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REVIEW ARTICLE

Smart sampling as the *"Spot-on"* Method for LC-MS protein analysis from dried blood spots

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This perspective explores the feasibility of smart sampling with dried blood spots for the determination of proteins and peptides from human biomatrices using liquid chromatography coupled to mass spectrometry for clinical purposes. The focus is on innovative approaches to transform filter paper from a mere sample carrier to an active element in sample preparation, with the aim of reducing the need for extensive and intensive sample preparation in the conventional sense. Specifically, we discuss the use of modified cellulose to integrate sample preparation at an early stage of sample handling. The use of paper immobilized with either trypsin or monoclonal antibodies for protein digestion and affinity clean-up is discussed as a potential benefit of starting sample preparation instantly at the moment of sampling to optimize time efficiency and enable faster analysis, diagnosis, and follow-up of patients.

KEYWORDS
DBS, lab-on-paper, LC-MS, microsampling, protein determination

1 | INTRODUCTION

The use of paper in the clinical analysis of biologically relevant substances has a long history. Already at the beginning of the 20th century, Ivar Bang showed the possibility of determining glucose from blood deposited on filter paper [1] (Figure 1). The development and publication of the phenylketonuria screening test in dried blood spots (DBS) from neonates in 1963 renewed the interest in filter paper as a sample carrier [2] (Figure 1). From then on, there have been an increasing number of publications on the determination of various biologically relevant substances from DBS. The electrophoretic separation of hemoglobin S and hemoglobin A in whole blood was one of the first reports on determining proteins from DBS [3] (Figure 1). However, it was not until the mid-eighties that the use of MS was described for the analysis of proteins from DBS [4]. This study successfully monitored specific amino acid mutations in hemoglobin with MS, however, a tedious sample preparation was needed. After the sample was solubilized, the following steps were carried out: preparative chromatography for protein isolation, protein denaturation, aminoethylation, and trypsination [4]. A chronological representation of such key events like the separation of hemoglobin from DBS can be seen in Figure 1. Despite significant advancements since then, the need for extensive and intensive sample preparation remains prevalent when proteins are determined from DBS using MS. That is why this perspective investigates possible alternatives for conventional sample preparation.

Article Related Abbreviations: bmAbs, biotinylated mAbs; DBS, dried blood spots; HCT, hematocrit; hCG, human chorionic gonadotropin; mAb, monoclonal antibodies; MIPs, molecularly imprinted polymers.

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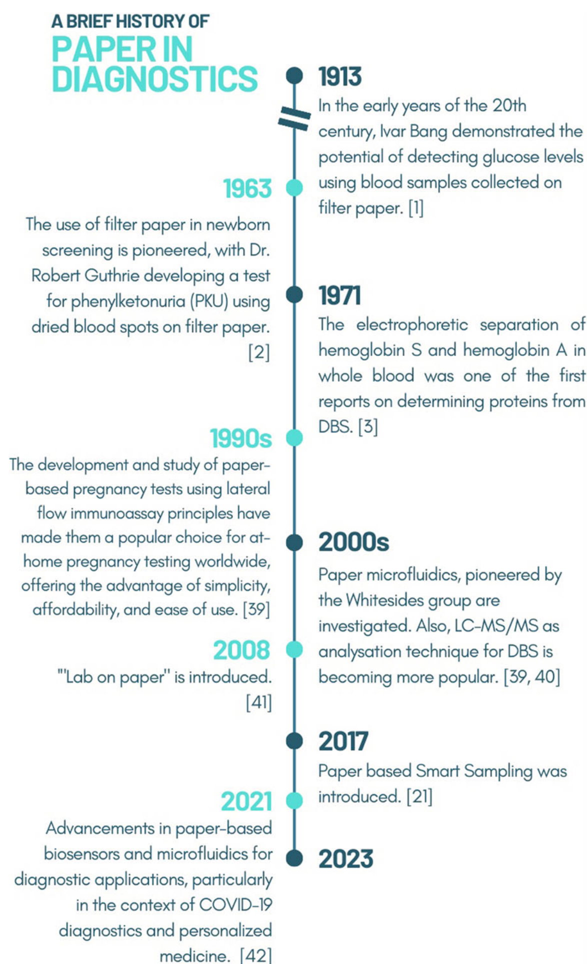

