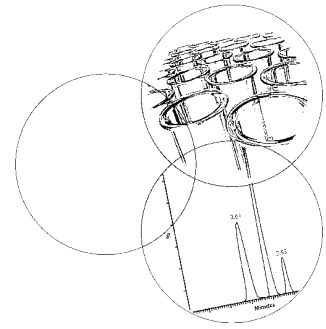

A powerful couple in the future of clinical biochemistry: *in situ* analysis of dried blood spots by ambient mass spectrometry

Since the early 1960s, dried blood spots (DBS) on filter paper have been used in clinical applications. The first key milestone in the use of DBS was the screening of phenylketonuria and other inborn errors of metabolism using microbiological and enzymatic analytical methods. 20 years after its introduction, advanced mass spectrometers and new soft ionization techniques have permitted the coupling of liquid chromatography with MS and tandem MS (MS/MS) and since the 1990s, DBS analysis by LC–MS/MS expanded screening to many inborn errors of metabolism simultaneously. Recently, DBS–LC–MS/MS analysis has been used in other fields such as pharmacology, toxicology and forensic sciences. Today, new ambient ionization techniques, coupled to MS, directly desorb/ionize molecules from solid samples. This presents new opportunities for the *in situ* analysis of DBS. Most likely, ambient MS methods will be used to analyze DBS, increasing the clinical applications of MS within the next 10 years.

Clinical and/or toxicological analyses are generally performed using blood/plasma obtained by venipuncture. In 1963, whole blood collected on filter paper was successfully introduced for the neonatal screening of phenylketonuria [1]. Briefly, the technique consists of peripheral blood collection by heel or fingerprick and blotting onto specially manufactured, high-quality filter paper. The blood spot is then air dried and stored in low gas permeable bags that contain desiccant to reduce humidity, at ambient temperature or frozen at -20 °C. Before analysis, a disc of **dried blood spots** (DBS) typically 3.0 or 6.0 mm in diameter, equivalent to 3.1 or 12.4 µl of whole blood, respectively, is punched either manually or by automated machine. In 1997, Rashed developed in-house a high-throughput automated method and a software program to read DBS sample IDs by barcode, and to map them into the 96-well microplate positions; amino acid and acylcarnitine profiles from DBS were obtained in less than 2 min per sample [2]. Today, fully automated equipment is commercially available that is able to identify by barcode, punch and insert the DBS samples into a 96-well microplate, creating an accurate record of the location of each sample [101,102]. Over the years, specific methods have been developed for the analysis of several compounds from DBS for chemical, serological and genetic applications, but primarily for the expanded newborn screening of **inborn errors of metabolism** (IEM) [3].

The DBS sample collection technique shows significant advantages over conventional blood collection (longer lifespan of samples with reduced need for refrigeration, less invasive, more cost effective, easy shipment and storage, and reduction of infection risks by deactivation of potential pathogens on the filter paper [4–6,103]). DBS has also achieved the same level of precision and reproducibility as standard methods that collect blood in vacuum tubes and capillary pipettes [4]; so DBS is simple to perform and can be collected in a variety of environments. It also has the advantage that it can be archived on a long-term basis and used in retrospective diagnosis. Thus, it can elucidate previously unexplainable cases of sudden infant death, for example, or can be used to indicate the true incidence value of many underestimated diseases.

Due to these advantages, DBS use in clinical and research settings has expanded to include testing for many diseases [7] and monitoring of therapeutic agents including antimalarials, antiepileptics, antiretrovirals, antidiabetics and antibiotics [8–13]. In principle, any compound measurable in whole blood, serum or plasma can also be measured in DBS. The dried blood matrix stabilizes many labile molecules, including DNA, thereby allowing measurement of both phenotype (biochemical marker) and genotype (mutation or polymorphism) from a small volume of blood [4,14]. DBS is also a potentially valid method for the screening

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Key terms

Dried blood spot: Drop of capillary blood, obtained from pricking the heel or finger, blotted onto specially manufactured absorbent filter paper and dried.

Inborn errors of metabolism: Inherited disorder due to a defect in a specific enzyme that impairs a specific metabolic pathway.

Mass spectrometers: Analytical technique for molecular mass determination that depends on the ionization of molecules followed by the measurement of mass-to-charge ratios.

Tandem MS: Integrated series of two mass spectrometers to improve analytical specificity and sensitivity.

Metabolic profiles: Group of metabolites analyzed in a single run using techniques such as gas chromatography, liquid chromatograpy, MS and NMR.

