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Opening the toolbox of alternative sampling strategies in clinical routine: a key-role for (LC-)MS/MS

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ABSTRACT

Alternative sampling strategies such as dried blood sampling, liquid microsampling and the sampling of oral fluid, hair, meconium, interstitial fluid, sweat, exhaled breath condensate and sputum offer interesting opportunities for many applications in clinical routine. Here, we provide an overview of different applications, with special attention to the pivotal role of LC-MS/MS in facilitating analysis of the collected matrices. Covered clinical fields include newborn screening, endocrinology, therapeutic drug monitoring, phenotyping, toxicology, proteomics and metabolomics. Furthermore, specific advantages, challenges and limitations of each alternative sampling strategy are discussed, along with recent advances and future trends that may contribute to routine implementation of these sampling strategies. Given the development of many recent potentially valuable clinical applications, the possibility of home sampling and the opportunity to obtain information that is hard to procure using traditional sampling, a well-balanced role for alternative sampling strategies can be envisaged in patient healthcare in the (near) future.

KEYWORDS

LC-MS/MS; liquid chromatography; tandem mass spectrometry; alternative sampling strategies; alternative matrices; dried blood spots; oral fluid; clinical laboratory

LIST OF ABBREVIATIONS

CDC: center for disease control and prevention

CDT: carbohydrate-deficient transferrin

COPD: chronic obstructive pulmonary disease

CYP: cytochrome P

DBS: dried blood spot

DPS: dried plasma spot

DRUID: driving under the influence of drugs, alcohol and medicines

EBC: exhaled breath condensate

GC-MS: gas chromatography-mass spectrometry

GH: growth hormone

HbA1c: hemoglobin A1c

HRMS: high resolution mass spectrometry

IGF-1: insulin-like growth factor-1

ISF: interstitial fluid

LC-MS/MS: liquid chromatography-tandem mass spectrometry

MS/MS: tandem mass spectrometry

NBS: newborn screening

PEth: phosphatidylethanol

POC: point-of-care

PT: proficiency testing

QC: quality control

SoHT: society of hair testing

SPE: solid phase extraction

T4: thyroxine

TDM: therapeutic drug monitoring

VAMS: volumetric absorptive microsampling

1. INTRODUCTION

Alternative sampling strategies include the collection of ‘traditional’ samples (blood, plasma, serum or urine) in an alternative way, as well as the collection of ‘alternative’ samples in all kind of ways. A typical example of the former is the collection of dried blood spots (DBS) (i.e. the collection of blood in an unconventional manner), while examples of the latter include sampling of oral fluid, hair and a wide variety of other matrices. Both the ‘alternative sampling’ and the ‘alternative samples’ offer interesting opportunities for clinical applications, as they do not only imply easier sample collection (particularly in special patient populations such as small children and neonates), but can also provide information that is impossible or hard to obtain using traditional sampling strategies, such as venipuncture and urine collection. Moreover, the use of alternative sampling strategies is often coupled to matrix-specific advantages such as increased analyte stability and/or the possibility of home sampling. However, the implementation of alternative sampling strategies in clinical routine requires sensitive analytical techniques, since generally only minute amounts of sample are available and/or low analyte levels may be present. For the quantitative analysis of traditional samples in clinical routine, detection methods such as gas chromatography-mass spectrometry (GC-MS) and particularly immunoassays have been and are still being employed. Over the last decade, a clear trend towards implementing liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been observed[1, 2]. Especially in larger clinical laboratories, the added-value of LC-MS/MS has been advocated. Also for the analysis of samples obtained via alternative sampling strategies, LC-MS/MS is the technique of choice when it comes to combining sufficient sensitivity with utmost selectivity.

2. ALTERNATIVE SAMPLING STRATEGIES

In this review we will focus on the implementation of patient-friendly, minimally or non-invasive alternative sampling strategies which are promising for clinical routine, with special attention to the role of LC-MS/MS. Microsampling of blood to collect liquid microsamples or to generate dried blood or dried plasma spots (DPS), as well as sampling of oral fluid, hair, meconium, interstitial fluid (ISF), sweat, exhaled breath condensate (EBC) and sputum are the alternative sampling strategies covered in this review. While there is a plethora of reports on

the use of alternative sampling strategies for research purposes, (pre)clinical and epidemiological studies, we focus here on those methods that are -in our opinion- most promising for application in clinical routine. The different areas in which alternative sampling strategies may have added-value will be highlighted and for each category some key examples will be discussed, without being comprehensive.

One of the best known alternative sampling strategies is DBS sampling. DBS are generally prepared by depositing a drop of capillary blood, obtained by a finger or heel prick, on a filter paper. The application field of DBS is highly diverse, going from newborn screening (NBS, the screening for inborn errors of metabolism), over therapeutic drug monitoring (TDM) to toxicology and pharmacokinetic studies in drug development. Other microsampling approaches closely related to DBS sampling are DPS and liquid microsampling, as well as volumetric absorptive microsampling (VAMS). The latter is performed using a handheld device consisting of a plastic handle and a hydrophilic polymer tip which absorbs a fixed volume of blood (±10 µL). The advantages and challenges posed by (dried) blood microsampling have been subject to many reviews[3-6]. Briefly, common advantages of dried samples in general include the ease of sampling and the convenient transport and storage under ambient conditions. In addition, these samples pose a reduced risk of infection due to deactivation of pathogens upon drying. In DBS analysis, the hematocrit issue is undoubtedly the most widely discussed challenge. Variations in hematocrit influence the spreading of blood on filter paper, thereby impacting the spot size and, possibly, homogeneity. Furthermore, hematocrit may also influence recovery and matrix effect. As outlined further (see section 6.1), several approaches have been developed that allow to cope with the issues imposed by varying hematocrit[3, 7, 8]. Aside from the hematocrit effect, DBS analysis is also affected by the volume of blood deposited on the filter paper and the punch location[9]. Another issue, which applies to all microsampling strategies, is the possible difference in concentration between capillary and venous blood.

Liquid microsampling is the sampling of liquid capillary blood using a precision capillary. It is used in the pharmaceutical industry, primarily in the preclinical phase of drug development, to obtain pharmacokinetic and toxicokinetic information, e.g. from laboratory animals. Also in the clinical lab, liquid microsamples (typically taken from children) are already being used, e.g. for hemoglobin A1c (HbA1c) monitoring. As the precision capillaries are filled with liquid blood, immediate analysis or processing (e.g. centrifugation, dilution in a stabilizing

buffer and/or freezing) after sampling is generally required, making transport and storage less practical when compared to DBS, DPS and VAMS.