FIGURE 1 Milestones in paper-based diagnostic techniques. This figure presents a chronological overview of key developments in the field of paper-based diagnostics, highlighting significant contributions and breakthroughs. It begins with Ivar Bang's demonstration of glucose level detection using blood samples on filter paper [1], Guthrie's work on using DBS for newborn screening, specifically for phenylketonuria (PKU), follows [2]. Thielmann's report on electrophoretic separation of hemoglobin S and hemoglobin A in whole blood showcases the determination of proteins from dried blood spots [3]. The figure also highlights the introduction of paper-based pregnancy tests based on lateral flow immunoassay principles by the Whitesides group [39]. Further, the adoption of LC-MS/MS for dried blood spot analysis is noted [40]. Notable advancements include the introduction of "Lab on paper" [41] and paper-based smart sampling [21]. The figure concludes by acknowledging recent advancements in paper-based biosensors and microfluidics for COVID-19 diagnostics and personalized medicine [42].

We introduce the feasibility of smart sampling with DBS, specifically on determination of proteins and peptides from dried biological samples using MS for clinical purposes. The focus will be on smart sampling as an innovative approach that transforms filter paper from a mere sample carrier into an active element in sample preparation.

1.1 | Modified cellulose in sample preparation of low molecular substances from biological samples

Among the advantages of using DBS in analytical workflows are the use of minute amounts of sample, increased stability of analytes in a dried sample, and the ability to sample everywhere without the need for trained personnel [5, 6]. Additionally, a growing focus has been on using sustainable materials in general, particularly paper in analytical applications. Many of these are reviewed by Gonzaléz-Bermúdez [7]. The information gathered in this article shows the potential of paper (cellulose) in sample preparation in a great variety of applications in all kinds of chemical analysis. The cellulose was modified with affinity materials such as molecularly imprinted polymers (MIPs) [8] or aptamers [9], or coated with covalent organic polymers [10]. Sample preparation of codeine and imipramine was performed using cellulose attached to a cotter pin placed in a liquid biosample. In this case, the extraction starts in the laboratory. This differs from the report of the carbamazepine extraction from whole blood: the cellulose modified with MIPs was used as DBS paper. In other words, the sample preparation starts at the moment of sample application. This way of integrating sample preparation already in an early stage of the sample handling is especially interesting for the determination of compounds from DBS, which required extensive sample preparation.

1.2 | Filter paper that changes the analytical workflow of protein determination from DBS

Most analytical workflows for protein determination using LC-MS are based on a bottom-up approach involving trypsin. This step is important to increase the sensitivity of the analytical method [11]. If needed, additional sensitivity is obtained when including an extra affinity clean-up step. The latter step also allows us to get rid of interfering high abundant proteins. Usually, an affinity step is included when detection limits in the low ng/mL range or lower need to be obtained [12–15]. However, both bottom-up digestion and affinity clean-up of proteins are time-consuming steps, especially for determining proteins from DBS. The transportation accounts for a significant portion of the total time consumed when it comes to analysis from DBS (Figure 2). It is not until it is delivered to a laboratory that the tedious and time-consuming sample preparation starts (up to 24 h or even longer). To optimize the time efficiency of this workflow, starting sample preparation already upon the time of sampling would be beneficial (Figure 2). The sample will then be processed

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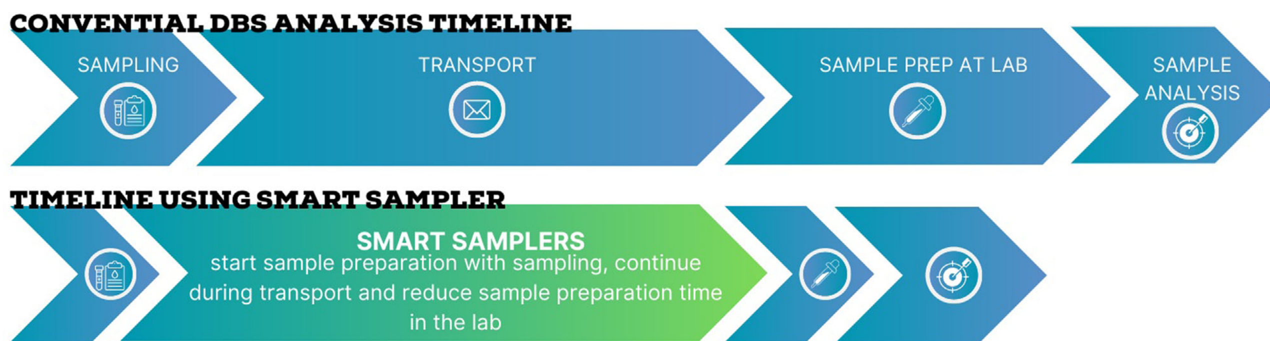

