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REVIEW ARTICLE

Dried blood spots in toxicology: from the cradle to the grave?

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Abstract
About a century after its first described application by Ivar Bang, the potential of sampling *via* dried blood spots (DBS) as an alternative for classical venous blood sampling is increasingly recognized. Perhaps best known is the use of DBS in newborn screening programs, ignited by the hallmark paper by Guthrie and Susi half a century ago. However, it is only recently that both academia and industry have recognized the many advantages that DBS sampling may offer for bioanalytical purposes, as reflected by the strong increase in published reports during the last few years. Currently, major DBS applications include newborn screening for metabolic disorders, epidemiological surveys (e.g. HIV monitoring), therapeutic drug monitoring (TDM), as well as toxicology. In this review, we provide a comprehensive overview of the distinct subdisciplines of toxicology for which DBS sampling has been applied. DBS sampling for toxicological evaluation has been performed from birth until autopsy, aiming at the assessment of therapeutic drugs, drugs of abuse, environmental contaminants, toxins, as well as (trace) elements, with applications situated in fields as toxicokinetics, epidemiology and environmental and forensic toxicology. We discuss the strengths and limitations of DBS in the different subdisciplines and provide future prospects for the use of this promising sampling technique in toxicology.

Keywords: Toxicology, dried blood spots (DBS), forensic, newborn, trace elements, filter paper, toxicokinetics, drugs of abuse

Contents

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Introduction

Already about a century ago, the potential of using dried blood spots (DBS) for biomonitoring was recognized by Ivar Bang, who demonstrated its usefulness for glucose monitoring (Bang, 1913). However, it took another 50 years before the use of DBS became more widespread, ignited by the seminal paper by Guthrie and Susi, who demonstrated the applicability of screening newborn DBS for phenylketonuria (Guthrie and Susi, 1963). Since then, an ever increasing amount of biomarkers has been included in DBS newborn screening programs worldwide (Seashore and Seashore, 2005; Watson et al., 2006; Garg and Dasouki, 2006; Bodamer et al., 2007; Chace, 2009). Apart from its use for newborn screening, DBS sampling has also been applied in animals, children, adults and even post mortem, its applications rising rapidly the last few years, covering the analysis of DNA (e.g. HIV, serotyping of bacteria, genotyping), proteins (e.g. enzyme activity or antibody-based analysis), small molecules (endogenous or exogenous, e.g. amino acids or therapeutic drugs), as well as trace elements (e.g. lead).

This review aims at covering the different subdisciplines of toxicology for which DBS sampling has been reported. More specifically, we will discuss the application of DBS for the analysis of therapeutic drugs (toxicokinetics), drugs of abuse, environmental contaminants, toxins and (trace) elements. Topics not covered by this review are the use of DBS for therapeutic drug monitoring (TDM) and for metabolic screening, two fields which, although having some overlap with toxicology because toxic effects may be encountered, are considered as distinct disciplines.

The interest in using DBS for the purpose of TDM (including clinical toxicology), with sampling either in the clinic or at the patient's home, has recently shown a strong increase. Also here, apart from follow-up and reassuring that therapeutic concentrations are reached in patients, toxicology may come into play when considering the purpose to monitor (and avoid) supratherapeutic (potentially toxic) concentrations. However, it is beyond the scope of this review to provide a full overview of all therapeutic drugs for which DBS sampling has been applied. The reader is referred to other, comprehensive reviews on this topic (e.g. Edelbroek et al., 2009; Li and Tse, 2010; Stolk and Edelbroek, 2010).

Metabolic screening programs based on DBS sampling aim at identifying disturbed balances in endogenous biomolecules, which may lead to toxic effects. A forensic application worth mentioning in this context is the "metabolic autopsy", which can be performed on DBS to screen for inherited metabolic disorders in cases of sudden infant death syndrome or sudden unexplained death syndrome (Chace et al., 2001). These DBS can either be obtained post mortem or can be those that were obtained at birth in cases where only later a metabolic disorder is suspected in deaths of previously unknown cause.

Dried blood spots – sources

In developed countries, typically five DBS, each corresponding to about 80 µl of blood, are obtained by heel stick from the vast majority (>95%) of newborns within the first 1–3 days of life. With the exception of the 'positive' cases, only a limited amount of this material is used for newborn screening programs, which primarily focus on inborn errors of metabolism. Thus, a substantial amount of valuable material is left behind. These remainders have proven to be a useful matrix for assessing certain exposures at birth. The prime aim of these assessments is to monitor prenatal exposure to toxic compounds capable of crossing the foetoplacental barrier. However, as in most cases no information is available with respect to breastfeeding, it needs to be remarked that postpartum exposure of the newborn *via* mother milk cannot be excluded. Moreover, when interpreting the results in the context of epidemiological studies, several potential sources of bias need to be kept in mind (Searles Nielsen et al., 2008). First, although newborn screening is almost universal in developed countries, non-participation is unlikely to be random (e.g. infants may have died before DBS sampling or parents may have refused DBS sampling). Second, less (or no) material may be left from those newborns that tested positive in newborn screening programs. As analyses are typically performed on material that has been archived up to several years, contamination has to be excluded and analyses are limited to analytes with long-term stability in DBS. In addition, parameters potentially influencing the analytical result, such as haematocrit, blood volume spotted and site of punching (peripheral *versus* central) (Holub et al., 2006; O'Mara et al., 2011) have not been examined in many cases. Nevertheless, keeping these limitations in mind, newborn DBS can be valuable material for screening and may provide important retrospective information on the extent of exposure to a wide array of chemicals or elements. Given the fact that these early life exposures may be relevant to disease later in life, this information may not only result in a close follow-up of 'positively scored' newborns, but may also form the basis for intervention studies, targeting women at specific locations and/or belonging to specific (social) groups.