Another widely used alternative matrix is oral fluid. Oral fluid is composed of saliva (an aqueous secretion produced by the major salivary glands), the secrete of the accessory glands, gingival fluid, enzymes, other proteins, electrolytes, bacteria, epithelial cells, ora-naso-pharyngeal secretions, and other debris[10]. Although oral fluid, as an alternative to plasma, has arisen as a potential alternative matrix for TDM, the best established oral fluid-based application to date is roadside drug testing. A major concern coupled to oral fluid analysis, is the risk of contamination. Indeed, contamination with food and/or beverages, other debris from the mouth or smoke are commonly seen[11]. In addition, oral fluid analysis is also prone to oral contamination. Therefore, sampling should be performed immediately before drug intake or after an adequate 'wash-out' period[12]. Furthermore, blood contamination of the oral fluid caused by leakage from the oral mucosa as a result of microinjuries, such as burns or abrasions and due to gingivitis and periodontitis or even following regular mouth hygiene, might compromise analyte quantitation in oral fluid. The latter can have a major impact on the analysis of compounds with a blood to oral fluid ratio which strongly deviates from 1, as was demonstrated for the measurement of e.g. salivary cortisol and testosterone[13-15]. Another important issue in oral fluid analysis is the fact that analyte levels may depend on a multitude of variables such as compound pKa, molecular weight, charge and lipid solubility, as well as oral fluid pH, flow rate and metabolism[10, 16]. Consequently, the measured oral fluid concentrations can be heavily influenced by the employed collection procedure[16, 17]. Oral fluid can either be collected without stimulation (e.g. via passive drooling) or with mechanical or chemical stimulation (e.g. by chewing on paraffin or by using citric acid, respectively)[16, 17]. Moreover, adsorption may occur to collection devices and -important when sample analysis is to be performed by LC-MS/MS- matrix effects may arise from incorporated buffers, preservatives or surfactants[16-18]. Due to the above-mentioned issues, careful selection and standardization of the oral fluid collection procedure is essential to obtain reliable and reproducible results.

The most important advantage of hair sampling is undoubtedly the wider window of detection, due to the fact that incorporated compounds are no longer subject to biotransformation. However, substances of interest can gradually leach out of the hair or can be removed to an important extent by hair damaging caused by cosmetic treatment, such as bleaching or dyeing, resulting in an underestimation of exposure or use[19]. Other advantages

are the non-invasive nature of the sampling and the fact that the collection of a hair sample does not pose any privacy issues. However, it needs to be mentioned that hair sampling can be considered somewhat intrusive. One of the main challenges in hair analysis is external contamination or passive drug exposure. Therefore, a decontamination step is an essential factor in hair analysis, although also the decontamination itself poses challenges, as outlined in section 4[20-22].

Sweat, having a detection or collection window that may range from 30 minutes up to 1-2 weeks, is commonly collected using transdermal absorptive sweat patches, typically applied to the back, upper arm or lower chest and generally worn for several days. While the measurement of chloride in sweat for diagnosis of cystic fibrosis is likely the best studied application, LC-MS/MS-based applications -offering the required sensitivity- include the determination of prescription drugs or drugs of abuse. The main disadvantages of this sampling technique are the potential influence of external contamination of the skin or the sweat patches and the unknown sample volume (rendering interpretation of a quantitative result challenging), as sweat production may vary in function of physical activity or ambient temperature. Given the limited potential of sweat analysis in the routine clinical lab, the interested reader is referred to other recent reviews[23, 24].

EBC collection only requires quiet breathing in a specially designed collection device for several minutes. Various measures can be taken to avoid EBC contamination, as recently reviewed by Konstantinidi et al.[25]. Generally, it is recommended to analyze EBC samples immediately after collection, otherwise immediate freezing, including inconvenient storage, is necessary[26].

The collection of sputum, mucus coughed up from the lungs (after induction or not), is considered a semi-invasive sampling method. Sputum is typically -although not on a routine basis- used to investigate chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD) and interstitial lung disease. LC-MS/MS has been used to measure a variety of analytes in sputum, amongst which leukotrienes, iso(desmosine), mucins, as well as therapeutic drugs[27-30].

Meconium, a neonate's first stool, is widely accepted as the matrix of choice for determining fetal drug exposure (other alternative matrices include umbilical cord, placenta, hair and nails). The wide detection window, covering approximately the last 2-3 months of pregnancy, is, just as the easy sampling, a major advantage[31, 32]. Although meconium is hitherto not used

in general clinical routine, its use can be beneficial for example for the analysis of ethanol markers in centres specialized in the follow-up of neonates from mothers at risk of alcohol abuse[33].

Finally, although less applied in clinical practice up till now, also ISF is an interesting alternative matrix. The composition of ISF, the fluid which surrounds tissue cells, is determined by the continuous exchange of water and small, non-protein bound solutes such as therapeutic drugs, between whole blood and the ISF under the influence of hydrostatic and osmotic pressure. Since ISF concentrations often closely reflect free plasma concentrations of drugs and endogenous substances, this matrix can be particularly valuable in the field of TDM. Over the years, different technologies have been developed for ISF sampling, such as reverse iontophoresis and microneedles[34, 35]. The latter have the advantage of being more convenient for patient self-sampling.

Despite the many advantages accompanying the various alternative sampling strategies, several challenges still remain. Table 1 summarizes the main advantages and challenges of the different types of alternative sampling strategies. Alternative sampling strategies readily being implemented on a routine basis in clinical labs, as well as strategies with potential for future implementation, will be discussed in the next section, which covers several subdisciplines of the clinical lab.

3. CLINICAL APPLICATIONS INVOLVING ALTERNATIVE SAMPLING STRATEGIES

3.1 Newborn screening

The use of DBS sampling as an alternative for conventional blood sampling in neonates has become a widespread technique in NBS programs, ignited by the demonstration of Guthrie and Susi in 1963 to use newborn DBS to determine phenylketonuria[36]. DBS-based NBS by (LC-)MS/MS has exponentially increased since the 1990s and has become an established procedure in developed countries[37]. Moreover, the experience gained from NBS has undoubtedly facilitated the development of DBS-based applications in other fields as well. NBS can be divided into primary screening tests and second-tier tests. The primary tests are designed to identify as many inborn errors as possible. Since diagnostic sensitivity is favored over specificity for disorder detection here, the number of false-positive tests increases.

Therefore, second-tier tests have been implemented, enabled by the introduction of MS/MS methods, to improve the specificity of disorder detection. A second-tier test is performed using the same DBS, is characterized by a lower sample throughput and is extremely suitable to confirm or refute an initial positive result, due to the measurement of additional metabolites[38]. In addition, a distinction has to be made between direct and indirect screening. Direct screening examines endogenous substances, while indirect screening focusses on the conversion of substrates by specific enzymes[39]. Current NBS programs screen for up to over 50 disorders[40, 41]. Of these, 20 to over 40 disorders can be screened for by LC-MS/MS[37]. However the exact number of disorders that is screened for varies strongly from country to country[37, 41]. A key advantage of the (LC-)MS/MS technology is that it is highly multiplexeable (e.g. a multiplex assay of lysosomal enzymes in DBS), making the procedure very attractive in routine NBS as a diagnostic platform for the early detection and confirmation of genetic disorders[42, 43]. As it is beyond the scope of this review to provide a full overview of the metabolic diseases that can be screened for by tandem MS, we refer to a comprehensive review by Lehotay et al. on this topic[37]. Furthermore, DBS can also be used at a later age, for the follow up of (treatment) of inborn errors of metabolism, as is currently applied for patients with e.g. maple syrup urine disease, phenylketonuria or tyrosinemia type 1[44].

