GHENT UNIVERSITY

FACULTY OF PHARMACEUTICAL SCIENCES

Novel direct dried blood spot-based approaches for forensic toxicology and clinical applications

Nele SADONES

2017

Promoters: Prof. Dr. Christophe Stove & Prof. Dr. Willy Lambert

Thesis submitted to obtain the degree of Doctor in Pharmaceutical Sciences

Copyright

Ghent, 2017,

The promoters,

Prof. Dr. Christophe Stove

Prof. Dr. Willy Lambert

The author,

Nele Sadones

Dankwoord

Een woord van dank aan iedereen die rechtstreeks of onrechtstreeks heeft bijgedragen tot dit werk.

Vooreerst wil ik mijn promotoren prof. Christophe Stove en prof. Willy Lambert bedanken. Zonder hen kon ik uiteraard nooit starten aan een doctoraat. Dank u wel om mij deze kans te geven! Jullie constructieve feedback heeft in belangrijke mate bijgedragen tot het realiseren van dit werk. Bedankt prof. Stove om het vele schrijfwerk telkens opnieuw kritisch te verbeteren. Gezien je overvolle agenda heb je hiervoor heel wat uren van je slaap opgeofferd. Prof. Lambert, bedankt voor de steun de voorbije jaren. Zowel op professioneel als op menselijk vlak ben je een voorbeeld.

Bedankt aan alle collega's waarmee ik heb samengewerkt. Dit werk is ook een deel van jullie. Jullie hebben er allemaal op een of andere manier voor gezorgd dat dit tot stand kwam. Ik kon steeds bij jullie terecht. In het bijzonder wil ik hierbij Jolien en Sofie bedanken om me te steunen! Jullie begrepen me met weinig woorden. Uitermate belangrijk aangezien ik niet zo'n grote spraakwaterval ben ;-) Ann-Sofie, bedankt om me in te leiden in de wereld van de GC. Filip, bedankt om de gasflessen te vervangen. Ook bij heel wat andere praktische problemen was een ingenieur soms iets handiger dan een apotheker ;-) Ook wil ik de thesisstudenten bedanken voor de vele analyses die ze uitgevoerd hebben en Annelies, sorry als ik jouw strakke planning moest doorbreken voor het uitvoeren van heel wat PCR-reacties.

Iedereen die betrokken was bij de verschillende studies wil ik natuurlijk ook bedanken. Ann-Sofie, Michelle Wood, Dr. Archer, Dr. Dargan, Dr. Wood en prof. Neels wil ik bedanken voor hun bijdrage aan de GHB studie. Eveneens een woord van dank aan prof. Van Bortel en Elien voor de vlotte samenwerking en het opzetten van de gabapentine studie. Pieter en Sara, dank u wel, om mee te helpen bij het verzamelen van stalen. Prof. Delanghe wens ik te bedanken voor het delen van zijn haptoglobine kennis. Het 24 uurs-labo van UZ Gent verdient ook een speciaal woord van dank voor de vele bloedafnames en het uitvoeren van de hematocriet bepalingen.

Jolien verdient natuurlijk nog een extra woord van dank. Zij was immers het creatieve brein die de kaft heeft ontworpen. Dikke merci Jolien! Zonder jou zou het een blanco kaft geweest zijn ;-)

Mama, papa en Ben, 1000x bedankt! Geen woorden om te beschrijven hoe dankbaar ik jullie ben.

Table of contents

List of abbreviations

1,2-BD	1,2-butanediol
1,4-BD	1,4-butanediol
6-MAM	6-monoacetylmorphine
% RE	% residual error
ACN	Acetonitrile
ACP	2-amino-5-chloropyridine
BE	Benzoylecgonine
BHB	Beta-hydroxybutyric acid
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
C	Complete DBS
cDBS	Capillary DBS
CE	Cocaethylene
d	Days
DAD	Diode array detection
DBS	Dried blood spot(s)
DEG	Diethylene glycol
DFSA	Drug-facilitated sexual assault
DNA	Deoxyribonucleic acid
DRUID	Driving under the influence of drugs, alcohol and medicines
DUID	Driving under the influence of drugs
EBF	European Bioanalysis Forum
ECME	Ecgonine methylester
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EMA	European Medicines Agency
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EMDP	2-ethyl-5-methyl-3,3-diphenylpyrroline
EME	Electromembrane extraction
FAEE	Fatty acid ethyl esters
FDA	Food and Drug Administration
FID	Flame ionization detection
GBL	Gamma-butyrolactone
GC	Gas chromatography
GC-MS	Gas chromatography coupled to mass spectrometry
GHB	Gamma-hydroxybutyric acid
h	Hours
Hct	Hematocrit

HFB	Heptafluorobutanol
Hp	Haptoglobin
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HS-GC-FID	Headspace sampling-gas chromatography-flame ionization detection
i.d.	Internal diameter
IQ Consortium	The International Consortium for Innovation and Quality in Pharmaceutical Development
IS	Internal standard(s)
LC	Liquid chromatography
LC-DAD	Liquid chromatography-diode array detection
LC-FLUO	Liquid chromatography-fluorescence detection
LC-HRMS(/MS)	Liquid chromatography-high resolution (tandem) mass spectrometry
LC-(MS/)MS	Liquid chromatography coupled to (tandem) mass spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxy-N-ethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MeOH	Methanol
MEPS	Microextraction packed sorbent
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MTBSTFA	N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
n.a.	Not applicable
NA	Not analyzed/ not available
NS	Not studied
P	Punch
PAE	Prenatal alcohol exposure
PCR	Polymerase chain reaction
PEth	Phosphatidylethanol
PG	Propylene glycol
PK	Pharmacokinetics
PP	Protein precipitation
QC	Quality control

RBC	Red blood cell
RECOV	Recovery
RIA	Radioimmunoassay
RT	Room temperature
SAMPLE PREP	Sample preparation
SCD	Sample collection device
SIM	Selected ion monitoring
SPE	Solid phase extraction
STA	Systematic toxicological analysis
TAE	Tris-acetate-ethylenediaminetetra-acetic acid
TBME	Tert-butyl methyl ether
TDM	Therapeutic drug monitoring
TFAA	Trifluoroacetic anhydride
THC	Δ^9-tetrahydrocannabinol
THC-COOH	Tetrahydrocannabinol carboxylic acid
THC-OH	11-hydroxy- Δ^9-tetrahydrocannabinol
U(H)PLC	Ultra-performance liquid chromatography
ULOQ	Upper limit of quantification
VAMS	Volumetric absorptive microsampling
vDBS	Venous DBS
w	Weeks

AIM AND OUTLINE OF THE THESIS

Dried blood spots (DBS) are the common thread throughout this work. They have been used in both an analytical context and for the development of a genotyping method.

DBS sampling is a microsampling technique in which a drop of capillary blood, derived from a finger or heel stick, is collected on special filter paper. This approach has been used successfully in newborn screening since it was introduced in the 1960s by Guthrie and Susi to determine phenylketonuria in neonates [1]. During the last decade, thanks to the development of more sensitive analytical techniques, DBS sampling -and microsampling in general- has gained its place in a lot of other fields, amongst which pharmaco- and toxicokinetics [2-5]. In toxicokinetics the relationship between the systemic exposure to a compound and the harmful effects of this compound is determined. In the preclinical context, DBS-based toxicokinetics offer both an improvement in animal welfare, as well as a financial benefit. Consequently, a lot of pharmaceutical companies and contract research organizations have undertaken efforts to utilize microsampling (liquid or DBS-based) as an alternative to plasma or serum in the (pre)clinical phases of the drug discovery process. DBS do not only have a potential for the analysis of therapeutic drugs, they are also suitable for the detection of drugs of abuse, as outlined in **Chapter 1, which serves as an Introductory Chapter to DBS in general, with a particular focus on DBS applications in toxicology**. More specifically, the analysis of abused substances is discussed, along with the associated benefits, limitations and challenges. While the focus of this Introductory Chapter primarily lies on forensic applications, it is clear that the determination of drugs of abuse may also have a potential for e.g newborn screening, more specifically to assess prenatal exposure to toxic compounds, or for therapeutic drug monitoring (TDM).

The increased use of DBS sampling in the clinical arena is the logical consequence of the many advantages associated with this sampling technique [6]. Indeed, DBS sampling is a very easy and inexpensive way of taking a representative sample, which can even be performed by the patient himself in his home environment or in remote areas, eliminating the need for a trained phlebotomist [7-9]. Moreover, the dried matrix improves the stability of most compounds [10-13], enabling more cost-effective transport and storage; DBS samples are generally transported via regular mail [9,14] and can often be stored at ambient temperature for prolonged periods of time. Since only small volumes of blood -typically between 10 and 80 µL- are collected, the DBS sampling technique is highly suitable for the collection of samples during preclinical and toxicokinetic studies involving animals. DBS sampling certainly confines to the principles of the 3R's (replacement, reduction and refinement), as fewer animals are needed and sampling procedures are refined using DBS [15,16]. In addition, the advantage of small blood volumes, combined with the possibility to collect samples by a minimally invasive finger- or heelprick, creates a high potential for the use of DBS in pediatric studies as well [17,18]. Another benefit of using DBS instead of liquid samples is that pathogens that may be present in blood are deactivated upon drying, strongly reducing the risk of infection [19]. Furthermore, from the analytical point of view, the process of generating DBS can also be considered as a simple sample preparation procedure [20,21]. Indeed, many analytes can selectively be extracted from the cards, often allowing straightforward and automatable processing and analysis [22-27]. Despite the numerous applications using DBS and the many advantages

associated with this sampling procedure, DBS sampling still faces some important challenges. Box 1 summarizes the advantages and challenges associated with DBS sampling [6].

Box 1. Advantages and challenges associated with DBS sampling.

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

A first concern is whether venous and capillary blood concentrations of an analyte are equivalent. Obviously, this is an aspect that needs to be evaluated on a case-by-case basis and may depend on the aim of the study (e.g. setting up a toxicokinetic profile versus measurement of trough levels). This concern, which is inherent to any non-venous sampling technique, has been addressed in several recent publications [7,28-37]. In these comparative studies, both capillary DBS (cDBS) (obtained by a fingerprick) and venous blood (obtained by classical venipuncture) are collected. The latter is analyzed as such and/or is used to generate venous DBS (vDBS). A cross-comparison between vDBS and venous blood allows to investigate the validity of the DBS approach. Although equal analyte concentrations are expected -there is only a difference in presentation- the entire process of spotting, drying and storing the samples may have an influence on the analytical result (a so called “DBS effect”). A cross-comparison between cDBS and vDBS concentrations allows to evaluate if there is a difference between capillary and venous blood. Capillary blood is a mixture of blood obtained from venules, arterioles and capillaries. Differences between capillary and venous blood may be due to the time of sampling as well as to the physicochemical characteristics of the analyte. E.g. small compounds which are highly lipid-soluble and have a low protein binding capacity can easily cross through the capillary walls into the interstitial and intracellulair fluids, inducing an arteriovenous difference in the early distribution phase. While for many abused substances, a proof-of-principle of “detectability” in DBS has been demonstrated, there is **a paucity of reports that have actually compared venous-capillary concentrations. In Chapter 2, we set out to do this for gamma-hydroxybutyric acid (GHB)**, a drug which is misused in the dance scene and which has also been associated with drug-facilitated sexual assaults [38].

Other concerns associated with DBS-based sampling are the acquisition of correctly obtained samples, contamination risk, the chromatographic or volcano effect, the site of punching and sensitivity issues in case of analytes at very low concentrations [6]. The latter implies the implementation of sensitive analytical techniques for DBS analysis. Although in many cases liquid chromatography coupled to (tandem) mass spectrometry (LC-MS(/MS)) is the most appropriate technique, gas chromatographic coupled to mass spectrometry (GC-MS) methods are still being used [39,40]. They offer the advantage that a wide range of compounds with widely varying polarity can be analyzed using one single standard configuration. However, to achieve adequate sensitivity with GC-MS, DBS analysis may involve derivatization [40]. As this additional sample preparation step is considered as laborious and time-consuming, improvements to simplify and/or shorten this step are warranted. In this context, we wished to speed up a procedure that had previously been established in our laboratory, coined "on-spot derivatization" in which derivatization reagents are directly applied to DBS [41]. Given the known advantages of "microwave derivatization" in terms of shortening derivatization times, **we aimed at combining "on-spot derivatization" with "microwave derivatization",** thereby ensuring a minimal, economic and fast sample workup and high sample throughput **(Chapter 3)**. We particularly focused on analytes where GC-MS may be competitive with LC-MS/MS because of challenges with detection and/or the need to use special configurations for LC-MS/MS-based detection. Apart from applying the newly developed approach on DBS obtained from potential GHB positive cases, we also **aimed at applying this approach on real samples for the assessment of other polar low molecular weight compounds, more particularly the anti-epileptic drug gabapentin (Chapter 4) and for the ketone body beta-hydroxybutyric acid (BHB) (Chapter 5).** While for gabapentin, the DBS-based approach is primarily relevant from a sampling-point-of-view, for BHB, the advantage primarily lays in the simplification of sample preparation.

We aimed at evaluating the DBS-based approach for a therapeutically applied compound, gabapentin, for a variety of reasons (**Chapter 4**). First, gabapentin is, just like GHB, structurally related to the inhibitory neurotransmitter gamma-amino butyric acid. Second, this application may add new and relevant data to the ongoing discussion about the implementation of DBS sampling in TDM. The use of dried matrix spots for TDM purposes, with sampling either in the clinic or at the patient's home, has many advantages. E.g. patients can easily collect their own samples at home. Since gabapentin is an anticonvulsant taken by many out-patients [42], DBS could be a useful tool for these patients: monitoring their gabapentin concentrations may be relevant to guarantee that therapeutic concentrations are reached and supratherapeutic concentrations are avoided. The determination of gabapentin may not only be useful for TDM (including clinical toxicology), but also for the determination in a forensic context. E.g. there is an increased illegal use of gabapentin in Britain's opiate-using and prison populations [43]. **A second aim of determining gabapentin in DBS was to evaluate the correlation between blood and serum concentrations (Chapter 4)**. This is relevant as therapeutic ranges for gabapentin are available in plasma or serum, but not in blood. In the context of a pharmacokinetic study for gabapentin we therefore conducted a so-called "bridging study". Such studies, in which a comparison is made between concentrations obtained from DBS and those obtained from plasma or serum, are an essential step for the implementation in routine practice (**Chapter 4**).

Second, we aimed at applying the "microwave-assisted on-spot derivatization" approach for the assessment of BHB, a molecule of relevance in forensic toxicology (**Chapter 5**). BHB is an endogenous compound structurally related to GHB. Elevated levels of this ketone body are found in ketotic diabetics and alcoholics and may induce ketoacidosis (i.e. reduced blood pH caused by an increased level of the ketone bodies). Although ketoacidosis may contribute to the cause of death, it has not been unequivocally established what the relevance is of routinely determining BHB in all cases in the forensic laboratory, when already having at hand the results of acetone, another ketone body. To address this question, **we aimed at setting up a retrospective analysis of a large number of biological forensic samples (Chapter 5).** Importantly, as BHB can be assessed in a variety of matrices, we wished to extend in this context the above-described DBS approach to a "dried matrix" approach, wherein we also evaluated dried urine and dried vitreous samples, which were volumetrically applied (**Chapter 5**).

In addition to the above-mentioned bio-analytical DBS-based applications, **we also wished to set up a DBS-based genotyping method**. The haptoglobin (*Hp*) gene, encoding for an abundant hemoglobin-binding plasma protein, was chosen, as polymorphisms in this gene -with 2 common alleles, denoted 1 and 2- may have important biological and clinical consequences [44,45]. As there is a perfect correlation between the *Hp* pheno- and genotype, PCR-based methods have emerged as a good alternative for time-consuming protein-based phenotyping methods. E.g. large-scale studies have unequivocally shown that diabetics with the *Hp 2-2* phenotype have a 2-5-fold increased risk for developing micro- and macrovascular complications [46-49]. In **Chapter 6**, **we wished to evaluate whether we could improve and simplify the current 'gold standard' of *Hp* genotyping** by developing an extractionless procedure, directly starting from DBS micropunches, allowing *Hp* genotyping in a high-throughput setting.

In **Chapter 7** the broader international context, the relevance and the future perspectives are described. **Chapter 8** gives a summary and a general conclusion. Box 2 outlines the aims of each Chapter in this thesis.

Box 2. Overview of the aims of each Chapter.

Chapter	Aim
1	Introductory Chapter to DBS
2	Correlation between venous and capillary blood concentrations of GHB
3	Set-up of a new derivatization strategy for DBS: microwave-assisted on-spot derivatization
4	Application of a new derivatization strategy: correlation between blood and serum concentrations of gabapentin (bridging study)
5	Application of a new derivatization strategy: relevance of BHB determination in routine forensic toxicology analysis
6	DBS application: set-up of an improved *Hp* genotyping method
7	Broader international context, relevance and future perspectives
8	Conclusion and summary

References

1. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 32(3), 338-343 (1963).
2. Edelbroek P, van der Heijden J, Stolk L. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Ther Drug Monit* 31(3), 327-336 (2009).
3. Li W, Tse FLS. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed Chromatogr* 24(1), 49-65 (2010).
4. Stove CP, Ingels ASME, De Kesel PMM, Lambert WE. Dried blood spots in toxicology: from the cradle to the grave? *Crit Rev in Toxicol* 42(3), 230-243 (2012).
5. Demirev PA. Dried blood spots: analysis and applications. *Anal Chem* 85(2), 779-789 (2013).
6. De Kesel PMM, Sadones N, Capiau S, Lambert WE, Stove CP. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis* 5(16), 2023-2041 (2013).
7. Fokkema MR, Bakker AJ, de Boer F, Kooistra J, de Vries S, Wolthuis A. HbA1c measurements from dried blood spots: validation and patient satisfaction. *Clin Chem Lab Med* 47(10), 1259-1264 (2009).
8. Kromdijk W, Mulder JW, Smit PM, Ter Heine R, Beijnen JH, Huitema AD. Therapeutic drug monitoring of antiretroviral drugs at home using dried blood spots: a proof-of-concept study. *Antivir Ther* 18(6), 821-825 (2013).
9. Ingels AS, Hertegonne KB, Lambert WE, Stove CP. Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home: an exploratory study. *CNS drugs* 27(3), 233-237 (2013).
10. Bowen CL, Hemberger MD, Kehler JR, Evans CA. Utility of dried blood spot sampling and storage for increased stability of photosensitive compounds. *Bioanalysis* 2(11), 1823–1828 (2010).
11. Bowen CL, Volpatti J, Cades J, Licea-Perez H, Evans CA. Evaluation of glucuronide metabolite stability in dried blood spots. *Bioanalysis* 4(23), 2823–2832 (2012).
12. D'Arienzo CJ, Ji QC, Discenza L *et al.* DBS sampling can be used to stabilize prodrugs in drug discovery rodent studies without the addition of esterase inhibitors. *Bioanalysis* 2(8), 1415–1422 (2010).
13. Faller A, Richter B, Kluge M, Koenig P, Seitz HK, Skopp G. Stability of phosphatidylethanol species in spiked and authentic whole blood and matching dried blood spots. *Int J Legal Med* 127(3), 603-610 (2013).
14. Hoogtanders K, van der Heijden J, Christiaans M, van de Plas A, van Hooff J, Stolk L. Dried blood spot measurement of tacrolimus is promising for patient monitoring. *Transplantation* 83(2), 237–238 (2007).
15. Burnett JE. Dried blood spot sampling: practical considerations and recommendation for use with preclinical studies. *Bioanalysis* 3(10), 1099–1107 (2011).
16. Dainty TC, Richmond ES, Davies I, Blackwell MP. Dried blood spot bioanalysis: an evaluation of techniques and opportunities for reduction and refinement in mouse and juvenile rat toxicokinetic studies. *Int J Toxicol* 31(1), 4–13 (2012).

17. Patel P, Mulla H, Tanna S, Pandya H. Facilitating pharmacokinetic studies in children: a new use of dried blood spots. *Arch Dis Child* 95(6), 484–487 (2010).
18. Pandya HC, Spooner N, Mulla H. Dried blood spots, pharmacokinetic studies and better medicines for children. *Bioanalysis* 3(7), 779–786 (2011).
19. Parker SP, Cubitt WD. The use of the dried blood spot sample in epidemiological studies. *J Clin Pathol* 52(9), 633-639 (1999).
20. Déglon J, Versace F, Lauer E *et al.* Rapid LC-MS/MS quantification of the major benzodiazepines and their metabolites on dried blood spots using a simple and cost-effective sample pretreatment. *Bioanalysis* 4(11), 1337-1350 (2012).
21. Versace F, Déglon J, Lauer E, Mangin P, Staub C. Automated DBS extraction prior to hilic/RP LC-MS/MS target screening of drugs. *Chromatographia* 76(19-20), 1281-1293 (2013).
22. Ooms JA, Knegt L, Koster EH. Exploration of a new concept for automated dried blood spot analysis using flow-through desorption and online SPE–MS/MS. *Bioanalysis* 3(20), 2311– 2320 (2011).
23. Heinig K, Wirz T, Bucheli F, Gajate-Perez A. Determination of oseltamivir (Tamiflu®) and oseltamivir carboxylate in dried blood spots using offline or online extraction. *Bioanalysis* 3(4), 421–437 (2011).
24. Abu-Rabie P, Spooner N. Dried matrix spot direct analysis: evaluating the robustness of a direct elution technique for use in quantitative bioanalysis. *Bioanalysis* 3(24), 2769–2781 (2011).
25. Déglon J, Thomas A, Daali Y *et al.* Automated system for on-line desorption of dried blood spots applied to LC–MS/MS pharmacokinetic study of flurbiprofen and its metabolite. *J Pharm Biomed Anal* 54(2), 359–367 (2011).
26. Déglon J, Thomas A, Mangin P, Staub C. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Anal Bioanal Chem* 402(8), 2485-2498 (2012).
27. Ganz N, Singrasa M, Nicolas L *et al.* Development and validation of a fully automated online human dried blood spot analysis of bosentan and its metabolites using the sample card and prep DBS system. *J Chrom B Analyt Technol Biomed Life Sci* 885–886, 50–60 (2012).
28. Ashley EA, Stepniewska K, Lindegardh N *et al*. Comparison of plasma, venous and capillary blood levels of piperaquine in patients with uncomplicated falciparum malaria. *Eur J Clin Pharmacol* 66(7), 705-712 (2010).
29. Mohammed BS, Cameron GA, Cameron L, Hawksworth GH, Helms PJ, Mclay JS. Can finger-prick sampling replace venous sampling to determine the pharmacokinetic profile of oral paracetamol? *Br J Clin Pharmacol* 70(1), 52-56 (2010).
30. de Boer T, Wieling J, Meulman E *et al*. Application of dried blood spot sampling combined with LC-MS/MS for genotyping and phenotyping of CYP450 enzymes in healthy volunteers. *Biomed Chromatogr* 25(10), 1112-1123 (2011).
31. Egier DA, Keys JL, Hall SK, McQueen MJ. Measurement of hemoglobin A1c from filter papers for population-based studies. *Clin Chem* 57(4), 577–585 (2011).

32. Wilhelm AJ, Klijn A, den Burger JC *et al*. Clinical validation of dried blood spot sampling in therapeutic drug monitoring of ciclosporin A in allogeneic stem cell transplant recipients: direct comparison between capillary and venous sampling. *Ther Drug Monit* 35(1), 92-95 (2013).
33. Hernández Redondo A, Schroeck A, Kneubuehl B, Weinmann W. Determination of ethyl glucuronide and ethyl sulfate from dried blood spots. *Int J Legal Med* 127(4), 769-775 (2013).
34. Affan ET, Praveen D, Chow CK, Neal BC. Comparability of HbA1c and lipids measured with dried blood spot versus venous samples: a systematic review and meta-analysis. *BMC Clin Pathol* doi: 10.1186/1472-6890-14-21 (2014).
35. Ellefsen KN, da Costa JL, Concheiro M, *et al.* Cocaine and metabolite concentrations in DBS and venous blood after controlled intravenous cocaine administration. *Bioanalysis* 7(6), 2041-2056 (2015).
36. Patteet L, Maudens KE, Stove CP, *et al*. Are capillary DBS applicable for therapeutic drug monitoring of commound antipsychotics? A proof of concept. *Bioanalysis* 7(16), 2119-2130 (2015).
37. Kummer N, Ingels AS, Wille SM, *et al*. Quantification of phosphatidylethanol 16:0/18:1, 18:1/18:1, and 16:0/16:0 in venous blood and venous and capillary dried blood spots from patients in alcohol withdrawal and control volunteers. *Anal Bioanal Chem* 408(3), 825-838 (2016).
38. Andresen H, Stimpfl T, Sprys N, Schnitgerhans T, Müller A. Liquid ecstasy – a significant drug problem. *Dtsch Arztebl Int* 105(36), 599-603 (2008).
39. Velghe S, Capiau S, Stove CP. Opening the toolbox of alternative sampling strategies in clinical routine: A key-role for (LC-)MS/MS. *Trends Analyt Chem* 84(B), 61-73 (2016).
40. Ingels A-SME, Sadones N, De Kesel PMM, Lambert WE, Stove CP. Derivatization techniques in dried blood spot analysis. In: *Dried blood spots: applications and techniques.* Li, W, Lee, MS (Eds.), John Wiley & Sons, Hoboken, NJ, USA, 344-354 (2014).
41. Ingels A, Lambert W, Stove C. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. *Anal Bioanal Chem* 398(5), 2173-2182 (2010).
42. Ikeda K, Ikawa K, Yokoshige S, Yoshikawa S, Morikawa N. Gas chromatography-electron ionization-mass spectrometry quantitation of valproic acid and gabapentin, using dried plasma spots, for therapeutic drug monitoring in in-home medical care. *Biomed Chromatogr* 28(12), 1756-1762 (2014).
43. Daly M. Down a stony road: the 2014 drugscope street drug survey. http://www.drugsandalcohol.ie/23282/1/DownAStonyRoadDrugTrendsSurvey2014.pdf
44. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem* 42(10), 1589-1600 (1996).
45. Levy AP, Asleh R, Blum S, *et al.* Haptoglobin: basic and clinical aspects. *Antioxid Redox Signal* 12(2), 293-304 (2010).
46. Levy AP, Hochberg I, Jablonski K, *et al.* Haptoglobin phenotype is an independent risk factor for cardiovascular disease in individuals with diabetes: The Strong Heart Study. *J Am Coll Cardiol* 40(11), 1984-1990 (2002).

47. Milman U, Blum S, Shapira C, *et al.* Vitamin E supplementation reduces cardiovascular events in a subgroup of middle-aged individuals with both type 2 diabetes mellitus and the haptoglobin 2-2 genotype: a prospective double-blinded clinical trial. *Arterioscler Thromb Vasc Biol* 28(2), 341-347 (2008).

48. Roguin A, Koch W, Kastrati A, Aronson D, Schomig A, Levy AP. Haptoglobin genotype is predictive of major adverse cardiac events in the 1-year period after percutaneous transluminal coronary angioplasty in individuals with diabetes. *Diabetes Care* 26(9), 2628-2631 (2003).

49. Suleiman M, Aronson D, Asleh R, *et al.* Haptoglobin polymorphism predicts 30-day mortality and heart failure in patients with diabetes and acute myocardial infarction. *Diabetes* 54(9), 2802-2806 (2005).

CHAPTER 1:
ANALYSIS OF ABUSED SUBSTANCES USING DRIED BLOOD SPOTS

Based on

Sadones N, Capiau S, De Kesel PMM, Lambert WE, Stove CP. Spot them in the spot: analysis of abused substances using dried blood spots. *Bioanalysis* 6(17), 2211-2227 (2014).

Abstract

During recent years dried blood spot (DBS) sampling and DBS analysis have increasingly received attention in various fields, amongst which in clinical, forensic and occupational toxicology. In this Chapter, we provide an overview of the different DBS-based methods that have been developed for detecting (markers of) abused substances. These include both legal and illegal drugs, belonging to different categories, amongst which cannabinoids, cocaine and metabolites, opioids, benzodiazepines and Z-drugs, amphetamines and analogues, gamma-hydroxybutyric acid, ketamine and new psychoactive substances such as cathinones. Also covered are markers of ethanol consumption and tobacco use. Since overall, the majority of published methods shows promising results, an interesting role for DBS analysis in diverse toxicological applications can be envisaged. For the distinct applications, we discuss the specific potential and benefits of DBS, the associated limitations and challenges, as well as recent developments and future perspectives.

1.1 Introduction

The use of dried blood spots (DBS) and DBS sampling is not new. DBS analysis was introduced over a century ago by Ivar Bang [1], who used DBS for glucose monitoring in rabbits, while it was Guthrie and Susi who spread the applicability of DBS by demonstrating its usefulness in the newborn screening of phenylketonuria and in other screening programs [2,3]. In the last few years, DBS applications have grown exponentially, not only in newborn screening, but also for the analysis of DNA, proteins, small molecules and trace elements in animals, children, adults, and even post-mortem samples [4].

The use of DBS can be looked at from different perspectives. On the one hand, DBS can be considered as a simple and cost-effective sample preparation procedure, as the filter paper can retain several matrix compounds [5-8]. Consequently, the use of DBS may simplify the extraction procedure, possibly allowing automated online extraction (see section 1.4). Alternatively, DBS can be considered as a useful matrix in clinical and forensic toxicology as they can be collected in a fast, simple and economical way (in contrast to blood samples obtained by venipuncture) and give information on the acute state of the patient (in contrast to urine, oral fluid and hair) [8]. However, in emergency toxicology -in cases where diagnosis and treatment of the patient may depend upon toxicological information- DBS will likely have little added-value since medical personnel should have rapid access to toxicological information and there is no time to wait for DBS to dry for at least two hours. When speed is not essential, DBS -either as a sampling or sample preparation technique- can be useful for screening purposes as well as for quantitative analysis. In this context, it should be noted that DBS may readily dry during transit from the sampling site to the laboratory, so in many cases there will be no delay after arrival at the laboratory.

The aim of this Chapter is to give an overview of applications of DBS in toxicology, more specifically for the analysis of abused substances (see Table 1.1 & Appendix 1). DBS sampling for therapeutic drug monitoring (TDM) and metabolic screening is beyond the scope of this Chapter, as is DBS sampling for the analysis of therapeutic drugs, environmental contaminants, toxins and (trace) elements. Abused substances include both legal and illegal drugs, belonging to different categories, amongst which cannabinoids, cocaine and metabolites, opioids, benzodiazepines and Z-drugs, amphetamines and analogues, gamma-hydroxybutyric acid (GHB), ketamine and new psychoactive substances such as cathinones. Also covered in this overview are markers of ethanol consumption and tobacco use.

Table 1.1 Overview of the different drug categories of abused substances determined in DBS, with referral to the utilized analytical techniques and their potential to measure cut-off concentrations proposed by DRUID.

Drug category	Technique	References	DRUID cut-off	Comments
Cannabinoids	LC-MS/MS GC-MS LC-HRMS	[7,11,12,93] [26] [94]	✗ ✗ ✗	Only 1 method attained sufficient sensitivity, yet still requiring complete 20-µL DBS [94]; no methods for synthetic cannabinoids in DBS
Cocaine and metabolites	RIA LC-MS/MS LC-FLUO GC-MS LC-HRMS	[19,97] [8,12,13,31,47,56,77,93,96,97] [6] [26] [63,94]	✓(a) ✓ ✓(a) ✗ ✓(b)	DBS need to be stored at freezing temperatures
Opiates	LC-MS/MS GC-MS	[8,11-13,31,40,45,47,61,67,79,93,96,100,101,116] [26]	✓ ✗	Stabilizing effect of DBS for 6-monoacetylmorphine
Non-opiate opioids	LC-MS/MS GC-MS LC-coulometry LC-MS	[8,11-13,30,36,40,45,47,93,101,103,116] [26] [20] [10]	✓(c) ✗ ✓(d) ✓(d)	Analytes include methadone, buprenorphine, tramadol, fentanyl, pethidine and their metabolites
Benzodiazepines	LC-MS/MS GC-MS LC-HRMS	[5,8,11-13,37,46,77,91,93,117-120,122] [26] [39]	✓(e) ✗ ✓(f)	Stabilizing effect of DBS for benzodiazepines
Z-drugs	LC-MS/MS GC-MS	[5,8,9,12,13,78,91,122] [26]	✓ ✗	Stabilizing effect of DBS for zopiclone
Amphetamine and analogues	LC-MS/MS GC-MS LC-HRMS	[8,12,13,31,41,47,79,80,91,93,121] [26] [94]	✓ ✗ ✗	
GHB	LC-MS/MS GC-MS	[44] [23-25]	n.a.	No *ex vivo* formation of GHB in DBS; similar concentrations in cDBS and vDBS
Ketamine and metabolite	LC-MS/MS LC-DAD	[47,80,104] [21]	✓ ✗	
New psychoactive substances	LC-MS/MS	[41,80]	n.a.	Analytes include cathinones, piperazines and mephedrone; stabilizing effect of DBS on cathinone derivatives when stored cooled

Table 1.1 Continued.

Drug category	Technique	References	DRUID cut-off	Comments
Alcohol markers	LC-MS/MS	[43,51,110-114]	n.a.	Analytes include ethylglucuronide, ethylsulphate and phosphatidylethanol; no *ex vivo de novo* formation of phosphatidylethanol
Markers of tobacco use	GC-MS LC-MS/MS Paper spray MS LC-HRMS/MS	[22] [29,49,72] [35] [42]	n.a.	Analytes include nicotine and metabolites, anabasine and anatabine; primary application lies in newborn DBS analysis

The 'DRUID cut-off' column indicates if the DBS methods attained sufficient sensitivity to allow detection at DRUID LLOQ levels, when starting from complete ≤ 10-µL DBS and/or ≤ 6.4-mm diameter DBS punches.

(a) only benzoylecgonine; (b) only cocaine and benzoylecgonine; (c) only methadone and tramadol; (d) only methadone; (e) only 7-aminoclonazepam, 7-aminoflunitrazepam, alprazolam, OH-alprazolam, clobazam, clonazepam, diazepam, desalkylflurazepam, desmethylflunitrazepam, flunitrazepam, flurazepam, lormetazepam, lorazepam, midazolam, nitrazepam, oxazepam, temazepam, triazolam and nordiazepam; (f) only midazolam

cDBS: capillary dried blood spots; DAD: diode array detection; DBS: dried blood spot; DRUID: driving under the influence of drugs, alcohol and medicines; FLUO: fluorescence detector; GC-MS: gas chromatography coupled to mass spectrometry; GHB: gamma-hydroxybutyric acid; HRMS: high resolution mass spectrometry; LC: liquid chromatography; n.a.: not applicable; RIA: radioimmunoassay; vDBS: venous dried blood spots

1.2 Paper used and volume spotted

Whatman® 903 paper is the predominant filter paper used for DBS collection and is manufactured from 100% pure cotton fibers. Other cellulose-based papers used for determination of drugs of abuse are Ahlström 226, Sartorius TFN, Whatman® BFC 180 and Whatman® FTA® (see Appendix 1). The latter paper is chemically treated to prevent degradation of genomic DNA. Bond Elut DMS is a non-cellulose collection material which would reduce non-specific binding, improving mass spectrometer analyte response and increasing signal to noise ratios [9]. Our experience, however, learned that the reduced rigidity of this paper makes it less suitable for manual punching. Eibak *et al.* obtained higher recoveries for methadone using alginate and chitosan foams as sampling media, when compared with Whatman® FTA® DMPK and Bond Elut DMS filter paper [10]. Although these foams, which can be fully solubilized in 1 mM HCl, seem promising, further experiments are warranted to investigate their utility in routine applications.

The volume of blood that has been used to generate DBS for detecting abused substances ranges from 5 to 100 µL. While 5-µL spots have primarily been used in a sample preparation context [5,8,11-13], it should be noted that 100-µL DBS are not easily obtained by fingerprick; hence an important advantage of DBS sampling is lost in that case. In case of volumetric application, often the complete DBS is analyzed. However, such volumetric application is difficult to sustain when it is to be performed by non-trained persons -although also here progress is underway. E.g. Lenk *et al.* developed a prototype of a 'metering chip' [14], whereas Leuthold *et al.* evaluated a microfluidic approach which consists of a microfluidic plate with sized capillaries and a DBS card holding element [15]. Another recently developed approach which allows the collection of a fixed volume of blood from a non-volumetrically deposited sample is volumetric absorptive microsampling (VAMS) [16,17] This alternative collection device wicks up a fixed volume of blood, independent of hematocrit (Hct), onto a porous substrate. When DBS are obtained by direct application from the fingertip onto filter paper, typically a smaller disk (e.g. 3- or 6-mm diameter) is punched from the DBS. Analyzing a complete spot largely avoids the Hct effect and facilitates validation. In contrast, analysis of a partial punch further reduces the amount available for analysis, resulting in an even higher requirement of sensitivity, and imposing a more elaborate validation (see section 1.6) [18]. It also needs to be remarked that the vast majority of available publications describes the analysis of DBS that have been obtained by pipetting existing venous blood samples, already available in the lab, rather than setting up a true capillary DBS (cDBS) approach. Lastly, it has to be noted that archived newborn DBS, which are obtained by heel stick within the first 1-3 days of life in the context of screening for inborn errors of metabolism and are in general larger than DBS obtained by fingerprick (newborn DBS correspond to about 80 µL of blood), have also been used to assess abused substances. Since in most cases a substantial left-over remains after the newborn screening procedure (except in those cases where newborn screening was positive), the remaining material can be used for assessing exposure to abused substances at birth.

1.3 Analytical techniques

An extra challenge associated with using small blood volumes is the requirement of highly sensitive techniques for identification and quantification of the compounds. The first report about the determination of abused substances in DBS was published by Henderson *et al.*, who applied a modified urinary benzoylecgonine radioimmunoassay screening test on newborn DBS in order to assess prenatal use of cocaine by the mother [19]. The obtained results correlated well with gas chromatography-mass spectrometric (GC-MS) measurements in blood. Although immunoassays can be used for DBS analysis in some cases, they are subject to non-specific binding and to cross-reactivity with endogenous components and/or structurally related drugs and metabolites. Chromatographic techniques, either liquid or gas chromatography, have been used in later studies. Mostly, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the most appropriate technique, preceded by either online or offline sample preparation (see section 1.4). Although MS/MS detection is usually required and preferred, also other detectors have been used for identifying and quantifying abused substances in DBS. Examples include spectrofluorimetric detection of natively fluorescent cocaine, benzoylecgonine and cocaethylene [6], and coulometric and diode array detection of methadone and (nor)ketamine, respectively [20,21]. Also GC-MS has been applied, more particularly for cotinine [22], GHB [23-25], amphetamine-like drugs, cocaine, benzodiazepines, zopiclone, buprenorphine, methadone, tramadol, Δ^9-tetrahydrocannabinol (THC) and THC-COOH [26]. However, GC-based analyses mostly require a derivatization step, which can be seen as a disadvantage.

1.4 Extraction

The desorption of analytes from DBS is most often performed using organic solvents, water or a mixture of both. Mixtures with a water content of over 25-30% will typically yield colored extracts, indicating release from hemoglobin -and also other proteins- from the filter paper. In contrast, mixtures containing less water will yield cleaner extracts, as is also the case when using pure organic solvents, as shown by Déglon *et al*. [27]. The desorption process can be performed by mere passive infusion of the filter paper, but is most often facilitated by sonication, agitation or heating. To this end, Saracino *et al.* even used microwave-assisted extraction [20].

Another possible simplification of the pre-analytical phase is the concept of “on-spot derivatization”, developed by Ingels *et al.* for the quantitation of GHB using GC-MS [23-25]. This approach consists of adding derivatization reagents directly onto the filter paper, without the use of an extraction solvent, followed by drying and re-dissolution in a suitable solvent, ensuring a minimal, less time-consuming sample pretreatment. In Chapter 3, we used this derivatization technique in combination with microwave-assisted derivatization for the determination of some polar low molecular weight compounds with clinical and forensic toxicological relevance [28].

A straightforward procedure for sample work-up consists of immediately subjecting the obtained extract to further analysis, either directly or following dilution. Unfortunately, depending on the elution solvent used, extra pre-analytical steps may be necessary for sample clean-up, such as protein precipitation, solid phase extraction (SPE) and/or liquid-liquid extraction. Since elaborate sample pretreatment is labor intensive and requires a lot of consumables, more efficient alternatives have been developed. For example, Saracino *et al.* used microextraction packed sorbent for the determination of methadone, ketamine and norketamine, drastically reducing the volume of solvents [20,21]. The use of automated sample preparation procedures has also been described: Murphy *et al.* performed SPE with a robotic system prior to cotinine analysis and Clavijo *et al.* and Saussereau *et al.* implemented online extraction for the analysis of a wide range of drugs of abuse, significantly reducing the number of manual steps needed [29-31]. Fully automated analysis of DBS cards has become possible with the development of automated DBS samplers, as described further in this section. Other recent developments include digital microfluidics [32] and paper spray MS [33-35].

If mere injection or dilution of the extract is not an option because of required sensitivity or because the extraction solvent is not compatible with the analyzing system, evaporation may be a necessary step. After this additional tedious step, which is often relatively time-consuming, the extract is reconstituted in a limited volume of a suitable solvent (e.g. the mobile phase), most often offering the advantage of concentrating the sample. An alternative has been proposed by Eibak *et al*., who used electromembrane extraction for sample clean-up and for enrichment of the target analyte (methadone) in an aqueous acceptor solution compatible with LC [10]. Important to note is that, as mentioned before, in this case DBS are not eluted from filter paper, but rather are dissolved, together with the matrix that consists of chitosan or alginate foam substrates. Electromembrane extraction was also used by Seip *et al.* for the analysis of methadone and pethidine [36].

Apart from the automated DBS samplers referred to in the next paragraph, several methods have been developed to achieve higher throughput without significant investment. One example is the simple offline procedure employed by Déglon *et al.* for the analysis of benzodiazepines and Z-drugs [5]. This procedure consists of infusing a whole DBS directly into the extraction solvent contained in a LC vial, immediately followed by injection of an aliquot of the supernatant onto the chromatographic system. A similar but more automated approach has been developed by Versace *et al*. for a rapid LC–MS/MS target screening on DBS [8]. To this end, a quick (2 min) and automated in-well extraction was performed in the LC autosampler itself, before injecting part of the supernatant. Another inventive approach was proposed by Johnson *et al.*, who used an improved punching device to punch DBS directly into filter tips of a liquid-handling device that was subsequently used to elute the analytes from the DBS [37]. This semi-automated procedure was used for the analysis of alprazolam, OH-alprazolam and midazolam, yielding similar results as an offline approach, whilst reducing sample preparation time by more than 50%.

Despite efforts to increase the efficiency of DBS sample pretreatment, its manual character remains an important hurdle to more widespread use of DBS analysis in routine practice. However, during the last few

years progress has been made in automating this process, increasing the throughput and making DBS analysis more appealing to modern laboratories. Déglon *et al.* were the first to describe the concept of online desorption of DBS [27], while Thomas *et al.*, from the same lab, were the first to use this concept for the analysis of drugs of abuse [11]. To this end, whole DBS were punched out and placed in a home-made desorption cell, integrating the sample directly into the LC device. Using a column switching procedure, the analytes were desorbed from the filter paper in the cell prior to online analysis. An improved prototype, based on an automated rotating plate that can host more manually punched samples, was implemented by Lauer *et al.* and Déglon *et al*. for the screening of a wide range of drugs in DBS, including cocaine and metabolites, opiates, amphetamines and benzodiazepines [12,13]. Three commercial suppliers have developed fully automated DBS samplers: CAMAG (DBS-MS 500), Prolab Instruments, GmbH (SCAP^{TM}) and Spark-Holland (DBS^{TM} Autosampler) [38]. In the context of (potential) abused substances, the SCAP^{TM} system was used by Oliveira *et al.* for the determination of midazolam, leading to a 5-fold improvement in sensitivity when compared with an offline procedure [39]. Verplaetse and Henion quantified opioids and stimulants by coupling the Spark-Holland Autosampler to online SPE-LC-MS/MS [40,41]. Such automated system was also utilized by Tretzel *et al.* for the determination of nicotine and its major metabolites in DBS [42].

1.5 Lower limit of quantification (LLOQ)

As mentioned in section 1.3, DBS sampling has the inherent disadvantage of providing only a small amount of target analyte, often requiring an analytical technique such as GC-MS, LC-MS or LC-MS/MS to reach sufficient sensitivity. When punches are used -which often is the most feasible approach in real practice as it does not necessitate volumetric application- the available amount of sample decreases even further. E.g. when envisaging low ng/mL detection of an analyte, starting from a 3-mm punch (corresponding to approximately 3 µL of blood), this means that only low pg amounts of analyte are available. In an attempt to increase the amount of starting material and hence improve the LLOQ, larger punch sizes can be used or multiple punches can be analyzed simultaneously [29,43-47]. As discussed above in section 1.4, the dilution introduced during the extraction process may require the incorporation of a concentrating step during sample pretreatment. Also in this respect, online extraction of DBS may be advantageous, as it allows complete transfer of the analyte of interest to the (LC-)MS/MS. E.g. Abu-Rabie and Spooner demonstrated a 10-fold increase in sensitivity using a DBS direct elution technique compared to manual DBS extraction [48].

In order for DBS analysis to be implemented in routine laboratories, the obtained LLOQs have to be relevant to clinical/toxicological practice. Obviously, the required sensitivity depends on the compound of interest. As newer abused substances are often more potent, their quantitation may require very low LLOQs, making the analysis of such compounds challenging, especially when using DBS. Moreover, the required LLOQ is also determined by the intended use of the method. For example, the LLOQ of cotinine in newborn whole blood for the detection of maternal smoking (6 ng/mL) is higher than the one to establish newborn exposure to secondhand smoke (0.02 ng/mL) [49]. Furthermore, expected concentration ranges depend on the intended

application, e.g. TDM, toxicological analysis, pharmacokinetics (PK) and analyses in the context of driving under the influence of drugs (DUID). In the latter case, the required LLOQs may vary between countries as well.

As DBS sampling may be an interesting alternative option in the context of DUID, we evaluated whether the methods that are currently present in literature are fit for this purpose, by comparing the stated LLOQs with the cut-offs proposed by the DRUID project [50] and the legally accepted limits in Belgium (Table 1.1 & Appendix 2). Especially for abused substances that can be used for therapeutic purposes as well, it is quite challenging to establish legal limits, as phenomena such as tolerance can occur, and, consequently, relatively high blood levels may not necessarily impair a person's ability to drive. Moreover, further studies are needed to establish a universal cut-off for phosphatidylethanol (PEth) as well. Kummer *et al.* already suggested a cut-off value at 221 ng/mL for PEth 16:0/18:1 to distinguish between inpatients in alcohol withdrawal and control volunteers [51]. However, more sensitive methods are required to determine optimal cut-off values for PEth 18:1/18:1 and PEth 16:0/16:0. When interpreting the LLOQs of the methods mentioned in Appendix 2, it is also important to note the different DBS sizes or volumes, ranging from a 2-mm flow-through area to complete 100-µL DBS. In the latter cases, the mentioned LLOQs might actually be underestimated, as DBS containing smaller blood volumes will be available in real practice. Considering the data in Table 1.1, Appendix 1 and Appendix 2, for the majority of analytes of the DRUID panel, there are methods available that achieve sufficient sensitivity to allow detection at the required level, when starting from complete ≤ 10-µL DBS and/or ≤ 6.4-mm diameter DBS punches. These methods can be found in the shaded columns of Appendix 2.

1.6 Validation

The choice between a partial- and whole-cut DBS method should ideally be made before setting up a DBS-based method, as it has important repercussions on what parameters should be included in the validation protocol. Validation of a DBS-based procedure using partial punches ideally encompasses extra validation parameters, e.g. punch location, blood volume spotted and Hct, as these factors may have an impact on the quantitative result [3,18,52,53]. More specifically, Hct may affect the precision, accuracy, extraction efficiency and the recovery of an analyte [54,55]. For instance, punches from DBS prepared from blood with a high Hct will contain larger volumes of blood than punches from DBS of blood with lower Hct, resulting in respectively an over- and underestimation when the calibration line is established in blood with intermediate Hct. Therefore, it is recommended to establish a calibration curve with a Hct in the middle of the range of the target population, to minimize the Hct effect. Sosnoff *et al*., for instance, prepared QCs in blood with a Hct close to that of newborns [56]. In conclusion, the combined effect of Hct, blood volume spotted and punch location has to be evaluated when using partial-cut DBS. Analyzing complete, volumetrically applied DBS, however, may overcome the Hct effect. Ideally, before broad implementation of the method, its validity should be tested by applying it on a set of real samples, thereby evaluating incurred sample reproducibility and -if applicable- the equivalence between capillary and venous concentrations. Relevant to mention in this context is that, for practical reasons, virtually all validations are performed by applying anticoagulated venous blood onto filter

paper. Although this blood differs from the one obtained in a real-life application by direct application after fingerprick, we are not aware of reports describing problems related to this inherent methodological problem. Furthermore, Hct needs to be taken into account when a correlation or conversion is to be made between DBS and plasma concentrations (see section 1.7). Additionally, a distinction has to be made between DBS analysis for qualitative (i.e. screening) and for quantitative purposes. Obviously, strict validation parameters are needed when aiming at quantitative analysis of DBS.

There are various ways of introducing an internal standard (IS) during the process of DBS sampling and analysis [55]. Ideally, the IS should be mixed homogenously with the blood sample prior to blood spotting, enabling correction for any analyte loss. However, this approach is only feasible when DBS are seen as an alternative sample preparation strategy. Alternative approaches are spraying [57-59] or application of IS to the DBS card prior to spotting [57,60,61]. An example of the latter is the procedure developed by Mommers *et al.*, who described two IS DBS pre-impregnation procedures, in which the DBS card is pretreated with IS (morphine-D3) [61]. Abu-Rabie *et al.* demonstrated that, when combined with accurate volume DBS sampling and whole-spot extraction, the IS spray technique may nullify the Hct-based assay bias [59]. However, in the vast majority of reports, the IS is added to the extraction solvent. As this means that there can be no compensation for varying recoveries, sufficient time should be spent in developing a robust extraction procedure.

1.7 Comparison with other biological matrices

The use of a DBS method as an alternative for techniques using (larger amounts of) whole blood or plasma requires some additional experiments, demonstrating the equivalence or correlation with results measured in whole blood or plasma. When analyzing cDBS, also the equivalence with concentrations in venous blood has to be evaluated, as mentioned above in section 1.6. However, such cross-comparison between concentrations in venous DBS (vDBS) and cDBS has often not been performed. Examples where this cross-comparison has been made include the work by de Boer *et al.*, who compared concentrations of midazolam and its main metabolite 1'-hydroxymidazolam in cDBS and plasma and whole blood, obtained by venipuncture [46], as well as our own work, in which we confirmed that capillary and venous concentrations of GHB are comparable [62 + Chapter 2]. The group of Huestis observed a discrepancy between cDBS and venous blood concentrations for cocaine and benzoylecgonine, although further studies are warranted as they also found a significant DBS variability [63]. Kummer *et al.* found a good correlation between the PEth concentrations in venous blood, vDBS and cDBS [51]. The agreement between DBS and other biological matrices can be evaluated by performing ordinary linear regression analysis. In this graphical method, the results of one method (for instance those obtained from the DBS method) are plotted against the results of another method (for instance those from whole blood analysis). However, ordinary linear regression analysis involves several assumptions which are seldomly met in practice and may lead to misleading results [64,65]. A better insight into the correlation between two methods can be obtained by Deming, Passing-Bablok and/or Bland-Altman analysis [64-66]. A Bland-Altman analysis plots the (percentage) differences between the results obtained by two measurement procedures (y-axis) against the

average of these results (x-axis). In this comparative graph the mean difference between both procedures is also plotted, together with its 95% confidence interval (corresponding to 2 standard deviations of the mean). Then, it should be evaluated whether the difference between both methods and the width of the 95% confidence interval are acceptable [66]. By using Bland-Altman analysis, Garcia Boy *et al.*, for instance, found a mean difference between vDBS and whole blood of 0.43 ± 0.67 ng/100 µL for morphine, meaning that DBS values are somewhat higher than blood values, with a 95% confidence interval spanning 2.6 ng/mL in total [67]. In the absence of an effect of the spotting *per se*, equivalent concentrations should be observed in venous blood and in DBS derived by spotting this blood. Deviations can be present because of the Hct effect (primarily when analyzing partial spots) and because of capillary-venous differences. When a comparison with plasma is to be made, one has to take into account the blood:plasma ratio, which is determined by the Hct and by the red blood cell (RBC):plasma distribution of a drug. Also here, deviations may occur, e.g. in certain disease states where altered plasma protein levels may affect the normal RBC:plasma distribution. Additionally, there is an effect of the particulate fraction present in blood: even when a compound is evenly distributed throughout plasma and RBCs, it will be present in blood at concentrations about 85% of those found in plasma, when considering blood with Hct of about 0.40. Hence, also here Hct plays a role, as blood with lower Hct will have a reduced particulate fraction. To make a quantitative comparison between plasma and DBS concentrations and/or to derive plasma concentrations from DBS concentrations, the latter are mostly multiplied with a mean correction factor, assuming a 'normal' Hct. For instance, Saracino *et al.* multiplied the methadone levels in DBS with 1.79, assuming a mean Hct of 0.44 [20], whereas Mercolini *et al.* introduced 1.62 and 1.92 as correction factor for THC, THC-OH and THC-COOH in women and men, assuming a Hct of respectively 0.38 and 0.48 [7]. However, one has to be aware of populations with deviating Hct levels, such as newborns or patients suffering from anemia. Ideally, the Hct of any sample should be known to determine a correct conversion factor. In this context, potassium measurements of DBS could be a promising strategy, as the potassium content of DBS allows prediction of the approximate Hct from non-volumetrically applied DBS [68]. In fact, it has been shown that this approach allows to correct for the Hct effect in samples with deviating Hct [69]. Also reflectance spectroscopy [70] and near-infrared spectroscopy [71] have recently been shown to allow Hct prediction of DBS. Furthermore, an even partitioning between RBCs and plasma is assumed in many cases. However, this distribution has to be investigated, as demonstrated for benzoylecgonine by Henderson *et al.* [19] or for cotinine by Sosnoff and Bernert [72]. Lastly, although whole blood analyses are common in toxicology, dried plasma spots may also be used. These can be prepared by spotting plasma on filter paper after conventional plasma preparation or, alternatively, via the use of dedicated cards [73-76]. Such plasma separator devices are multilayered extraction cards onto which a drop of blood is applied. The multilayered polymeric filter membrane consists of a separation and collection membrane and a removable top layer. The upper membrane traps the RBCs and allows plasma to penetrate through onto the bottom collection membrane. After waiting for a few minutes, the top layer onto which the cellular fraction is trapped, can be peeled off and plasma spots are generated onto the lower membrane.

1.8 Stability

In the forensic toxicology laboratory, it is not unusual that analytes need to be detected a considerable time after sampling. An important limitation to be considered is that only analytes which remain stable for an extended period of time, may be detected. The use of DBS could be an advantage here since DBS may improve analyte stability, as pointed out in multiple publications. Examples of unstable analytes include benzodiazepines, zopiclone and cocaine, which are subject to hydrolysis of the amide or ester function, respectively. They rapidly degrade in biological fluids, such as whole blood, whereas the degradation process is reduced or minimized in DBS, as demonstrated in a number of publications [19,77,78]. A stabilizing effect of DBS is also seen for 6-monoacetylmorphine (6-MAM), a heroin metabolite whose detection points at heroin use. Whereas filter paper stabilizes 6-MAM, in blood it is rapidly metabolized into morphine, which is also a metabolite of prescribed opiates such as codeine [67,79]. In untreated whole blood samples, cathinones are also prone to degradation, whereas in DBS all cathinone derivatives (with the exception of cathinone itself) are stable for 2 weeks when stored at 4°C [80-82]. Verplaetse and Henion observed stability problems for mephedrone which could be counteracted by storage of DBS under a N_2 flow and/or at cooled temperature [41]. A second remark to be considered when analysis does not take place immediately after sampling, is the possibility of *ex vivo* formation of some compounds upon storage of biofluids. Also here, DBS may increase the reliability of the result: although *ex vivo* generation of PEth and GHB may take place in biological fluids [43,83-86], this phenomenon is not seen in DBS [24,43].

1.9 Applications

1.9.1 Driving under the influence of drugs

Currently, the use of on-site drug testing in oral fluid is described in the legislation of many countries, and in some of these, oral fluid is effectively used for DUID testing [87]. However, this matrix suffers from the drawback that contamination may not be excluded and is sometimes criticized as measured concentrations not necessarily reflect those present in blood (although this is of less relevance in *per se* legislation). Therefore, in selected cases, DBS sampling may be considered as a possible alternative. However, several factors need to be taken into account when considering DBS sampling in the context of DUID. First, it should be noted that cDBS sampling is also inherently associated with a contamination risk. For example, when a fingerprick is made from an individual who touched drug powder or fluid without actively ingesting the drug, traces of the substance may be found when analyzing DBS (if the finger is insufficiently decontaminated). As is the case for hair analysis, identification of metabolites could be a possible solution [88,89]. For instance, when a DBS only contains cocaine, and no benzoylecgonine is detected, external contamination may be likely. External contamination and false positive results can also arise upon incorrect handling of the filter paper. This can be checked for by evaluating blank paper material, adjacent to the DBS. Another point of attention is that blood spot on filter paper must be dried adequately. Bringing the DBS in a standardized container with desiccant may

be a convenient way to allow them to dry safely during street controls, as has been applied for remote sampling in animals [90]. While DBS prepared from authentic DUID venous blood samples have been analyzed [5,31,91], overall giving comparable results as those obtained by routine analytical methods (see further), we are not aware of reports describing the use of true cDBS for DUID roadside testing. If cDBS are to be used, again, it will be important to demonstrate equivalence between capillary and venous concentrations for the analyte of interest. Moreover, cut-offs in e.g. Belgian legislation (mentioned in Appendix 2) refer to plasma levels rather than to blood levels, so this is an additional aspect that needs to be taken into account.

1.9.2 Cannabinoids

Cannabinoid determination in DBS may offer a promising approach in the follow-up of drug addicts or in the context of DUID [7]. When considering the detection of different abused substances in DBS, cannabinoids are likely amongst the more challenging compounds. Analytes typically monitored are THC, as well as its metabolites THC-OH and THC-COOH (Figure 1.1). As THC-OH has a short half-life, it can be considered as a marker of recent cannabis use. In contrast, detection of THC-COOH, displaying a very long half-life, generally indicates past use of cannabis [92].

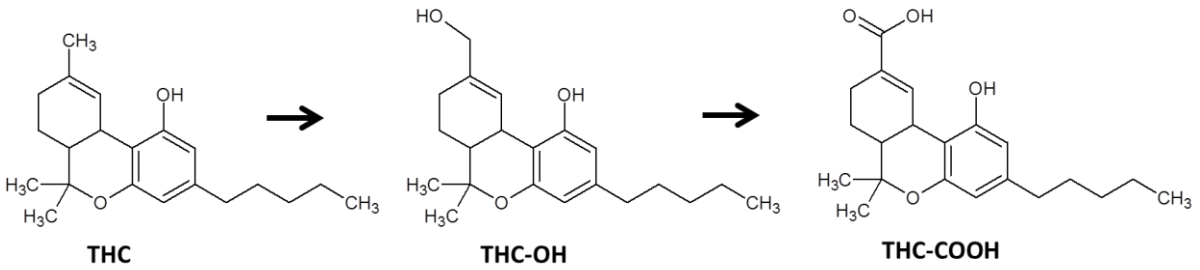


Figure 1.1 Structures of THC, THC-OH and THC-COOH.

Current methods describing the detection of cannabinoids in DBS require high blood volumes (100 µL of a 5-ng/mL DBS) or have insufficient sensitivity [7,11,12,26,93]. Using a high-end MS combining a quadrupole mass filter, a higher energy collision dissociation cell and an Orbitrap detector, Thomas *et al.* succeeded in attaining a LOD respectively LLOQ of 0.25 respectively 1 ng/mL for THC, starting from a complete 20-µL DBS [94]. An enhanced sensitivity for cannabinoids (in the range of sub-ng/mL) is relevant in e.g. the context of DUID, where the legal cut-off in Belgium is currently set at 1 ng/mL THC in plasma.