Apart from DBS sampling for newborn screening, more recently, this sampling technique has increasingly gained interest for its use in both animal and human studies. In humans (both adults and children), DBS are mostly obtained by finger prick. The resulting DBS, which may be directly applied from the finger onto the filter paper or *via* a precision capillary, are in general smaller than those obtained by heel stick. Advantages associated with the sampling itself primarily include its ease and its minimal invasiveness, facilitating sampling in remote areas and in paediatric studies by non-specialized individuals. Sampling from animals at specified time intervals after administration of a given drug is generally performed using microsampling devices (e.g. precision capillaries).

The blood collected with these devices can be used to generate DBS, or it can be frozen, diluted in another solution or centrifuged to prepare plasma (Smith et al., 2011; Stokes et al., 2011). Applications include pharmaco- and toxicokinetics, TDM and clinical, forensic and environmental toxicology.

Analysis of therapeutic drugs–toxicokinetics

Currently, many pharmaceutical companies undertake major efforts to implement DBS rather than classical plasma samples as starting material for bioanalytical measurements. These efforts are situated in the preclinical phase of the drug discovery process (e.g. toxicokinetics) as well as in later phases (pharmacokinetics and TDM). Here, we will primarily focus on the use of DBS for toxicokinetics, determining the relationship between systemic exposure of an animal to a compound and the harmful effects (toxicity) of this compound. A preliminary safety assessment can be derived from parameters such as bioavailability and dose proportionality, serving as a basis to decide which doses can be used in future studies.

From the point of view of animal welfare, DBS sampling conforms very well to the '3R principle (Replacement, Reduction, Refinement)' in toxico- and pharmacokinetic studies. The fact that finer needles can be used to obtain DBS and that there is no need to warm the animals prior to sampling, causes less distress to the animals. Besides this refinement, resulting in less animal burden, the implementation of DBS sampling also leads to a strong reduction in the number of animals needed in early drug discovery and preclinical studies. More specifically, DBS sampling requires less blood to be taken at each time point than is the case when analyses are performed using the 'classical' matrices plasma or serum. For the latter, the number of samples that can be collected per animal is limited by both ethical and physiological constraints. These constraints are greatly relieved by 'microsampling', resulting in the generation of DBS and allowing serial sampling from a strongly reduced amount of laboratory animals, including small rodents such as mice. This allows the replacement of composite pharmaco- or toxicokinetic profiles (obtained from different animals) by serial profiles (obtained within individual animals), which leads to higher data quality (Clark et al., 2010; Turpin et al., 2010; Crawford et al., 2011). Apart from a large improvement in animal welfare, DBS sampling is also beneficial for the (pharmaceutical) companies involved. From a financial point of view, there is a serious reduction in costs associated with animal studies (including amount of test compound that should be available) and with sample handling. The latter includes both transportation costs to a bioanalytical facility and storage costs. Both transportation and storage are often facilitated, as experience has learned that stability, though requiring analyte-specific evaluation, is generally good. Thus, in many cases prolonged storage, even at ambient temperatures, is possible. Despite these many advantages, however, pharmaceutical companies initially somewhat restrained from utilizing DBS as an alternative to plasma or serum. Importantly, the use of DBS instead of plasma or serum necessitated a rethinking of bioanalytical procedures, particularly in the pre-analytical phase. This not only includes the selection of the filter paper card, but ideally also encompasses evaluation of the influence of spotting temperature, anticoagulant, the spotting device used, the volume spotted, the site of punching and the haematocrit, in addition to evaluation of the "on spot stability" and the effect of drying and storage conditions. Additionally, one should also dispose of means for detecting contamination, such as evaluation of blanks and/or incurred sample reanalysis (Spooner et al., 2009; Denniff and Spooner, 2010a; Denniff and Spooner, 2010b; Barfield and Wheller, 2011; Barfield et al., 2011; Timmerman et al., 2011). However, setting up new bioanalytical procedures is (was) not the main problem for the 'switch' from plasma to DBS. More important are regulatory constraints and the fact that plasma and serum have been used for decades as the gold standard, with all currently available toxico- and pharmacokinetic data having been obtained in these matrices. The latter implies that care should be taken, not only in evaluating how plasma concentrations correlate with blood or DBS concentrations, but also if and how capillary concentrations correlate with venous concentrations (Emmons and Rowland, 2010). This may be particularly relevant when evaluating early time points in kinetic experiments (Mohammed et al., 2010). Another factor to consider is the anticipated concentration: when in the low- or sub-ng/ml range, the limited amount of available material may impose analytical challenges that have to be dealt with. Recent improvements in analytical equipment, with primarily LC-MS/MS becoming more widespread available, have catalyzed progress in this field. Currently, major efforts are also being undertaken to render DBS analysis high-throughput-capable. Examples include the automated analysis of DBS, the on-line extraction and analysis of DBS ('direct elution') and direct desorption of DBS (e.g. Crawford et al., 2011; Déglon et al., 2011a). These new developments have recently been reviewed elsewhere (Déglon et al., 2011b; Abu-Rabie, 2011).

Analysis of drugs of abuse

Several publications and meeting abstracts demonstrate (or suggest) the potential of DBS for detecting exposure to drugs of abuse. Analytes measured include both legal drugs (scheduled drugs available on prescription) and illegal drugs. It needs to be mentioned, though, that some of these reports focus on the potential of determining these drugs for TDM (e.g. narcotic painkillers) or for newborn screening (e.g. monitoring exposure to cocaine), rather than for forensic purposes. In addition, several reports have demonstrated the possibility to identify drugs of abuse, as well as ethyl glucuronide, a

marker for alcohol abuse, in blood spotted on different surfaces, rather than on filter paper. As in these cases the resulting blood spots are bloodstains rather than DBS, we do not consider these as true 'DBS applications' (Schütz et al., 2002; Fuller and Pisana, 2009; Winkler et al., 2011). Overall, two sources of DBS can be distinguished for monitoring drugs of abuse: firstly DBS obtained from adults, where the application can be classified under 'forensic toxicology', and secondly DBS from newborns, where the aim is to assess exposure prior to birth. Owing to the low concentrations to be detected in minute amounts of material, LC-MS/MS has been the method of choice in the vast majority of applications, although also GC-MS has been applied in some cases (Table 1).

