen aan te pakken die dit verhinder(d)en. Deze problemen omvatten het gebrek aan een validatierichtlijn voor kwantitatieve DBS-methoden en het gebrek aan geschikte manieren om op een adequate manier om te gaan met het hematocriet (HT) effect.

In **'General background, structure and objectives'** wordt een algemene inleiding gegeven over DBS *sampling* en analyse. DBS worden bereid door een (doorgaans niet-volumetrische) druppel bloed te collecteren op filterpapier. Deze bloeddruppel wordt verkregen *via* vinger- of hielprik, afhankelijk van de leeftijd van de patiënt. Staalname via DBS gaat gepaard met een aantal voordelen ten opzichte van de klassieke venapunctie, zoals de minimaal invasieve staalafname, het zeer klein staalvolume dat vereist is, de doorgaans verhoogde stabiliteit van de analyten en de eenvoudige manier van staalafname, transport en bewaring. DBS-analyse kent toepassingen in diverse gebieden en wordt gebruikt voor het bepalen van een brede waaier aan analyten. Het gebruik van DBS kent echter ook nog een aantal uitdagingen en problemen, waarvan het HT effect een van de meest belangrijke is. Het HT effect kan onderverdeeld worden in analytische en fysiologische deelaspecten. De analytische aspecten verwijzen naar het feit dat een analytisch DBS-gebaseerd resultaat afhankelijk kan zijn van het HT van een patiënt, terwijl het fysiologisch aspect verwijst naar de impact die het HT kan hebben op de interpretatie van een DBS-gebaseerd resultaat. In deze sectie wordt ook het gebruik van *volumetric absorptive microsampling* (VAMS) besproken. Bij VAMS wordt met behulp van een absorberende tip die vastzit aan een plastic staafje een accuraat bloedvolume gecollecteerd vertrekkende van een niet-volumetrische hoeveelheid bloed. Aangezien het volume bloed dat gecollecteerd wordt onafhankelijk is van het HT, zouden VAMS-gebaseerde resultaten in theorie ook HT-onafhankelijk moeten zijn.

Een meer gedetailleerd overzicht van bovenvermelde staalafnametechnieken, alsook andere alternatieve staalafnametechnieken, wordt gegeven in **Hoofdstuk 1**. Deze alternatieve staalafnametechnieken omvatten zowel patiëntvriendelijke manieren voor het collecteren van niet-conventionele matrices als het collecteren van de klassieke matrices (bloed, serum en plasma) op een niet-conventionele manier. Matrices die besproken worden, zijn: DBS, VAMS, gedroogde plasmaspots, gedroogde matrixspots, speeksel, interstitiële vloeistof, haar, tranen, adem, zweet en nasale mucus. Voor elke matrix worden de specifieke voordelen, nadelen en beperkingen besproken, alsook een aantal relevante toepassingen. Deze

toepassingen situeren zich in diverse gebieden, zoals *therapeutic drug monitoring* (TDM), neonatale screening, endocrinologie, toxicologie, fenotypering, proteomics en metabolomics. Er wordt hierbij bijzondere aandacht besteed aan de analyse van gedroogde bloedstalen aangezien dit het centrale onderwerp vormen van deze doctoraatsthesis. Bloed blijft namelijk de voorkeursmatrix voor vele toepassingen, aangezien er doorgaans een goede correlatie bestaat tussen de concentratie van een analyt in het bloed en het (therapeutisch) effect van deze component. TDM-gerelateerde toepassingen worden ook uitgebreider besproken, aangezien kwantitatieve DBS-methoden binnen dit domein reeds in beperkte mate in routine worden gebruikt.

