LABORATORY MEDICINE

The dried blood spot sampling method in the laboratory medicine

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ABSTRACT
The minimization is currently expanding in all fields of human life. The smaller size of analytical instruments and their higher sensitivity enables an analysis of small amounts of a biological material with a very low consumption of chemicals, what is economically and also environmentally beneficial. A patient friendly and minimal invasive sample collection is therefore more than required. The dried blood spot (DBS) sampling standardly used in the newborn screening (NS) may be an option. The sample collection is simple, non-invasive, does not require trained medical personnel assistance and also storing and transportation of these samples is much easier in comparison with the whole blood samples. The DBS sampling method is used in the therapeutic drug monitoring or in the diagnosis of infectious diseases and its usage is still spreading. In some cases, it is still only a research object, but it shows a big potential for a future use. It could completely replace the whole venous blood collection in some cases, for example in the metabolic screening of diabetic patients or monitoring of treatment response, and it can overall simplify the sample collection and the transportation process (Tab. 1, Ref. 152). Text in PDF www.elis.sk.
KEY WORDS: dried blood spot, DBS, dried samples, sample collection, laboratory medicine.

Introduction

The dried blood spot (DBS), a new sampling technique, was first introduced by Ivar Christian Bang in 1913 for the determination of blood sugar levels in rabbits (1). Subsequently, several scientists used this type of sample collection in their studies, but it became popular only in 1963, thanks to Robert Guthrie, who used it for sample collection in the neonatal screening of phenylketonuria. The capillary blood from a newborn’s heel was saturated onto cellulose filter paper to detect levels of phenylalanine that is typically elevated in the disease. A disc was punched out from DBS sample and transferred to an agar plate containing Bacillus subtilis and a competitive inhibitor (beta-2-thienylalanine). The phenylalanine from the sample competed with the inhibitor, and the disease was detected by a zone of growth around the disc (2).

Currently, DBS is a standard sampling method in newborn screening of different diseases all over the world (the number and type of screened diseases vary from country to country). It allows us to analyze not only biochemical markers, but also nucleic acids. The whole process of blood collection, sample handling and transport conditions, to ensure the maximal quality, is published in a standard manual (3).

Advantages and disadvantages

Due to a lot of advantages, in comparison with plasma or whole blood samples, DBS has become a very interesting sampling method in other analytical fields as well, like therapeutic drug monitoring (TDM), diagnostics of infectious diseases, metabolomics and so on. In contrast to newborn screening, the methodology of sample collection, handling and processing in these cases has not been unified yet. If the analytes of interest are defined with long-term stability, also the cards from newborn screening, that have been stored for years, could be used for retrospective studies (4, 5).

Advantages

In a comparison with classic liquid samples, DBS sampling has several advantages. One of the most important is a less invasive sample collection that is acceptable mainly in case of newborns, small children, and older people or needle-phobic patients. The small incision of a finger pad by a special lancelet is nearly painless and without special practicing can be performed by patients themselves or by other adults. In the case of newborns, even parents can prick the heel, thus, the need for qualified medical personnel is minimized. DBS cards can be stored very easily. After drying and eventual overlaying with a protective layer, the cards can be put on top of each other, thereby requiring less space. With the drying process, the most analytes become more stable and the need for low temperatures during storage then disappears. So, the transportation of those samples is much easier. It can be said, with exaggeration, that DBS samples can be collected by patients themselves and sent to a laboratory by post without the need of freezing. However, particular attention must be taken, because the temperature and humidity in mailboxes can rise according to the

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weather conditions, which could harm the samples. In one of the studies van Amsterdam and Waldrop pointed out that depending on local conditions, distance and weight, more or less 200 euros per shipment could be saved by shipping DBS samples instead of plasma. It is also mentioned that approximately 30% of all plasma samples are shipped in an incorrect way (incorrect packaging, documentation and so on) (6). On account of drying, viruses, such as HIV (human immunodeficiency virus) or HBV (hepatitis B virus), are not able to survive due to the disruption of the virus envelope, thus the risk of the transfer of blood borne diseases is minimized.