Forensic toxicology

There is a substantial number of reports describing DBS applications for drugs of abuse (for an overview, see Table 1). Analytes of particular forensic interest that have been measured in DBS include benzodiazepines (alprazolam, clonazepam, diazepam, flunitrazepam, flurazepam, lorazepam, midazolam, nitrazepam, nordiazepam, oxazepam, phenazepam, temazepam), zolpidem, zopiclone, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDEA), amphetamine, methamphetamine, cocaine, tetrahydrocannabinol (THC), opiates (6-monoacetylmorphine, morphine, codeine, hydromorphone, hydrocodone, oxycodone, noroxycodone), tramadol, methadone, buprenorphine, fentanyl, ketamine and their respective metabolites and γ-hydroxybutyric acid (GHB) (Henderson et al., 1993; Sosnoff et al., 1996; Henderson et al., 1997; Alfazil and Anderson, 2008; Garcia Boy et al., 2008; Moll et al., 2009; Clavijo et al., 2010; Havard et al., 2010; Ingels et al., 2010; Marin et al., 2010; Mercolini et al., 2010; Thomas et al., 2010; Clavijo et al., 2011a; Clavijo et al., 2011b; Hudson et al., 2011; Ingels et al., 2011; Jantos and Skopp, 2011; Jantos et al., 2011a; Jantos et al., 2011b; Langel et al., 2011; Lauer et al., 2011). Also interesting from a forensic point of view is the potential to monitor alcohol abuse *via* the determination of ethylglucuronide and ethylsulfate or phosphatidylethanol in DBS (Faller et al., 2011; Jones et al., 2011; Redondo et al., 2011).

Although most reports have included patient samples, two important remarks need to be made. First, a substantial amount of these reports utilizes DBS prepared by pipetting venous blood onto a paper card (e.g. Garcia Boy et al., 2008; Ingels et al., 2010; Faller et al., 2011; Jantos and Skopp, 2011; Jantos et al., 2011b; Jones et al., 2011), with only a limited amount of reports describing the analysis of true capillary DBS (e.g. Sosnoff et al., 1996; Mercolini et al., 2010; Ingels et al., 2011). Although the latter can be obtained by using a precision capillary (Mercolini et al., 2010), our experience learned that this significantly complicates the procedure and ideally requires some training. Instead, direct application of the blood drop from the pricked fingertip onto the paper is generally found to be easy, also for a non-trained individual. However, as DBS obtained in this way do not represent a fixed volume, quantitative evaluation requires the analysis of DBS punches rather than of complete DBS. This brings us to the second remark. Analysis of (very) large spots, obtained from spotting up to 100 µl, has been performed in a substantial number of publications (e.g. Alfazil and Anderson, 2008; Garcia Boy et al., 2008; Faller et al., 2011; Jantos and Skopp, 2011; Jantos et al., 2011b). These volumes are not easily obtained by finger prick; in our experience a spot applied directly from a pricked fingertip onto paper corresponds typically to less than 40 µl of blood. Moreover, the non-volumetric application also has the consequence that disks (typically 3, 6 or 6.35 mm diameter) need to be punched from the paper, further reducing the amount of material available for analysis. Important to mention in this context is that the validation of methods starting from disk punches rather than from complete blood spots requires the evaluation of additional parameters such as punch location, haematocrit and volume spotted (Li and Tse, 2010; Ingels et al., 2011). Thus, although promising results have been obtained, suggesting more widespread applicability in forensic toxicology, true 'on-field' studies are needed for a substantial amount of compounds, in which DBS are obtained by a finger prick. Below we discuss more into detail two specific advantages associated with DBS sampling and the opportunities these offer for implementation in forensic toxicology: the ease of sampling (facilitating rapid sampling) and the stabilizing effect of DBS.

Ease of sampling

Although legislation in most countries does not (yet) allow non-medical staff to obtain DBS from someone else, the ease with which DBS can be taken renders it in principle possible to acquire a blood sample with a minimal loss of time. As this would imply that, at least in some instances, the sampling is done 'on-field', also care has to be taken to let the filter paper dry properly, as an analyte's stability may be impacted by the drying time and drying conditions. In such situations, the paper can be dried by e.g. putting it in a box or bag with desiccant, taking care that the blood spots do not come into contact with other surfaces. This approach of pro-active drying has already been applied in field studies where blood was sampled from wild birds (Trudeau et al., 2007).

Rapid sampling is particularly relevant in cases in which the half-life of a drug is short. Examples include cocaine, heroin (and its metabolite 6-monoacetylmorphine, 6-MAM) and GHB (Henderson et al., 1993; Sosnoff et al., 1996; Henderson et al., 1997; Alfazil and Anderson, 2008; Garcia Boy et al., 2008; Mercolini et al., 2010; Ingels et al., 2010; Ingels et al., 2011). Whereas cocaine intake can be demonstrated by virtue of its metabolite benzoylecgonine, heroin abuse cannot be simply deduced from the presence of its hydrolysis end-product, morphine (see also below). An even more difficult case is presented by GHB, which is also endogenously present and is rapidly

Table 1. Overview of the analytes discussed in this review, with referral to the utilized analytical techniques.