3.2 Endocrinology

The measurement of sex steroids, testosterone and especially estradiol, serves as a key tool in the diagnosis or management of a wide range of disorders, such as hypogonadism, polycystic ovary syndrome, amenorrhea, disorders of puberty, male and female infertility and tumors of prostate, testes, breast and ovary[45]. Sex steroid testing has known a noticeable transition from colorimetric assays using urine, over manual radio-immunoassays and automated immunoassays using serum to LC-MS/MS methods[45]. Improved precision, sensitivity and selectivity compared to automated immunoassays and the capability of multiplexing methods has resulted in substitution of traditional sex steroid immunoassays by LC-MS/MS methods in large reference clinical laboratories.

Oral fluid serves, next to plasma and serum, as a matrix for sex steroid determination. Progress in LC-MS/MS has allowed to -at least partly- cope with the low hormone concentration, one of the challenges coupled to oral fluid analysis. However, still, sensitivity remains the limiting factor when considering e.g. the assessment of testosterone in children

and women, and, to a lesser extent, in hypogonadal men via alternative sampling strategies. Furthermore, cortisol and progesterone are also detectable in oral fluid by LC-MS/MS[46]. Cortisol has been determined in oral fluid for the diagnosis of Cushing's syndrome and numerous stress-related disorders, as it is considered a 'stress' biomarker. Importantly, oral fluid can be used for home sampling, which can be of interest for example in the diagnosis of Cushing's syndrome, since late-night measuring of cortisol levels in oral fluid is recommended as a first-line screening test[47]. Furthermore, additional stress -which could affect cortisol test results- due to venous sampling and/or hospital visits is avoided in this way. Progesterone, a hormone that plays a pivotal role in the regulation of the menstrual cycle and in the maintenance of pregnancy, also has been measured in oral fluid, to determine luteal and placental functions in non-pregnant and pregnant women, respectively[48]. Besides oral fluid, also DBS can be used for the LC-MS/MS-based determination of steroid hormones, including corticosterone, deoxycorticosterone, progesterone, 17-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, testosterone, dihydrotestosterone and cortisol, although not in all cases the required sensitivity will be achievable[49]. An upcoming tool for the assessment of long-term cortisol secretion, as a biomarker of chronic stress in various settings, is the analysis of hair cortisol concentrations via LC-MS/MS[50].

Anti-Müllerian hormone is a predictor of the ovarian response in women undergoing ovarian stimulation for *in vitro* fertilization. Since it can be quantified in DBS, this minimally invasive sampling strategy could be another possible future LC-MS/MS-based application in women undergoing fertility treatment[51]. Other hormones that have been measured in oral fluid and/or DBS via LC-MS/MS include 25-hydroxyvitamin D, melatonin and thyroxine (T4)[46, 52, 53]. T4 determination in oral fluid may serve as a simple and cost-effective alternative to free T4 measurement in serum, used in the diagnosis of thyroid disorders. Clinical application of the method could be an interesting future prospect, since T4 measurement in oral fluid turned out to be useful in the diagnosis of Graves disease[46]. Total T4 can also be measured in DBS via MS/MS, along with immunoassay-based determination of thyroid stimulating hormone and antithyroid antibodies[54-56].

Overall, oral fluid- and DBS-based hormone tests are an upcoming tool allowing patient friendly evaluation of endocrine functions. While undoubtedly immunoassays will continue to be used for routine measurement of hormones, LC-MS/MS methods are increasingly being integrated in clinical routine due to the disadvantages (e.g. sometimes poor specificity and accuracy) associated with immunoassays. Clearly, for the accurate determination of hormones

in oral fluid and DBS, LC-MS/MS is the method of choice. For oral fluid it needs to be remarked, though, that although the concentrations measured in this matrix may correlate with the serum/plasma free fraction, they are not necessarily equivalent. For example, Fiers et al. nicely demonstrated that salivary testosterone concentrations measured by LC-MS/MS are not identical to free testosterone concentrations in serum[57]. Moreover, as mentioned in section 2, pre-analytical issues, amongst which contamination of the oral fluid with blood, as well as the choice of the collection method, may have an impact on the result[58]. Hence, the decision whether oral fluid may truly serve as a more convenient and inexpensive alternative to serum/plasma for free active hormone testing may actually depend on the clinical question.

3.3 Toxicology

Alternative samples like hair and oral fluid have become an established part of toxicological investigations in many countries, with analyses being performed in both forensic and clinical labs. Although these samples can provide valuable information, their analysis is also accompanied by some challenges, as discussed in section 2. As already mentioned above (see section 2), in several countries, oral fluid has become -or will soon become- the matrix of choice for immunoassay-based on-site drug screening. Whereas blood is the classical matrix for unequivocal MS-based confirmation of a positive on-site screening test, also oral fluid can serve this purpose. For this confirmation the toxicology section of clinical labs may play a role. Given the potentially high sample-throughput, focus has been put on the development of automated procedures, e.g. applying automated solid phase extraction (SPE) (on-line or off-line) and on-line sample clean-up procedures, typically followed by LC-MS/MS[59]. Another matrix often considered in the context of drugs and driving -albeit covering another time frame- is hair. Indeed, hair analysis is increasingly being used for demonstrating drug abuse or for the determination of (bio)markers, such as the alcohol markers ethyl glucuronide and fatty acid ethyl esters. This approach is currently readily being applied on a routine basis in several countries (e.g. in Sweden, Switzerland and Germany) in driving license regranting programs, providing some labs with a throughput of several thousands of samples per year[60-62]. Such throughputs, combined with the need of ultimate sensitivity, are offered by LC-MS/MS.

Also the use of DBS for toxicology purposes has been advocated[6]. Samples can be obtained from adults or from newborns, e.g. to assess exposure to drugs, alcohol and other xenobiotics prior to birth. For an overview of analytes of particular forensic interest that have been

measured in DBS the interested reader is referred to previous work of our group[6, 63]. The ease of (rapid) sampling and the stabilizing effect are two significant advantages coupled to DBS sampling in (forensic) toxicology. In newborn DBS, benzoylecgonine and cotinine, respectively metabolites of cocaine and nicotine, have been determined to evaluate the use of cocaine and tobacco products among childbearing woman[6]. For most applications, the limited amount of material, combined with the required sensitivity, imposes the need for a dedicated LC-MS/MS configuration. Recent progress in this field includes the set-up of hands-off on-line systems (see also section 5.1)[64]. When considering the cut off for driving under the influence of drugs, alcohol and medicines (DRUID), for most analytes procedures have been described that are able to achieve the required sensitivity when starting from a ≤6.4-mm DBS punch or when starting from 10 µl or less dried blood[63]. Like oral fluid, DBS may offer more convenient sample collection in the context of driving under the influence of drugs, as the usual urine sampling is coupled to privacy issues and the collection of a conventional blood sample by summoned medical staff is cumbersome and time-consuming[65]. Also in the context of driving license regranting programs, in which drivers with a history of alcohol abuse are followed up, DBS sampling may be beneficial. We recently demonstrated this for phosphatidylethanol (PEth), a direct alcohol marker that is used to monitor alcohol consumption during the past few weeks: capillary concentrations of PEth were equivalent to those found in venous blood, demonstrating that capillary DBS are a valid alternative for venous blood for this purpose[66]. Since the sampling procedure does not require dedicated staff and PEth outperforms indirect markers like carbohydrate-deficient transferrin (CDT), capillary DBS sampling offers a promising avenue for routine follow-up of drivers with a history of alcohol abuse. As is the case for CDT now, PEth determination might become a routine procedure integrated in the clinical lab. Again, LC-MS/MS is the method of choice, combining both high-throughput and sensitivity. In the toxicology lab, DBS can also be used as a sampling preparation strategy. We routinely use DBS (as well as other dried matrix spots) for quantitative determination of gamma- and beta-hydroxybutyric acid. Although for these particular analyses we use "on-spot derivatization" and GC-MS[67-69], the use of dried (blood, urine, …) spots as an analytical tool (also allowing automation - see section 5.1) prior to LC-MS/MS can be applied for other compounds as well[6, 70, 71]. When considering toxicology screening in an acute setting, liquid microsampling, coupled to e.g. on-line sample cleanup procedures like turbulent flow chromatography and MS/MS detection, are more likely to be used than DBS, since in most cases it would not make sense to wait for a sample to dry. Yet, it is conceivable that for screening purposes approaches like paper spray-MS/MS or -HRMS

(see further in section 6.4) might be employed in future, to get an instant identification of an intoxicant. Again, a drop of blood might suffice.

