Analysis of Biofluids by Paper Spray Mass Spectrometry: Advances and Challenges

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Abstract

Paper spray mass spectrometry (MS) is part of a cohort of ambient ionization or direct analysis methods that seek to analyze complex samples without prior sample preparation. Extraction and electrospray ionization occur directly from the paper substrate upon which a dried matrix spot is stored. Paper spray MS is capable of detecting drugs directly from dried blood, plasma, and urine spots at the low ng/mL to pg/mL levels without sample preparation. No front end separation is performed, so tandem mass spectrometry (MS/MS) or high resolution MS is required. Here, we discuss paper spray methodology, give a comprehensive literature review of the use of paper spray MS for bioanalysis, discuss technological advancements and variations on this technique, and discuss some of its limitations.

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Abstract

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Keywords

Dried blood spots
Dried matrix spots
Dried plasma spots
Dried Urine Spots
DBS
Chromatography
Ion Mobility
Ambient Ionization
Direct Analysis

Executive Summary

Introduction

- Paper spray is an ambient ionization technique used to analyze biological samples directly from dried matrix spots stored on paper.
- Detection limits in the low ng/mL to high pg/mL range are routinely achieved without sample preparation
- No chromatography is performed.

Methodology

- Solvent wicks through a biological sample stored on paper, extracting the analyte it moves to the tip of the paper.
- A high voltage applied to the paper, initiating an electrospray at the sharp tip of the wet paper.
- Volume and viscosity should be considered when sampling a biological matrix.
- Because entire blood spots are being analyzed, hematocrit should have minimal impact.
- Solvent system must be able to penetrate biological matrices, extract relevant analytes, and form a stable electrospray.
- A variety of types paper substrates have been used with differing porosities and spray tip angles.
- Quantitation typically requires matrix matched calibrators and a stable isotope label as an internal standard.
- An automated source is available which utilizes disposable cartridges that contain the paper and a metal contact to conduct the spray current.

Applications

- Paper spray has been used to analyze a wide variety of xenobiotics and biomolecules.
- Drugs have been measured in a variety of matrices ranging from blood and plasma to tissue homogenates.
- Select pharmaceuticals have been measured at biologically relevant levels for therapeutic drug monitoring.
- A number of different biomolecules have been measured in a variety of biological matrices.
- Microalgae and bacteria have been differentiated by studying their lipid profiles using paper spray.

Technological Developments

- Using paper substrates with optimized properties, such as pore size, thickness and hydrophobicity, can improve limits of detection.

- Coating the paper substrate with different materials can lead to improved sensitivity and lower ionization voltages.
- Leaves can be used to emit a spray in a similar process for direct analysis of its chemical contents.
- Other solid matrices, such as wooden tips and TLC plates, can also be used for ambient ionization.
- Disposable cartridges facilitate sampling and analysis by providing an easily manipulated scaffold to hold the paper.
- 3D printing allows for rapid prototyping of cartridges.
- Integrated solid phase extraction material has been shown to significantly improve limits of detection.
- Miniature mass spectrometers have been shown to be adequate for the detection of a number of analytes using paper spray.
- Alternate sampling techniques for paper spray have also been demonstrated in the form of surface swabbing and continuous sampling methods.

Challenges/Limitations

- The elimination of chromatography shifts the burden of selectivity onto the mass spectrometer.
- Analytes with identical mass spectra such as diastereomers or fragile metabolites that decompose into the parent drug pose a problem for paper spray.
- Reactive paper spray incorporates a derivatization agent, either on the spray substrate or solvent, which can help differentiate similar structures and/or improve sensitivity.
- Ion mobility has been shown to improve the selectivity of paper spray by separating structurally similar isomers.
- Matrix effects occur when analyzing complex biological matrices.
- Quantitation is still possible despite matrix effects so long as matrix matched calibrators and stabile isotope label internal standards are employed.
- Detection limits for paper spray may be insufficient for certain potent drugs and small molecules with low biologically relevant concentrations.