Using an online DBS extraction device coupled with a hydrophilic interaction/reversed-phase LC-MS/MS system, Thomas *et al.* were able to detect THC, the most important compound in cannabis, as well as its metabolites THC-OH and THC-COOH (and its glucuronide) in 5-µL DBS, albeit at very high concentrations [11]. The same group developed a screening method based on an automated DBS extraction for the detection of a wide range of abused substances, including THC (50 ng/mL in a 5-µL DBS) [12]. An UHPLC-MS/MS screening method developed by Kyriakou *et al.* [93] and a GC-MS method developed by Langel *et al.* [26] also had

insufficient sensitivity for the detection of cannabinoids (15 ng/mL in 30-µL DBS and 5 ng/mL in 100-µL DBS, respectively). Mercolini *et al.* developed a method for the quantitative analysis of THC, THC-OH and THC-COOH in DBS [7]. The good correlation between plasma concentrations that were calculated from DBS concentrations and those that were actually measured in corresponding plasma samples of 5 cannabis users was based upon several assumptions. First, it was assumed that a 7-mm punch corresponds to 10 µL blood; second, an even partitioning of the different analytes of interest between plasma and RBCs was assumed, which is actually not really the case [95]. Hence, although these results are promising, studies including larger groups are warranted.

1.9.3 Cocaine and metabolites

Cocaine is an illicit drug whose effects (increased self-confidence, a sense of omnipotence and sometimes a sensory impairment) explain its relevance in DUID studies [6]. Given the short half-life of cocaine, cocaine use is generally demonstrated by detecting its main metabolite benzoylecgonine (Figure 1.2). Multiple methods -either GC- or LC-based- have been developed for detection and quantification of cocaine and/or benzoylecgonine in DBS [8,12,13,26,47,56,63,77,93,94,96]. Cocaethylene, a metabolite formed after concomitant use of cocaine and ethanol-containing drinks, was also included in the DBS-based methods of Mercolini *et al.* and Saussereau *et al.* [6,31]. Saussereau *et al.* and Odoardi *et al.* were able to quantify the metabolite ecgonine methyl ester as well [31,47]. The metabolite norcocaine was included in the method of Ellefsen *et al.* [63].

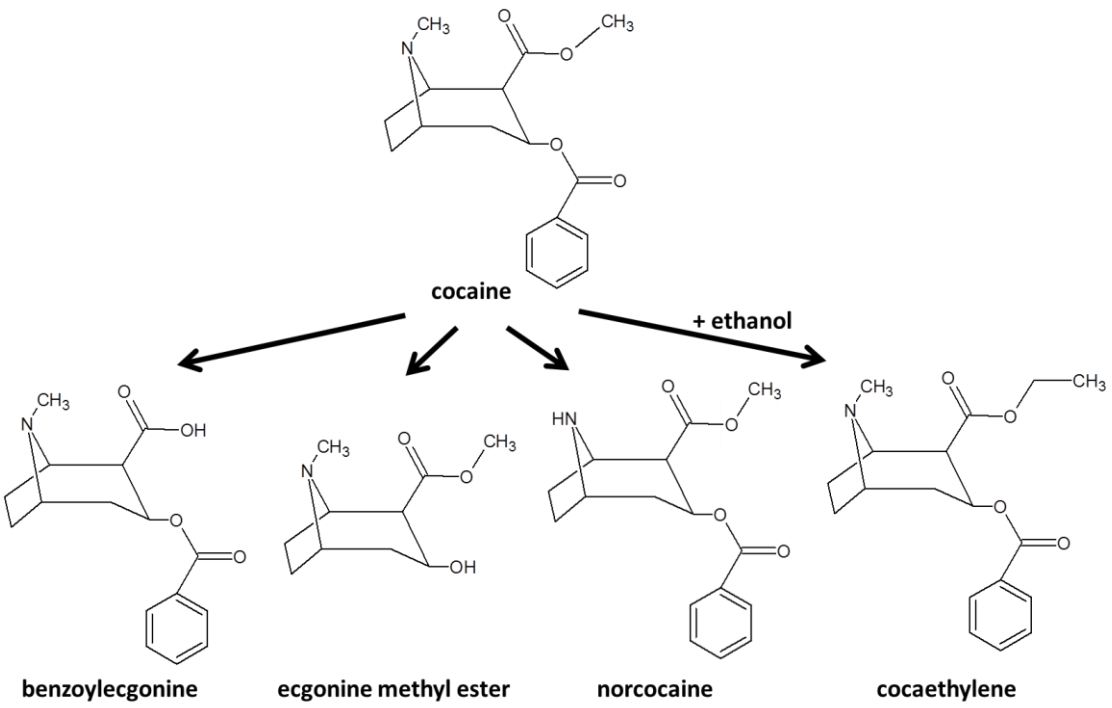


Figure 1.2 Structures of cocaine and its metabolites benzoylecgonine, ecgonine methyl ester, norcocaine and cocaethylene.

Cocaine abuse among pregnant women is associated with neonatal and obstetrical complications. Therefore, benzoylecgonine concentrations have been measured in newborn DBS to examine the prevalence and demographic aspects of cocaine use during pregnancy [19,56,97]. It has to be noted that detection in newborn DBS will only be possible if maternal use of cocaine took place at the end of the pregnancy.

As cocaine is subject to hydrolysis in whole blood samples, potential degradation needs to be taken into account when interpreting blood concentrations. Henderson *et al.,* for example, detected increasing amounts of benzoylecgonine upon incubation of cocaine-spiked blood [19]. These authors reported that in DBS, in contrast, degradation was minimal: no detectable benzoylecgonine concentrations were found when cocaine-containing DBS were incubated for 108 h at 45°C [19]. Kyriakou *et al.* observed no significant alteration of cocaine and benzoylecgonine DBS concentrations when stored at room temperature for two weeks [93]. Saussereau *et al*. evaluated long-term stability of cocaine and its metabolites in DBS and found that after a 6-month storage at -20°C concentrations had changed less than 20%. However, after a 6-month storage of DBS at 4°C, cocaine concentrations had decreased by 55% and ecgonine methylester concentrations by 88% [31]. Therefore, DBS for cocaine analysis need to be stored at freezing temperatures.

Mercolini *et al.* determined cocaine, benzoylecgonine and cocaethylene in cDBS obtained from cocaine and ethanol abusers [6]. They used an arbitrary correction factor to convert DBS concentrations to plasma concentrations, taking into account expected Hct levels for males and females, and reported a good correlation, although only documented for one case. Rather than starting from DBS prepared from spiked blood, these authors used blank DBS to which standards were spiked during validation. Ellefsen *et al.* investigated the correlation between cocaine and metabolite concentrations in DBS and venous blood following controlled intravenous cocaine administration. They observed slightly lower concentrations in DBS and, importantly, large intra- and inter-subject variability for both cocaine and benzoylecgonine. According to the authors, this variability could be attributed to nonhomogeneity of the blood spots, different blood spot volumes and different extraction efficiencies of the methods used for blood and DBS analysis. Since sampling occurred rapidly after cocaine administration, the authors also believe that the observed discrepancies between cDBS and venous blood concentrations may be due to inherent concentration differences between these two matrices [63]. Saussereau *et al.*, utilizing a limited set of authentic samples, reported a good correlation between benzoylecgonine concentrations in venous blood and DBS prepared thereof, assuming that a 3-mm DBS contains a volume of 3 µL blood [31]. Also Antelo-Dominguez *et al.* and Odoardi *et al*., analyzing limited sets of authentic venous samples by a DBS-based procedure and by a routine procedure for blood analysis, reported a good correlation between both methods [47,96]. However, in both approaches the use of DBS should be considered as an alternative sample preparation procedure: whereas Odoardi *et al.* used 100-µL DBS, in the approach of Antelo-Dominguez *et al.* the blood samples need to be diluted first with water before spotting them on filter paper [47,96]. Starting from 5-µL DBS and utilizing a new-generation MS, Versace *et al.* detected cocainics equally well as when using routine screening procedures which require larger amounts

of sample (about 1 mL) [8]. Overall, to our opinion, more studies illustrating the suitability of DBS as a valid alternative for routine procedures using conventional matrices, are warranted.

1.9.4 Opiates

Morphine is a metabolite of several opiates, either illicit (heroin) or licit (codeine). Consequently, detection of morphine alone does not allow to find out the parent molecule, whereas detection of 6-MAM indicates heroin use. A high prevalence of viral infections is seen in heroin abusers as this illicit drug is mainly abused intravenously. Consequently, special safety precautions have to be taken when handling these samples. Here, DBS may offer an advantage as DBS minimize the risk of transmitting blood-borne viruses since these lose their infectivity upon drying [98,99]. Furthermore, whereas 6-MAM degrades rapidly in whole blood at any storage temperature different from -20°C, Skopp reported it to be stable in DBS for at least 6 days at any storage temperature [79]. Kyriakou *et al.* also observed a decrease of less than 10% when the DBS samples were stored at room temperature for two weeks [93]. Garcia Boy *et al.* confirmed this stabilizing effect of DBS for 6-MAM: although the 6-MAM concentration in DBS still decreased by 50% within 5 days at 40°C, storage in whole blood resulted in complete disappearance of 6-MAM [67]. Clavijo *et al.* demonstrated increased stability of the 3β- and 6β-glucuronide metabolites of morphine in DBS [100]. Whereas in plasma, the glucuronides were stable for only 24 h at 4°C and for less than 8 h at room temperature, in DBS, these metabolites were stable for at least 7 days at 4°C and for 3 days at room temperature.

DBS sampling for opiate analysis may have several applications: for compliance monitoring in chronic pain patients, reliable quantification of opiates in the context of DUID, doping control or for the follow-up of drug addicts. The structures of most important opiates are given in Figure 1.3. Garcia Boy *et al.*, using authentic samples, found a good correlation between morphine concentrations in 100 µL blood and 100-µL DBS, albeit with a somewhat positive bias for the DBS [67]. Jantos *et al.*, also starting from complete 100-µL DBS, reported similar concentrations of the opiates morphine, hydromorphone, oxycodone and noroxycodone in DBS and whole blood [101]. Similar results were obtained by Odoardi *et al.* [47]. Using spiked blood samples, Goggin *et al.* determined DBS:plasma ratios for various opiates, including codeine, dihydrocodeine, morphine, hydrocodone, hydromorphone, oxycodone and oxymorphone in 9 samples [45]. Although not holding true for all analytes, the overall DBS:plasma ratios were more or less in line with expected ratios. While in only one sample of the report by Antelo-Dominguez *et al.* morphine concentrations were high enough to allow comparison between a DBS-based method and a GC-MS-based screening method, the results of both methods were essentially the same [96]. The automated DBS extraction procedure set up by Versace *et al.* for screening DBS for drugs of abuse showed limited sensitivity for opiates, as exemplified by the fact that the procedure frequently missed morphine and codeine in authentic samples [8]. On the other hand, the fully automated DBS-SPE-LC-MS/MS method developed by Verplaetse and Henion, seems promising for the quantification of morphine, codeine, oxycodone and hydrocodone in a real setting [40].

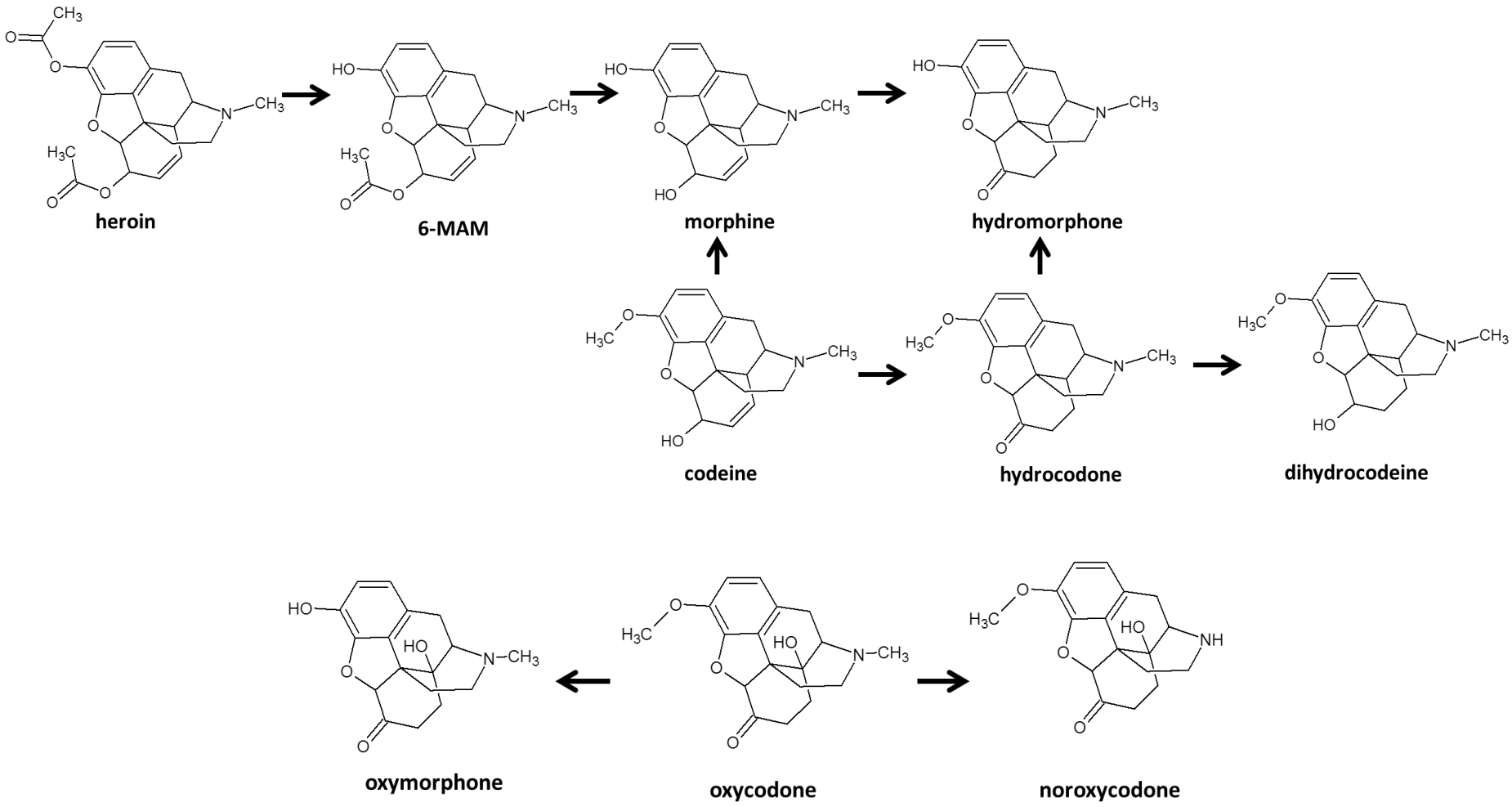


Figure 1.3 Structures of heroin, 6-MAM, morphine, hydromorphone, codeine, hydrocodone, dihydrocodeine, oxymorphone, oxycodone and noroxycodone.

1.9.5 Non-opiate opioids

Methadone is a long-acting synthetic μ-opioid receptor agonist used in rehabilitation programs by opiate addicts, in particular heroin addicts. This heroin substitute reduces craving and withdrawal symptoms, resulting in less tendency to use heroin. Similar to morphine, DBS sampling may facilitate compliance monitoring. Saracino *et al.* determined methadone concentrations in cDBS and in the corresponding plasma samples in methadone maintenance patients [20]. Bland-Altman plots showed a good correlation between both matrices, however, requiring the following: i) calculation of the blood volume contained within the DBS (calculated from the DBS surface, assuming an average Hct), ii) application of a correction factor to calculate the plasma concentration from the DBS concentration (again assuming an average Hct), and iii) assuming an even distribution of methadone between RBCs and plasma. The latter assumption in fact may not hold true, as methadone partitions more in plasma than in RBCs [102]. A deviating correlation was indeed observed by Goggin *et al.*, who compared methadone DBS concentrations with plasma levels [45]. Odoardi *et al.* observed similar DBS and blood concentrations in an authentic post-mortem sample [47]. The structures of methadone as well as its metabolites 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) are given in Figure 1.4.

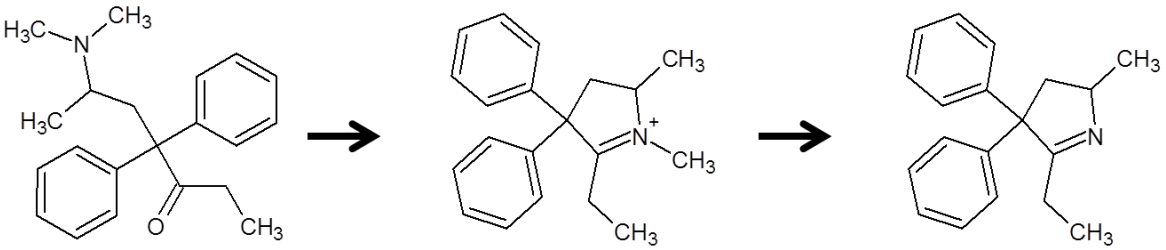


Figure 1.4 Structures of methadone, EDDP and EMDP.

Other synthetic opioids analyzed in DBS are the analgesics tramadol and fentanyl (also agonists) and buprenorphine, a partial agonist used in opioid addiction treatment (Figure 1.5). Using an online LC-MS/MS method, Thomas *et al.* were able to monitor the *in vivo* metabolism of buprenorphine by repeated DBS-based sampling following intraperitoneal injection of this molecule in a rat [11]. Verplaetse and Henion developed a method for the quantification of fentanyl using flow-through elution of the DBS card, followed by online-SPE [40]. Clavijo *et al.* successfully quantified fentanyl and its major metabolites, norfentanyl and despropionylfentanyl, in DBS of neonates for the assessment of PK [103]. Odoardi *et al.* used DBS as a sample preparation step for the determination of alfentanyl, fentanyl, norfentanyl and sufentanyl in post-mortem samples [47]. Jantos *et al.* found similar concentrations of fentanyl and norfentanyl in DBS and whole blood [101]. From a bridging study performed by Goggin *et al.*, where DBS and plasma concentrations of a variety of opioids were compared (e.g. tramadol, fentanyl and buprenorphine), it can be concluded that, although for some analytes a DBS:plasma ratio conform expectations was observed, more in-depth studies are needed [45].

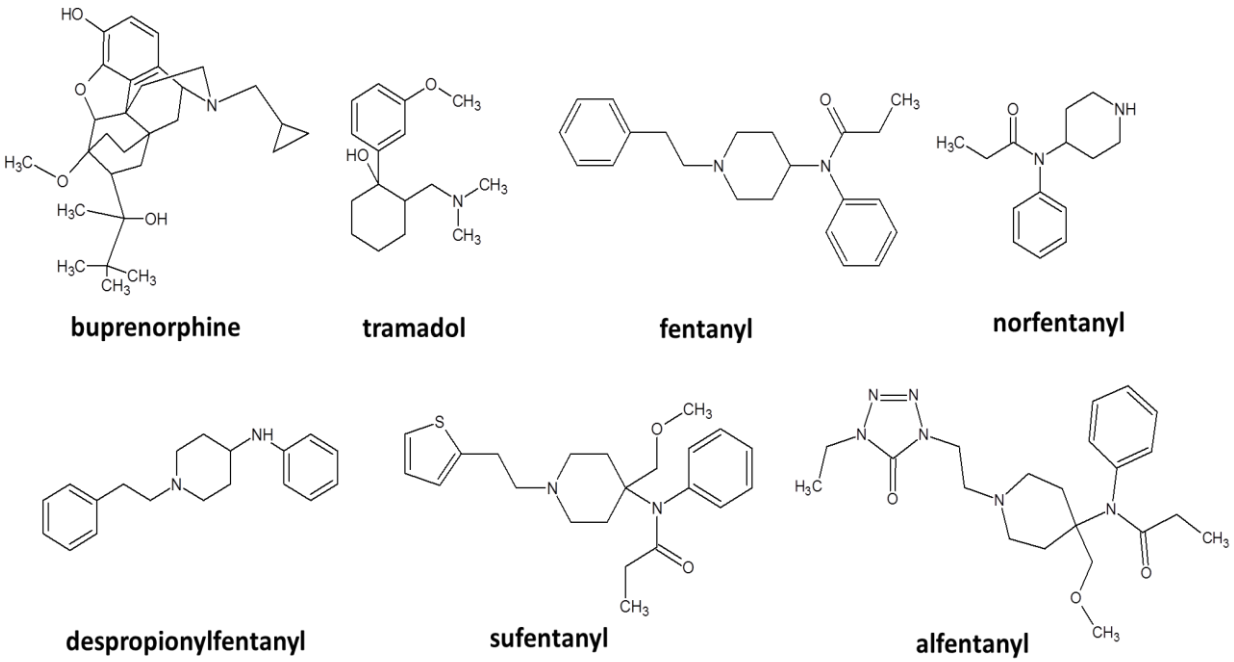


Figure 1.5 Structures of buprenorphine, tramadol, fentanyl, norfentanyl, despropionylfentanyl, sufentanyl and alfentanyl.

1.9.6 Benzodiazepines and Z-drugs

Benzodiazepines and Z-drugs (Figure 1.6) are prescribed for the treatment of insomnia or anxiety disorders. However, as these are addictive drugs and given their sleep-inducing effect, they are of interest in clinical and forensic toxicology and in the context of DUID. The amide in zopiclone and in most benzodiazepines renders these compounds subject to degradation, more particularly hydrolysis. Again, the use of DBS may offer a stability advantage, as has been demonstrated for zopiclone. Whereas in whole blood only 25% of the initial zopiclone concentrations was left after 8 days at room temperature, in low-level DBS which were stored for 30 days at room temperature still 77% of the initial concentration could be found [78]. These findings were corroborated by Déglon *et al.*, who reported stability of a multi-analyte panel after 30 days storage at room temperature [5]. These authors developed a simplified offline LC-MS/MS method, allowing detection of the major benzodiazepines (except bromazepam) and Z-drugs, along with selected metabolites, in 5-µL DBS.

To investigate the equivalence of DBS methods and whole blood methods, Jantos and Skopp analyzed blood and 100-µL DBS from volunteers participating in driving simulation experiments and who had ingested drugs, amongst which alprazolam [91]. They found a mean blood/DBS ratio of 0.99 for alprazolam, which means that the concentrations found in DBS do not differ from those in blood. Déglon *et al.* applied their method on DBS prepared from venous blood samples that had been collected in the context of DUID [5]. Again, a good correlation was seen between the quantitative results obtained from DBS and those obtained from blood that had been analyzed by routine LC-MS, resulting in the implementation of the simplified method in routine

analysis. Versace *et al.* also found similar results between a newly developed screening method based on an automated extraction of 5-µL DBS and routine screening procedures starting from 1 mL [8]. With the aim of phenotyping the liver enzyme CYP 3A4, de Boer *et al.* determined the PK profile of midazolam in plasma, whole blood, cDBS and vDBS. Regression analyses indicated a strong correlation between the concentrations in the corresponding samples. However, these analyses also demonstrated that, apart from the expected difference in concentration between blood and plasma, there was also a difference between concentrations in venous blood and DBS derived thereof, as well as between concentrations in vDBS and cDBS [46]. Midazolam has also been measured in rat DBS, using a fully automated system, coupling online DBS extraction to 2D LC and high resolution MS [39].

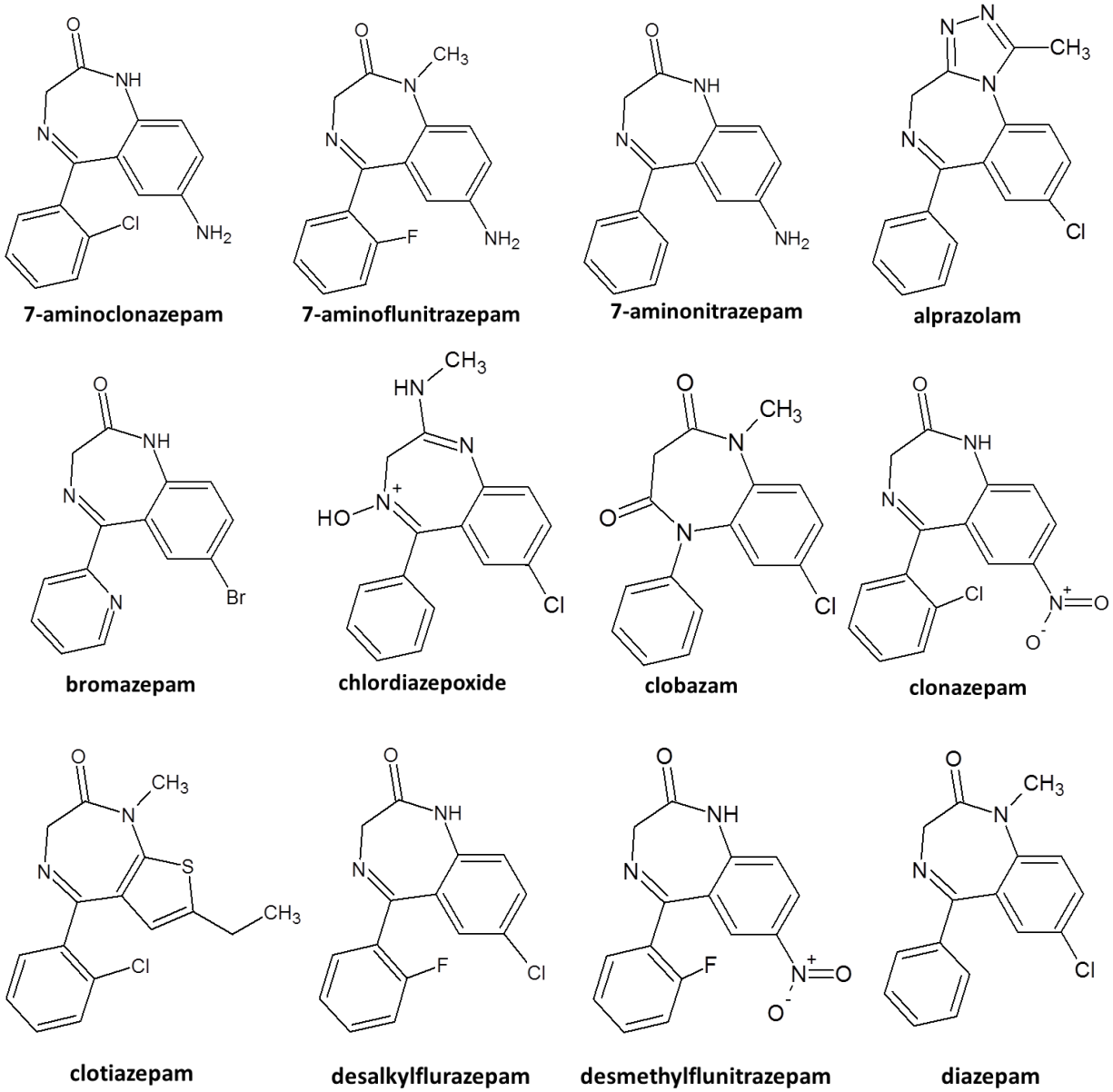


Figure 1.6 Structures of benzodiazepines and Z-drugs.

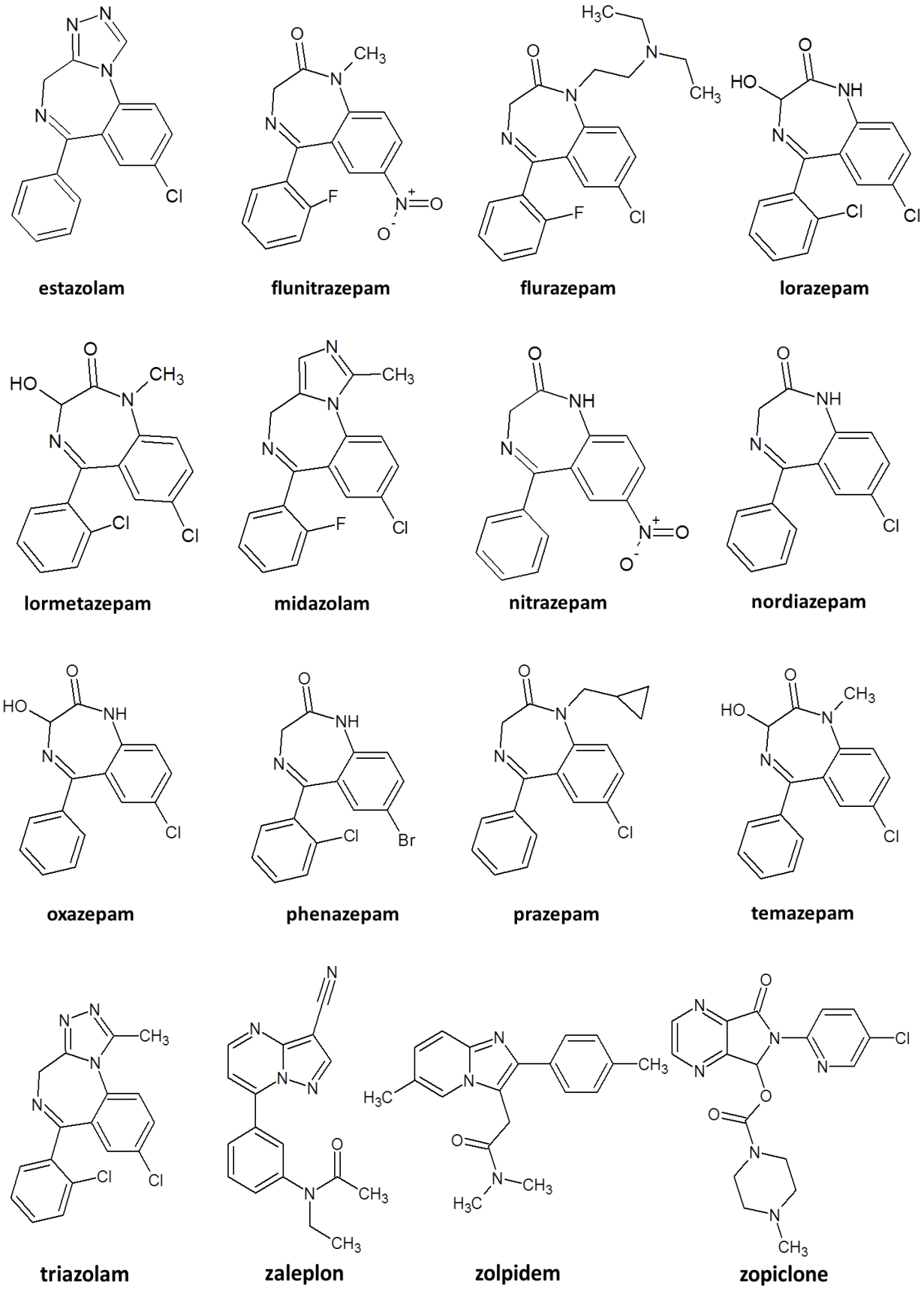


Figure 1.6 Continued.

1.9.7 Amphetamine and analogues (methamphetamine, MDMA, MDA and MDEA)

Methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxy-N-ethylamphetamine (MDEA) are amphetamine-like drugs (Figure 1.7). Determination of these drugs is relevant in the forensic context and in cases of DUID as consumption of MDMA may affect risk assessment and may result in a disturbed balance and motor coordination.

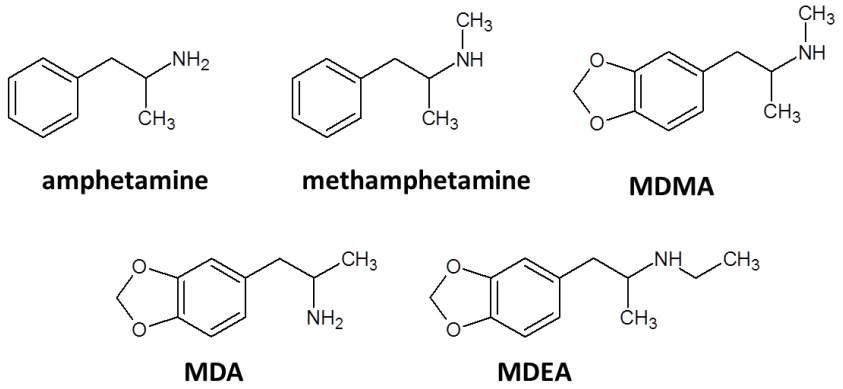


Figure 1.7 Structures of amphetamine and analogues.

Déglon *et al.*, Versace *et al.*, Ambach *et al.* as well as Kyriakou *et al.* developed DBS-based screening methods -either for classic drugs of abuse or for new psychoactive substances- enabling the detection of amphetamine-like drugs [8,12,80,93]. Thomas *et al.* focused on determination of prohibited drugs for doping controls [94]. For the quantitative analysis of amphetamine and analogues in DBS, Jantos and Skopp, Odoardi *et al.* as well as Saussereau *et al.*, developed an LC-MS/MS method, whereas Langel *et al.* applied GC-MS [26,31,47,91]. Using an automated DBS system, Verplaetse and Henion were able to quantify amphetamine, methamphetamine and MDMA [41].

Jantos and Skopp and Saussereau *et al.* performed a cross-comparison between vDBS and whole blood and concluded that both methods gave similar results [31,91]. Ambach *et al.* and Versace *et al.* also did not observe major discrepancies between DBS-based and blood-based screening, taking into account different sensitivities of both methods -the DBS method starting from 50 or even 200x less material but utilizing a higher-end MS than the methods for routine analysis [8,80].

1.9.8 Gamma-hydroxybutyric acid

GHB is primarily notorious for its use as club and date-rape drug (Figure 1.8). Unfortunately, interpretation of GHB concentrations is not always straightforward. First of all, the presence of GHB in a blood sample can be attributed either to illicit use of this substance or to its endogenous presence. Furthermore, GHB may be

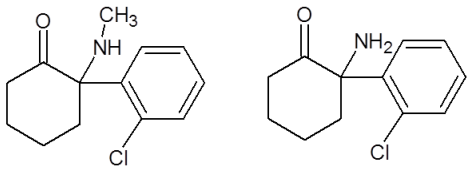
formed *de novo* post-mortem as well as *ex vivo* in stored samples [83,84]. This *ex vivo* formation, however, is not observed in DBS [24]. Additionally, GHB is quickly cleared from the circulation, making rapid sampling imperative. Here as well, DBS sampling may offer an interesting alternative since no medical staff is needed for the sampling procedure, hence allowing more prompt sampling. This way, GHB concentrations representative of the intoxication state at the moment of interest can be measured. Particularly in cases of drug-facilitated sexual assault (DFSA) a rapid sampling method may prove to be of crucial importance as there is most often a delay before the victim reports the assault. The good correlation between concentrations in venous blood, DBS prepared from venous blood and cDBS [24 and Chapter 2], suggests that the DBS sampling technique for GHB analysis in toxicological cases may be recommended in routine practice. In our laboratory, we routinely use dried matrix spots (DBS, but also dried plasma or dried urine spots) for GHB analysis in a forensic context. To this end, we prepare spots volumetrically and, after adding labeled IS, subject the complete spots to "on-spot derivatization" and analysis via GC-MS [23,24].

GHB

Figure 1.8 Structure of GHB.

1.9.9 Ketamine and norketamine

Ketamine is a popular drug for sedation and anesthesia in children which requires proper knowledge of PK and pharmacodynamics for choosing the right dose and dosing interval (Figure 1.9). DBS sampling is an attractive method for sampling blood for PK studies in neonates, as only a minimal amount of blood is needed [104]. Ketamine is also used as a recreational drug and in cases of DFSA. In these cases as well, DBS sampling may be a valuable option as sampling should be done as fast as possible (see GHB, subsection 1.9.8).



ketamine **norketamine**

Figure 1.9 Structures of ketamine and norketamine.

With the aim to improve the understanding of PK in neonates, Moll *et al.* developed a method for the quantification of ketamine and its main metabolite norketamine in DBS [104]. Ketamine was also included in the multi-analyte DBS-based procedures developed by Ambach *et al*. and Odoardi *et al.* [47,80]. Furthermore, utilizing a microextraction packed sorbent procedure followed by HPLC-diode array detection analysis, Saracino

et al. could also successfully quantify ketamine and norketamine in DBS [21]. Studies are still in progress to investigate the equivalence with whole blood.

1.9.10 New psychoactive substances

During the last decade, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has observed a significant increase of new (psychoactive) substances, mainly cathinones and synthetic cannabinoids [105]. Mephedrone, a cathinone analogue (Figure 1.10), was successfully quantified by Verplaetse and Henion using an online SPE-LC-MS/MS method, following flow-through desorption [41]. Ambach *et al.* developed an LC-MS/MS method for the screening of 64 established and new psychoactive substances (including e.g. amphetamines, cathinones, piperazines), using complete 10-µL DBS [80]. Samples that scored positive in this screening were quantified with a validated whole blood method used in routine analysis. As overall similar results were obtained with both methods and DBS may simplify sample preparation, the authors concluded that DBS are a useful matrix for rapid screening of new psychoactive substances. Furthermore, DBS have a stabilizing effect on cathinone derivatives when stored cooled [80]. Mercolini *et al.* demonstrated that other alternative matrices like hair, oral fluid, dried urine and dried plasma also have potential for the determination of new psychoactive substances [106,107].

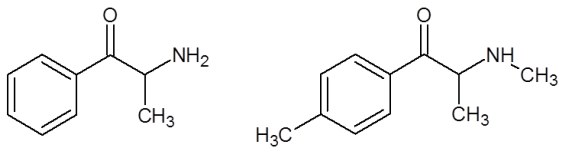


Figure 1.10 Structures of cathinone and mephedrone.

1.9.11 Alcohol markers

It has been well established that alcohol misuse has social and behavioral effects, as well as effects on mental and physical health. Consequently, tracing ethanol consumption or abstinence is relevant, either for clinical, forensic or traffic cases (e.g. in driver's license regranting), or in the context of workplace monitoring. Assessing whether someone is under the influence of alcohol can be done by breath testing and by measuring blood alcohol concentrations. However, when the aim is to evaluate whether ethanol consumption took place in the recent past, the small detection window of ethanol itself may not suffice. There are several alternative markers available for this purpose. Examples include ethylglucuronide and ethylsulfate, which are highly sensitive and specific markers of either recent (blood, urine) or historic (hair, nails) intake of ethanol [108,109]. Redondo *et al.* successfully developed a method allowing detection of these biomarkers in complete 10-µl DBS [110]. As long-term alcohol biomarker, however, ethylglucuronide and ethylsulfate are not widespread yet. An alternative marker is PEth, the collective term for a group of ethanol-derived phospholipids formed from

phospathidylcholine after ethanol consumption. Formation of these products is catalyzed by phospholipase D in the membranes of RBCs. PEth has gained popularity as biomarker of alcohol misuse during the last few years and should offer a wider window of detection than ethylglucuronide and ethylsulfate [43,111-114]. However, PEth is susceptible to post-sampling synthesis during storage of ethanol-containing samples and could generate false positive results [43,112]. Consequently, blood samples have to be stored at -80°C and should be analyzed within 30 days [111]. DBS, however, lack *ex vivo de novo* formation of PEth, as demonstrated by Jones *et al.*: PEth 16:0/18:1 (a specific isomer) could not be detected in DBS stored in ethanol vapor or in DBS which were spiked to significant ethanol concentrations [43]. According to Kummer *et al.*, PEth 16:0/18:1 and PEth 18:1/18:1 are stable for 6 months in DBS when stored at room temperature. They developed an UHPLC-MS/MS method for the quantification of PEth species (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 (see Figure 1.11)) in blood and DBS and found a good correlation between venous blood, vDBS and cDBS concentrations, suggesting that DBS are a valid alternative to venous blood for the detection of alcohol consumption [51].

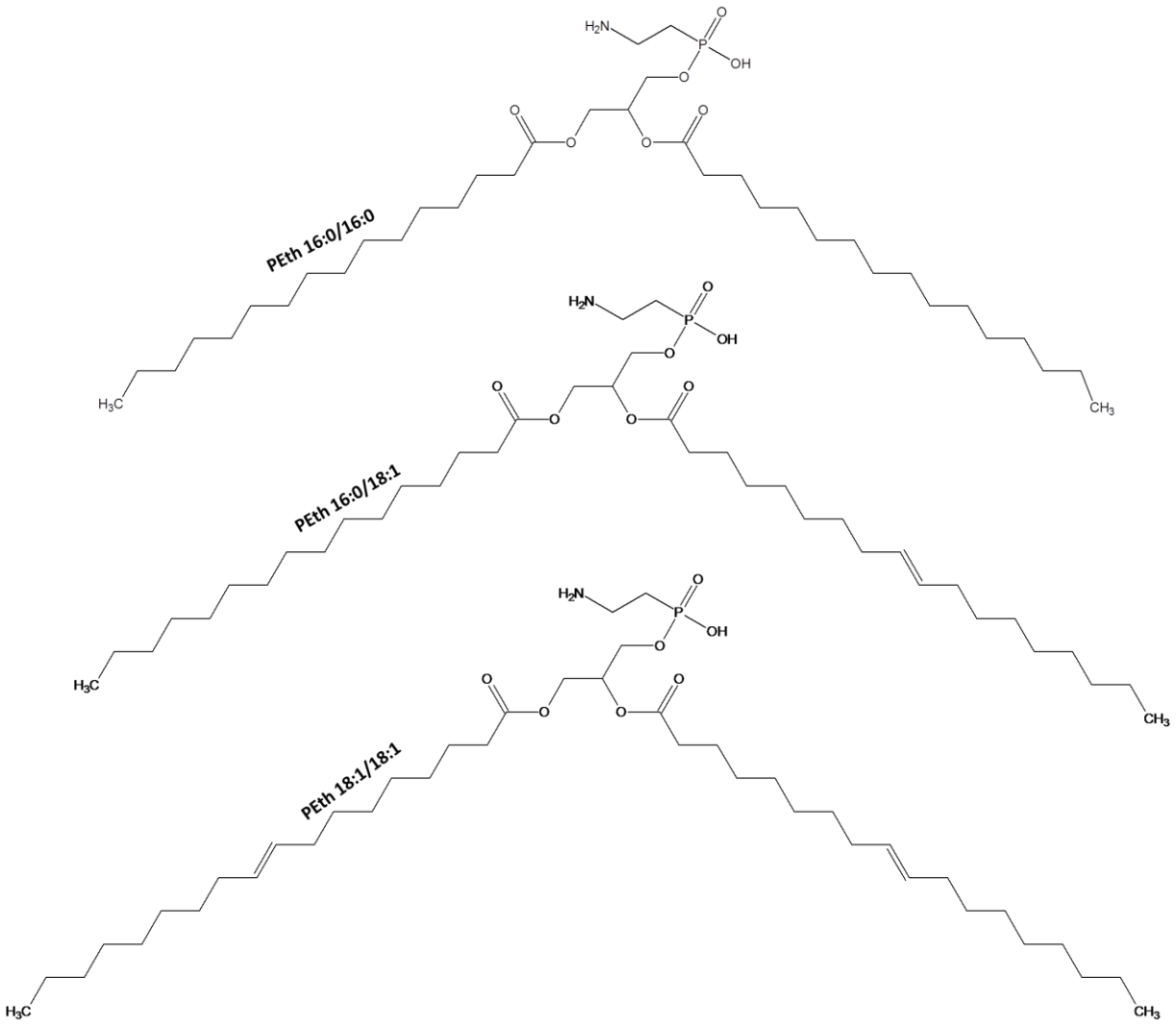


Figure 1.11 Structures of PEth 16:0/16:0, PEth 18:1/18:1 and PEth 16:0/18:1.

Given the risk of neurocognitive problems in children exposed to alcohol during pregnancy, attention is paid to screening newborns for prenatal alcohol exposure (PAE) [113]. Therefore, meconium fatty acid ethyl esters (FAEE) are, to date, measured to confirm PAE. However, this approach suffers from some limitations, amongst which low sensitivity and possibility of post-collection synthesis [43,114]. Detection of PEth in newborn DBS may be an interesting alternative for FAEE in meconium as post-collection synthesis does not occur on DBS cards [43] and no extra sampling is needed since DBS are already taken from newborns to screen for metabolic disorders. Although first reports confirm that accurate detection of PEth in conjunction with other biomarkers facilitates detection of PAE, other studies are warranted to further examine the utility of determining PEth in newborn DBS for identifying cases of PAE [113]. It has to be pointed out that, given the relatively short detection window (about 2-3 weeks), PEth in DBS is a biomarker of PAE during the last stage of pregnancy, whereas meconium FAEE allow detection of alcohol consumption during the 2^{nd} and 3^{rd} trimester [114]. However, in spite of the longer detection window of the latter biological matrix, it is still not able to identify maternal alcohol consumption in early pregnancy. Additionally, detection of PEth in DBS may allow more sensitive detection of PAE than meconium FAEE, despite the much shorter detection window [113].

1.9.12. Cotinine

The occurrence of low birth weight, childhood cancers and premature death has been associated with the active use of tobacco and exposure to second-hand smoke during pregnancy [22,29,49,72,115]. To assess exposure to tobacco or second-hand smoke, cotinine concentrations are measured as cotinine is the primary metabolite of nicotine. Given the good correlation between cotinine concentrations in DBS and in plasma, Murphy *et al.* proposed DBS cotinine as a reliable biomarker of tobacco metabolism [29]. Furthermore, several studies in newborns demonstrated that cotinine levels in newborn DBS may be used to identify maternal smoking at the very end of pregnancy [22,49,115]. However, several limitations have to be considered. Given the small sample volume, cotinine in newborn DBS could not be used as a biomarker of maternal second-hand smoke because of sensitivity issues [49,72]. Moreover, one has to be aware that cotinine concentrations will only be increased when DBS are collected within 72 h after birth and exposure was close to the time of delivery [22,29,49,72]. Yang *et al.* also found a good correlation between DBS cotinine levels and umbilical cord blood cotinine concentrations in a large set of samples (n = 100) from a newborn screening program, although concentrations in umbilical cord blood were somewhat higher [49]. Wang *et al*. also reported similar cotinine concentrations in blood and DBS from a rat that had been injected with nicotine [35]. These authors used the interesting approach of paper spray MS, a technique in which a spray, that is generated by applying a high voltage directly onto filter paper, directly enters the MS. Using a fully automated online SPE LC-HRMS/MS approach, Tretzel *et al.* were able to quantify nicotine, its major metabolites nornicotine, cotinine and trans-3'-hydroxycotinine and the tobacco alkaloids anabasine and anatabine for sports drug testing purposes [42]. The structures of tobacco markers are given in Figure 1.12.

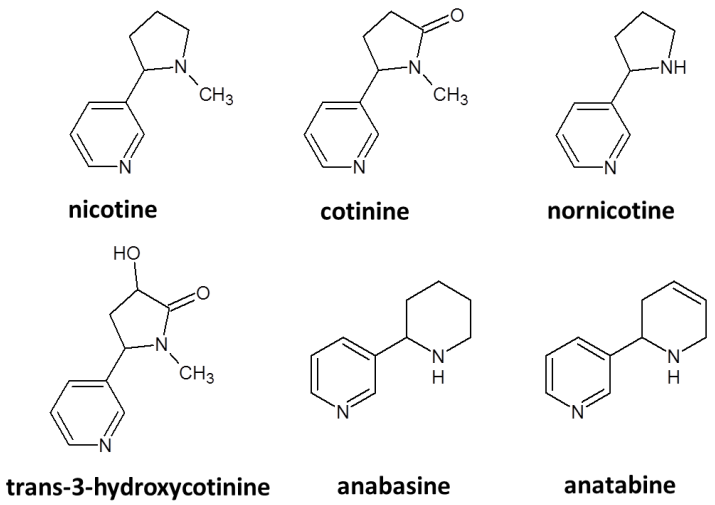


Figure 1.12 Structures of tobacco markers.

1.10 Conclusion

A substantial number of DBS methods is available to detect and quantify a vast array of abused substances. The majority of these methods shows sufficient sensitivity to be relevant in the context of DUID, despite the fact that some compounds (e.g. THC) are clearly more challenging than others. Moreover, DBS sampling provides several benefits over conventional sampling techniques that can be advantageous for the analysis of abused substances. First, this technique is minimally invasive and represents a fast, simple and economic way to collect a specimen that provides information on the acute intoxication state of a person. Second, as DBS sampling can be performed by non-medical personnel, the time until sample collection is minimized, which is especially of importance in the context of DUID and DFSA. Third, DBS offer a very stable matrix, reducing degradation and preventing *de novo* formation, often even at room temperature, thereby facilitating storage and transportation. As discussed above (section 1.8), benzodiazepines, cocaine, zopiclone, cathinones and 6-MAM are susceptible to degradation in whole blood, whereas the degradation process is reduced or minimized in DBS. It should be noted though, that for several analytes, storage will still require freezing at -20°C or flushing with N_2 [41]. Furthermore, *ex vivo* formation of GHB or of the alcohol biomarker PEth is not seen in DBS, in contrast to other biological fluids. Last, but certainly not least, given the higher prevalence of viral infections in drug abusers, DBS sampling may provide an appealing alternative to venipuncture, as the dried matrix of DBS drastically reduces biohazard.

Aside from quantitative applications, DBS can be used for rapid toxicological screening as well, since DBS may simplify sample preparation and reduce the necessary sample volume, which in (forensic) toxicological analysis can be limited. Additionally, DBS, which are already collected in the context of newborn screening programs for metabolic diseases, also provide a very interesting matrix for the assessment of newborn exposure to tobacco, ethanol and certain drugs during (the last part of) pregnancy. This facilitates extensive studies evaluating the

effect of these factors on the occurrence of e.g. premature death and childhood cancers. Moreover, these methods can also be used in a more clinical context, e.g. for the diagnosis of PAE.

Despite the numerous advantages associated with DBS sampling and the wide range of applications, this approach also faces some challenges. Given the small blood volume that is available, highly sensitive detection techniques are often required. For instance, cannabinoid quantitation proved to be a real challenge as, to the best of our knowledge, only one method, using high-end equipment, attained sufficient sensitivity, yet still requiring complete 20-µL DBS. Other challenges associated with DBS sampling are contamination risk, the acquisition of correctly obtained samples, the influence of the site of punching and of the blood volume spotted, the chromatographic effect and the Hct effect, the most widely discussed DBS-related problem. With respect to the latter, several solutions have been proposed [54]. Moreover, the number of applications performed in a real setting and the number of applications demonstrating the equivalence with results in other biological fluids are limited. Based on the available data, it can be concluded that in most cases no "DBS effect" *per se* is present, meaning that equivalent concentrations are present in DBS and in whole blood. Whether this also holds true for cDBS and venous blood, remains to be established in most cases. Conclusively, although current DBS approaches for the detection of abused substances seem promising, more experiments are warranted for the implementation of DBS sampling in routine analysis.

1.11 References

1. Bang I. *Der Blutzucker*, Wiesbaden, Germany (1913).
2. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 32(3), 338-343 (1963).
3. Li W, Tse F. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed Chromatogr* 24(1), 49-65 (2010).
4. Stove C, Ingels A, De Kesel P, Lambert W. Dried blood spots in toxicology: from the cradle to the grave? *Crit Rev Toxicol* 42(3), 230-243 (2012).
5. Déglon J, Versace F, Lauer E *et al.* Rapid LC-MS/MS quantification of the major benzodiazepines and their metabolites on dried blood spots using a simple and cost-effective sample pretreatment. *Bioanalysis* 4(11), 1337-1350 (2012).
6. Mercolini L, Mandrioli R, Gerra G, Raggi M. Analysis of cocaine and two metabolites in dried blood spots by liquid chromatography with fluorescence detection: a novel test for cocaine and alcohol intake. *J Chromatogr A* 1217(46), 7242-7248 (2010).
7. Mercolini L, Mandrioli R, Sorella V *et al.* Dried blood spots: liquid chromatography-mass spectrometry analysis of Delta9-tetrahydrocannabinol and its main metabolites. *J Chromatogr A* 1271(1), 33-40 (2013).
8. Versace F, Déglon J, Lauer E, Mangin P, Staub C. Automated DBS extraction prior to hilic/RP LC-MS/MS target screening of drugs. *Chromatographia* 76(19-20), 1281-1293 (2013).
9. Hudson W, Yong B, Boguszewski P. Analysis of clozapine, nortriptyline, paroxetine and zolpidem using dried blood spots. Agilent Technologies. http://www.chem.agilent.com/Library/applications/5990-8033EN.pdf.10.
10. Eibak L, Hegge A, Rasmussen K, Pedersen-Bjergaard S, Gjelstad A. Alginate and chitosan foam combined with electromembrane extraction for dried blood spot analysis. *Anal Chem* 84(20), 8783-8789 (2012).
11. Thomas A, Déglon J, Steimer T, Mangin P, Daali Y, Staub C. On-line desorption of dried blood spots coupled to hydrophilic interaction/reversed-phase LC/MS/MS system for the simultaneous analysis of drugs and their polar metabolites. *J Sep Sci* 33(6-7), 873-879 (2010).
12. Déglon J, Thomas A, Mangin P, Staub C. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Anal Bioanal Chem* 402(8), 2485-2498 (2012).
13. Lauer E, Déglon J, Versace F, Thomas A, Mangin P, Staub C. Target screening of drugs from dried blood spot samples based on LC-MS/MS and on-line desorption. Presented at: *49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT)*. San Francisco, CA, USA, 25-30 September 2011.
14. Lenk G, Sandkvist S, Pohanka A, Stemme G, Beck O, Roxhed N. A disposable sampling device to collect volume-measured DBS directly from a fingerprick onto DBS paper. *Bioanalysis* 7(16), 2085-2094 (2015).

15. Leuthold LA, Heudi O, Déglon J, *et al*. New microfluidic-based sampling procedure for overcoming the hematocrit problem associated with dried blood spot analysis. *Anal Chem* 87(4), 2068-2071 (2015).
16. Denniff P, Spooner N. Volumetric absorptive microsampling: a dried sample collection technique for quantitative bioanalysis. *Anal Chem* 86(16), 8489-8495 (2014).
17. Spooner N, Denniff P, Michielsen L, *et al.* A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. *Bioanalysis* 7(6), 653-659 (2015).
18. Denniff P, Spooner N. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. *Bioanalysis* 2(8), 1385-1395 (2010).
19. Henderson L, Powell M, Hannon W *et al.* Radioimmunoassay screening of dried blood spot materials for benzoylecgonine. *J Anal Toxicol* 17(1), 42-47 (1993).
20. Saracino M, Marcheselli C, Somaini L *et al.* A novel test using dried blood spots for the chromatographic assay of methadone. *Anal Bioanal Chem.* 404(2), 503-511 (2012).
21. Saracino M, Marcheselli C, Bugamelli F, Morganti E, Serpelloni G, Raggi M. Club drugs: analysis of ketamine and norketamine in dried blood spots by HPLC-DAD-MEPS. Presented at: *24th International Symposium on Pharmaceutical and Biomedical Analysis.* Bologna, Italy, 30 June - 3 July 2013.
22. Spector L, Hecht S, Ognjanovic S, Carmella S, Ross J. Detection of cotinine in newborn dried blood spots. *Cancer Epidemiol Biomarkers Prev* 16(9), 1902-1905 (2007).
23. Ingels A, Lambert W, Stove C. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. *Anal Bioanal Chem* 398(5), 2173-2182 (2010).
24. Ingels A, De Paepe P, Anseeuw K *et al.* Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis* 3(20), 2271-2281 (2011).
25. Ingels A, Hertegonne K, Lambert W, Stove C. Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home: an exploratory study. *CNS drugs* 27(3), 233-237 (2013).
26. Langel K, Uusivirta H, Ariniemi K, Lillsunde P. Analysis of drugs of abuse by GC-MS in dried blood spot sample matrix. Presented at: *49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT)*. San Francisco, CA, USA, 25-30 September 2011.
27. Déglon J, Thomas A, Cataldo A, Mangin P, Staub C. On-line desorption of dried blood spot: a novel approach for the direct LC/MS analysis of micro-whole blood samples. *J Pharm Biomed Anal* 49(4), 1034-1039 (2009).
28. Sadones N, Van Bever E, Archer JRH, *et al*. Microwave-assisted on-spot derivatization for gas chromatography-mass spectrometry based determination of polar low molecular weight compounds in dried blood spots. *J Chromatogr A* 1465, 175-183 (2016).
29. Murphy S, Wickham K, Lindgren B, Spector L, Joseph A. Cotinine and trans 3'-hydroxycotinine in dried blood spots as biomarkers of tobacco exposure and nicotine metabolism. *J Expo Sci Environ Epidemiol* 23(5), 513-518 (2013).

30. Clavijo C, Thomas J, Hammer G, Christians U, Galinkin J. A sensitive LC-MS/MS assay for quantification of methadone and its metabolites in dried blood spots. Presented at: *2010 Annual Meeting of the American Society of Anesthesiologists (ASA).* San Diego, CA, USA, 16-20 October 2010.
31. Saussereau E, Lacroix C, Gaulier J, Goulle J. On-line liquid chromatography/tandem mass spectrometry simultaneous determination of opiates, cocainics and amphetamines in dried blood spots. *J Chromatogr B Analyt Technol Biomed Life Sci* 885-886, 1-7 (2012).
32. Lafrenière N, Shih S, Abu-Rabie P, Jebrail M, Spooner N, Wheeler A. Multiplexed extraction and quantitative analysis of pharmaceuticals from DBS samples using digital microfluidics. *Bioanalysis* 6(3), 307-318 (2014).
33. Liu J, Wang H, Manicke N, Lin J, Cooks R, Ouyang Z. Development, characterization, and application of paper spray ionization. *Anal Chem* 82(6), 2463-2471 (2010).
34. Zhang Z, Xu W, Manicke N, Cooks R, Ouyang Z. Silica coated paper substrate for paper-spray analysis of therapeutic drugs in dried blood spots. *Anal Chem* 84(2), 931-938 (2012).
35. Wang H, Ren Y, McLuckey M *et al.* Direct quantitative analysis of nicotine alkaloids from biofluid samples using paper spray mass spectrometry. *Anal Chem* 85(23), 11540-11544 (2013).
36. Seip K, Gjelstad A, Pedersen-Bjergaard S. Electromembrane extraction from aqueous samples containing polar organic solvents. *J Chromatogr A* 1308, 37-44 (2013).
37. Johnson C, Christianson C, Sheaff C, Laine D, Zimmer J, Needham S. Use of conventional bioanalytical devices to automate DBS extractions in liquid-handling dispensing tips. *Bioanalysis* 3(20), 2303-2310 (2011).
38. Henion J, Oliveira R, Fumin L, Foley T, Pomponio R. Dried blood spots: the future. In: *Microsampling in Pharmaceutical Bioanalysis (ebook).* Zane P, Emmons GT (Eds), Future Science Ltd, 48-66 (2013).
39. Oliveira R, Henion J, Wickremsinhe E. Fully-automated approach for online dried blood spot extraction and bioanalysis by two-dimensional-liquid chromatography coupled with high-resolution quadrupole time-of-flight mass spectrometry. *Anal Chem* 86(2), 1246-1253 (2014).
40. Verplaetse R, Henion J. Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS. *Drug Test Anal* 8(1), 30-38 (2016).
41. Verplaetse R, Henion J. Hematocrit-independent quantitation of stimulants in dried blood spots: pipet versus microfluidic-based volumetric sampling coupled with automated flow-through desorption and online solid phase extraction-LC-MS/MS bioanalysis. *Anal Chem* 88(13), 6789-6796 (2016).
42. Tretzel L, Thomas A, Piper T, *et al.* Fully automated determination of nicotine and its major metabolites in whole blood by means of a DBS online-SPE LC-HR-MS/MS approach for sports drug testing. *J Pharm Biomed Anal* 123, 132-140 (2016).
43. Jones J, Jones M, Plate C, Lewis D. The detection of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol in human dried blood spots. *Anal Methods* 3(5), 1101-1106 (2011).
44. Forni S, Pearl P, Gibson K, Yu Y, Sweetman L. Quantitation of gamma-hydroxybutyric acid in dried blood spots: feasibility assessment for newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency. *Mol Genet Metab* 109(3), 255-259 (2013).