Over the last years, meconium has proven to be a valuable matrix in the assessment of prenatal exposure to drugs of abuse and has gained a lot of interest due to the higher sensitivity, the easier sample collection and the larger detection window than traditional matrices, such as neonatal hair and urine[32]. To date, effort has been put in the development of advanced broad-spectrum screening methods using LC-MS/MS, facilitating the use of meconium in clinical routine screening for drugs of abuse. Ristimaa et al. developed in this context an LC-MS/MS-based targeted analysis method for a wide range of drugs of abuse, amongst which MDMA, MDA and THC-COOH[72]. Another application of meconium analysis is the quantification by LC-MS/MS of meconium fatty acid ethyl esters, ethyl glucuronide and ethyl sulfate, three alcohol markers used for the identification of *in utero* alcohol exposure[73]. Furthermore, the use of other non-invasive matrices in toxicology, such as nails, sweat and breast milk, has been enabled by the introduction of sensitive analytical techniques. Although these matrices can be useful in some instances (e.g. doping control, determination of exposure to environmental contaminants), their more widespread implementation in clinical routine is less likely, given the specialized nature of these samples.

3.4 TDM

TDM serves as an excellent tool in the optimization and individualization of drug therapy in both the general and special populations. Most often, TDM is performed on venous blood samples (whole blood, plasma or serum). Unfortunately, these samples are collected in an invasive way and the amounts of blood that are required are relatively large for e.g. neonates or anemic patients. In addition, samples need to be obtained by a phlebotomist, which obliges patients to visit a hospital or doctor's office for a blood draw. Therefore, there is a growing interest in the use of non- and minimally invasive alternative sampling strategies for TDM.

The most widely used alternative matrix in this regard is DBS[4, 5]. The use of DBS for TDM offers several benefits. As DBS are mostly obtained by a finger prick, the patient himself can perform the finger prick at home. In addition, as DBS are considered non-contagious, they can be sent via regular mail to the clinical laboratory[5]. This way, laboratory results may already be available before a patient visits the clinician for routine follow-up. However, the small sample size (typically 3 - 12 µL) associated with DBS imposes the need for sensitive instrumentation[5]. This need can generally be met with LC-MS/MS. Whereas throughput can

be considered a limitation of DBS analysis - at least when considering manual handling of DBS - the emergence of automated DBS analyzers could be of great benefit for clinical routine, as outlined further (see section 5.1).

Table 2 provides an overview of therapeutic drug classes, with selected examples, for which DBS-based TDM via LC-MS/MS has been reported[5, 74-83]. It needs to be mentioned, though, that to date in clinical routine only few therapeutic drugs are determined in DBS via LC-MS/MS. A search throughout lab guides of different clinical laboratories only yielded 4 drug classes for which DBS are used for TDM in clinical routine: tricyclic antidepressants, antibiotics, anticonvulsants and immunosuppressants. Especially in the Netherlands, several hospitals have put major efforts to implement DBS for TDM (and other applications) in clinical routine[84-88]. As mentioned above for the use of DBS in an acute toxicology setting, also for TDM, liquid microsampling might be preferred over DBS sampling when feedback on the sample concentration is urgent. Still, in a hospital context, where staff is acquainted with traditional sampling and where patients are sampled anyway for evaluation of a variety of parameters, the implementation of alternative sampling strategies may not be a logical option in many cases. Microsampling may be a valuable option in those cases that require repeated measurement of drug levels e.g. for the abbreviated area under the curve estimation for the follow-up of tacrolimus treatment[89]. Outside the hospital, TDM may also play a crucial role in assessing patient adherence to prescription regimens of medication, as patient non-adherence is a worldwide problem and leads to serious consequences (e.g. additional use of scarce healthcare sources and higher costs of care, negative impact on the efficacy of treatments and patient’s wellbeing)[90]. In this context, TDM via DBS also fits within the concept of “precision medicine”, where a patient should not only get the right drug, but also at the right dosage to achieve the right concentration, eventually leading to optimized medication usage.

Although the small sample volume is one of the main advantages of DBS, it can be a limiting factor in certain cases as well, e.g. when a physician wants to evaluate various parameters in the same blood sample during treatment follow-up. The simultaneous determination of the kidney function, for example, can be of great importance given the fact that many drugs are excreted by the kidneys and/or may cause renal failure. In this case, creatinine (endogenous) or iohexol (administered) can be determined to assess the glomerular filtration rate. Koster et al. recently developed an LC-MS/MS method for the combined analysis of creatinine and several immunosuppressants in the same DBS extract, which is of great importance given the

risk of renal failure associated with immunosuppressant use[91]. Since iohexol, a contrast agent, has already been analyzed in DBS, its determination in a DBS together with a drug of interest appears another feasible future perspective[92]. In summary, DBS can offer a lot of advantages in the context of TDM. However, the choice to switch from traditional sampling to DBS sampling needs to be well-balanced, taking into account the clinical question and the context in which both sampling and analysis need to take place.

Another alternative matrix which has been extensively evaluated in the context of TDM is oral fluid. Since oral fluid is often regarded as a natural ultrafiltrate of whole blood, its use has been advocated as a convenient alternative to ultrafiltration or equilibrium dialysis to assess the free concentrations of therapeutic drugs[93]. In addition, oral fluid has the advantage of being obtained in a non-invasive manner, yielding the possibility of home sampling. Obviously, the latter is only feasible when a compound is sufficiently stable under ambient conditions, which needs to be evaluated during method development and validation. The stability of newer anti-epileptic drugs, for example, has proven to be adequate, allowing samples to be sent to the laboratory via postal service[94]. Furthermore, preservatives can be added to collection devices to enhance analyte stability[95].

Although oral fluid levels are suggested to correlate with the plasma free fraction of a drug, a correlation between both (or between oral fluid levels and total plasma levels) is often lacking. Moreover, even when a correlation is observed, the latter might be time- or concentration dependent and/or intra- and interpatient variability may be too large to allow reliable use in clinical practice[17]. Therefore, oral fluid is probably not suitable for TDM of most therapeutic drugs[96]. Generally, non-ionizable drugs (at least within the pH range of oral fluid) are considered the best candidates[10, 16]. However, this always needs to be evaluated on a case-by-case basis. Antiepileptics are one of the drug classes for which oral fluid-based TDM can be performed successfully. Specific antiepileptic drugs for which oral fluid provides a good alternative include carbamazepine, clobazam, ethosuximide, gabapentin, lacosamide, lamotrigine, levetiracetam, oxcarbazepine, phenobarbital, phenytoin, primidone, topiramate, and zonisamide. For valproic acid on the other hand, this approach is not useful[12].