Introduction

Paper spray mass spectrometry, first described in 2010[1], was conceived because of the growing interest in two areas: dried blood spot analysis and ambient ionization mass spectrometry. The idea of ambient ionization, or direct analysis, took hold with the publication of papers on DESI (desorption electrospray ionization)[2] and DART (direct analysis in real time)[3] about 10 years ago. Since then, the number of methods and the application space has grown rapidly. Most of the ambient ionization methods are designed for surface analysis and showed poor sensitivity toward dried blood spots, in which the sample is distributed through the three dimensional porous structure of the blood card paper. Paper spray garnered interest largely because it was able to achieve useful detection limits from the direct analysis of dried blood spots (sub ng/mL in many cases) while maintaining the simplicity and lack of sample preparation of ambient ionization approaches. The primary advantages of paper spray compared to traditional mass spectrometry based approaches that are typically cited include:

- Paper spray requires no sample preparation. Single digit ng/mL or sub-ng/mL detection limits for drugs, pharmaceuticals and other small molecules from blood, plasma, urine, and oral fluids have been demonstrated by multiple groups.
- The sample volume consumed by paper spray is low. Methods published in the literature use between 0.5 μL and 15 μL of sample.
- Storage of samples as dried spots improves sample stability at room temperature in many cases.
- The paper substrate is inexpensive and readily available.
- Clogging, which commonly occurs in conventional capillary electrospray ionization, does not occur in paper spray due to the multi-porous nature of the substrate
- Carry-over is eliminated because the ion source and the entire fluid path that contacts the sample is discarded after each analysis
- The amount of solvent required per sample is low (less than 100 μL) and all of the solvent is consumed so there is practically no solvent waste to dispose.
- Liquid chromatography is removed, which simplifies the analysis and removes common sources of failure in HPLC-MS assays such as leaks and clogged columns.

This article gives a description of paper spray methodology, a comprehensive review of published paper spray applications for bioanalysis, an overview of technological innovations related to paper spray, and, finally, a discussion of some of the limitations of the technique.

Methodology

General Description

A diagram of a typical experimental setup for paper spray is shown in Figure 1. The biofluid sample is spotted onto paper with a pipette and allowed to dry. The paper is cut to a sharp point either before or after sample application, with the dried matrix spot positioned 5-10 mm away from the tip. The sharp tip is positioned in front of the atmospheric pressure inlet of the mass spectrometer (normally 3 to 10 mm away). An aliquot of solvent is applied to the paper where that it interacts with the sample and extracts any soluble components.

The solvent volume varies with the size and properties of the paper (like thickness and porosity); the volume must be large enough to saturate the paper. An excessive amount, which will lead to solvent pooling underneath or running off of the paper, will cause spray instability and lower MS signals. Electrospray ionization is initiated from the tip of the paper by application of a high voltage (in the 3 -5 kV range typically) (Figure 2).

Sample considerations

Analysis of blood, plasma, urine, saliva, and tissue by paper spray MS has been reported in the literature (see Table 1). Samples are typically analyzed as dried spots after drying at room temperature. In some cases, immediate sample analysis is desired which makes room temperature drying undesirable. Accelerated drying by blowing warm air across the samples or placing the samples in an incubator at moderate temperatures (40-50°C) has been reported[4]. Analysis of wet samples has also been described, but the method must be modified to prevent cellular material from reaching the tip of the paper where ionization occurs. For the analysis of nicotine from wet blood, print paper was found to give better performance than typical chromatography paper. Print paper has pore sizes about 100 times smaller than chromatography papers, and the authors theorize that the smaller pore size helped to trap blood cells on the paper[5]. Another approach to analyze wet blood samples used chromatography paper pretreated with the coagulating agent alum, which also served to prevent cellular material from flowing to the tip of the paper[6].

When spotting samples for paper spray, the sample should not reach the tip of the paper or be very close (within 1 or 2 mm). Moving the sample farther away from the spray tip decreases ion suppression, but there is also an increase in analyte loss[7]. Also, for thicker papers such as dried blood card paper, the best performance is obtained when the sample volume is large enough that the sample spans the complete width and depth of the paper. This ensures that the extraction solvent wicks through the dried matrix spot rather than the neat paper. This is less important for thin papers. The sample volume required for paper spray varies depending on the matrix and paper type. Sample volumes less than 1 µL are routinely used on relatively thin papers such as Whatman 1. Thicker and more absorbent dried blood card papers such as Whatman 31ET and Ahlstrom 226 require large blood volumes (10 to 15 µL range is often reported). Plasma and urine samples are less viscous than blood samples, so a smaller volume is required to fill the desired paper volume.