45. Goggin M, Lundberg R, Janis G. Measuring circulating levels of 13 opiate and opioid analgesics from dried blood spot samples. Presented at: *American Association of Pharmaceutical Scientists (AAPS).* Chicago, USA, 14-17 October 2012.
46. de Boer T, Wieling J, Meulman E *et al.* Application of dried blood spot sampling combined with LC-MS/MS for genotyping and phenotyping of CYP450 enzymes in healthy volunteers. *Biomed Chromatogr* 25(10), 1112-1123 (2011).
47. Odoardi S, Anzillotti L, Strano-Rossi S. Simplifying sample pretreatment: application of dried blood spot (DBS) method to blood samples, including postmortem, for UHPLC-MS/MS analysis of drugs of abuse. *Forensic Sci Int* 243, 61-67 (2014).
48. Abu-Rabie P, Spooner N. Direct quantitative bioanalysis of drugs in dried blood spot samples using a thin-layer chromatography mass spectrometer interface. *Anal Chem* 81(24), 10275-10284 (2009).
49. Yang J, Pearl M, Jacob Pr *et al.* Levels of cotinine in dried blood specimens from newborns as a biomarker of maternal smoking close to the time of delivery. *Am J Epidemiol* 178(11), 1648-1654 (2013).
50. Verstraete A, Knoche A, Jantos R, *et al.* DRUID, deliverable 1.4.2. per se limits- methods of defining cut-off values for zero tolerance (2011).
51. Kummer N, Ingels AS, Wille SM, *et al.* Quantification of phosphatidylethanol 16:0/18:1, 18:1/18:1, and 16:0/16:0 in venous blood and venous and capillary dried blood spots from patients in alcohol withdrawal and control volunteers. *Anal Bioanal Chem* 408(3), 825-838 (2016).
52. Edelbroek P, van der Heijden J, Stolk L. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Ther Drug Monit* 31(3), 327-336 (2009).
53. O'Mara M, Hudson-Curtis B, Olson K, Yueh Y, Dunn J, Spooner N. The effect of hematocrit and punch location on assay bias during quantitative bioanalysis of dried blood spot samples. *Bioanalysis* 3(20), 2335-2347 (2011).
54. De Kesel P, Sadones N, Capiau S, Lambert W, Stove C. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis* 5(16), 2023-2041 (2013).
55. van Baar B, Verhaeghe T, Heudi O *et al.* IS addition in bioanalysis of DBS: results from the EBF DBS-microsampling consortium. *Bioanalysis* 5(17), 2137-2145 (2013).
56. Sosnoff C, Ann Q, Bernert JJ *et al.* Analysis of benzoylecgonine in dried blood spots by liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry. *J Anal Toxicol* 20(3), 179-184 (1996).
57. Abu-Rabie P, Denniff P, Spooner N, Brynjolffssen J, Galluzzo P, Sanders G. Method of applying internal standard to dried matrix spot samples for use in quantitative bioanalysis. *Anal Chem* 83(22), 8779-8786 (2011).
58. Zimmer D, Hassler S, Betschart B, Sack S, Fankhauser C, Loppacher M. Internal standard application to dried blood spots by spraying: investigation of the internal standard distribution. *Bioanalysis* 5(6), 711-719 (2013).

59. Abu-Rabie P, Denniff P, Spooner N, Chowdhry BZ, Pullen FS. Investigation of different approaches to incorporating internal standard in DBS quantitative bioanalytical workflows and their effect on nullifying hematocrit-based assay bias. *Anal Chem* 87(9), 4996-5003 (2015).

60. Manicke NE, Abu-Rabie P, Spooner N, Ouyang Z, Cooks RG. Quantitative analysis of therapeutic drugs in dried blood spot samples by paper spray mass spectrometry: an avenue to therapeutic drug monitoring. *J Am Soc Mass Spectrom* 22(9), 1501-1507 (2011).

61. Mommers J, Mengerink Y, Ritzen E, Weusten J, van der Heijden J, van der Wal S. Quantitative analysis of morphine in dried blood spots by using morphine-d3 pre-impregnated dried blood spot cards. *Anal Chim Acta* 774, 26-32 (2013).

62. Sadones N, Archer JR, Ingels AM, *et al*. Do capillary dried blood spot concentrations of gamma-hydroxybutyric acid mirror those in venous blood? A comparative study. *Drug Test Anal* 7(4), 336-340 (2015).

63. Ellefsen KN, da Costa JL, Concheiro M, *et al.* Cocaine and metabolite concentrations in DBS and venous blood after controlled intravenous cocaine administration. *Bioanalysis* 7(16), 2041-2056 (2015).

64. Bland J, Altman D. Stastistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1(8476), 307-310 (1986).

65. Stockl D, Dewitte K, Thienpont L. Validity of linear regression in method comparison studies: is it limited by the statistical model or the quality of the analytical input data? *Clin Chem* 44(11), 2340-2346 (1998).

66. Dewitte K, Fierens C, Stockl D, Thienpont L. Application of the Bland-Altman plot for interpretation of method-comparison studies: A critical investigation of its practice. *Clin Chem* 48(5), 799-801 (2002).

67. Garcia Boy R, Henseler J, Mattern R, Skopp G. Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots. *Ther Drug Monit* 30(6), 733-739 (2008).

68. Capiau S, Stove V, Lambert W, Stove C. Prediction of the hematocrit of dried blood spots via potassium measurement on a routine clinical chemistry analyzer. *Anal Chem* 85(1), 404-410 (2013).

69. De Kesel PM, Capiau S, Stove VV, Lambert WE, Stove CP. Potassium-based algorithm allows correction for the hematocrit bias in quantitative analysis of caffeine and its major metabolite in dried blood spots. *Anal Bioanal Chem* 406(26), 6749-6755 (2014).

70. Capiau S, Wilk LS, Aalders MC, Stove CP. A novel, nondestructive, dried blood spot-based hematocrit prediction method using noncontact diffuse reflectance spectroscopy. *Anal Chem* 88(12), 6538-6546 (2016).

71. Oostendorp M, El Amrani M, Diemel EC, Hekman D, van Maarseveen EM. Measurement of hematocrit in dried blood spots using near-infrared spectroscopy: robust, fast, and nondestructive. *Clin Chem* 62(11), 1534-1536 (2016).

72. Sosnoff C, Bernert J. Analysis of cotinine in dried blood spots by LC APCI tandem mass spectrometry. *Clin Chim Acta* 388(1-2), 228-229 (2008).

73. Li Y, Henion J, Abbott R, Wang P. The use of a membrane filtration device to form dried plasma spots for the quantitative determination of guanfacine in whole blood. *Rapid Commun Mass Spectrom* 26(10), 1208-1212 (2012).

74. Kim JH, Woenker T, Adamec J, Regnier F. Simple, miniaturized blood plasma extraction method. *Anal Chem* 85(23), 11501-11508 (2013).

75. Sturm R, Henion J, Abbott R, Wang P. Novel membrane devices and their potential utility in blood sample collection prior to analysis of dried plasma spots. *Bioanalysis* 7(16), 1987-2002 (2015).

76. Ryona I, Henion J. A book-type dried plasma spot card for automated flow-through elution coupled with on-line SPE-LC-MS/MS bioanalysis of opioids and stimulants in blood. *Anal Chem* in press (2016).

77. Alfazil A, Anderson R. Stability of benzodiazepines and cocaine in blood spots stored on filter paper. *J Anal Toxicol* 32(7), 511-515 (2008).

78. Jantos R, Vermeeren A, Sabljic D, Ramaekers J, Skopp G. Degradation of zopiclone during storage of spiked and authentic whole blood and matching dried blood spots. *Int J Legal Med* 127(1), 69-76 (2013).

79. Skopp G. Blood spot analysis. Presented at: *Joint Meeting of the ICADTS and TIAFT*. Seattle, WA, USA, 26-30 August 2007.

80. Ambach L, Redondo H, Konig S, Weinmann W. Rapid and simple LC-MS/MS screening of 64 novel psychoactive substances using dried blood spots. *Drug Test Anal* 6(4), 367-375 (2013).

81. Sorensen L. Determination of cathinones and related ephedrines in forensic whole-blood samples by liquid-chromatography-electrospray tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 879(11-12), 727-736 (2011).

82. Johnson R, Botch-Jones S. The stability of four designer drugs: MDPV, mephedrone, BZP and TFMPP in three biological matrices under various storage conditions. *J Anal Toxicol* 37(2), 51-55 (2013).

83. Berankova K, Mutnanska K, Balikova M. Gamma-hydroxybutyric acid stability and formation in blood and urine. *Forensic Sci Int* 161(2-3), 158-162 (2006).

84. Zorntlein S, Kopp A, Becker J, Kaufmann T, Rohrich J, Urban R. In vitro production of GHB in blood and serum samples under various storage conditions. *Forensic Sci Int* 214(1-3), 113-117 (2012).

85. Aradottir S, Seidl S, Wurst F, Jonsson B, Alling C. Phosphatidylethanol in human organs and blood: a study on autopsy material and influences by storage conditions. *Alcohol Clin Exp Res* 28(11), 1718-1723 (2004).

86. Helander A, Zheng Y. Molecular species of the alcohol biomarker phosphatidylethanol in human blood measured by LC-MS. *Clin Chem* 55(7), 1395-1405 (2009).

87. Huestis M, Verstraete A, Kwong T, Morland J, Vincent M, de la Torre R. Oral fluid testing: promises and pitfalls. *Clin Chem* 57(6), 805-810 (2011).

88. De Kesel PMM, Lambert WE, Stove CP. The role of (bio)markers in hair analysis. In: *New sampling strategies in toxicology and therapeutic drug monitoring* (ebook), Stove CP (Ed), Future Science Ltd, 121-134 (2015).

89. Cooper G, Kronstrand R, Kintz P. Society of Hair Testing guidelines for drug testing in hair. *Forensic Sci Int* 218(1-3), 20-24 (2012).

90. Trudeau S, Mineau P, Cartier G *et al.* Using dried blood spots stored on filter paper to measure cholinesterase activity in wild avian species. *Biomarkers* 12(2), 145-154 (2007).

91. Jantos R, Skopp G. Comparison of drug analysis in whole blood and dried blood spots. *Toxichem Krimtech* 78, 268-275 (2011).

92. Huestis M, Henningfield J, Cone E. Blood cannabinoids. I. Absorption of THC and formation of 11-OH-THC and THCCOOH during and after smoling marijuana. *J Anal Toxicol* 16(5), 276-282 (1992).

93. Kyriakou C, Marchei E, Scaravelli G, Garcia-Algar O, Supervia A, Graziano S. Identification and quantification of psychoactive drugs in whole blood using dried blood spot (DBS) by ultra-performance liquid chromatography tandem mass spectrometry. *J Pharm Biomed Anal* 128, 53-60 (2016).

94. Thomas A, Geyer H, Schanzer W *et al.* Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. *Anal Bioanal Chem* 403(5), 1279-1289 (2012).

95. Huestis MA. Human cannabinoid pharmacokinetics. *Chem Biodivers* 4(8), 1770-1804 (2007).

96. Antelo-Dominguez A, Cocho J, Tabernero M, Bermejo A, Bermejo-Barrera P, Moreda-Pineiro A. Simultaneous determination of cocaine and opiates in dried blood spots by electrospray ionization tandem mass spectrometry. *Talanta* 117, 235-241 (2013).

97. Henderson L, Powell M, Hannon W *et al.* An evaluation of the use of dried blood spots from newborn screening for monitoring the prevalence of cocaine use among childbearing women. *Biochem Mol Med* 61(2), 143-151 (1997).

98. Parker S, Cubitt W. The use of the dried blood spot sample in epidemiological studies. *J Clin Pathol* 52(9), 633-639 (1999).

99. Mei J, Alexander J, Adam B, Hannon W. Use of filter paper for the collection and analysis of human whole blood specimens. *J Nutr* 131(5), 1631S-1636S (2001).

100. Clavijo C, Hoffman K, Thomas J *et al.* A sensitive assay for the quantification of morphine and its active metabolites in human plasma and dried blood spots using high-performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem* 400(3), 715-728 (2011).

101. Jantos R, Schumacher M, Skopp G. Comparison of opioid analysis in whole blood and dried blood spots. Presented at: *49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT)*. San Francisco, CA, USA, 25-30 September 2011.

102. Baselt R. In: *Disposition of toxic drugs and chemicals in man (9th edition)* Biomedical Publications, Foster City, CA, USA (2011).

103. Clavijo C, Thomas J, Cromie M *et al.* A low blood volume LC-MS/MS assay for the quantification of fentanyl and its major metabolites norfentanyl and despropionyl fentanyl in children. *J Sep Sci* 34(24), 3568-3577 (2011).

104. Moll V, Clavijo C, Cohen M, Christians U, Galinkin J. A analytical method to determine ketamine and norketamine levels in dried blood spots. Presented at: *Annual Meeting of the American Society of Anesthesiologists (ASA).* New Orleans, LA, USA, 17-21 October 2009.

105. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report. http://www.emcdda.europa.eu/attachements.cfm/att_239505_EN_TDAT15001ENN.pdf

106. Mercolini L, Protti M, Catapano MC, Rudge J, Sberna AE. LC-MS/MS and volumetric absorptive microsampling for quantitative bioanalysis of cathinone analogues in dried urine, plasma and oral fluid samples. *J Pharm Biomed Anal* 123, 186-194 (2016).

107. Mercolini L, Protti M. Biosampling strategies for emerging drugs of abuse: towards the future of toxicological and forensic analysis. *J Pharm Biomed Anal* 130, 202-219 (2016).

108. Maenhout T, De Buyzere M, Delanghe J. Non-oxidative ethanol metabolites as a measure of alcohol intake. *Clin Chim Acta* 415, 322-329 (2013).

109. Crunelle C, Yegles M, van Nuijs A *et al.* Hair ethyl glucuronide levels as a marker for alcohol use and abuse: a review of the current state of the art. *Drug Alcohol Depend* 134, 1-11 (2014).

110. Hernández Redondo A, Schroeck A, Kneubuehl B, Weinmann W. Determination of ethyl glucuronide and ethyl sulfate from dried blood spots. *Int J Legal Med* 127(4), 769-775 (2013).

111. Faller A, Richter B, Kluge M, Koenig P, Seitz H, Skopp G. Stability of phosphatidylethanol species in spiked and authentic whole blood and matching dried blood spots. *Int J Legal Med* 127(3), 603-610 (2013).

112. Faller A, Richter B, Kluge M *et al.* LC-MS/MS analysis of phosphatidylethanol in dried blood spots versus conventional blood specimens. *Anal Bioanal Chem* 401(4), 1163-1166 (2011).

113. Bakhireva L, Leeman L, Savich R *et al.* The validity of phosphatidylethanol in dried blood spots of newborns for the identification of prenatal alcohol exposure. *Alcohol Clin Exp Res* 38(4), 1078-1085 (2014).

114. Bakhireva L, Savich R, Raisch D *et al.* The feasibility and cost of neonatal screening for prenatal alcohol exposure by measuring phosphatidylethanol in dried blood spots. *Alcohol Clin Exp Res* 37(6), 1008-1015 (2013).

115. Searles Nielsen S, Dills R, Glass M, Mueller B. Accuracy of prenatal smoking data from Washington Statebirth certificates in a population-based sample with cotinine measurements. *Ann Epidemiol* 24(3), 236-239 (2014).

116. Marin LJ, Tyrrel-Pawlowic CA, Moroney EC, Coopersmith BI, Shet MS. Comparative analysis of buprenorpine and oxycodone in human dried blood spots (DBS) using LC-MS/MS. Presented at: *American Association of Pharmaceutical Scientists (AAPS)- Pharmaceutical Sciences World Congress.* New Orleans, LA, USA, 14-18 November 2010.

117. Havard G, Théberge MC, Boudreau N, Lévesque A, Massé R. Development and validation of a dried blood spot assay for the determination of midazolam in human whole blood by LCMSMS. Presented at: *American Association of Pharmaceutical Scientists (AAPS)-Pharmaceutical Sciences World Congress.* New Orleans, LA, USA, 14-18 November 2010.

118. Havard G, Théberge MC, Boudreau N, Lévesque A, Pellerin B. Determination of midazolam in human plasma and whole blood: comparison of dried blood spot and different collection procedure. Presented at: *American Association of Pharmaceutical Scientists (AAPS).* Washington, USA, 23-27 October 2011.

119. Lad R. Validation of individual quantitative methods for determination of cytochrome P450 probe substrates in human dried blood spots with HPLC-MS/MS. *Bioanalysis* 2(11), 1849-1861 (2010).

120. Mather J, Rainville P, Spooner N, Evans C, Smith N, Plumb R. Rapid analysis of dried blood spot samples with sub-2-microm LC-MS/MS. *Bioanalysis* 3(4), 411-420 (2011).

121. Jantos R, Veldstra J, Mattern R, Brookhuis K, Skopp G. Analysis of 3,4-methylenedioxymetamphetamine: whole blood versus dried blood spots. *J Anal Toxicol* 35(5), 269-273 (2011).

122. Lee H, Park Y, Jo J, *et al.* Analysis of benzodiazepines and their metabolites using DBS cards and LC-MS/MS. *Forensic Sci Int* 255, 137-145 (2015).

CHAPTER 2:
CORRELATION OF GHB CONCENTRATIONS BETWEEN vDBS AND cDBS

Based on

Sadones N, Archer JR, Ingels AM, Dargan PI, Wood DM, Wood M, Neels H, Lambert WE, Stove CP. Do capillary dried blood spot concentrations of gamma-hydroxybutyric acid mirror those in venous blood? A comparative study. *Drug Test Anal* 7(4), 336-340 (2015).

Abstract

Gamma-hydroxybutyric acid (GHB) is a well-known illicit club and date-rape drug. Dried blood spot (DBS) sampling is a promising alternative for classical venous sampling in cases of (suspected) GHB intoxication since it allows rapid sampling, which is of interest for the rapidly metabolized GHB. However, there is limited data if -and how- capillary DBS concentrations correlate with venous concentrations. We conducted a comparative study in 50 patients with suspected GHB intoxication, to determine and to correlate GHB concentrations in venous DBS (vDBS) and capillary DBS (cDBS). This is the first study that evaluates in a large cohort the correlation between capillary and venous concentrations of an illicit drug in real-life samples. Of the 50 paired samples, 7 were excluded: the vDBS concentration was below the LLOQ of 2 µg/mL in 3 cases and 4 samples were excluded after visual inspection of the DBS. Bland-Altman analysis revealed a mean % difference of -2.8% between cDBS and vDBS concentrations, with the zero value included in the 95% confidence interval of the mean difference in GHB concentration. A paired sample T-test confirmed this observation ($p = 0.17$). Also the requirement for incurred sample reproducibility was fulfilled: for more than 2/3 of the samples the concentrations obtained in cDBS and those in vDBS were within 20% of their mean. Since equivalent concentrations were observed in cDBS and vDBS, blood obtained by fingerprick can be considered a valid alternative for venous blood for GHB determination.

2.1 Introduction

Gamma-hydroxybutyric acid (GHB) and its precursor gamma-butyrolactone (GBL) are used as recreational drug and date-rape drug, particulary in a nightclub environment and in men having sex with men. There have been many cases of GHB intoxication, also in combination with other drugs of abuse [1-4]. Toxicological interpretation of GHB concentrations in clinical and forensic samples is impeded by its endogenous presence. Furthermore, given the short half-life of GHB, rapid sampling is needed [5]. Dried blood spot (DBS) sampling has been proposed as a possible alternative for classical venipuncture, offering several advantages, in particular rapid and easy collection of a representative sample and easy sample transfer and storage [6]. A number of studies have provided a proof-of-concept of the potential to determine abused substances, including GHB, in DBS samples [7]. However, these studies have generally not assessed the developed methodology in a large cohort of patients, comparing capillary samples with the gold standard of venous samples. In addition, in most reports a cross-comparison between venous DBS (vDBS) and capillary DBS (cDBS) concentrations is lacking [7]. Therefore, although DBS sampling is an interesting and promising alternative to classical venous sampling, it remains to be demonstrated if cDBS concentrations correlate with those in venous samples. While we previously demonstrated in our laboratory that GHB can be quantitatively determined in DBS of patients with a GHB/GBL intoxication and in narcoleptic patients taking the medication Xyrem® (sodium oxybate, the sodium salt of GHB) [8-10], the aim of this study was to compare GHB concentrations in vDBS and cDBS in a large cohort of patients with acute GHB intoxication.

2.2 Materials and methods

The derivatization reagents trifluoroacetic anhydride and heptafluorobutanol, as well as the sodium salt of GHB were purchased from Sigma-Aldrich (Diegem, Belgium). The internal standard GHB-D6 was obtained from Lipomed (Arlesheim, Switzerland). Suprasolve methanol and ethyl acetate were provided by Merck (Darmstadt, Germany). The sodium fluoride/potassium oxalate (NaF-KOx) blood collection tubes (9 mL tubes with 100 mg sodium fluoride and 22.50 mg potassium oxalate) were obtained from Terumo (Leuven, Belgium).

2.2.1 Samples

This study was approved by The UK National Research Ethics Service (Reference 11/LO/0976). All patients presenting to the emergency department at Guy's and St Thomas' Hospital, London between February and December 2013 with either a clinical diagnosis or other indications of GHB/GBL intoxication were considered for inclusion. cDBS were generated by non-volumetric direct application of a blood drop from the fingertip onto Whatman filter paper following a fingerprick. vDBS were obtained by pipetting 25 µL of venous blood from the NaF-KOx blood tubes in which the venous samples were collected and stored. DBS were dried for at least 2 hours before storage at room temperature in zip-closure plastic bags with desiccant. Paired cDBS and venous whole blood samples were collected at the time of admission from 99 patients. As these patients were

generally drowsy or agitated at presentation, informed consent was not possible prior to the collection of samples and therefore delayed consent was employed. Of the patients sampled, 50 provided delayed consent. The paired samples obtained from these 50 patients were used in this study.

2.2.2 Analytical procedures

GHB concentrations in DBS were determined using a validated gas chromatography coupled to mass spectrometry (GC-MS)-based procedure [9]. Briefly, 6-mm partial-spot punches were taken from the DBS. After adding the internal standard (IS) GHB-D6, the DBS punches were subjected to "on-spot derivatization" by direct application of a mixture of 50 µL trifluoroacetic acid anhydride and heptafluorobutanol (2:1) and heating for 10 min at 60°C. After evaporation under a gentle stream of nitrogen, the dried extract was re-dissolved in 100 µL ethyl acetate and 1 µL was injected into an Agilent 6890 GC system coupled to a 5973 MS. Of those DBS with a GHB concentration above 100 µg/mL, 10 µL of the final derivatized extract was diluted to 100 µL with ethyl acetate.

Venous whole blood samples were analyzed according to the procedure of Van hee *et al.* [11]. Briefly, after adding the IS 1,3-propylene glycol and the catalyst dimethylformamide to 20 µL of whole blood, the samples were directly derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 70°C for 15 min. Following centrifugation, an aliquot was injected into a GC-MS sytem.

2.2.3 Storage experiment

To evaluate GHB stability in venous whole blood (collected in NaF-KOx tubes), we added two different concentrations (10 and 100 µg/mL) in whole blood and stored the samples at different temperatures (4°C and room temperature) for up to 2 weeks. At different time points, 25-µL DBS were generated from this spiked blood, which were analyzed using the above-mentioned DBS-based GC-MS method of Ingels *et al.* [9].

2.2.4 Data analysis

To evaluate the correlation between venous and capillary concentrations, we performed a paired sample T-test, using Microsoft Excel® 2010 (Microsoft, Redmond, WA, USA), and Bland-Altman and Passing-Bablok analysis, using MedCalc® (MedCalc software bvba, Ostend, Belgium).

A p-value ≤ 0.05 was considered statistically significant. In the Bland-Altman plot, the differences between venous and capillary concentrations were plotted against the average of both measurements. Indicated in this plot are the mean difference between both concentrations and the limits of agreement (1.96 SD), along with the respective 95% confidence intervals. In the Passing-Bablok scatter plot diagram, capillary concentrations

were plotted against venous concentrations. If the confidence interval of the slope includes 1, there is no proportional difference between both measurements. When the confidence interval of the intercept value includes 0, it can be concluded that there is no systematic difference between both methods.

2.3 Results and discussion

Of the 50 paired samples, 7 were excluded: the venous blood concentration was below the LLOQ in 3 cases, while 4 samples were excluded after visual inspection of the DBS (in 3 cases cDBS samples were too small (< 6 mm diameter) and in one case vDBS had an irregular shape). cDBS concentrations of GHB ranged from 41 to 646 µg/mL, whereas vDBS concentrations ranged from 48 to 705 µg/mL. Clinically, all patients -apart from the 3 patients with a venous concentration below LLOQ- had clinical symptoms consistent with acute GHB intoxication.

In literature, a cut-off GHB concentration of 4-5 µg/mL in blood has been proposed to differentiate between endogenous and exogenous GHB [5,12-14]. In our study, 43 patients were screened positive for GHB: both venous and capillary concentrations were well above these cut-off levels. In the 3 cases of our study that did not have symptoms of acute GHB intoxication, venous concentrations were below the limit. However, GHB intake is likely as clearly higher signals were detected than those found in GHB-naïve persons (see Figure 2.1).

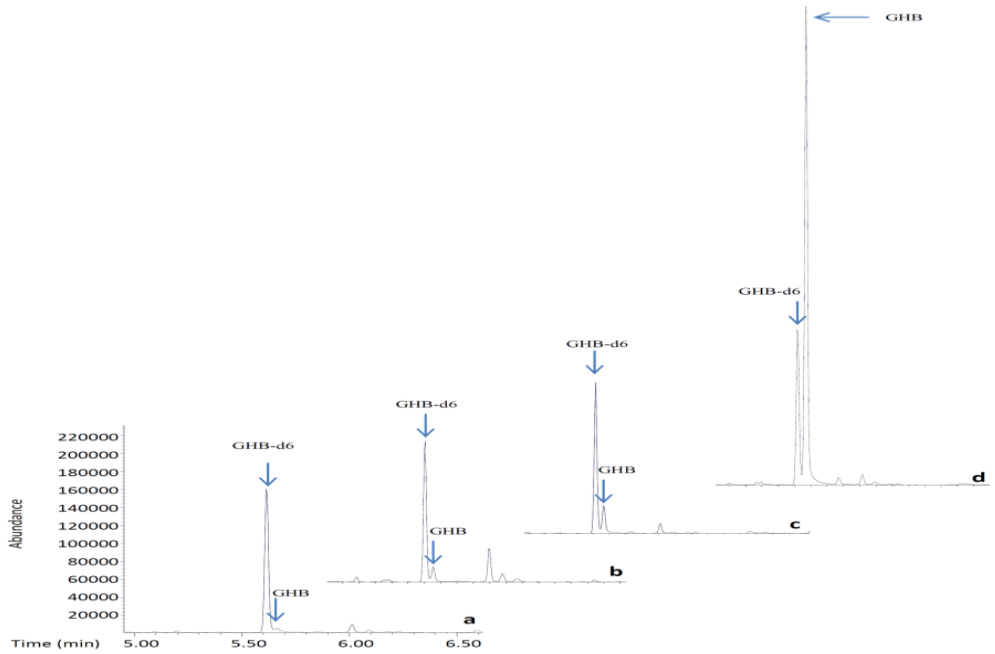


Figure 2.1 Overlay of representative chromatograms obtained by analyzing (a) vDBS of a GHB-naïve person, (b) vDBS of a patient with assumed GHB intake (GHB concentration < 2 µg/mL), (c) vDBS with a GHB concentration of 2 µg/mL (LLOQ), (d) vDBS of a GHB-intoxicated person (GHB concentration of 48 µg/mL), using a DBS-based GC-MS method [9].

These cases presented to the hospital with clinical symptoms consistent with recent stimulant use or secondary to GHB withdrawal rather than acute intoxication. In all 3 cases, there was some evidence that GHB use may have taken place more than 5 hours before sampling (e.g. history of GHB abuse, declared GHB use,…). However, it was not possible to formally confirm GHB use in these patients, as urine samples were not routinely collected. As suggested by these 3 cases, rapid sampling is of utmost importance since GHB is metabolized rapidly, with a half-life of less than one hour, resulting in blood concentrations below proposed cut-off levels within a few hours after use [15,16]. Shima and colleagues proposed lowering the cut-off to 1 µg/mL in blood in cases where in-life blood specimens can be collected aseptically and stored at 4°C or lower before timely analysis [17]. In legal cases, cDBS samples may provide the advantage that they may not only allow rapid sampling, but also stabilise the sample, allowing long-term storage at room temperature [9].

We performed Bland-Altman analysis to compare cDBS and vDBS concentrations. As can be seen in Figure 2.2a, we found a mean % difference of -2.8%, with the zero value included in the 95% confidence interval of the mean difference in GHB concentration. From this, it can be concluded that there is no significant difference between cDBS and vDBS concentrations. A paired sample T-test confirmed this conclusion ($p = 0.17$). We also applied the European Medicines Agency (EMA) guideline for incurred sample reanalysis in bioanalytical method validation [18]. These state that the concentration obtained for the initial analysis and the concentration obtained by reanalysis should be within 20% of their mean for at least 67% of the repeats. Although this requirement actually concerns reanalysis of the *same* samples, this condition was still fulfilled when analyzing *different* (cDBS *versus* vDBS) samples: in 72% of cases the concentrations obtained in cDBS and those in vDBS were within 20% of the mean GHB concentrations obtained with the 2 methods. As can be seen in Figure 2.2b, a Passing-Bablok scatter plot also demonstrated a good overall correlation between cDBS and vDBS GHB concentrations, although 1 was just not included in the 95% confidence interval of the slope.

In addition to performing a pairwise comparison between cDBS and vDBS, we also performed reanalysis of both cDBS ($n = 29$) and vDBS ($n = 28$). Again, the EMA requirement for incurred sample reanalysis was fulfilled: we found that for more than two-thirds (i.e. 70%) of the samples the initial concentration and the concentration obtained by reanalysis, were within 20% of the mean of the first and repeat measurement.

When comparing DBS values with those obtained from venous blood (using another procedure in another laboratory), the latter were significantly lower ($p < 0.005$) although the differences remained limited: Bland-Altman analysis revealed a mean % difference of -9.2% and -13% between venous whole blood and respectively cDBS and vDBS (Figures 2.3 and 2.4). Also here, the EMA guidelines for incurred sample reanalysis were fulfilled: in 77% of the cases the concentrations obtained in cDBS and those in venous whole blood were within 20% of the mean GHB concentrations; also for more than two-thirds of the samples, the vDBS concentrations and the concentrations obtained from venous whole blood were within 20% of their mean. It is unclear if the observed difference between concentrations in venous blood and in DBS prepared thereof can be ascribed to

the DBS approach *per se* or rather is the result of differences in calibration between the distinct analyzing laboratories.

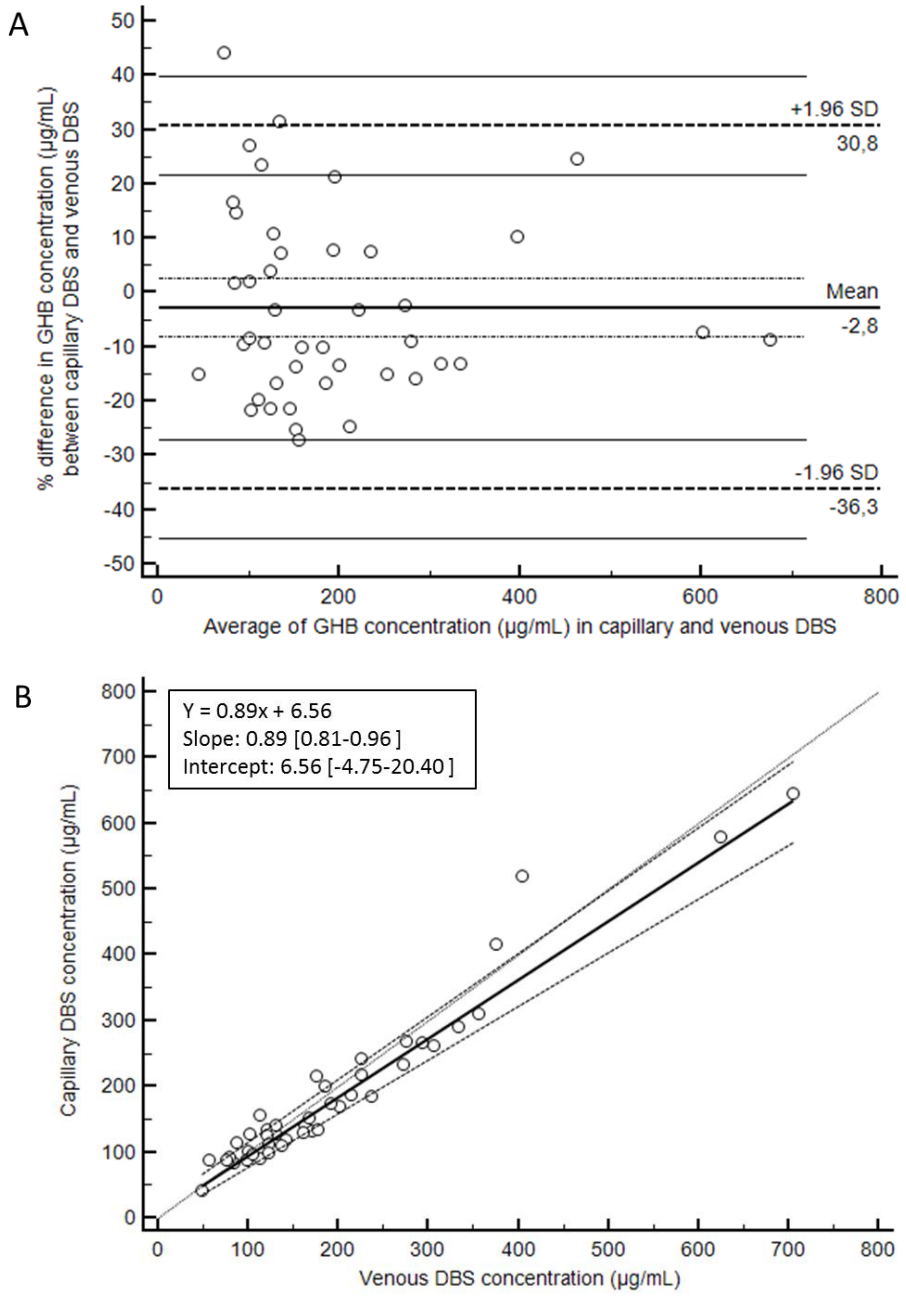


Figure 2.2 (a) Bland-Altman analysis of cDBS and vDBS, plotting the % differences between both GHB concentrations (y-axis) against the average of these results (x-axis). The mean difference and the limits of agreement (set to 1.96 SD) are also indicated with its 95% confidence interval. (b) Passing-Bablok regression analysis of cDBS and vDBS, plotting the concentrations in both matrices against each other. The solid line illustrates the regression line, the dashed lines indicate the confidence interval for the regression line and the dotted line corresponds to the identity line.

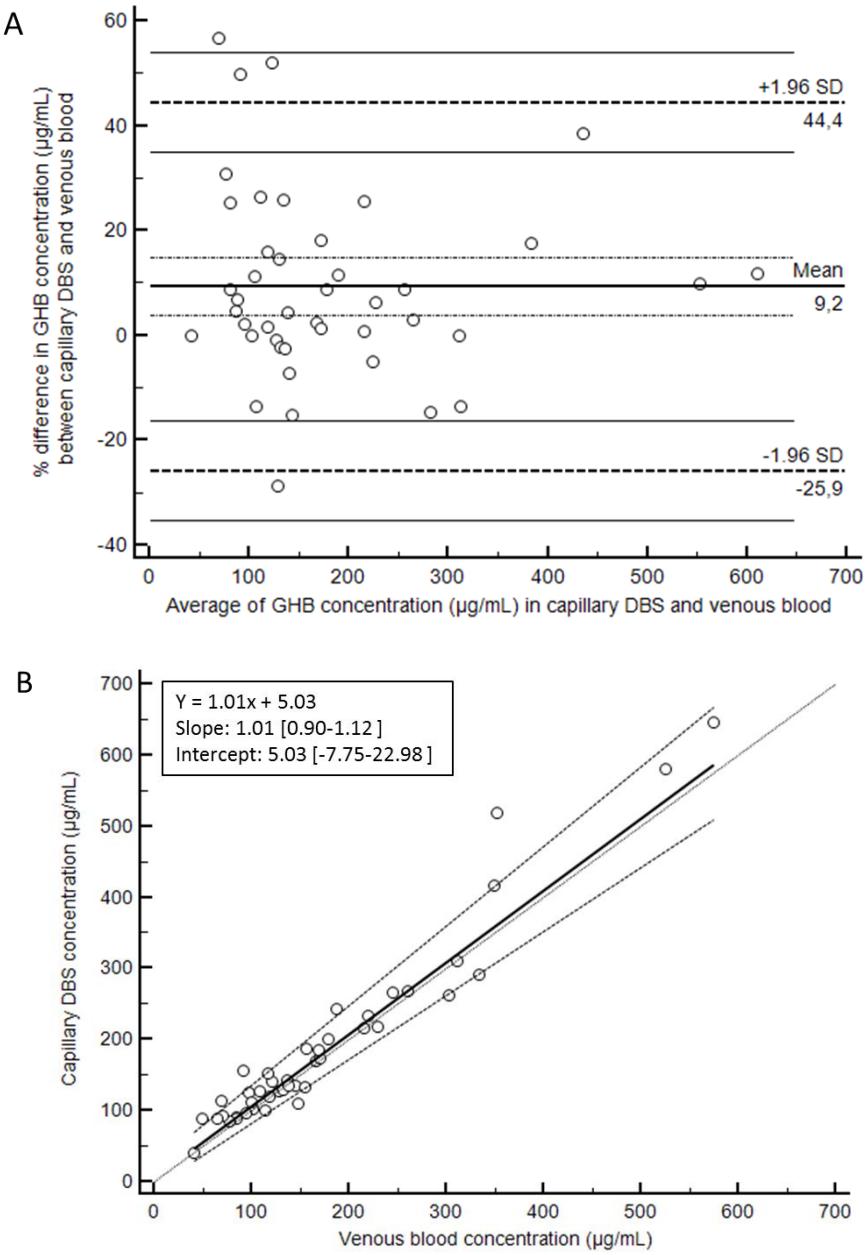


Figure 2.3 (a) Bland-Altman analysis of cDBS and venous whole blood, plotting the % differences between both GHB concentrations (y-axis) against the average of these results (x-axis). The mean difference and the limits of agreement (set to 1.96 SD) are also indicated with its 95% confidence interval. (b) Passing-Bablok regression analysis of cDBS and venous whole blood, plotting the concentrations in both matrices against each other. The solid line illustrates the regression line, the dashed lines indicate the confidence interval for the regression line and the dotted line corresponds to the identity line.

It should be noted that, while cDBS and venous blood were sampled at almost the same time point, in some cases, there was a delay of several days before vDBS were prepared from the venous blood. To exclude that this may have an effect on our results, we evaluated GHB stability in spiked venous whole blood collected in NaF-KOx tubes that were stored for up to two weeks at 4°C and at room temperature before DBS preparation.

The bias from nominal concentrations (10 or 100 µg/mL) did not exceed 11%. This suggests that no substantial alteration takes place in GHB concentration if venous blood, collected in NaF-KOx tubes, is stored under these conditions. Although this finding is consistent with previously published data [19-21], it should be noted that in incurred (real-life) samples a contribution from e.g. hydrolysis of GHB glucuronide might not be excluded. However, such contribution is expected to be limited [22].

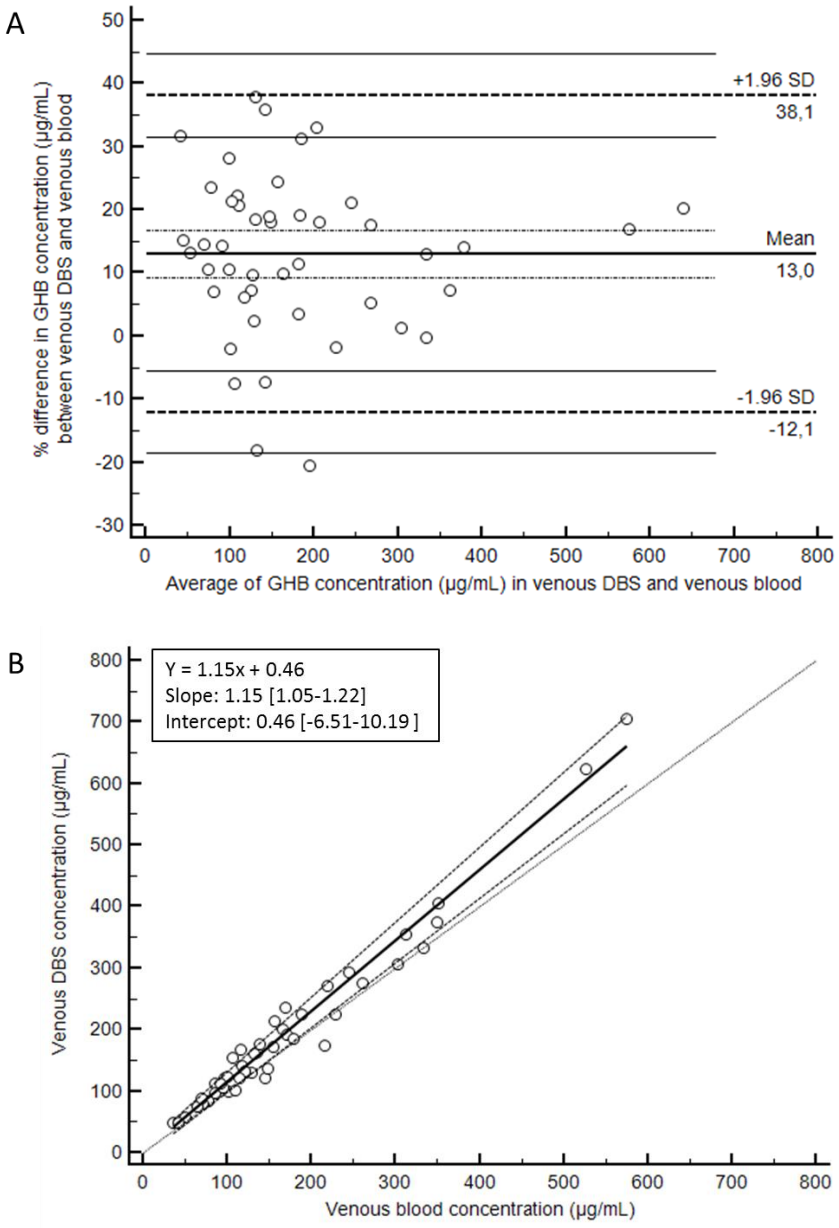


Figure 2.4 (a) Bland-Altman analysis of vDBS and venous whole blood, plotting the % differences between both GHB concentrations (y-axis) against the average of these results (x-axis). The mean difference and the limits of agreement (set to 1.96 SD) are also indicated with its 95% confidence interval. (b) Passing-Bablok regression analysis of vDBS and venous whole blood, plotting the concentrations in both matrices against each other. The solid line illustrates the regression line, the dashed lines indicate the confidence interval for the regression line and the dotted line corresponds to the identity line.

2.4 Conclusion

DBS sampling is a promising alternative for classical venous sampling in cases of (suspected) GHB intoxication: the DBS sampling technique allows rapid sampling -which is of interest for the rapidly metabolized GHB- and DBS are also easier to store and transport than venous samples. The study reported here is the largest comparative study to date evaluating capillary and venous concentrations of an illicit drug. In a large cohort of patients with acute GHB intoxication we observed equivalent GHB concentrations and an excellent correlation between cDBS and vDBS. In conclusion, blood obtained by fingerprick is a valid alternative for venous blood for GHB determination.

2.5 References

1. Abanades S, Farré M, Segura M, *et al*. Gamma-hydroxybutyrate (GHB) in humans: pharmacodynamics and pharmacokinetics. *Ann N Y Acad Sci* 1074, 559-576 (2006).
2. Wood D, Nicolaou M, Dargan P. Epidemiology of recreational drug toxicity in a nightclub environment. *Subst Use Misuse* 44(11), 1495-1502 (2009).
3. Wood D, Greene S, Dargan P. Five-year trends in self-reported recreational drugs associated with presentation to a UK emergency department with suspected drug-related toxicity. *Eur J Emerg Med* 20(4), 263-267 (2013).
4. Brennan R, Van Hout M. Gamma-hydroxybutyrate (GHB): a scoping review of pharmacology, toxicology, motives for use, and user groups. *J Psychoact Drugs* 46(3), 243-251 (2014).
5. Ingels A, Wille S, Samyn N, Lambert W, Stove C. Screening and confirmation methods for GHB determination in biological fluids. *Anal Bioanal Chem* 406(15), 3553-3577 (2014).
6. Stove C, Ingels A, De Kesel P, Lambert W. Dried blood spots in toxicology: from the cradle to the grave? *Crit Rev Toxicol* 42(3), 230-243 (2012).
7. Sadones N, Capiau S, De Kesel P, Lambert W, Stove C. Spot them in the spot: analysis of abused substances using dried blood spots. *Bioanalysis* 6(17), 2211-2227 (2014).
8. Ingels A, Lambert W, Stove C. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct “on-spot” derivatization. *Anal Bioanal Chem* 398(5), 2173-2182 (2010).
9. Ingels A, De Paepe P, Anseeuw K, *et al*. Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis* 3(20), 2271-2281 (2011).
10. Ingels A, Hertegonne K, Lambert W, Stove C. Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home: an exploratory study. *CNS Drugs* 27(3), 233-237 (2013).
11. Van hee P, Neels H, De Doncker M, *et al.* Analysis of γ-hydroxybutyric acid, DL-lactic acid, glycolic acid, ethylene glycol and other glycols in body fluids by a direct injection gas chromatography-mass spectrometry assay for wide use. *Clin Chem Lab Med* 42(11), 1341-1345 (2004).
12. Elian A. Determination of endogenous gamma-hydroxybutyric acid (GHB) levels in antemortem urine and blood. *Forensic Sci Int* 128(3), 120-122 (2002).
13. Elliott S. Gamma hydroxybutyric acid (GHB) concentrations in humans and factors affecting endogenous production. *Forensic Sci Int* 133(1-2), 9-16 (2003).
14. Andresen H, Sprys N, Schmoldt A, Mueller A, Iwersen-Bergmann S. Gamma-hydroxybutyrate in urine and serum: additional data supporting current cut-off recommendations. *Forensic Sci Int* 200(1-3), 93-99 (2010).
15. Baselt R. In: *Disposition of toxic drugs and chemicals in man (9th edition)* Biomedical Publications, Foster City, CA, USA (2011).

16. Schröck A, Hari Y, König S, Auwärter V, Schürch S, Weinmann W. Pharmacokinetics of GHB and detection window in serum and urine after single uptake of a low dose of GBL -an experiment with two volunteers. *Drug Test Anal* 6(4), 363-366 (2013).
17. Shima N, Miki A, Kamata T, Katagi M, Tsuchihashi H. Endogenous level and in vitro production of GHB in blood from healthy humans, and the interpretation of GHB levels detected in antemortem blood samples. *J Health Sci* 51(2), 147-154 (2005).
18. European Medicines Agency. Guideline on bioanalytical method validation. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf.
19. Berankova K, Mutnanska K, Balikova M. Gamma-hydroxybutyric acid stability and formation in blood and urine. *Forensic Sci Int* 161(2-3), 158-162 (2006).
20. Johansen S, Windberg C. Simultaneous determination of gamma-hydroxybutyrate (GHB) and its analogues (GBL, 1.4-BD, GVL) in whole blood and urine by liquid chromatography coupled to tandem mass spectrometry. *J Anal Toxicol* 35(1), 8-14 (2011).
21. Sørensen L, Hasselstrøm J. A hydrophilic interaction liquid chromatography electrospray tandem mass spectrometry method for the simultaneous determination of gamma-hydroxybutyrate and its precursors in forensic whole blood. *Forensic Sci Int* 222(1-3), 352-359 (2012).
22. Petersen I, Tortzen C, Kristensen J, Pedersen D, Breindahl T. Identification of a new metabolite of GHB: gamma-hydroxybutyric acid glucuronide. *J Anal Toxicol* 37(5), 291-297 (2013).

CHAPTER 3:
MICROWAVE-ASSISTED ON-SPOT DERIVATIZATION OF POLAR LOW MOLECULAR WEIGHT COMPOUNDS

Based on

Sadones N, Van Bever E, Archer JRH, Wood DM, Dargan PI, Van Bortel L, Lambert WE, Stove CP. Microwave-assisted on-spot derivatization for gas chromatography-mass spectrometry based determination of polar low molecular weight compounds in dried blood spots. *J Chromatogr A* 1465, 175-183 (2016).

Abstract

Dried blood spot (DBS) sampling and analysis is increasingly being applied in bioanalysis. Although the use of DBS has many advantages, it is also associated with some challenges. E.g. given the limited amount of available material, highly sensitive detection techniques are often required to attain sufficient sensitivity. In gas chromatography coupled to mass spectrometry (GC-MS), derivatization can be helpful to achieve adequate sensitivity. Because this additional sample preparation step is considered as time-consuming, we introduce a new derivatization procedure, i.e. "microwave-assisted on-spot derivatization", to minimize sample preparation of DBS. In this approach the derivatization reagents are directly applied onto the DBS and derivatization takes place in a microwave instead of via conventional heating. In this Chapter we evaluated the applicability of this new concept of derivatization for the determination of two polar low molecular weight molecules, gamma-hydroxybutyric acid (GHB) and gabapentin, in DBS using a standard GC-MS configuration. The method was successfully validated for both compounds, with imprecision and bias values within acceptance criteria (< 20% at LLOQ, < 15% at 3 other QC levels). Calibration lines were linear over the 10-100 µg/mL and 1-30 µg/mL range for GHB and gabapentin, respectively. Stability studies revealed no significant decrease of gabapentin and GHB in DBS upon storage at room temperature for at least 84 days. Furthermore, DBS-specific parameters, including hematocrit and volume spotted, were evaluated. As demonstrated by the analysis of GHB and gabapentin positive samples, "microwave-assisted on-spot derivatization" proved to be reliable, fast and applicable in routine toxicology. Moreover, other polar low molecular weight compounds of interest in clinical and/or forensic toxicology, including vigabatrin, beta-hydroxybutyric acid, propylene glycol, diethylene glycol, 1,4-butanediol and 1,2-butanediol, can also be detected using this method.

3.1 Introduction

Dried blood spot (DBS) sampling has been associated with many advantages. It is a minimally invasive sampling technique enabling rapid (home-)sampling and convenient transport and storage of samples [1,2]. Moreover, it offers a reduced risk of infection and in many instances leads to improved compound stability. Furthermore, DBS are a convenient sample preparation strategy: they may simplify sample preparation procedures and they are suitable for automation of sample processing and analysis [3,4]. Consequently, DBS analysis is an increasing field of research, which can be deduced from the rapidly increasing number of published studies on DBS in the last decade [2,3].

DBS have been applied in many disciplines such as preclinical and clinical studies, epidemiological research, phenotyping, therapeutic drug monitoring and toxicology [3,5-8]. In these applications, mostly liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been used [9-11]. Adequate sensitivity has also been achieved with other analytical methods, such as direct MS/MS, LC coupled to fluorescence detection, LC with ultraviolet detection or gas chromatography coupled to (tandem) mass spectrometry (GC-MS(/MS)) [10].

GC-MS based analysis of DBS has been described for metabolomic profiling as well as for the detection of a wide range of analytes, amongst which pesticides, drugs of abuse, anti-epileptic and antidepressant drugs [11-16]. GC-MS also still has its place in many forensic toxicology laboratories, for a variety of reasons. First, GC-MS remains an important confirmation method in systematic toxicological analysis, complementing LC-MS/MS results [17-19]. Second, one single configuration can be used for a variety of compounds with widely varying polarity, not requiring a dedicated configuration for determination of a given compound. E.g. while LC-MS/MS procedures for the determination of gamma-hydroxybutyric acid (GHB) have been described, these typically require the use of hydrophilic interaction liquid chromatography, although reversed phase C_{18} columns with acidified mobile phases have been used as well. However, the latter poses limitations with respect to method sensitivity and selectivity [20]. Third, the use of a standard GC-MS configuration also offers the advantage that it is a robust and highly selective technique which is widely available at reasonable prices, requiring less specialized handling than LC-MS. Additionally, in emerging countries, some laboratories cannot afford buying or maintaining expensive LC-MS equipment but do often have a GC-MS system at their disposal.

In many GC-MS based procedures (including those starting from DBS), a derivatization reaction is needed to improve the chromatographic properties of the analytes of interest and to achieve adequate method sensitivity [10,21]. Although the integration of derivatization techniques may offer several advantages -higher molecular weight compounds can more easily be discerned from interfering signals and the chromatographic and/or mass spectrometric properties of the target analyte may be improved- this additional sample preparation step is often experienced as laborious and tedious. In order to overcome this rate limiting step, we further simplified the concept of “on-spot derivatization” that we introduced in Chapter 2 [22]. In this concept, we add the

derivatization reagents directly to a DBS, without the use of a separate extraction step. Here, we extend this concept towards "microwave-assisted on-spot derivatization". Microwave derivatization is increasingly being applied in a toxicological context [23-30]. To the best of our knowledge, we are the first to combine microwave derivatization with "on-spot derivatization". By doing so, the derivatization step should no longer be experienced as a rate-limiting step of the sample workup protocol. An approach bearing some resemblance to the "microwave-assisted on-spot derivatization" used here is actually already being applied in proteomics, where DBS are subjected to a direct enzymatic digestion in a microwave, allowing quantification of therapeutic proteins [31] .

We evaluated the validity of "microwave-assisted on-spot derivatization" in a real setting for the GC-MS based determination of two distinct polar low molecular weight compounds, GHB and gabapentin, in DBS (Figure 3.1). GHB and its precursors gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) are well-known illicit club and date-rape drugs which are often abused in combination with other drugs of abuse [32-35]. Consequently, quantification of GHB is important in forensic and clinical toxicology. While determination of the anti-epileptic drug gabapentin may be done in the context of therapeutic drug monitoring, this drug is also increasingly encountered in the forensic lab, given the increased illegal use of gabapentin [36]. Additionally, to demonstrate that our methodology is not limited to these two compounds, we also assessed the applicability of our method for the determination of some other polar low molecular weight molecules with relevance in forensic and clinical toxicology, including the ketone body beta-hydroxybutyric acid (BHB), the GHB precursor 1,4-BD and its isomer 1,2-butanediol (1,2-BD), as well as the glycols propylene glycol (PG) and diethylene glycol (DEG) and the anti-epileptic vigabatrin, which is often prescribed together with gabapentin (Figure 3.1).

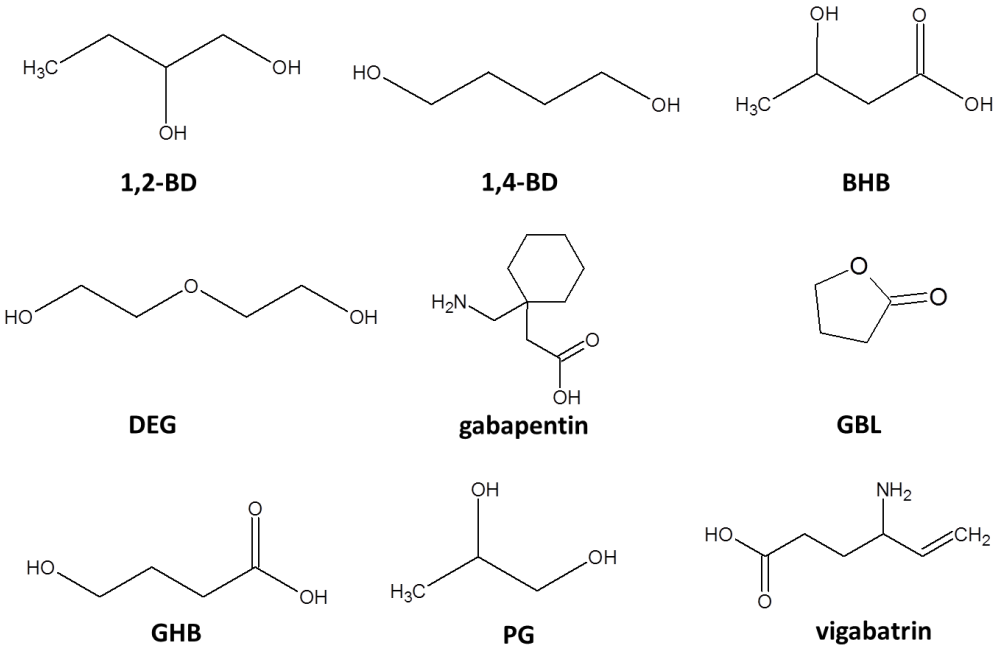


Figure 3.1 Structures of 1,2-BD, 1,4-BD, BHB, DEG, gabapentin, GBL, GHB, PG and vigabatrin.

3.2 Materials and methods

3.2.1 Chemicals and reagents

1,4-BD, 1,2-BD, DEG, PG, vigababatrin, gabapentin, the sodium salt of BHB and GHB, as well as the derivatization reagents (trifluoroacetic anhydride (TFAA), acetic anhydride, pyridine and heptafluorobutanol (HFB)) were purchased from Sigma-Aldrich (Diegem, Belgium). Suprasolve methanol, ethyl acetate, toluene and hexane were provided by Merck (Darmstadt, Germany). The internal standards (IS) GHB-D6 and gabapentin-D10 were obtained from Lipomed (Arlesheim, Switzerland) and Sigma-Aldrich (Diegem, Belgium), respectively.

3.2.2 Preparation of calibrators and quality control samples

Stock solutions were prepared by dissolving respectively 50 mg of BHB, 10 mg of GHB and 10 mg of gabapentin in one mL of methanol. For DEG, PG, 1,2-BD and 1,4-BD, we prepared 100 mg/mL stock solutions in methanol. Vigabatrin was available as a 1 mg/mL solution in methanol. Quality control samples (QCs) were prepared from independent stock solutions. All these stock solutions were stored at -20°C. At the day of analysis, working solutions were prepared by dilution of the stock solutions with methanol. These working solutions were used to prepare a multi-analyte mix, i.e. a mix containing all of the above-mentioned analytes, being PG, DEG, 1,2-BD, 1,4-BD, GHB, BHB, vigabatrin and gabapentin. Using this mix, 6 calibration standards (5, 20, 50, 100, 150 and 200 µg/mL for PG, 1,2-BD and 1,4-BD; 2.5, 10, 20, 40, 75 and 100 µg/mL for DEG; 5, 15, 20, 50, 100 and 300 µg/mL for BHB; 10, 15, 25, 50, 75 and 100 µg/mL for GHB; 1, 5, 10, 17.5, 22.5 and 30 µg/mL for gabapentin; 5, 10, 12.5, 17.5, 20 and 30 µg/mL for vigabatrin) and 4 QCs (5, 15, 85 and 175 µg/mL for PG, 1,2-BD and 1,4-BD; 2.5, 7.5, 50 and 90 µg/mL for DEG; 5, 10, 125 and 250 µg/mL for BHB; 10, 12.5, 30 and 85 µg/mL for GHB; 1, 2.5, 15 and 25 µg/mL for gabapentin; 5, 7.5, 15 and 25 µg/mL for vigabatrin) were prepared in blood. The percentage organic solvent used to prepare calibrators and QCs did not exceed 5%. Finally, DBS were prepared by spotting 25 µL of venous whole blood, which was spiked with the above-mentioned polar low molecular weight molecules, onto filter paper. For quantification of GHB and gabapentin, we used the IS GHB-D6 and gabapentin-D10, which were mixed to obtain final concentrations of 60 and 12 µg/mL, respectively. For the quantification of BHB, GHB-D6 was used as IS, whereas for PG, DEG, 1,2- and 1,4-BD and vigabatrin we used gabapentin-D10.

3.2.3 Instrumentation

Analytical standards and QCs were prepared using an AT261 DeltaRange balance of Mettler Toledo (Zaventem, Belgium). Three different filter papers, being Whatman 903, Munktell 2460 and Ahlstrom 237, were evaluated. Microwave-assisted derivatization was performed in a Samsung ME711K household microwave. Samples were centrifuged at room temperature and at 4°C in respectively a MSE Mistral 2000 (Anderlecht, Belgium) and a 5804R Eppendorf centrifuge (Hamburg, Germany). A Branson 1510 ultrasonic bath (Danbury, Connecticut, USA)

was used for sonication of the samples. Evaporation under nitrogen took place at 25°C in a TurboVap LC evaporator of Zymark (Hopkinton, Massachusetts, USA). Chromatographic separation was achieved on a 30 m x 0.25 mm i.d. x 0.25 µm Agilent HP-5MS column in an Agilent 6890-5973 GC-MS system.