Analyte	Technique	Selected references
(Markers of) Drugs of abuse		
Amphetamine	LC-MS/MS	Jantos and Skopp, 2011; Lauer et al., 2011
	GC-MS	Langel et al., 2011
MDMA, MDA	LC-MS/MS	Jantos and Skopp, 2011; Jantos et al., 2011b; Lauer et al., 2011
	GC-MS	Langel et al., 2011
MDEA	LC-MS/MS	Lauer et al., 2011
Methamphetamine	LC-MS/MS	Lauer et al., 2011
	GC-MS	Langel et al., 2011
Cocaine, benzoylecgonine and other metabolites	RIA, GC-MS	Henderson et al., 1993
	RIA, LC-MS	Henderson et al., 1997
	LC-FLUO	Mercolini et al., 2010
	LC-MS/MS	Sosnoff et al., 1996; Alfazil and Anderson, 2008; Lauer et al., 2011
	GC-MS	Langel et al., 2011
Benzodiazepines	LC-MS/MS	Alfazil and Anderson, 2008; Havard et al., 2010; Thomas et al., 2010; Jantos and Skopp, 2011; Lauer et al., 2011
	GC-MS	Langel et al., 2011
Zolpidem	LC-MS/MS	Hudson et al., 2011; Lauer et al., 2011
Zopiclone	LC-MS/MS	Jantos and Skopp, 2011; Lauer et al., 2011
	GC-MS	Langel et al., 2011
Ketamine and norketamine	LC-MS/MS	Moll et al., 2009
Gamma-hydroxybutyric acid	GC-MS	Ingels et al., 2010; Ingels et al., 2011
Opiates and metabolites	LC-MS/MS	Garcia Boy et al., 2008; Thomas et al., 2010; Marin et al., 2010; Clavijo et al., 2011a; Jantos et al., 2011a; Lauer et al., 2011
	GC-MS	Langel et al., 2011
Buprenorphine and metabolites	LC-MS/MS	Thomas et al., 2010; Marin et al., 2010, Lauer et al., 2011
	GC-MS	Langel et al., 2011
Methadone and metabolites	LC-MS/MS	Clavijo et al., 2010, Lauer et al., 2011
	GC-MS	Langel et al., 2011
Fentanyl and metabolites	LC-MS/MS	Clavijo et al., 2011b; Jantos et al., 2011a, Lauer et al., 2011
Tramadol	GC-MS	Langel et al., 2011
Tetrahydrocannabinol and metabolites	LC-MS/MS	Thomas et al., 2010
	GC-MS	Langel et al., 2011
Cotinine	GC-MS	Spector et al., 2007
Ethylglucuronide – ethylsulfate	LC-MS/MS	Redondo et al., 2011
Phosphatidylethanol	LC-MS/MS	Faller et al., 2011; Jones et al., 2011
Environmental contaminants		
Benzene oxide	GC-MS	Funk et al., 2008
Organochlorine pesticides	GC-ECD	Dua et al., 1996; Burse et al., 1997
	GC-HRMS	Shlosberg et al., 2011b
Perfluoroalkyl compounds	LC-MS/MS	Spliethoff et al., 2008; Kato et al., 2009; Shlosberg et al., 2011b
Polychlorinated biphenyls	GC-HRMS	Shlosberg et al., 2011b; Lu et al., 2011
Polybrominated diphenyl esters	GC-HRMS	Shlosberg et al., 2011b; Lu et al., 2011
Perchlorate	IC-MS/MS	Otero-Santos et al., 2009
Bisphenol A	LC-MS/MS	Leonard et al., 2011
Cholinesterase inhibitors	Cholinesterase activity measurement	Heilbronn, 1953; Augustinsson and Heimburger, 1953; Augustinsson and Holmstedt, 1965; Holmstedt and Oudart, 1966; Collombel and Perrot, 1970; Oudart and Holmstedt, 1970; Augustinsson et al., 1978; Eriksson and Faijersson, 1980; Rhyänen et al., 1984; Hilborn and Padilla, 2004; Trudeau et al., 2007; Quandt et al., 2010
Biotoxins		
Domoic acid	C-ELISA	Maucher and Ramsdell, 2005
Brevetoxins	R-binding assay	Fairey et al., 2001; Woofter et al., 2003
	RIA	Woofter et al., 2003; Woofter et al., 2005
	C-ELISA	Maucher et al., 2007
Ciguatoxin	Cytotox. assay	Bottein Dechraoui et al., 2005

(*continued*)

Table 1. Continued

Analyte	Technique	Selected references
(Trace) elements		
As	ICP-MS	Shlosberg et al., 2011b
Ba	LA-ICP-MS	Hsieh et al., 2011
Be	LA-ICP-MS	Hsieh et al., 2011
Bi	LA-ICP-MS	Hsieh et al., 2011
Ca	LA-ICP-TOF-MS	Cizdziel, 2007
	SF-ICP-MS	Langer et al., 2010
Cd	ICP-MS	Chaudhuri et al., 2009; Shlosberg et al., 2011b
	SF-ICP-MS	Langer et al., 2010
	LA-ICP-MS	Hsieh et al., 2011
Co	LA-ICP-MS	Hsieh et al., 2011
Cr	SF-ICP-MS	Langer et al., 2010
Cs	SF-ICP-MS	Langer et al., 2010
Cu	LA-ICP-TOF-MS	Cizdziel, 2007
	SF-ICP-MS	Langer et al., 2010
	LA-ICP-MS	Hsieh et al., 2011
Fe	LA-ICP-TOF-MS	Cizdziel, 2007
	SF-ICP-MS	Langer et al., 2010
Hg	ICP-MS	Chaudhuri et al., 2009; Shlosberg et al., 2011b
K	SF-ICP-MS	Langer et al., 2010
Li	SF-ICP-MS	Langer et al., 2010
Mg	SF-ICP-MS	Langer et al., 2010
	LA-ICP-MS	Hsieh et al., 2011
Mn	LA-ICP-MS	Hsieh et al., 2011
Mo	SF-ICP-MS	Langer et al., 2010
Na	SF-ICP-MS	Langer et al., 2010
Ni	SF-ICP-MS	Langer et al., 2010
	LA-ICP-MS	Hsieh et al., 2011
P	SF-ICP-MS	Langer et al., 2010
Pb	AAS	Cernik et al., 1971; Verebey et al., 1991
	GFAAS	Shen et al., 2003
	SS-GFAAS	Resano et al., 2007
	ICP-MS	El-Hajjar et al., 2007; Chaudhuri et al., 2009; Shlosberg et al., 2011b
	LA-ICP-TOF-MS	Cizdziel, 2007
	SF-ICP-MS	Langer et al., 2010
	LA-ICP-MS	Hsieh et al., 2011
Rb	SF-ICP-MS	Langer et al., 2010
S	SF-ICP-MS	Langer et al., 2010
Sb	LA-ICP-MS	Hsieh et al., 2011
Se	AAS	Lombeck et al., 1989
	ICP-MS	Shlosberg et al., 2011b
Tl	LA-ICP-MS	Hsieh et al., 2011
V	LA-ICP-TOF-MS	Cizdziel, 2007
Zn	LA-ICP-TOF-MS	Cizdziel, 2007
	SF-ICP-MS	Langer et al., 2010
	LA-ICP-MS	Hsieh et al., 2011