Where animal experiments are necessary, DBS sample collection confines and reduces them. So, instead of taking several milliliters of blood, only few drops are necessary. For example, the total blood volume of a laboratory mouse is only 1.5 ml on average, so DBS sampling is suitable if more than one sample is needed. The low volume of biological material requires more sensitive analytical techniques, but, on the other hand, the amount of chemicals used in the processing of such samples is significantly lower, which is environmentally beneficial. Due to a very low sensitivity or problematic stability, the DBS sampling method is not 100% appropriate for all analytes and the determination of some of them can be challenging. Most of all, the influence of other blood components must be considered (mainly different haematocrit levels among individuals – see Effect of matrix). A DBS sample is the mixture of plasma and blood elements, thus the concentration values cannot correspond to routinely used plasma reference levels and must be set, when establishing a method.

**DBS sample collection**

The skin must be disinfected with alcohol before being punctured and then allowed to air-dry. The blood can be taken from the finger, heel or ear lobe, by pricking with an automatic lancet. The first drop should be wiped away. The other blood drop can then be applied onto a sampling paper/card within the marked area that should be touched neither before, nor after blood application (especially while the sample is still wet). The indicated area should be homogeneously filled with blood (preferably with only one drop if it is possible) and show the same red color from both sides of filter paper. A DBS sample prepared like this should be left to air-dry in the horizontal position at ambient temperature for a minimum of 2–3 hours. Contact with other surfaces, direct sunlight exposure or the presence of any other heat sources, should be avoided (4, 7–9).

DBS sample can also be prepared by pipetting whole-venous blood from the tube with an anticoagulant in the middle of the indicated area. The blood is dosed in a particular amount with a calibrated pipette and disposable plastic tip. Thus, the error, due to the variable volume of blood on the filter paper, is minimized. The DBS sample must be prepared as soon as possible after anticoagulated whole-venous blood collection (not more than 24 hours after venepuncture). Drying conditions are the same as in the previous case. With this approach, the benefit of a less invasive sample collection is cancelled, but, on the other hand, there are benefits if venepuncture is required for some other examination. For example, collection of samples for research can be done in this way in a medical laboratory, with only the written consent of the patient, and without any additional punctures (4, 7).

**Type of a filter paper**

The DBS samples can be collected on the different types of filter papers that are available on the market. They are all made from 100% pure cotton linters, but can vary in pore size or thickness and influence the absorptivity of blood (7). It was investigated if the type of filter paper can influence the results of DBS analysis. Some studies indicate that differences can be observed, mainly at extremely high or low analyte concentrations or extreme haematocrit levels (10, 11). However, according to Koster et al, all types of filter papers tested reported the same haematocrit level and the same pattern of recovery dependence on concentration. Mei et al propose that the results of analysis among different DBS cards are comparable (12). In the newborn screening programs, the untreated filter papers are mostly used and they are controlled also among different lots of the same manufacturer to ensure that the same conditions are preserved (8, 13). The untreated papers can be modified when soaked with various chemical solutions and allowed to air-dry in order to increase stability of analytes or in any other way to positively affect the character of DBS sample. The chemically-treated papers can also be found in the market. They were initially designed for nucleic acid analysis and they should cause cell lysis, protein denaturation or pathogen inactivation (13). The use of alternative filter paper for DBS sample collection, for example chromatographic (14), or even non-cellulose-based materials, like glass (15), are also mentioned in certain studies, but their usage is not significant.

**DBS storage and stability**

The quality of DBS samples can be negatively affected by moisture and humidity, which can cause bacterial growth, a degradation of molecules of interest or a variation in extraction recovery. Complete drying is therefore necessary before storing. The DBS samples should be protected even during storage either by adding desiccant to gas permeable zip-closure packaging or by overlaying them with paper. With the aim to increase stability, samples with less stable analytes should be stored at lower temperatures (4 °C, −20 °C or −80 °C), but if the molecules in the DBS samples are stable, room temperature is also appropriate (7–9).