3.2.4 Sample preparation

First, a 6-mm disc was punched out of a 25-µL DBS spot. To this punch, 5 µL IS-mix was added, followed by evaporation under nitrogen at 25°C for 10 min. Then, the punches were subjected to "microwave-assisted on-spot derivatization" by direct application of the derivatization reagents onto the spot. We varied the amount of derivatization reagent and the time of heating to evaluate which parameters gave the best results (see subsection 3.3.1.1). After the derivatization step, samples were subjected to a short-spin centrifugation at 4°C. The excess derivatization reagent was removed by evaporation under nitrogen. Subsequently, 100 µL of injection solvent were added to the sample (hexane, toluene and ethyl acetate were tested as injection solvent, as described in subsection 3.3.2.1), followed by centrifugation at room temperature for 5 min. Finally, one µL of the supernatant of this derivatized extract was injected into the GC-MS system.

3.2.5 Analytical parameters

Optimization of the analytical parameters was performed as described in subsection 3.3.2. Helium was used as carrier gas for the chromatography. We utilized a non-pulsed splitless injection (see subsection 3.3.2.5). In the optimized procedure, the flow rate was set at 1.1 mL/min and the injection temperature at 250°C, as described in subsections 3.3.2.3 and 3.3.2.2, respectively. The initial oven temperature was 60°C, which was held for 2 minutes. Then, the temperature ramped at 8°C/min to 110°C, raised 30°C/min until 230°C, followed by an increase of 50°C/min to 300°C, which was held for 2 minutes. The transfer line temperature, MS ion source temperature and MS quadrupole temperature were set at 300, 230 and 150°C, respectively. The MS was used in the electron impact mode. First, the MS was operated in full-SCAN mode to obtain the mass spectrum of the derivatized analytes (Figure 3.2). Then, quantifier and qualifier ions were selected for each analyte, allowing to use the MS in SIM mode. Quantification was performed in SIM mode using *m/z* 87, 100 and 116 for PG, 227, 268 and 285 for BHB, 72, 86, 101 and 117 for 1,2-BD, 227, 240, 268 and 285 for GHB, 231, 245, 273 and 291 for GHB-D6, 86, 101 and 114 for 1,4-BD, 84, 111, 125 and 153 for vigabatrin, 87, 117 and 182 for DEG, 153, 167 and 195 for gabapentin and 163, 177 and 205 for gabapentin-D10. Quantifier ions are underscored.

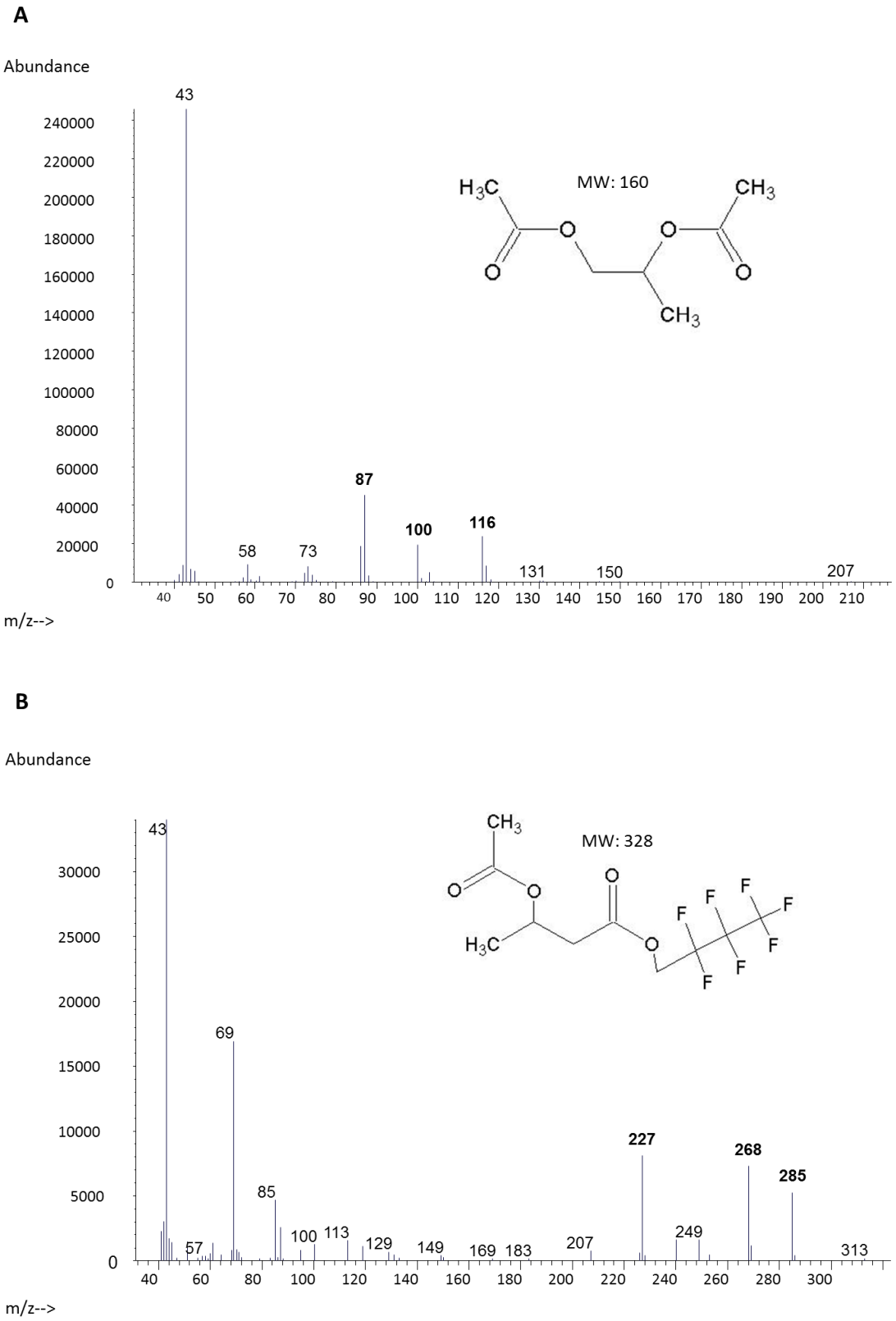
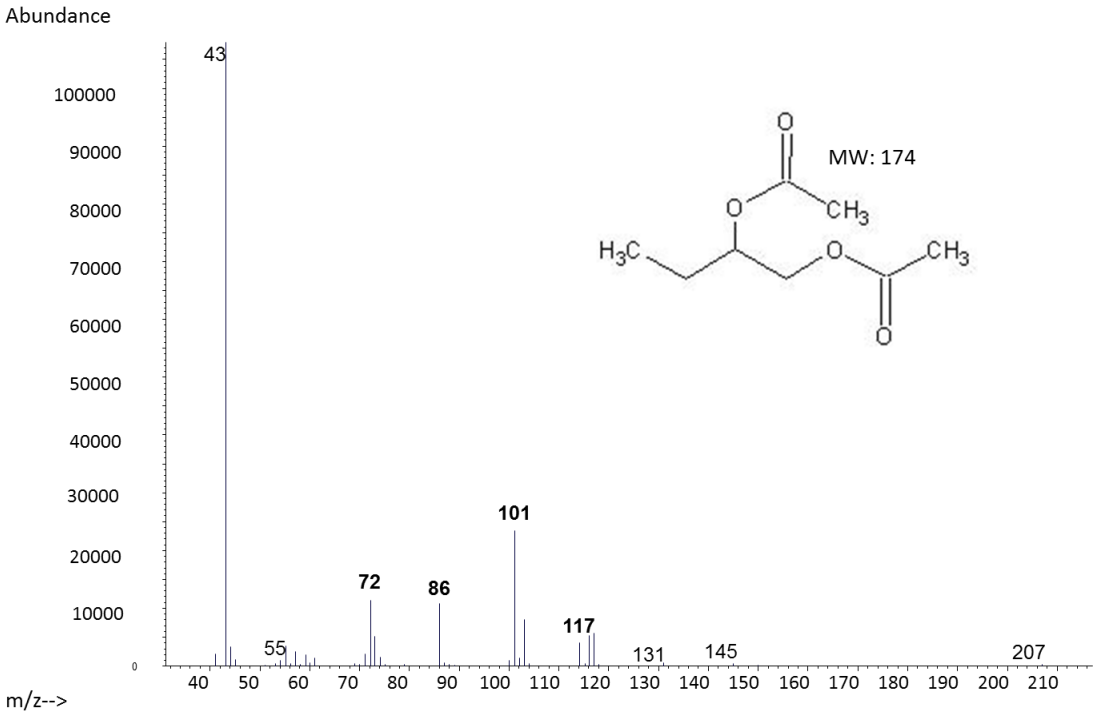


Figure 3.2 Full scan mass spectrum of (a) PG, (b) BHB, (c) 1,2-BD, (d) GHB, (e) 1,4-BD, (f) vigabatrin, (g) DEG and (h) gabapentin derivatized with acetic anhydride, pyridine and heptafluorobutanol.

C



D

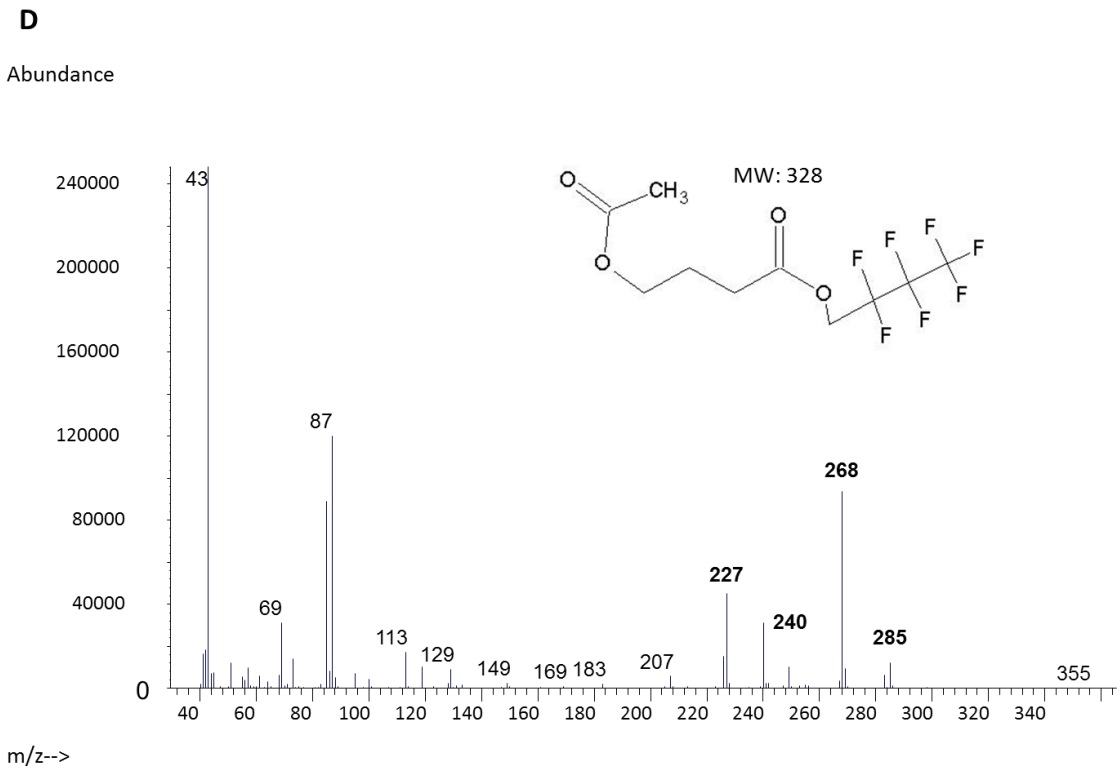
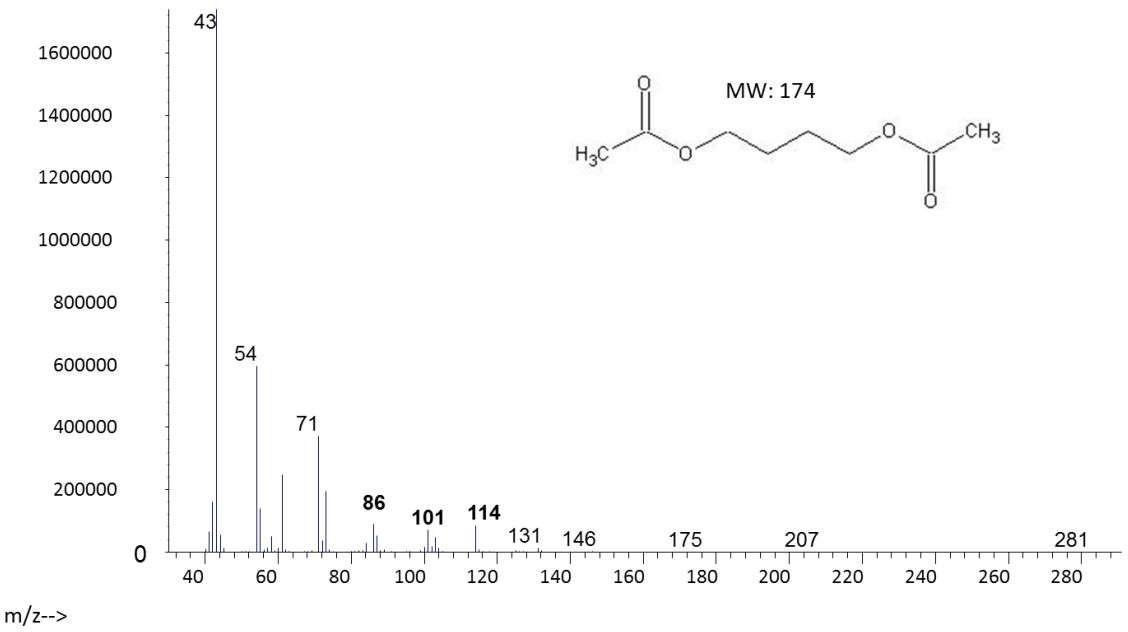


Figure 3.2 Continued.

E



F

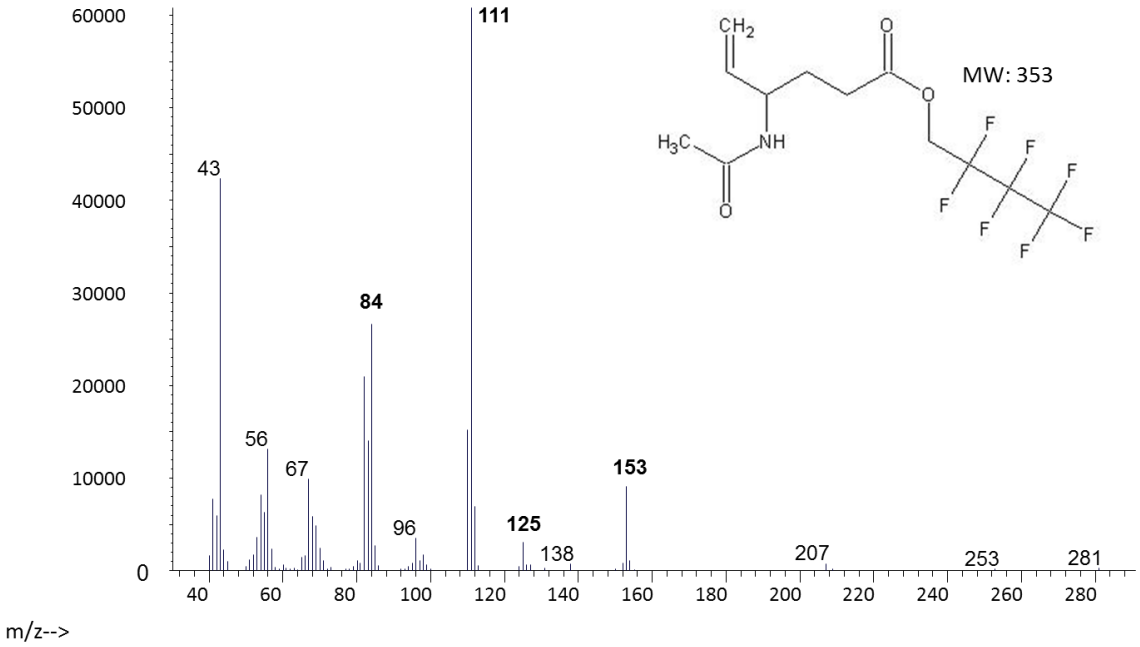
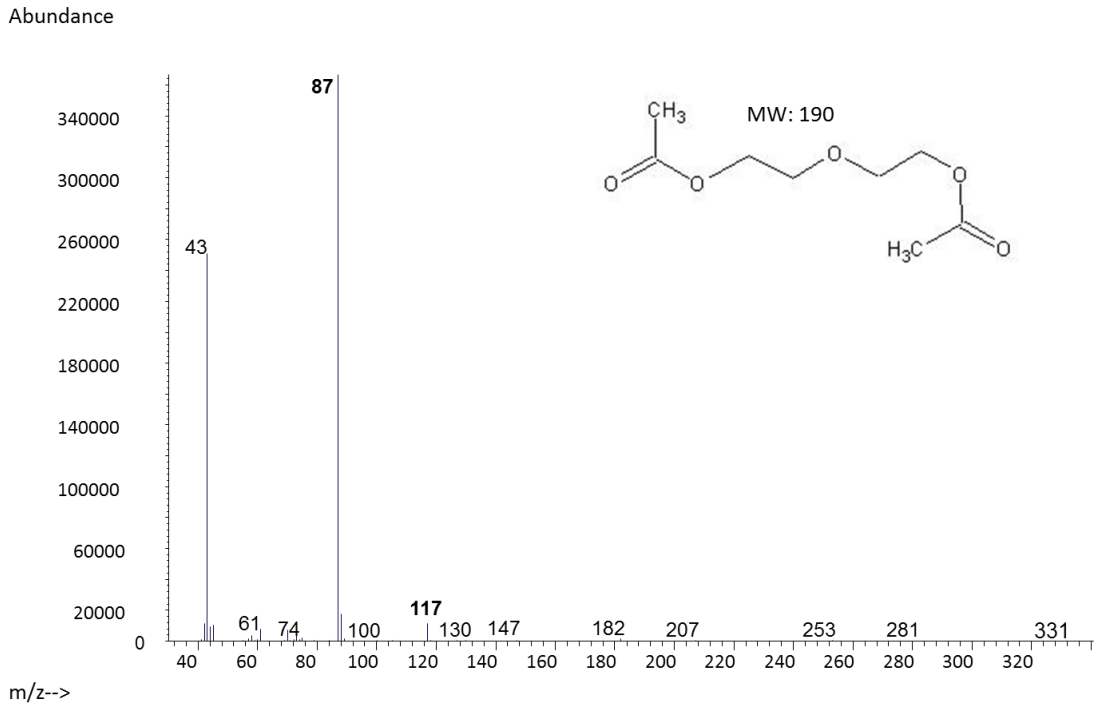


Figure 3.2 Continued.

G



H

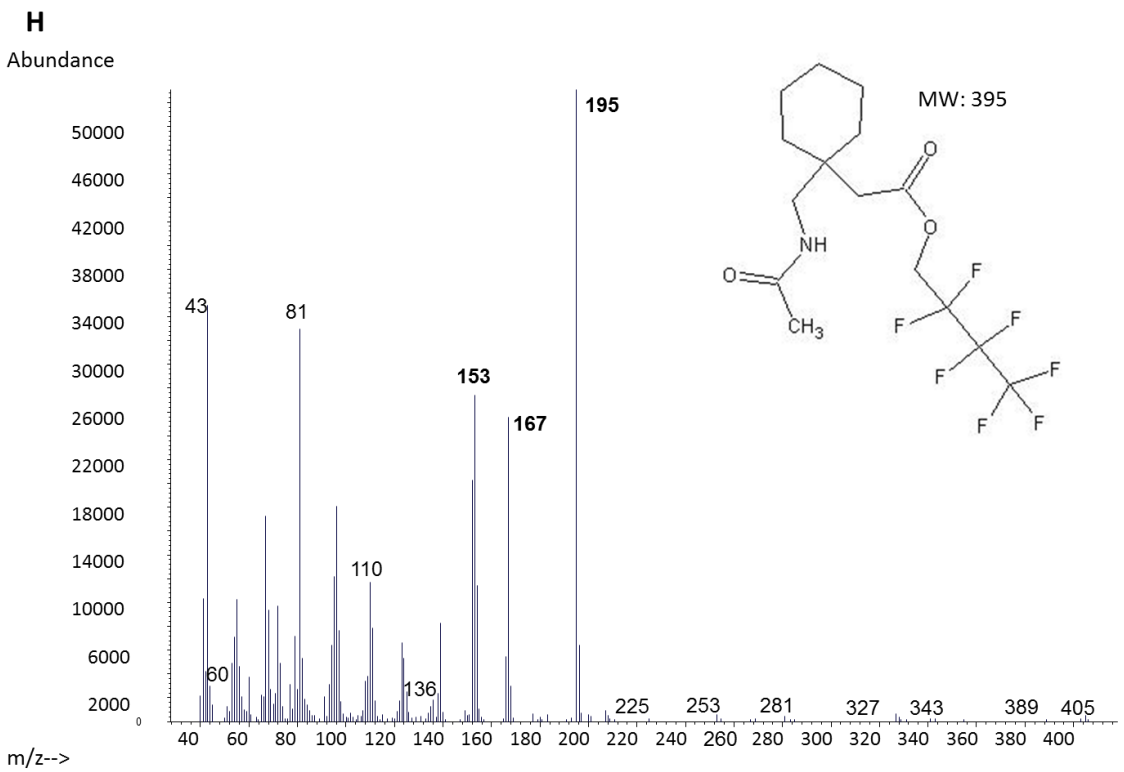


Figure 3.2 Continued.

3.2.6 Validation

3.2.6.1 Microwave calibration

A calorimetric methodology was used to calibrate the microwave (see subsection 3.3.3.1) [37]. To this end, we measured the temperature rise of 1 L water after heating for 90 s at 800W (maximum power) for 14 days. Then, the power of the microwave required for heating the water was calculated using the following formula:

$$\Delta T = \frac{P \times t}{V \times \mathrm{Cp} \times \rho}$$

with ΔT = temperature rise after heating (K); P = microwave power (W); V = volume of water which is heated (m^3); Cp = heat capacity (J/(kg K)); ρ = density (kg/m^3); t = time of heating (s).

In this Chapter, we refer to the effective absorbed power, i.e. 602 ± 15W, when the microwave was set at maximum power (see subsection 3.3.3.1).

3.2.6.2 Analytical evaluation

We validated our newly developed method based upon the Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [38,39]. For that purpose, we evaluated linearity, accuracy, precision, selectivity, carry-over, dilution integrity and stability, as well as two DBS-specific parameters, i.e. hematocrit (Hct) and volume effect [2,40]. For GHB and gabapentin, our method was performed in a real setting and labeled IS were used. Validation parameters were also evaluated for the other polar low molecular weight molecules, however, the use of labeled IS is recommended for quantification in a real-life application.

To evaluate linearity, six-point calibration lines were constructed in duplicate on three non-consecutive days. These calibration lines were prepared by spotting 25 µL of venous whole blood (Hct 0.4) which was spiked with our multi-analyte mix. Of these DBS, 6-mm discs were analyzed. The best fitting calibration model was evaluated by statistical analysis. Therefore, the ratio of the peak area of the analyte to the peak area of the IS was plotted versus the concentration. Then, the slopes and intercepts of the calibration curves were calculated using weighted and unweighted linear regression. As described by Almeida *et al.* [41], a test of homoscedasticity should be performed in linear regression analysis. Therefore, we plotted the residuals versus the nominal concentrations and performed an F-test at the 99% confidence level. To select the most appropriate calibration model, we calculated the sum % residual errors (% RE) and plotted the % RE versus the concentration. The % RE was obtained by dividing the difference between found and nominal concentrations by the nominal concentration and multiplying by 100%. Linearity was assessed by performing the Fisher's test.

Samples were considered positive based on retention time and the ratio of the ion fragments. For the evaluation of sensitivity, we defined the lower limits of quantification (LLOQs) as the lowest concentrations

with an acceptable accuracy and precision. Accuracy, expressed as % bias, was calculated as % deviation from the nominal value. Within-day and between-day precision are expressed as % relative standard deviation and were calculated using one-way ANOVA, as described by Wille *et al.* and Peters *et al.* [42,43]. To this end, QCs were prepared at four levels (i.e. LLOQ, low, medium and high level) in duplicate on 3 separate days. Accuracy and precision should be within 15%, except for LLOQ where it should be within 20%. The limits of detection (LODs) have not been studied. For GHB, a LOD is not so relevant, given the endogenous nature of this compound. For gabapentin, the aim was to demonstrate the potential of using capillary DBS (cDBS) in a pharmacokinetic study, so also here, demonstrating its presence (far) below the therapeutic range was not considered relevant.

To assess selectivity, we evaluated if interfering peaks were seen after analysis of DBS from six different sources. Carry-over was evaluated by injecting 3 blank (ethyl acetate) samples following injection of the highest concentration of the calibration curve. According to the EMA guidelines, carry-over in the blank sample following the high concentration standard should not exceed 20% of the LLOQ and 5% for the IS [39].

We also evaluated dilution integrity by diluting the final derivatized extract instead of diluting the original sample as in case of DBS, especially cDBS, the sample cannot be diluted with blank matrix. To this end, we spiked blood with an analyte concentration 2x higher than the upper limit of quantification (ULOQ) and prepared 25-µL DBS. Of these DBS, a 1:4 dilution of the final derivatized ethyl acetate extract was made. Precision and accuracy of these diluted samples should be within 15%, taking the dilution factor into account.

Stock solution stability was evaluated by analyzing low and high QCs (n = 3) after three freeze-thaw cycles and after storage for one week at -20°C. Long-term stability in DBS was assessed by analyzing these spots immediately after drying and after storage at room temperature for a longer period of time in a zip-closure plastic bag with desiccant. All stability studies were analyzed using a freshly prepared calibration curve.

We also assessed the impact of DBS-specific parameters, like Hct and volume effect, on the analytical results. This was evaluated by analyzing DBS prepared from blood samples with different Hct values (0.30, 0.40 and 0.49), of which different volumes of blood (i.e. 25, 35 and 50 µL) were spotted onto the filter paper.

3.2.7 Application

The applicability of our new approach was demonstrated using DBS obtained from patients and volunteers. For GHB, DBS were obtained from patients presenting at the emergency department of the Guy's and St Thomas' hospital in London with a suspected GHB/GBL intoxication (see subsection 2.2.1). From those patients who had a history of GHB abuse or declared GHB use, both venous DBS (vDBS) (prepared by spotting 25 µL of venous blood on filter paper) and cDBS (direct application on filter paper following fingerprick) were analyzed. This study was approved by the UK National Research Ethics Service (Reference 11/LO/0976). Additionally, our

method was applied on cDBS obtained from healthy volunteers participating in a cross-over study investigating the bioequivalence of two commercial gabapentin preparations (see subsection 4.2.2). This study was approved by the Ethics Committee of Ghent University Hospital (NCT01821235).

3.3 Results and discussion

3.3.1 Sample preparation

3.3.1.1 Optimization of the sample preparation

Microwave-assisted derivatization is increasingly being applied in bioanalysis, including forensic and clinical toxicology, to drastically reduce the derivatization time. E.g. Meyer *et al.* successfully quantified glycols and GHB in emergency toxicology using microwave-assisted trimethylsilylation [23]. Microwave-asssisted derivatization procedures have also been used for the determination of amphetamines, codeine, morphine, tetrahydrocannabinol and new psychoactive substances [24-28, 30].

For the derivatization of our DBS samples, we combined microwave-assisted derivatization, which results in an accelerated time of derivatization and an increased sample throughput, with the approach of "on-spot derivatization", allowing minimization of the DBS sample preparation. The latter concept was previously developed in our lab for the GC-MS based determination of the party drug GHB in DBS and is currently applied in our routine forensic toxicology cases [44]. Generally, in DBS methods utilizing derivatization, analytes are first eluted from the DBS. After evaporation under nitrogen, the extract is then derivatized. Subsequently, the excess of derivatization reagent is removed by evaporation, followed by reconstitution of the sample. The "on-spot derivatization" is a modification to this general procedure. It is a direct derivatization technique in which an excess of derivatization reagent is added directly to the matrix (i.e. the DBS), without the use of an additional extraction step: the reagent acts both as extracting and derivatizing reagent [10]. Mess *et al.* also utilized "on-spot derivatization" by the pre-treatment of DBS cards with the derivatization reagent prior to blood spotting [45].

When setting up a DBS based method involving "on-spot derivatization", it needs to be taken into account that the filter paper is derivatized as well. We therefore evaluated different blank filter papers to check which filter paper showed the lowest number of interferences and found that Whatman 903 filter paper scored best. We also opted to use a domestic microwave as we wished to set up a method that would be applicable in a routine toxicological laboratory without the use of expensive, dedicated instruments.

Based on Ingels *et al.* [22] and Damm *et al.* [24], combinations of TFAA, HFB, acetic anhydride and/or pyridine were evaluated to derivatize as many as possible of the above-mentioned polar low molecular weight molecules with toxicological relevance (Table 3.1).

Table 3.1 Evaluation of different derivatization reagents for the derivatization of various polar low molecular weight compounds.

derivatization reagents	GHB	Gabapentin	BHB	1,4-BD	1,2-BD	DEG	PG	Vigabatrin
[TFAA + HFB]	+	+	+	-	-	+	-	+
[$(AcO)_2O$ + pyridine]	-	-	-	+	+	+	+	-
[TFAA + HFB + $(AcO)_2O$ + pyridine]	NA	NA	NA	NA	NA	NA	NA	NA
[$(AcO)_2O$ + pyridine] & [HFB]	+	+	+	+	+	+	+	+
[$(AcO)_2O$ + HFB]	-	-	-	-	-	-	-	-

-: not detected +: detected NA: not analyzed (not injected)

Whereas a mix of TFAA and HFB could be used for the derivatization of DEG and analogues of gamma-aminobutyric acid (including GHB, BHB, gabapentin and vigabatrin), acetic anhydride and pyridine could be used for the derivatization of the glycols. The simultaneous addition of TFAA, HFB, acetic anhydride and pyridine generated a product which could not be injected because of the generation of a dark clot (TFAA being the cause). Theoretically, a first derivatization with TFAA and HFB, followed by evaporation and then a second derivatization with acetic anhydride and pyridine, seems to be the best choice. However, since this would render the method more time-consuming and would be at the expense of the advantages of fast microwave-assisted derivatization, we did not consider this as an option. Consequently, best results were obtained when first an acetylation reaction was performed using acetic anhydride and pyridine, followed by an alkylation step with HFB. Two separate reactions were needed as no adequate sensitivity was achieved when acetic anhydride, pyridine and HFB were simultaneously added. Although a silylation derivatization could be used as well, this derivatization reaction was not chosen as the injection of the excess of derivatization product contaminates the whole system and leads to in-situ derivatization of all injected compounds [46,47].

We optimized the main parameters for derivatization, i.e. the time of derivatization and the amount of derivatization reagent. To this end, we derivatized our analytes with a derivatization mixture consisting of different amounts of acetic anhydride and pyridine. All these combinations were evaluated at different lenghts of derivatization time (60, 90, 120 and 180 s), as can be seen in Figure 3.3. Since no significant differences were observed between 50 and 60 µL of a mix of acetic anhydride and pyridine (1:1 ratio, i.e. 25 or 30 µL of each reagent), we selected the lowest amount (i.e. 50 µL). When comparing a 3:2 with a 1:1 ratio of acetic anhydride and pyridine, also here, no significant differences were observed. Finally, we chose an equal amount of both reagents. Although longer derivatization times led to higher signals, results became less reproducible as -because of pressure build-up- the plugs closing the test tubes tended to come off.

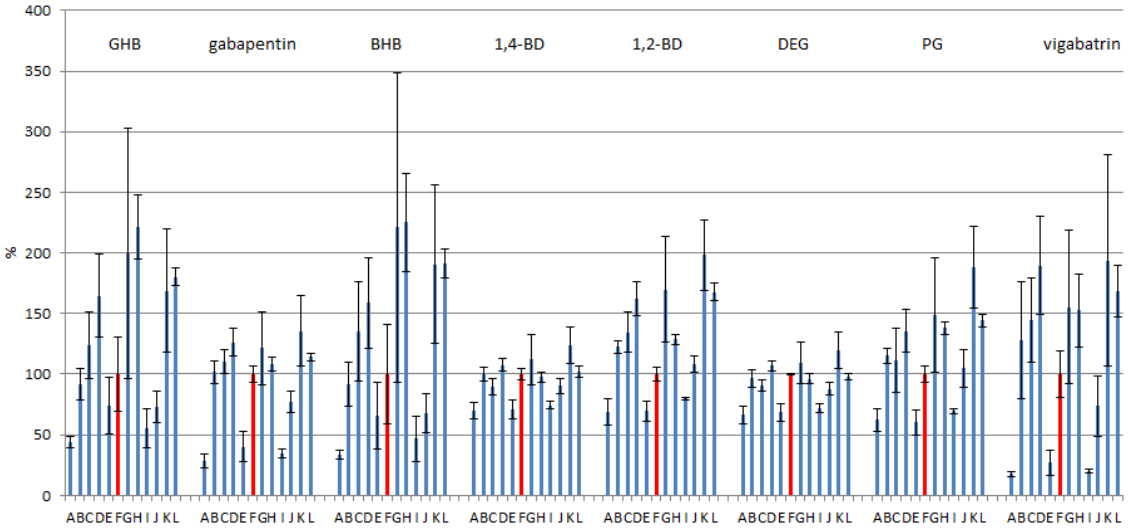


Figure 3.3 Influence of different derivatization times and different amounts of acetic anhydride and pyridine on the peak area of each analyte (n = 3). Data are presented as percentage of peak area of samples which were derivatized for 90 s with 25 µL of acetic anhydride and pyridine, followed by a second derivatization with 25 µL of heptafluorobutanol for 90 s. The condition finally chosen is highlighted in red. (A, E, I = 2 x 60 s; B, F, J = 2 x 90 s; C, G, K = 2 x 120 s; D, H, L = 1 x 180 s, 1x 120 s; A, B, C, D = 30 µL acetic anhydride + 30 µL pyridine + 25 µL HFB; E, F, G, H = 25 µL acetic anhydride + 25 µL pyridine + 25 µL HFB; I, J, K, L = 30 µL acetic anhydride + 20 µL pyridine + 25 µL HFB)

We also evaluated the influence of the amount of HFB, a derivatization reagent which is added to derivatize GHB, BHB, vigabatrin and gabapentin (Figure 3.4).

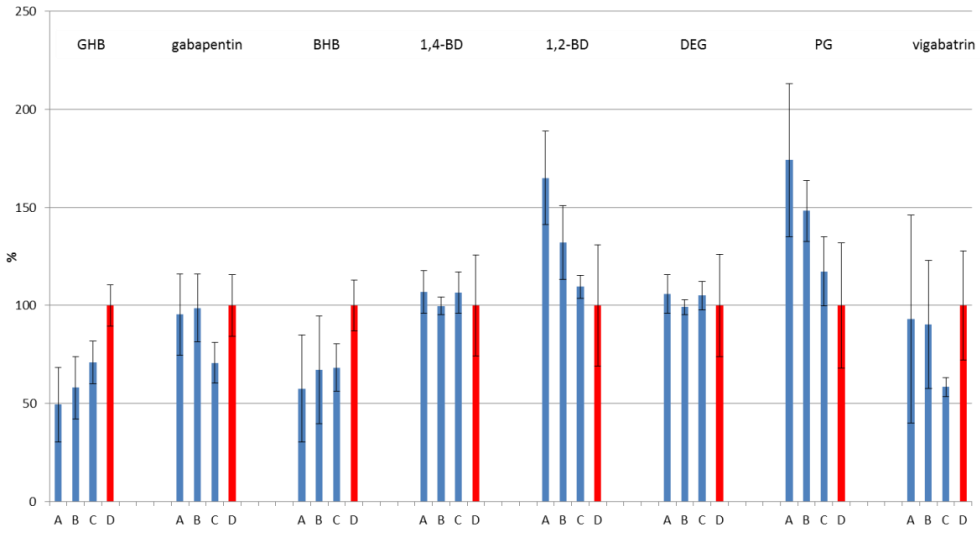


Figure 3.4 Influence of four different amounts of heptafluorobutanol (A = 10 µL, B = 15 µL, C = 20 µL and D = 25 µL) on the peak area of each analyte. Data are presented as percentage of normalized samples which are derivatized with 25 µL of heptafluorobutanol (n = 3). The condition finally chosen is highlighted in red.

For the anti-epileptics, no significant differences were observed between the largest amount of HFB, i.e. 25 µL, and the smallest amount of derivatization reagent, i.e. 10 µL. However, for BHB and especially for GHB, best results were obtained with 25 µL of HFB. Consequently, in our optimized procedure, the same amount of acetic anhydride, pyridine and HFB, i.e. 25 µL of each reagent, is added. It is worth noting that diols only had to be acetylated to improve their volatility. Consequently, the second derivatization step in our protocol is no added-value for the glycols. Therefore, if one is not interested in GHB, BHB, vigabatrin or gabapentin, the second derivatization could be omitted. In that case, the sample preparation is even faster and also allows the detection of other glycols, like ethylene glycol.

3.3.1.2 Final sample preparation

The final optimized protocol is as follows: first a 6-mm disc is punched out of a 25-µL DBS. To this punch, 5 µL IS mix, containing 60 µg/mL GHB-D6 and 12 µg/mL gabapentin-D10, is added, followed by evaporation under nitrogen at 25°C for 10 min. Then, the punches are subjected to "microwave-assisted on-spot derivatization" by direct application of 25 µL acetic anhydride and 25 µL pyridine to the punches and microwave heating for 90 s, followed by a short-spin centrifugation at 4°C. Subsequently, a second microwave derivatization is performed for 90 s by adding 25 µL HFB, after which the samples are subjected to a short-spin centrifugation at 4°C. After evaporation under nitrogen, the spots are reconstituted in 100 µL ethyl acetate and centrifuged at room temperature for 5 min. Finally, one µL of the supernatant of this derivatized extract is injected into the GC-MS system.

Noteworthy is that our derivatization step only takes 3 min (2 x 90 s), twice followed by a short-spin centrifugation step, whereas, without microwave assistance, derivatization times of 30 min are not unusual. Following the removal of the excess of derivatization reagent by evaporation, ethyl acetate was added to the samples. The latter sample preparation step takes about 10 min (5 min sonication + 5 min centrifugation). Conclusively, we have developed a fast sample preparation method for DBS (estimated time of sample preparation is about 15 min (time of evaporation not included)).

3.3.2 Analytical parameters

3.3.2.1 Optimization of injection solvent

Ethyl acetate, hexane and toluene were tested as injection solvent. However, it has to be noted that the choice of injection solvent may have an influence on the GC temperature program as the start temperature should be 20°C lower than the boiling point of the injection solvent. Consequently, the higher the boiling point, the higher the start temperature, the shorter the total run time. More specifically, the temperature program should start at 45, 60 and 90°C with respectively hexane, ethyl acetate and toluene. We observed that only ethyl acetate

could be used as injection solvent as GHB and vigabatrin could not be detected when using hexane as injection solvent, whereas PG was not detectable if toluene was used.

3.3.2.2 Optimization of injection temperature

The injection temperature should allow vaporization of the analytes and avoid degradation of the compounds and/or tailing peaks. Therefore, injection temperatures of 200, 250 and 300°C were evaluated. As can be seen in Figure 3.5, similar results were obtained with an injection temperature of 200, 250 and 300°C. Eventually, we opted for an injection temperature of 250°C.

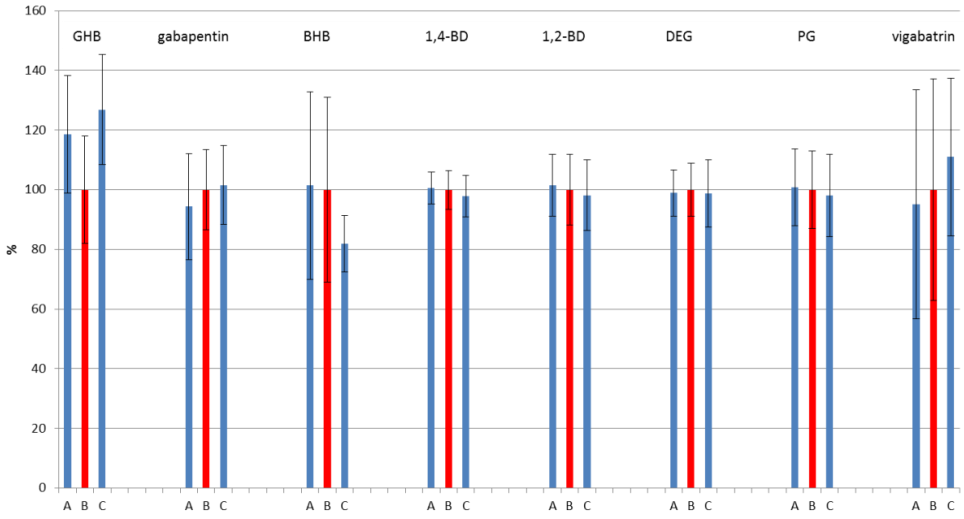


Figure 3.5 Influence of three different injection temperatures (A = 200°C, B = 250°C and C = 300°C) on the peak area of each analyte. Data are presented as percentage of normalized samples which are injected with an injection temperature of 250°C (n = 3). The condition finally chosen is highlighted in red.

3.3.2.3 Optimization of flow rate

The flow rate corresponds to the speed of the carrier gas (i.e. helium) through the column and this may influence the resolution. Since different flow rates had no influence on the baseline separation, peak areas were compared to each other (Figure 3.6). Data are presented as percentage of peak areas obtained for samples analyzed at a flow rate of 1.1 mL/min. Flow rates of 1, 1.3 and 1.5 mL/min resulted in lower peak areas for GHB, whereas a flow rate of 1.3 mL/min resulted in lower peak areas for 1,4-BD. For PG and DEG, the higher the flow rate, the higher the peak areas. Finally, we chose a flow rate of 1.1 mL/min, although the differences with other flow rates were minimal.

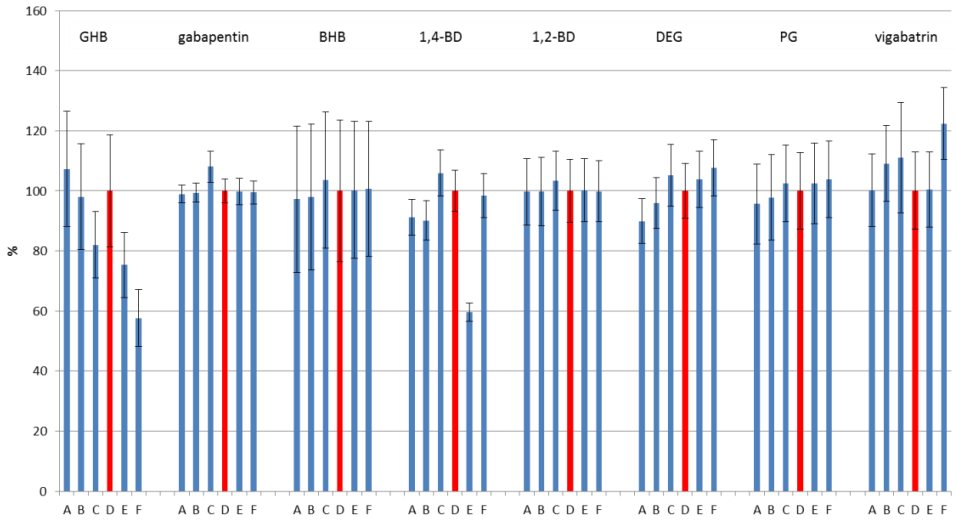


Figure 3.6 Influence of different flow rates (A = 0.7 mL/min, B = 0.9 mL/min, C = 1 mL/min, D = 1.1 mL/min, E = 1.3 mL/min and F = 1.5 mL/min) on the peak area of each analyte. Data are presented as percentage of normalized samples with a flow rate of 1.1 mL/min (n = 3). The condition finally chosen is highlighted in red.

3.3.2.4 Optimization of purge activation time

The purge activation time is the time that the split line opens when using a splitless injection. Best results were obtained with a purge activation time of 120 s (Figure 3.7).

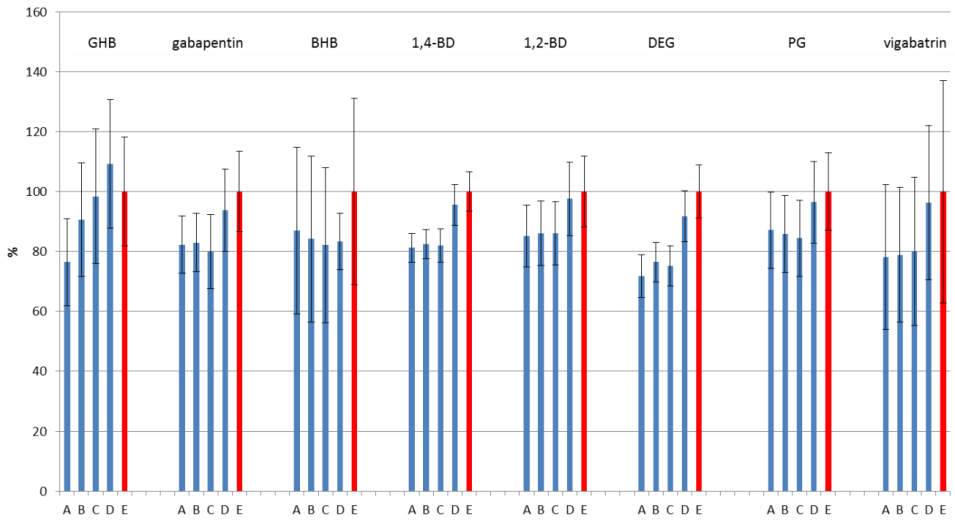


Figure 3.7 Influence of different purge activation times (A = 45 s, B = 60 s, C = 75 s, D = 90 s and E = 120 s) on the peak area of each analyte. Data are presented as percentage of normalized samples with a purge activation time of 120 s (n = 3). The condition finally chosen is highlighted in red.

3.3.2.5 Optimization of pulsed splitless injection and pulse time

We also evaluated the influence of an elevated pressure on the top of the column and the resulting sample transfer. We concluded that a higher increase of the pressure results in a shorter run. However, this gain in time is outweighted by the higher abundances obtained with a non-pulsed splitless injection, as can be seen in Figure 3.8.

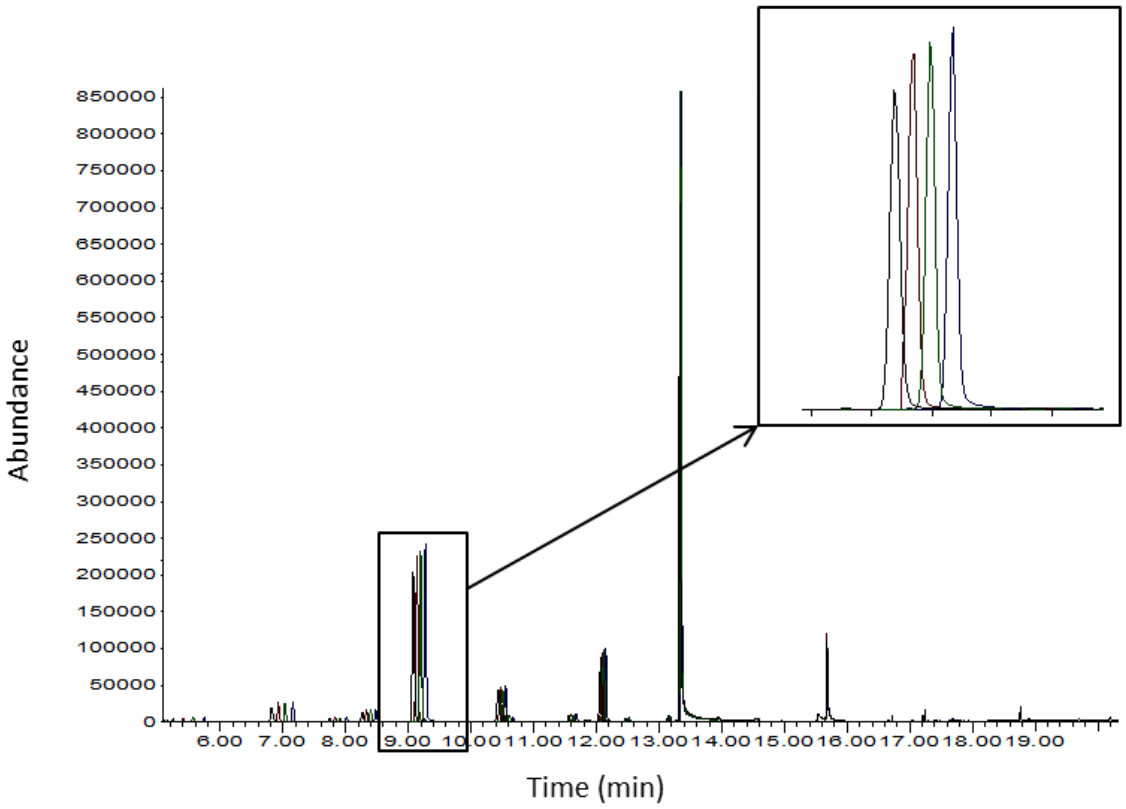


Figure 3.8 Influence of pulsed splitless injection and pulse time. (inset exemplifies the observed elution order, with respectively pulsed splitless injection (30 psi) (black color); pulsed splitless injection (25 psi) (red color); pulsed splitless injection (20 psi) (green color); non-pulsed splitless injection (blue color))

3.3.3 Validation

3.3.3.1 Microwave calibration

When monitoring the power of the microwave through time, a mean power of 602 ± 15 W was observed. The obtained values were plotted in a Shewhart graph (Figure 3.9) and the Westgard rules were applied. These rules were not broken. Consequently, the performance of the microwave was considered to be in-control.

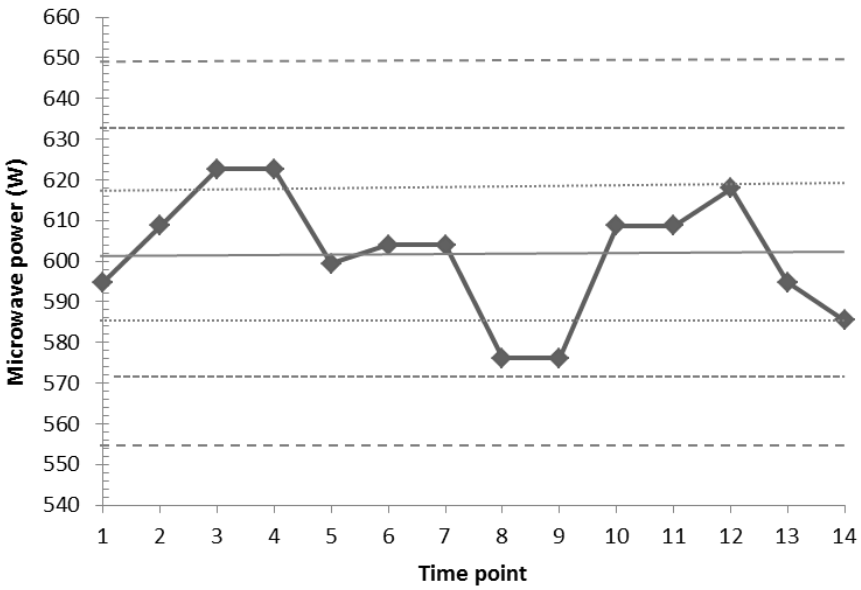


Figure 3.9 Shewhart graph obtained when plotting the obtained values of the microwave power through time. The mean power ± SD (·····), the mean power ± 2 SD (----) and the mean power ± 3 SD (- - -) are displayed.

3.3.3.2 Linearity, precision and accuracy

Linearity was determined by constructing six-point calibration curves in duplicate on three non-consecutive days. In addition, blank and zero samples (blank samples spiked with IS) were injected. A representative chromatogram of a spiked blood sample at LLOQ level is given in Figure 3.10.

Table 3.2 summarizes calibration and sensitivity data. Although the R^2 values were in some cases below the preferred value of 0.99, a lack-of-fit test to evaluate the goodness of fit demonstrated that the calibration curves were linear. Additionally, we also applied the EMA guidelines for the evaluation of the calibration curve. These state that the target back-calculated concentrations of the calibration standards should be within 15% of the nominal value (20% at LLOQ and ULOQ) for at least 75% of calibration standards. This criterion was fulfilled for all compounds. The LLOQ of 10 µg/mL for GHB can be considered as a limitation of our procedure as the cut-off levels in ante-mortem blood are set at 10, 5 or 4 µg/mL, the lower values being applied most for samples taken from living subjects [48-51]. In case of doubt, i.e. GHB concentrations around the LLOQ, the original "on-spot derivatization" with TFAA and HFB using conventional heating, which offers a somewhat higher sensitivity with an LLOQ at 2 µg/mL, could be performed on a replicate DBS. For the anti-epileptics, our calibration ranges cover the therapeutic intervals of 2-20 and 5-25 µg/mL for gabapentin and vigabatrin, respectively. BHB concentrations lower than 50 µg/mL are considered as normal, whereas BHB concentrations higher than 250 µg/mL are considered as high and pathologically significant [52]. Our concentration range covers these cut-off levels. Also concerning 1,4-BD, our concentration range is in line with other quantification methods [53,54]. Our method is also appropriate in case of intoxications with DEG and PG since intoxications with these glycols are in the high-µg/mL range.

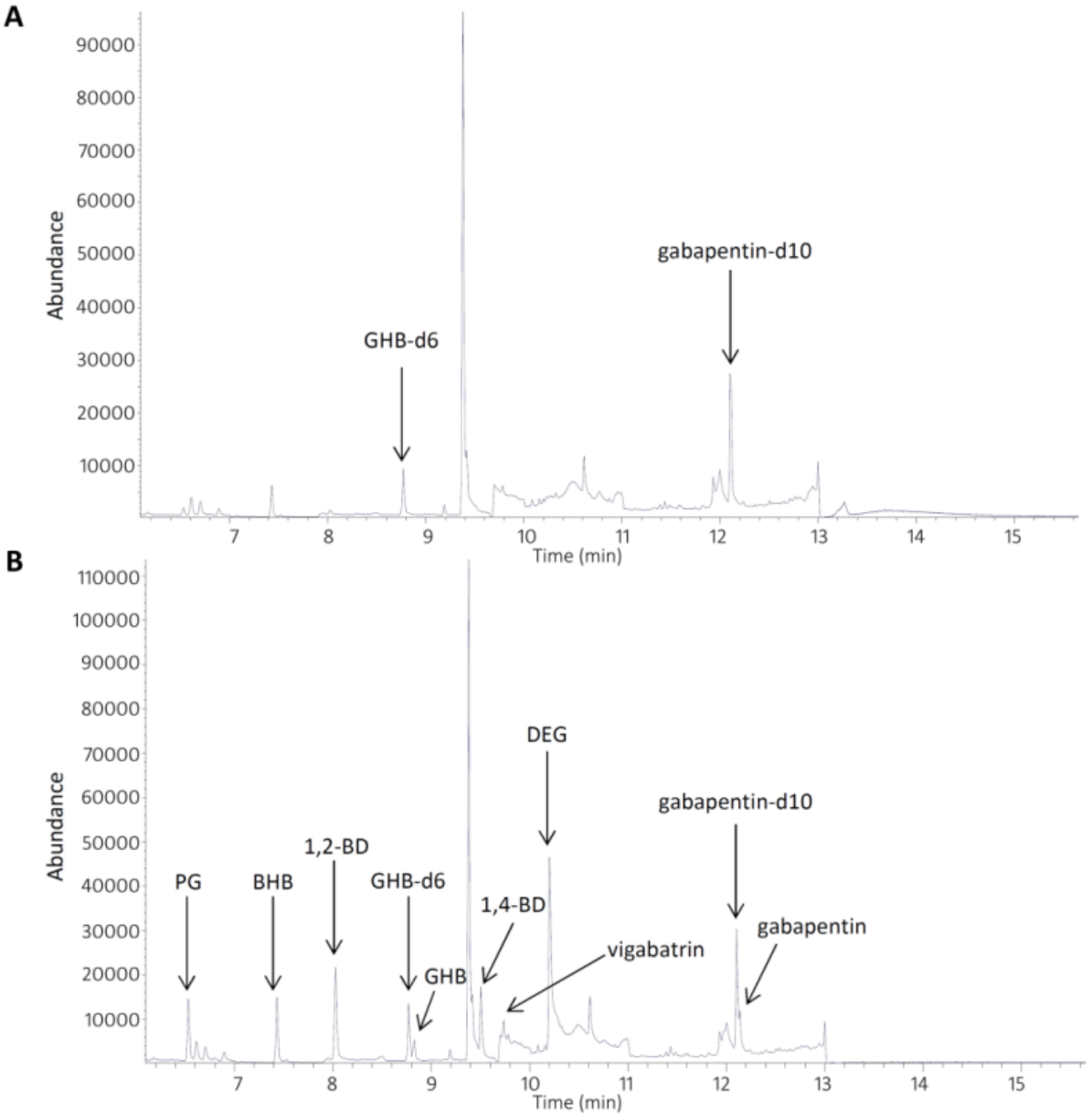


Figure 3.10 Representative chromatogram of (a) a DBS spiked with IS and (b) a DBS spiked with all studied compounds at LLOQ level.

An overview of accuracy, within-day and between-day precision data is given in Table 3.3. For the analytes for which deuterated analogues were used, being GHB and gabapentin, accuracy and precision criteria were met for all QC levels. For the other analytes, accuracy and precision were also evaluated although the use of stable labeled analogues as IS is recommended for quantification in real practice. While within-day precision criteria were met in all instances, slight exceedances of between-day precision criteria (> 15% but still below 20%) were seen for 1,4- and 1,2-BD, DEG, PG and vigabatrin. For both BHB and DEG, the bias was slightly above the acceptance criteria for two QC levels. For 1,4- and 1,2-BD, as well as for PG, the bias exceeded 20%. Hence, we consider the procedure semi-quantitative for these analytes.

Table 3.2 Calibration and sensitivity data for the determination of GHB, gabapentin, BHB, 1,4-BD, 1,2-BD, DEG, PG and vigabatrin in DBS using GC-MS (n = 3x2).

	Slope mean ± SD [95% CI]	**Intercept** mean ± SD [95% CI]	**R^2**	**Range** (µg/mL)	**LLOQ** (µg/mL)	**Weighting factor**
GHB	0.033 ± 0.002 [0.032-0.035]	0.067 ± 0.058 [0.020-0.113]	0.993	10-100	10	1/x
Gabapentin	0.226 ± 0.008 [0.220-0.232]	0.009 ± 0.024 [-0.010-0.028]	0.981	1-30	1	1/x
BHB	0.074 ± 0.004 [0.071-0.078]	-0.018 ± 0.075 [-0.078-0.042]	0.993	5-300	5	1/x
1,4-BD	0.065 ± 0.005 [0.060-0.069]	-0.011 ± 0.096 [-0.088-0.066]	0.986	5-200	5	1/x
1,2-BD	0.112 ± 0.010 [0.104-0.120]	-0.074 ± 0.103 [-0.156-0.008]	0.986	5-200	5	1/x
DEG	2.212 ± 0.148 [2.093-2.330]	-1.921 ± 1.584 [-3.188- -0.654]	0.988	2.5-100	2.5	1/x
PG	0.068 ± 0.006 [0.063-0.073]	-0.100 ± 0.055 [-0.144- -0.056]	0.979	5-200	5	1/x
Vigabatrin	0.062 ± 0.006 [0.058-0.067]	-0.029 ± 0.036 [-0.058- -0.000]	0.984	5-30	5	1/x

Table 3.3 Accuracy, intra- and interbatch precision for QCs at four concentration levels (n = 3x2). Exceedances of the criteria are set in italic.