Ambient MS: Group of new MS techniques by which compounds in their native sample are ionized outside the mass analyzer.

of cardiometabolic risk factors in developing countries [15] and for the screening of certain inherited diseases that require the measurement of enzyme activities [16,17]. Obviously, the molecules of interest must remain stable when dried and must be released from the paper during the analysis. Many studies on the stability and measurability of compounds in the dried matrix have been performed [18–26].

An important analytical aspect linked to DBS procedure is that the limited sample volume may be problematic in terms of sensitivity required for assay development and validation. It should also be systematically assessed for the potential impact of various blood-sample properties on the accurate quantification of the analyte of interest. The limited sample volume is compensated for by the use of highly sensitive **mass spectrometers (MS)** or **tandem MS** (MS/MS), coupled to liquid chromatography (LC) as a separating method, and by use of the isotope-dilution technique for the accurate quantification of **metabolic profiles** (e.g., acylcarnitines and amino acids) [19,27–29]. In fact, DBS–LC–MS/MS has emerged as an important method for quantitative analysis of a myriad of small molecules [29]. Another disadvantage of the DBS procedure is the offline solvent extraction step. Recently, an online DBS sample procedure has been proposed that allows direct extraction and analysis, using a conventional column switching LC–MS system, to be integrated into a single process [30,31]. However, all procedures described above are time consuming owing to sample preparation and chromatography steps.

In the past 2 years, a number of direct elution approaches have been published in the literature. Abu-Rabie and Spooner reported a TLC–MS interface as a potential and effective tool for the direct analysis of drugs in DBS samples at physiologically relevant concentrations, with or without HPLC separation [32]. This bioanalytical procedure was considered to be more time and cost effective, as well as much simpler, when compared with current manual practices (punching of DBS and elution with solvent prior to HPLC–MS analysis). Furthermore, they showed an increased sensitivity compared with that of manual extraction, potentially enabling the analysis of analytes not currently amenable to DBS sampling due to limitations in assay sensitivity. Van Berkel and Kertesz described the application of a liquid-extraction-based sealing-surface-sampling probe (SSSP) for the direct mass spectrometric analysis of targeted drugs and metabolites in DBS [33]. More recently, the authors reported fully automated liquid extraction-based surface sampling and ionization using a chip-based robotic nanoelectrospray platform [34]. The system was tested with three types of sample surface: spotted sample arrays on a MALDI plate, DBS on paper, and whole-body thin tissue sections from drug-dosed mice. The results were consistent with previous studies employing other liquid-extraction-based surface sampling techniques.

From these studies, it follows that the trend towards automation of the DBS procedure is making analysis faster and more reproducible. The next step consequently leads to the development of new methods for the direct analysis of DBS, with the aim of obtaining results from a greater number of samples per minute.

Ambient ionization MS

The main limitation in using the mass spectrometer as a detector is the introduction of ionized sample compounds into the vacuum environment of the instrument. This problem was solved with the introduction of 'soft' atmospheric pressure ionization (API) methods such as electrospray ionization (ESI), which is applicable to large and fragile polar molecules, such as oligonucleotides and proteins [35,36], or atmospheric pressure chemical ionization (APCI), a more versatile chemical ionization device used to analyze less polar small compounds [37] and the matrix-assisted laser desorption/ionization (MALDI) technique [38–40]. API techniques and the development of more versatile and sensitive mass spectrometers (MS and MS/MS), coupled with LC, dominate all analytical tools owing to their superior specificity, sensitivity, efficiency and efficacy, producing results at faster rates per sample. However, before performing LC–MS and LC–MS/MS analysis, it is necessary to perform a suitable sample preparation procedure (deproteinization, internal standard addition, centrifugation, extraction and derivatization). Thereafter, the chromatographic conditions must be optimized to provide the required separation selectivity, the best analysis time and good compatibility with the MS detection [41,42].

Recently, some advanced API techniques have been introduced. In 2000, Laiko *et al.* introduced a variant of the MALDI technique that allows ionization in an atmospheric environment (AP-MALDI) [43].

The next major ionization advance is based on the availability of MS sources that operate in the ambient environment, allowing the ionization

of ordinary/native samples, without sample preparation or preseparation, by creating ions outside the instrument [44]. The term **ambient MS** (AMS) [45] is used to describe capabilities that are broader than those encompassed by the term atmospheric pressure MS [46]; it is an extension to MS that allows the analysis of samples at atmospheric pressure under ambient conditions. In AMS, samples are exposed to agents that cause desorption (or extraction) and ionization of compounds present at or near the surface [47]. **Figure 1** shows a comparison between standard LC–MS and AMS procedure for a DBS sample. MS analysis without sample preparation could lead to the development of high-throughput analysis. AMS techniques have been used to perform analysis in different fields for biological [48–51], forensic [52,53] and pharmaceutical [54–56] applications. All of these are based on two fundamental principles described by Cooks *et al.* and by Cody *et al.*, termed desorption ESI (DESI) and direct analysis in real-time (DART), respectively [44,45,57]. Prior to the DESI and DART methods, samples analyzed by MS were not in the free ambient environment and could not be subjected to arbitrarily chosen processing actions or conditions during the mass spectra acquisition [46].