In traditional punch and extract workflows for dried matrix spotting, nonuniformity within the sample spot is a concern. For dried blood spots in particular, variation in hematocrit affects sample viscosity which in turn affects how the blood sample spreads through the paper. Both of these cases could cause bias and imprecision when a punch is taken from the dried matrix spot for analysis. Paper spray avoids this problem because the entire sample spot is extracted and analyzed. Hematocrit should not result in bias in the blood concentration measurement, therefore, because the volume of sample applied to the paper is independent of hematocrit. This is analogous in some ways to punching out the entire dried blood spot in traditional punch and extract methods. This does introduce the requirement that the sample volume applied to the paper must be controlled, for example by a pipette.

Extraction/spray solvent

Some spray solvents commonly found in the literature include 90:10:0.01 methanol:water:acetic acid[8], 90:10 acetonitrile:water[5, 9], and mixtures of methanol and chloroform[4, 10]. Acetic or formic acid is often added in the spray solvent to encourage analyte protonation and to improve spray stability. When sodium or ammonium adduct ions are desired, small concentrations (0.02% to 0.1%) of sodium or ammonium acetate are added to the spray solvent[4, 8].There are several considerations when selecting the extraction/spray solvent: the solvent must effectively extract the analyte, it must form a stable electrospray, it must adequately penetrate the dried sample matrix, and it should not dissolve an excessive amount of matrix components.

Some solvents may extract the analyte well, but not form a stable electrospray. For example, chloroform is a good solvent for extracting lipophilic analytes, but it does not form a stable spray. Using a mixture of 40:60 (v:v) methanol:chloroform instead will give a stable spray while still effectively extracting lipophilic analytes[4]. The selected solvent must also be able to effectively wick through the dried sample matrix by capillary action. This can be a problem with dried blood samples, but not with dried plasma, urine, or saliva in our experience. If the extraction solvent beads up on the dried blood spot rather than wicking into the sample, then the wicking rate of the extraction solvent through the sample may not be sufficient to maintain a stable spray. In our experience, this occurs when using either a high proportion of water (>50%) or acetonitrile. Acetonitrile based extraction solvents are excellent solvents for plasma and urine samples, but do not penetrate dried blood spots particularly well in our experience. On the other hand, other researchers have reported the use of acetonitrile based solvents on dried blood spots with good results[9]. High proportions of water are generally not used for bioanalysis by paper spray MS. Solvents with high proportions of water will tend to wick through dried bloods spots slowly or not at all. In dried plasma and urine spots, performance also tends to be poor presumably because of the larger amounts of matrix material such as salts and water soluble proteins that are co-extracted into the aqueous solvent. Practically all of the solvents reported in the literature for bioanalysis by paper spray use less than 20% water.

Solvent optimization should be done using analyte-spiked biofluids in order to mimic the final samples as closely as possible. Testing solvents using neat samples (i.e. analyte spiked into solvent and spotted onto matrix free paper) will reveal if the solvent forms a stable electrospray, but says nothing about the effectiveness of the solvent in extracting the analyte from the dried matrix spot or the level of ion suppression from co-extracted matrix components. Ion suppression levels and recovery can be estimated by spiking stable isotope labeled analogs of the analyte into the spray solvent[7].

Substrate

A number of different papers have been reported in the literature. The two most common are Whatman 1 and Whatman 31ET. Whatman 1, a thin and inexpensive paper, is advantageous for several reasons. The required volume of sample and spray solvent is low due to the thinness of the paper. Another advantage is that the blood samples are dry and ready for analysis in a few minutes. Whatman 31ET is a thick, fast chromatography paper that can accommodate larger sample volumes. The detection limits obtainable with this paper are better than Whatman 1 as a result. Other advantages of the more absorbent papers are that the reproducibility of the absolute signal intensity is somewhat better and the duration of the signal is longer. A larger volume of sample and extraction solvent is required,

however. More time is also needed for the sample to dry (about 1-2 hours under ambient conditions). Other paper types have also been reported, including print paper (discussed above) and silica coated paper, which was shown to have better recovery and lower detection limits in comparison with chromatography papers for some analytes[11]. Liu *et al.* compared four filter papers with different pore sizes (3-11μm), glass fiber paper, and chromatography paper. Highest quality spectrum with the highest S/N value for the target analyte (cocaine peak *m/z*, 304) was obtained with chromatography paper[12].