		GHB	**Gabapentin**	**BHB**	**1,4-BD**	**1,2-BD**	**DEG**	**PG**	**Vigabatrin**
		1/x	**1/x**	**1/x**	**1/x**	**1/x**	**1/x**	**1/x**	**1/x**
Within-day precision (%RSD)	**LLOQ**	4.45	10.20	19.30	5.47	0.16	11.52	6.63	13.47
	L QC	7.22	4.88	6.79	11.06	5.22	8.04	5.25	5.50
	M QC	4.59	4.99	8.10	8.03	11.16	5.97	12.19	9.31
	H QC	8.93	9.95	11.58	3.46	5.88	4.11	5.97	12.33
Between- day precision (%RSD)	**LLOQ**	15.71	11.88	19.30	15.69	0.74	15.97	6.63	13.47
	L QC	12.53	6.88	9.00	14.40	*16.53*	*16.83*	*18.46*	*16.11*
	M QC	4.93	6.93	9.92	*16.77*	11.16	14.67	12.19	9.31
	H QC	8.93	8.86	11.58	10.01	7.95	9.05	8.09	12.33
Accuracy (% bias)	**LLOQ**	19.44	-6.94	2.25	15.96	15.37	*20.58*	19.89	-8.19
	L QC	2.04	-6.02	*18.41*	*22.49*	*32.14*	0.33	*25.95*	-5.05
	M QC	5.88	7.99	6.54	9.50	*16.40*	*18.40*	10.13	-5.32
	H QC	6.41	3.73	*16.36*	14.71	*17.86*	9.18	13.21	-9.81

3.3.3.3 Selectivity, carry-over and dilution integrity

No interfering peaks or carry-over were observed. For the evaluation of the dilution integrity, we analyzed 6 DBS which were prepared from 25 µL venous blood which was spiked with analyte concentrations at twice the ULOQ level. For all analytes, accuracy and precision were within the acceptance criteria of 15%, as can be seen in Table 3.4.

Table 3.4 Accuracy and precision of samples which were diluted 4-fold.

	Dilution integrity (n = 6)	
	Accuracy (% bias)	**Precision** (% CV)
GHB *	12.29	3.64
Gabapentin	-4.93	4.88
BHB	8.18	9.00
1,4-BD	-6.79	6.63
1,2-BD	-3.35	9.96
DEG	10.26	13.74
PG	9.69	7.51
Vigabatrin	9.02	14.59

* n = 5

3.3.3.4 Stability

Stock solutions were stable for at least one week at -20°C. Three freeze-thaw cycles did also not affect the stock solution. GHB and gabapentin were found to be stable in DBS stored at room temperature for at least 84 days, with measured concentrations not deviating more than 7.4 and 4.9% of the concentrations measured at time point zero for GHB and gabapentin, respectively. The other analytes were also found to be stable for an extended period of time in DBS stored at room temperature.

3.3.3.5 DBS-specific parameters

For the evaluation of the Hct effect, we prepared blood samples with a low (0.30), medium (0.40) and high Hct (0.49). To evaluate the volume effect, different volumes of blood (25, 35 and 50 µL) were spotted onto the Whatman filter paper. For the effect of both Hct and volume, the results obtained from 25-µL DBS with a Hct of 0.40 were taken as a reference. For gabapentin, both a Hct and volume effect were observed, i.e. we observed lower concentrations at the low Hct level and higher concentrations at the higher Hct and a trend of increasing concentrations with increased blood volumes (Figure 3.11). For GHB, a volume effect was seen for the low QC levels at the high Hct level, whereas the Hct effect was less pronounced (Figure 3.11). These results are in line with the experiments performed during the validation of our original GHB method utilizing “on-spot derivatization”, i.e. in that method, we observed little or no influence in the Hct range of 0.39 to 0.51 and we

concluded that the best blood volume spotted was between 20 and 35 µL since higher blood volumes result in an overload of the filter paper when using a calibration line based upon 25-µL DBS [44]. The influence of volume and Hct did not show a pronounced trend for the other analytes (see Figure 3.11). The impact of the punch location, also a DBS-specific parameter, was not evaluated since there is no true peripheral localization when taking a 6-mm disc from a 25-µL DBS.

3.3.4 Application

In a first application, retrospective analysis of DBS from patients with a suspected GHB/GBL intoxication was performed. These DBS samples (both vDBS and cDBS) were collected to evaluate the correlation between venous and capillary GHB concentrations [55 and Chapter 2], using a validated GC-MS method for the quantification of GHB in DBS [44]. When analyzing a subset of these samples (n = 10) with our new method, we obtained similar GHB concentrations, with a mean difference of 1.21%. Table 3.5 gives an overview of the GHB concentrations measured in the DBS following the method of Ingels *et al.* [44] ("on-spot derivatization") and those obtained using "microwave-assisted on-spot derivatization". This observation confirms the applicability of our method in cases of (suspected) GHB intoxication. Our method is even faster than the original "on-spot derivatization" method by utilizing a microwave instead of a 'classic' heating block. In addition, our method also allows the simultaneous detection of other polar low molecular weight molecules.

In a second application, gabapentin concentrations were measured in treated volunteers. Of these volunteers, paired cDBS and serum samples were obtained at different time points. We quantified these cDBS concentrations, whereas the obtained serum concentrations were measured using an independent method [56]. The pharmacokinetic profile obtained from both fingerprick blood and serum of one volunteer is given in Figure 3.12. These measurements served as the basis for a larger study evaluating the correlation between serum and blood concentrations and to investigate the correlation between our method and an independent method (see Chapter 4). Our observation that DBS concentrations are somewhat lower than those in serum is in line with the expectation: although gabapentin is assumed to be evenly distributed between plasma/serum and blood, the blood-to-plasma(serum) ratio is slightly lower than 1 due to the presence of solid constituents in blood. Additionally, this study demonstrated the ease of DBS sampling: whereas a phlebotomist was needed to obtain serum samples, DBS sampling does not require trained staff and can be performed by the patient himself, even at home.

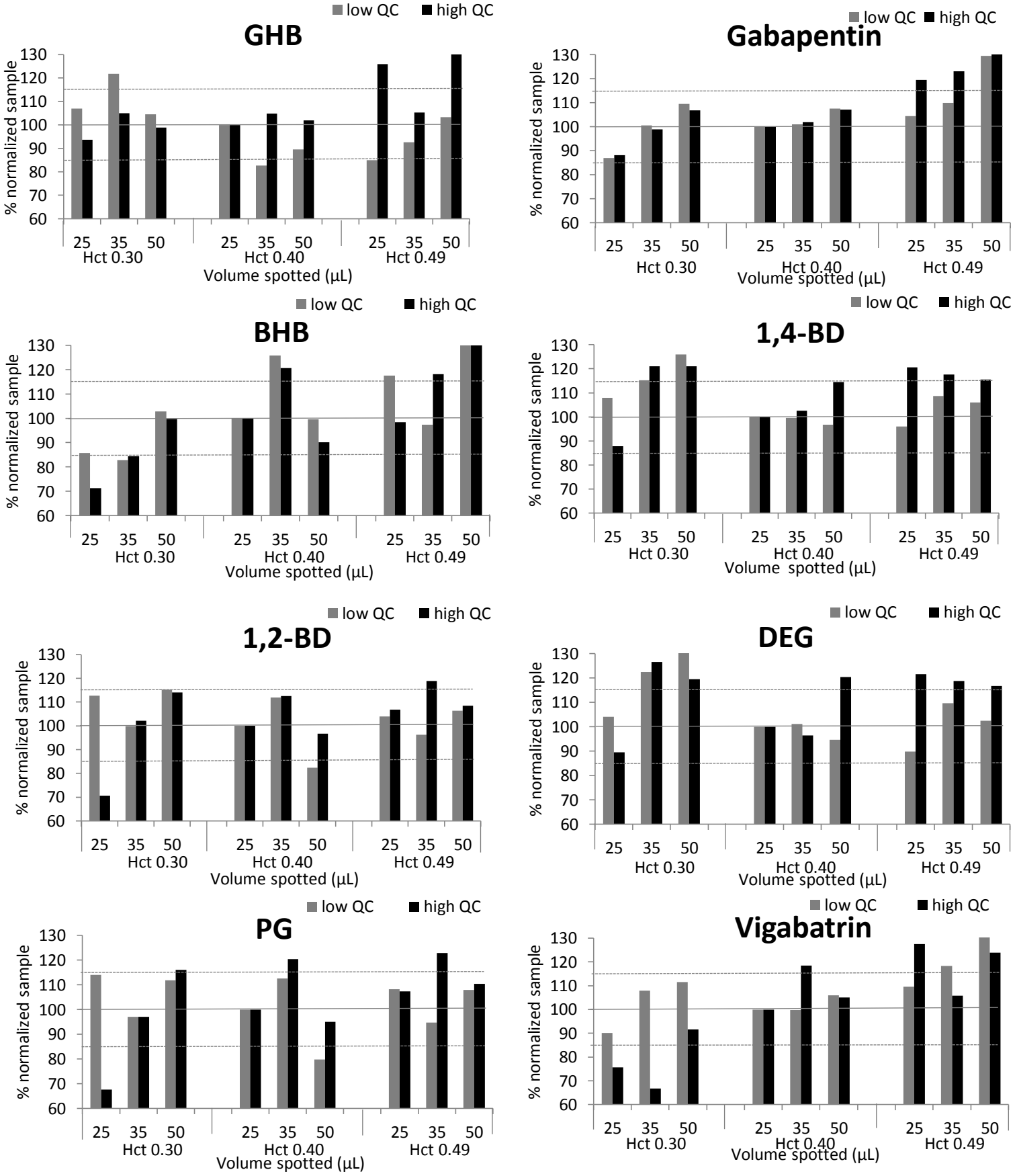


Figure 3.11 Influence of both the Hct and the blood volume spotted on GHB, gabapentin, BHB, 1,4-BD, 1,2-BD, DEG, PG and vigabatrin concentrations. Data are presented as percentage of normalized samples with a Hct of 0.40 and a spotted blood volume of 25 µL. The ± 15% deviation limits are indicated by dotted lines.

Table 3.5 GHB concentrations measured in cDBS and vDBS of patients with a suspected GHB intoxication using our method versus the concentrations obtained following the method of Ingels *et al.* [44].

		GHB concentration (µg/mL)	
		"microwave-assisted on-spot derivatization"	**"on-spot derivatization"**
1	vDBS	186.0	167.5
2	vDBS	67.1	75.2
3	vDBS	272.6	271.4
4	vDBS	43.6	48.1
5	vDBS	229.2	191.6
6	cDBS	242.2	233.7
7	cDBS	162.8	151.4
8	cDBS	41.1	41.4
9	cDBS	172.0	187.1
10	cDBS	206.1	200.3

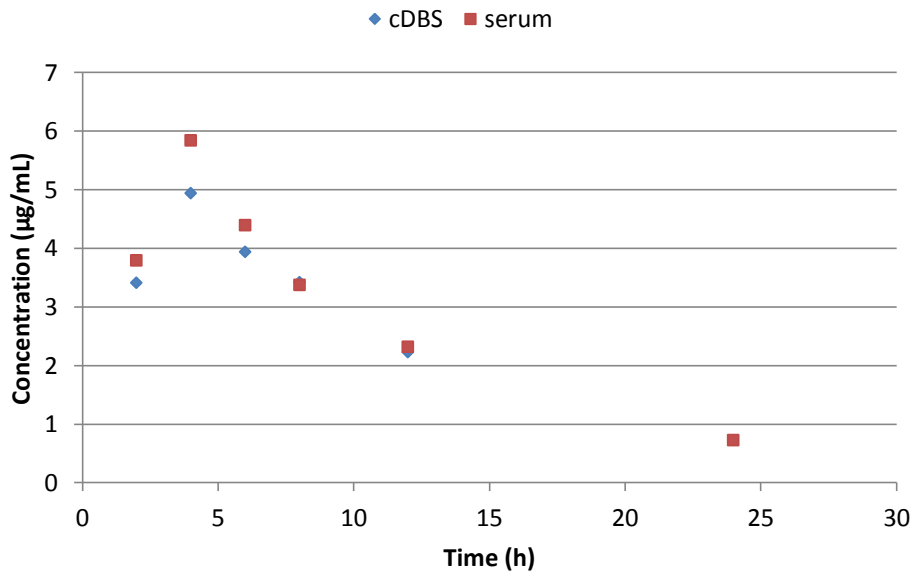


Figure 3.12 Pharmacokinetic profile of a volunteer who was administered gabapentin. This profile was obtained by analyzing cDBS and serum samples which were collected at different time points.

3.4 Conclusion

We have developed and validated a GC-MS method for the determination of GHB and gabapentin in DBS, utilizing "microwave-assisted on-spot derivatization". This approach combines microwave derivatization with direct application of the derivatization reagents onto the DBS ("on-spot derivatization"). Consequently, the derivatization step should no longer be experienced as a laborious and time-consuming sample preparation step. To the best of our knowledge, we are the first using this direct derivatization technique.

In our method, the use of DBS could be considered as a sample preparation strategy. However, many other advantages have been associated with DBS, amongst which convenient storage and transport, reduced risk of infection, easy sampling, and so on. Another non-negligible advantage of our method is that no dedicated equipment is needed as the standard GC-MS configuration utilized in routine forensic toxicology, could be used.

Our method proved to be reliable, fast and applicable in routine toxicology, as exemplified by the analysis of gabapentin- and GHB-positive samples. Additionally, our method also allows the detection of other polar low molecular weight compounds with relevance in the forensic and clinical toxicological context, including vigabatrin, 1,4-BD, BHB, DEG, PG and 1,2-BD.

3.5 References

1. Ingels AS, Hertegonne KB, Lambert WE, Stove CP. Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home: an exploratory study. *CNS Drugs* 27(3), 233-237 (2013).
2. De Kesel PMM, Sadones N, Capiau S, Lambert WE, Stove CP. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis* 5(16), 2023-2041 (2013).
3. Demirev PA. Dried blood spots: analysis and applications. *Anal Chem* 85(2), 779-789 (2013).
4. Stove C, Spooner N. DBS and beyond. *Bioanalysis* 7(16), 1961-1962 (2015).
5. Edelbroek PM, van der Heijden J, Stolk LML. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Ther Drug Monit* 31(3), 327-336 (2009).
6. Li W, Tse FLS. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed Chromatogr* 24(1), 49-65 (2010).
7. Stove CP, Ingels A-SME, De Kesel PMM, Lambert WE. Dried blood spots in toxicology: from the cradle to the grave? *Crit Rev in Toxicol* 42(3), 230-243 (2012).
8. De Kesel PM, Lambert WE, Stove CP. Why dried blood spots are an ideal tool for CYP1A2 phenotyping. *Clin Pharmacokinet* 53(8), 763-771 (2014).
9. Velghe S, Capiau S, Stove CP. Opening the toolbox of alternative sampling strategies in clinical routine: A key-role for (LC-)MS/MS. *Trends Analyt Chem* 84(B), 61-73 (2016).
10. Ingels A-SME, Sadones N, De Kesel PMM, Lambert WE, Stove CP. Derivatization techniques in dried blood spot analysis. In: *Dried blood spots: applications and techniques.* Li, W, Lee, MS (Eds.), John Wiley & Sons, Hoboken, NJ, USA, 344-354 (2014).
11. Sadones N, Capiau S, De Kesel PMM, Lambert WE, Stove CP. Spot them in the spot: analysis of abused substances using dried blood spots. *Bioanalysis* 6(17), 2211-2227 (2014).
12. Batterman SA, Chernyak S, Su FC. Measurement and comparison of organic compound concentrations in plasma, whole blood, and dried blood spot samples. *Front Genet* 7, 64 (2016).
13. Kong ST, Lim SH, Lee WB *et al.* Clinical validation and implications of dried blood spot sampling of carbamazepine, valproic acid and phenytoin in patients with epilepsy. *PLoS One* 9(9), e108190 (2014).
14. Kong ST, Lin HS, Ching J, Ho PC. Evaluation of dried blood spots as sample matrix for gas chromatography/mass spectrometry based metabolomic profiling. *Anal Chem* 83(11), 4314-4318 (2011).
15. Rhoden L, Antunes MV, Hidalgo P, da Silva CA, Linden R. Simple procedure for determination of valproic acid in dried blood spots by gas chromatography-mass spectrometry. *J Pharm Biomed Anal* 96, 207-212 (2014).
16. Déglon J, Lauer E, Thomas A, Mangin P, Staub C. Use of the dried blood spot sampling process coupled with fast gas chromatography and negative-ion chemical ionization tandem mass spectrometry: application to fluoxetine, norfluoxetine, reboxetine, and paroxetine analysis. *Anal Bioanal Chem* 396(7), 2523-2532 (2010).

17. Andrew P, Doig MV. Gas chromatography: what it is and how we use it. In: *Principles and practice of bioanalysis.* Venn, RF (Ed.), Taylor & Francis Group, Abingdon-on-Thames, Oxfordshire, UK, 123-140 (2008).
18. Maurer HH. Current role of liquid chromatography-mass spectrometry in clinical and forensic toxicology. *Anal Bioanal Chem* 388(7), 1315-1325 (2007).
19. Maurer HH. Position of chromatographic techniques in screening for detection of drugs or poisons in clinical and forensic toxicology and/or doping control. *Clin Chem Lab Med* 42(11), 1310-1324 (2004).
20. Ingels A-SME, Wille SMR, Samyn N, Lambert WE, Stove CP. Screening and confirmation methods for GHB determination in biological fluids. *Anal Bioanal Chem* 406(15), 3553-3577 (2014).
21. Ingels AS, Neels H, Lambert WE, Stove CP. Determination of gamma-hydroxybutyric acid in biofluids using a one-step procedure with "in-vial" derivatization and headspace-trap gas chromatography-mass spectrometry. *J Chromatogr A* 1296, 84-92 (2013).
22. Ingels A-SME, Lambert WE, Stove CP. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. *Anal Bioanal Chem* 398(5), 2173-2182 (2010).
23. Meyer MR, Weber AA, Maurer HH. A validated GC-MS procedure for fast, simple, and cost-effective quantification of glycols and GHB in human plasma and their identification in urine and plasma developed for emergency toxicology. *Anal Bioanal Chem* 400(2), 411-414 (2011).
24. Damm M, Rechberger G, Kollroser M, Kappe CO. An evaluation of microwave-assisted derivatization procedures using hyphenated mass spectrometric techniques. *J Chromatogr A* 1216(31), 5875-5881 (2009).
25. De Brabanter N, Van Gansbeke W, Hooghe F, Van Eenoo P. Fast quantification of 11-nor-Delta 9-tetrahydrocannabinol-9-carboxylic acid (THCA) using microwave-accelerated derivatisation and gas chromatography-triple quadrupole mass spectrometry. *Forensic Sci Int* 224(1-3), 90-95 (2013).
26. Chung LW, Liu GJ, Li ZG, Chang YZ, Lee MR. Solvent-enhanced microwave-assisted derivatization following solid-phase extraction combined with gas chromatography-mass spectrometry for determination of amphetamines in urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 874(1-2), 115-118 (2008).
27. Chung LW, Lin KL, Yang TC, Lee MR. Orthogonal array optimization of microwave-assisted derivatization for determination of trace amphetamine and methamphetamine using negative chemical ionization gas chromatography-mass spectrometry. *J Chromatogr A* 1216(18), 4083-4089 (2009).
28. Meng P, Zhu D, He H, Wang Y, Guo F, Zhang L. Determination of amphetamines in hair by GC/MS after small-volume liquid extraction and microwave derivatization. *Anal Sci* 25(9), 1115-1118 (2009).
29. Söderholm SL, Damm M, Kappe CO. Microwave-assisted derivatization procedures for gas chromatography/mass spectrometry analysis. *Mol Divers* 14(4), 869-888 (2010).
30. Margalho C, Castanheira A, Real FC, Gallardo E, Lopez-Rivadulla M. Determination of "new psychoactive substances" in postmortem matrices using microwave derivatization and gas

chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 1020, 14-23 (2016).

31. Olah TV, Sleczka BG, D'Arienzo C, Tymiak AA. Quantitation of therapeutic proteins following direct trypsin digestion of dried blood spot samples and detection by LC-MS-based bioanalytical methods in drug discovery. *Bioanalysis* 4(1), 29-40 (2012).
32. Abanades S, Farre M, Segura M *et al.* Gamma-hydroxybutyrate (GHB) in humans: pharmacodynamics and pharmacokinetics. *Ann N Y Acad Sci* 1074, 559-576 (2006).
33. Wood DM, Nicolaou M, Dargan PI. Epidemiology of recreational drug toxicity in a nightclub environment. *Subst Use Misuse* 44(11), 1495-1502 (2009).
34. Wood DM, Greene SL, Dargan PI. Five-year trends in self-reported recreational drugs associated with presentation to a UK emergency department with suspected drug-related toxicity. *Eur J Emerg Med* 20(4), 263-267 (2013).
35. Brennan R, Van Hout MC. Gamma-hydroxybutyrate (GHB): a scoping review of pharmacology, toxicology, motives for use, and user groups. *J Psychoactive Drugs* 46(3), 243-251 (2014).
36. Daly M. Down a stony road: the 2014 drugscope street drug survey. http://www.drugsandalcohol.ie/23282/1/DownAStonyRoadDrugTrendsSurvey2014.pdf
37. Housova J, Hoke K. Microwave heating - the influence of oven and load parameters on the power absorbed in the heated load. *Czech J Food Sci* 20(3), 117-124 (2002).
38. U.S. Department of Health and Humans Services. Food and Drug Administration. Center for Drug Evaluation and Research. Center for Veterinary Medicine. Draft Guidance for Industry. Bioanalytical Method Validation. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation /guidances/ucm368107.pdf
39. European Medicines Agency. Guideline on bioanalytical method validation. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf
40. De Kesel PM, Capiau S, Lambert WE, Stove CP. Current strategies for coping with the hematocrit problem in dried blood spot analysis. *Bioanalysis* 6(14), 1871-1874 (2014).
41. Almeida AM, Castel-Branco MM, Falcao AC. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. *J Chromatogr B Analyt Technol Biomed Life Sci* 774(2), 215-222 (2002).
42. Wille SMR, Peters FT, Di Fazio V, Samyn N. Practical aspects concerning validation and quality control for forensic and clinical bioanalytical quantitative methods. *Accred Qual Assur* 16(6), 279-292 (2011).
43. Peters FT, Drummer OH, Musshoff F. Validation of new methods. *Forensic Sci Int* 165(2-3), 216-224 (2007).
44. Ingels A-S, De Paepe P, Anseeuw K *et al.* Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis* 3(20), 2271-2281 (2011).

45. Mess J-N, Taillon M-P, Cote C, Garofolo F. Dried blood spot on-card derivatization: an alternative form of sample handling to overcome the instability of thiorphan in biological matrix. *Biomed Chromatogr* 26(12), 1617-1624 (2012).

46. Preu M, Guyot D, Petz M. Development of a gas chromatography mass spectrometry method for the analysis of aminoglycoside antibiotics using experimental design for the optimisation of the derivatisation reactions. *J Chromatogr A* 818(1), 95-108 (1998).

47. Preu M, Petz M. Development and optimisation of a new derivatisation procedure for gas chromatographic mass spectrometric analysis of dihydrostreptomycin - Comparison of multivariate and step-by-step optimisation procedures. *J Chromatogr A* 840(1), 81-91 (1999).

48. Villain M, Cirimele V, Ludes B, Kintz P. Ultra-rapid procedure to test for gamma-hydroxybutyric acid in blood and urine by gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 792(1), 83-87 (2003).

49. Elliott SP. Gamma hydroxybutyric acid (GHB) concentrations in humans and factors affecting endogenous production. *Forensic Sci Int* 133(1-2), 9-16 (2003).

50. Elian AA. Determination of endogenous gamma-hydroxybutyric acid (GHB) levels in antemortem urine and blood. *Forensic Sci Int* 128(3), 120-122 (2002).

51. Andresen H, Sprys N, Schmoldt A, Mueller A, Iwersen-Bergmann S. Gamma-hydroxybutyrate in urine and serum: additional data supporting current cut-off recommendations. *Forensic Sci Int* 200(1-3), 93-99 (2010).

52. Elliott S, Smith C, Cassidy D. The post-mortem relationship between beta-hydroxybutyrate (BHB), acetone and ethanol in ketoacidosis. *Forensic Sci Int* 198(1-3), 53-57 (2010).

53. Dahl SR, Olsen KM, Strand DH. Determination of gamma-hydroxybutyrate (GHB), beta-hydroxybutyrate (BHB), pregabalin, 1,4-butane-diol (1,4BD) and gamma-butyrolactone (GBL) in whole blood and urine samples by UPLC-MSMS. *J Chromatogr B Analyt Technol Biomed Life Sci* 885-886, 37-42 (2012).

54. Sorensen LK, Hasselstrom JB. A hydrophilic interaction liquid chromatography electrospray tandem mass spectrometry method for the simultaneous determination of gamma-hydroxybutyrate and its precursors in forensic whole blood. *Forensic Sci Int* 222(1-3), 352-359 (2012).

55. Sadones N, Archer JRH, Ingels A-SME *et al.* Do capillary dried blood spot concentrations of gamma-hydroxybutyric acid mirror those in venous blood? A comparative study. *Drug Test Anal* 7(4), 336-340 (2015).

56. Chahbouni A, Sinjewel A, den Burger JCG *et al.* Rapid quantification of gabapentin, pregabalin, and vigabatrin in human serum by ultraperformance liquid chromatography with mass-spectrometric detection. *Ther Drug Monit* 35(1), 48-53 (2013).

CHAPTER 4:
CORRELATION BETWEEN GABAPENTIN BLOOD AND SERUM CONCENTRATIONS

Based on

Sadones N, Van Bever E, Van Bortel L, Lambert WE, Stove CP. Dried blood spot analysis of gabapentin as a valid alternative for serum: a bridging study. *J Pharm Biomed Anal* 132, 72-76 (2017).

Abstract

We evaluated the applicability of a validated GC-MS method for the determination of gabapentin in dried blood spots (DBS). Important for the acceptance of DBS sampling as an alternative sampling strategy is the possibility to base solid conclusions on the quantification. Therefore, bridging studies -studies in which the correlation between both DBS and a reference matrix (e.g. serum) is evaluated statistically- need to be conducted. To this end, a comparative study was set up to quantify gabapentin in both blood (DBS) and serum samples. Statistically significant differences between DBS and serum concentrations were found ($p < 0.001$). A mean blood-to-serum ratio of 0.85 was observed, which is in line with expectations. Calculated serum concentrations (obtained by dividing the DBS concentrations by 0.85) demonstrated a good correlation with measured serum concentrations, with 87% of samples fulfilling the criterion for incurred sample reanalysis. Furthermore, our data indicate a good correlation between capillary and venous concentrations. Conclusively, this study demonstrated that DBS are a valid alternative to serum for the determination of gabapentin.

4.1 Introduction

Gabapentin, an anticonvulsant structurally related to the inhibitory neurotransmitter gamma-amino butyric acid, is used for the treatment of epilepsy and neuropathic pain. Monitoring gabapentin blood concentrations may be relevant in assessing compliance and in avoiding potential toxicity. Furthermore, the illicit use of gabapentin as street drug also increases. E.g. in the UK, there is a significant rise in prescribing of gabapentin, especially among opiate users and prisoners [1]. These populations often mix gabapentin with other depressants, resulting in life-threatening effects.

Since its introduction in neonatal screening in the 1960s by Guthrie and Susi [2], dried blood spot (DBS) sampling has increasingly received interest as an alternative sampling strategy and has been applied in various disciplines, including toxicology, phenotyping and therapeutic drug monitoring [3-7]. Many advantages have been associated with this minimally invasive sampling technique, amongst which easy transport and storage of the samples, simplification of sample preparation procedures and automation of sample processing. Moreover, the dried matrix may improve compound stability and reduce the risk of infection [3,8-11]. Other biological matrices have also been collected as a spot on (cellulose) filter paper, e.g. urine and plasma. The latter has already been used for the quantification of gabapentin [12,13]. However, these dried plasma spots were generated by pipetting plasma which was prepared by centrifugation of whole blood, obtained via venipuncture. Consequently, these methods are less feasible for sampling at home. Fingerprick sampling is a more convenient technique in a real-life setting and can be performed by non-experienced individuals. To the best of our knowledge, we are the first to describe the application of a procedure to quantify gabapentin in DBS obtained by fingerprick sampling. In this method, we applied gas chromatography coupled to mass spectrometry (GC-MS), following “microwave-assisted on-spot derivatization”. This new derivatization strategy simplifies and minimizes sample preparation by combining fast microwave-assisted derivatization with direct “on-spot derivatization”. Additionally, no dedicated equipment is needed since a standard GC-MS configuration, present in most routine toxicological and clinical laboratories, is used. We also opted for a GC-MS system since some laboratories in emerging countries cannot afford buying or maintaining expensive LC-MS equipment whereas they may have a GC-MS system at their disposal.

When setting up a DBS-based method, several parameters may complicate the interpretation of DBS results. Besides analytical issues like the impact of hematocrit (Hct) on DBS-based quantitation, an important factor that needs to be taken into consideration is the fact that therapeutic ranges for drugs are mostly based on plasma and serum concentrations. Consequently, a conversion factor is needed for the adequate interpretation of these blood concentrations, and thus also of gabapentin. Furthermore, for the acceptance and implementation of capillary DBS (cDBS) as an alternative (minimally invasive) sampling strategy in a routine laboratory, the execution of bridging studies is essential [10,14-16]. Bridging studies are studies in which concentrations determined in DBS are compared to those obtained in a reference matrix, e.g. serum. To this end, we set up a comparative study to investigate how serum and blood (DBS) concentrations are correlated.

4.2 Experimental

4.2.1 Chemical reagents and stock solutions

Gabapentin and its deuterated analogue gabapentin-D10 (available as a 100 µg/mL solution in methanol) were purchased from Sigma-Aldrich (Diegem, Belgium), as well as the derivatization reagents acetic anhydride, pyridine and heptafluorobutanol. Suprasolve ethyl acetate was delivered by Merck (Darmstadt, Germany).

A 10 mg/mL gabapentin stock solution in methanol was used to prepare calibration standards of 1, 5, 10, 17.5, 22.5 and 30 µg/mL in venous whole blood. DBS were prepared by spotting 25 µL of this spiked blood onto Whatman 903 filter paper (GE Healthcare, Dassel, Germany). Quality control samples (QCs) were prepared in a similar way from an independent stock solution.

4.2.2 Study set-up and sample collection

A comparative study was set up to determine gabapentin concentrations in both cDBS and serum samples. This study was approved by the Ethics Committee of Ghent University Hospital (NCT01821235). Samples from 15 healthy volunteers aged between 18 and 55 years and with a Hct between 0.37 and 0.48 were included. These subjects received either a single oral dose of 800 mg Neurontin® (Pfizer, Freiburg, Germany) or 800 mg Gabasandoz® (Salutas Pharma GmbH, Barleben, Germany). Patients were hospitalized up to 12 hours after administration, followed by a visit to the clinical research unit 24 hours after dosing. Prior to sampling, all volunteers provided informed consent. At 7 time points (predose, 2, 4, 6, 8, 12 and 24 hours postdose), paired serum samples and cDBS were collected. cDBS were generated by (non-volumetric) application of a drop of blood onto Whatman 903 filter paper following a fingerprick with an automated lancet. These spots were then dried for at least 2 hours before storage at room temperature in a plastic bag with desiccant until analysis. Assistance was provided during DBS sampling to ensure correct sampling (e.g. the first drop of blood was wiped off (because of the presence of tissue fluid), only one drop of blood was used for every spot and direct contact between the fingertip and the DBS card was avoided). Furthermore, some volunteers were drowsy after gabapentin administration and were not in a condition to prepare DBS themselves. At the same time, blood was taken via a cannula and serum was prepared within 1 hour after blood sampling. Serum samples were stored at -80°C and quantified in the Department of Clinical Pharmacology and Pharmacy of the VU University Medical Center (Amsterdam, The Netherlands) according to a validated UPLC-MS/MS method [17].

4.2.3 DBS analysis

The DBS were analyzed utilizing the validated GC-MS procedure described in Chapter 3 [18]. This method can be summarized as follows: first, a central 6-mm disc was taken from the dried spot. Then, after adding the internal standard (5 µL of 12 µg/mL gabapentin-D10), the punches were subjected to “microwave-assisted on-

spot derivatization" by direct application of 25 µL acetic anhydride and 25 µL pyridine and microwave heating at maximum power for 90 s, followed by a second microwave-assisted derivatization for 90 s with 25 µL heptafluorobutanol. After every derivatization step, the samples were subjected to a short-spin centrifugation step at 4°C. Following evaporation under nitrogen at 25°C, 100 µL ethyl acetate was added to the samples. Finally, one µL of this derivatized extract was injected into an Agilent 6890 GC system coupled to a 5973 MS (Agilent Technologies, Avondale, PA, USA). Chromatographic separation was achieved on a 30 m x 0.25 mm i.d. x 0.25 µm Agilent HP-5MS column. The injection temperature was set at 250°C, and a splitless injection was performed with a purge activation time of 120 s. The initial oven temperature was set at 60°C, which was held for 2 minutes. Then, the temperature was ramped at 8°C/min to 110°C, raised 30°C/min until 230°C, followed by an increase of 50°C/min to 300°C, which was held for 2 minutes. High-purity helium was used as the carrier gas with a constant flow rate of 1.1 mL/min. The transfer line temperature, ion source temperature and MS quadrupole temperature were set at 300, 230 and 150°C, respectively. Quantification of gabapentin and gabapentin-D10 was performed in SIM mode using *m/z* 153, 167 and <u>195</u> for derivatized gabapentin and 163, 177 and <u>205</u> for derivatized gabapentin-D10. Quantifier ions are underscored.

This method was validated based upon the Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation as described in subsection 3.2.6.2 [19,20]. Calibration lines were linear between 1 and 30 µg/mL, applying 1/x weighted linear regression. Within-day and between-day precision (% RSD) and accuracy (% bias), evaluated at 4 different QC levels (LLOQ, low, medium and high level), were below 11, 12 and 8%, respectively, thereby meeting pre-set acceptance criteria. No interferences or carry-over were observed. Gabapentin was found to be stable in the DBS stored for at least 84 days at room temperature. Matrix effects and recovery could not be assessed, given the nature of the utilized approach ("on-spot derivatization"). The use of a stable labeled internal standard prior to derivatization allows to compensate for variations that may occur throughout the procedure. DBS-specific parameters may also have an impact on the analytical DBS result when starting from partial-spot punches [9]. Therefore, the impact of the Hct in a range which covers the Hct values of our study population was evaluated. Although a Hct effect was observed in the 0.30-0.49 Hct range, % bias remained within ± 15% of the concentrations of the normalized sample for the low QC level, a concentration close to the concentrations measured in the participants of this study. When evaluating the impact of different spotted blood volumes (i.e. 25, 35 *vs.* 50 µL), increased concentrations with increasing blood volumes were observed. The influence of even smaller blood volumes was not evaluated as these spots are too small to obtain a 6-mm punch [18]. For the evaluation of incurred sample reanalysis, 66 cDBS were re-analyzed after a storage of 141 days at room temperature. The EMA criterion for incurred sample reanalysis, which states that the percent difference between the initial concentration and the concentration measured during the repeat analysis should not be greater than 20% of their mean for at least 67% of the repeats, was fulfilled as for 80% of the re-analyzed cDBS, the initial analysis and the concentration obtained by reanalysis were within 20% of their mean.

4.2.4 Data analysis

To evaluate the correlation between gabapentin concentrations in different biological matrices, a paired sample T-test was performed using Microsoft Excel® 2010 (Microsoft, Redmond, WA, USA). MedCalc® (MedCalc software bvba, Ostend, Belgium) was used to calculate the Pearson's correlation coefficient (with p-value), and to perform Passing-Bablok and Bland-Altman analyses.

The correlation coefficient measures the strength and direction of a relationship between two variables. A p-value ≤ 0.05 is considered statistically significant. Passing-Bablok and Bland-Altman analyses were performed to evaluate the correlation between the gabapentin concentrations in both matrices (i.e. blood *vs*. serum concentrations).

4.3 Results and discussion

Of the 105 paired samples, 38 cDBS were excluded: 3 cDBS were too small (< 6-mm diameter), while the other spots were excluded because their gabapentin concentration was below the LLOQ (i.e. < 1 µg/mL). A detailed overview of the obtained DBS concentrations is given in Table 4.1.

Table 4.1 Overview of the DBS concentrations at different time points.

	Pre-dose	**2 h post-dose**	**4 h post-dose**	**6 h post-dose**	**8 h post-dose**	**12 h post-dose**	**24 h post-dose**
Number of samples included	0	14	15	13	15	10	0
Number of samples excluded:							
< LLOQ	14	0	0	1	0	5	15
< 6-mm	1	1	0	1	0	0	0
Concentration range (µg/mL)	/	2.30-6.10	1.84-5.52	1.92-4.19	1.52-4.38	1.49-2.97	/
Median concentration (µg/mL)	/	3.50	3.49	3.02	2.38	1.88	/

When plotting the DBS *vs.* the serum concentrations (Figure 4.1), the blood (DBS) concentrations were found to be significantly lower than the serum concentrations ($p < 0.001$). This was in line with our expectation: gabapentin has been reported to freely enter red blood cells without binding to cellular or plasma proteins. Consequently, an equal blood-to-plasma ratio is expected [21]. However, when taking into account the fraction of the solid constituents in blood, the blood concentration is expected to lie approximately 15% lower than the plasma/serum concentrations [7,22,23]. We indeed found a mean blood-to-serum ratio of 0.85 ± 0.12, meaning that concentrations in DBS are about 85% of those found in serum. Consequently, serum concentrations can be calculated from DBS concentrations as follows: [serum] = [DBS]/0.85.

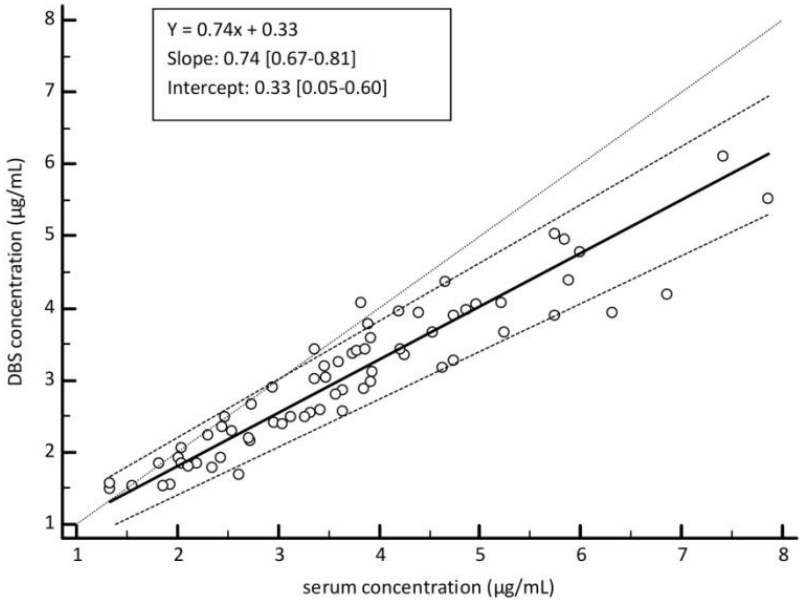


Figure 4.1 Passing-Bablok regression analysis plotting the DBS concentrations against the serum concentrations. The slope and intercept of the regression line (solid line) are calculated with their 95% confidence interval (dashed line). The dotted line corresponds to the identity line.

Figure 4.2 shows a Passing-Bablok scatter plot in which the serum concentrations calculated from DBS analysis are plotted *vs.* measured serum concentrations. A good overall correlation was observed, although 1 was just not included in the 95% confidence interval of the slope. Bland-Altman analysis revealed a mean difference of only 0.12 µg/mL, with the 0 value included in the 95% confidence interval (Figure 4.3). This observation was confirmed by the Pearson's correlation coefficient ($r = 0.9353$; $p < 0.0001$), demonstrating a strong correlation between the measured serum concentrations and the serum concentrations based on DBS results.

Importantly, these differences may also be dependent on the sampling time, as exemplified for acetaminophen by Mohammed *et al.* [24]. We therefore plotted the % difference between the calculated and measured serum concentrations *vs.* the sampling time (Figure 4.4). This revealed that the differences between both concentrations were randomly distributed around 0, with no indication of a time-dependent effect.

Another important factor that needs to be kept in mind is that the serum samples were obtained following traditional blood sampling by venipuncture, whereas the blood samples were collected by a capillary sampling technique using an automated lancet. cDBS-blood differences have been described for e.g. caffeine, acetaminophen, artemesinin, ethanol, lignocaine,… [7,24-26]. Although the set-up of this study did not really intend to compare venous and capillary blood concentrations, the data do suggest that -given the good correlation between the measured serum concentrations (obtained by venous sampling) and the calculated serum concentrations (from DBS obtained by capillary sampling)- there are no considerable capillary-venous differences.

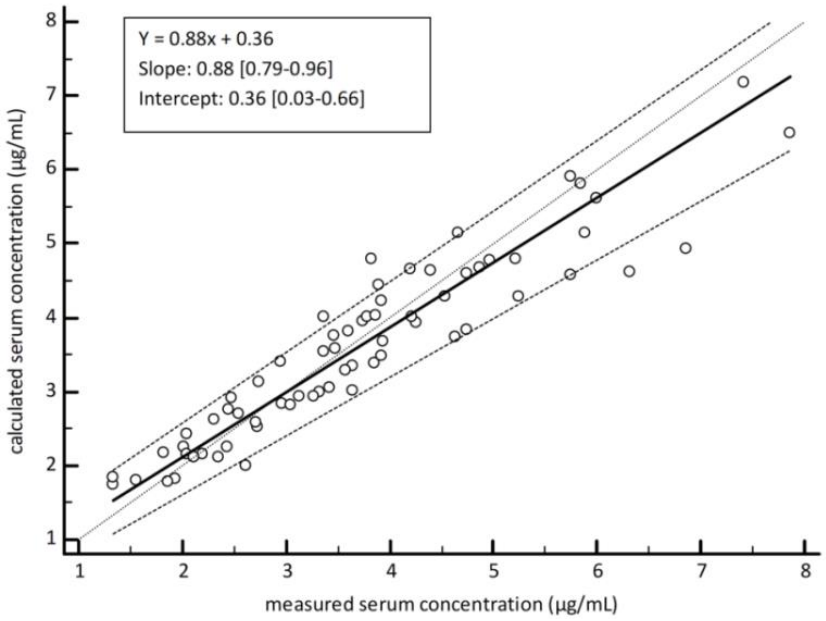


Figure 4.2 Passing-Bablok regression analysis plotting the serum concentrations calculated from the blood concentrations against the measured serum concentrations. The slope and intercept of the regression line (solid line) are calculated with their 95% confidence interval (dashed line). The dotted line corresponds to the identity line.

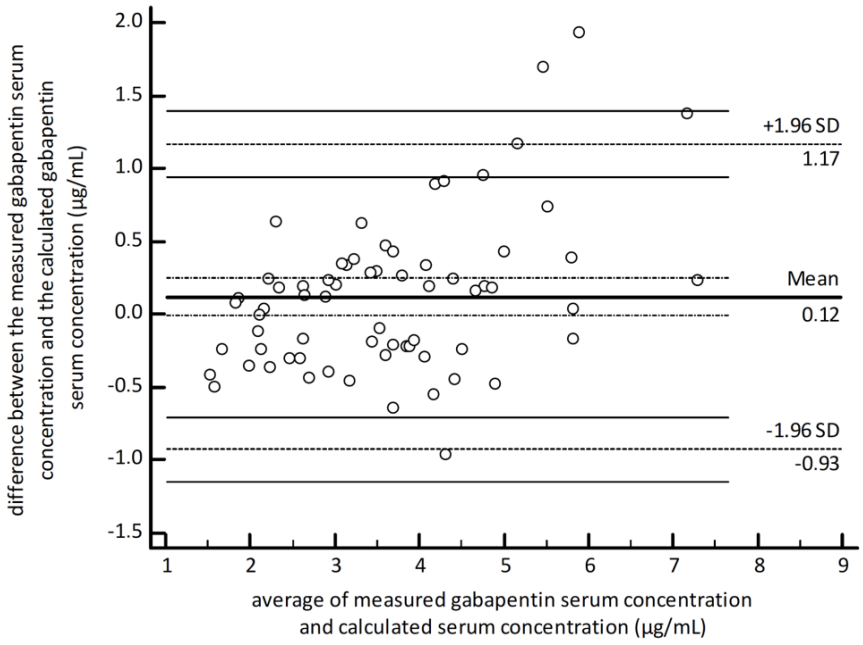


Figure 4.3 Bland-Altman analysis plotting the differences between the serum concentrations calculated from the blood concentrations and the measured concentrations (y-axis) against the average of both measurements (x-axis). The mean difference and the limits of agreements (set to 1.96 SD) are also indicated with its 95% confidence interval.

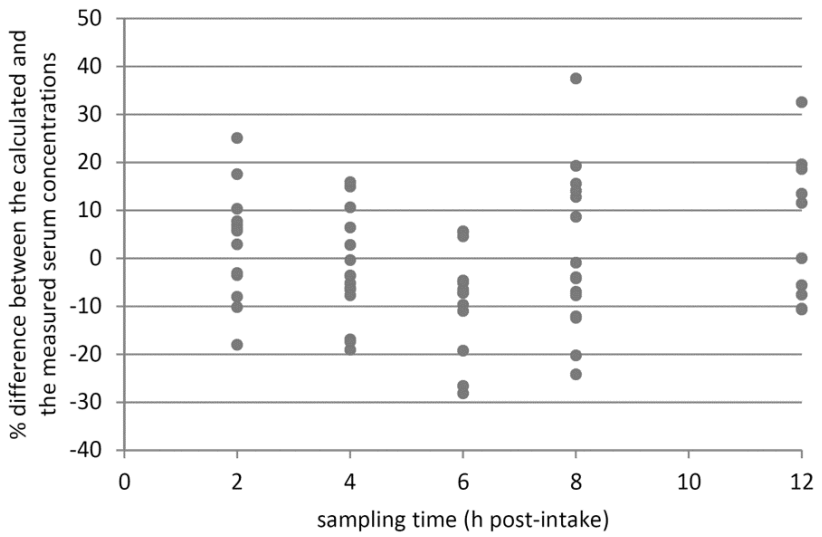


Figure 4.4 % difference between the calculated and measured serum concentrations *vs.* the time of sampling.

As described in subsection 4.2.3, the EMA guidelines for incurred sample reanalysis were fulfilled [20]. Although these criteria are actually intended for reanalysis of the same samples, this guideline was also applied for the evaluation of the measured and the calculated serum concentrations. Again, this criterion was fulfilled as for more than two-thirds (i.e. 87%) of the samples the two results lay within 20% of their mean. This requirement was also fulfilled when comparing serum concentrations obtained with both the LC- and the GC-method, excluding that the observed differences between blood and serum concentrations are caused by a bias between both methods.

4.4 Conclusion

We successfully applied a validated GC-MS method for the determination of gabapentin in DBS in a real-life setting. Based upon the observation that a mean blood-to-serum ratio of 0.85 was obtained, we established a conversion factor to allow interpretation of gabapentin blood concentrations since reference intervals concerning therapeutic and toxic gabapentin concentrations are only available in plasma/serum. Taking this conversion factor into consideration, serum concentrations could be calculated from DBS concentrations. By doing so, a good correlation was found between measured serum concentrations and serum concentrations calculated from DBS concentrations. Additionally, our data suggest that there is a good correlation between capillary and venous gabapentin concentrations.

4.5 References

1. Daly M. Down a stony road: the 2014 drugscope street drug survey.http://www.drugsandalcohol.ie/23282/1/DownAStonyRoadDrugTrendsSurvey2014.pdf
2. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborns infants. *Pediatrics* 32(3), 338-343 (1963).
3. Demirev PA. Dried blood spots: analysis and applications. *Anal Chem* 85(2), 779-789 (2013).
4. Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Ther Drug Monit* 31(3), 327-336 (2009).
5. Li W, Tse FL. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed Chromatogr* 24(1), 49-65 (2010).
6. Stove CP, Ingels AS, De Kesel PM, Lambert WE. Dried blood spots in toxicology: from the cradle to the grave? *Crit Rev Toxicol* 42(3), 230-243 (2012).
7. De Kesel PM, Lambert WE, Stove CP. Why dried blood spots are an ideal tool for CYP1A2 phenotyping. *Clin Pharmacokinet* 53(8), 763-771 (2014).
8. Ingels AS, Hertegonne KB, Lambert WE, Stove CP. Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home: an exploratory study. *CNS Drugs* 27(3), 233-237 (2013).
9. De Kesel PMM, Sadones N, Capiau S, Lambert WE, Stove CP. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis* 5(16), 2023-2041 (2013).
10. Sadones N, Capiau S, De Kesel PM, Lambert WE, Stove CP. Spot them in the spot: analysis of abused substances using dried blood spots. *Bioanalysis* 6(17), 2211-2227 (2014).
11. Stove C, Spooner N. DBS and beyond. *Bioanalysis* 7(16), 1961-1962 (2015).
12. Kolocouri F, Dotsikas Y, Loukas YL. Dried plasma spots as an alternative sample collection technique for the quantitative LC-MS/MS determination of gabapentin. *Anal Bioanal Chem* 398(3), 1339-1347 (2010).
13. Ikeda K, Ikawa K, Yokoshige S, Yoshikawa S, Morikawa N. Gas chromatography-electron ionization-mass spectrometry quantitation of valproic acid and gabapentin, using dried plasma spots, for therapeutic drug monitoring in in-home medical care. *Biomed Chromatogr* 28(12), 1756-1762 (2014).
14. Jager NG, Rosing H, Schellens JH, Beijnen JH, Linn SC. Use of dried blood spots for the determination of serum concentrations of tamoxifen and endoxifen. *Breast Cancer Res Treat* 146(1), 137-144 (2014).
15. Patteet L, Maudens KE, Stove CP *et al.* Are capillary DBS applicable for therapeutic drug monitoring of common antipsychotics? A proof of concept. *Bioanalysis* 7(16), 2119-2130 (2015).
16. Berm EJ, Odigie B, Bijlsma MJ, Wilffert B, Touw DJ, Maring JG. A clinical validation study for application of DBS in therapeutic drug monitoring of antidepressants. *Bioanalysis* 8(5), 413-424 (2016).
17. Chahbouni A, Sinjewel A, den Burger JCG *et al.* Rapid quantification of gabapentin, pregabalin, and vigabatrin in human serum by ultraperformance liquid chromatography with mass-spectrometric detection. *Ther Drug Monit* 35(1), 48-53 (2013).

18. Sadones N, Van Bever E, Archer JRH *et al.* Microwave-assisted on-spot derivatization for gas chromatography-mass spectrometry based determination of polar low molecular weight compounds in dried blood spots. *J Chromatogr A* 1465, 175-183 (2016).

19. U.S. Department of Health and Humans Services. Food and Drug Administration. Center for Drug Evaluation and Research. Center for Veterinary Medicine. Draft Guidance for Industry. Bioanalytical Method Validation. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation /guidances/ucm368107.pdf

20. European Medicines Agency. Guideline on bioanalytical method validation. http://www.ema.europa.e u/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf

21. Baselt R. *Disposition of toxic drugs and chemicals in man (9th edition).* Biomedical Publications, California, (2011).

22. Rainey PM. Relation between serum and whole-blood ethanol concentrations. *Clin Chem* 39(11 Pt 1), 2288-2292 (1993).

23. Rowland M, Emmons GT. Use of dried blood spots in drug development: pharmacokinetic considerations. *AAPS J* 12(3), 290-293 (2010).

24. Mohammed BS, Cameron GA, Cameron L, Hawksworth GH, Helms PJ, McLay JS. Can finger-prick sampling replace venous sampling to determine the pharmacokinetic profile of oral paracetamol? *Br J Clin Pharmacol* 70(1), 52-56 (2010).

25. Gordi T, Hai TN, Hoai NM, Thyberg M, Ashton M. Use of saliva and capillary blood samples as substitutes for venous blood sampling in pharmacokinetic investigations of artemisinin. *Eur J Clin Pharmacol* 56(8), 561-566 (2000).

26. Chiou WL. The phenomenon and rationale of marked dependence of drug concentration on blood sampling site. Implications in pharmacokinetics, pharmacodynamics, toxicology and therapeutics (Part I). *Clin Pharmacokinet* 17(3), 175-199 (1989).

CHAPTER 5:
RELEVANCE OF BHB ANALYSIS IN FORENSIC TOXICOLOGY

Based on

Sadones N, Lambert WE, Stove CP. The (non)sense of routinely analyzing beta-hydroxybutyric acid in forensic toxicology casework. *Forensic Sci Int* doi: 10.1016/j.forsciint.2017.01.002 (2017).

Abstract

Beta-hydroxybutyric acid (BHB) is a ketone body which is generated from fatty acids as an alternative energy source when glucose is not available. Determination of this compound may be relevant in the forensic laboratory as ketoacidosis -an elevated level of ketone bodies- may contribute to the cause of death. In this study, we aimed at determining the relevance of routinely implementing BHB analysis in the forensic toxicological laboratory, as BHB analysis typically requires an additional workload. We therefore performed an unbiased retrospective analysis of BHB in 599 cases, comprising 553 blood, 232 urine and 62 vitreous humour samples. Cases with BHB concentrations above 100 µg/mL (in blood, urine and/or vitreous humour) were invariably associated with elevated levels of acetone, another ketone body, the detection of which is already implemented in most forensic laboratories using the gas chromatographic procedure for ethanol quantification. Our retrospective analysis did not reveal any positive case that had been missed initially and confirms that BHB analysis can be limited to acetone positive cases.

5.1 Introduction

Ketoacidosis is a metabolic disturbance caused by an elevated blood level of the ketone bodies acetoacetate, acetone and beta-hydroxybutyric acid (BHB). These compounds are generated by the liver when there is insufficient glucose available. Under these circumstances, lipids -stored as triglycerides in fatty tissue- are an alternative energy source. During lipolysis, triglycerides are degraded into fatty acids, resulting in the generation of acetyl-CoA. This co-enzyme is converted into acetoacetate, a precursor molecule of BHB and acetone. The latter compound is generated by decarboxylation of acetoacetate and is eliminated by urine and breath, resulting in a typical sweet odor of breath and urine. Since acetone may be reduced to isopropanol, the latter may also be found in case of ketoacidosis. On the other hand, acetoacetate may also be converted enzymatically into BHB. This small low molecular weight compound is readily water-soluble and can easily cross the blood brain barrier. There, BHB is converted into acetyl-CoA, which is *via* the Krebs cycle converted to ATP, an energy-carrying molecule. Consequently, high levels of ketone bodies indicate deficient sugar metabolism which may be caused by uncontrolled diabetes. The use of atypical antipsychotics may also induce ketoacidosis. Other sources of ketoacidosis are chronic alcoholism, starvation, hypothermia or infection [1]. In conclusion, determination of ketone bodies is relevant in a forensic context as they may elucidate pathological findings or the cause of death. Yet, there remains a lot of uncertainty about the interpretation of (concentrations of) ketone bodies. E.g. it is unclear if ketone bodies (i.e. acetone, acetoacetate and/or BHB) should routinely be measured in all cases or if measurement can be limited to some specific cases. It is also unclear if all ketone bodies should be measured or if the determination of one ketone body suffices. Up till now, acetone is already co-detected routinely in blood and/or urine using a standard ethanol quantification procedure via headspace sampling-gas chromatography-flame ionization detection (HS-GC-FID). Even a simple dipstick test readily sheds some light on the extent of positivity of acetone and acetoacetate in urine. On the other hand, BHB analysis is not performed routinely. Mostly, BHB is determined when there is a suspicion of ketoacidosis, based on background information. However, sometimes there is no access to background information. Additionally, the presence of alcoholism and/or diabetes -two important factors causing ketoacidosis- is not always known. There is also some controversy about the preferred biological matrix. E.g. can vitreous humour be considered as an alternative for blood?

In this Chapter, we evaluate the relevance of routine implementation of BHB analysis in a forensic toxicological laboratory, taking into account that a dedicated analysis is required for the quantification of this ketone body, as this analyte is typically not covered by other routinely applied liquid chromatography (LC) or GC-based general screening procedures. To this end, we applied a recently developed dried spot-based method to perform retrospective BHB analysis of a large number of samples which were received for forensic toxicological analysis. Noteworthy is that in this retrospective analysis we did not select samples based upon background information (e.g. known diabetic or alcoholic) and earlier obtained toxicological results.

5.2 Materials and methods

5.2.1 Chemicals and stock solutions

Heptafluorobutanol, acetic anhydride, pyridine and the sodium salt of BHB were provided by Sigma-Aldrich (Diegem, Belgium). Suprasolve ethyl acetate and methanol were obtained from Merck (Darmstadt, Germany). Deuterated GHB (GHB-D6) was used as internal standard (IS) and provided by Lipomed (Arlesheim, Switzerland).

A BHB stock solution was prepared by dissolving 50 mg BHB in one mL of methanol. This stock solution was diluted and used to prepare calibration standards of 5, 15, 25, 50, 100 and 300 µg/mL. Quality control samples (QCs) of 65, 125 and 250 µg/mL were prepared from an independent stock solution. For the IS, we made a 50 µg/mL GHB-D6 solution in methanol. All solutions were stored at -20°C.

5.2.2 Samples

We performed retrospective BHB analysis of routine samples -mostly post-mortem samples- which were received for forensic toxicological analysis in the Laboratory of Toxicology of Ghent University and stored at -20°C. Since BHB has been found to be stable in post-mortem samples [2], the age of the samples was not an issue. All available samples were analyzed, irrespective of background information and earlier obtained (toxicological) results. Finally, we had access to 599 cases, comprising 553 blood samples, 232 urine samples and 62 vitreous humour samples. Of these samples, dried matrix spots were prepared by pipetting 15 µL of biofluid onto a 7-mm pre-punched disc of Whatman 903 filter paper. These spots were dried for at least 2 hours and stored at room temperature in a zip-closure plastic bag with desiccant before analysis. Acetone and isopropanol were qualitatively assessed in the procedure for routine ethanol quantification (ISO/IEC 17025 accredited), applied at the day of arrival using HS-GC-FID.

5.2.3 BHB analysis

For the quantification of BHB, the GC coupled to mass spectrometry (GC-MS) method described in Chapter 3 was slightly adapted [3]. This adaptation consisted of using 7-mm pre-punched discs, onto which a fixed volume of 15 µL biofluid was applied, rather than using 6-mm partial punches from a dried blood spot (DBS). The protocol used can be summarized as follows: first, a fixed volume of 15 µL of blood, urine or vitreous humour is spotted onto a 7-mm pre-punched disc of Whatman 903 filter paper. After adding 5 µL IS (50 µg/mL GHB-D6 in methanol) to the dried spots, the punches are subjected to “microwave-assisted on-spot derivatization”, as described in subsection 3.3.1.2 [3]. Briefly, derivatization is achieved by direct application of 25 µL acetic anhydride and 25 µL pyridine onto the spots and microwave heating for 90 s at maximum power, followed by a second derivatization for 90 s at maximum power with 25 µL heptafluorobutanol. Following

evaporation under nitrogen, 100 µL of ethyl acetate was added to the samples. Finally, one µL of the derivatized extract is injected into an Agilent 6890 GC coupled to a 5973 MS system. Chromatographic separation is achieved on a 30 m x 0.25 mm i.d. x 0.25 µm Agilent HP-5MS column. Helium is used as carrier gas with a flow rate of 1.1 mL/min. A splitless injection is chosen. The injection temperature is set at 250°C. The initial oven temperature is 60°C, which is held for 2 minutes. Then, the temperature ramped at 8°C/min to 110°C, raised 30°C/min until 230°C, followed by an increase of 50°C/min to 300°C, which is held for 2 minutes. The transfer line temperature, MS ion source temperature and MS quadrupole temperature are set at 300, 230 and 150°C, respectively. Quantification of BHB and GHB-D6 is performed in SIM mode using *m/z* 227, 268 and 285 for BHB and 231, 245, 273 and 291 for GHB-D6. Quantifier ions are underscored.

Validation of the procedure starting from a 6-mm disc of a 25-µL DBS prepared from fresh whole blood [3] encompassed the following (see subsections 3.3.3.2, 3.3.3.3 and 3.3.3.4): calibration lines were linear over the 5-300 µg/mL concentration range, applying a 1/x weighted linear regression. Within-day and between-day precision criteria (< 20% at LLOQ, < 15% at 3 other QC levels) were fulfilled for all QC levels, whereas accuracy was slightly above the acceptance criterion for two QC levels, i.e. > 15% but still below 20%. No carry-over was observed following injection of the highest concentration of the calibration curve. Samples could be diluted 4-fold without influencing precision and accuracy. Stability studies revealed no significant alteration of BHB concentration in DBS which were stored in a zip-closure plastic bag with desiccant for 84 days at room temperature [3].