Abbreviations: AAS: atomic absorption spectrometry; C-ELISA: competitive enzyme-linked immunosorbent assay; GC-ECD: gas chromatography with electron capture detection; GC-HRMS: gas chromatography with high resolution mass spectrometric detection; GC-MS: gas chromatography with mass spectrometric detection; GFAAS: graphite furnace atomic absorption spectrometry; IC-MS/MS: ion chromatography with tandem mass spectrometric detection; ICP-MS: inductively coupled plasma mass spectrometry; LA-ICP-MS: laser-ablation inductively coupled plasma mass spectrometry; LA-ICP-TOF-MS:laser-ablation inductively coupled plasma time-of-flight mass spectrometry; LC-FLUO: liquid LC-MS: liquid chromatography with mass spectrometric detection; chromatography with fluorescence detection; LC-MS/MS: liquid chromatography with tandem mass spectrometric detection; R-binding assay: receptor-binding assay; RIA: radioimmunoassay; SF-ICP-MS: sector-field inductively coupled plasma mass spectrometry; SS-GFAAS: solid sampling graphite furnace atomic absorption spectrometry.

cleared from the circulation. GHB or one of its precursors is sometimes used in cases of drug-facilitated sexual assault (DFSA). In these cases, there is most often readily a delay before the victim presents at the police station, thus sampling should be done as fast as possible (without needing to wait for a doctor to arrive). DBS sampling may be a good option in these cases.

The ease and speed of sampling also allows to investigate the epidemiology of drug abuse in a nightclub environment, where DBS could be obtained in "first aid" rooms (e.g. Wood et al., 2009). As in these cases informed consent needs to be obtained from individuals who are under the influence of drugs, this may pose bio-ethical issues, which can be dealt with *via* informed consent from a relative or *via* delayed informed consent.

DBS sampling in the context of DUID (driving under the influence of drugs) has only been evaluated to a limited extent (DRUID, 2011). Although for DUID testing, oral fluid has become the matrix of choice for both screening and confirmation in many countries, some controversy exists whether the obtained concentrations always closely mirror blood concentrations (supposedly best correlating with intoxication) and whether falsification by e.g. mouth washing may be possible (Bosker and Huestis, 2009; Huestis et al., 2011). DBS sampling does not suffer from these drawbacks and combines the advantages (relevance and reliability) of obtaining the ultimate specimen for determination of drug concentrations – i.e. blood – with an easy and rapid collection procedure by non-specialized staff. A key issue related to DBS sampling in this context is the exclusion of contamination.

For the follow-up of drug and alcohol addicts, DBS sampling may be useful to control abstinence from drugs and/or intake of substitution medication. Here, the use of DBS, though offering a more restricted window of detection, may offer an alternative for urine testing, which is now routinely used. Importantly, DBS sampling is gender neutral and is not hampered by privacy issues, which often lead to unsupervised sampling (and possibly adulteration) of urine. As there is also no need for medical staff, one may envisage a system in which unwittingly (former) addicts get a phone call at irregular time intervals and have to present themselves at a given centre to provide DBS under supervision. Moreover, given the higher prevalence of viral infections (e.g. hepatitis and HIV) in people with a history of intravenous drug abuse, the low biohazard risk posed by the resulting DBS is an additional important advantage. No manipulation of the blood is required (in contrast to e.g. the preparation of plasma, requiring centrifugation) and DBS can be transported *via* regular mail with no risk of breaking or leaking, thus minimizing the risk of transmitting blood-borne viruses and overcoming the need for taking special safety precautions (SCDHEC, 2011). In fact, viruses such as HIV-1 lose their infectivity as their envelope is disrupted upon drying, which has led to the use of DBS for routine HIV monitoring in screening and follow-up programs in developing countries (Johannessen, 2010).

Stabilizing effect

Multiple publications have pointed out that DBS may have a stabilizing effect (e.g. Bowen et al., 2010; D'Arienzo et al., 2010). In a forensic context, this can be exemplified by several examples. First, the stabilizing effect on drugs having e.g. an ester function was demonstrated by the increased stability (reduced hydrolysis) of cocaine and 6-monoacetylmorphine (6-MAM, a metabolite of heroin) in DBS, as compared to whole blood (Henderson et al., 1993; Alfazil and Anderson, 2008; Garcia Boy et al., 2008). This is of particular importance as identification of 6-MAM unequivocally demonstrates heroin use (whereas the presence of morphine alone doesn't). Secondly, DBS may also overcome the problem of *ex vivo* formation of a given compound. Whereas *ex vivo* formation of the club drug GHB in whole blood has been reported (Beránková et al., 2006; Zörntlein et al., 2012), prolonged storage of DBS at room temperature (up to 6 months) demonstrated no significant changes (Ingels et al., 2011). Similarly, whereas the presence of ethanol in blood may result in the *ex vivo* generation of phosphatidylethanol (1-palmitoyl-2-oleyl-sn-glycero-4-phosphoethanol) upon storage (Aradóttir et al., 2004; Jones et al., 2011), this *ex vivo* formation does not take place in DBS (Jones et al., 2011). Thus, DBS sampling is able to counter a serious drawback associated with classical venous sampling, thereby increasing the trustworthiness of the result.