For example, enzymes can be degraded very quickly, especially with higher temperatures (16, 17). However, lysosomal enzymes show a very good stability even at ambient temperature, so the DBS technique can be easily used for the diagnosis of lysosomal storage diseases (18, 19). The stability of nucleic acids in DBS varies depending on the type of nucleic acid. Studies for HIV detection show that DNA is more stable in DBS samples than RNA. Whereas DNA is stable at least one year even, when stored at 37 °C, in most cases RNA is unstable under these conditions. Otherwise, RNA shows a good stability at −70 °C (6–9 months, depending on viral load) (20, 21). C-reactive protein (CRP) lev-
els decrease quickly in DBS, when stored at ambient temperature, since its stability under these conditions is only 3 days. However, storage at −20 °C prolongs stability for at least one year (22, 23). Immunoglobulins (IgG, IgM, IgA) for hypogammaglobulinemia detection are stable in DBS for 14 days at ambient temperature, but they degrade after 3 days, when stored at 37 °C (24).

The knowledge of analyte stability is crucial, when using the DBS method. Stable molecules are well preserved by drying, but the degradation or modification of less stable molecules presents a problem. Then, it must be considered very carefully if DBS is a suitable method for sample collection (7, 25–27). The stability can be positively affected by adding other substances to the filter paper before sample collection. For example, glutathione is subjected to non-enzymatic autoxidation and enzymatic conversion that can be stopped by pre-treating the filter paper with a reducing agent (for instance dithiothreitol) (28). Similarly, peroxidation of polyunsaturated fatty acids can be limited by adding butylated hydroxytoluene (BHT) (29).

Effect of the matrix

The most frequently reported component of blood, affecting the DBS samples, is haematocrit (HCT) (30–37). Its value indicates the percentage of erythrocytes in total blood volume and differs not only among individuals, but also during different disease states of one organism. The typical HCT level for men is 46 ± 6 % and for women is 41 ± 5 %, but it can vary from 28–67 % in some certain populations (neonates, 2–12 year old children) (7, 32, 38, 39). Moreover, capillary blood tends to have a higher HCT level than venous blood (40). HCT is closely related to blood properties, and that is why it can affect its behaviour, when spotting onto filter paper and can cause significant assay bias, mainly at very low or at very high concentrations. The blood becomes more viscous with high HCT levels, which means that the absorptivity and spreading of blood on filter paper is very poor in comparison with low HCT levels and the volume of blood in a punched disc may vary (7, 9, 25, 37, 39). The recommended, but not frequently used, manner of how to overcome this problem is an involvement of different HCT levels in method validation. The interval, in which the results are still valid, should be defined. Then, it should be decided if the HCT of the DBS samples is within this range or not. The measurement of the HCT level is not problematic, when using venous anticoagulated blood for preparation of DBS samples. However, this practice excludes the possibility of blood sampling by patients themselves at home, which is reported as one of the main advantages of using the DBS sampling method (32, 37). An alternative approach must be used, when samples are prepared from capillary blood or are already dried. The measurement of some other parameter that correlates with the HCT level (for example, potassium) can be performed (30, 41). Moreover, with the aim to minimize the HCT effect, the calibrators should be prepared from the blood with HCT levels corresponding to the expected median of the screened population. Another recommendation of how to deal with haematocrit problem is to analyse the whole DBS sample after pipetting the particular volume of blood on filter paper. However, again, this approach is not approved for the patient self-sampling procedure (37).

Some studies suggest that the punch location is also an important factor affecting the assay bias (33, 35). Holub et al inferred that the location of disc punch influences the levels of some analytes in conjunction with HCT levels. For example, in case the level of HCT is low in the blood used for a DBS sample preparation, higher concentrations of amino acids can be found on the edge of a blood spot. In the center of the blood spot, their concentration is lower. It is due to the chromatographic effect causing different distribution of the analyte in the DBS sample (35).

If the extraction procedure is not optimal, particularity of the DBS sampling method can result in other problems and it is a problem with recovery at different concentration levels. Because of the presence of −OH groups on the surface of cellulose filter paper, some analytes can soak into the paper in higher concentrations and stay on the surface in lower concentrations. This could lead to lower extraction recovery in higher concentrations, and conversely, higher extraction recovery in lower concentrations. The recovery of the method can be improved by impregnating the paper with some agents that decrease the hydrogen-bond activity (7, 42).