FIGURE 2 Schematic illustration of the timelines of the two different workflows. The top illustrates the conventional timeline when DBS are sampled and analyzed while the bottom portrays the contrast to the timeline when smart samplers are used.

to a great extent before it reaches the laboratory. Enabling quick analyses of samples and, therewith, faster diagnosis (up to 10 times faster) and follow-up of patients. In the sections below, this possibility is addressed through the concept of smart sampling. In Figure 2, it is shown how smart sampling can reduce the overall time consumption in comparison to the conventional DBS analysis time.

1.2.1 | Trypsin

To perform the digestion of proteins on paper, it is advantageous that the proteolytic enzyme is bound to the paper. The attachment of enzymes to cellulose has been described for many applications, either through adsorption or covalent binding [16, 17]. In the first study to describe trypsin/α-chymotrypsin binding to cellulose, simple adsorption to crystalline cellulose was used for enzyme attachment with the goal to study their immobilization through adsorption. Nikolic et al. showed that covalent binding of trypsin to cotton yarn through oxidation of cellulose made it possible to produce bioactive fibers with anti-inflammatory properties relevant in the treatment of wounds [18, 19]. The potential in LC-MS-based protein analysis of trypsin bound to cellulose was shown by Sun et al. [20]. They performed surface adsorption of trypsin to hydrophobic cellulose-decorated nanoparticles and showed better proteolytic activity under varying conditions than trypsin in solution. Although this was carried out on cellulose, it was not suitable for remote paper sampling as the immobilized trypsin on these particles were intended to perform digestion of proteins in solution.

Skjærvø et al. were the first to integrate trypsin in filter paper with the intention to initiate the time-consuming protein digestion for bottom-up protein analysis at the moment of sampling. In addition to just dripping trypsin (which was immobilized on Sepharose beads) onto filter paper [21], covalently bound protease on paper with HEMA-VDM chemistry was also used [22, 23]. This prelab proteolysis made quantitation of a low abundant small cell lung cancer biomarker down to 500 pg/mL possible [22]. Although covalently bound trypsin on HEMA-VDM functionalized filter paper shows good activity, the filter paper lost much of its absorbing capacity and became more rigid. The latter is circumvented when, under controlled conditions, a simple oxidation with IO_4^- to functionalize cellulose was used: the filter paper keeps its absorbing properties [24]. Oxidized paper allows trypsin to bind covalently and can potentially be used for proteomic applications and for simple bottom-up protein determinations [25, 26]. A limited stability study even shows that filter paper with immobilized trypsin in the dried state kept refrigerated did not lose its activity over 4 months [25].

These findings are promising, and the workflow is simplified compared to a conventional protein determination from DBS (see Figure 3A). However, there is still a long way to go before smart proteolytic samplers, as described above, can be used for protein determination or routine proteomics analysis. One of the key factors still to be investigated is the potentially altered accessibility of tryptic sites compared to in-solution digestion. Reduction and alkylation needs to be carried out postdigestion, causing changes in the type of peptide generated [25]. Also, potential differences between the activity of immobilized and solubilized trypsin might give rise to changes in peptide production. The latter probably influences sensitivity and protein coverage. Other factors to be studied are the influence of sample pH and protein:trypsin ratios on the digestion performance as well as the extraction recoveries of the generated peptides from cellulose.