Additionally, also other alternative matrices such as tears, hair, sweat, exhaled breath and ISF have been evaluated for TDM purposes. Although some of these (e.g. ISF sampling via microneedles[34]) seem promising, currently, their use in routine TDM laboratories is non-existing or limited at best.

3.5 Phenotyping

Phenotyping aims at determining the exact actual enzymatic activity of Cytochrome P (CYP) 450 enzymes. In general, phenotyping for a drug-metabolizing enzyme consists of administering a selective probe drug of the enzyme, followed by determining a specific pharmacokinetic metric (e.g. systemic clearance of the probe drug, single-point concentrations or metabolite/parent drug concentration ratios)[97]. CYP450 enzymes mainly catalyze phase I metabolism reactions, the first step in the enzymatic biotransformation which is chiefly responsible for the elimination of drugs and other xenobiotics. Interindividual variability is seen in CYP450 enzyme expression and function, which is determined by genetic, epigenetic and non-genetic host factors (e.g. sex, age, pathophysiological conditions) and by environmental influences, such as tobacco smoke, drug intake and diet[97]. Therefore, every person has his own CYP450 enzyme activity profile, resulting in variability in drug metabolism and -consequently- variability in drug response. This variability is the prime reason why CYP450 phenotyping can be implemented in clinical routine for a selection of drugs, including tricyclic antidepressants (imipramine, nortriptyline), antipsychotics (haloperidol, risperidone, clozapine, olanzapine), opioid analgesics (codeine, tramadol), proton-pump inhibitors (omeprazole, lansoprazole) and antithrombotic agents (clopidogrel, warfarine)[97]. The use of alternative sampling strategies for CYP450 phenotyping in combination with LC-MS/MS is currently confined to oral fluid and DBS. Although data obtained using EBC for CYP450 phenotyping are in some respect comparable with those obtained during oral fluid-based phenotyping, the use of EBC in this context is less obvious due to the highly specialized equipment necessary for the measurement of $^{13}CO_2/^{12}CO_2$ ratios in breath. To date, reliable phenotyping methods for various clinically relevant CYP450 enzymes, including CYP1A2, CYP2C19 and CYP2D6 are available using alternative sampling strategies. For CYP3A4 on the other hand, additional studies are necessary[97]. For more details, the interested reader is referred to the recent comprehensive review by De Kesel et al.[97].

3.6 Metabolomics, proteomics and protein analysis

The use of alternative sampling strategies in the 'omics' arena is to date limited to DBS, oral fluid and EBC. In a clinical setting, metabolomics and proteomics are typically targeted approaches, following a discovery phase in which a selected set of biomarkers (proteins and small molecules, respectively) has been identified.

Metabolomics, the global study of metabolites in human body fluids, is an emerging 'omics' science which intends, just as proteomics, to discover specific disease biomarkers. Clinical metabolome studies by LC-MS/MS have been performed for transplantation, cancer, diabetes, lipid profiling and coronary heart disease. LC-MS/MS is an indispensable partner for performing quantitative metabolomics[98]. Alternative sampling strategies in the metabolomics field include EBC as well as oral fluid, with as an example the measurement of salivary biomarkers for the early diagnosis of various types of cancer[99-101].

EBC is an example of an alternative sampling strategy for which proteomic analysis has been performed for the detection of biomarkers related to asthma and COPD. Here, untargeted proteomics plays a distinct role in the search for the underlying pathobiology of these two most common chronic airway diseases. Analysis of EBC by an LC-MS/MS method revealed in this context the promising possibility of using a panel of proteins in the quest for the etiology of COPD[102].

Currently, clinical proteomics can be defined as the (large-scale) study of peptides/proteins in human biological matrices, aiming at validating and/or implementing biomarkers for the diagnosis, prognosis and/or therapeutic monitoring of diseases. Improvements in MS technology partly explain the increased focus on proteomics and protein analysis over the past decade, with applications in a variety of disciplines[103]. Recently, Chambers et al. published a multiplexed approach for the (semi-)quantification of a panel of 97 proteins in DBS[104]. However, the performance of proteomics and protein analysis in clinical routine, both by the use of conventional and alternative biological matrices (e.g. DBS), has remained rather limited to date and mainly focusses on studying one or several proteins. DBS-based protein analysis by MS includes quantitative and qualitative hemoglobin analysis, used in the diagnosis of sickle cell disease and other clinically relevant hemoglobinopathies[105]. Furthermore, Dewilde et al. developed a method for the determination of ceruloplasmin, a biomarker for Wilson's disease, in DBS using LC-MS/MS[106]. Also proteins used in doping can be determined in DBS via LC-MS/MS. Examples are insulin-like growth factor-1 (IGF-1), a biomarker of growth hormone (GH) abuse and Synacthen®, a synthetic human adrenocorticotropic hormone, causing increased plasma levels of cortisol[107, 108]. Further, the rapid emergence of protein therapeutics will likely bring along the need in some clinical labs to measure these proteins in at least a subset of patients. This is typically done by targeted LC-MS/MS-based analysis of a representative set of peptides, generated by proteolytic digestion of a sample, such as a DBS.

Next to LC-MS/MS methods, several immunoassays were developed for the determination of relevant proteins in alternative samples, including DBS. Examples include thyroglobulin and prostate-specific antigen[109, 110]. Although more challenging to set up and implement, LC-MS/MS assays offer the advantage over immunoassays that they do not suffer from false positive results, caused by autoantibodies (e.g. against thyroglobulin) and rely on unequivocal identification rather than on antibody-based recognition. Another disadvantage coupled to many immunoassays is the lack of reliable reference methodologies, sometimes causing incomparable results. In this context, LC-MS/MS methods could be developed as reference measurement procedures[111]. An example of such a reference method is the determination of HbA1c, a fundamental biomarker in the long-term follow-up of the glycemic state of diabetic patients, in whole blood. Several publications readily unveiled the advantages of HbA1c determination in DBS[112]. As blood lipids are also important risk determinants in patients with diabetes, a combined LC-MS/MS determination of HbA1c and lipids in DBS could be beneficial. Since LC-MS/MS-based quantification of cholesterol and related metabolites in DBS has already been performed in the screening of inborn errors, we believe that a combined method is certainly possible in the monitoring of diabetes patients[113].

4. LIMITING FACTORS

Although the use of alternative sampling strategies may be appealing for certain applications, their use in clinical routine is hampered by some practical hurdles, technical challenges and inherent (minor) disadvantages. First of all, the development of methods using alternative matrices generally takes longer, since more variables need to be evaluated. Examples include the evaluation of hematocrit, volume and chromatographic effect in DBS analysis and the influence of the collection method and collection device used in oral fluid analysis. In addition, method development may be further complicated due to interferences originating from e.g. DBS filter paper or oral fluid collection devices[18, 114]. Unfortunately, the above-mentioned additional variables are often not included in standard validation guidelines and matrix-specific guidelines are not always available.