The method of solvent and sample application varies somewhat depending on the substrate. In the case of thick papers like Whatman 31ET, care should be taken to ensure that the sample bridges the entire width of the paper, and the solvent should be added to the rear of the paper behind the dried matrix spot. If these criteria are met, the solvent must wick through the dried matrix sample before reaching the paper tip, which improves recovery and reproducibility. With thin papers like Whatman 1 that use small (sub μL) volumes of sample, controlling the application of the sample and solvent is not as straight-forward. Adequate extraction can be obtained anyway, however, because of the high surface area to volume ratio of the dried matrix spot. The sample is typically spotted in the middle of the paper triangle, and the spray solvent is applied to the paper all at once with a pipette.

The geometry of the paper is an important factor in the analysis, and some investigations into this area have been reported. In varying the angle from 30° to 150°, for example, 90° was found to give the highest MS signal intensity. A higher applied voltage was required, however, which could hurt robustness by increasing the chance of electrical discharge. Also, paper position with respect to the MS inlet was found to be more sensitive at larger angles[13]. A paper cone spray ionization method has also been developed in which a pyramidal shaped paper tip was used instead of a planar triangular-shaped paper[14].

Quantitative Analysis

There are numerous examples in the literature using paper spray MS to obtain quantitative results, virtually all using matrix matched calibrators and internal standardization. The best results are obtained using a stable isotope labeled (SIL) analog of the analyte. If a SIL analog is not available or is prohibitively expensive, other compounds can be explored as internal standards. The imprecision of the assay will likely be higher, with relative standard deviations (RSD) of 10% or greater, than obtained using SIL-IS, If a structural analog is to be used as an IS rather than a SIL analog, performance can be maximized by matching the chemical and physical properties of the IS to analyte as much as possible, particularly the pKa, logP, and molecular mass.

Another important methodological question is how to incorporate the IS into the sample. Best analytical performance is obtained via true internal standardization: i.e. thoroughly mixing the IS into the liquid sample prior to spotting the sample onto the paper. Pseudo-internal standardization can also be explored. These include prespotting a SIL internal standard onto the paper prior to sample deposition[6, 8, 12], applying an IS solution to the dried sample prior to analysis, and using internal standard coated glass capillaries for drawing up and spotting the blood sample onto the paper[15]. The precision obtained using these methods are worse than for true internal standardization, but may nevertheless be adequate for the application.

A robust quantitative assay also requires demonstration of adequate selectivity. This topic is discussed in below.

Automation and throughput

Most of the publications in the literature perform paper spray manually. Paper is cut into triangle shapes by hand using scissors or razor blades. The paper triangle is positioned in front of the MS with electrical contact provided by an alligator clip, and the spray solvent is applied to the paper using a pipette. Such an approach allows for a great deal of flexibility in choosing the substrate, but is laborious and requires a lot of hands-on time from the analyst. A paper spray autosampler and a disposable cartridge is available from Prosolia, Inc. (Figure 3). The cartridge consists of a plastic clam shell part containing the paper substrate (a thick blood card type cellulosic paper) and a metal ball for electrical contact. The paper spray autosampler attaches to the front of the mass spectrometer inlet in place of the commercial pneumatically assisted electrospray source typically used downstream of the HPLC. The autosampler loads the sample cartridges from a stack of cartridges contained in a magazine, applies the solvent to the cartridge, positions the spray tip in front of the inlet, and applies the spray voltage. The spent cartridge is ejected after analysis. Several papers have been published using this automated system[4, 10, 16].

Sample throughput varies depending on experimental parameters. The automated source analyzes about one sample every 90 seconds. The rate limiting step is the time required for the solvent to wick through the paper substrate and the dried matrix spot. Sample analysis and solvent addition occur in parallel for different sample cartridges in order to save time. Other methods using thinner papers and smaller sample volumes report faster analysis times, although most of these are manual processes that requires cutting the paper by hand, applying the solvent with a pipette, and manually positioning the substrate in front of the MS. Shen *et al.* reported a high-throughput semi-automated device that analyzed paper samples in rapid succession (7s/sample) without any detectable carryover[17].