5.3 Results and discussion

BHB analysis was based on the GC-MS method for the detection of several polar low molecular weight compounds with relevance in forensic and clinical toxicology described in Chapter 3 [3]. This validated procedure (with punching a 6-mm disc from a 25-µL DBS) was slightly modified in our application as we wished to apply the method on a divergent set of matrices (urine, vitreous humour, post-mortem blood,…) which have a different spreading on the filter paper. To this end, a whole-cut approach with application of a fixed volume of 15 µL of biofluid onto a 7-mm pre-punched disc was utilized instead of using partial punches. To ensure the validity of the slightly adapted method we applied, a calibration line and QC samples were run every day of analysis. % CV was below 20% and % bias below 13% for 3 QC levels (65, 125 and 250 µg/mL). These QC concentrations were based on proposed cut-off levels (see next paragraph).

The BHB concentrations obtained in blood, vitreous humour and urine were arbitrarily grouped in different classes (Figure 5.1): BHB concentrations below 50 µg/mL were considered as ‘low’, concentrations between 50 and 100 µg/mL as ‘slightly elevated’, concentrations between 100 and 250 µg/mL as ‘moderately elevated’, and concentrations above 250 µg/L as ‘high and pathologically significant’. Although this classification is rather based on decision levels for BHB in blood and vitreous humour, we also applied these ranges for urine, as suggested by Elliott *et al.* [4].

As can be seen in Figure 5.1a, a 'slightly or moderately elevated' BHB blood level was found in 28 cases (5%) and a 'high' BHB level in 5 (0.9%) blood samples. Most vitreous humour samples (89%) also had a 'low' BHB concentration (Figure 5.1b). There were no vitreous humour samples with a BHB concentration higher than 250 µg/mL, while 7 (11%) had a 'slightly or moderately elevated' BHB concentration. Of the 232 analyzed urine samples, 8 (3.5%) had a 'slightly or moderately elevated' urinary BHB concentration and 6 (2.5%) had a urinary concentration above 250 µg/mL (Figure 5.1c). The slightly higher percentage of vitreous humour samples with an elevated BHB concentration may be owing to the fact that, while vitreous humour is not always routinely collected by the forensic pathologist, it is likely to be sampled if there is a suspicion of an electrolyte imbalance or ketoacidosis. Despite this inherent bias during sample collection, only three cases in which we could perform the retrospective BHB analysis in vitreous humour yielded a concentration near or above 100 µg/mL. For these samples, the sum of glucose and lactate, as a measure of antemortem glycemia [5], was within the normal range.

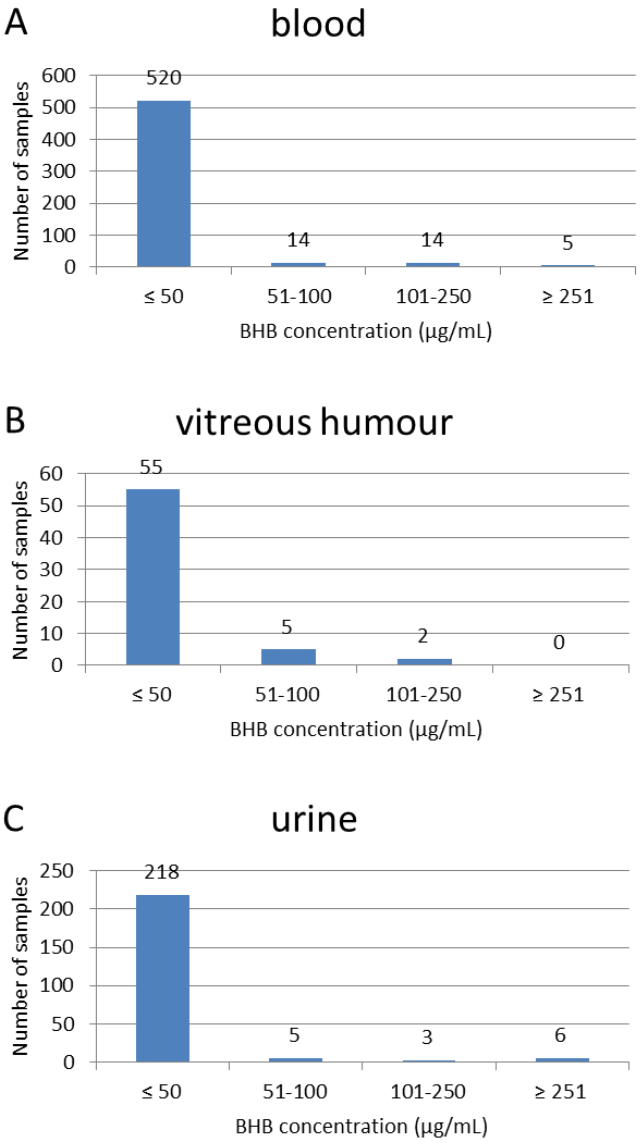


Figure 5.1 BHB concentrations obtained in (a) blood, (b) vitreous humour and (c) urine, respectively.

Table 5.1 gives an overview of the cases with a BHB concentration above 100 µg/mL (25 cases, numbered from 1 to 25, encompassing samples considered as 'moderately elevated' and 'high and pathologically significant') in blood, vitreous and/or urine. Cases with the highest BHB blood levels are first reported, followed by the cases with the highest BHB urine concentrations. Semi-quantitative acetone results as well as some additional information which may be relevant for the interpretation are also given for each case.

Table 5.1 Overview of the cases with a BHB concentration above 100 µg/mL.

Case n°	BHB concentration (µg/mL)			Acetone		Remarks
	Blood	Vitreous	Urine	Blood	Urine	
1	1466	NA	NA	+++	NA	Post-mortem Diabetic; known alcohol abuse [Lactate + glucose] = 990 mg/dL in vitreous humour
2	848	NA	NA	++	NA	Post-mortem Diabetic; known alcohol abuse
3	600	NA	NA	++	NA	Post-mortem Alcoholic
4	306	NA	NA	++	NA	Non-fatal
5	282	NA	NA	++	NA	Non-fatal
6	247	NA	NA	++	NA	Post-mortem Alcoholic
7	206	NA	NA	+	NA	Post-mortem Neurotrauma; lactic acidosis
8	185	NA	NA	+	NA	Non-fatal Olanzapine
9	177	NA	NA	++	NA	Post-mortem Anorexia
10	171	NA	1194	+	+++	Non-fatal
11	168	NA	NA	NA	NA	Non-fatal Quetiapine
12	153	157	1074	+	+++	Post-mortem Alcohol intoxication [Lactate + glucose] = 292 mg/dL in vitreous humour
13	131	NA	NA	+	NA	Non-fatal Morphine intoxication
14	129	NA	NA	+	NA	Non-fatal
15	117	NA	1053	+	+++	Post-mortem Meningitis
16	110	NA	<LLOQ	NA	+	Post-mortem Quetiapine
17	109	NA	NA	+	NA	Non-fatal
18	103	94	NA	+	NA	Post-mortem Alcoholic [Lactate + glucose] = 220 mg/dL in vitreous humour
19	102	NA	29	+	+	Post-mortem Suicide: zopiclone + alprazolam + alcohol

NA: not analyzed/not available; +++: acetone concentration > 20 mg/dL;
++: acetone concentration > 3 mg/dL; +: acetone concentration < 3 mg/dL

Table 5.1 Continued.

Case n°	BHB concentration (µg/mL)			Acetone		Remarks
	Blood	Vitreous	Urine	Blood	Urine	
20	13	NA	347	+	+++	Non-fatal
21	80	191	333	++	++	Post-mortem [Lactate + glucose] = 194 mg/dL in vitreous humour
22	14	NA	285	+	++	Post-mortem
23	55	NA	136	+	+	Non-fatal Methadon intoxication (coma); Hyperglycemia (prolonged stress)
24	93	NA	119	+	+	Post-mortem
25	22	NA	110	+	++	Post-mortem (suicide)

NA: not analyzed/not available; +++: acetone concentration > 20 mg/dL;
++: acetone concentration > 3 mg/dL; +: acetone concentration < 3 mg/dL

Of the 553 analyzed blood samples, five had a 'high and pathologically significant' BHB blood concentration (i.e. > 250 µg/mL). Two of these were non-fatal cases (cases n° 4 and 5) and three were fatalities, the latter encompassing one known alcoholic (case n° 3) and two diabetics with a known alcohol abuse (cases n° 1 and 2) (see below).

The highest BHB concentration (i.e. 1466 µg/mL) was found in blood from a diabetic with known alcohol abuse (case n° 1). Also in the headspace chromatogram, a very pronounced peak was observed for acetone in blood. Semi-quantitative analysis revealed an acetone concentration higher than 20 mg/dL. Vitreous humour analysis had revealed a pronounced hyperglycemia ([glucose + lactate] = 990 mg/dL) and confirmed the diagnosis of diabetic ketoacidosis. Unfortunately, there was no vitreous humour left for this retrospective BHB study.

At least three fatalities could be associated with alcoholic ketoacidosis (case n° 2 with a BHB blood concentration of 848 µg/mL, case n° 3 with a BHB blood concentration of 600 µg/mL and case n° 6 with a BHB blood concentration of 247 µg/mL, i.e. only marginally below the 250 µg/mL cut-off). Not only a 'high and pathologically significant' BHB blood concentration, but also a clear acetone peak, corresponding with an acetone concentration between 3 and 20 mg/dL, was observed in blood of these three alcoholics who had suddenly died. In case n° 2, ethanol and isopropanol were detected as well. Unfortunately, also here, no vitreous humour was available for BHB, glucose and lactate determination. Cases n° 4 and 5 clearly illustrate that the interpretation of BHB concentrations may not always be straightforward in forensic cases. In these cases 'high and pathologically significant' BHB concentrations were found, although these were not associated with fatalities but were linked to a theft and a drug control, respectively. Whether in these cases the ketoacidosis was stress-related or whether these individuals were diabetics is not known (no availability of background information or urine for e.g. glucose testing).

In the 14 cases with a BHB blood concentration between 100 and 250 µg/mL, there were eight post-mortem samples. In four out of these post-mortem cases an alternative cause of death (i.e. suicide (case n° 19), neurotrauma (case n° 7), meningitis (case n° 15) or acute alcohol intoxication (case n° 12)) was found, whereas two samples were from known alcoholics who had suddenly died (cases n° 6 (described in the previous paragraph) and 18). In case n° 18, BHB concentrations were moderately elevated in blood and vitreous humour and, also here, acetone was detected, albeit at a lower concentration than in the cases with the highest BHB concentrations. In this case, the available results do not allow to firmly conclude that this is a fatal alcoholic ketoacidosis. Ketoacidosis may be a contributing factor to the cause of death, but since no autopsy has been performed, we could not conclusively determine the cause of death. Also in cases n° 9 and 16 the cause of death remained unexplained although malnutrition and the use of quetiapine (an atypical antipsychotic drug) may have contributed to the increased BHB levels in these cases. Two out of the six non-fatal cases could be associated with the use of atypical antipsychotics (cases n° 8 and 11). The other non-fatal cases were related to a morphine intoxication (case n° 13), a theft (case n° 17), a fight (case n° 10) and an assassination attempt (case n° 14). Traces of isopropanol were found in cases n° 7, 9, 12 and 15, whereas acetone was detected in blood of all of the 14 cases with a BHB blood concentration between 100 and 250 µg/mL. Noteworthy is that, whereas in the cases with a BHB concentration between 100 and 250 µg/mL, acetone was invariably present, it was mostly only present to a limited extent in the blood. In contrast, a clear acetone peak (> 3 mg/dL) was observed in the blood from all cases with a BHB concentration above 250 µg/mL. Therefore, we consider acetone as a good initial marker to decide if BHB analysis should be performed. This finding is in line with the observation of Hockenhull *et al*. [6]. These authors reported the presence of acetone (> 2 mg/dL) in all cases with a 'significant' BHB concentration (> 250 µg/mL) and readily suggested that acetone analysis is a good screening procedure to identify alcoholic and diabetic ketoacidosis. Acetone analysis is preferred as screening procedure as this analyte is easily detected using a procedure (HS-GC-FID) which is already performed routinely in every case. However, as readily pointed out by Hockenhull *et al.* [6], acetone analysis is not enough to justify the contribution of ketoacidosis to the cause of death. In the acetone positive samples, the measurement of BHB is advised to obtain a complete metabolic overview and to exclude acetone and/or isopropanol intoxications. Furthermore, these results should ideally be combined with autopsy findings, other toxicological results, the person's background, as well as with the circumstances in which the deceased was found -if these data are available to the toxicologist. Since we analyzed all available forensic samples without taking into consideration any background information, our retrospective BHB study also included some non-fatal cases, in which there was no reason at all to expect elevated BHB levels. These cases demonstrate that in a forensic context 'high and pathologically significant' BHB concentrations and strongly elevated acetone concentrations are not always associated with fatalities and that other situations may also involve ketoacidosis.

We also agree with Hockenhull *et al.* [6] that acetone analysis should be performed in all cases and not only in those cases with a (suspected) history of alcoholism or diabetes. One should also pay attention to the presence of isopropanol, as this compound can be generated from acetone via alcohol dehydrogenase. However, the absence of isopropanol does not preclude ketoacidosis. If diabetic ketoacidosis is suspected, it is also

recommended to estimate antemortem hyperglycemia by biochemical analysis of vitreous for glucose and/or lactate. While vitreous glucose may be the most reliable marker to diagnose hyperglycemia in fresh corpses, the use of the sum of glucose and lactate (also known as the Traub formula) has also been used in cases where there is a prolonged interval between death and sampling (as in several of our cases) [7-9]. Indeed, in our experience, in many of these cases even no or only very low glucose remains detectable.

Teresinski *et al.* observed that elevated BHB levels are not always associated with elevated acetone concentrations [10]. In hypothermia cases, for instance, ketoacidosis appears to be characterized by less important levels of blood ketones. Both Palmiere *et al.* and Teresinski *et al.* observed an inverse relationship between blood acetone and ethanol levels in hypothermia cases [10-13]. Therefore, these authors questioned whether acetone may be a suitable marker for ketoacidosis in hypothermia cases with a high blood alcohol concentration. However, it is unclear to what extent ketoacidosis did play a contributing role to the cause of death in these hypothermia cases. Additionally, as we did not find any cases in our large-scale study with elevated BHB levels without elevated acetone concentrations, we believe that an additional BHB analysis should only be performed if acetone is detected.

Of the 54 cases in which corresponding blood and vitreous humour samples were available, vitreous humour BHB concentrations exceeded or approached 100 µg/mL in only 3 cases. As suggested by Elliott *et al.* [4], the same ranges can be used for vitreous humour as for blood. Although the number of vitreous humour samples was limited, our data do support an overall good accordance between BHB concentrations in blood and the corresponding vitreous humour. This is in line with the conclusions of Elliott *et al*. [4], Kadis *et al.* [14], Felby *et al.* [15], Osuna *et al.* [16] and Pounder *et al*. [17]. On the other hand, Teresinski *et al.* and Palmiere *et al.* argued that the vitreous humour concentration may not be a true reflection of the blood concentration since the equilibrium between both matrices may not have been attained in case of rapidly occurring ketonemia [10,18].

When comparing urinary and blood concentrations, we observed that the blood concentration was below 100 µg/mL for the three samples with a urinary concentration between 100 and 250 µg/mL (cases n° 23, 24 and 25). Also in the three cases with a urinary BHB concentration between 250 and 1000 µg/mL, the corresponding BHB blood concentration did not exceed 100 µg/mL (cases n° 20, 21 and 22). Only in the three cases with a very high urinary BHB concentration, i.e. above 1000 µg/mL (cases n° 10, 12 and 15), a blood concentration between 100 and 250 µg/mL was observed. Conclusively, unless in more extreme cases (urinary BHB above 1000 µg/mL), elevated or high urinary concentrations are not necessarily reflected by elevated or high blood concentrations. Conversely, elevated blood concentrations are neither always reflected by elevated urinary concentrations: in two out of the five cases with a BHB blood concentration higher than 100 µg/mL, the corresponding urinary BHB concentration was not elevated (cases n° 16 and 19). As already mentioned, the other three cases had a very high urinary concentration (> 1000 µg/mL) (cases n° 10, 12 and 15). These findings confirm the conclusion of Elliott *et al.* that a urinary BHB concentration cannot be solely used to diagnose pathologically significant ketoacidosis [4]. We also agree with Teresinski *et al.* and Palmiere *et al.* that the

interpretation of ketone body concentrations in urine is not straightforward and that blood is the most appropriate matrix for the determination of ketone bodies [10,18]. Not only in a forensic setting, but also in a clinical context (i.e. for the evaluation of the metabolic status of the patient in the treatment of diabetic ketoacidosis), blood levels are more reliable than urinary concentrations [19].

5.4 Conclusion

An elevated level of the ketone bodies is called ketoacidosis and may be a contributing factor to the cause of death. Therefore, determination of the ketone bodies may be helpful to explain pathological and/or toxicological findings. Qualitative acetone analysis is already implemented in most routine laboratories using a HS-GC-FID method, as routinely applied for ethanol quantification. Quantification of acetoacetate is less useful as this molecule is rapidly converted into BHB or acetone. One of the aims of this study was to evaluate whether BHB analysis should be implemented in routine analysis in all cases or could be limited to those cases where, based upon acetone analysis or background information (e.g. diabetic or alcoholic), there was a reason to specifically test for BHB. This is relevant when considering a limitation of the workload, without risking to miss positive cases (i.e. avoid false negatives).

Retrospective BHB analysis of blood, urine and vitreous humour samples which were received for forensic toxicological analysis demonstrated that the BHB concentrations measured in vitreous humour are overall comparable to the concentrations obtained in blood, suggesting that vitreous humour is a good alternative for blood. Urinary concentrations, on the other hand, show a lower correlation with blood levels. We also observed that acetone was found in all cases with a BHB concentration higher than 100 µg/mL. Consequently, our findings confirm that acetone is a good initial marker for ketoacidosis and our findings support the recommendation by Elliott *et al.* that first a HS-GC-FID analysis should be performed to detect acetone (and isopropanol) [4,6]. This analysis does not impose an additional workload and is already performed for every forensic case. If acetone is detected, we advise to perform BHB analysis, even if no ketoacidosis is suspected based on background information received at case submission. Conclusively, our data suggest that if one limits BHB analysis to acetone positive samples, no cases with ketoacidosis will be missed.

5.5 References

1. Kanetake J, Kanawaku Y, Mimasaka S *et al*. The relationship of a high level of serum beta-hydroxybutyrate to cause of death. *Leg Med (Tokyo)* 7(3), 169-174 (2005).
2. Iten PX, Meier M. Beta-hydroxybutyric acid - An indicator for an alcoholic ketoacidosis as cause of death in deceased alcohol abusers. *J Forensic Sci* 45(3), 624-632 (2000).
3. Sadones N, Van Bever E, Archer JRH *et al.* Microwave-assisted on-spot derivatization for gas chromatography-mass spectrometry based determination of polar low molecular weight compounds in dried blood spots. *J Chromatogr A* 1465, 175-183 (2016).
4. Elliott S, Smith C, Cassidy D. The post-mortem relationship between beta-hydroxybutyrate (BHB), acetone and ethanol in ketoacidosis. *Forensic Sci Int* 198(1-3), 53-57 (2010).
5. Traub F. Method for the detection of lethal glucose metabolism disorders in the corpse (diabetes mellitus and hypoglycemia). *Zentralbl Allg Pathol* 112(4), 390-399 (1969).
6. Hockenhull J, Dhillo W, Andrews R, Paterson S. Investigation of markers to indicate and distinguish death due to alcoholic ketoacidosis, diabetic ketoacidosis and hyperosmolar hyperglycemic state using post-mortem samples. *Forensic Sci Int* 214(1-3), 142-147 (2012).
7. Hess C, Musshoff F, Madea B. Disorders of glucose metabolism-post mortem analyses in forensic cases: part I. *Int J Legal Med* 125(2), 163-170 (2011).
8. Palmiere C, Sporkert F, Vaucher P *et al*. Is the formula of Traub still up to date in antemortem blood glucose level estimation? *Int J Legal Med* 126(3), 407-413 (2012).
9. Keltanen T, Sajantila A, Palo JU, Partanen T, Valonen T, Lindroos K. Assessment of Traub formula and ketone bodies in cause of death investigations. *Int J Legal Med* 127(6), 1131-1137 (2013).
10. Teresinski G, Buszewicz G, Madro R. The influence of ethanol on the level of ketone bodies in hypothermia. *Forensic Sci Int* 127(1-2), 88-96 (2002).
11. Teresinski G, Buszewicz G, Madro R. Biochemical background of ethanol-induced cold susceptibility. *Leg Med* 7(1), 15-23 (2005).
12. Teresinski G, Buszewicz G, Madro R. Acetonaemia as an initial criterion of evaluation of a probable cause of sudden death. *Leg Med* 11(1), 18-24 (2009).
13. Palmiere C, Mangin P. Postmortem chemistry update part I. *Int J Legal Med* 126(2), 187-198 (2012).
14. Kadis P, Balazic J, Ferlan-Marolt V. Alcoholic ketoacidosis: a cause of sudden death of chronic alcoholics. *Forensic Sci Int* 103(1), S53-S59 (1999).
15. Felby S, Nielsen E, Thomsen JL. The postmortem distribution of ketone bodies between blood, vitreous humor, spinal fluid, and urine. *Forensic Sci Med Pathol* 4(2), 100-107 (2008).
16. Osuna E, Vivero G, Conejero J *et al*. Postmortem vitreous humor beta-hydroxybutyrate: its utility for the postmortem interpretation of diabetes mellitus. *Forensic Sci Int* 153(2-3), 189-195 (2005).
17. Pounder DJ, Stevenson RJ, Taylor KK. Alcoholic ketoacidosis at autopsy. *J Forensic Sci* 43(4), 812-816 (1998).

18. Palmiere C, Mangin P, Werner D. Postmortem distribution of 3-beta-hydroxybutyrate. *J Forensic Sci* 59(1), 161-166 (2014).

19. Turan S, Omar A, Bereket A. Comparison of capillary blood ketone measurement by electrochemical method and urinary ketone in treatment of diabetic ketosis and ketoacidosis in children. *Acta Diabetol* 45(2), 83-85 (2008).

CHAPTER 6:
HAPTOGLOBIN GENOTYPING BY DIRECT PCR ON DRIED BLOOD SPOTS

Based on

Sadones N, Onderbeke L, Cannaert A, De Kesel PMM, Delanghe JR, Lambert WE, Stove CP. Development of a new haptoglobin genotyping method by direct PCR on dried blood spots. *In preparation*

Abstract

Haptoglobin (Hp) is a glycoprotein which prevents iron loss and renal damage by binding free hemoglobin which is released during hemolysis. It is synthesized by the liver and is encoded by two alleles, resulting in two homozygous genotypes (*Hp 1-1* and *Hp 2-2*), and one heterozygous genotype (*Hp 2-1*). *Hp* genotyping may help in preventing fatal anaphylactic reactions and in establishing the relationships between the *Hp* genotypes and diseases. Current 'gold standard' methods for *Hp* genotyping suffer from the drawback that mostly two PCR reactions are needed to score the *Hp* genotype. Mostly a second PCR reaction is also required in cases of extensively degraded DNA or small amounts of DNA. Therefore, we aimed to set up an improved PCR-based assay for *Hp* genotyping, directly starting from a dried blood spot (DBS). To this end, we carefully selected our primers, allowing selective amplification of each *Hp* allele, resulting in a 2100 and 1800 bp fragment for *Hp 1* and *2*, respectively. Using a Phusion Blood Direct PCR kit, our method is directly applicable to (dried) whole blood without the need for a separate DNA extraction step. *Hp* genotyping of 221 DBS samples demonstrated that the genotypes obtained with our method were fully consistent with the genotypes obtained using a reference method for *Hp* genotyping. Moreover, we are also able to detect simultaneously the single nucleotide *Hp* A-61C polymorphism in the promoter region by amplification of a 1200 bp fragment, followed by restriction enzyme analysis with *Bpm*I. We found 3 heterozygotes for the *Hp* A-61C polymorphism, which was confirmed by DNA sequencing.

6.1 Introduction

Haptoglobin (Hp) is a glycoprotein with an anti-oxidative capacity by binding free hemoglobin and by reducing iron loss following hemolysis. The *Hp* gene locus is located on chromosome 16, the two alleles giving rise to the *Hp 1-1*, *Hp 2-2* and *Hp 2-1* genotypes. This polymorphism has been associated with (increased risk of developing) distinct diseases, as demonstrated in Table 6.1 [1-3]. E.g. the *Hp* phenotype is an independent risk factor for complications in diabetes, via a mechanism involving oxidative stress.

Table 6.1 Examples of diseases associated with the *Hp* polymorphism [2,3].

Hp 1-1	Overrepresented in breast cancer and cervix carcinoma
Hp 2-2	Diabetics have an increased risk for developing micro- and macrovascular complications
Hp 2-1	Excess in patients with family history of ovarian carcinoma

Although historically, phenotyping by gel electrophoresis was the gold standard, this method requires fresh serum samples and trained staff for the interpretation. Given the perfect correlation between geno- and phenotype, PCR-based methods have emerged as a good alternative. To date, the method of Koch *et al.* [4] is considered the gold standard for *Hp* genotyping. However, different intensities of different bands, provoked by the large difference in band size, may lead to the false homozygous scoring of a heterozygote, which is the major drawback of this method. Accordingly, two PCR reactions are often needed to conclusively determine the *Hp* genotype. Furthermore, the method starts from DNA extracts, rather than directly from raw biological material. Therefore, we have set up an improved (conventional) PCR-based method, directly starting from a micropunch from a dried blood spot (DBS), allowing to discriminate between both *Hp* alleles. In addition, our method is also suitable for the simultaneous detection of the *Hp* A-61C polymorphism, a single nucleotide polymorphism in the promoter region of the *Hp* gene that has been associated with ahaptoglobinaemia [5].

6.2 Materials and methods

6.2.1 Samples

A study was set up to evaluate the applicability of our newly developed *Hp* genotyping method. This study was approved by the Ethics Committee of Ghent University Hospital (B670201317381). Written informed consent was obtained from all participants. Capillary DBS were generated on Whatman 903 filter paper following a fingerprick using an automatic lancet (Becton Dickinson ref n^{o} VAC366594, Franklin Lakes, NJ, USA). Venous DBS were prepared by spotting 25 µL of venous blood (obtained from left-over samples) onto the filter paper.

6.2.2 Hp genotyping

6.2.2.1 Primers

The sequences of the newly designed primers for *Hp* genotyping are listed in Table 6.2. We combined the forward primer of the method of Koch *et al.* [4] with two carefully selected reverse primers. Combination with the first reverse primer results in the amplification of a *Hp 1*-specific sequence of ± 2100 bp and a *Hp 2*-specific fragment of ± 3500 bp. However, the latter is too large to be easily amplified. The second reverse primer in the reaction generates a *Hp 2*-specific sequence of 1800 bp. All primers were provided by Eurofins Genomics (Ebersberg, Germany).

Table 6.2 Overview of the primers used for *Hp* genotyping.

Forward primer (F)	5'-GAGGGGAGCTTGCCTTTCCATTG-3'
Reverse primer 1 (R1)	5'-GAGATTATGGTGGGAAACCATCTTAGC-3'
Reverse primer 2 (R2)	5'-CCGAATAGAAGCTCGCGAACTG-3'

6.2.2.2 PCR reaction

We utilized a Phusion Blood Direct PCR Kit obtained from Thermo Fisher Scientific (Erembodegem, Belgium). This kit contains a Phusion Blood II DNA Polymerase which exhibits high resistance to inhibitors present in blood and eliminates the need for a separate DNA extraction step prior to the PCR reaction.

Our 20-µL PCR reaction contained a 0.5 mm DBS punch, 0.4 µL Phusion Blood DNA II Polymerase, 10 µL Phusion Blood PCR Buffer, and our three primers for *Hp* genotyping (see Table 6.2), which had a final concentration of 0.5 µM. After an initial denaturation -allowing lysis of leukocytes and thus release of DNA- of 5 min at 98°C, denaturation and annealing/elongation were performed for 1 s at 98°C and 30 s at 72°C, respectively. These steps were repeated for 35 cycles. The final elongation step took 1 min at 72°C. This PCR reaction was performed in a Mastercycler® Nexus Thermal Cycler (Eppendorf, Hamburg, Germany).

6.2.2.3 Gel electrophoresis and visualization

The PCR products were separated using agarose gel electrophoresis. Therefore, we prepared a 1.5% agarose (Sigma Aldrich, Diegem, Belgium) gel in 50x Tris-acetate-ethylenediaminetetra-acetic acid (TAE) buffer (Thermo Fisher Scientific, Erembodegem, Belgium). Following the addition of 6x DNA loading dye (Thermo Fisher Scientific, Erembodegem, Belgium), a GeneRuler DNA ladder mix (Thermo Fisher Scientific, Erembodegem, Belgium) and 10 µL of each sample were loaded. Finally, the DNA fragments were visualized under UV light using a GelRed™ staining solution (Biotium, Hayward, CA, USA).

6.2.3 Determination of the Hp A-61C polymorphism

Using restriction enzyme analysis, we are also able to detect the *Hp* A-61C polymorphism. To this end, a PCR-product of ± 1200 bp which encloses position -61 in the *Hp* gene was amplified using the forward primer 5'-CTCCTGTTGATGGGCATTTGTCTTG-3' and the reverse primer 5'-GGAGCTGATGACATACCCTATAAAGTC-3' (with a final concentration of 1.125 µM), followed by a restriction digest with 0.5 µL *Bpm*I (Thermo Fisher Scientific, Erembodegem, Belgium) for 15 min at 30°C.

6.3 Results and discussion

As discussed by Ko *et al.* [6] current Hp genotyping methods utilizing conventional PCR methods suffer from the drawback that more than one PCR reaction is needed. Therefore, we have selected our primers so that one PCR reaction suffices in all cases. Our first reverse primer allows amplification of a *Hp 1*-specific fragment, whereas our second reverse primer binds to the unique part between exon 4 and exon 5 of *Hp* allele 2, resulting in a *Hp 2*-specific sequence of 1800 bp (see Figure 6.1).

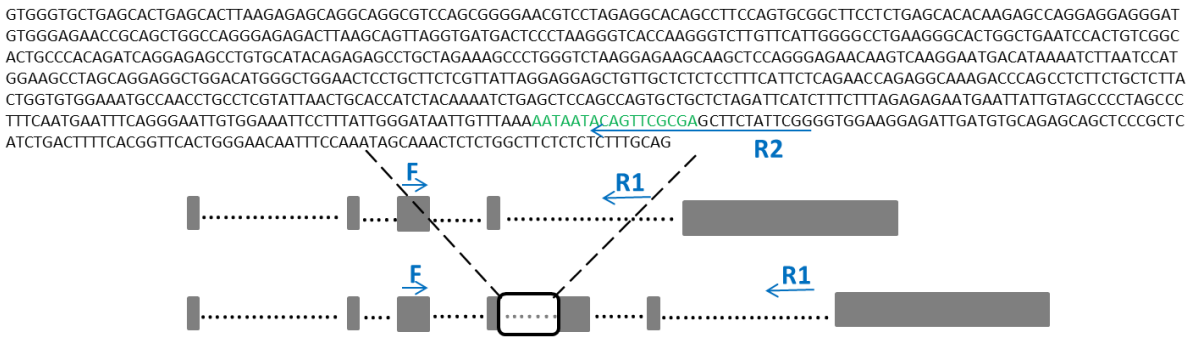


Figure 6.1 Scheme of the primers in the new *Hp* genotyping method.

The similar lengths of the *Hp* allele-specific sequences, combined with similar efficiencies of both PCR reactions, results in heterozygotes in bands with similar intensities, irrespective of the amount of DNA. Consequently, one PCR reaction suffices in all cases in our method, in contrast to other genotyping methods, which are characterized by large-sized products which may be difficult to amplify and to detect. E.g. using the method of Koch *et al.* [4], it is easier to amplify the *Hp 1*-specific band of 1700 bp than the *Hp 2*-specific band of 3400 bp in heterozygotes. Consequently, the smallest band is considerably more intense than the larger band, which may hamper *Hp* genotyping of heterozygotes (i.e. heterozygotes could be falsely scored as *Hp 1-1*), as demonstrated in Figure 6.2. Therefore, a second PCR reaction which generates a *Hp 2*-specific PCR product of about 350 bp, is warranted when using the method of Koch *et al.* [4].

Another advantage of our assay is that it is directly applicable to a 0.5 mm punch from a DBS, without prior DNA extraction. We only need a limited amount of starting material (one drop of blood suffices) and sampling

can be performed at home without dedicated staff. Furthermore, we demonstrated that our method is also applicable to dried oral fluid or lyzed blood (direct PCR on 1 µL) and that hemoglobin and anticoagulants do not hamper the PCR reaction (see Figure 6.3).

To ensure the validity of our new *Hp* genotyping method, we performed *Hp* genotyping of 221 DBS using both the method of Koch *et al.* [4] and our new method. We found a 100% correlation between the genotypes obtained with both methods.

	Reference method	New method
Hp 1 allele	**± 1700 bp**	**± 2100 bp**
Hp 2 allele	**± 3400 bp**	**± 1800 bp**

Sample	Genotype
A	blank
B	*Hp 2-1*
C	*Hp 2-1*
D	*Hp 1-1*
E	*Hp 1-1*
F	*Hp 2-2*
G	*Hp 2-1*
H	*Hp 2-1*
I	*Hp 1-1*
J	*Hp 2-1*

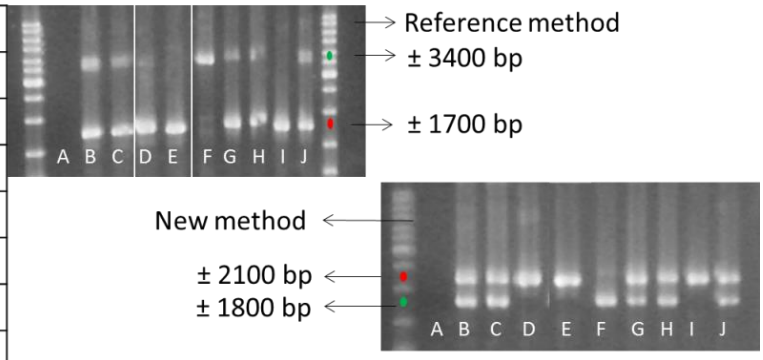


Figure 6.2 Genotyping of DBS samples using the reference method of Koch *et al.* [4] and our new method.

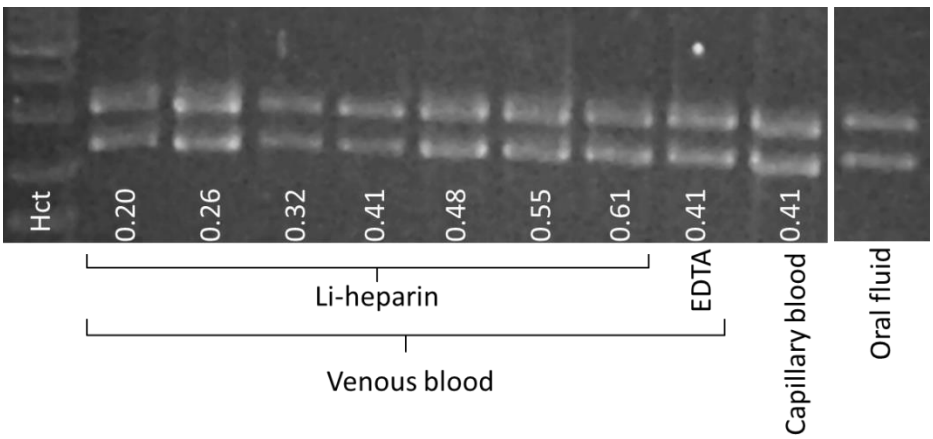


Figure 6.3 Influence of hematocrit (Hct) and anticoagulants on our newly developed *Hp* genotyping method.

Using restriction enzyme analysis, we are also able to detect *Hp* A-61C polymorphism. To this end, we generated a 1200 bp-fragment, enclosing position -61, followed by a restriction enzyme digestion with *Bpm*I. In absence of this *Hp* promoter polymorphism, *Bpm*I will recognize one cleavage site, whereas in the presence of this polymorphism, two cleavage sites will be recognized (see Figure 6.4). Finally, we found 3 heterozygotes. This observation was confirmed by DNA sequencing.

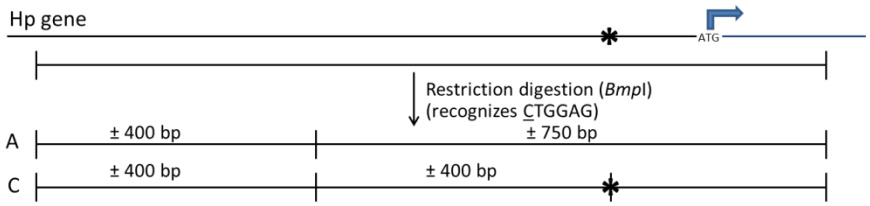


Figure 6.4 *Hp* A-61C polymorphism.

What can be considered a disadvantage of our method (and other conventional PCR methods for *Hp* genotyping) is the need for a laborious and time-consuming post-amplification step (i.e. running a gel). Although the use of labeled probes and real-time PCR may overcome this issue, this may be at the expense of the simplicity of our assay, which is important for high-throughput analyses.

We believe that it would be useful to determine the *Hp* genotype for every person. Since the *Hp* genotype has been associated with various infections and diseases, *Hp* genotyping may increase life expectancy and reduce health care costs by preventing complications. As the genotype always remains the same, this determination only needs to be done once per patient.

6.4 Conclusion

In conclusion, a new, simple and rapid (45 min) PCR method, allowing *Hp* genotyping in a high-throughput setting was developed. There is no need for a time-consuming and expensive DNA extraction and only a limited amount of biological material is required, resulting in a minimal contamination risk. Furthermore, sampling can be done by the patient himself. Additionally, in contrast to other genotyping methods, only one PCR reaction is needed to come to a conclusive result in all instances, the length of the fragments allowing simple amplification and detection. Using the restriction fragment length polymorphism technique, we are also able to detect simultaneously the *Hp* A-61C polymorphism in the same reaction.

6.5 References

1. Costacou T, Levy AP. Haptoglobin genotype and its role in diabetic cardiovascular disease. *J Cardiovasc Transl Res* 5(4), 423-435 (2012).
2. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem* 42(10), 1589-1600 (1996).
3. Levy AP, Hochberg I, Jablonski K, *et al*. Haptoglobin phenotype is an independent risk factor for cardiovascular disease in individuals with diabetes: The Strong Heart Study. *J Am Coll Cardiol* 40(11), 1984-1990 (2002).
4. Koch W, Latz W, Eichinger M, *et al*. Genotyping of the common haptoglobin Hp 1/2 polymorphism based on PCR. *Clin Chem* 48(9), 1377-1382 (2002).
5. Teye K, Quaye IKE, Koda Y, *et al*. A-61C and C-101G Hp gene promoter polymorphisms are, respectively, associated with ahaptoglobinaemia and hypohaptoglobinaemia in Ghana. *Clin Genet* 64(5), 439-443 (2003).
6. Ko DH, Chang HE, Kim TS, *et al*. A review of haptoglobin typing methods for disease association study and preventing anaphylactic transfusion reaction. *Biomed Res Int* 2013, 390630 (2013).

CHAPTER 7:
BROADER INTERNATIONAL CONTEXT, RELEVANCE AND FUTURE PERSPECTIVES

The use of dried blood spots (DBS) as an alternative sampling strategy has gained popularity during the last several years. This can be deduced from the increasing number of papers dealing with DBS (see Figure 7.1) and from the many fields in which DBS have been implemented, covering the analysis of DNA, proteins, small molecules (endogenous compounds, therapeutic drugs and drugs of abuse) as well as trace elements.

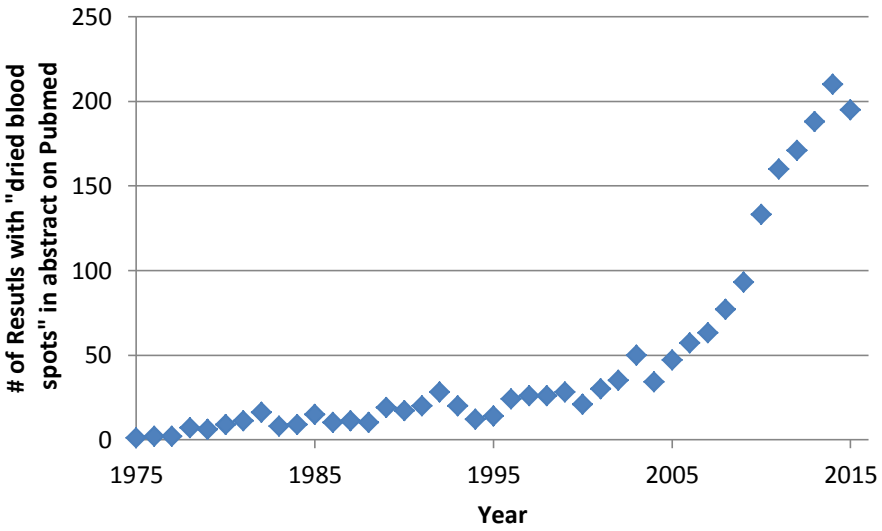


Figure 7.1 Overview of the number of publications dealing with DBS in Pubmed during the last 40 years.

Several committees have focused on the potential and limitations of DBS and have provided recommendations for the validation of bioanalytical methods for DBS with the aim to contribute to the harmonization and standardization of alternative matrix analysis. E.g. the European Bioanalysis Forum (EBF), which brings together different pharmaceutical companies and contract research organizations, with the aim to share, discuss and optimize different bioanalytical topics, has a topic team formed for blood microsampling and DBS approaches: different subteams have evaluated DBS-related issues like hematocrit (Hct), stability, dilution and the use of internal standard (IS). Also in the USA, there is a microsampling working group within The International Consortium for Innovation and Quality in Pharmaceutical Development (IQ Consortium). Such cross-company consortia increase the understanding and improve the processes concerning DBS sampling.

In this thesis, we also gave some recommendations and/or points of attention when setting up a DBS-based method. Although promising results have been obtained with DBS, its widespread use, application and acceptance in practice is limited. This is due to some practical hurdles, technical challenges and inherent disadvantages which have been associated with DBS sampling (see Chapter 1). From Chapter 2 till Chapter 5, we tackled some of these DBS-related issues. The choice of the analytes which were used for this purpose, has been discussed in Chapter 3. In addition to tackling DBS-related issues, each Chapter in this thesis also demonstrated the advantages of DBS sampling. E.g. in Chapter 5 we demonstrated the potential of DBS in post-mortem forensic toxicology as a convenient sample preparation strategy.

The Hct problem is a generally acknowledged theme in DBS analysis. The effect of the Hct on quantification of an analyte in DBS samples is a compound-dependent matter and is influenced by many factors. It is primarily an issue when dealing with non-volumetrically applied spots, where discs are punched from the DBS. In Chapter 3, we also evaluated the influence of the Hct on the analytical results obtained with our GC-MS method, following "microwave-assisted on-spot derivatization". This DBS-specific parameter should be part of the validation procedure for any DBS-based procedure using partial-spot approaches. However, this requires some additional validation work and novel and/or improved strategies to cope with the Hct effect will be required to support the growing and widespread use of DBS in bioanalysis. Therefore, continuous efforts to further understand and tackle this issue will likely be made in the future. Recently, new approaches have been developed to overcome the Hct problem by allowing the collection of a fixed volume of blood from a non-volumetrically applied sample. E.g. Lenk *et al.* and Leuthold *et al.* investigated the usefulness of microfluidic-based sampling procedures [1,2]. The first developed a 'disposable metering device' prototype [1], whereas the latter utilize a microfluidic device which consists of a foldable support system. After applying a blood of drop onto the inlet of a microfluidic channel, the channel is filled by capillary force. Then the support system is closed, allowing contact between the outlet of the channel and a DBS card. Using this microfluidic-based sampling procedure, no Hct effect was observed within the Hct range 0.26-0.62 for the test compound Mavoglurant [2]. Verplaetse and Henion confirmed that this device is an easy-to-operate instrument for acquiring volumetrically applied spots and considered it as a promising tool for point-of-care patient self sampling, doping control, workplace drug testing and in the context of driving under the influence of drugs (DUID) [3]. HemaSpot^{TM}-HF (Spot-On Sciences) is another recently developed volumetric blood sampling device in which one drop of blood suffices. Other interesting new approaches that overcome area bias and homogeneity issues associated with analyzing subpunches from DBS samples are volumetric absorptive microsampling (VAMS) devices. These allow the collection of a fixed volume of blood from a non-volumetrically deposited sample onto a porous substrate, independent of the Hct. These devices are on the march and can be used for other biological matrices as well. Mercolini *et al.*, for instance, quantified cathinone analogues in dried urine, plasma and oral fluid using VAMS [4]. Many research groups are also developing different kinds of devices to generate dried plasma spots following non-volumetric application of whole blood since the use of dried plasma spots are another way to avoid the Hct problem. Additionally, the pharmaceutical industry prefers microsampling of plasma rather than DBS samples for pharmacokinetic and pharmacodynamic studies. Spot-On-Sciences, for instance, designed the HemaSpot^{TM}-SE device that allows the separation of serum/plasma from blood cells. Lenk *et al.* also developed a microfluidic device to collect a specific volume of plasma from an unknown blood volume [5]. Another focus to cope with the Hct problem may lie in the exploration of the potential of various endogenous compounds to function as surrogate Hct markers. These compounds allow to estimate or predict the Hct of a DBS and allows one to determine which analytical results are reliable and which analyte concentrations might need a correction. E.g. in our laboratory potassium was demonstrated to be a good reliable marker for Hct prediction: application of a potassium-based algorithm for the quantification of caffeine and paraxanthine allows correction for the Hct bias [6]. Other laboratories are using this strategy as well, e.g. den Burger *et al.* performed Hct corrected analysis of creatinine in DBS through potassium

measurement [7]. Recently, another new interesting approach has been developed in our laboratory to predict the Hct from a DBS. It concerns a nondestructive method in which the total hemoglobin content is measured via noncontact diffuse reflectance spectroscopy [8]. The use of near-infrared spectroscopy also allows a fast, robust and nondestructive quantification of the Hct [9]. Development of innovative materials/substrates that are less affected by Hct-dependent spreading of blood and allow more homogenous distribution across spots will also contribute to the widespread use and acceptance of DBS. E.g. Mengerink *et al.* demonstrated that new DBS cards which consist of hydrophilically coated woven polyester fibers give spot sizes independent of the Hct [10]. Conclusively, this is a highly dynamic field in which various groups are busy with the development of new approaches to solve or to minimize the Hct issue. Pricing may be an important determinant factor when considering which of the proposed approaches will eventually turn out to be competitive.

The addition of IS in DBS analysis is also challenging. Also here, new IS addition techniques have been developed. As recently studied by Abu-Rabie *et al*. [11], an interesting approach is spraying the IS onto the DBS card. They investigated different approaches to incorporate the IS in the DBS quantitative bioanalytical workflow and evaluated their influence on the Hct-based assay bias. They observed that when the IS spray addition technique is coupled with accurate volume sampling and whole spot extraction, the Hct-based assay bias is nullified.

In order for DBS methods to become integrated in routine toxicological practice, efforts have also been made to automate DBS procedures, allowing high throughput analysis. E.g. Spark-Holland developed the DBS^{TM} Autosampler which enables automation of the entire workflow for DBS analysis without any manual intervention. First a digital picture is taken from the DBS card to detect the spot and to clamp the card. Then, the compounds are desorbed using automated flow-through elution, followed by online solid phase extraction. Verplaetse and Henion nicely demonstrated that the direct coupling of automated online DBS samplers with new generation LC-MS/MS equipment may prove valuable to obtain sufficient sensitivity with limited amount of starting material [3]. Furthermore, they opted for whole spot analysis of blood samples which were obtained using a microfluidic-based volumetric sampling approach, resulting in a Hct-independent fully automated analysis.

Automation in DBS bioanalysis, however, mostly requires an investment in dedicated equipment, which may not be considered feasible by an average laboratory. E.g. only large, well-funded laboratories will have access to such automated DBS systems or robotic systems. Digital microfluidic systems, on the other hand, are more accessible while they also reduce extraction time and the amount of solvents. Other devices/approaches which minimize sample preparation have been developed as well. E.g. different suppliers developed instruments to automate the punching of DBS samples. Perkin Elmer, for instance, developed the DBS Puncher® device which automatically punches dried blood samples into microtitration plates. Manicke *et al.* demonstrated that paper spray MS may also be useful to detect drugs directly from DBS without sample preparation [12]. As time

management is of utmost importance in any laboratory, automation and DBS direct elution techniques will continue to be an important part of future DBS research.

Another challenge associated with DBS sampling is to obtain sufficient sensitivity since only a limited sample volume is available. This is relevant in e.g. the context of DUID where the lower limit of quantification (LLOQ) of the method should be below proposed cut-off levels or legal limits. DBS-based methods for quantification of cannabinoids, for instance, may be promising in the context of DUID. However, up till now, there is only one method attaining sufficient sensitivity to measure cut-off levels proposed by DRUID. As newer drugs (e.g. synthetic cannabinoids) tend to be more potent, even higher sensitivity will be required. Up till now, no DBS methods for synthetic cannabinoids are available. Here as well, the direct coupling of automated online DBS samplers with new generation LC-MS/MS equipment may prove valuable. Also other innovative approaches, such as the use of alginate or chitosan foam substrates, which may allow higher recoveries, may aid to achieve the necessary sensitivity [13]. There will be continuous improvements in analytical equipment and efforts to render DBS analysis high-throughput-capable.

Other difficulties may lie in establishing legal limits, e.g. in driver's licence regranting processes. Currently, indirect alcohol biomarkers are used to monitor the abstinence period in case of fitness to drive decisions. These analyses, however, have a low sensitivity and specificity. Therefore, Kummer *et al.* demonstrated that the use of direct ethanol markers obtained by non- or minimally invasive sampling techniques has potential to assess the alcohol consumption in cases of fitness to drive assessment [14]. They suggest to quantify ethylglucuronide and ethylsulfate in urine, ethylglucuronide in hair and phosphatidylethanol in capillary DBS (cDBS) to give an insight into the evolution of the alcohol consumption prior to sampling.

An important limitation of most DBS studies is the fact that, while they demonstrated the proof-of-principle, the developed methods have often not yet been applied on (true patient) capillary samples or only a limited number of samples was included in these studies, with often no cross-comparison between venous DBS and cDBS. In Chapter 2, we coped with this DBS-associated issue by setting up a comparative study in which capillary gamma-hydroxybutyric acid concentrations were correlated to venous concentrations. To the best of our knowledge, this was the first study evaluating at a large scale the correlation between venous and capillary concentrations for a drug of abuse. This study served as the basis for similar studies which have been set up for other drugs of abuse. Ellefsen *et al.*, for instance, compared capillary and venous concentrations for cocaine and metabolites [15], whereas Kummer *et al.* evaluated venous-capillary concentrations for alcohol markers [16].

In addition, before the use of DBS as a sampling technique can move to a next stage, more bridging studies, comparing concentrations in cDBS with those in venous blood or plasma, need to be conducted. In Chapter 4 such a bridging study was set up for gabapentin. Our study was the first to evaluate the use of DBS sampling for gabapentin monitoring and has the potential to contribute to the acceptance of DBS as a valid alternative for

current quantification methods. Furthermore, it adds new and relevant data to the ongoing discussion about the implementation of DBS sampling in therapeutic drug monitoring (TDM). Currently, serum/plasma is the most common matrix in routine TDM and reference intervals are based on serum/plasma levels. However, the use of DBS for TDM may have many advantages: 1) sampling can be performed at home without the need for skilled personnel, 2) sampling can be performed at any desired time, 3) monitoring results are available when the patient goes to the clinician for routine control, 4) samples can be easily sent by post without any biohazard risk, and 5) there in an increased stability of analytes in dried spots. Therefore, bridging studies are gaining more and more interest as they allow to convert DBS concentrations into interpretative serum/plasma values.

Given the many advantages which have been associated with DBS, there will also be an increased use of other dried biological matrices such as urine, vitreous humour, synovial and oral fluid. We already use dried blood and urine spots in a routine forensic toxicological context to quantify gamma-hydroxybuytric acid and beta-hydroxybutyric acid using GC-MS and we aim to use dried matrix spots for the detection of other compounds as well, e.g. for the quantification of anti-epileptics, cocaine (and metabolites),… In the future, the use of "microwave-assisted on-spot derivatization" could be extended to more compounds and other derivatization reagents could be tested as well. Multi-analyte procedures such as our newly developed GC-MS method are also suitable in a routine laboratory since they increase throughput by the simultaneous detection of multiple compounds in a single run. The use of VAMS as well as fully automated DBS analyses will also be encouraged.

Not only DBS sampling, but also other microsampling techniques, in which small amounts of blood are collected, are gaining interest as alternative for classical venous blood sampling. E.g. in an acute setting where feedback on sample concentration is urgent, whole wet blood liquid microsampling is more feasible than DBS sampling. Liquid microsampling techniques may also play an important role in the pharmaceutical industry. Therefore, there will be continuous improvements and new ways to collect small amounts of blood. E.g. Theranos designed a Sample Collection Device (SCD) to collect blood from a fingerprick into Nanotainer™ tubes, which are about 1.29 cm. Liquid microsamples have the advantage that they do not differ from traditional liquid samples, although also here this needs to be evaluated on a case-by-case basis, as differences have been reported for various analytes [17]. Other alternative sampling strategies, such as the sampling of oral fluid, hair, meconium, interstitial fluid, sweat, exhaled breath condensate and sputum, offer interesting opportunities as well. It has to be noted that in the latter sampling approaches, "alternative" samples are collected, whereas in DBS sampling, a traditional matrix (i.e. blood) is collected in an alternative way. Analysis of alternative biological matrices may be a good complement to blood and urine analysis, but cannot be considered as an alternative for blood and urine analysis. Although alternative sampling strategies (including DBS sampling) will never replace traditional sampling approaches, we do believe that there is a widely underexplored potential for these alternative sampling strategies, either in medicine, health management or in (forensic) toxicology.

References

1. Lenk G, Sandkvist S, Pohanka A, Stemme G, Beck O, Roxhed N. A disposable sampling device to collect volume-measured DBS directly from a fingerprick onto DBS paper. *Bioanalysis* 7(16), 2085-2094 (2015).
2. Leuthold LA, Heudi O, Déglon J, *et al*. New microfluidic-based sampling procedure for overcoming the hematocrit problem associated with dried blood spot analysis. *Anal Chem* 87(4), 2068-2071 (2015).
3. Verplaetse R, Henion J. Hematocrit-independent quantitation of stimulants in dried blood spots: pipet versus microfluidic-based volumetric sampling coupled with automated flow-through desorption and online solid phase extraction-LC-MS/MS bioanalysis. *Anal Chem* 88(13), 6789-6796 (2016).
4. Mercolini L, Protti M, Catapano MC, Rudge J, Sberna AE. LC-MS/MS and volumetric absorptive microsampling for quantitative bioanalysis of cathinone analogues in dried urine, plasma and oral fluid samples. *J Pharm Biomed Anal* 123, 186-194 (2016).
5. Lenk G, Hansson J, van der Wijngaart W, Stemme G, Roxhed N. Capillary driven and volume-metred blood-plasma separation. Presented at: *18th International Conference on Solid-State Sensors, Actuators and Microsystems (Transducers).* Alaska, USA, 21-25 June 2015.
6. De Kesel PM, Capiau S, Stove VV, Lambert WE, Stove CP. Potassium-based algorithm allows correction for the hematocrit bias in quantitative analysis of caffeine and its major metabolite in dried blood spots. *Anal Bioanal Chem* 406(26), 6749-6755 (2014).
7. den Burger JC, Wilhelm AJ, Chahbouni AC, Vos RM, Sinjewel A, Swart EL. Haematocrit corrected analysis of creatinine in dried blood spots through potassium measurement. *Anal Bioanal Chem* 407(2), 621-627 (2015).
8. Capiau S, Wilk LS, Aalders MC, Stove CP. A novel, nondestructive, dried blood spot-based hematocrit prediction method using noncontact diffuse reflectance spectroscopy. *Anal Chem* 88(12), 6538-6546 (2016).
9. Oostendorp M, El Amrani M, Diemel EC, Hekman D, van Maarseveen EM. Measurement of hematocrit in dried blood spots using near-infrared spectroscopy: robust, fast, and nondestructive. *Clin Chem* 62(11), 1534-1536 (2016).
10. Mengerink Y, Mommers J, Qiu J, Mengerink J, Steijger O, Honing M. A new DBS card with spot sizes independent of the hematocrit value of blood. *Bioanalysis* 7(16), 2095-2104 (2015).
11. Abu-Rabie P, Denniff P, Spooner N, Chowdhry BZ, Pullen FS. Investigation of different approaches to incorporating internal standard in DBS quantitative bioanalytical workflows and their effect on nullifying hematocrit-based assay bias. *Anal Chem* 87(9), 4996-5003 (2015).
12. Manicke NE, Abu-Rabie P, Spooner N, Ouyang Z, Cooks RG. Quantitative analysis of therapeutic drugs in dried blood spot samples by paper spray mass spectrometry: an avenue to therapeutic drug monitoring. *J Am Soc Mass Spectrom* 22(9), 1501-1507 (2011).

13. Eibak L, Hegge A, Rasmussen K, Pedersen-Bjergaard S, Gjelstad A. Alginate and chitosan foam combined with electromembrane extraction for dried blood spot analysis. *Anal Chem* 84(20), 8783-8789 (2012).
14. Kummer N, Wille SM, Poll A, Lambert WE, Samyn N, Stove CP. Quantification of EtG in hair, EtG and EtS in urine and PEth species in capillary dried blood spots to assess the alcohol consumption in driver's licence regranting cases. *Drug Alcohol Depend* 165, 191-197 (2016).
15. Ellefsen KN, da Costa JL, Concheiro M, *et al.* Cocaine and metabolite concentrations in DBS and venous blood after controlled intravenous cocaine administration. *Bioanalysis* 7(16), 2041-2056 (2015).
16. Kummer N, Ingels AS, Wille SM, *et al*. Quantification of phosphatidylethanol 16:0/18:1, 18:1/18:1, and 16:0/16:0 in venous blood and venous and capillary dried blood spots from patients in alcohol withdrawal and control volunteers. *Anal Bioanal Chem* 408(3), 825-838 (2016).
17. Bond MM, Richards-Kortum RR. Drop-to-drop variation in the cellular components of fingerprick blood: implications for point-of-care diagnostic development. *Am J Clin Pathol* 144 (6), 885-894 (2015).

CHAPTER 8:
CONCLUSION AND SUMMARY

In this work we focus on dried blood spots (DBS). During the last few years, there is an increased interest in the use of DBS, with different kinds of applications in various fields. E.g. DBS have been applied for the analysis of DNA, proteins, small molecules and trace elements, with applications situated in the field of epidemiology, therapeutic drug monitoring (TDM), toxicology, phenotyping, and so on [1]. This variety in DBS applications is also reflected in this work. On the one hand, we used DBS in an analytical context, on the other hand, we developed a DBS-based genotyping method.

DBS sampling is a microsampling strategy in which the sample (i.e. ≤ 100 µL of whole blood obtained via a heel- or fingerprick) is collected as a spot on a (cellulose) filter paper. Many advantages have been associated with this alternative sampling technique [2]. First, DBS sampling is a minimally invasive sampling technique, enabling collection of a representative sample from animals, newborns, children and adults with minimal discomfort. Furthermore, it facilitates sampling at home and in remote areas as there is no need for dedicated medical staff [3-5]. Secondly, the dried matrix reduces the risk of infection and improves compound stability [6-10]. Third, DBS samples can easily be transported and stored [5,11]. In addition, DBS may simplify sample preparation procedures (e.g. via "on-spot derivatization") and they are suitable for automation of sample processing and analysis, which renders DBS analysis high-throughput-capable [12-19]. Consequently, there has been a growing interest in the use of DBS applications for a variety of purposes (e.g. in a pharmaceutical, clinical or toxicological context), which, on its turn, has been a driving force for moving the field forward.

In **Chapter 1** an overview of the DBS applications in toxicology, more specifically for the analysis of abused substances, is given. These include therapeutic drugs which are often abused, illegal drugs and markers for alcohol and tobacco use. Benefits as well as limitations and challenges which have been associated with these diverse toxicological applications are discussed in that Chapter. In the following Chapters, we tackled several of the limitations which have been associated with DBS sampling.