Desorption ESI is an MS technique where charged droplets of solvent are directed onto a solid sample placed on a surface. The impact of the charged particles on the surface produces gaseous ions from material originally deposited on the surface. The resulting mass spectra are similar to those obtained by ESI, where the produced ions are mainly singly or multiply charged molecules [44–46]. In the DART process, neutral gas molecules, including metastable species, are produced by an electrical discharge in a gas phase that reacts with water and air molecules, resulting in a gas-phase chemical reaction that in combination with a desorption step, ionizes molecules directly from the surface. DESI, DART and other recently introduced AMS techniques, such as desorption APCI (DAPCI) [58], electrospray-assisted laser desorption/ionization (ELDI) [59], and atmospheric solids analysis probe (ASAP) [60], can be considered atmospheric pressure versions of secondary ion MS (MS based on the collection and analysis of secondary ions emitted when a surface, solid or, sometimes, liquid, is bombarded by a focused primary ion beam).

To date, many different AMS techniques have been proposed, of which the main ones are reported in **Table 1**. Readers interested in the

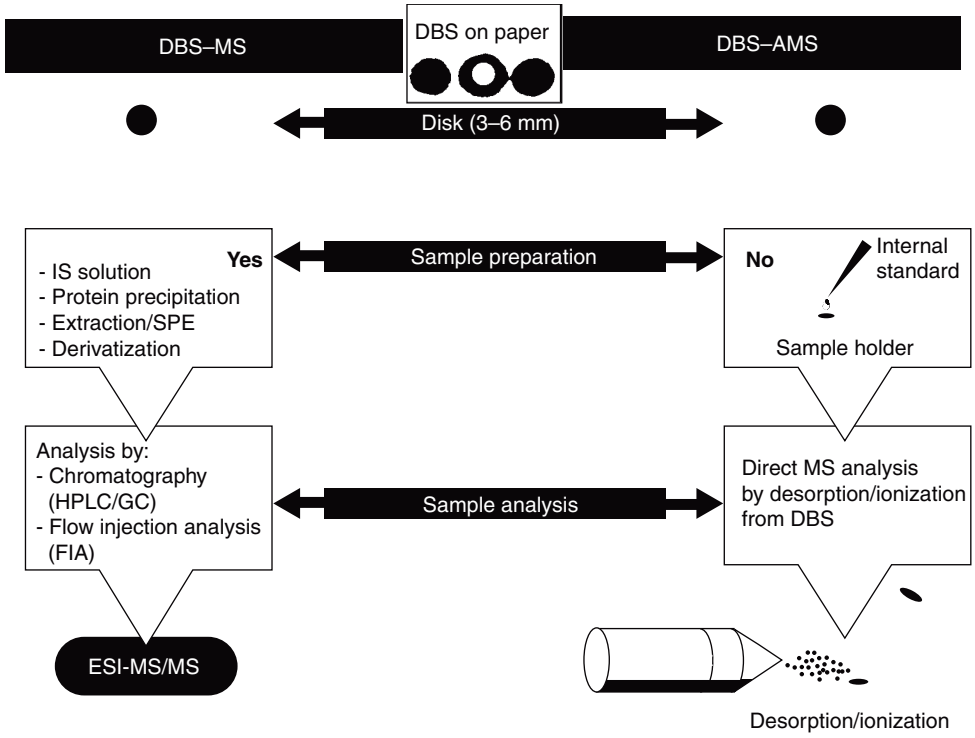

Figure 1. Methods comparison for dried blood spots analysis by classical LC–MS and ambient MS.
AMS: Ambient MS; DBS: Dried blood spots; IS: Internal standard.