Use of the internal standard

An internal standard (IS) is required for monitoring the behaviour of analytes in a sample and for compensation of losses during sample handling or due to the matrix effect. Ideally, it should be a stable isotope-labelled substance, but a more cost-effective solution is to use any substance with the structure and properties most similar to the tested analyte, that is not naturally presented in the sample. The proper addition of an IS into the DBS sample is challenging. Even if the most commonly used technique – addition of an IS into an extraction solvent – has reproducible results and is very easy to perform, it actually does not fully reflect the behaviour of the analyte. Firstly, it cannot reflect the changes of the analyte during storage, and, secondly, it does not indicate the losses during the extraction procedure. From an analytical point of view, an IS should be added to the sample as soon as possible; that means before spotting blood onto filter paper. Ideally, it should perfectly interact with blood and paper matrix, without influencing the original sample (for example with chromatographic effects). Therefore, some other approaches of IS additions have been investigated (43–46).

According to van Baar et al, slightly better results, in a comparison with the widely used technique of adding an IS into an extraction solution, are achieved by adding an IS directly to a liquid blood sample. The complete incorporation of the IS into the blood matrix is supposed to occur this way. In practice, this approach is difficult to perform, because of increased complexity of all procedures and untrained personnel (44, 45). Liu et al proposed the use of small glass tubes covered with the IS on the inner wall, for blood collection and DBS preparation, which could simplify the process of sample-handling (47).

The results, comparable with those, when the IS is a part of an extraction solvent, are obtained by an application of the IS via
Tab. 1. Examples of the usage of DBS samples (except of newborn screening).