Om ervoor te zorgen dat de kwaliteit van nieuw ontwikkelde DBS-methoden adequaat is, werd een validatierichtlijn voor DBS-methoden geschreven in samenwerking met het *Alternative Sampling Strategies Committee van de International Association of Therapeutic Drug Monitoring and Clinical Toxicology* (IATDMCT). Deze richtlijn wordt weergegeven in **Hoofdstuk 2**. De goede kwaliteit van DBS methoden is namelijk een *conditio sine qua non* om DBS-methoden te integreren in de routine. Aangezien de kwaliteit van een methode start met hoe de methode wordt opgezet, is een doordachte methode-ontwikkeling essentieel. Dit zorgt er namelijk voor dat een methode geschikt is voor het doel waarvoor ze werd ontworpen, wat ook de kans op een succesvolle methodevalidatie verhoogt. De kwaliteit van een methode moet geëvalueerd worden door middel van een analytische en een klinische validatie en moet voldoen aan vooraf vastgelegde acceptatiecriteria. Het allerbelangrijkste bij een methodevalidatie is dat elke parameter moet geëvalueerd worden op een manier die representatief is voor hoe de methode gebruikt zal worden in de praktijk. Doorheen deze richtlijn wordt natuurlijk bijzondere aandacht besteed aan de matrix-specifieke aspecten, zoals het HT-, volume- en chromatografisch effect. Bovendien wordt ook gesuggereerd hoe met deze effecten kan omgegaan worden. Hoewel deze richtlijn geschreven werd met het oog op de validatie van kwantitatieve DBS methoden die gebruik maken van LC-MS/MS en die gebruikt worden voor TDM, bevat ze vele aspecten die ook van toepassing zijn op andere methoden voor de analyse van gedroogde bloedstalen.

In de daaropvolgende hoofdstukken wordt het HT effect aangepakt. Dit gebeurde enerzijds door het ontwikkelen van methoden die toelaten het HT te bepalen (voorspellen) uitgaande van een gedroogd bloedstaal en anderzijds door het evalueren van VAMS, een HT-onafhankelijke staalafnametechniek.

De eerste methode die ontwikkeld werd in deze context wordt beschreven in **Hoofdstuk 5** en was in staat om het HT van een DBS te bepalen uitgaande van het kaliumgehalte in een DBS-extract. Kalium werd geselecteerd als HT merker, aangezien diens intracellulaire concentraties ongeveer 35 keer hoger zijn dan de extracellulaire concentraties en de kaliumspiegel fysiologisch nauw gecontroleerd wordt. Aangezien de meeste cellen in bloed rode bloedcellen zijn, dragen deze (en dus het HT) het meest bij tot de K^+-concentratie in volbloed. De methode omvat een tweestapsextractie van een 3 mm DBS *punch* met MilliQ-water dat 2.5 mM KCl bevat en het kaliumgehalte werd bepaald door middel van een routine *analyzer* die gebruik maakt van indirecte potentiometrie. Na een succesvolle analytische validatie, werd de methode toegepast op 111 veneuze DBS van patiënten met een HT tussen 0.19 en 0.63, wat goede resultaten opleverde. Gezien zijn eenvoud en snelheid, kan deze kalium-gebaseerde methode eenvoudig toegepast worden in elk geautomatiseerd klinisch laboratorium. De HT waarde die op deze manier bepaald wordt, kan gebruikt worden om na te gaan of het HT van een DBS binnen het gevalideerde HT-interval ligt of kan gebruikt worden om een HT-afhankelijke correctiefactor toe te passen op een DBS-resultaat om de impact van het HT effect te compenseren. Daarnaast kan deze HT waarde gebruikt worden om DBS-gebaseerde resultaten om te rekenen naar de overeenkomstige plasma- of serumwaarden.

Deze kalium-gebaseerde HT-- predictiemethode werd aangepast, zodat deze ook kan gebruikt worden voor VAMS stalen. Hoewel VAMS-gebaseerde resultaten in theorie onafhankelijk zijn van het HT van een patiënt, blijkt dit in de praktijk niets steeds zo te zijn, aangezien VAMS gevoelig is voor HT-afhankelijke extractieproblemen. Daarom kan het nuttig zijn om na te gaan of het HT van een VAMS staal zich binnen het gevalideerde HT-interval bevindt. Daarnaast kan het HT ook hier gebruikt worden om VAMS-gebaseerde resultaten om te zetten in de corresponderende plasma- of serumwaarden. In **Hoofdstuk 4** worden twee eenvoudige methoden beschreven die toelaten het HT te bepalen op basis van het kaliumgehalte van een VAMS extract. De eerste methode maakt gebruik van een waterige extractie, terwijl de tweede methode gebaseerd is op een organische extractieprocedure. Beide methoden kunnen geïntegreerd worden in de meeste VAMS-analyseprocedures, waardoor zowel de eigenlijke doelanalyt als het HT bepaald kunnen worden op één VAMS-extract. De methoden werden succesvol gevalideerd en toegepast op 95 veneuze VAMS stalen van patiënten met een HT tussen 0.21 en 0.45.