An important limitation for the implementation of DBS sampling in the industry and in routine analysis is the fact that a cross-comparison between capillary and venous samples is often lacking. Such studies are essential for the acceptance of DBS as a valid alternative for classical venous sampling. In addition, although a large number of studies have demonstrated the theoretical potential of DBS applications (not using true capillary DBS (cDBS) samples), the developed methodologies often have not been applied on a large cohort of real-life samples [20]. To this end, we have set up a large-scale comparative study for gamma-hydroxybutyric acid (GHB) (**Chapter 2**). We evaluated in 50 patients with a suspected GHB intoxication how capillary GHB concentrations (obtained by fingerprick) were correlated with venous concentrations (obtained by venipuncture). DBS sampling is a promising alternative for classical venous sampling in cases of (suspected) GHB intoxication since it allows rapid sampling, which is of interest for the rapidly metabolized GHB. However, as a cross-comparison between venous DBS (vDBS) and cDBS concentrations is lacking, paired cDBS and venous whole blood samples -of which vDBS were prepared- were collected from patients presenting at the emergency department of the Guy's and St Thomas' Hospital in London with a suspected GHB intoxication. cDBS were

generated by non-volumetric direct application of a drop of blood onto Whatman 903 filter paper following a fingerprick, whereas vDBS were prepared by pipetting 25 µL of venous blood onto the filter paper. Following a drying period of at least 2 hours, the DBS samples were transported to our lab in zip-closure plastic bags with desiccant. GHB concentrations were determined using a validated gas chromatography coupled to mass spectrometry (GC-MS)-based procedure previously developed in our lab [21]. The results of this study indicated that GHB concentrations in cDBS and vDBS are equivalent. From this, we concluded that fingerprick blood can be considered as a valid alternative for venous blood for GHB determination.

Given the small blood volume, there are also some sensitivity issues associated with DBS analysis. Continuous improvements and advancements in analytical equipment and sample preparation technology, however, allow quantitative determination of most analytes at low ng/mL level, starting from ± 3 µL blood. To this end, liquid chromatography coupled to (tandem) mass spectrometry (LC-MS(/MS)) is mostly preferred, although GC methods also have their place [20,22-23]. To achieve sufficient sensitivity, the latter technique, however, often involves the introduction of a derivatization step, an additional sample preparation step which is considered as laborious and time-consuming. Therefore, we introduced a new derivatization strategy by combining "on-spot derivatization" (in which derivatization reagents are directly applied to DBS) with "microwave derivatization", thereby ensuring a minimal, economic and fast sample workup and high sample throughput.

In **Chapter 3**, we successfully validated and evaluated the applicability of this newly developed method for GHB and the anti-epileptic drug gabapentin. During validation, we evaluated classical parameters like linearity, precision, accuracy, selectivity, carry-over, dilution integrity and stability as well as DBS-specific parameters like the influence of the hematocrit and spotted blood volume, which may have an impact on the analytical results. In many cases, the benefits of DBS sampling outweigh this additional validation work. The calibration lines were linear between 10 and 100 µg/mL for GHB and from 1 to 30 µg/mL for gabapentin, applying a 1/x weighing factor. Accuracy and precision criteria were fulfilled for both compounds. Stability studies revealed that GHB and gabapentin were stable in DBS when stored at room temperature for 84 days. Analysis of patient samples demonstrated the applicability of "microwave-assisted on-spot derivatization", followed by GC-MS analysis, in a routine setting. Furthermore, this newly developed GC-MS method also allows the detection of other polar low molecular weight molecules with clinical and/or forensic toxicological relevance, amongst which vigabatrin, beta-hydroxybutyric acid (BHB), 1,4- and 1,2-butanediol, propylene glycol and diethylene glycol.

In many cases, there is also a need for studies evaluating the correlation between blood concentrations and plasma/serum concentrations, as reference intervals are typically only available for plasma (or serum). Such studies are coined "bridging studies". We have set up a study like this for the anti-epileptic drug gabapentin by collecting paired cDBS and serum samples from gabapentin-positive patients, in the framework of a pharmacokinetic study (**Chapter 4**). For the quantification of gabapentin in DBS, our newly developed GC-MS procedure following "microwave-assisted on-spot derivatization" was used, whereas serum concentrations were determined using an independent validated LC-MS/MS method developed by Chahbouni *et al.* [24]. We

observed a mean blood-to-serum ratio of 0.85. Taking this conversion factor into account, a good correlation was found between measured serum concentrations and serum concentrations calculated from DBS results. Consequently, our data lend support to the potential of DBS for deriving plasma concentrations of this compound, rendering DBS-based TDM for gabapentin a feasible option, using plasma reference values as a guide.

Our "microwave-assisted on-spot derivatization" approach is also suitable for BHB analysis. Elevated levels of this ketone body are found in ketotic diabetics and alcoholics and may be a contributing factor to the cause of death [25]. However, the sense of routinely analyzing BHB in a forensic toxicological context is unclear. Therefore, we performed retrospective BHB analysis of 553 blood samples, 232 urine samples and 62 vitreous humour samples (**Chapter 5**). In this unbiased study, we observed that cases with BHB concentrations above 100 µg/mL are invariably associated with elevated levels of acetone. Analysis of the latter ketone body is already implemented in most routine laboratories using a headspace GC configuration for ethanol quantification and does not require an additional workload. Therefore, we concluded that acetone is a good initial marker for ketoacidosis and that BHB analysis can be limited to acetone-positive cases. We also observed that vitreous humour is a good alternative for blood for BHB analysis and that the interpretation of urinary BHB concentrations is not straightforward. The suitability of dried matrix samples for BHB analysis was also demonstrated with this study.

The above-mentioned DBS applications concern the analysis of small polar molecules, including illegal drugs (such as GHB), therapeutic drugs (like gabapentin) and endogenous molecules (BHB). However, not only small molecules, but also trace elements, proteins, mRNA and DNA can be analyzed using DBS. The latter was demonstrated in **Chapter 6**, by setting up a DBS-based genotyping method for haptoglobin (Hp).

Hp is an abundant hemoglobin-binding plasma protein, encoded by 2 common alleles, denoted 1 and 2. This *Hp* polymorphism has been reported to have important biological and clinical consequences [26]. In this respect, Chapter 6 can somewhat be linked to Chapter 5, as both are related to diabetes. E.g. large-scale studies have unequivocally shown that diabetics with the *Hp 2-2* phenotype have a 2-5-fold increased risk for developing micro- and macrovascular complications. Since there is a perfect correlation between the *Hp* pheno- and genotype, PCR-based methods have emerged as a good alternative for time-consuming phenotyping methods. We therefore set up an improved PCR-based strategy, directly starting from DBS micropunches, allowing *Hp* genotyping in a high-throughput setting. Current *Hp* genotyping methods utilizing conventional PCR reactions suffer from the drawback that in most cases more than one PCR reaction is needed to conclusively determine the *Hp* genotype [27]. To this end, we have developed a genotyping method in which one PCR reaction suffices in all cases. Furthermore, our method is directly applicable to DBS and does not require a separate time-consuming and expensive DNA extraction step. In addition, our method also allows the simultaneous detection of the *Hp* A-61C polymorphism, a polymorphism in the promoter region of the *Hp* gene which has been associated with ahaptoglobinemia [28].

Conclusively, there are numerous new developments which are rejuvenating the “old technique” of DBS sampling and which support the increased use of DBS. However, one should always bear in mind that, despite great progress which has been made in the context of DBS sampling, there will always be some limitations and disadvantages associated with DBS (sampling). E.g. in some circumstances rapid access to analytical results is required and there is no time to wait until the sample is dry. In these cases, the use of wet (micro)sampling techniques is still recommended. The use of DBS sampling could be encouraged for home monitoring, in remote areas,… The encouraging results that have been obtained with DBS, combined with the particular advantages of DBS (sampling), such as the possibility of prompt and convenient sampling and the increased stability of DBS -both of which can be of key importance in driving under the influence of drugs cases- suggest that DBS analysis could also play a role in e.g. future road-side drug testing. Furthermore, a wide range of other toxicological applications can be envisaged for DBS analysis as well, such as analyses in the context of drug-facilitated sexual assault, the follow-up of drug or alcohol addicts, workplace monitoring, TDM and toxicological analysis of post-mortem samples.

References

1. Stove C, Ingels A, De Kesel P, Lambert W. Dried blood spots in toxicology: from the cradle to the grave? *Crit Rev Toxicol* 42(3), 230-243 (2012).
2. De Kesel P, Sadones N, Capiau S, Lambert W, Stove C. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis* 5(16), 2023-2041 (2013).
3. Fokkema MR, Bakker AJ, de Boer F, Kooistra J, de Vries S, Wolthuis A. HbA1c measurements from dried blood spots: validation and patient satisfaction. *Clin Chem Lab Med* 47(10), 1259-1264 (2009).
4. Kromdijk W, Mulder JW, Smit PM, Ter Heine R, Beijnen JH, Huitema AD. Therapeutic drug monitoring of antiretroviral drugs at home using dried blood spots: a proof-of-concept study. *Antivir Ther* 18(6), 821-825 (2013).
5. Ingels AS, Hertegonne KB, Lambert WE, Stove CP. Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home: an exploratory study. *CNS drugs* 27(3), 233-237 (2013).
6. Bowen CL, Hemberger MD, Kehler JR, Evans CA. Utility of dried blood spot sampling and storage for increased stability of photosensitive compounds. *Bioanalysis* 2(11), 1823–1828 (2010).
7. Bowen CL, Volpatti J, Cades J, Licea-Perez H, Evans CA. Evaluation of glucuronide metabolite stability in dried blood spots. *Bioanalysis* 4(23), 2823–2832 (2012).
8. D'Arienzo CJ, Ji QC, Discenza L *et al.* DBS sampling can be used to stabilize prodrugs in drug discovery rodent studies without the addition of esterase inhibitors. *Bioanalysis* 2(8), 1415–1422 (2010).
9. Faller A, Richter B, Kluge M, Koenig P, Seitz HK, Skopp G. Stability of phosphatidylethanol species in spiked and authentic whole blood and matching dried blood spots. *Int J Legal Med* 127(3), 603-610 (2013).
10. Parker SP, Cubitt WD. The use of the dried blood spot sample in epidemiological studies. *J Clin Pathol* 52(9), 633-639 (1999).
11. Hoogtanders K, van der Heijden J, Christiaans M, van de Plas A, van Hooff J, Stolk L. Dried blood spot measurement of tacrolimus is promising for patient monitoring. *Transplantation* 83(2), 237–238 (2007).
12. Déglon J, Versace F, Lauer E *et al.* Rapid LC-MS/MS quantification of the major benzodiazepines and their metabolites on dried blood spots using a simple and cost-effective sample pretreatment. *Bioanalysis* 4(11), 1337-1350 (2012).
13. Versace F, Déglon J, Lauer E, Mangin P, Staub C. Automated DBS extraction prior to hilic/RP LC-MS/MS target screening of drugs. *Chromatographia* 76(19-20), 1281-1293 (2013).
14. Ooms JA, Knegt L, Koster EH. Exploration of a new concept for automated dried blood spot analysis using flow-through desorption and online SPE–MS/MS. *Bioanalysis* 3(20), 2311– 2320 (2011).
15. Heinig K, Wirz T, Bucheli F, Gajate-Perez A. Determination of oseltamivir (Tamiflu®) and oseltamivir carboxylate in dried blood spots using offline or online extraction. *Bioanalysis* 3(4), 421–437 (2011).

16. Abu-Rabie P, Spooner N. Dried matrix spot direct analysis: evaluating the robustness of a direct elution technique for use in quantitative bioanalysis. *Bioanalysis* 3(24), 2769–2781 (2011).
17. Déglon J, Thomas A, Daali Y *et al.* Automated system for on-line desorption of dried blood spots applied to LC–MS/MS pharmacokinetic study of flurbiprofen and its metabolite. *J Pharm Biomed Anal* 54(2), 359–367 (2011).
18. Déglon J, Thomas A, Mangin P, Staub C. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Anal Bioanal Chem* 402(8), 2485-2498 (2012).
19. Ganz N, Singrasa M, Nicolas L *et al.* Development and validation of a fully automated online human dried blood spot analysis of bosentan and its metabolites using the sample card and prep DBS system. *J Chrom B Analyt Technol Biomed Life Sci* 885–886, 50–60 (2012).
20. Sadones N, Capiau S, De Kesel PMM, Lambert WE, Stove CP. Spot them in the spot: analysis of abused substances using dried blood spots. *Bioanalysis* 6(17), 2211-2227 (2014).
21. Ingels A, De Paepe P, Anseeuw K *et al.* Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis* 3(20), 2271-2281 (2011).
22. Velghe S, Capiau S, Stove CP. Opening the toolbox of alternative sampling strategies in clinical routine: A key-role for (LC-)MS/MS. *Trends Analyt Chem* 84(B), 61-73 (2016).
23. Ingels A-SME, Sadones N, De Kesel PMM, Lambert WE, Stove CP. Derivatization techniques in dried blood spot analysis. In: *Dried blood spots: applications and techniques.* Li, W, Lee, MS (Eds.), John Wiley & Sons, Hoboken, NJ, USA, 344-354 (2014).
24. Chahbouni A, Sinjewel A, den Burger JCG *et al.* Rapid quantification of gabapentin, pregabalin, and vigabatrin in human serum by ultraperformance liquid chromatography with mass-spectrometric detection. *Ther Drug Monit* 35(1), 48-53 (2013).
25. Kanetake J, Kanawaku Y, Mimasaka S, Sakai J, Hashiyada M, Nata M, Funayama M. The relationship of a high level of serum beta-hydroxybutyrate to cause of death. *Leg Med (Tokyo)* 7(3), 169-174 (2005).
26. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem* 42(10), 1589-1600 (1996).
27. Ko DH, Chang HE, Kim TS, *et al*. A review of haptoglobin typing methods for disease association study and preventing anaphylactic transfusion reaction. *Biomed Res Int* 2013, 390630 (2013).
28. Teye K, Quaye IKE, Koda Y, *et al*. A-61C and C-101G Hp gene promoter polymorphisms are, respectively, associated with ahaptoglobinaemia and hypohaptoglobinaemia in Ghana. *Clin Genet* 64(5), 439-443 (2003).

SAMENVATTING

Gedroogde bloedspots vormen de rode draad doorheen dit werk. Ze kennen de laatste jaren een enorme opmars en bieden tal van mogelijkheden. Zo kunnen ze gebruikt worden voor het opsporen van heel wat verschillende stoffen, gaande van kleine exogene (lichaamsvreemde) tot grote endogene (lichaamseigen) substanties. Ook is er een toegenomen interesse vanuit diverse disciplines. De verscheidenheid aan toepassingen waarvoor gedroogde bloedspots gebruikt kunnen worden, wordt eveneens gereflecteerd in dit werk. Enerzijds zullen wij gedroogde bloedspots gebruiken voor het ontwikkelen van analytische methodes in een toxicologische context. Anderzijds tonen wij aan dat gedroogde bloedspots eveneens gebruikt kunnen worden voor genotypering. Genotypering is een proces waarbij de genetische samenstelling van een individu bepaald wordt door een bepaalde DNA sequentie te onderzoeken.

Gedroogde bloedspots worden gevormd door capillair bloed, verkregen via een vinger- of hielprik, op te vangen op een filterpapier. Deze manier van staalafname werd in de jaren ’60 geïntroduceerd voor neonatale screeningsdoeleinden, meer bepaald voor het opsporen van zeldzame metabole stoornissen in pasgeborenen via een hielprik. Nadien won deze alternatieve staalafname aan populariteit en werd deze techniek ook gebruikt in o.a. (pre-)klinische studies, farmacokinetiek, toxicologie,… De toegenomen interesse in deze alternatieve staalafname is te danken aan de vele voordelen van gedroogde bloedspots. De staalafname is namelijk weinig invasief en kan gemakkelijk worden uitgevoerd in afwezigheid van medisch personeel. Bovendien zorgt de gedroogde matrix voor een verhoogde stabiliteit en een verminderd risico op infectie. Het vergemakkelijkt eveneens de staalvoorbereiding, de bewaring en het transport. Vermits slechts een kleine hoeveelheid bloed wordt afgenomen, zijn gedroogde bloedspots ook erg geschikt voor het uitvoeren van studies op proefdieren en/of kinderen.

In **hoofdstuk 1** wordt een gedetailleerd overzicht gegeven van het gebruik van gedroogde bloedspots voor het opsporen van drugs, cotinine (afbraakproduct van nicotine), alcoholmerkers en geneesmiddelen die vaak misbruikt worden. Naast de vele voordelen worden ook de beperkingen, tekortkomingen en uitdagingen die gepaard gaan met deze diverse toxicologische toepassingen besproken. In de daaropvolgende hoofdstukken zullen we deze limitaties ook aanpakken.

Een eerste beperking is dat heel wat methodes niet worden toegepast op reële patiëntenstalen en dat er ook vaak niet wordt nagegaan of er een verschil is tussen de concentraties bekomen in capillair bloed (i.e. bloed bekomen via een vinger- of hielprik) en deze in veneus bloed (i.e. bloed afkomstig van een klassieke bloedafname via de ader). **Hoofdstuk 2** beschrijft een studie die werd opgezet om na te gaan of dergelijke capillaire-veneuze verschillen konden worden waargenomen voor de drug gamma-hydroxyboterzuur (GHB). Hiervoor werkten we samen met het St Thomas ziekenhuis in Londen aangezien ze daar op de spoedafdeling vaak geconfronteerd worden met GHB-geïntoxiceerde personen. Er werden van 50 patiënten met een vermoedelijke GHB-intoxicatie zowel veneuze als capillaire bloedspots aangemaakt. De capillaire spots werden bekomen door een druppel bloed bekomen via een vingerprik op te vangen op filterpapier. De veneuze spots werden bereid door 25 µL bloed, bekomen via een klassieke bloedafname, aan te brengen op filterpapier.

Zowel de capillaire als de veneuze bloedspots werden nadien minstens 2 uur gedroogd vooraleer ze in een plastiek zakje met droogstof bewaard werden en opgestuurd werden naar het laboratorium. Wij analyseerden deze bloedspots dan m.b.v. een accurate en gevoelige methode voor de bepaling van GHB. Hierbij maakten wij gebruik van "on-spot derivatisatie", een techniek die resulteert in een snelle en efficiënte staalvoorbereiding, gevolgd door analyse m.b.v. gaschromatografie gekoppeld aan massaspectrometrie (GC-MS). Deze studie toonde aan dat er voor GHB een goede correlatie kan worden gevonden tussen de veneuze en de capillaire concentraties. Bijgevolg kan worden geconcludeerd dat, in het geval van GHB, capillair bloed een volwaardig alternatief is voor veneus bloed.

Één van de voordelen van gedroogde bloedspots is dat er slechts een kleine hoeveelheid bloed wordt afgenomen. Dit leidt echter ook tot een bijkomende uitdaging bij het ontwikkelen van analytische methodes, nl. het bereiken van voldoende gevoeligheid (i.e. de mogelijkheid om lage concentraties op te sporen). Bijgevolg is erg gevoelige apparatuur nodig. Alhoewel de voorkeur meestal gegeven wordt aan vloeistofchromatografie gekoppeld aan (tandem) massaspectrometrie, kan in bepaalde gevallen de gewenste gevoeligheid ook bereikt worden m.b.v. GC. Hiervoor dient echter wel een derivatisatiestap geïntroduceerd te worden tijdens de staalvoorbereiding. Aangezien deze extra staalvoorbereidingsstap als tijdrovend beschouwd wordt, introduceerden we in **hoofdstuk 3** een nieuwe derivatisatiestrategie. Hierbij combineerden we "on-spot derivatisatie" met "microgolf-geassisteerde derivatisatie". In dit concept worden de derivatisatiereagentia rechtstreeks toegevoegd aan de gedroogde bloedspots en wordt de derivatisatietijd, i.e. de tijd die nodig is om de derivatisatiereactie te laten doorgaan, ingekort door de chemische reactie te laten doorgaan in een conventionele microgolfoven. De toepasbaarheid van deze nieuw ontwikkelde techniek werd nagegaan voor de kwantitatieve bepaling van GHB en het anti-epilepticum gabapentine m.b.v. GC-MS. Voor het valideren van de methode werden lineariteit, precisie, accuraatheid, selectiviteit, overdracht, mogelijkheid tot verdunnen, stabiliteit alsook de invloed van het bloedvolume en hematocriet geëvalueerd. De calibratielijnen waren lineair van 10 tot 100 µg/mL voor GHB en van 1 tot 30 µg/mL voor gabapentine. De methode was voldoende accuraat en precies. Stabiliteitsstudies toonden aan dat zowel gabapentine als GHB stabiel waren in gedroogde bloedspots die gedurende 84 dagen bij kamertemperatuur bewaard werden. Analyse van patiëntenstalen toonde aan dat "microgolf-geassisteerde on-spot derivatisatie", gevolgd door GC-MS, een snelle en betrouwbare aanpak is die ook toepasbaar is in routine toxicologie. Bovendien is de door ons ontwikkelde GC-MS methode ook geschikt voor het detecteren van andere polaire laagmoleculaire verbinden met klinische en/of forensische toxicologische relevantie, zoals vigabatrine, beta-hydroxyboterzuur (BHB), 1,4- en 1,2-butaandiol, propyleenglycol en diethyleenglycol.

Behalve een gebrek aan patiëntenstalen en de afwezigheid van onderzoek naar de correlatie tussen veneuze en capillaire concentraties, wordt er ook vaak geen vergelijking gemaakt met andere biologische vloeistoffen zoals serum of plasma. Opdat gedroogde bloedspots echter als een alternatieve staalafname zouden kunnen worden beschouwd, dient de correlatie tussen concentraties bekomen in bloedspots en deze in een referentiematrix te worden nagegaan. In **hoofdstuk 4** deden we dit voor het anti-epilepticum gabapentine. Vermits de

referentiewaarden voor deze component enkel beschikbaar zijn in plasma/serum, worden gabapentine bepalingen steeds uitgevoerd in plasma/serum. Bereiden van plasma/serum vereist echter de aanwezigheid van medisch personeel en labo-apparatuur. Aangezien het gebruik van gedroogde bloedspots hier dus zeker een voordeel zou bieden, hebben wij een vergelijkende studie opgezet waarbij de concentratie aan gabapentine zowel in capillaire bloedspots als in serum werd bepaald. Tijdens deze studie merkten we op dat de concentraties in bloed, zoals verwacht, ongeveer 15% lager lagen dan de concentraties in serum. Er bleek echter wel een goede correlatie te bestaan tussen de gemeten serum concentraties en de serum concentraties berekend uit de bloedconcentraties. Hiermee toonden we aan dat capillaire bloedspots een goed alternatief kunnen vormen voor de bepaling van gabapentine.

Een andere polaire laagmoleculaire verbinding die kan worden opgespoord met onze nieuw ontwikkelde methode is BHB. Dit ketonlichaam is een structuuranaloog van GHB en wordt in verhoogde concentraties teruggevonden bij o.a. alcoholici en diabetici. Alhoewel sterk verhoogde BHB concentraties ook lethaal kunnen zijn, wordt de bepaling van BHB niet routinematig uitgevoerd in een forensisch toxicologisch laboratorium. Enkel wanneer er o.b.v. de verkregen achtergrondinformatie een vermoeden is van ketoacidose (i.e. een sterk verhoogde BHB concentratie in bloed) wordt een BHB analyse uitgevoerd. In **hoofdstuk 5** onderzochten we of het relevant is om BHB routinematig te bepalen. Hierbij voerden we een retrospectieve BHB analyse uit op 553 bloedstalen, 232 urinestalen en 62 oogvochtstalen en merkten we op dat in het overgrote deel van de gevallen een lage BHB concentratie teruggevonden werd. We merkten echter ook op dat in de stalen waarbij de BHB concentratie hoger was dan 100 µg/mL steeds aceton, een ander ketonlichaam, teruggevonden werd. De bepaling van aceton gebeurt al routinematig en vereist geen bijkomende analyse. Hieruit concludeerden we dat aceton een goede initiële merker is voor ketoacidose en dat de bepaling van BHB beperkt kan worden tot aceton-positieve stalen.

In bovenstaande toepassingen worden gedroogde bloedspots steeds gebruikt voor het opsporen van kleine polaire moleculen m.b.v. analytische methoden, meer bepaald voor het opsporen van drugs (GHB), therapeutische geneesmiddelen (gabapentine) of lichaamseigen moleculen (BHB). Gedroogde bloedspots kunnen echter ook gebruikt worden voor het opsporen van heel wat andere componenten, zoals (sporen)elementen, proteïnen, mRNA en DNA. Laatstgenoemde toonden we aan in **hoofdstuk 6** door, vertrekkend van gedroogde bloedspots, een nieuwe methode voor haptoglobine (Hp) genotypering te ontwikkelen.

Hp is een glycoproteïne dat bindt aan vrij hemoglobine en op die manier bescherming biedt tegen weefselschade. Net zoals hoofdstuk 5 kan ook hoofdstuk 6 gelinkt worden aan diabetes. Grootschalige studies hebben immers aangetoond dat diabetici met het fenotype *Hp 2-2* een verhoogd risico hebben op het ontwikkelen van complicaties. Dit toont het belang aan van *Hp* fenotypering. Aangezien er een perfecte correlatie is tussen het *Hp* geno- en fenotype, vormen *Hp* genotyperingsmethoden een volwaardig alternatief voor tijdrovende fenotyperingsmethoden. Bestaande methodes voor *Hp* genotypering hebben echter als

nadeel dat meer dan 1 PCR amplificatie-reactie nodig is. Bijgevolg hebben we een verbeterde PCR-reactie ontwikkeld waarmee het *Hp* genotype eenvoudig kan worden bepaald a.d.h.v. 1 PCR-reactie. Bovendien is onze methode direct toepasbaar op gedroogde bloedspots en is geen voorafgaande tijdrovende en dure DNA extractie vereist. Ook laat onze methode toe om tegelijkertijd het *Hp* A-61C polymorfisme, i.e. een polymorfisme dat kan worden gelinkt aan ahaptoglobinemie (= afwezigheid van Hp in het bloed), op te sporen.

APPENDIX

Appendix 1 Overview of abused substances determined in DBS, as discussed in Chapter 1.

Drug category	Analyte	Reference	Analytical Technique	LLOQ Assay Range	C = complete DBS / P = DBS punch Sample Preparation Recovery	Application	Stability of DBS
Cannabinoids	THC, THC-COOH and its glucuronide, THC-OH	[11]	LC-MS/MS	NS	C: 5 µL (10 mm ∅ punch covers complete spot) PAPER: Whatman® 903 SAMPLE PREP: online extraction with ACN:15mM CH_3COONH_4 (97:3)	No	NS
	THC and THC-COOH	[26]	GC-MS	5-100 ng/mL (THC) 5-500 ng/mL (THC-COOH)	C: 100 µL PAPER: NS SAMPLE PREP: - 500 µL saturated borate buffer (pH 10) + 2 mL butyl acetate - separation in fraction A & B - evaporated, reconstituted and derivatized with MTBSTFA/ MSTFA (fraction A/B) RECOV: 41.2% (THC) 32.2% (THC-COOH)	No	NS
	THC	[12]	LC-MS/MS	± 50 ng/mL	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	No	NS
	THC and THC-COOH	[94]	LC-HRMS	1 ng/mL	C: 20 µL PAPER: Sartorius TFN SAMPLE PREP: - 100 µL MeOH + 400 µL TBME - 300 µL acetone (2nd extraction) - solvents evaporated and re-dissolved in ACN:H_2O (60:40) RECOV: 19% (THC) 36% (THC-COOH)	No	7 d at 4°C
	THC, THC-OH and THC-COOH	[7]	LC-MS/MS	2.5-2000 ng/mL (THC) 5-2000 ng/mL (THC-OH & THC-COOH) !! [ ]'s are those in a MeOH solution spiked to the DBS	P: 7 mm ∅ PAPER: Whatman® 903 SAMPLE PREP: - 1 mL MeOH - evaporated and re-dissolved in mobile phase RECOV: 81-85%	Yes (capillary DBS of subjects undergoing drug addiction treatment) Comparison with plasma	3 months at RT
	THC, THC-OH and THC-COOH	[93]	LC-MS/MS	15 ng/mL	C: ± 30 µL PAPER: Whatman® 903 Protein Saver cards	Yes (capillary DBS of drug abusers) Comparison with urine immunoassay	3 months at RT

					SAMPLE PREP: - 10 µL IS + 990 µL MeOH - evaporated and re-dissolved in mobile phase RECOV: 81.1% (THC) 79.0% (THC-OH) 78.3% (THC-COOH)		
Cocaine and metabolites (benzoylecgonine (BE), cocaethylene (CE), ecgonine methylester (ECME), norcocaine)	BE	[19]	RIA	10-600 ng/mL	P: 6.4 mm ∅ PAPER: Whatman® 903 SAMPLE PREP: 200 µL PBS:Tween 20 RECOV: >95%	Yes (autopsy samples & newborn DBS)	>1024 h at 25, 45, 55°C (BE) 108 h at 45°C (cocaine)
	BE	[56]	LC-MS/MS	± 2-166 ng/mL	P: 6.4 mm ∅ (reconstituted blood) SAMPLE PREP: - 200 µL 2mM aqueous CH_3COONH_4 - PP: 1 mL MeOH - supernatant filtered, evaporated and re-dissolved in mobile phase	Yes (capillary DBS from drug abusers and DBS from newborns & mothers) Comparison with RIA screening	NS
	BE	[97]	RIA, LC-MS	/	P: 6.4 mm ∅ Procedures of Henderson *et al.*, 1993 [19] & Sosnoff *et al.*, 1996 [56]	Yes (newborn DBS)	/
	Cocaine	[77]	LC-MS/MS	24.6 ng/mL 50-2000 ng/mL	C: 100 µL (reconstituted blood) PAPER: Whatman® 903 SAMPLE PREP: - phosphate buffer (3.5 mL, pH6) - SPE - evaporated and re-dissolved in mobile phase RECOV: 90-97%	No	1 month at -20°C & 4°C 1 month at RT: 19.9% ↓ (DBS from spiked blood)
	Cocaine, BE and CE	[6]	LC-FLUO	20-1000 ng/mL (cocaine & BE) 12-1000 ng/mL (CE)	C: 10 µL (10 mm ∅ punch) PAPER: Whatman® 903 SAMPLE PREP: - 500 µL MeOH - evaporated and re-dissolved in mobile phase RECOV: 92-93%	Yes (capillary DBS of drug abusers) Comparison with plasma	NS
	Cocaine	[26]	GC-MS	50-1000 ng/mL	C: 100 µL PAPER: NS SAMPLE PREP: - 500 µL saturated borate buffer	No	NS

					(pH 10) + 2 mL butyl acetate - separation in fraction A & B - evaporated, reconstituted and derivatized with MTBSTFA/ MSTFA (fraction A/B) RECOV: 83.2%		
	Cocaine and BE	[12,13]	LC-MS/MS	± 1 ng/mL	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	No	NS
	Cocaine, CE, BE and ECME	[8]	LC-MS/MS	NS (screening technique) LOD < 1 ng/mL (cocaine and CE) LOD 1 ng/mL (BE) LOD 10 ng/mL (ECME)	C: 5 µL (6 mm ∅ punch) PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (automated) RECOV: 49-60% (cocaine) 47-58% (CE) 10-34% (BE) 77% (ECME)	Yes (post-mortem samples) Comparison with routine STA screening on whole blood, as well as with GC-MS(MS) and LC-MS/MS quantitative analyses	NS
	Cocaine, BE, CE and ECME	[31]	LC-MS/MS	5-200 ng/mL	P: 3 mm ∅ of 30 µL DBS PAPER: Whatman® 903 SAMPLE PREP: 150 µL H_2O RECOV: 100% (cocaine) 99.4% (BE) 85.9% (ECME)	Yes (venous) (BE) (DUID) Comparison with whole blood	6 months at: -20°C (cocaine, BE, ECME) 4°C (BE) 4°C: ±55% ↓ (cocaine) 4°C: ±90% ↓ (ECME)
	Cocaine	[94]	LC-HRMS	0.25 ng/mL	C: 20 µL PAPER: Sartorius TFN SAMPLE PREP: - 100 µL MeOH + 400 µL TBME - 300 µL acetone (2nd extraction) - solvents evaporated and re-dissolved in ACN:H_2O (60:40) RECOV: 67%	No	7 d at 4°C (cocaine)
	Cocaine, BE	[96]	LC-MS/MS	3.5 ng/mL (cocaine) 2.3 ng/mL (BE) 20-400 ng/ml (cocaine & BE)	C: 20 µL PAPER: Whatman® 2012-10 filter cards SAMPLE PREP: - 5 mL MeOH - evaporated and re-dissolved in mobile phase RECOV:103% (cocaine) 100% (BE)	Yes (venous DBS of polydrug abusers) Comparison with whole blood	NS
	Cocaine, BE, CE and ECME	[47]	LC-MS/MS	2 ng/mL	3 x 3 mm ∅ punches PAPER: Bond Elut Dried Matrix Spotting cards	Yes (post-mortem samples) Comparison with routine analysis	NS

					SAMPLE PREP: - 900 µL MeOH/0.1% formic acid - evaporated and re-dissolved in 100 µL of water with 0.1% of formic acid RECOV: 12-54% (cocaine) 20-44% (BE) 11-45% (CE) 25-34% (ECME)		
	Cocaine, BE and norcocaine	[63]	LC-HRMS	1-200 ng/mL	P: 3 mm ∅ PAPER: Whatman® 903 SAMPLE PREP: - 1 mL 1% formic acid in water - SPE - evaporated and re-dissolved in mobile phase RECOV: 43.3-52.1% (cocaine) 48.6-64.5% (BE) 35.2-48.6% (norcocaine)	Yes (capillary DBS following controlled intravenous cocaine administration) Comparison with venous blood	24 h at RT, 72 h at 4°C, 72 h at -20°C, 3 freeze-thaw cycles
	Cocaine and BE	[93]	LC-MS/MS	5 ng/mL	C: ± 30 µL PAPER: Whatman® 903 Protein Saver cards SAMPLE PREP: - 10 µL IS + 990 µL MeOH - evaporated and re-dissolved in mobile phase RECOV: 90.8% (cocaine) 89.9% (BE)	Yes (capillary DBS of drug abusers) Comparison with urine immunoassay	3 months at RT
Opiates	6-monoacetylmorphine (6-MAM)	[79]	LC-MS/MS	NS	C: 100 µL PAPER: Whatman® 903 SAMPLE PREP: 0.5% NH_4OH ⇒ LLE (chloroform/isopropanol)	No	6 d at 4°C, RT & 40°C
	Morphine and 6-MAM	[67]	LC-MS/MS	14 ng/mL (morphine) 27 ng/mL (6-MAM) 50-500 ng/mL (morphine & 6-MAM)	C: 100 µL PAPER: Whatman® BFC 180 SAMPLE PREP: - 1mL 0.1M borate buffer (pH 8.5) - 1.5 mL ethyl acetate (LLE) - evaporated and reconstituted in suitable solvent RECOV: 23-37% (morphine) 76-86% (6-MAM)	Yes (venous DBS) (drug abusers) Comparison with whole blood	10% ↓ in both morphine & 6-MAM upon drying Morphine: 7 d at 4°C, -20°C; 5 d at 40°C 6-MAM: 5 d at 40°C: ±50% ↓
	Morphine, codeine and their glucuronides	[11]	LC-MS/MS	NS	C: 5 µL (10 mm ∅ punch covers complete spot)	No	NS

					PAPER: Whatman® 903 SAMPLE PREP: online extraction with ACN:15 mM CH_3COONH_4 (97:3)		
	Oxycodone	[116]	LC-MS/MS	100 pg/mL	RECOV: ±85%	No	NS
	Morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G)	[100]	LC-MS/MS	1-1000 ng/mL (morphine & M3G) 2.5-1000 ng/mL (M6G)	P: 6.4 mm ∅ from 50 µL spot PAPER: Whatman® 903 SAMPLE PREP: - 100 µL H_2O - PP RECOV: 99.6-108.3% (morphine) 95.6-102% (M3G) 99.7-103.3% (M6G)	Yes (method used in PK studies)	≥ 3 freeze-thaw cycles; ≥ 3 d at RT 7 d at 4°C, -20°C, -80°C
	Morphine, hydro-morphone, oxycodone and noroxycodone	[101]	LC-MS/MS	NS	C: 100 µL PAPER: Whatman® 903	Yes (venous DBS) Comparison with whole blood	NS
	Morphine and codeine	[26]	GC-MS	10-1000 ng/mL	C: 100 µL PAPER: NS SAMPLE PREP: - 500 µL saturated borate buffer (pH 10) + 2 mL butyl acetate - separation in fraction A & B - evaporated, reconstituted and derivatized with MTBSTFA/ MSTFA (fraction A/B) RECOV: 50% (morphine) >75% (codeine)	No	NS
	Morphine, codeine, 6-MAM and hydrocodone	[12,13]	LC-MS/MS	± 20 ng/mL (morphine) ± 5 ng/mL (codeine) ± 1 ng/mL (6-MAM, hydrocodone)	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	No	NS
	Morphine, 6-MAM, M3G and codeine	[8]	LC-MS/MS	NS (Screening technique) LOD 100 ng/mL (morphine) LOD 5 ng/mL (6-MAM) LOD 10 ng/mL (M3G) LOD 50 ng/mL (codeine)	C: 5 µL (6 mm ∅ punch) PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (automated) RECOV: 20% (morphine) 38% (6-MAM) 17% (M3G) 40% (codeine)	Yes (post-mortem samples) Comparison with routine STA screening on whole blood, as well as with GC-MS(MS) and LC-MS/MS quantitative analyses	NS
	Morphine, hydromorphone, codeine, hydrocodone, oxymorphone, dihydrocodeine and oxycodone	[45]	LC-MS/MS	5-1000 ng/mL Except oxycodone 10-1000 ng/mL	2 x 5 mm ∅ punches PAPER: Ahlstrom 226 SAMPLE PREP: 5% formic acid RECOV: ±65%	Yes Comparison with plasma	32 d

	Morphine, codeine, M3G and M6G	[31]	LC-MS/MS	5-200 ng/mL (morphine, codeine) 10-200 ng/mL (M3G & M6G)	P: 3 mm ∅ of 30 µL DBS PAPER: Whatman® 903 SAMPLE PREP: 150 µL H_2O RECOV: 90% (morphine) 93.2% (codeine)	Yes (venous DBS) Comparison with whole blood (morphine)	6 months at -20°C
	Morphine	[61]	LC-MS/MS	4-1000 ng/mL	P: 3 mm ∅ PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH:H_2O (1:9)	No	NS
	Codeine, morphine and 6-MAM	[96]	LC-MS/MS	9.4 ng/mL (codeine) 10 ng/mL (morphine) 11 ng/mL (6-MAM) 100-2000 ng/mL	C: 20 µL PAPER: Whatman® 2012-10 filter cards SAMPLE PREP: - 5 mL MeOH - evaporated and re-dissolved in mobile phase RECOV: 101% (codeine) 98% (morphine) 100% (6-MAM)	Yes (venous DBS of polydrug abusers) Comparison with whole blood and plasma	NS
	Morphine and 6-MAM	[47]	LC-MS/MS	2 ng/mL	3 x 3 mm ∅ punches PAPER: Bond Elut Dried Matrix Spotting cards SAMPLE PREP: - 900 µL MeOH/0.1% formic acid - evaporated and re-dissolved in 100 µL of water with 0.1% of formic acid RECOV: 16-33% (morphine) 17-46% (6-MAM)	Yes (post-mortem samples) Comparison with routine analysis	NS
	Morphine, codeine, oxycodone and hydrocodone	[40]	LC-MS/MS	1-500 ng/mL	P: 2 mm ∅ ≈ clamp size PAPER: Ahlstrom 226 SAMPLE PREP: DBSA: - automated flow-through elution of DBS cards - online SPE - elution from the SPE cartridge onto the LC column using the LC mobile phase RECOV: 77.3-78.1% (morphine) 68.8-70.1% (codeine) 72.9-78.0% (oxycodone) 71.7-74.0% (hydrocodone)	Yes (capillary DBS of volunteer who was administered 10 mg hydrocodone)	Morphine: 30 d at RT, 55 d at 4°C, 95 d at -20°C Codeine, oxycodone, hydrocodone: 15 d at RT, 95 d at 4°C & -20°C
	Morphine, codeine and 6-MAM	[93]	LC-MS/MS	5 ng/mL	C: ± 30 µL PAPER: Whatman® 903 Protein Saver	Yes (capillary DBS of drug abusers)	3 months at RT

					cards SAMPLE PREP: - 10 µL IS + 990 µL MeOH - evaporated and re-dissolved in mobile phase RECOV: 83.6% (morphine) 88.0% (codeine) 89.2% (6-MAM)	Comparison with urine immunoassay	
Non-opiate opioids	Methadone and metabolites (EDDP, EMDP)	[30]	LC-MS/MS	0.1-100 ng/mL	P: 6.4 mm ∅ PAPER: Whatman® 903 SAMPLE PREP: - 100 µL H_2O - PP - online extraction RECOV: NS	No	NS
	Methadone	[26]	GC-MS	10-1000 ng/mL	C: 100 µL PAPER: NS SAMPLE PREP: - 500 µL saturated borate buffer (pH 10) + 2 mL butyl acetate - separation in fraction A & B - evaporated, reconstituted and derivatized with MTBSTFA/ MSTFA (fraction A/B) RECOV: 78.5%	No	NS
	Methadone and EDDP	[12,13]	LC-MS/MS	± 1 ng/mL (methadone) ± 5 ng/mL (EDDP)	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	NS	NS
	Methadone	[8]	LC-MS/MS	NS Screening technique LOD < 1 ng/mL	C: 5 µL (6 mm ∅ punch) PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (automated) RECOV: 36-56% (methadone)	Yes (post-mortem samples) Comparison with routine STA screening on whole blood, as well as with GC-MS(MS) and LC-MS/MS quantitative analyses	NS
	Methadone	[45]	LC-MS/MS	5-1000 ng/mL	2 x 5 mm ∅ punches PAPER: Ahlstrom 226 SAMPLE PREP: 5% formic acid RECOV: ±65%	Yes Comparison with plasma	32 d
	Methadone	[20]	LC-Coulometry	4 ng/mL 4-500 ng/mL (on-column concentration)	C: Theoretical volume calculated from ∅ PAPER: Whatman® FTA® classic SAMPLE PREP: - 250 µL phosphate buffer/ACN	Yes (capillary DBS from methadone maintenance patients) Comparison with plasma	1 month at RT

					(20:80): microwave-assisted elution - evaporated and re-dissolved in 250µL phosphate buffer - SPE: MEPS - evaporated and re-dissolved in mobile phase RECOV: 90.1-94.8%		
	Methadone	[10]	LC-MS	2 ng/mL 17.5-560 ng/mL	C: 10 µL (8 mm Ø) PAPER: alginate and chitosan foam SAMPLE PREP: - foam dissolved in 300 µL 1 mM HCl - EME: 10 min, 21µL 10 mM HCOOH - diluted 1:1 with mobile phase RECOV: 105% (alginate) 115% (chitosan)	No	50 d at -18°C, 4°C, RT & 37°C
	Methadone Pethidine	[36]	LC-MS/MS	0.3 ng/mL	P: 3 mm ∅ PAPER: Agilent Bond Elut DMS SAMPLE PREP: - 500 µL MeOH:0.1% HCOOH (80:20) - diluted with 10 mM HCl (1:1) - EME (25 min; 25 µL 10 mM HCl) RECOV: 48% (methadone) 17% (pethidine)	No	NS
	Methadone	[47]	LC-MS/MS	0.2-50 ng/mL	3 x 3 mm ∅ punches PAPER: Bond Elut Dried Matrix Spotting cards SAMPLE PREP: - 900 µL MeOH/0.1% formic acid - evaporated and re-dissolved in 100 µL of water with 0.1% of formic acid RECOV: 15-31%	Yes (post-mortem samples) Comparison with routine analysis	NS
	Methadone and EDDP	[93]	LC-MS/MS	5 ng/mL	C: ± 30 µL PAPER: Whatman® 903 Protein Saver cards SAMPLE PREP: - 10 µL IS + 990 µL MeOH - evaporated and re-dissolved in mobile phase	Yes (capillary DBS of drug abusers) Comparison with urine immunoassay	3 months at RT

					RECOV: 81.7% (methadone) 79.5% (EDDP)		
	Buprenorphine and metabolites (norbuprenorphine and their glucuronides)	[11]	LC-MS/MS	NS	C: 5 µL (10 mm ∅ punch covers complete spot) PAPER: Whatman® 903 SAMPLE PREP: online extraction with ACN:15 mM CH_3COONH_4 (97:3)	Yes (animal study)	NS
	Buprenorphine	[116]	LC-MS/MS	NS (>25 pg/mL)	RECOV: ±45%	No	NS
	Buprenorphine	[26]	GC-MS	5-100 ng/mL	C: 100 µL PAPER: NS SAMPLE PREP: - 500 µL saturated borate buffer (pH 10) + 2 mL butyl acetate - separation in fraction A & B - evaporated, reconstituted and derivatized with MTBSTFA/ MSTFA (fraction A/B) RECOV: 49.8%	No	NS
	Buprenorphine	[13]	LC-MS/MS	NS	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	No	NS
	Buprenorphine	[45]	LC-MS/MS	1-100 ng/mL	2 x 5 mm ∅ punches PAPER: Ahlstrom 226 SAMPLE PREP: 5% formic acid RECOV: ±65%	Yes Comparison with plasma	21 d
	Fentanyl and metabolites (norfentanyl and despropionylfentanyl)	[103]	LC-MS/MS	0.1-100 ng/mL (fentanyl) 0.25-100 ng/mL (norfentanyl & despropionylfentanyl)	P: 6.4 mm ∅ from 50 µL spot PAPER: Whatman® 903 SAMPLE PREP: - 100 µL H_2O - PP RECOV: 75-78%	Yes (PK study in neonates & children)	DBS from spiked blood 6 d at -20°C & -80°C 3 d at RT (all 3 analytes) 5 d at RT (fentanyl)
	Fentanyl and norfentanyl	[101]	LC-MS/MS	NS	C: 100 µL PAPER: Whatman® 903	Yes (venous DBS) Comparison with whole blood	NS
	Fentanyl	[12,13]	LC-MS/MS	± 0.2 ng/mL	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	NS	NS
	Fentanyl	[45]	LC-MS/MS	0.5-100 ng/mL	2 x 5 mm ∅ punches PAPER: Ahlstrom 226 SAMPLE PREP: 5% formic acid RECOV: ±65%	Yes Comparison with plasma	32 d
	Fentanyl, alfentanyl, norfentanyl, sufentanyl	[47]	LC-MS/MS	0.2 ng/mL	3 x 3 mm ∅ punches	Yes (post-mortem samples)	NS

					PAPER: Bond Elut Dried Matrix Spotting cards SAMPLE PREP: - 900 µL MeOH/0.1% formic acid - evaporated and re-dissolved in 100 µL of water with 0.1% of formic acid RECOV: 14-54% (fentanyl) 14-52% (alfentanyl) 22-48% (sufentanyl) 21-44% (norfentanyl)	Comparison with routine analysis	
	Fentanyl	[40]	LC-MS/MS	0.1-50 ng/mL	P: 2 mm ∅ ≈ clamp size PAPER: Ahlstrom 226 SAMPLE PREP: DBSA: - automated flow-through elution of DBS cards - online SPE - elution from the SPE cartridge onto the LC column using the LC mobile phase RECOV: 73.5-74.6%	No	Fentanyl: 30 d at RT, 55 d at 4°C, 95 d at -20°C
	Tramadol	[26]	GC-MS	50-5000 ng/mL	C: 100 µL PAPER: NS SAMPLE PREP: - 500 µL saturated borate buffer (pH 10) + 2 mL butyl acetate - separation in fraction A & B - evaporated, reconstituted and derivatized with MTBSTFA/ MSTFA (fraction A/B) RECOV: 78%	No	NS
	Tramadol	[12]	LC-MS/MS	± 0.5 ng/mL	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	NS	NS
	Tramadol and ODM-tramadol	[45]	LC-MS/MS	5-1000 ng/mL	2 x 5 mm ∅ punches PAPER: Ahlstrom 226 SAMPLE PREP: 5% formic acid RECOV: ±65%	Yes Comparison with plasma	32 d
Benzodiazepines	Diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam and temazepam	[77]	LC-MS/MS	9.9 ng/mL (diazepam) 15.8 ng/mL (flunitrazepam) 11 ng/mL (lorazepam) 18 ng/mL (nitrazepam)	C: 100 µL (reconstituted blood) PAPER: Whatman® 903 SAMPLE PREP: - phosphate buffer (3.5 mL, pH6)	No	(DBS from spiked blood) 1 month at -20°C 1 month at 4°C 1 month at RT: Diazepam: 12.3% ↓

				20.6 ng/mL (oxazepam) 10.8 ng/mL (temazepam) 50-2000 ng/mL	- SPE - evaporated and re-dissolved in mobile phase RECOV: 81-106% (diazepam)		Flunitrazepam: 15% ↓ Lorazepam: 11% ↓; Nitrazepam: 15.5% ↓ Oxazepam: 12% ↓ Temazepam: 15% ↓
	Midazolam	[117,118]	LC-MS/MS	100 pg/mL	P: 4 mm ∅ of 20 µL DBS SAMPLE PREP: MeOH	Yes	At -80°C, -20°C and at RT
	Diazepam, nordiazepam, oxazepam and oxazepam glucuronide	[11]	LC-MS/MS	NS	C: 5 µL (10 mm ∅ punch covers complete spot) PAPER: Whatman® 903 SAMPLE PREP: online extraction with ACN:15 mM CH_3COONH_4 (97:3)	No	NS
	Midazolam	[119]	LC-MS/MS	0.35 ng/mL 0.35-72 ng/mL	P: 3 mm ∅ PAPER: Whatman® FTA® Elute™ SAMPLE PREP: 100 µL MeOH	Yes (clinical samples)	5 d at RT
	Alprazolam	[120]	LC-MS/MS	0.1-500 ng/mL	P: 4 mm ∅ PAPER: DMPK A, B and C cards SAMPLE PREP: - 100 µl MeOH - supernatant diluted with H_2O (1:1)	No	NS
	Midazolam	[46]	LC-MS/MS	0.1-100 ng/mL Except when using a 3-mm ∅ punch: 0.3-100 ng/mL	P: 6 mm ∅ (fully validated) 3 mm ∅ (partially validated) 2 x 3 mm ∅ (partially validated) PAPER: Ahlstrom 226 SAMPLE PREP: - 50 µL H_2O to pre-wet - 1000 µL MeOH - evaporated and re-dissolved in a suitable solvent RECOV: ±40%	Yes (phenotyping study) Comparison with plasma and whole blood	4 w at RT
	Alprazolam	[91]	LC-MS/MS	0.7 ng/mL 2.5-50 ng/mL	C: 100µL (18 mm ∅ punch covers complete spot) PAPER: Whatman® 903 SAMPLE PREP: - 1 mL borate buffer pH 8.5 + 1mL toluene/isoamylalc. (95:5) => LLE - evaporated and re-dissolved in mobile phase	Yes (venous DBS) (DUID study) Comparison with whole blood	NS
	Lorazepam, alprazolam, clonazepam, midazolam, nitrazepam, phenazepam, nordiazepam, oxazepam,	[26]	GC-MS	5-250 ng/mL (lorazepam) 5-500 ng/mL (alprazolam & clonazepam) 10-1000 ng/mL (midazolam &	C: 100 µL PAPER: NS SAMPLE PREP: - 500 µL saturated borate buffer (pH 10) + 2 mL butyl acetate	No	NS

	diazepam and temazepam)			nitrazepam) 20-1000 ng/mL (phenazepam) 20-2000 ng/mL (nordiazepam) 50-1250 ng/mL (oxazepam) 50-2500 ng/mL (diazepam) 50-5000 ng/mL (temazepam)	- separation in fraction A & B - evaporated, reconstituted and derivatized with MTBSTFA/ MSTFA (fraction A/B) RECOV: 87.4-96.6%		
	Midazolam, alprazolam and OH-alprazolam	[37]	LC-MS/MS	1-500 ng/mL	P: 3 mm ∅ PAPER: Ahlstrom 226 SAMPLE PREP: - 250 µL MeOH (semi-automated extraction) - evaporated and re-dissolved in a suitable solvent	No	NS
	Prazepam, clobazam, flurazepam, midazolam, triazolam, alprazolam, 7-aminoflunitrazepam, nitrazepam, nordiazepam, temazepam, OH-midazolam, flunitrazepam, lorazepam, clonazepam, diazepam and oxazepam	[12,13]	LC-MS/MS	± 0.1 ng/mL (prazepam) ± 0.5 ng/mL (clobazam, flurazepam, midazolam, triazolam); ± 1 ng/mL (alprazolam, 7-aminoflunitrazepam, nitrazepam, nordiazepam, temazepam), ± 2 ng/mL (OH-midazolam) ± 5 ng/mL (flunitrazepam, lorazepam) ± 10 ng/mL (clonazepam, diazepam, oxazepam)	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	No	NS
	Diazepam, 7-aminoclonazepam, midazolam and OH-midazolam	[8]	LC-MS/MS	NS Screening technique LOD 50 ng/mL (diazepam) LOD 100 ng/mL (7-aminoclonazepam) LOD <1 ng/mL (midazolam) LOD 5 ng/mL (OH-midazolam)	C: 5 µL (6 mm ∅ punch) PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (automated) RECOV: 42% (diazepam) 25% (7-aminoclonazepam) 48% (midazolam) 40% (OH-midazolam)	Yes (post-mortem samples) Comparison with routine STA screening on whole blood, as well as with GC-MS(MS) and LC-MS/MS quantitative analyses	NS
	Desmethylflunitrazepam, flurazepam,	[5]	LC-MS/MS	1ng/mL 0.5-100 ng/mL	C: 5 µL (6 mm ∅ punch covers complete spot)	Yes (venous DBS) (DUID)	30 d at -20°C & RT

	lormetazepam, triazolam, alprazolam, OH-alprazolam, clonazepam, 7-aminoclonazepam, desalkylflurazepam, flunitrazepam, 7-aminoflunitrazepam, lorazepam, midazolam, OH-midazolam, clobazam, diazepam, nitrazepam, nordiazepam, oxazepam, prazepam and temazepam			(desmethylflunitrazepam, flurazepam, lormetazepam, triazolam) 4 ng/mL 2-500 ng/mL (alprazolam, OH-alprazolam, clonazepam, 7-aminoclonazepam, desalkylflurazepam, flunitrazepam, 7-aminoflunitrazepam, lorazepam, midazolam, OH-midazolam) 40 ng/mL 20-5000 ng/mL (clobazam, diazepam, nitrazepam, nordiazepam, oxazepam, prazepam, temazepam)	PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (*in-vial* extraction) RECOV: 51.9-98%	Comparison with routine LC-MS method	
	Midazolam	[39]	LC-HRMS	5-1000 ng/mL (online)	P: 2 mm ∅ ≈ clamp size SAMPLE PREP: - SCAP: DBS cards were automatically positioned into the mobile phase flow path, followed by online extraction, using the mobile phase as extraction solvent. RECOV: 80.6% DMPK-C 66.2% Auto DBS cards	Yes (animal study)	50 d at RT
	Alprazolam, bromazepam, clordiazepoxide, clobazam, clonazepam, clotiazepam, diazepam, estazolam, flunitrazepam, flurazepam, lorazepam, midazolam, nitrazepam, nordiazepam, oxazepam, prazepam, temazepam, triazolam, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, OH-	[122]	LC-MS/MS	4 ng/mL (alprazolam, clonazepam) 2 ng/mL (bromazepam, chlordiazepoxide, clobazam, diazepam, estazolam, flunitrazepam, flurazepam, lorazepam, midazolam, nitrazepam, prazepam, triazolam, OH-alprazolam, OH-midazolam, OH-triazolam, desalkylflurazepam) 3.5 ng/mL (nordiazepam)	P: 6 mm ∅ PAPER: FTA DMPK-C SAMPLE PREP: - 300 µL MeOH:ACN (1/1) - evaporated and re-dissolved in 100 µL mobile phase RECOV: 62.1-82.9% (alprazolam) 63.5-84.2% (bromazepam) 59.3-97.5% (chlordiazepo-xide) 75.6-78.6% (clobazam) 59.1-79.3% (clonazepam) 69.9-86.4% (diazepam) 71.4-86.4% (estazolam) 74.4-85.3% (flunitrazepam)	No	NS

	alprazolam, OH-midazolam,OH-triazolam and desalkylflurazepam			3 ng/mL (oxazepam, temazepam) 2.5 ng/mL (7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam)	62.7-78.1% (flurazepam) 53.6-74.9% (lorazepam) 65.0-89.9% (midazolam) 85.5-92.2% (nitrazepam) 66.7-85.1% (nordiazepam) 67.8-94.1% (oxazepam) 71.3-92.6% (prazepam) 72.5-87.1% (temazepam) 75.6 -82.7% (triazolam) 65.1-78.3% (7-aminoclonazepam) 64.3-70.1% (7-aminoflunitrazepam) 60.7-79.8% (7-aminonitrazepam) 67.6-89.2% (OH-alprazolam) 67.4-79.0% (OH-midazolam) 58.7-79.0% (OH-triazolam) 73.2-93.7% (desalkylflurazepam)		
	Medazepam, oxazepam, lorazepam, alprazolam, clonazepam, midazolam, nordiazepam, lormetazepam and diazepam	[93]	LC-MS/MS	1 ng/mL (medazepam, lorazepam, alprazolam, clonazepam, midazolam, nordiazepam, lormetazepam and diazepam) 5 ng/mL (oxazepam)	C: ± 30 µL PAPER: Whatman® 903 Protein Saver cards SAMPLE PREP: - 10 µL IS + 990 µL MeOH - evaporated and re-dissolved in mobile phase RECOV: 84.8% (medazepam) 81.5% (oxazepam) 88.0% (lorazepam) 89.6% (alprazolam) 83.7% (clonazepam) 81.2% (midazolam) 88.4% (nordiazepam) 88.6% (lormetazepam) 89.3% (diazepam)	Yes (capillary DBS of drug abusers) Comparison with urine immunoassay	3 months at RT
Z-drugs	Zolpidem	[9]	LC-MS/MS	(0.1-500 ng/mL)	P: 3 mm ∅ of 15 µL DBS PAPER: Bond Elut DMS SAMPLE PREP: - 300 µL 0.1% HCOOH in 80% MeOH - evaporated and reconstituted in mobile phase RECOV: 102-110%	No	NS
	Zolpidem	[12,13]	LC-MS/MS	± 1 ng/mL	C: 5 µL	No	NS

					PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)		
	Zolpidem	[8]	LC-MS/MS	NS Screening technique LOD < 1 ng/mL	C: 5µL (6 mm ∅ punch) PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (automated) RECOV: 35%	Yes (post-mortem samples) Comparison with routine STA screening on whole blood, as well as with GC-MS(MS) and LC-MS/MS quantitative analyses	NS
	Zolpidem	[5]	LC-MS/MS	4 ng/mL 2-500 ng/mL	C: 5 µL (6 mm ∅ punch covers complete spot) PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (*in-vial* extraction) RECOV: 66.4-81.6%	Yes (venous DBS) (DUID) Comparison with routine LC-MS method	30 d at -20°C & RT
	Zolpidem	[122]	LC-MS/MS	2.5 ng/mL	P: 6 mm ∅ PAPER: FTA DMPK-C SAMPLE PREP: - 300 µL MeOH:ACN (1/1) - evaporated and re-dissolved in 100 µL mobile phase RECOV: 63.4-83.3%	No	NS
	Zopiclone	[26]	GC-MS	10-1000 ng/mL	C: 100 µL PAPER: NS SAMPLE PREP: - 500 µL saturated borate buffer (pH 10) + 2 mL butyl acetate - separation in fraction A & B - evaporated, reconstituted and derivatized with MTBSTFA/ MSTFA (fraction A/B) RECOV: 79.3%	No	NS
	Zopiclone	[12,13]	LC-MS/MS	± 2 ng/mL	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	No	NS
	Zopiclone	[91]	LC-MS/MS	1.2 ng/mL 2.5-50 ng/mL	C: 100 µL PAPER: Whatman® 903 SAMPLE PREP: - 1 mL borate buffer pH 8.5 + 1mL toluene/isoamylalc. (95:5) => LLE - evaporated and re-dissolved in mobile phase	Yes (venous DBS) (DRUID study) Comparison with whole blood	NS