| Analytes and ions | Sample type | Time | Temperature | Stability | Extraction | Derivatization | Separation and detection | Source |
|-------------------|-------------|------|-------------|-----------|------------|---------------|--------------------------|--------|
| **Elements and ions** | animal | N.A. | N.A. | 2y-30°C | HNO₃ | - | ICP-MS | (113) |
| Na, Mg, K, Ca, Fe, Co, Cu, Zn, As, Se, Cd, Pb | human | finger prick | 3m | -20°C | N.A. | Triton X-100 and HNO₃ mixture | - | ICP-MS | (114) |
| As, Pb, Hg, Cd | human | finger prick | prior to shipment | -20°C | N.A. | Triton X-100 and acetic acid mixture | - | ICP-MS | (115) |
| Hg | animal | venous anticoagulated/ heparinized capillary tube blood | prior to analysis | RT | 1y at RT/3m at 30°C | - | - | AAS | (116) |
| Pb, Hg | human | newborns heel prick | N.A. | N.A. | 8.5m at RT | 5% double-distilled HCl solution containing 0.05% 2-mercaptoethanol, 0.001% L-cysteine and 10 p.p.b. Ir and Rh | - | ICP-MS | (117) |
| K⁺ | human | venous anticoagulated blood | 2d | RT | 2d RT/20/60°C | 2.5m MCl in ultrapure water | - | ISE | (118) |
| K⁺ | animal | venous anticoagulated heparinized capillary tube blood | prior to analysis | RT | minimal 2w at 25°C | MetOH:formic acid:DIT | - | LC-MS/MS | (119) |
| **Amino acids** | human | N.A. | N.A. | N.A. | IS solution in 30% citrate phosphate buffer 0.1 M in MatOH | - | BuOH/acetylimidazole (50:1) | LC-MS/MS | (120) |
| 23 amino acids | human | N.A. | N.A. | N.A. | IS solution in MatOH | - | acetyl chloride:BuOH (10:90,v/v) | ESI-MS | (121) |
| Pho, Tyr, Trp | human | finger prick | N.A. | N.A. | 70% BHOH | AQC | HPLC-MS/MS | (122) |
| Pho, Tyr, Trp, Ile | human | finger prick | N.A. | N.A. | MetOH, water | Acrlylidene in BuOH | LC-MS/MS | (123) |
| cystathionine, bHcy, Met | human | N.A. | prior to analysis | RT | minimal 2w at 25°C | MetOH:formic acid:DIT | - | LC-MS/MS | (124) |
| Val, Leu, B, Met, Phe, Tyr | human | newborns heel prick | N.A. | prior to analysis | -/4°C | N.A. | HC3/HPO₄/HCO₃/C₃H₂O₂ / 5-sulfosalicylic acid/ MatOH/citric acid | ORA | HPLC-FL | (125) |
| 4 fatty acids | human | newborns heel prick | N.A. | prior to analysis | -/4°C | N.A. | MatOH/HCl | GC-FID | (126) |
| 17 fatty acids | human | N.A. | 15d | 4°C | N.A. | saline and isopropylalcohol (1:1) | MatOH/acetylimidazole (50:1) | GC-FID | (127) |
| 7 fatty acids | human | finger prick | N.A. | -25°C | N.A. | - | MatOH/HCl | GC-FID | (128) |
| γ-hydroxybutyric acid | human | finger prick/venous anticoagulated blood | 28d | 4°C/20°C | 1m/20°C | - | TFAA:heptafluorobutanol (2:1) | GC-MS | (129) |
| 4 fatty acids | human | finger prick | N.A. | prior to analysis | RT | N.A. | - | BF3-MetOH | GC-FID | (130) |
| 24 fatty acids | human | newborns heel prick | 17d | N.A. | - | HCl/acetylimidazole | DMAE | ESI-MS/MS | (131) |
| 22 fatty acids | human | finger prick | 1d | -30°C | N.A. | - | BF3-MetOH | GC-FID | (132) |
| CRP | human | finger prick/venous blood with no additives | 3w then another 7/14/28 42d | -20°C then 22°C 37°C | 12°C | up to 1w at 22°C | assay buffer (0.01M PBS, 0.5M NaCl, 0.1% v/v Tween20, pH 7.2) | - | ELSA | (133) |
| CRP | human | finger prick | N.A. | N.A. | N.A. | assay buffer (0.01M PBS, 0.5M NaCl, 0.1% v/v Tween20, pH 7.2) | - | ELSA | (134) |
| thyroglobulin | human | finger prick | prior to analysis | -20°C | N.A. | N.A. | - | - | ELSA | (135) |
| hCG | human | venous anticoagulated blood | 7/14/45d | RT/8°C | 25°C | minimal 45 d at 8°C | PBS (pH 7.4) | - | LC-MS/MS | (136) |
| hCG | human | N.A. | none | none | none | PBS (pH 7.4) | - | nanoLC-MS | (137) |
| 97 proteins | human | venous anticoagulated blood | overnight | RT | majority 22w at -20°C | 25mM NH₄HCO₃, 10% (v/v) sodium deoxycholate and 5mM mix[2-carboxyethyl]phosphine mixture | - | IUPHLC-MS/MS | (138) |
| measles specific IgG | human | heel/finger prick | 15-20d, then up to 12m | 10-12°C then -20°C | N.A. | N.A. | PBS | - | ELSA | (139) |
| IgG, IgM, IgA | human | finger prick | N.A. | N.A. | 14-4/4d 14-2,5 °C 3-4/0.3°C 10-14 d/-25°C | PBS, 0.05% Tween-20 | - | ELSA | (140) |
| Analyte                          | Sample type         | Stability | Extraction            | Derivatization          | Source       |
|--------------------------------|---------------------|-----------|-----------------------|-------------------------|--------------|
| S. pneumoniae DNA             | human               | N.A.      | 3 m at RT             | MetOH                   | (145)        |
| THC, THC-OH, THC-COOH         | human               | N.A.      | 3 m at RT             | MetOH                   | (83)         |
| PETH                          | human               | N.A.      | 110 d at 22°C         | MetOH                   | (146)        |
| salicylic acid                | human               | N.A.      | -                     | DESI-MS                  | (59)         |
| tricyclic antidepressants     | human               | N.A.      | 1-3 m at RT           | acetosulfate MetOH (1:3)| (147)        |
| antiangiotic (cinoxam,        | human               | N.A.      | 1-3 m at 8°C          | IS solution in MetOH    | (81)         |
| metronidazole,                 | human               | N.A.      | 3 d at 60°C           | IS solution in MetOH    | (142)        |
| everolimus                    | human               | overnight | 3 m at RT             | MetOH                   | (143)        |
| oseltamivir and oseltamivine  | human               | N.A.      | minimal 7 d at RT     | MetOH                   | (144)        |
| carboxylate                   | human               | -         | -                     | MetOH                   | (145)        |
| rifampicin                    | human               | -         | -                     | MetOH                   | (146)        |
| N-acetyl galactosamin-6-       | human               | N.A.      | 4°C                   | 3 h at 50°C substrate   | (150)        |
| salicylic acid                | human               | N.A.      | 4°C                   | substrate buffer and NaCl| (151)        |
| glycoconitropeptide            | animal              | up to 1 m | 4°C                   | distilled water           | (16)         |
| PDE6A activity                | human               | 34-72 h   | RT                    | substrate buffer and phosphate-citrate buffer | (16)         |
| glucose-6-phosphate dephosphoroxytransferase 1 | human | N.A. | 2 m at 4°C | - spectral fluorimeter | (18) |
| alpha-galactosidase A, acid   | human               | up to 1 m | 4°C                   | distilled water           | (16)         |
| alpha-galactosidase            | human               | up to 1 m | 3 m at 4°C | distilled water           | (16)         |
| alpha-glucosidase and galactosylceramidase activity | human | N.A. | 2 m at 4°C | distilled water           | (16)         |
| lysosomal acid lipase activity | human               | up to 1 w/ longer periods | 4°C ≤ 30°C | 20M sodium phosphate buffer (pH 7.1) | (151) |
| lysosomal acid lipase activity | human               | up to 2 w | -20°C | - spectral fluorimeter | (152) |
| pyridoxamine 5-phosphate      | human               | up to 1 w prior to | RT/20°C - 40°C | 40mM Triphospho-phosphate buffer (pH 7.6) | (153) |