		Mechanisms		
Name	**Acronyms**	**Desorption**	**Ionization**	**Ref.**
Ambient solid analysis probe	ASAP	Thermal desorption (heated gas flow)	Chemi- and chemical ionization	[60]
Atmospheric pressure thermal desorption chemical ionization	APTDCI	Thermal desorption (heated gas flow)	Chemi- and chemical ionization	[76]
Atmospheric pressure-thermal desorption/ electrospray ionization	AP-TD/ESI	Thermal desorption (heated gas flow)	ESI	[79]
Desorption atmospheric pressure chemical ionization	DAPCI	Thermal desorption (charged droplets and heated gas flow)	Chemi- and chemical ionization	[80]
Desorption atmospheric pressure photoionization	DAPPI	Thermal desorption (charged droplets and heated gas flow)	Photoionization	[81]
Desorption electrospray ionization	DESI	Spray desorption (charged droplets)	ESI	[44]
Easy ambient sonic-spray ionization	EASI	Neutral spray desorption (neutral droplets)	ESI	[82]
Direct analysis in real time	DART	Thermal desorption (heated gas flow)	Chemi- and chemical ionization	[57]
Electrospray laser desorption/ionization	ELDI	Laser desorption	ESI	[59]
Laser-ablation electrospray ionization	LAESI	Laser desorption	ESI	[83]
Low temperature plasma	LTP	Electric discharge	Chemi- and chemical ionization	[84]
Neutral desorption extractive electrospray ionization	ND-EESI	Ambient gas jet	ESI	[85]
Paper spray	PS	High voltage	ESI	[78]

mechanisms of these techniques should refer to recent reviews elsewhere [61–63]. As is clear from the literature, compounds ranging from nonpolar small molecules, such as alkaloids and drugs, to polar compounds, such as peptides and proteins, can be analyzed selectively using DESI on solids, biological matrices and *in vivo* [45,64]. Instead, to analyze lipophilic compounds (such as cholesterol) that are not easily ionized by ESI and by DESI because of their lower proton affinity, other AMS techniques have been developed, such as reactive DESI, which is used for the analysis of free cholesterol from human serum [65], and the ASAP to ionize sterols [66].

However, the quantitative performance of AMS techniques has not yet been sufficiently investigated. In fact, few works have demonstrated that AMS could be used for quantitative analysis. In 2008, Ifa *et al.* demonstrated that DESI-MS could be successfully employed for routine quantitative analysis by adding an internal standard [67], while in the same year Fernandez *et al.* used reactive DESI-MS to quantify active drugs (artesunate and antimalarials) in solid pharmaceutical tablets using D_4-artesunic acid as an internal standard [68]. In 2009, Manicke *et al.* described a new high-throughput DESI source able to perform quantitative analysis of 96-sample arrays containing pharmaceuticals in various matrices [69]. Finally, in 2010, Paglia *et al.* showed that the atmospheric pressure thermal desorption chemical ionization (APTDCI) technique, which easily ionizes sterols, can be used for quantitative analysis of DBS from patients affected by the Smith–Lemli–Opitz syndrome [70], while Wiseman *et al.* described the quantitative direct analysis of xenobiotics from DBS by DESI-MS without sample preparation or chromatography [71].

Coupling of DBS & AMS for clinical biochemistry

Since the 1990s the application of DBS analysis by MS/MS to obtain metabolic profiles expanded the screening of IEM worldwide as a routine method [27]. Today, the fields of application of DBS are varied and not exclusive to newborn screening or expanded newborn screening. Some more recent examples include the use of DBS in surveillance studies [72], for medical anthropological research [73], pharmacokinetic/pharmacodynamic studies [74] and genetic studies [75]. As mentioned above, the utilization of DBS samples offers many advantages regarding collecting, processing, infectious hazard risk,

storage and transport. For these reasons, together with the development of new methodologies employing mass spectrometry and new ambient ionization, DBS analysis is becoming more prevalent in clinical and research laboratories.

The next step is the *in situ* analysis of DBS by AMS. To date, three studies have demonstrated the direct analysis of DBS by AMS methods. Paglia and co-workers proposed the direct analysis of sterols from dried blood/plasma spots by APTDCI-MS/MS [70]. The method was first employed in 2008 for the analysis of acylcarnitines extracted from a pathological and control DBS samples [76]. Using this approach, the sterols were directly desorbed and ionized by APTDCI from DBS and quantified by MS using D_6-cholesterol as a labeled internal standard. The implemented method was able to differentiate normal subjects from Smith–Lemli–Opitz syndrome patients [70]. The direct analysis of DBS by DESI-MS proposed by Wiseman *et al.* allows the quantitative determination of xenobiotics in whole blood samples without sample preparation or chromatography, which are usually used to minimize suppression effects on ionization and to preconcentrate the compounds [71]. Using sitamaquine, terfenadine and prazosin as model compounds, with verapamil as an internal standard, this methodology could detect individual compound down to 10 ng/ml from DBS. The authors examined the effect of each surface on the recovery of each compound from various treated and untreated blood cards, showing that the untreated papers provide the best substrates for DBS analysis by DESI [71]. More recently, Wang *et al.* described a method, termed paper spray (PS), which combines the characteristics of both ESI and ambient ionization methods. The method provides analyte transportation by applying a voltage to the wet paper, which is held in front of a mass spectrometer. PS was shown to be useful for fast, qualitative and quantitative analysis of complex mixtures. In particular, PS-MS and PS-MS/MS were used for the direct analysis of DBS from whole bovine blood enriched with the drug imatinib or atenolol, with labeled compounds added as internal standard to obtain a quantitative analysis. The procedure was also applied to analyze illicit drugs from dried urine spots, which yielded PS-MS and PS-MS/MS spectra almost instantaneously [77,78].