Furthermore, matrix-specific issues exist, which can lead to erroneous results or may complicate data interpretation. Hair analysis, for example, is subject to several issues, as already mentioned in section 2. First of all, it has been shown that external contamination can lead to false positive results, since contaminants can be introduced in the hair matrix of a non-

user in various ways, including during washing steps carried out in the lab (Cuypers et al., unpublished data)[21, 22]. Secondly, for certain compounds a single dose may yield positive results not only in the hair segment corresponding to the moment of intake, but throughout the entire length of the hair, falsely indicating chronic use[115]. Thirdly, cut-offs employed for toxicological hair analysis are not available for every compound or may vary between different guidelines[116]. In DBS analysis on the other hand, the hematocrit effect is the most prominent issue affecting data accuracy and interpretation[3]. Another practical issue in interpreting DBS results is the fact that existing reference intervals and therapeutic ranges are generally established using serum or plasma. So either new reference intervals need to be set up for the specific matrix or bridging studies have to be conducted to correlate alternative matrix levels to systemic plasma or serum levels, whenever a correlation between both is assumed[117]. However, thorough clinical validation is often lacking, as generally only a limited number of samples are included in these studies or the included samples are not true patient samples. In addition, studies on the effect of the use of alternative matrix analysis on patient outcome have not been conducted to the best of our knowledge.

Moreover, as mentioned above, the analysis of alternative matrices requires sensitive equipment, since only a limited sample volume may be available and/or concentrations present may be very low. Hence, when obtaining sufficient sensitivity using traditional matrices (e.g. 100 µL of plasma) is already challenging, analysis of a DBS, for example, may not be feasible. Furthermore, the analysis of alternative matrices such as DBS and hair often includes a lot of manual steps and hands-on time, limiting sample throughput and increasing turn-around-time. Also the set-up of a quality control (QC) program is particularly challenging for alternative matrices (as outlined in section 5.2). Furthermore, it needs to be taken into account that in the case of home sampling of e.g. DBS or oral fluid there is no control of sample collection and storage conditions, as the collection is carried out by the patient or his caregiver, instead of by trained personnel. Although the collection of these samples is not that hard, quality issues with patient samples may pose problems. Also important in a clinical setting is the fact that these alternative tests may not be included in nomenclature and hence may not be reimbursed by the public healthcare system.

5. TOWARDS ROUTINE IMPLEMENTATION

To overcome the above-mentioned hurdles a lot of work has been done and is still ongoing, including the development of new, sensitive and robust analysis techniques, new sampling formats, automated analyzers, and a surrounding support system comprising e.g. proficiency testing (PT) programs and matrix-specific best practice guidelines.

5.1 Automation

An important step in incorporating the analysis of alternative samples in routine laboratories is automation; not only to increase throughput and safety, but also to decrease hands-on time and to exclude human errors. Ideally, automation encompasses the pre-analytical, analytical and post-analytical phase. More specifically in the case of DBS or DPS, this means a lab technician would only have to introduce a patient's card into an analyzer after which the sample is automatically analyzed and the obtained result communicated into a laboratory management information system. Promising advancements have been made in this regard. The tedious punching step can be replaced by (semi-)automated punching devices, whilst sample preparation can be automated using e.g. readily available liquid handling systems[118]. Furthermore, completely automated DBS/DPS analyzers have become commercially available and can be directly coupled to standard LC-MS/MS configurations. These analyzers have accessories such as e.g. barcode readers that allow for sample registration and traceability. Every type of DBS/DPS automated analyzer uses solvents to elute a fixed area of matrix from a collection card, obviating the need for punching. The way the elution is performed depends on the type of analyzer: the DBS Autosampler™ and the Sample Card and Prep system™ both employ flow through desorption, whilst in the DBS-MS 500 system extraction solvent is guided horizontally through the DBS during a surface sealed extraction, after which the extract is guided into a sample loop[119-121]. After elution, the extract can be subjected to on-line sample clean-up and/or separation on an LC column or even direct injection into the MS, depending on the chosen configuration[8, 64, 119-121]. Importantly, using automated DBS analyzers, the entire DBS extract is introduced into the (LC-)MS/MS system, thereby increasing method sensitivity, since in off-line approaches only part of the extract is injected into the analyzing system. The DBS-MS 500 system is currently the only analyzer in which internal standard can be sprayed onto the DBS before extraction[119]. In the two other types of analyzers the internal standard is automatically added to the elution solvent. To verify whether the correct portion of the card has been analyzed these analyzers can take a picture of the sample before and after analysis. In addition, it is also possible to use the DBS analyzers as automated sample preparation devices that are not coupled to an LC-MS/MS system. To

reduce hands-on-time even further, also the preparation and spotting (in the case of DBS/DPS) of calibrators, QCs and blanks can be automated using a commercially available liquid handling system[59, 122]. This procedure showed similar accuracy and precision as manual preparation, but was safer, more efficient and yielded samples of predictable quality.

Also for the analysis of other alternative matrices, automation is important to allow convenient implementation in a routine setting. Therefore, commercially available workstations can be employed to automate laborious sample pretreatment steps as much as possible. This was e.g. done for the analysis of drugs of abuse in preserved oral fluid samples collected using Quantisal™ devices[59]. For this application the workstation was not only used for IS addition, but also for automated SPE. The only remaining manual step during sample pretreatment was the evaporation of the SPE eluate which was automatically collected in LC vials. However, for some matrices automation may prove challenging. In the case of hair analysis for example, the inability to handle the solid hair sample limits the degree of potential automation[123].

Importantly, when using LC-MS/MS in a routine setting, not only the sample handling and the on-line sample preparation need to be automated, also the LC-MS/MS modules and software programs should become more user-friendly. More specifically, to make LC-MS/MS technology as convenient as possible for lab technicians, ideally a sort of "black box" LC-MS/MS unit should be integrated in existing chemistry analyzers. One suggestion that has been made in this regard is the development of an analyzing module with hybrid technology combining the characteristics of immunoassays and tandem MS[124], potentially omitting the need for LC.

Another, complementary way of increasing throughput to which LC-MS/MS lends itself perfectly, is to multiplex different analytes in a single run. The latter also aids in retrieving as much information as possible from a limited sample. However, in hair analysis the development of such multi-analyte procedures might prove challenging, since authentic hair samples are required for e.g. extraction optimization during method development[123]. Additionally, multiple LC systems can be multiplexed on one MS, further allowing a more economic use of the MS. This can be via staggered analysis (in which the chromatographic eluate only enters the MS/MS system during the time window where the compounds of interest elute) and/or via a more convenient switching between different methods, since no hardware changes need to be performed. Even sample multiplexing (i.e. the simultaneous

introduction of two samples that were differentially derivatized) may be considered an option to increase throughput[125].