**AAS** – atomic absorption spectrometry; **AQC** – 6-aminoquinoly-n-Hydroxycinnamyl carbamate; **Arg** – arginine; **BzOH** – butanol; **Cit** – citrulline; **CRP** – C-reactive protein; **DESI** – description electrospray ionization ion; **DMAE** – N,N-dimethylethanolamine; **DNA** – deoxyribonucleic acid; **DIT** – dithiothreitol; **ELISA** – enzyme-linked immunosorbent assay; **ESI** – electrospray ionization; **E0H** – ethanol; **FID** – flame ionization detector; **FL** – fluorescence detector; **GC** – gas chromatography; **GLC** – gas-liquid chromatography; **Hb** – human hemoglobin; **HCV** – hepatitis C virus; **HIV** – human immunodeficiency virus; **HPLC** – high-performance liquid chromatography; **Ig** – immunoglobulin; **Ile** – isoleucine; **IS** – internal standard; **ISE** – ion selective electrode; **LC** – liquid chromatography; **Leu** – leucine; **m** – month(s); **Met** – methionine; **MetOH** – methanol; **MS** – mass spectrometry; **MS/MS** – tandem mass spectrometry; **N.A.** – not available; **OPA** – o-phthaldialdehyde; **Orn** – ornithine; **PBS** – phosphate-buffered saline; **PCR** – polymerase chain reaction; **PDA** – photodiode array; **PETH** – phosphatidylethanol; **Phe** – phenylalanine; **Hep** – total homocysteine; **Trp** – tryptophan; **Tyr** – tyrosine; **RNA** – ribonucleic acid; **RT-PCR** – reverse transcription polymerase chain reaction; **TFAA** – trifluoroacetic anhydride; **THC** – 9-tetrahydrocannabinol; **UHPLC** – ultra-high performance liquid chromatography; **Val** – valine; **w** – week(s); **y** – year(s).
spray in separated step either before or after blood spotting onto filter paper. This technique results in a more homogenous distribution, when compared to IS pipetting. Additionally, potential chromatographic effects are avoided. It is assumed that better results could be achieved by applying the IS onto filter paper before blood spotting, hence avoiding time differences emerging from variable time between sample collection and IS application (43–45).

Calibration standards and quality of the control samples

Just as in all other types of samples, analysis of DBS samples also requires calibration standards and the quality control (QC) samples most similar to the real ones. It means that fresh blood, with haematocrit level within the range of the tested population, should be used for DBS QC and calibration standard sample preparation. It is not difficult to find non-interfering blood for the analysis of new molecules (for example, new pharmaceutical drugs), but it could be a problem in the case of small biomarker molecules, which are presented endogenously and whose level can vary from human to human or according to biorhythms (7). A possible solution to this problem is the preparation of artificial “whole blood” by adding washed blood cells to an artificial plasma or serum (48, 49). Since the calibration and QC stock/working solutions are not solved in the blood, their volume in the artificial whole blood should be minimized as much as possible (maximum 5 % of the final volume) (7).