Apart from the aforementioned "metabolic autopsy" (Chace et al., 2001), which can be performed on DBS samples taken at autopsy, we found only one report in which analysis of DBS (obtained by spotting venous blood) from a post-mortem sample has been described (Henderson et al., 1993). Although obviously for post mortem analysis there is no sampling advantage anymore, the increased stability of some analytes may still warrant DBS sampling. Yet, another difficulty is posed by the fact that post mortal blood may be lyzed or coagulated, leading to a different spread of the blood on the filter paper. Indeed, DBS generated from lyzed blood have been shown to differ ultrastructurally from those obtained from fresh blood (Cizdziel, 2007), which, dependent on the analyte to be measured, may (or may not) lead to skewing of the results (Cizdziel, 2007; Abu-Rabie and Spooner, 2010).

In the forensic toxicology laboratory, the use of DBS may also offer the possibility to preserve small amounts of sample in an economical way in 'closed cases', where all other evidence is to be discarded. When, for one reason or another, a case is to be reopened, there is at least some material left, potentially allowing targeted analysis. A similar approach has also been suggested for other biological matrices, such as urine (DuBey and Caplan, 1996). Obviously, a limitation is that only analytes can be detected that remain stable for an extended period of time (DuBey and Caplan, 1996).

Newborn screening

Benzoylecgonine and cotinine, which are metabolites of cocaine and nicotine, respectively, have been determined in newborn DBS to assess the prevalence of the use of cocaine and tobacco products among childbearing women (Henderson et al., 1993; Sosnoff et al., 1996; Henderson et al., 1997; Spector et al., 2007). An inherent limitation here is that positive results will only indicate the mother's use of cocaine or tobacco near the time of delivery, thus only offering a limited view on the use during pregnancy. On the other hand, a factor likely extending the interval for detecting positive cases, is the immature liver function in newborns. Although immunological assays have been found to be a useful tool for initial screening of benzoylecgonine in DBS, confirmation is required using other techniques, such as mass spectrometry (GC-MS or LC-MS/MS) (Henderson et al., 1993; Sosnoff et al., 1996; Henderson et al., 1997). With respect to decision-making, any positive signal (above the limit of detection, LOD, or lower limit of quantification, LLOQ) may raise an alert. This implies that the lower the LOD or LLOQ of a given method, the higher the expected detection rate. Implementing a cut-off value in DBS testing of newborns for drugs of abuse may facilitate the inter-laboratory comparison of prevalences. While defining this decision limit, the potential error caused by the possible effect of e.g. varying haematocrit and volume spotted should be taken into account. However, as the cut-off would necessarily be above the LOD or LLOQ, the % of false negatives will undoubtedly increase.

Analysis of environmental contaminants

Screening for environmental contaminants has been performed using DBS from both humans (primarily newborns) and animals. Examples of analytes that have been monitored include environmental pollutants such as benzene oxide (a metabolite of benzene, monitored *via* its adducts with haemoglobin) (Funk et al., 2008), organochlorine pesticides (Dua et al., 1996; Burse et al., 1997; Shlosberg et al., 2011b), perfluoroalkyl compounds (PFCs) (Spliethoff et al., 2008; Kato et al., 2009; Shlosberg et al., 2011b), polychlorinated biphenyls (PCBs) (Lu et al., 2011; Shlosberg et al., 2011b), polybrominated diphenyl esters (PBDEs) used as flame-retarding chemicals (Lu et al., 2011; Shlosberg et al., 2011b), perchlorate (Otero-Santos et al., 2009), heavy metals, as well as certain toxins. Although no published reports are available, yet, DBS have also been suggested to be useful for monitoring bisphenol A (Leonard et al., 2011).

An alternative, indirect way for assessing the exposure to a contaminant, is the monitoring of a biological activity directly influenced by this contaminant (via a so-called 'biomarker of effect'). Insecticides like organophosphates and carbamates are good candidates for this approach, as exposure can be assessed by virtue of their inhibition of cholinesterase activity. The first reports on the determination of cholinesterase activity in blood samples absorbed on filter paper readily date back to 1953 (Augustinsson and Heimburger, 1953; Heilbronn, 1953), a decade before Guthrie and Susi published on the detection of phenylalanine in newborn DBS (Guthrie and Susi, 1963). It needs to be remarked, though, that sensitivity is rather limited and ideally one should know an individual's enzyme activity prior to exposure, with only considerable intoxications resulting in a significant decrease in enzyme activity. Yet, multiple other publications have shown the potential to use (dried) blood and plasma spots for monitoring cholinesterase activity, primarily for occupational surveillance of exposed workers (Augustinsson and Holmstedt, 1965; Holmstedt and Oudart, 1966; Collombel and Perrot, 1970; Oudart and Holmstedt, 1970; Augustinsson et al., 1978; Eriksson and Faijersson, 1980; Rhyänen et al., 1984; Hilborn and Padilla, 2004; Quandt et al., 2010). Several of the DBS applications for monitoring environmental contaminants are discussed more into depth below.

Newborn screening

The organochlorine dichlorodiphenyldichloroethylene (DDE, a metabolite of DDT) and the PFCs perfluorooctane sulfonate (PFOS) and perfluorooctanate (PFOA), as well as benzene oxide and perchlorate, have been detected in all evaluated newborns' DBS (Burse et al., 1997; Funk et al., 2008; Spliethoff et al., 2008; Kato et al., 2009; Otero-Santos et al., 2009), mirroring their general spread in ecosystems and their presence in virtually 100% of the adult population, including pregnant women (Woodruff et al., 2011). Interestingly, a sharp decline in perfluoroalkyl content in DBS from newborns after the year 2000, coinciding with the phasing-out of PFOS in the US, nicely demonstrates the utility of this approach for assessing temporal trends in exposure to environmental chemicals (Spliethoff et al., 2008). Newborn DBS have also been demonstrated to have the potential for monitoring exposure to supraphysiological levels of trace elements (e.g. lead), allowing the extraction of (semi)quantitative information (Langer et al., 2010). However, most of these studies have not been performed within the context of newborn screening and are therefore discussed in a separate paragraph.