1.2.2 | Antibodies

Another important step that can be beneficial to integrate early in the DBS workflow is affinity capture and sample clean up, with particular interest for target proteins that occur at low concentrations in blood. Affinity capture

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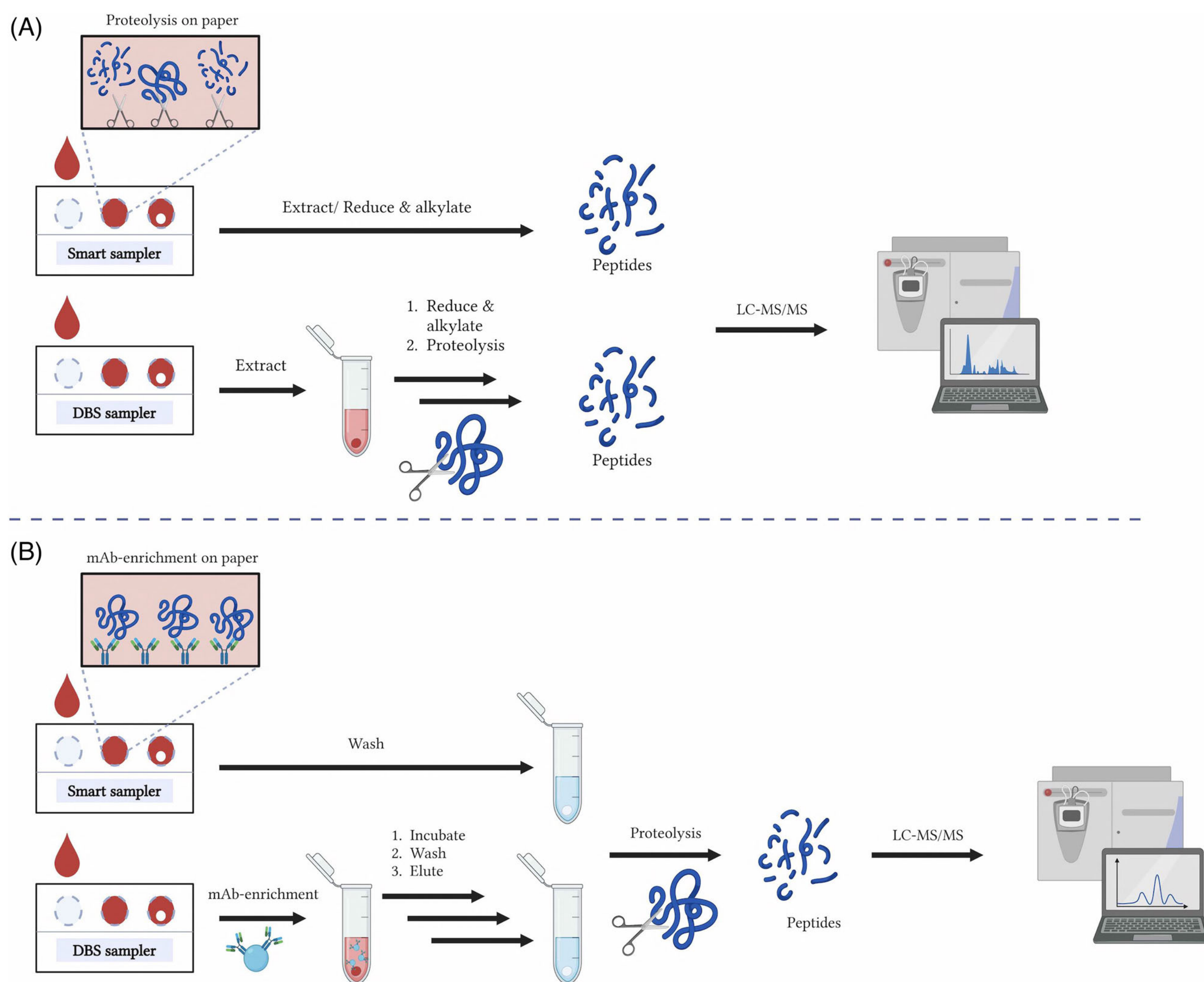

FIGURE 3 Schematic illustrations showing the general workflow using smart samplers compared to commercial DBS samplers. The illustrations display the sampling and sample preparation of proteins from whole blood. (A) Displays a general workflow where protein digestion has been integrated into paper-based sampling. (B) Displays a general workflow where affinity capture has been integrated into paper-based sampling. These illustrations were created using BioRender.