Extraction procedure

The extraction from solid to the liquid phase represents one of the most critical steps in DBS sample processing. The extraction of whole DBS, prepared from the exact amount of blood (50), or usage of these so-called perforated (51) or even pre-cut (52) DBS are mentioned in literature, but their usage is rare. Usually, the extraction procedure consists of punching out a disc, extraction and derivatization (if necessary), while the latter two mentioned can be performed in any order. The diameter of the disc punched out from DBS can range from 3 to 8 mm (9, 27, 53) and the number of discs used can vary according to the sensitivity of the analytical method. The extraction is performed by adding a specific volume of the extraction solvent that plays a crucial role in re-solubilizing the analytes to the disc. For better effectiveness, shaking, vortexing or ultrasound can be used. The choice of the extraction solvent depends on the physicochemical properties. It should be strong enough to interrupt the bindings between the molecule of interest and the paper matrix (7, 9, 27). Usually, organic solvents (methanol, hexane etc.) (54, 55), aqueous buffers (saline, phosphate buffers etc.) (56, 57) or mixtures of organic and aqueous solvents, and alternatively the commercial kits (58), are used for this purpose. To increase the extraction efficiency, pH altering substances, surfactants (TWEEN Triton X) or chelating agents (EDTA) can be added (27). Manual extraction of DBS samples is a bit laborious and time-consuming and therefore an alternative approach would be necessary. Either automation or sample analysis that does not require sample preparation at all (or at least minimizes it) (43).

Direct analysis without a previous extraction could be an option, for example: desorption electrospray ionization (DESI) (59–61), direct analysis in real time (DART) (15, 62), matrix-assisted laser desorption/ionization (MALDI) (63) etc. Unfortunately, direct analysis means the loss of the separation step, which may result in lower sensitivity and possible interferences, while current technological status does not allow automatic analysis of a huge number of samples (46, 53, 64). That is the reason why most of the studies prefer manual offline extraction. The extracts can be directly injected into an instrument (58) or derivatized considering the properties of the analyte and the chosen detector (7, 65). Alternatively, the derivatization reagent can be added directly to the DBS sample and then, just prepared derivatives are extracted (66). This procedure is worthy of discussion because it is not known what reactions could occur between the derivatization reagent and the paper matrix. Especially very aggressive derivatization reagents, for example acetyl chloride, could cause some unexpected damage of the paper material and negatively affect the sample processing and analysis.

DBS and liquid sample results comparison

The essential part of all research on the application of DBS samples should be a comparison of the levels of tested analyte(s) between routinely used types of sample (most often liquid) and DBS samples and a search for possible correlations in the results. The finding of strong correlations indicates that DBS samples can be used as an effective alternative to sample collection (7, 67–69).

Applications of the DBS samples

The DBS sample is standardly used for the early detection of different genetic and metabolic disorders in newborns, to avoid irreversible changes or at least significantly improve symptoms of the disease. The capillary blood from a newborn’s heel is spotted onto a filter paper card, optimally 24–48 hours after birth, and analyzed by tandem mass spectrometry. The changes in the concentration of individual molecules might not have such an informative character as the monitoring of several biomarker molecules and their comparison in relation to each other. These metabolic patterns are evaluated in the context of different disease states (7, 9, 70–73).

Metabolomics is a relatively new discipline that reflects the actual state of the organism and that can show diverse variations in time. The qualification and quantification of metabolites in time has a great potential in the early detection of cancer, in the prediction of disease-development or in the monitoring of the treatment response. With huge number of samples that must be analyzed to find new biomarkers, DBS seems to be a good alternative to sample collection (65, 74, 75). As Kang et al and Zukunft et al demonstrated in their studies, DBS samples could fully replace plasma in metabolite profiling (76, 77). Chace et al proved that DBS samples can also be used in post-mortem metabolomics screening in cases of unexpected or unknown infant and child death (78).