Biomonitoring of animals

Intoxication of animals with cholinesterase inhibitors (e.g. organophosphate and carbamate insecticides) may occur *via* ingestion (e.g. of exposed prey) or *via* dermal contact. Assessment of cholinesterase activity in DBS of avian species has been found sensitive enough to serve as a diagnostic tool for identifying exposure to cholinesterase-inhibiting pesticides. DBS sampling of animals allows the collection of samples at remote areas and in non-specialized centres, where no special equipment like a centrifuge is available and where proper storage of a blood sample is difficult (Trudeau et al., 2007).

A recent initiative in the context of monitoring exposure of animals to toxic substances, somewhat paralleling the efforts done for evaluating a newborn's exposure to environmental contaminants *via* DBS, is DABSE ("Database for avian blood spot examination") (Shlosberg et al., 2011a). This biomonitoring project aims at setting up reference values for exposure of wild birds to five groups of environmental contaminants: trace elements, organochlorine pesticides, PCBs, PFCs and PBDEs. Referral to the values within this database should help to pinpoint a possible cause in cases in which an individual bird or a bird population presents with a problem. A first application of this biomonitoring project was performed on griffon vultures, demonstrating detectable levels of several contaminants in DBS obtained from these birds (Shlosberg et al., 2011b).

Environmental health can also be assessed by monitoring the exposure of top predators, acting as sentinels. In coastal waters in the Gulf of Mexico, this approach has been used for monitoring the exposure of bottlenose dolphins to the marine algal biotoxins domoic acid and brevetoxins, respectively produced by members of the diatom genus *Pseudo-nitzschia* and by the dinoflagellate *Karenia brevis* (Twiner et al., 2011). Both biotoxins have been measured in DBS, obtained by spotting filter paper cards with 100 µl of blood, obtained from either exposed laboratory test animals (mice, rats or the fish species striped mullet) or from free-living dolphins (Fairey et al., 2001; Woofter et al., 2003; Woofter et al., 2005; Schwacke et al., 2010; Twiner et al., 2011). Toxin detection in DBS extracts has been performed using receptor-binding assays (Fairey et al., 2001) and radioimmunoassay (RIA, for brevetoxins) (Woofter et al., 2003) and, more recently, by competitive ELISA, either detecting brevetoxins and their metabolites, or domoic acid (Maucher and Ramsdell, 2005; Maucher et al., 2007). Ciguatoxins are another class of highly potent neurotoxins, sharing with brevetoxins the binding site 5 on the α-subunit of voltage-gated sodium channels as effector site (Wang, 2008). Using a neuroblastoma cytotoxicity assay, ciguatoxins have been determined in DBS extracts from exposed mice (Bottein Dechraoui et al., 2005).

Elemental analysis

Biomonitoring of toxic trace elements (metals and metalloids) in human blood has been applied for decades. Examples include lead, which exerts neurological toxicity, and arsenic, cadmium, mercury, chromium, copper, nickel and vanadium, all of which have distinct toxicity profiles. When aiming at (primarily) single-element analysis, analysis is usually performed by atomic absorption spectrometry (AAS), or more recently by (solid sampling) graphite furnace atomic absorption spectrometry ((SS-)GFAAS) (Verebey et al., 1991; Resano et al., 2007). Inductively coupled plasma mass spectrometry (ICP-MS) has been used for both single-element and for multi-element analysis, with more recent developments being laser ablation-ICP-(TOF-)MS and sector-field-ICP-MS (Cizdziel, 2007; Chaudhuri et al., 2009; Langer et al., 2010; Hsieh et al., 2011).

Considering the analysis of trace elements in DBS, most attention has been given to the determination of Pb in DBS obtained from children. Although the determination of venous Pb concentrations is considered the gold standard, venipuncture of infants and toddlers is impractical, may be traumatic for the children and in many countries is not widely accepted by the parents as a screening test for asymptomatic children (Shen et al., 2003). As children are particularly sensitive to Pb and in most countries Pb concentrations peak at approximately 2 years of age (American Academy of Pediatrics; Committee on Environmental Health, 2005, Chandran and Cataldo, 2010), a minimally invasive technique such as DBS sampling offers many advantages for obtaining a representative blood sample. Microsampling of blood for Pb determination in DBS was first reported in the early seventies (Delves, 1970; Cernik and Sayers, 1971). Although since then, many reports have been published on the determination of Pb in DBS, this approach has also been the subject of controversy, given the risk of contamination that may take place, as opposed to blood collection by venipuncture (Verebey et al., 1991; Stanton et al., 1999; Moyer et al., 1999; Verebey, 2000; Moyer et al., 2000; Stanton et al., 2000). Indeed, given the ubiquitous presence of Pb in the environment, special care has to be taken to avoid contamination at every step, from paper handling, sampling, and drying, over transport to analysis. More specifically, falsely elevated Pb concentrations may result from contamination by Pb present on the skin (thus necessitating suitable cleansing before sampling) and/or by improper paper handling (El-Hajjar et al., 2007; Moyer et al., 1999; Moyer et al., 2000). In contrast to a controlled clinical environment, in which the issue of contamination can be dealt with from sampling to analysis, one has to be aware that DBS sampling 'on-field', with less control on pre-analytical variables, potentially suffers from an increased risk of contamination. Yet, especially in developing countries, where studies have shown that the threshold limit of Pb poisoning is exceeded in a large percentage of children (Shen et al., 1996; Ahamed and Siddiqui, 2007; Zhang et al., 2009), the lack of resources renders DBS sampling one of the most feasible ways for screening large populations (Shen et al., 2003). Sampling can be done on-site by a relatively untrained collector and samples can be sent by mail to an analytical laboratory. To correct for possibly inhomogeneous Pb distribution on the filter paper, analysis of 5 replicates (3.2-mm punches obtained from a single 50-µl blood spot) has been recommended by Resano and colleagues (Resano et al., 2007). However, in practice, blood spots often correspond to smaller blood volumes, which may limit the number of punches and/or may pose a problem when