5.2 Quality assurance and harmonization

LC-MS/MS methods, including those for alternative matrix analysis, are typically developed in-house in clinical laboratories and are (at least up till now) generally not approved by the Food and Drug Administration[126]. Therefore, every laboratory is completely responsible for each test it implements. Unfortunately, QC materials for alternative matrices - necessary to help guarantee method quality - are generally not commercially available. QCs should be prepared in native matrix and whenever this is not feasible, should at least be commutable with true samples. Although a few standard kits exist for LC-MS/MS, these are not necessarily suitable for e.g. DBS methods, since in that case calibrator and QC materials should also have the same viscosity as true blood to have similar spreading properties. Therefore, calibrators and QCs are currently often prepared in-house from different (non-)certified starting materials. The development of more LC-MS/MS kits, encompassing calibrators and QCs in a suitable matrix, as well as e.g. internal standards, extraction solvents and mobile phases would hence be a tremendous step forward. Although such a kit has, for example, already been developed for the analysis of amino acids and acylcarnitines in DBS, it needs to be pointed out that it was developed for MS/MS analysis and not specifically for LC-MS/MS analysis[127]. Some alternative matrices also have special QC requirements. For DBS, for example, it is advisable to include different hematocrit levels[128], whilst for hair analysis it is important to include different hair types[129]. In addition, to ensure appropriate accuracy, methods should ideally be traceable to a higher order reference method. However, these reference methods are often lacking.

Concerning external quality assurance, the NBS quality assurance program has played an important role for DBS analysis, since it offers both certified PT materials and external QCs for (MS/MS-based) NBS assays[130]. However, external QC and PT materials are not yet available for all performed NBS tests. Furthermore, an initiative has been launched by The Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology to set up a pilot PT program for TDM of immunosuppressive drugs in DBS[131]. For hair analysis the Society of Hair Testing (SoHT), HAIRVEQ and the German Society of Toxicological and Forensic Chemistry organize PT programs for some drugs of abuse and ethanol markers[116]. Also for oral fluid and sweat PT schemes exist for drug testing. An extra

complicating factor in PT programs for alternative matrices are the different types of substrates that are used to collect samples (e.g. different types of filter paper cards in DBS sampling and different collection devices in oral fluid sampling)[131]. Therefore, to facilitate these types of programs, harmonization/standardization will be important in the future.

Not only the analytical method itself, but also the patient samples (even when collected at home) have to be of sufficient quality. A first step towards achieving this is by guaranteeing the quality of the substrate on which the sample is collected. For DBS sampling this is achieved via the Filter Paper Evaluation Project, set up by the center for disease control and prevention (CDC) which also offers its services to filter paper manufacturers[130]. The quality criteria postulated by the CDC resulted in the laboratory standard, Clinical and Laboratory Standards Institute NBS01-A6, which has greatly contributed to the reduction of analytical imprecision due to batch-to-batch variability in filter paper. Furthermore, the quality of a DBS is also evaluated before analysis, either by experienced laboratory personnel or in an automated fashion using an optical scanning instrument[132]. Such instruments objectively evaluate spot area, circularity, convexity and consistency (i.e. DBS size, symmetry and uniformity). In this way, acceptable, marginally acceptable, and unacceptable DBS are distinguished and acceptable punch areas identified. To maximize the amount of acceptable spots in the event of home sampling, patient education via demonstration folders and movies has proven to be essential. To evaluate whether contamination occurred during sample collection, incurred sample reanalysis or analysis of a blank filter paper area close to the DBS has been advocated[6, 133]. Also for other matrices which are usable for home monitoring, evaluation of sample quality is essential. Therefore, it can for example be advised to perform oral fluid home sampling using collection devices with volume indicators.

To help harmonization and standardization of alternative matrix analysis, best practice guidelines have been proposed by several committees (e.g. SoHT or the European Bioanalysis Forum consortium) highlighting important pitfalls and validation requirements[116, 128]. Also harmonization of data interpretation is an essential goal of these committees. The SoHT and the DRUID project, for example, have played an important role in the determination of cut-offs for toxicological analysis of hair and oral fluid, respectively[116, 134].

6. FUTURE TRENDS

6.1 Development of new formats

New formats and approaches have been developed to improve the acceptance of dried blood (spot) analysis by tackling the crucial hematocrit issue. Strikingly, most of these approaches have been designed to be compatible with automated analysis. First of all, volumetric approaches have been suggested which are able to produce fixed-volume DBS from a non-volumetric whole blood droplet. Whilst some of these approaches use formats which are compatible with existing DBS analyzers[135, 136], others such as VAMS employ a different type of collection device[137, 138]. However, also in this case, sample preparation can still be automated, more particularly via readily available liquid handling systems. Secondly, others have suggested the use of DPS instead of DBS. In such instance the former can be prepared via filtration of a blood drop over a size-exclusion membrane, which withholds blood cells[139, 140]. The DPS cards developed by Sturm et al. are compatible with commercially available automated analyzers[141]. Unfortunately, also DPS appear to be subject to a certain hematocrit effect[141]. A third possibility is to estimate the hematocrit of a DBS and to correct for the anticipated hematocrit effect[142]. Recently, we developed a non-contact hematocrit estimation method (Capiau et al., unpublished data) which could potentially be more easily automated than the potassium-based hematocrit estimation method we previously established[7].

Also for other alternative matrices innovation is ongoing. An example is the development of a new exhaled breath collection device for the analysis of drugs of abuse, which allows to standardize the volume of matrix collected (at least to some degree) and facilitates remote collection by non-trained personnel[143]. The device is composed of a filter which is located in a filter holder and a mouth piece which is attached to a plastic bag. When a person breathes into the device, aerosol particles will be collected onto the filter and the plastic bag will inflate. When the plastic bag is full, sufficient matrix has been collected onto the filter. Subsequently, the filter holder and the mouthpiece can be detached from one another and the filter holder can be closed with plugs and sent to a laboratory via regular mail.

6.2 Microfluidics

Microfluidics have been employed for alternative matrix collection and sample preparation in conjunction with tandem MS. One example includes the use of a lab-on-a-chip for sample preparation of either a DBS punch or a directly applied capillary blood droplet[144]. Multiplexed extraction of DBS using this type of chips was demonstrated by Lafrenière et al. using automated droplet control.[145] Due to its relative simplicity this technique has even been suggested to be able to bring clinical analysis closer to the patient. In this context, a proof of

principle was published by Kirby et al. who used digital microfluidics to extract dried urine spots and to transport the extract to a nanoelectrospray emitter to allow tandem MS-based detection of drugs of abuse using a portable mass spectrometer[146]. Evans et al. on the other hand, employed capillary-scale LC to analyze DBS extracts, since this increases assay sensitivity[147]. To avoid problems with column connections, a chip can be employed onto which column, connections and MS emitter and spray are co-located[148]. Although no published examples could be found by the authors, chip-based microfluidic extraction could potentially be made compatible with LC by integrating the required technology into a single chip format for a seamless workflow. Notwithstanding the promising nature of these new developments, latest technological advancements are often only steadily implemented in a routine setting, as robustness first has to be well established.

6.3 High resolution mass spectrometry (HRMS)

Aside from its use in e.g. biomarker discovery research (possibly in oral fluid or DBS), untargeted screening using HRMS is increasingly becoming a valuable tool for toxicological purposes, e.g. allowing detection of new psychoactive compounds and their metabolites[149]. Also in other cases where untargeted screening is advised, e.g. for the evaluation of the chemical and biological exposure of humans (which has been suggested to become more important in the future)[150], HRMS may be an ideal screening tool. In addition, for the detection of inborn errors of metabolism, the use of HRMS has been advocated as an alternative for the traditionally used electrospray ionization-MS/MS, since multivariate pattern recognition analysis would lead to better specificity and the identification of comorbidities and interferences caused by medical treatment or damaged DBS[151]. Last, although LC-MS/MS is likely to remain the workhorse for quantitative bio-analysis during the next couple of years, LC-HRMS/MS is increasingly advocated as a suitable alternative, further facilitating quantitative analysis of complex mixtures.