	Zopiclone and ACP (2-amino-5-chloropyridine)	[78]	LC-MS/MS	6.4 ng/mL (zopiclone) 0.14 ng/mL (ACP)	C: 100 µL PAPER: Whatman® 903 SAMPLE PREP: - 1 mL borate buffer pH 8.5 + 1mL toluene/isoamylalc. (95:5) => LLE - evaporated and re-dissolved in mobile phase RECOV: 67.1-79.7% (zopiclone) 80.4-83.8% (ACP)	Yes	Authentic & spiked DBS 30 d at -20°C 22 d at 4°C 8-30 d at 20°C 3 d at 40°C
	Zopiclone	[5]	LC-MS/MS	4 ng/mL	C: 5 µL (6 mm ∅ punch covers complete spot) PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (*in-vial* extraction) RECOV: 61.6-67.2%	No	30 d at -20°C & RT
	Zaleplon	[5]	LC-MS/MS	4 ng/mL	C: 5 µL (6 mm ∅ punch covers complete spot) PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (*in-vial* extraction) RECOV: 77.4-89.8%	No	30 d at -20°C & RT
Amphetamine and analogues	Amphetamine and MDMA	[79]	LC-MS/MS	NS	C: 100 µL PAPER: Whatman® 903 SAMPLE PREP: 0.1M NaOH ⇒ LLE (ethyl acetate)	Yes (venous) Comparison with whole blood	NS
	Amphetamine, MDMA and MDA	[91]	LC-MS/MS	3.0 ng/mL 5-50 ng/mL (amphetamine) 5.7 ng/mL 50-400 ng/mL (MDMA) 0.4 ng/mL 2.5-30 ng/mL (MDA)	C: 100 µL (18 mm ∅ punch covers complete spot) PAPER: Whatman® 903 SAMPLE PREP: - 1 mL 0.01 M NaOH - LLE: 1.5 mL ethyl acetate - acidified, evaporated and re-dissolved in mobile phase	Yes (venous DBS) (DRUID study) Comparison with whole blood	NS
	MDMA and MDA	[121]	LC-MS/MS	5.7 ng/mL 5-40 & 50-400 ng/mL (MDMA) 0.40 ng/mL 0.25-3 & 2.5-30 ng/mL (MDA)	C: 100 µL (18 mm ∅ punch covers complete spot) PAPER: Whatman® 903 SAMPLE PREP: - 1 mL 0.01 M NaOH - LLE: 1.5 mL ethyl acetate - acidified, evaporated and re-dissolved in mobile phase RECOV: >85% (MDMA) >95% (MDA)	Yes (venous DBS) (DRUID study) Comparison with whole blood	NS
	Amphetamine,	[26]	GC-MS	20-2000 ng/mL	C: 100 µL	No	NS

	methamphetamine, MDA and MDMA				PAPER: NS SAMPLE PREP: - 500 µL saturated borate buffer (pH 10) + 2 mL butyl acetate - separation in fraction A & B - evaporated, reconstituted and derivatized with MTBSTFA/ MSTFA (fraction A/B) RECOV: 87.3-98.6%		
	Amphetamine, MDMA, MDA, MDEA and methamphetamine	[12,13]	LC-MS/MS	± 40 ng/mL (amphetamine) ± 2 ng/mL (MDMA) ± 20 ng/mL (MDA) ± 2 ng/mL (MDEA) ± 50 ng/mL (methamphetamine)	C: 5 µL PAPER: NS SAMPLE PREP: 100% MeOH (online extraction)	No	NS
	Amphetamine, MDMA and methamphetamine	[8]	LC-MS/MS	NS Screening technique LOD 1 ng/mL (amphetamine & MDMA) LOD 10 ng/mL (methamphetamine)	C: 5 µL (6 mm ∅ punch) PAPER: Whatman® 903 SAMPLE PREP: 100 µL MeOH (automated) RECOV: 63% (amphetamine) 74% (MDMA) 105% (methamphetamine)	Yes (post-mortem samples) Comparison with routine STA screening on whole blood, as well as with GC-MS(MS) and LC-MS/MS quantitative analyses	NS
	MDMA and MDA	[94]	LC-HRMS	(0.5-20 ng/mL) (qualitative)	C: 20 µL PAPER: Sartorius TFN SAMPLE PREP: - 100 µL MeOH + 400 µL TBME - 300 µL acetone (2nd extraction) - solvents evaporated and re-dissolved in ACN:H_2O (60:40) RECOV: 62% (MDMA) 78% (MDA)	No	7 d at 4°C
	Amphetamine, MDMA, MDA, MDEA and methamphetamine	[31]	LC-MS/MS	5-200 ng/mL	P: 3 mm ∅ of 30 µL DBS PAPER: Whatman® 903 SAMPLE PREP: 150 µL H_2O RECOV: 97.4% (amphetamine) 101.6% (MDMA) 121.6% (MDA) 88.6% (methamphetamine) NS (MDEA)	Yes (venous DBS) (DUID) Comparison with whole blood	6 months at -20°C 6 months at 4°C: ± 40% ↓ (amphetamine) ±25-30% ↓ (MDMA, MDA) ±40-50% ↓ (methamphetamine)
	Amphetamine, MDMA, MDA, MDEA and methamphetamine	[80]	LC-MS/MS	NS Screening technique LOD 5.0 ng/mL (amphetamine) LOD 2.5 ng/mL (MDMA &	C: 10 µL (10 mm ∅) PAPER: Bioanalysis cards 226 SAMPLE PREP: - 500 µL MeOH - add to 10 µL 0.25% HCl in	Yes (venous DBS) Comparison with blood	2 w at RT & 4°C

				MDA) LOD 1.0 ng/mL (MDEA) LOD 1.0 ng/mL (methamphetamine)	MeOH - evaporated and re-dissolved in mobile phase RECOV: 87.6-98.3%		
	Amphetamine, MDA, MDEA, MDMA and methamphetamine	[47]	LC-MS/MS	2 ng/mL	3 x 3 mm ∅ punches PAPER: Bond Elut Dried Matrix Spotting cards SAMPLE PREP: - 900 µL MeOH/0.1% formic acid - evaporated and re-dissolved in 100 µL of water with 0.1% of formic acid RECOV: 18-46% (amphetamine) 20-47% (MDA) 19-50% (MDEA) 19-54% (MDMA) 22-48% (methamphetamine)	Yes (post-mortem samples) Comparison with routine analysis	NS
	Amphetamine, methamphetamine, MDMA and phentermine	[41]	LC-MS/MS	5-1000 ng/mL	C: 5 µL (6 mm clamp covers complete spot) PAPER: Ahlstrom 226 SAMPLE PREP: DBSA: - automated flow-through elution of DBS cards - online SPE - elution from the SPE cartridge onto the LC column using the LC mobile phase RECOV: 90.3-100%	Yes (capillary DBS of volunteer who was administered 25 mg of phentermine)	15 d at RT (amphetamine, methamphetamine, phentermine) 30 d at RT (MDMA) 15 d at RT under N_2 flow (amphetamine, methamphetamine, phentermine) 50 d at RT under N_2 flow (MDMA) 30 d at 4°C (amphetamine, methamphetamine) 50 d at 4°C (MDMA, phentermine) 50 d at -20°C (amphetamine, methamphetamine, MDMA, phentermine)
	Amphetamine, methamphetamine, MDMA and MDA	[93]	LC-MS/MS	5 ng/mL	C: ± 30 µL PAPER: Whatman® 903 Protein Saver cards SAMPLE PREP: - 10 µL IS + 990 µL MeOH - evaporated and re-dissolved in mobile phase RECOV: 97.1% (amphetamine) 89.5% (methamphetamine)	Yes (capillary DBS of drug abusers) Comparison with urine immunoassay	3 months at RT

					99.8% (MDMA) 95.5% (MDA)		
GHB	GHB	[44]	LC-MS/MS	1-128 µg/mL	3 x 3.2 mm ∅ punches PAPER: Whatman® 903 SAMPLE PREP: - 200 µL MeOH - evaporated and re-dissolved in H_2O RECOV: > 70%	Yes (newborn DBS)	NS
	GHB	[23]	GC-MS	2-100 µg/mL	C: 50 µL PAPER: Whatman® 903 SAMPLE PREP: - *on-spot derivatization* with 100 µL TFAA & HFB (2:1) - evaporated and re-dissolved in 200 µL ethyl acetate	Yes (venous DBS) (drug abuser)	> 7 d at RT > 14 d at -20°C
	GHB	[24,25]	GC-MS	2-100 µg/mL	P: 6 mm ∅ PAPER: Whatman® 903 SAMPLE PREP: - *on-spot derivatization* with 50 µL TFAA & HFB (2:1) - evaporated and re-dissolved in 100 µL ethyl acetate	Yes (fingerprick) Comparison with venous blood & DBS from venous blood	> 148 d at RT
Ketamine and norketamine	Ketamine and norketamine	[104]	LC-MS/MS	5 ng/mL 5-2500 ng/mL	P: 3.2 mm ∅ from 19 µL spot PAPER: NS SAMPLE PREP: MeOH/0.2M $ZnSO_4$ (7:3) ⇒ diluted with H_2O (1:1)	No	NS
	Ketamine	[80]	LC-MS/MS	NS Screening technique LOD 1.0 ng/mL	C: 10 µL (10 mm ∅) PAPER: Bioanalysis cards 226 SAMPLE PREP: - 500 µL MeOH - add to 10 µL 0.25 % HCl in MeOH - evaporated and re-dissolved in mobile phase RECOV: 96.3%	Yes (venous DBS) Comparison with blood	2 w at RT & 4°C
	Ketamine and norketamine	[21]	LC-DAD	NS	SAMPE PREP: ultrasound-assisted extraction followed by MEPS	No	NS
	Ketamine and norketamine	[47]	LC-MS/MS	2 ng/mL	3 x 3 mm ∅ punches PAPER: Bond Elut Dried Matrix Spotting cards SAMPLE PREP: - 900 µL MeOH/0.1% formic acid - evaporated and re-dissolved	Yes (post-mortem samples) Comparison with routine analysis	NS

					in 100 µL of water with 0.1% of formic acid RECOV: 18-45% (ketamine) 18-41% (norketamine)		
New psychoactive substances	64, e.g. cathinones and piperazine derivatives	[80]	LC-MS/MS	NS Screening technique LODs included for each compound	C: 10 µL (10 mm ∅) PAPER: Bioanalysis cards 226 SAMPLE PREP: - 500 µL MeOH - add to 10 µL 0.25 % HCl in MeOH - evaporated and re-dissolved in mobile phase	Yes (venous DBS) Comparison with blood	2 w at RT & 4°C
	Mephedrone	[41]	LC-MS/MS	5-1000 ng/mL	C: 5 µL (6 mm clamp covers complete spot) PAPER: Ahlstrom 226 SAMPLE PREP: DBSA: - automated flow-through elution of DBS cards - online SPE - elution from the SPE cartridge onto the LC column using the LC mobile phase RECOV: 90.3-100%	No	5 d at RT: 50% ↓ 15 d at RT under N_2 flow 15 d at 4°C
Alcohol markers	Ethylglucuronide & ethylsulfate	[110]	LC-MS/MS	0.1-10 µg/mL	C: 10 µL (1 cm ∅ punch covers the whole spot) PAPER: 226-1004 Bioanalysis cards SAMPLE PREP: - 500 µL MeOH - evaporated and re-dissolved in mobile phase RECOV: 43% (ethylglucuronide) 48% (ethylsulfate)	Yes Comparison with whole blood	3 w at 4°C & RT
	PEth	[111,112]	LC-MS/MS	22.7 ng/mL (PEth 18:1/18:1) 87.3 ng/mL (PEth 16:0/18:1) 50-5000 ng/mL	C: 100 µL PAPER: Whatman® 903 SAMPLE PREP: - 400 µL 0.5 M CH_3COONa (pH 5) + 600 µL isopropanol - 2 x 700 µL n-hexane - evaporated and re-dissolved in mobile phase RECOV: 26.6-42.5% (PEth 18:1/18:1) 68.9-90.7% (PEth 16:0/18:1)	Yes (subjects in alcohol detoxification program) Comparison with whole blood	> 30 d at -20°C & 20°C
	PEth	[43,113,114]	LC-MS/MS	8 ng/mL (PEth 16:0/18:1)	P: 3 X 3.2 mm ∅ punches of 30 µL spot PAPER: Whatman® 903	Yes (venous DBS) Comparison with whole blood	No *ex vivo de novo* formation

					SAMPLE PREP: - 0.5 mL MeOH - evaporated and re-dissolved in mobile phase RECOV: 56.0-82.9%		
	PEth	[51]	LC-MS/MS	10-2000 ng/mL (PEth 16:0/18:1) 10-1940 ng/mL (PEth 18:1/18:1) 19-3872 ng/mL (PEth 16:0/16:0)	C: 30 µL (venous DBS) P: 3 x 3 mm ∅ (capillary DBS) PAPER: Whatman® 903 SAMPLE PREP: - 250 µL solution A (isopropanol, 10 mM ammonium acetate buffer and formic acid (6:4:0.2)) + 50 µL IS - 1 mL n-hexane - evaporated and re-dissolved in 250 µL (venous DBS) or 100 µL (capillary DBS) solution B (50% mobile phase A and 50 % mobile phase B) RECOV: 58-74% (PEth 16:0/18:1) 59-77% (PEth 18:1/18:1) 55-78% (PEth 16:0/16:0)	Yes Comparison between whole blood, venous DBS and capillary DBS	6 months at RT
Cotinine	Cotinine	[22]	GC-MS	NS	¼ of filled (± 200 µL) circle ⇒ ± 50 µL PAPER: NS SAMPLE PREP: - 0.5 mL 0.2N NaOH - heated for 1h at 80°C - LLE: K_2CO_3 (0.5 mL, 25% aqueous) + 1 mL CH_2Cl_2 - CH_2Cl_2.-layer transferred and 25 µL MeOH added - evaporated to ±25 µL	Yes (newborn DBS)	NS
	Cotinine	[72]	LC-MS/MS	NS	P: 6.35 mm ∅ PAPER: standard neonatal screening cards SAMPLE PREP: - eluted with 200 µL H_2O - 1 mL MeOH added - supernatant filtered, evaporated and re-dissolved in ± 200 µL MeOH - evaporated and re-dissolved in 100 µL H_2O	No	4 years at 4°C
	Cotinine and trans 3'-OH-cotinine	[29]	LC-MS/MS	0.2-0.3 ng cotinine/g (3 x 4.6 mm punches)	P: 3.2 or 4.8 mm ∅ (multiple punches)	Yes Comparison with plasma	11-26 months at -20°C 9-11 months at RT

				0.3-102 ng cotinine/g	PAPER: Ahlstrom 226 SAMPLE PREP: - 400 µL H_2O - SPE: Oasis MCX 96-well plate - evaporated and re-dissolved in a suitable solvent		
	Cotinine	[49]	LC-MS/MS	3.13 ng/mL	P: 6.35 mm (multiple punches) PAPER: Whatman® 903 SAMPLE PREP: - 450 µL H_2O - sonicated 75 min at 55°C - PP: 50 µL 30% $HClO_4$ - LLE: 1 mL 3.6M K_3PO_4 + CH_2Cl_2 - acidified, evaporated and re-dissolved in suitable solvent	Yes (newborn DBS) Comparison with umbilical cord blood	7 months at RT
	Cotinine	[115]		NS	P: 2 x 6.35 mm PAPER: NS SAMPLE PREP: NS	Yes	NS
	Nicotine Cotinine Trans-3'-OH-cotinine Anabasine	[35]	Paper spray MS	1 ng/mL 3 ng/mL 2 ng/mL 1 ng/mL	Print paper (Xerox) NO SAMPLE PREP	Yes (rat DBS) Comparison with blood	NS
	Nicotine, nornicotine, cotinine, trans-3'-OH-cotinine, anabasine and anatabine	[42]	LC-HRMS/MS	15-2000 ng/mL	P: 6 mm ∅ ≈ clamp size PAPER: FTA® DMPK-C cards SAMPLE PREP: DBSA: - automated flow-through elution of DBS cards - online SPE - elution from the SPE cartridge onto the LC column using the LC mobile phase RECOV: 43.4% (nicotine) 38.1% (cotinine) 39.0% (trans-3'-OH-cotinine) 44.4% (nornicotine) 42.2% (anabasine) 25.4% (anatabine)	Yes (authentic samples)	30 d at RT

6-MAM: 6-monoacetylmorphine; ACN: acetonitrile; ACP: 2-amino-5-chloropyridine; BE: benzoylecgonine; C: complete DBS; CE: cocaethylene; d: days; DAD: diode array detection; DBS: dried blood spot(s); DRUID: driving under the influence of drugs, alcohol and medicines; DUID: driving under the influence of drugs; ECME: ecgonine methylester; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EMDP: 2-ethyl-5-methyl-3,3-diphenylpyrroline; EME: electromembrane extraction; FID: flame

ionization detection; GC-MS: gas chromatography coupled to mass spectrometry; GHB: gamma-hydroxybutyric acid; h: hours; HFB: heptafluorobutanol; HRMS: high resolution mass spectrometry; IS: internal standard(s); LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry; LLE: liquid-liquid extraction; LLOQ: lower limit of quantification; LOD: limit of detection; MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxy-N-ethylamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; MeOH: methanol; MEPS: microextraction packed sorbent; MS: mass spectrometry; MSTFA: N-methyl-N-(trimethylsilyl)trifluoroacetamide; MTBSTFA: N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide; M3G: morphine-3-glucuronide; M6G: morphine-6-glucuronide; NS: not studied; P: punch; PEth: phosphatidylethanol; PK: pharmacokinetics; PP: protein precipitation; RECOV: recovery; RIA: radioimmunoassay; RT: room temperature; SAMPLE PREP: sample preparation; SPE: solid phase extraction; STA: systematic toxicological analysis; TBME: tert-butyl methyl ether; TFAA: trifluoroacetic anhydride; THC: Δ^9-tetrahydrocannabinol; THC-COOH: tetrahydrocannabinol carboxylic acid; THC-OH: 11-hydroxy- Δ^9-tetrahydrocannabinol; w: weeks

Appendix 2 Cut-off concentrations for abused substances proposed by DRUID [50]. A distinction is made between analyses of complete DBS and analyses of DBS punches. The shaded columns contain these DBS methods achieving sufficient sensitivity to allow detection at DRUID LLOQ levels, when starting from complete ≤ 10-µL DBS and/or ≤ 6.4-mm diameter DBS punches.

COMPOUND	DRUID CUT-OFF in ng/mL IN BLOOD (BELGIAN LEGAL LIMIT in ng/mL IN PLASMA)	COMPLETE DBS LLOQ in ng/mL (blood volume) [reference]		DBS PUNCH LLOQ in ng/mL (∅ punch) [reference]	
THC	1 (1)		1 (20 µL) [94] 5 (100 µL) [26] 50 (5 µL) [12] 15 (30 µL) [93]		2.5 (7 mm ∅) [7]
THC-COOH	5		1 (20 µL) [94] 5 (100 µL) [26] 15 (30 µL) [93]		5 (7 mm ∅) [7]
THC-OH	1		15 (30 µL) [93]		5 (7 mm ∅) [7]
Benzoylecgonine	50 (25)	1 (5 µL) [12,13] 1 (5 µL) [8] 20 (10 µL) [6]	2.3 (20 µL) [96] 5 (30 µL) [93]	2 (6.4 mm ∅) [56] 5 (3 mm ∅) [31] 10 (6.4 mm ∅) [19] 1 (3 mm ∅) [63] 2 (3 x 3 mm ∅) [47]	
Cocaine	10 (25)	< 1 (5 µL) [8] 1 (5 µL) [12,13]	0.25 (20 µL) [94] 3.5 (20 µL) [96] 20 (10 µL) [6] 24.6 (100 µL) [77] 50 (100 µL) [26] 5 (30 µL) [93]	5 (3 mm ∅) [31] 1 (3 mm ∅) [63] 2 (3 x 3 mm ∅) [47]	
Ecgonine methylester	5		10 (5 µL) [8]	5 (3 mm ∅) [31]	2 (3 x 3 mm ∅) [47]
6-MAM	10 (10)	1 (5 µL) [12,13] 5 (5 µL) [8]	11 (20 µL) [96] 27 (100 µL) [67] 5 (30 µL) [93]	2 (3 x 3 mm ∅) [47]	
Codeine	10	5 (5 µL) [12,13]	9.4 (20 µL) [96] 10 (100 µL) [26] 50 (5 µL) [8] 5 (30 µL) [93]	5 (2 x 5 mm ∅) [45] 5 (3 mm ∅) [31] 1 (2mm ∅) [40]	
Morphine	10 (10)		10 (100 µL) [26] 10 (20 µL) [96] 14 (100 µL) [67]	1 (6.4 mm ∅) [100] 4 (3 mm ∅) [61] 5 (2 x 5 mm ∅) [45]	

			20 (5 µL) [12,13] 100 (5 µL) [8] 5 (30 µL) [93]	5 (3 mm ∅) [31] 1 (2mm ∅) [40] 2 (3 x 3 mm ∅) [47]	
Buprenorphine	1		5 (100 µL) [26]		1 (2 x 5 mm ∅) [45]
Methadone	10	< 1 (5 µL) [8] 1 (5 µL) [12,13] 2 (10 µL) [10] 4 (theoretical volume calculated from ∅) [20]	10 (100 µL) [26] 5 (30 µL) [93]	0.1 (6.4 mm ∅) [30] 0.3 (3 mm ∅) [36] 5 (2 x 5 mm ∅) [45] 0.2 (3 x 3 mm ∅) [47]	
Tramadol	50	0.5 (5 µL) [12]	50 (100 µL) [26]	5 (2 x 5 mm ∅) [45]	
7-aminoclonazepam	10	4 (5 µL) [5]	100 (5 µL) [8]	2.5 (6 mm ∅) [122]	
7-aminoflunitrazepam	2	1 (5 µL) [12,13]	4 (5 µL) [5]		2.5 (6 mm ∅) [122]
Alprazolam	10	1 (5 µL) [12,13] 4 (5 µL) [5]	0.7 (100 µL) [91] 5 (100 µL) [26] 1 (30 µL) [93]	0.1 (4 mm ∅) [120] 1 (3 mm ∅) [37] 4 (6 mm ∅) [122]	
Clobazam	5	0.5 (5 µL) [12,13]	40 (5 µL) [5]	2 (6 mm ∅) [122]	
Clonazepam	10	4 (5 µL) [5] 10 (5 µL) [12,13]	5 (100 µL) [26] 1 (30 µL) [93]	4 (6 mm ∅) [122]	
Desalkylflurazepam	2		4 (5 µL) [5]	2 (6 mm ∅) [122]	
Desmethylflunitrazepam	1	1 (5 µL) [5]			
Diazepam	20	10 (5 µL) [12,13]	9.9 (100 µL) [77] 40 (5 µL) [5] 50 (100 µL) [26] 50 (5 µL) [8] 1 (30 µL) [93]	2 (6 mm ∅) [122]	
Flunitrazepam	2		4 (5µL) [5] 5 (5 µL) [12,13] 15.8 (100 µL) [77]	2 (6 mm ∅) [122]	
Flurazepam	2	0.5 (5 µL) [12,13] 1 (5 µL) [5]		2 (6 mm ∅) [122]	
Lorazepam	10	4 (5 µL) [5] 5 (5 µL) [12,13]	5 (100 µL) [26] 11 (100 µL) [77] 1 (30 µL) [93]	2 (6 mm ∅) [122]	
Lormetazepam	1	1 (5 µL) [5]	1 (30 µL) [93]		
Midazolam	10	0.5 (5 µL) [12,13] <1 (5 µL) [8]	10 (100 µL) [26] 1 (30 µL) [93]	0.1 (4 mm ∅) [117,118] 0.1 (6 mm ∅ or	

		4 (5 µL) [5]		2 x 3 mm ∅) [46] 0.3 (3 mm ∅) [46] 0.35 (3 mm ∅) [119] 1 (3 mm ∅) [37] 5 (2 mm ∅) [39] 2 (6 mm ∅) [122]	
Nitrazepam	1	1 (5 µL) [12,13]	10 (100 µL) [26] 18 (100 µL) [77] 40 (5 µL) [5]		2 (6 mm ∅) [122]
Nordiazepam	20	1 (5 µL) [12,13]	20 (100 µL) [26] 40 (5 µL) [5] 1 (30 µL) [93]	3.5 (6 mm ∅) [122]	
OH-alprazolam	1		4 (5 µL) [5]	1 (3 mm ∅) [37]	2 (6 mm ∅) [122]
OH-midazolam	1		2 (5 µL) [12,13] 4 (5 µL) [5] 5 (5 µL) [8]		2 (6 mm ∅) [122]
Oxazepam	50	10 (5 µL) [12,13] 40 (5 µL) [5]	20.6 (100 µL) [77] 50 (100 µL) [26] 5 (30 µL) [93]	3 (6 mm ∅) [122]	
Temazepam	20	1 (5 µL) [12,13]	10.8 (100 µL) [77] 40 (5 µL) [5] 50 (100 µL) [26]	3 (6 mm ∅) [122]	
Triazolam	1	0.5 (5 µL) [12,13] 1 (5 µL) [5]			2 (6 mm ∅) [122]
Zolpidem	20	< 1 (5 µL) [8] 1 (5 µL) [12,13] 4 (5 µL) [5]		0.1 (3 mm ∅) [9] 2.5 (6 mm ∅) [122]	
Zopiclone	10	2 (5 µL) [12,13] 4 (5 µL) [5]	1.2 (100 µL) [91] 6.4 (100 µL) [78] 10 (100 µL) [26]		
Amphetamine	20 (25)	1 (5 µL) [8] 5 (10 µL) [80] 5 (5 µL) [41]	3 (100 µL) [91] 20 (100 µL) [26] 40 (5 µL) [12,13] 5 (30 µL) [93]	5 (3 mm ∅) [31] 2 (3 x 3 mm ∅) [47]	
MDA	20	2.5 (10 µL) [80] 20 (5 µL) [12,13]	0.4 (100 µL) [91] 0.4 (100 µL) [121] 20 (100 µL) [26] 5 (30 µL) [93]	5 (3 mm ∅) [31] 2 (3 x 3 mm ∅) [47]	

MDEA	20	1 (10 µL) [80] 2 (5 µL) [12,13]		5 (3 mm ∅) [31] 2 (3 x 3 mm ∅) [47]	
MDMA	20 (25)	1 (5 µL) [8] 2 (5 µL) [12,13] 2.5 (10 µL) [80] 5 (5 µL) [41]	5.7 (100 µL) [91] 5.7 (100 µL) [121] 20 (100 µL) [26] 5 (30 µL) [93]	5 (3 mm ∅) [31] 2 (3 x 3 mm ∅) [47]	
Methamphetamine	20	1 (10 µL) [80] 10 (5 µL) [8] 5 (5 µL) [41]	20 (100 µL) [26] 50 (5 µL) [12,13] 5 (30 µL) [93]	5 (3 mm ∅) [31] 2 (3 x 3 mm ∅) [47]	
Ketamine	20	1 (10 µL) [80]		5 (3.2 mm ∅) [104] 2 (3 x 3 mm ∅) [47]	

6-MAM: 6-monoacetylmorphine; DBS: dried blood spot(s); DRUID: driving under the influence of drugs, alcohol and medicines; LLOQ: lower limit of quantification; MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxy-N-ethylamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; THC: Δ^9-tetrahydrocannabinol; THC-COOH: tetrahydrocannabinol carboxylic acid; THC-OH: 11-hydroxy- Δ^9-tetrahydrocannabinol;

References

1. Bang I. *Der Blutzucker*, Wiesbaden, Germany (1913).
2. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 32(3), 338-343 (1963).
3. Li W, Tse F. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed Chromatogr* 24(1), 49-65 (2010).
4. Stove C, Ingels A, De Kesel P, Lambert W. Dried blood spots in toxicology: from the cradle to the grave? *Crit Rev Toxicol* 42(3), 230-243 (2012).
5. Déglon J, Versace F, Lauer E *et al.* Rapid LC-MS/MS quantification of the major benzodiazepines and their metabolites on dried blood spots using a simple and cost-effective sample pretreatment. *Bioanalysis* 4(11), 1337-1350 (2012).
6. Mercolini L, Mandrioli R, Gerra G, Raggi M. Analysis of cocaine and two metabolites in dried blood spots by liquid chromatography with fluorescence detection: a novel test for cocaine and alcohol intake. *J Chromatogr A* 1217(46), 7242-7248 (2010).
7. Mercolini L, Mandrioli R, Sorella V *et al.* Dried blood spots: liquid chromatography-mass spectrometry analysis of Delta9-tetrahydrocannabinol and its main metabolites. *J Chromatogr A* 1271(1), 33-40 (2013).
8. Versace F, Déglon J, Lauer E, Mangin P, Staub C. Automated DBS extraction prior to hilic/RP LC-MS/MS target screening of drugs. *Chromatographia* 76(19-20), 1281-1293 (2013).
9. Hudson W, Yong B, Boguszewski P. Analysis of clozapine, nortriptyline, paroxetine and zolpidem using dried blood spots. Agilent Technologies. http://www.chem.agilent.com/Library/applications/5990-8033EN.pdf.10.
10. Eibak L, Hegge A, Rasmussen K, Pedersen-Bjergaard S, Gjelstad A. Alginate and chitosan foam combined with electromembrane extraction for dried blood spot analysis. *Anal Chem* 84(20), 8783-8789 (2012).
11. Thomas A, Déglon J, Steimer T, Mangin P, Daali Y, Staub C. On-line desorption of dried blood spots coupled to hydrophilic interaction/reversed-phase LC/MS/MS system for the simultaneous analysis of drugs and their polar metabolites. *J Sep Sci* 33(6-7), 873-879 (2010).
12. Déglon J, Thomas A, Mangin P, Staub C. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Anal Bioanal Chem* 402(8), 2485-2498 (2012).
13. Lauer E, Déglon J, Versace F, Thomas A, Mangin P, Staub C. Target screening of drugs from dried blood spot samples based on LC-MS/MS and on-line desorption. Presented at: *49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT)*. San Francisco, CA, USA, 25-30 September 2011.
14. Lenk G, Sandkvist S, Pohanka A, Stemme G, Beck O, Roxhed N. A disposable sampling device to collect volume-measured DBS directly from a fingerprick onto DBS paper. *Bioanalysis* 7(16), 2085-2094 (2015).

15. Leuthold LA, Heudi O, Déglon J, *et al*. New microfluidic-based sampling procedure for overcoming the hematocrit problem associated with dried blood spot analysis. *Anal Chem* 87(4), 2068-2071 (2015).
16. Denniff P, Spooner N. Volumetric absorptive microsampling: a dried sample collection technique for quantitative bioanalysis. *Anal Chem* 86(16), 8489-8495 (2014).
17. Spooner N, Denniff P, Michielsen L, *et al.* A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. *Bioanalysis* 7(6), 653-659 (2015).
18. Denniff P, Spooner N. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. *Bioanalysis* 2(8), 1385-1395 (2010).
19. Henderson L, Powell M, Hannon W *et al.* Radioimmunoassay screening of dried blood spot materials for benzoylecgonine. *J Anal Toxicol* 17(1), 42-47 (1993).
20. Saracino M, Marcheselli C, Somaini L *et al.* A novel test using dried blood spots for the chromatographic assay of methadone. *Anal Bioanal Chem.* 404(2), 503-511 (2012).
21. Saracino M, Marcheselli C, Bugamelli F, Morganti E, Serpelloni G, Raggi M. Club drugs: analysis of ketamine and norketamine in dried blood spots by HPLC-DAD-MEPS. Presented at: *24th International Symposium on Pharmaceutical and Biomedical Analysis.* Bologna, Italy, 30 June - 3 July 2013.
22. Spector L, Hecht S, Ognjanovic S, Carmella S, Ross J. Detection of cotinine in newborn dried blood spots. *Cancer Epidemiol Biomarkers Prev* 16(9), 1902-1905 (2007).
23. Ingels A, Lambert W, Stove C. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. *Anal Bioanal Chem* 398(5), 2173-2182 (2010).
24. Ingels A, De Paepe P, Anseeuw K *et al.* Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis* 3(20), 2271-2281 (2011).
25. Ingels A, Hertegonne K, Lambert W, Stove C. Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home: an exploratory study. *CNS drugs* 27(3), 233-237 (2013).
26. Langel K, Uusivirta H, Ariniemi K, Lillsunde P. Analysis of drugs of abuse by GC-MS in dried blood spot sample matrix. Presented at: *49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT)*. San Francisco, CA, USA, 25-30 September 2011.
27. Déglon J, Thomas A, Cataldo A, Mangin P, Staub C. On-line desorption of dried blood spot: a novel approach for the direct LC/MS analysis of micro-whole blood samples. *J Pharm Biomed Anal* 49(4), 1034-1039 (2009).
28. Sadones N, Van Bever E, Archer JRH, *et al*. Microwave-assisted on-spot derivatization for gas chromatography-mass spectrometry based determination of polar low molecular weight compounds in dried blood spots. *J Chromatogr A* 1465, 175-183 (2016).
29. Murphy S, Wickham K, Lindgren B, Spector L, Joseph A. Cotinine and trans 3'-hydroxycotinine in dried blood spots as biomarkers of tobacco exposure and nicotine metabolism. *J Expo Sci Environ Epidemiol* 23(5), 513-518 (2013).

30. Clavijo C, Thomas J, Hammer G, Christians U, Galinkin J. A sensitive LC-MS/MS assay for quantification of methadone and its metabolites in dried blood spots. Presented at: *2010 Annual Meeting of the American Society of Anesthesiologists (ASA).* San Diego, CA, USA, 16-20 October 2010.
31. Saussereau E, Lacroix C, Gaulier J, Goulle J. On-line liquid chromatography/tandem mass spectrometry simultaneous determination of opiates, cocainics and amphetamines in dried blood spots. *J Chromatogr B Analyt Technol Biomed Life Sci* 885-886, 1-7 (2012).
32. Lafrenière N, Shih S, Abu-Rabie P, Jebrail M, Spooner N, Wheeler A. Multiplexed extraction and quantitative analysis of pharmaceuticals from DBS samples using digital microfluidics. *Bioanalysis* 6(3), 307-318 (2014).
33. Liu J, Wang H, Manicke N, Lin J, Cooks R, Ouyang Z. Development, characterization, and application of paper spray ionization. *Anal Chem* 82(6), 2463-2471 (2010).
34. Zhang Z, Xu W, Manicke N, Cooks R, Ouyang Z. Silica coated paper substrate for paper-spray analysis of therapeutic drugs in dried blood spots. *Anal Chem* 84(2), 931-938 (2012).
35. Wang H, Ren Y, McLuckey M *et al.* Direct quantitative analysis of nicotine alkaloids from biofluid samples using paper spray mass spectrometry. *Anal Chem* 85(23), 11540-11544 (2013).
36. Seip K, Gjelstad A, Pedersen-Bjergaard S. Electromembrane extraction from aqueous samples containing polar organic solvents. *J Chromatogr A* 1308, 37-44 (2013).
37. Johnson C, Christianson C, Sheaff C, Laine D, Zimmer J, Needham S. Use of conventional bioanalytical devices to automate DBS extractions in liquid-handling dispensing tips. *Bioanalysis* 3(20), 2303-2310 (2011).
38. Henion J, Oliveira R, Fumin L, Foley T, Pomponio R. Dried blood spots: the future. In: *Microsampling in Pharmaceutical Bioanalysis (ebook).* Zane P, Emmons GT (Eds), Future Science Ltd, 48-66 (2013).
39. Oliveira R, Henion J, Wickremsinhe E. Fully-automated approach for online dried blood spot extraction and bioanalysis by two-dimensional-liquid chromatography coupled with high-resolution quadrupole time-of-flight mass spectrometry. *Anal Chem* 86(2), 1246-1253 (2014).
40. Verplaetse R, Henion J. Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS. *Drug Test Anal* 8(1), 30-38 (2016).
41. Verplaetse R, Henion J. Hematocrit-independent quantitation of stimulants in dried blood spots: pipet versus microfluidic-based volumetric sampling coupled with automated flow-through desorption and online solid phase extraction-LC-MS/MS bioanalysis. *Anal Chem* 88(13), 6789-6796 (2016).
42. Tretzel L, Thomas A, Piper T, *et al.* Fully automated determination of nicotine and its major metabolites in whole blood by means of a DBS online-SPE LC-HR-MS/MS approach for sports drug testing. *J Pharm Biomed Anal* 123, 132-140 (2016).
43. Jones J, Jones M, Plate C, Lewis D. The detection of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol in human dried blood spots. *Anal Methods* 3(5), 1101-1106 (2011).
44. Forni S, Pearl P, Gibson K, Yu Y, Sweetman L. Quantitation of gamma-hydroxybutyric acid in dried blood spots: feasibility assessment for newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency. *Mol Genet Metab* 109(3), 255-259 (2013).

45. Goggin M, Lundberg R, Janis G. Measuring circulating levels of 13 opiate and opioid analgesics from dried blood spot samples. Presented at: *American Association of Pharmaceutical Scientists (AAPS).* Chicago, USA, 14-17 October 2012.
46. de Boer T, Wieling J, Meulman E *et al.* Application of dried blood spot sampling combined with LC-MS/MS for genotyping and phenotyping of CYP450 enzymes in healthy volunteers. *Biomed Chromatogr* 25(10), 1112-1123 (2011).
47. Odoardi S, Anzillotti L, Strano-Rossi S. Simplifying sample pretreatment: application of dried blood spot (DBS) method to blood samples, including postmortem, for UHPLC-MS/MS analysis of drugs of abuse. *Forensic Sci Int* 243, 61-67 (2014).
48. Abu-Rabie P, Spooner N. Direct quantitative bioanalysis of drugs in dried blood spot samples using a thin-layer chromatography mass spectrometer interface. *Anal Chem* 81(24), 10275-10284 (2009).
49. Yang J, Pearl M, Jacob Pr *et al.* Levels of cotinine in dried blood specimens from newborns as a biomarker of maternal smoking close to the time of delivery. *Am J Epidemiol* 178(11), 1648-1654 (2013).
50. Verstraete A, Knoche A, Jantos R, *et al.* DRUID, deliverable 1.4.2. per se limits- methods of defining cut-off values for zero tolerance (2011).
51. Kummer N, Ingels AS, Wille SM, *et al.* Quantification of phosphatidylethanol 16:0/18:1, 18:1/18:1, and 16:0/16:0 in venous blood and venous and capillary dried blood spots from patients in alcohol withdrawal and control volunteers. *Anal Bioanal Chem* 408(3), 825-838 (2016).
52. Edelbroek P, van der Heijden J, Stolk L. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Ther Drug Monit* 31(3), 327-336 (2009).
53. O'Mara M, Hudson-Curtis B, Olson K, Yueh Y, Dunn J, Spooner N. The effect of hematocrit and punch location on assay bias during quantitative bioanalysis of dried blood spot samples. *Bioanalysis* 3(20), 2335-2347 (2011).
54. De Kesel P, Sadones N, Capiau S, Lambert W, Stove C. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis* 5(16), 2023-2041 (2013).
55. van Baar B, Verhaeghe T, Heudi O *et al.* IS addition in bioanalysis of DBS: results from the EBF DBS-microsampling consortium. *Bioanalysis* 5(17), 2137-2145 (2013).
56. Sosnoff C, Ann Q, Bernert JJ *et al.* Analysis of benzoylecgonine in dried blood spots by liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry. *J Anal Toxicol* 20(3), 179-184 (1996).
57. Abu-Rabie P, Denniff P, Spooner N, Brynjolffssen J, Galluzzo P, Sanders G. Method of applying internal standard to dried matrix spot samples for use in quantitative bioanalysis. *Anal Chem* 83(22), 8779-8786 (2011).
58. Zimmer D, Hassler S, Betschart B, Sack S, Fankhauser C, Loppacher M. Internal standard application to dried blood spots by spraying: investigation of the internal standard distribution. *Bioanalysis* 5(6), 711-719 (2013).

59. Abu-Rabie P, Denniff P, Spooner N, Chowdhry BZ, Pullen FS. Investigation of different approaches to incorporating internal standard in DBS quantitative bioanalytical workflows and their effect on nullifying hematocrit-based assay bias. *Anal Chem* 87(9), 4996-5003 (2015).
60. Manicke NE, Abu-Rabie P, Spooner N, Ouyang Z, Cooks RG. Quantitative analysis of therapeutic drugs in dried blood spot samples by paper spray mass spectrometry: an avenue to therapeutic drug monitoring. *J Am Soc Mass Spectrom* 22(9), 1501-1507 (2011).
61. Mommers J, Mengerink Y, Ritzen E, Weusten J, van der Heijden J, van der Wal S. Quantitative analysis of morphine in dried blood spots by using morphine-d3 pre-impregnated dried blood spot cards. *Anal Chim Acta* 774, 26-32 (2013).
62. Sadones N, Archer JR, Ingels AM, *et al*. Do capillary dried blood spot concentrations of gamma-hydroxybutyric acid mirror those in venous blood? A comparative study. *Drug Test Anal* 7(4), 336-340 (2015).
63. Ellefsen KN, da Costa JL, Concheiro M, *et al.* Cocaine and metabolite concentrations in DBS and venous blood after controlled intravenous cocaine administration. *Bioanalysis* 7(16), 2041-2056 (2015).
64. Bland J, Altman D. Stastistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1(8476), 307-310 (1986).
65. Stockl D, Dewitte K, Thienpont L. Validity of linear regression in method comparison studies: is it limited by the statistical model or the quality of the analytical input data? *Clin Chem* 44(11), 2340-2346 (1998).
66. Dewitte K, Fierens C, Stockl D, Thienpont L. Application of the Bland-Altman plot for interpretation of method-comparison studies: A critical investigation of its practice. *Clin Chem* 48(5), 799-801 (2002).
67. Garcia Boy R, Henseler J, Mattern R, Skopp G. Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots. *Ther Drug Monit* 30(6), 733-739 (2008).
68. Capiau S, Stove V, Lambert W, Stove C. Prediction of the hematocrit of dried blood spots via potassium measurement on a routine clinical chemistry analyzer. *Anal Chem* 85(1), 404-410 (2013).
69. De Kesel PM, Capiau S, Stove VV, Lambert WE, Stove CP. Potassium-based algorithm allows correction for the hematocrit bias in quantitative analysis of caffeine and its major metabolite in dried blood spots. *Anal Bioanal Chem* 406(26), 6749-6755 (2014).
70. Capiau S, Wilk LS, Aalders MC, Stove CP. A novel, nondestructive, dried blood spot-based hematocrit prediction method using noncontact diffuse reflectance spectroscopy. *Anal Chem* 88(12), 6538-6546 (2016).
71. Oostendorp M, El Amrani M, Diemel EC, Hekman D, van Maarseveen EM. Measurement of hematocrit in dried blood spots using near-infrared spectroscopy: robust, fast, and nondestructive. *Clin Chem* 62(11), 1534-1536 (2016).
72. Sosnoff C, Bernert J. Analysis of cotinine in dried blood spots by LC APCI tandem mass spectrometry. *Clin Chim Acta* 388(1-2), 228-229 (2008).

73. Li Y, Henion J, Abbott R, Wang P. The use of a membrane filtration device to form dried plasma spots for the quantitative determination of guanfacine in whole blood. *Rapid Commun Mass Spectrom* 26(10), 1208-1212 (2012).

74. Kim JH, Woenker T, Adamec J, Regnier F. Simple, miniaturized blood plasma extraction method. *Anal Chem* 85(23), 11501-11508 (2013).

75. Sturm R, Henion J, Abbott R, Wang P. Novel membrane devices and their potential utility in blood sample collection prior to analysis of dried plasma spots. *Bioanalysis* 7(16), 1987-2002 (2015).

76. Ryona I, Henion J. A book-type dried plasma spot card for automated flow-through elution coupled with on-line SPE-LC-MS/MS bioanalysis of opioids and stimulants in blood. *Anal Chem* in press (2016).

77. Alfazil A, Anderson R. Stability of benzodiazepines and cocaine in blood spots stored on filter paper. *J Anal Toxicol* 32(7), 511-515 (2008).

78. Jantos R, Vermeeren A, Sabljic D, Ramaekers J, Skopp G. Degradation of zopiclone during storage of spiked and authentic whole blood and matching dried blood spots. *Int J Legal Med* 127(1), 69-76 (2013).

79. Skopp G. Blood spot analysis. Presented at: *Joint Meeting of the ICADTS and TIAFT*. Seattle, WA, USA, 26-30 August 2007.

80. Ambach L, Redondo H, Konig S, Weinmann W. Rapid and simple LC-MS/MS screening of 64 novel psychoactive substances using dried blood spots. *Drug Test Anal* 6(4), 367-375 (2013).

81. Sorensen L. Determination of cathinones and related ephedrines in forensic whole-blood samples by liquid-chromatography-electrospray tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 879(11-12), 727-736 (2011).

82. Johnson R, Botch-Jones S. The stability of four designer drugs: MDPV, mephedrone, BZP and TFMPP in three biological matrices under various storage conditions. *J Anal Toxicol* 37(2), 51-55 (2013).

83. Berankova K, Mutnanska K, Balikova M. Gamma-hydroxybutyric acid stability and formation in blood and urine. *Forensic Sci Int* 161(2-3), 158-162 (2006).

84. Zorntlein S, Kopp A, Becker J, Kaufmann T, Rohrich J, Urban R. In vitro production of GHB in blood and serum samples under various storage conditions. *Forensic Sci Int* 214(1-3), 113-117 (2012).

85. Aradottir S, Seidl S, Wurst F, Jonsson B, Alling C. Phosphatidylethanol in human organs and blood: a study on autopsy material and influences by storage conditions. *Alcohol Clin Exp Res* 28(11), 1718-1723 (2004).

86. Helander A, Zheng Y. Molecular species of the alcohol biomarker phosphatidylethanol in human blood measured by LC-MS. *Clin Chem* 55(7), 1395-1405 (2009).

87. Huestis M, Verstraete A, Kwong T, Morland J, Vincent M, de la Torre R. Oral fluid testing: promises and pitfalls. *Clin Chem* 57(6), 805-810 (2011).

88. De Kesel PMM, Lambert WE, Stove CP. The role of (bio)markers in hair analysis. In: *New sampling strategies in toxicology and therapeutic drug monitoring* (ebook), Stove CP (Ed), Future Science Ltd, 121-134 (2015).

89. Cooper G, Kronstrand R, Kintz P. Society of Hair Testing guidelines for drug testing in hair. *Forensic Sci Int* 218(1-3), 20-24 (2012).

90. Trudeau S, Mineau P, Cartier G *et al.* Using dried blood spots stored on filter paper to measure cholinesterase activity in wild avian species. *Biomarkers* 12(2), 145-154 (2007).

91. Jantos R, Skopp G. Comparison of drug analysis in whole blood and dried blood spots. *Toxichem Krimtech* 78, 268-275 (2011).

92. Huestis M, Henningfield J, Cone E. Blood cannabinoids. I. Absorption of THC and formation of 11-OH-THC and THCCOOH during and after smoling marijuana. *J Anal Toxicol* 16(5), 276-282 (1992).

93. Kyriakou C, Marchei E, Scaravelli G, Garcia-Algar O, Supervia A, Graziano S. Identification and quantification of psychoactive drugs in whole blood using dried blood spot (DBS) by ultra-performance liquid chromatography tandem mass spectrometry. *J Pharm Biomed Anal* 128, 53-60 (2016).

94. Thomas A, Geyer H, Schanzer W *et al.* Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. *Anal Bioanal Chem* 403(5), 1279-1289 (2012).

95. Huestis MA. Human cannabinoid pharmacokinetics. *Chem Biodivers* 4(8), 1770-1804 (2007).

96. Antelo-Dominguez A, Cocho J, Tabernero M, Bermejo A, Bermejo-Barrera P, Moreda-Pineiro A. Simultaneous determination of cocaine and opiates in dried blood spots by electrospray ionization tandem mass spectrometry. *Talanta* 117, 235-241 (2013).

97. Henderson L, Powell M, Hannon W *et al.* An evaluation of the use of dried blood spots from newborn screening for monitoring the prevalence of cocaine use among childbearing women. *Biochem Mol Med* 61(2), 143-151 (1997).

98. Parker S, Cubitt W. The use of the dried blood spot sample in epidemiological studies. *J Clin Pathol* 52(9), 633-639 (1999).

99. Mei J, Alexander J, Adam B, Hannon W. Use of filter paper for the collection and analysis of human whole blood specimens. *J Nutr* 131(5), 1631S-1636S (2001).

100. Clavijo C, Hoffman K, Thomas J *et al.* A sensitive assay for the quantification of morphine and its active metabolites in human plasma and dried blood spots using high-performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem* 400(3), 715-728 (2011).

101. Jantos R, Schumacher M, Skopp G. Comparison of opioid analysis in whole blood and dried blood spots. Presented at: *49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT)*. San Francisco, CA, USA, 25-30 September 2011.

102. Baselt R. In: *Disposition of toxic drugs and chemicals in man (9th edition)* Biomedical Publications, Foster City, CA, USA (2011).

103. Clavijo C, Thomas J, Cromie M *et al.* A low blood volume LC-MS/MS assay for the quantification of fentanyl and its major metabolites norfentanyl and despropionyl fentanyl in children. *J Sep Sci* 34(24), 3568-3577 (2011).

104. Moll V, Clavijo C, Cohen M, Christians U, Galinkin J. A analytical method to determine ketamine and norketamine levels in dried blood spots. Presented at: *Annual Meeting of the American Society of Anesthesiologists (ASA).* New Orleans, LA, USA, 17-21 October 2009.

105. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report. http://www.emcdda.europa.eu/attachements.cfm/att_239505_EN_TDAT15001ENN.pdf

106. Mercolini L, Protti M, Catapano MC, Rudge J, Sberna AE. LC-MS/MS and volumetric absorptive microsampling for quantitative bioanalysis of cathinone analogues in dried urine, plasma and oral fluid samples. *J Pharm Biomed Anal* 123, 186-194 (2016).

107. Mercolini L, Protti M. Biosampling strategies for emerging drugs of abuse: towards the future of toxicological and forensic analysis. *J Pharm Biomed Anal* 130, 202-219 (2016).

108. Maenhout T, De Buyzere M, Delanghe J. Non-oxidative ethanol metabolites as a measure of alcohol intake. *Clin Chim Acta* 415, 322-329 (2013).

109. Crunelle C, Yegles M, van Nuijs A *et al.* Hair ethyl glucuronide levels as a marker for alcohol use and abuse: a review of the current state of the art. *Drug Alcohol Depend* 134, 1-11 (2014).

110. Hernández Redondo A, Schroeck A, Kneubuehl B, Weinmann W. Determination of ethyl glucuronide and ethyl sulfate from dried blood spots. *Int J Legal Med* 127(4), 769-775 (2013).

111. Faller A, Richter B, Kluge M, Koenig P, Seitz H, Skopp G. Stability of phosphatidylethanol species in spiked and authentic whole blood and matching dried blood spots. *Int J Legal Med* 127(3), 603-610 (2013).

112. Faller A, Richter B, Kluge M *et al.* LC-MS/MS analysis of phosphatidylethanol in dried blood spots versus conventional blood specimens. *Anal Bioanal Chem* 401(4), 1163-1166 (2011).

113. Bakhireva L, Leeman L, Savich R *et al.* The validity of phosphatidylethanol in dried blood spots of newborns for the identification of prenatal alcohol exposure. *Alcohol Clin Exp Res* 38(4), 1078-1085 (2014).

114. Bakhireva L, Savich R, Raisch D *et al.* The feasibility and cost of neonatal screening for prenatal alcohol exposure by measuring phosphatidylethanol in dried blood spots. *Alcohol Clin Exp Res* 37(6), 1008-1015 (2013).

115. Searles Nielsen S, Dills R, Glass M, Mueller B. Accuracy of prenatal smoking data from Washington Statebirth certificates in a population-based sample with cotinine measurements. *Ann Epidemiol* 24(3), 236-239 (2014).

116. Marin LJ, Tyrrel-Pawlowic CA, Moroney EC, Coopersmith BI, Shet MS. Comparative analysis of buprenorpine and oxycodone in human dried blood spots (DBS) using LC-MS/MS. Presented at: *American Association of Pharmaceutical Scientists (AAPS)- Pharmaceutical Sciences World Congress.* New Orleans, LA, USA, 14-18 November 2010.

117. Havard G, Théberge MC, Boudreau N, Lévesque A, Massé R. Development and validation of a dried blood spot assay for the determination of midazolam in human whole blood by LCMSMS. Presented at: *American Association of Pharmaceutical Scientists (AAPS)-Pharmaceutical Sciences World Congress.* New Orleans, LA, USA, 14-18 November 2010.

118. Havard G, Théberge MC, Boudreau N, Lévesque A, Pellerin B. Determination of midazolam in human plasma and whole blood: comparison of dried blood spot and different collection procedure. Presented at: *American Association of Pharmaceutical Scientists (AAPS).* Washington, USA, 23-27 October 2011.

119. Lad R. Validation of individual quantitative methods for determination of cytochrome P450 probe substrates in human dried blood spots with HPLC-MS/MS. *Bioanalysis* 2(11), 1849-1861 (2010).

120. Mather J, Rainville P, Spooner N, Evans C, Smith N, Plumb R. Rapid analysis of dried blood spot samples with sub-2-microm LC-MS/MS. *Bioanalysis* 3(4), 411-420 (2011).

121. Jantos R, Veldstra J, Mattern R, Brookhuis K, Skopp G. Analysis of 3,4-methylenedioxymetamphetamine: whole blood versus dried blood spots. *J Anal Toxicol* 35(5), 269-273 (2011).

122. Lee H, Park Y, Jo J, *et al.* Analysis of benzodiazepines and their metabolites using DBS cards and LC-MS/MS. *Forensic Sci Int* 255, 137-145 (2015).

CURRICULUM VITAE

NELE SADONES

Personal information

Address	Grote Herreweg 242, 9690 Kluisbergen
Date and place of birth	01-09-1988, Ronse
Email address	nele_sadones@hotmail.com
Telephone number	+ 32 496 62 18 30

Education

2011-2016	PhD in Pharmaceutical Sciences, Ghent University
2009-2011	Master in Pharmaceutical Care, Ghent University (Magna cum laude)
2006-2009	Bachelor in Pharmaceutical Sciences, Ghent University (Magna cum laude)
2000-2006	Sciences-mathematics, Sint-Jan Berchmanscollege, Avelgem

Work experience 2011-2016

PhD-student at the Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University (Prof. W. Lambert/ Prof. C. Stove)

Extra-scientific contributions

- Education
    - Practical courses Toxicology (2nd Master in Pharmaceutical Care or Drug Development)
    - Practical courses Bio-analysis (3rd Bachelor in Pharmaceutical Sciences)
    - Support of demonstrations for courses Chemical Criminalistics (students Criminology)
    - Theoretical courses Gas Chromatography (students Biotechnology (Panta Rhei))
    - Supervisor of Pharmaceutical Bachelor dissertations (3rd Bachelor in Pharmaceutical Sciences)
    - Supervisor of Master dissertations (1st Master in Pharmaceutical Care or Drug Development)
- Laboratory
    - Support in forensic toxicological services

Scientific curriculum

A1-publications

De Kesel PMM*, Sadones N*, Capiau S*, Lambert WE, Stove CP. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis* 5(16), 2023-2041 (2013).
* equally contributing authors
IF (2013): 3.027; IF (2015): 2.813; 2nd Quartile (20/75) of 'Chemistry, Analytical'; Times Cited: 48

Sadones N, Capiau S, De Kesel PMM, Lambert WE, Stove CP. Spot them in the spot: analysis of abused substances using dried blood spots. *Bioanalysis* 6(17), 2211-2227 (2014).
IF (2014): 3.003; IF (2015): 2.813; 2nd Quartile (20/75) of 'Chemistry, Analytical'; Times Cited: 18

Sadones N, Archer JR, Ingels AM, Dargan PI, Wood DM, Wood M, Neels H, Lambert WE, Stove CP. Do capillary dried blood spot concentrations of gamma-hydroxybutyric acid mirror those in venous blood? A comparative study. *Drug Test Anal* 7(4), 336-340 (2015).
IF (2015): 2.859; 1st Quartile (18/75) of 'Chemistry, Analytical'; Times Cited: 6

Sadones N, Van Bever E, Archer JRH, Wood DM, Dargan PI, Van Bortel L, Lambert WE, Stove CP. Microwave-assisted on-spot derivatization for gas chromatography-mass spectrometry based determination of polar low molecular weight compounds in dried blood spots. *J Chromatogr A* 1465, 175-183 (2016).
IF (2015): 3.926; 1st Quartile (11/75) of 'Chemistry, Analytical'; Times Cited: 1

Sadones N, Van Bever E, Van Bortel L, Lambert WE, Stove CP. Dried blood spot analysis of gabapentin as a valid alternative for serum: a bridging study. *J Pharm Biomed Anal* 132, 72-76 (2017).
IF (2015): 3.169; 1st Quartile (14/75) of 'Chemistry, Analytical'; Times Cited: 0

Sadones N, Lambert WE, Stove CP. The (non)sense of routinely analyzing beta-hydroxybutyric acid in forensic toxicology casework. *Forensic Sci Int* doi: 10.1016/j.forsciint.2017.01.002 (2017).
IF (2015): 1.950; 2nd Quartile (5/15) of 'Medicine, Legal'

Abouchedid R, Hudson S, Thurtle N, Yamamoto T, Ho JH, Bailey G, Wood M, Sadones N, Stove CP, Dines A, Archer JRH, Wood DM, Dargan PI. Analytical confirmation of Synthetic Cannabinoid Receptor Agonists (SCRAs) in a cohort of 179 presentations with acute recreational drug toxicity to an Emergency Department in London, UK. *Clin Toxicol* in press (2017).
IF (2015): 2.886; 2nd Quartile (26/90) of 'Toxicology'

Book Chapter

Derivatization techniques in dried blood spot analysis. In "Dried Blood Spots: Applications and Techniques". Ingels ASME, Sadones N, De Kesel PMM, Lambert WE, Stove CP. Eds. Li W and Lee M, John Wiley & Sons, Chapter 27.

Submitted manuscript

Taylor O, Van Laeken N, Polis I, Dockx R, Vlerick L, Dobbeleir A, Goethals I, Saunders J, Sadones N, Baeken C, De Vos F, Peremans K. Optimization of the dosing regimen of escitalopram in dogs: a dose occupancy study with [^{11}C]DASB. *PLoS One*
IF (2015): 3.057; 1st Quartile (11/63) of 'Multidisciplinary sciences'

Membership of scientific organizations

TIAFT (The International Association of Forensic Toxicologists)

BLT (Belgium Luxembourg Toxicological Society)

IATDMCT (International Association of Therapeutic Drug Monitoring and Clinical Toxicology)

Congress participation

Poster presentation: Development of a new haptoglobin genotyping method by direct PCR on dried blood spots, Annual meeting of the Belgian Society of Clinical Biology, Brussels, Belgium, 5th October 2013

Oral presentation: Do capillary dried blood spot concentrations of gamma-hydroxybutyric acid mirror those in venous blood? A comparative study, Belgium Luxembourg Toxicological Society (BLT) scientific session, Brussels, Belgium, 29th April 2014

Oral presentation: Development of a new haptoglobin genotyping method by direct PCR on dried blood spots, Alternative sampling strategies in toxicology and therapeutic drug monitoring symposium, Ghent, Belgium, 18th-19th September 2014

Oral presentation: Do capillary dried blood spot concentrations of gamma-hydroxybutyric acid mirror those in venous blood? A comparative study, 52nd Annual meeting of The International Association of Forensic Toxicologists (TIAFT), Buenos Aires, Argentina, 9th-13th November 2014

Oral presentation: Microwave-assisted on-spot derivatization for the GC-MS based determination of polar low molecular weight molecules in dried blood spots, Belgium Luxembourg Toxicological Society (BLT) meeting, Koksijde, Belgium, 27th-28th March 2015

Oral presentation: Microwave-assisted on-spot derivatization for the GC-MS based determination of polar low molecular weight molecules -amongst which GHB and BHB- in dried blood spots, 53rd Annual meeting of The International Association of Forensic Toxicologists (TIAFT), Florence, Italy, 30th August – 4th September 2015

Oral presentation: Microwave-assisted on-spot derivatization for the GC-MS based determination of polar low molecular weight molecules -amongst which gabapentin- in dried blood spots, 14th international congress of therapeutic drug monitoring and clinical toxicology (IATDMCT), Rotterdam, The Netherlands, 11th-15th October 2015

Oral presentation: The (non)sense of routinely analyzing beta-hydroxybutyric acid in forensic toxicology casework, Belgium Luxembourg Toxicological Society (BLT) meeting, Echternach, Luxembourg, 21st-22nd October 2016