typically relies on monoclonal antibodies (mAb) which can selectively bind to target proteins. It is, thus, required to immobilize these to cellulose. Often described applications are innovative paper-based immunometric assays where mAbs are either noncovalently [27, 28] or covalently [29, 30] bound to cellulose. In all of these cases, the modified paper is part of the analysis and not a part of the sample preparation as in LC-MS-based protein analysis from DBS. The first efforts in the sense of using DBS as part of the sample preparation were reported in 2019: cellulose functionalized with HEMA-VDM or HEMA-Tosyl was used for the immobilization of human chorionic gonadotropin (hCG) antibodies [31, 32]. When used as DBS, that is,dripping on sample followed by air drying, the paper spots only needed to be washed and digested before nano LC-MS/MS (MRM mode) analysis. Not only good linearity was obtained [31, 32], but also a detection limit down to 630 pg/mL in a dried blood spot (from 20 uL whole blood) could be achieved with this approach [32]. In this way, tailor-made DBS papers were produced, allowing LC-MS-based determination of low-abundant proteins. Figure 3B shows a comparison between smart affinity sampling and the conventional affinity capture from DBS.

Tailoring DBS paper for targeted protein analysis became even simpler by utilizing the strength of the interaction between streptavidin and biotin. Johannsen et al. [33] showed that covalent binding of streptavidin allowed biotinylated mAbs (bmAbs) to attach to paper. Furthermore, a five times higher signal for the model protein hCG was obtained when bmAbs were used compared to nonbmAbs. One advantage of utilizing streptavidin-biotin-mediated binding of bmAbs over direct covalent binding of mAbs is the flexibility it offers in tailoring DBS samples. With the streptavidin paper being produced, individual

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laboratories can customize their own DBS by incubating it with the desired bmAbs, providing a more versatile and adaptable approach.

1.2.3 | Other possibilities

The above-mentioned examples are mainly performed on filter paper used in DBS cards. Lately, new variants of the conventional DBS cards have become available [34]. Some of these are based on sample collection on cellulose, while others are based on sample collection on other polymers. Reubsaet et al. showed that the concept of smart sampling is not limited to cellulose as a base material: Mitra® tips were modified through covalent binding of trypsin. The trypsin-modified Mitra® tips showed their potential in the bottom-up determination of single proteins as well as proteomic approaches [35].

Smart sampling is not only limited to mAb enrichment and proteolysis as sample preparation principles. For example, the combination of MIPs on filter paper, which potentially allows prelab sample preparation, is shown for low molecular substances [8]. Furthermore, since there are many reports on MIPs for peptide and protein determination using LC-MS [36], it should be feasible to integrate such MIPs in the DBS workflow to allow for prelaboratory extractions. Integration might also be possible for other affinity materials like aptamers.

2 | CONCLUDING REMARKS

Integrating proteolysis, mAb-based affinity clean up, or other sample preparation steps in samplers makes LC-MS-based protein determination from DBS faster and less laborious. With the concept of smart sampling, the preparation starts already at the moment of sampling, enabling both targeted and global proteomic experiments. Smart affinity samplers allow sufficient sample clean up to measure low ng/mL levels of proteins in dried blood. However, the stability of the samplers has had limited attention, with promising results, but further investigation is necessary to determine the stability of both the sampler as well as the processed sample.

Smart proteolytic samplers are promising, but efforts need to be made to show that proteotypic peptides' production are reproducible from sampler to sampler. Standardization of proteolytic samplers also requires attention, as variability in tryptic activity will occur and a form of internal calibration can be necessary to be able to use them for quantitative determinations. A major challenge for DBS sampling has been in overcoming the hematocrit (HCT) effect. VAMS is the most promising solution for the HCT bias due to the fact that the tip absorbs a specific volume quickly and accurately, delivering a more dependable HCT reading without DBS variability [37, 38]. In addition, the approaches discussed in this perspective paper hold potential for application to other dried biological samples. The use of modified cellulose in sample preparation, smart proteolytic samplers, and smart affinity samplers are also tested with dried serum [25] and might also be used for other dried biological matrices like plasma, urine, or saliva. Despite the fact that smart sampling techniques are considered low maintenance and less laborious, it is worth noting that in the end they rely on a highly sophisticated analytical method, LC-MS.

All in all, there is solid scientific proof for the concept of smart sampling. However, to become the next-generation DBS, there is a need for implementation in realistic projects that will allow assessment of their usability.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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