larger punches (e.g. 6 or 6.35 mm diameter) are to be analyzed (Peck et al., 2009). The Pb concentrations determined in DBS have been shown to be independent from the volume spotted and from the site of punching (excluding the area near the perimeter, where concentrations are higher owing to a higher amount of red blood cells). Moreover, a good correlation was found between Pb concentrations in DBS and those in venous blood (El-Hajjar et al., 2007; Resano et al., 2007). DBS obtained from subjects with strongly deviating haematocrit values, however, may give rise to discordant results (El-Hajjar et al., 2007).

Apart from Pb, also other toxic metals, as well as elements of clinical or forensic interest, have been determined (or have been shown to be detectable) in DBS, including As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Rb, S, Sb, Se, Tl, V and Zn (Lombeck et al., 1989, Chaudhuri et al., 2009, Langer et al., 2010, Hsieh et al., 2011; Shlosberg et al., 2011b). Quantification of several of these elements may lead to the generation of an individual's "metallic profile", from which exposure to a certain contamination source may be deduced (Goullé et al., 2010). An important obstacle for fully quantitative analysis of a substantial amount of elements, however, is the variable contribution by the filter paper (both within and between lots) and possible contamination, requiring adequate control of blank filter paper. This implies the control of different lots of unexposed blank paper, directly from the manufacturer, as well as the control of paper ("internal blanks") at some distance from the DBS. Yet, still, one cannot fully exclude the scenario in which contamination within, but not near the DBS took place (Chaudhuri et al., 2009; Langer et al., 2010). The background values obtained from the controls can either be used for subtracting (possibly causing a negative bias) or can merely be used for evaluating the overall extent of contamination. Either way, replicate analysis of the same DBS (punch) and/or analysis of another DBS (punch) from positive cases is recommended to reduce the reporting of false positives (Cizdziel, 2007; Chaudhuri et al., 2009). In this respect, the technique of laser ablation ICP-TOF-MS, providing a "line scan" with several data points per blank and per DBS, allows easy discrimination of potential random contamination (Cizdziel, 2007). Moreover, as reported by Cizdziel, the use of isotope ratio's determined by this technique may also allow to discriminate contamination extraneous to the blood sample (Cizdziel, 2007). To overcome the major problem of contamination encountered in elemental analysis of DBS and, at the same time, to account for possible variations in haematocrit and/or volume spotted, normalization may be another possible future improvement. This can be done using one or multiple elements, having a narrow physiological distribution and/or being (almost) absent in blank filter paper. As suggested by Langer et al., one such candidate could be potassium (Langer et al., 2010). Finally, it is important to mention that decision-making in the case of environmental pollution (including the analysis of trace elements) is somewhat distinct from that in the case of drugs of abuse. Whereas for the latter any positive signal (above the LOD, LLOQ or a certain cut-off) can raise an alert, positivity for the former can in many cases be considered as 'normal', with only levels exceeding a certain threshold warranting further follow-up.

Conclusion and future perspectives

DBS sampling is being applied in a wide range of applications in toxicology, covering fields as toxicokinetics, epidemiology and environmental and forensic toxicology. The analytes measured in DBS include therapeutic drugs, drugs of abuse, environmental contaminants and (trace) elements. Among the advantages associated with DBS sampling, the ease of collecting a representative sample with minimal discomfort is of particular importance for its application in toxicology. This holds true for sampling of animals, newborns, children, but also for adults, considering the potential of DBS sampling at home or in the context of DFSA, DUID or the follow-up of drug addicts. The stabilizing effect of DBS, largely preventing both *ex vivo* degradation and *de novo* formation of analytes, is another significant advantage associated with this sampling technique, facilitating sample handling and transport and often allowing long-term storage of samples. Despite these as well as other important advantages, also some remarks should be made with respect to the use of DBS for toxicological purposes. A first remark is the issue of contamination, which primarily (but not only) is a problem in the field of elemental analysis. Although this issue can be largely dealt with in a tightly controlled environment, contamination can never be excluded, especially in the case of 'on-field' sampling. Given the bioanalyst's awareness of this problem, various avenues are being explored to increase the confidence one may have in a positive result. As mentioned above, these include e.g. the analysis of blank controls, the acquisition of multiple data points from a single spot or from replicate spots and/or attempts to normalize for e.g. haematocrit using one or multiple elements. A second remark is that for many analytes the influence of parameters such as haematocrit, volume spotted and site of punching has not been examined. Lack of knowledge about the influence of these (as well as other) parameters adds an additional, often neglected, factor of uncertainty to the reported analytical result. Thirdly, although promising results have been obtained in e.g. forensic toxicology, the approaches followed are often not fully compatible with the collection of true 'on-field' capillary blood samples, requiring more extensive validation. Apart from these points of attention, requiring more work to be done, it is our feeling that the largest contribution of DBS sampling in toxicology may lie in the field of drug development. There, its implementation of 'refinement' and 'reduction', allowing "small sampling of small animals" closely follows the 3R principle and is even accompanied by improved data quality. Also in

(pre)clinical studies, the implementation of DBS sampling may be an incentive, e.g. by facilitating patient recruitment. As this evolution will evidently lead to a large amount of samples to be analyzed, current efforts are now being focused on automation and rapid, direct analyses of DBS.

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Declaration of interest

The author's affiliations are as shown on the cover page. This paper was prepared during the normal course of employment by Ghent University. The authors have sole responsibility for the writing and content of the paper.

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