While the gold standard approach to DBS analysis still requires the removal of a core from the DBS, compound(s) extraction and analysis by LC–MS/MS, the direct desorption/ionization approach, using DESI, APTDCI and PS, shows that the direct analysis of DBS samples is possible through the elimination of extensive sample preparation or cleanup. This could potentially lead to the development of high-throughput analysis; in fact, these results have wide implications for several applications including IEM, clinical analyses, therapeutic drug and substance abuse monitoring and clinical and forensic toxicology. Furthermore, the use of specialty absorbent papers is not limited to whole blood; hence, the collection and analysis of plasma, urine and saliva as other volume-limited biological fluids on specialty papers are areas of further research, together with the development of papers for DBS that protect compounds in the dry state, and of papers more specific for DBS–AMS methods.

Optimization of AMS methods and comparison with reference methods, improvement of quantitative analysis and validation of DBS–AMS methods, as well as automation of DBS–AMS methods, are the major points to be developed.

Some automated devices for AMS analysis are already available [104–106], even though some automated steps (i.e., internal standard addition) for the direct analysis of DBS must still be developed, optimized and validated. In addition, the lack of conventional sample separation (LC) could lead to possible issues with labile metabolites and often produce complex ion populations that can impact on the selective detection of isobaric species. As already reported, the DBS stabilizes many labile molecules, and AMS paired with an ion mobility/MS platform has shown the advantage of postsource (gas phase) electrophoretic analyte separation and should make a positive impact on AMS [55,63]. Finally, the lack of sample cleanup could lead to a dirty instrument, but more attention to cleaning the ion source entrance after each analytical session would be sufficient. Only when the sample concentration is above the higher limit of quantification would sample extraction and dilution be necessary.

Conclusion

The introduction of different AMS techniques to perform direct sampling/ionization and analysis of many compounds from raw samples provides the stimulating concept of direct MS analysis without sample pretreatment and separation. The analysis of DBS using AMS potentially offers several advantages, resulting in significant savings in time and cost, with no

requirement for the arduous sample handling procedures currently employed for conventional DBS analysis. Thus, this promising couple could represent a new mass spectrometric approach for the analysis of metabolic profiles directly *in situ*. Moreover, the combination of AMS with miniaturized mass spectrometers could make it applicable in nonlaboratory environments or usable by non-experts in MS (point-of-care devices) for clinical applications such as a bedside testing, or *in vivo* on the skin surface.

Future perspective

Dried blood spots can be qualitatively analyzed directly by AMS, in particular for small polar and nonpolar molecules. Many direct methods for the analysis of biomarkers will be implemented by DBS–AMS in several clinical fields such as diagnostic, epidemiology, pharmacology, forensic and environmental sciences. These methods will form the basis for the *in vivo* application of AMS in the near future.

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Key conclusions

Dried blood spots (DBS) as a sampling procedure have been in use for around 50 years, and specific paper/cards have been produced and tested by different analytical methods.

The employment of DBS has followed the technological progress, and in the last 10 years DBS has been coupled to LC–MS/MS, while in the last 3 years DBS has been analyzed *in situ* using ambient MS (AMS; DBS–AMS).

For accurate quantification of a compound, the DBS–AMS results must be compared with those of reference methods to validate this new procedure.

Unresolved issues

Full automation of some pre-analytical steps such as addition of internal standard, handling of cards and verification of blood spots must still be developed.

Analytical variability of DBS is strictly linked to the chemical nature of compounds, and their stability in the paper depends on time and temperature of DBS storage, and distribution/chromatography of compounds in the paper during the collection and drying of blood.

Accuracy and precision of DBS–AMS methods depend on internal standard addition, efficiency, efficacy and optimization of the desorption/ionization processes.

Point for emphasis for future work

Papers for DBS that more specifically protect compounds in the dry state and that are more specific for DBS–AMS methods must be developed.

Optimization of DBS–AMS methods and comparison with reference methods is needed.

Improvement of quantitative analysis and validation of DBS–AMS methods.

Development of new devices for the full automation of DBS–AMS methods.

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