Hoewel de kalium-gebaseerde aanpak eenvoudig, betrouwbaar en goedkoop was en enkel instrumentatie vereiste die in elk klinisch laboratorium aanwezig is, verbruikte het wel (een deel van) een gedroogd bloedstaal. Aangezien het beschikbaar staalvolume sowieso al zeer beperkt is, werd een niet-destructieve methode ontwikkeld om het HT van een DBS te bepalen. Deze methode, die beschreven wordt in **Hoofdstuk 5**, kan het HT van een DBS bepalen op basis van zijn Hb gehalte en maakt gebruik van *non-contact diffuse reflectance spectroscopy.* Dit houdt in dat halogeen licht naar het oppervlak van de DBS wordt geleid met behulp van een *fiber probe* en dat het deel van dat licht dat wordt gereflecteerd door de DBS *via* diezelfde probe naar een spectrometer wordt geleid. De reflectiespectra die op die manier worden verkregen, worden gefit op een licht transportmodel dat rekening houdt met de aanwezigheid van zowel oxyhemoglobine (OxyHb), methemoglobine (MetHb) en hemichroom (HC). Dit is essentieel, aangezien Hb in een DBS oorspronkelijk aanwezig is onder de vorm van OxyHb, vervolgens geoxideerd wordt tot MetHb en nadien gedenatureerd wordt tot HC. Deze reacties zijn ook de reden waarom DBS van kleur veranderen bij bewaring. Het algoritme dat instaat voor deze fitting zal, afhankelijk van hoeveel van de verschillende Hb derivaten aanwezig is, een bepaalde waarde (in arbitraire eenheden) toekennen aan elk Hb derivaat. Het is de som van die waarden die gebruikt wordt als een surrogaatmerker voor het totaal Hb en het HT. Na een uitgebreide analytische validatie, werd de methode met succes toegepast op 233 veneuze DBS van patiënten. Deze non-contactmethode elimineert de nood aan enige staalvoorbereiding, wat resulteert in een zeer korte analysetijd, een verminderd risico op menselijke fouten en natuurlijk in het feit dat een DBS volledig kan gebruikt worden voor de eigenlijke analyse van de *target analyte*. Het volstaat namelijk om een DBS als het ware te scannen om het HT te achterhalen.

In **Hoofdstuk 6** wordt een vereenvoudiging van deze non-contactmethode beschreven. Bij deze vereenvoudigde methode wordt het HT bepaald aan de hand van de reflectie gemeten bij één enkele golflengte, namelijk bij het quasi-isosbestisch punt van 589 nm. Bij deze golflengte is de gemeten reflectie onafhankelijk van het type Hb derivaat dat aanwezig is en daardoor enkel afhankelijk van het totaal Hb en dus het HT. Door deze vereenvoudiging is er geen complex algoritme meer nodig om het totaal Hb af te leiden uit het reflectiespectrum. Bovendien leverde deze vereenvoudigde methode zelfs iets betere resultaten op dan de oorspronkelijke, meer complexe, spectrum-gebaseerde methode. Daarnaast werd ook aangetoond dat het HT dat op deze manier werd bepaald, gebruikt kan worden om een HT-

afhankelijke correctiefactor toe te passen op DBS-gebaseerde resultaten om op die manier de HT-bias weg te werken. Hiertoe werd cafeïne gebruikt als modelcomponent.