6.4 MS(/MS)-based point-of-care testing (POC)

Another important trend in clinical analysis is the development of MS(/MS)-based POC testing and/or near-patient analysis. The latter may be performed at a professional healthcare center, at the emergency unit or even in operating theatres. However, development of these decentralized analyses is most often -and ideally- still under the supervision of the clinical laboratory, which is responsible for e.g. quality assurance. In general, these POC techniques do not require sample preparation steps nor a separation step and employ ambient ionization

techniques (although a multitude of ambient ionization techniques have been employed, selected examples will be discussed). In this context as well, alternative sampling strategies have been employed. Examples include the use of paper spray MS for the analysis of blood or oral fluid collected on filter paper for e.g. TDM purposes and abstinence monitoring[152, 153]. In paper spray MS, a drop of the biological sample is deposited on a triangle-shaped filter paper which is part of a disposable collection cartridge. After the cartridge has been positioned in front of the MS, a solvent is applied to the filter paper, as well as a high voltage. This causes a spray to be formed at the tip of the triangle, which is then transmitted into the MS. Subsequently, the resulting signal is recorded for a fixed period of time, which results in a signal vs. time plot (called a chronogram). To quantify the amount of the target compound present, the area under the curve of this chronogram is employed. To be workable in a POC-setting, the blood on the filter paper is either analyzed when it is still wet or after it has been quickly dried using either pre-spotted coagulants or heat application[152-154]. A first step towards automation has been accomplished by the development of a tray, which can hold multiple filter paper triangles which are consecutively analyzed[155]. In addition, to render POC-MS feasible, portable MS systems have been developed[156]. The mini 12, for example, has been employed for the analysis of the therapeutic drug amitriptyline using paper spray MS[157]. The goal of this instrument is to be able to offer a sample to a miniature MS after which analysis and data analysis are performed automatically and a result is directly generated on the screen. Similarly, the concept of touch spray MS has been developed. This refers to the direct analysis of oral fluid collected on a medical swab. In this case as well, a solvent and a voltage are applied to the collection device after which a spray is formed. Applications of the technique include the semi-quantification of various drugs and the detection of lipids which are specific to *S. pyogenes* to quickly diagnose strep throat[158, 159]. In addition, even surgical smoke has been sampled via a surgical knife to help differentiate between cancerous and non-cancerous tissue during surgery[160].

7. CONCLUSION

Although ample clinically valuable applications have been developed using alternative sampling strategies, their adoption in routine clinical laboratories is still limited. Aside from the vast use of DBS in NBS programs, only few examples can be found which employ alternative sampling strategies for routine analyses using LC-MS/MS. Examples include the

determination of salivary cortisol levels, the determination of drugs of abuse in oral fluid, and the use of DBS for TDM purposes. The growing automation of alternative matrix analysis will definitely contribute to the acceptance and introduction in routine practice. In addition, matrix-dependent issues are (successfully) being tackled and new, more robust formats are being developed, bridging the gap between research and clinical laboratories. To guarantee the quality of alternative matrix-based assays, initiatives to set up guidelines for the development and validation of these assays (which take into account matrix-specific requirements) as well as matrix-specific PT programs are essential. Although alternative sampling strategies will never replace traditional sampling, they should definitely be regarded as a complementary approach, which may be particularly valuable to extend the window of detection and/or to allow specific applications such as home monitoring, sampling of special patient populations (such as neonates, children and elderly), and sample collection in remote or resource limited areas. In this context, alternative sampling strategies, combined with LC-MS/MS, can be looked at as an additional tool, with the potential to provide high quality results where adequate information cannot be (conveniently) obtained using traditional approaches.

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	Dried blood spots	Dried plasma spots	Capillary liquid sampling	Volumetric absorptive microsampling (VAMS)	Oral fluid	Hair	Meconium	Interstitial fluid (micro-needles)	Sweat	Exhaled breath condensate	Sputum
Patient comfort (e.g. ease of sampling, invasiveness)	++	+	+	++	++	+	+++	++	++	+++	+
Small sample volume	++	++	++	+++	++	+	-	+++	NA	-	++
Analyte stability	+++	+++	+++	+++	+/-	+/-	++	+	++	-	-

Table 1: The main advantages and challenges of the different types of alternative sampling strategies.

Convenient storage and transport	+++	+++	-	+++	+	+++	-	-	+	-	-
Reduced infection risk (compared to blood samples)	+	+/-	+	++	+++	+++	+	+	+++	+++	+++
Resistance to contamination	-	-	+	-	-	-	++	++	-	+	++
Resistance to hematocrit effect	-*	+	++	++	NA	NA	NA	NA	NA	NA	NA

+++ / ++ / + / - : indication to what extent the statement on the left holds true; NA: not applicable

* The hematocrit effect is a major issue, but to date, several attempts to cope with the issue have been explored.

Table 2: Overview of therapeutic drug classes, with selected examples of drugs, for which DBS-based TDM via LC-MS/MS has been reported.

Drug class	Medicines	
Anticonvulsant[75, 82]	Phenobarbital	Clobazam
	Topiramate	Clonazepam
	Rufinamide	Phenytoin
	Carbamazepine	Valproic acid
	Lamotrigine	
Antiviral[5, 74, 76]	Atazanavir	Efavirenz
	Nevirapine	Darunavir
	Indinavir	Ribavarine

	Ritonavir	Tenofovir
	Saquinavir	Etravirine (TMC125)
	Nelfinavir	Raltegravir
	Lopinavir	(Val)Ganciclovir
Immunosuppressant[5, 76, 83]	Cyclosporine	Sirolimus
	Tacrolimus	Everolimus
	Mycophenolic acid	
Cytotoxic[5, 76, 78]	Actinomycin-D	Imatinib
	Vincristine	Nilotinib
	Busulfan	Dasatinib
	Paclitaxel	Tamoxifen
Analgetic[5]	Acetaminophen	
Angiotensin II receptor antagonist[5]	Losartan	
Antibiotic[5, 74, 81]	Ertapenem	Moxifloxacin
	Linezolid	Rifaximin
	Claritromycin	Rifampicin
	Ramoplanin	
Antidepressant[5]	Venlafaxine	
Antimalarial[5]	Mefloquine	

Antimycotic[5, 74]	Voriconazole	Posaconazol
	Fluconazole	Metronidazole
Diuretic[5]	Canrenone	
Histamine H_2-receptor antagonist[5]	Ranitidine	
β-blocker[5]	Propranolol	
Antipsychotic[79]	Amisulpride	(Dehydro-)Aripiprazole
	Asenapine	Bromperidol
	(Nor)Clozapine	(OH-)iloperidone
	Haloperidol	Lurasidone
	(Levo)Sulpiride	Paliperidone
	Risperdone	Pipamperone
	Sertindole	Quetiapine
	Zuclopenthixol	
Antidiabetics[80]	Metformin	Sitagliptin
Enzyme-inhibitors[77]	Nitisinone	