Toxicology is a wide field, including preclinical drug discovery process (toxicokinetics), as well as latter phases (pharmacokinetics...
and TDM). The aim is to determine the relation between a chemical substance entering the body with a specific rate and its fate in an organism (the way of metabolizing and excreting it). Very often animal experiments are needed, and DBS sampling meets the requirements of the 3R principle (replacement, reduction, and refinement). The number of samples per one animal is restricted physiologically and from an ethical point of view. DBS not only reduces the volume of blood needed and thus the number of animals needed, but it also reduces the cost of experiments for pharmaceutical companies (79). TDM is used in humans to optimize the drug dosage for individuals, so that the highest effectiveness and security is preserved. The self-sampling and almost painless sample collection is a big advantage of DBS, even if the samples can be possibly contaminated, if the person preparing them and dosing the drug is the same. The results obtained by the DBS method are comparable with plasma, so DBS sampling is an excellent alternative (80–82). DBS can be used also in forensic toxicology, for example in screening the abuse substances, like cannabinoids (83), cocaine (84), opiates (85) and so on (86). Also the screening of phosphatidylethanol, the direct ethanol metabolite, in DBS samples, could be a promising way of controlling the alcohol level of drivers. The same metabolite can be analyzed also in newborns, if prenatal alcohol exposure is suspected (86–88).

The simplicity and affordability of DBS samples is advantageous also in epidemiological studies. The disruption of the virus envelope during the DBS drying process ensures biosafety, whereas well preserved antibodies and nucleic acids enable the detection of various infectious diseases (89), for example: measles (90), rubella (91) or even Ebola (92). DBS samples could also help with the actual summary of epidemiological situations concerning asymptomatic or long-incubation period diseases (for example, AIDS or chronic hepatitis, via HIV or HCV monitoring) (93).

Some of the concrete examples of huge usage of DBS samples can be seen in the Table 1.

**Similar types of samples**

Recently, there is a great interest in a new form of simple, rapid and non-invasive sample collection. The unique character of DBS sample collection inspires the search for new types of samples in dried form.

Dried plasma spot (DPS) or dried serum spot (DSS) samples are prepared exactly like DBS samples, but from patients’ plasma/serum pipetted onto filter paper, which requires the additional step of blood centrifugation and automatically means the loss of the non-invasive sample collection status. However, DPS/DSS samples, which are more homogenous in comparison with DBS, do not contain blood cells and thus do not show the haematocrit dependence. These samples are often used in different fields of clinical and pre-clinical analyses (94–99).

The collection of dried urine spot (DUS) samples is even less invasive and in the case of newborns, the filter paper can be simply inserted into the diaper. This type of sample collection is suitable for all analytes excreted in urine. It can be used for example in the diagnosis of different diseases or for drug screening (100–105).

Some drugs and metabolites can be present also in human saliva, therefore dried saliva spot (DSSs) samples were created (106–108).

Also, a research on human milk samples in dried form could be truly beneficial for mothers staying at home. Self-sampling, simple collection and ease of transportation are big pluses of this method. Dried milk spot (DMS) samples can be used also for TDM (109–111).

Another biological fluid, in which analytes can be determined, is sweat. Dried sweat spot (DSwS) samples can be used for example in the diagnosis of disturbances in lipid metabolism as they show a very good correlation with plasma samples in the analysis of fatty acids (112).

**Conclusions**

The advantages of the usage of DBS samples are noticeable. The minimization of the amount of biological material needed for analysis, the enhancement of biosafety and the simplification of the whole collection and transportation process are all the reasons, why DBS samples have spread so quickly (not only in newborn screening programs). The influence of HCT and the absence of the automation step for a huge number of samples can be the main pitfall causing the lack of common use in clinical practice and as a primary method for research. The analysis of some molecules can be challenging due to a very small sample volume and the need for more sensitive analytical methods. However, this problem appears to gradually fade with the development of new methods, like mass spectrometry. Some molecules are not even appropriate for DBS sample analysis due to their instability or the fast alteration of their structure. Thus, DBS samples would never be able to completely replace the whole blood or plasma samples, but in some specific cases, DBS can simplify all logistics around sample handling and storage, which is beneficial mainly for long-term studies. In animal experiments, it helps to follow the principle of the 3Rs (replacement, reduction and refinement) and, what is more, it is economical and environmentally-friendly (the low amount of samples means a low amount of chemicals needed for analysis).

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