Naast het ontwikkelen van HT-predictiemethoden om te compenseren voor het HT-effect, werd, zoals eerder vermeld, ook een staalafnametechniek geëvalueerd die in principe onafhankelijk zou moeten zijn van het HT van een staal. I**n Hoofdstuk** 7 wordt meer bepaald een kwantitatieve VAMS-gebaseerde methode beschreven die gebruikt kan worden om, door middel van inductief gekoppeld plasma in combinatie met massaspectrometrie (ICP-MS), Co te monitoren in patiënten met metaal-op-metaal (MoM) prothesen. Bij deze patiënten worden de Co-spiegels opgevolgd als een merker van protheseslijtage. Bij het op punt stellen van deze methode werd duidelijk dat VAMS-gebaseerde methoden toch onderhevig kunnen zijn aan een HT-effect, meer bepaald aan een HT-effect op de extractie-efficiëntie. Deze bevinding benadrukt dat het van belang is om het HT-effect toch te evalueren bij VAMS-methoden en dit zowel op verse als oudere stalen. Na optimalisatie van de staalvoorbereidingsprocedure, werd uiteindelijk een robuuste methode bekomen die onafhankelijk was van het HT of de ouderdom van de VAMS-stalen. Toepassing van deze methode op patiëntenstalen (n = 78) toonde een goede overeenkomst tussen de resultaten bekomen op volbloed en veneuze VAMS. Ook bij het ontwikkelen van deze methode werd er op gelet dat deze zo eenvoudig mogelijk was, toepasbaar was in de praktijk en gebruik maakte van toestellen die aanwezig zijn in een klinisch laboratorium dat ICP-MS analyses uitvoert.

In de sectie '**Broader international context, relevance and future perspectives'** wordt een overzicht gegeven van de verschillende strategieën die tijdens de laatste jaren werden ontwikkeld om om te gaan met het HT-effect in DBS-analyse. Er zijn drie belangrijke trends: i) de ontwikkeling van systemen die toelaten om een volumetrisch gedroogd bloedstaal te verkrijgen uitgaande van een niet-volumetrische bloeddruppel; ii) de ontwikkeling van systemen die toelaten *in situ* DPS te genereren; en iii) de ontwikkeling van andere HT predictie- en/of HT correctiemethoden. Er blijkt tot op heden geen 'beste' manier te zijn om om te gaan met het HT effect. Meerdere strategieën hebben hun meerwaarde reeds aangetoond en een combinatie van verschillende strategieën lijkt nog steeds de meest volledige oplossing te bieden. Welke strategie of combinatie van strategieën het meest geschikt is, hangt af van de context waarin een methode moet toegepast worden. Naast de evoluties op het vlak van omgaan met het HT effect, worden ook een aantal andere recente ontwikkelingen aangehaald die kunnen bijdragen tot het meer wijdverspreid gebruik van

gedroogde bloedstalen. Verder wordt ook de huidige status besproken van het routinematig gebruik van DBS in verschillende toepassingsgebieden. Om aan te tonen dat er momenteel een veralgemeende interesse is in alternatieve staalafnamestrategieën die het mogelijk maken om stalen te collecteren in de thuisomgeving, worden bovendien een aantal nieuwe *formats* kort overlopen.

Curriculum Vitae

Personalia

Last name:	Capiau
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Education

2013 - present:	Master of Science in Clinical Biology
2018 – 2019:	Internship clinical biology – AZ Sint-Jan Brugge, Belgium
2017 – 2018:	Internship clinical biology – ASZ Aalst, Belgium
2013 - 2019:	Doctoral Training Programme Ghent University
2011 - 2013:	Master of Drug Development (summa cum laude; Scientific prize of the Bank van Breda)
2008 - 2011:	Bachelor of Pharmaceutical Sciences (summa cum laude)
2002 – 2008:	Mathematics-Sciences, Sint-Aloysiuscollege, Ninove

Work experience

Scientific experience

2013 – 2017:	PhD-student in the Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Belgium (Prof. Christophe Stove)
2014:	5 month research stay at the Department of Biomedical Engineering and Physics of the Academic Medical Center, University of Amsterdam, The Netherlands (Prof. Maurice Aalders).

Educational experience

2013 – 2015:	Support of practical courses and demonstrations for Toxicology, Master in Pharmaceutical Care and Drug Development
2014 – 2017:	Support of practical courses and demonstrations for Bioanalytical Practicum, Bachelor in Pharmaceutical Sciences
2014 – 2015:	Support of Master dissertations, Master in Pharmaceutical Care: Saar Favoreel. De ontwikkeling van een HPLC-MS/MS multi-analytmethode voor de detectie en kwantificatie van drugs in volbloed.
2015 – 2016:	Support of Master dissertations, Master in Pharmaceutical Care: Stefanie Marcos-Cayuelas. Vergelijkende studie: klassieke volumetrische DBS-staalname *versus microfluidics*-gebaseerde DBS staalname.

2015 – 2016:	Tutor Pharmaceutical Bachelor dissertation
2016 – 2017:	Support of Master dissertations, Master in Pharmaceutical Care: Elise Van Belleghem. The use of potassium as a HT marker in volumetric absorptive microsampling samples.
2017 – 2018:	Support of Bachelor dissertations, Bachelor in Medical Laboratory Technology: Nina Van Londerseele. Validatie van het STA-® NeoPTimal reagens voor de bepaling van de protrombinetijd met het STA-R® Evolution stollingstoestel.

Scientific curriculum

A1 publications

1. **Capiau S**, Stove VV, Lambert WE, Stove CP. Prediction of the hematocrit of dried blood spots via potassium measurement on a routine clinical chemistry analyzer. *Analytical Chemistry*. 2013;85(1):404-10. (Q1: 4/76)

2. 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. (Q2: 27/78)

3. De Kesel PM, **Capiau S**, Lambert WE, Stove CP. Current strategies for coping with the hematocrit problem in dried blood spot analysis. *Bioanalysis*. 2014;6(14):1871-4. (Q2: 25/79)

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8. **Capiau S,** Wilk LS, De Kesel PMM, Aalders MCG, Stove CP. Correction for the hematocrit bias in dried blood spot analysis using a nondestructive, single-wavelength

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B2. publications

1. **Capiau S**, Alffenaar J-WC, Stove CP. Chapter 13 Alternative sampling strategies for therapeutic drug monitoring. *Clinical Challenges in Therapeutic Drug Monitoring, 1st edition. Ed. Clarke W. and Dasgupta A. 2016. Elsevier.*

Oral presentations

1. 1st European Congress of Mass Spectrometry: Applications to the Clinical Lab (MSACL), Salzburg, Austria, September 2-5, 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)

2. Alternative Sampling Strategies in Toxicology and Therapeutic Drug Monitoring, Ghent, Belgium, September 18-19, 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)

3. 14th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology (IATDMCT), Rotterdam, The Netherlands, October 11-15, 2015. An innovative method to estimate the hematocrit of dried blood spots. (Capiau S, Wilk LS, Aalders MCG, Stove CP).

4. European Bioanalysis Forum, 2nd Young Scientist Symposium (YSS), Barcelona, Spain, November 17th, 2015. A novel non-contact method to estimate the hematocrit of dried blood spots. (Capiau S, Wilk LS, Aalders MCG, Stove CP).

5. European Bioanalysis Forum, 8th Open Symposium (EBF), Barcelona, Spain, November 18-20, 2015. Different strategies for coping with the hematocrit effect in dried blood micro-sampling. (Capiau S, Stove CP)

6. 9th International meeting of the international society of newborn screening & the 10th ISNS European Regional Meeting (ISNS), The Hague, The Netherlands. September 11 – 14, 2016.. A novel non-contact method to determine the hematocrit of dried blood spots. (Capiau S, Wilk LS, Aalders MCG, Stove CP).

7. 3rd Young scientist symposium of the European Bioanalysis Forum (YSS), Barcelona, Spain. November 15th, 2016. Development and validation of an ICP-MS method to quantitatively determine cobalt in volumetric absorptive microsampling (VAMS) devices for the follow-up of metal-on-metal prosthesis patients. (Capiau S, Bolea-Fernandez E, Balcaen L, Van Der Straeten C, Verstraete A, Vanhaecke F, Stove CP)

8. 3rd Young scientist symposium of the European Bioanalysis Forum (YSS), Barcelona, Spain. November 15th, 2016. Feedback from the pre-competitive collaborative work on liquid microsampling between AstraZeneca, Charles River, Covance, GlaxoSmithKline, Ghent University, Janssen R&D LGC and Q2-solutions. (Ahmad S, Swinnen A, Naseer H, Bell E, Capiau S, Lucey R, Dawson I and De Ruijter D, on behalf of the Young Scientist Microsampling consortium)

9. 15th international congress of therapeutic drug monitoring and clinical toxicology (IATDMCT), Kyoto, Japan, September, 24 – 28, 2017. Analytical perspective on developing a DBS assay for daily routine. (Capiau S, Veenhof H, Stove CP and Alffenaar J-WC, on behalf of the IATDMCT alternative sampling strategies committee)

10. Stago users meeting, Antwerp, Belgium, May 17, 2018. Verificatie van het STA® NeoPTimal reagens (Capiau S, Van Londerseele N, Meuleman P).

11. 18e orthopediedag, Lede, Belgium, September 8, 2018. Optimalisatie van het transfusiebeleid: een multi-disciplinaire aanpak (Capiau S, Bril T)

Poster presentations

1. Annual meeting of Belgian Clinical Biologists (BVKB), Ghent, Belgium, October, 2012. Prediction of the hematocrit of dried blood spots via potassium measurement on a routine clinical chemistry analyzer. (Capiau S, Stove VV, Lambert WE, Stove CP)

2. 2nd Annual European Congress of Mass Spectrometry: Applications to the Clinical Lab (MSACL), Salzburg, Austria, September 8-11, 2015. The development, validation and

application of a novel non-contact method for the hematocrit prediction of dried blood spots. (Capiau S, Wilk LS, Aalders MCG, Stove CP)

3. The Eurachem workshop on method validation in analytical science: current practices and future challenges, Ghent, Belgium, May 9-10, 2016. Validation of a novel non-contact method to estimate the hematocrit of dried blood spots. (Capiau S, Wilk LS, Aalders MCG, Stove CP)

4. The 15th International Congress of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT), Kyoto, Japan, September 24 – 28, 2017. Development and validation of an ICP-MS method to quantitatively determine cobalt in volumetric absorptive microsampling (VAMS) devices for the follow-up of metal-on-metal prosthesis patients. (Capiau S, Bolea-Fernandez E, Balcaen L, Van Der Straeten C, Verstraete A, Vanhaecke F, Stove CP)

5. The Joint National Symposium of the Royal Belgian Society of Laboratory Medicine (RBSLM) and the Belgian Thyroid Club, Brussels, Belgium, November 16th, 2018. Development, validation and application of a method to determine cobalt in metal-on-metal prosthesis patients using volumetric absorptive microsampling. (Capiau S, Bolea-Fernandez E, Balcaen L, Van Der Straeten C, Verstraete A, Vanhaecke F, Stove CP)

6. The Joint National Symposium of the Royal Belgian Society of Laboratory Medicine (RBSLM) and the Belgian Thyroid Club, Brussels, Belgium, November 16th, 2018. The Evaluation of STA® NeoPTimal, A new reagent for prothrombin time determination. (Capiau S, Van Londerseele N, Meuleman P)

7. The Joint National Symposium of the Royal Belgian Society of Laboratory Medicine (RBSLM) and the Belgian Thyroid Club, Brussels, Belgium, November 16th, 2018. The comparison of an antigen test with several molecular assays for the detection of RSV and Influenza A/B in nasopharyngeal specimens. (Capiau S, Troch T)

Other

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- Interview on dried matrices in bioanalysis for Bioanalysis Zone. March 2016. http://www.bioanalysis-zone.com/videos-podcasts/

- Patent: US10222324B1 en EPA15188354: Dried blood sample analysis; Inventors: Maurice Aalders, Leah Wilk, Sara Capiau, Christophe Stove; Current Assignee: Ghent University.

Scientific prizes

Poster prize at the annual meeting of Belgian Clinical Biologists (BVKB), Ghent, Belgium, October, 2012

Membership of scientific organizations

International Association for Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT)
Toxicological Society of Belgium and Luxemburg (BLT)
The Royal Belgian Society of Laboratory Medicine (RBSLM)

ResearchGate page

https://www.researchgate.net/profile/Sara_Capiau