Applications

Analysis of xenobiotics, such as drugs and their metabolites, and biomolecules in biofluids often requires time consuming extraction and chromatography steps. As a result, analysis of these analytes is cumbersome and often expensive. Ambient ionization techniques, such as paper spray mass spectrometry, allow for the simplification or elimination of sample preparations and can facilitate rapid facile analysis. The initial publications describing paper spray demonstrate the broad spectrum of analytes that can be analyzed, including amino acids, peptides, proteins, herbicides, therapeutic drugs and fatty acids[12],[1]. Also investigated in these first papers was the potential of the method to quantify drugs in biofluids such as blood and urine. Here, we review the publications that have used paper spray MS for the analysis of xenobiotics in biofluids (whether they be pharmaceuticals, drugs of abuse or their metabolites) and for different types of biomolecules (such as proteins and lipids) with minimal sample preparation.

A. Xenobiotics

Since the initial characterization of paper spray MS in 2010, a number of papers have been published demonstrating new applications. Although the methodology and specific aims of papers differ, they have a common theme in showing that PS-MS can detect and

quantify therapeutic drugs[12],[1],[8],[18],[6],[11],[17],[4],[19],[15],[20],[21] and drugs of abuse[9],[5],[10] (including designer drugs[22],[23]). In many cases, these drugs and metabolites are detected at clinically or physiologically relevant concentrations with good quantitative performance directly from biofluid. In Table 1, we show compiled information on the limits of detection and/or quantitation and linear range of different xenobiotics analyzed in a variety of biofluids. This information gives some insight into the areas that paper spray MS has been applied and what its capabilities are. Depending on the molecule, it has been shown that the limits of detection can be in the sub-ng/mL range[8],[6],[11],[4],[19],[9],[5],[10]. These low level detection limits are especially useful in drugs such as tacrolimus where the therapeutic range in blood is low and narrow and important to monitor to avoid serious side effects[4]. An adequate detection limit is important beyond therapeutic drug monitoring in applications such as law enforcement monitoring for drugs of abuse or court mandated medication in matrices such as blood[9], [10], saliva and urine[5].

It should be noted that the methods used in paper spray are not standardized and most of the studies in Table 1 use home-built apparatus. As such, the substrates, solvents, and sample volumes vary considerably. For example, the detection limit reported for citalopram differed by about an order of magnitude between two publications[8, 11] despite analyzing from the same matrix on the same instrument. The paper substrate and the extraction solvent are different between these two publications, which presumably accounts for the difference.

The most common matrices analyzed have been blood, plasma, and urine. There has also been some work analyzing drugs from tissues. Wang *et al.* demonstrated analysis of hydralazine and imatinib from tissue homogenate from a rat's kidney, liver and spinal cord[24], while analysis of atenolol was reported for mouse liver homogenate[20]. Detection of several drugs from beef homogenate down to the nanogram per gram level has also been reported[17]. Studies have also branched into living subjects with animal studies on nicotine alkaloids in rats[5] and a pharmacokinetics study on sunitinib, benzethonium and their metabolites in mice[25].

B. Biomolecules

Analysis of biomolecules can be useful for applications including identification of microorganisms and detecting biomarkers for metabolic diseases. As such, there has been interest in developing paper spray MS methods for the detection of biomolecules in a variety of matrices. In studies involving wet or dried blood spots the heme[6] group and the protein hemoglobin have been identified[26]. Peptides such as bradykinin 2-9[12] or angiotensin I[1] have been analyzed from neat solvents. Paper spray has been demonstrated for the detection of metabolic disease markers. Underivatized acylcarnitines have been measured directly from serum and blood spots[27]. Similarly, carnitines and acylcarnitines were measured in urine in a paper also without sample pre-treatment or derivatization[28]. Analysis of proteins has been demonstrated from both biological and non-biological matrices. Cytochrome C, lysozyme and myoglobin were analyzed directly from polyacrylamide gel utilizing paper modified with carbon nanotubes[29]. Non-covalent protein complexes were detected using paper spray and ion mobility MS/MS[30]. As for measuring proteins from a biological matrix, the relatively high detection limits for proteins by paper spray MS is a limiting factor. The hemoglobin tetramer could be identified from

whole blood[30]. The detection limit problem was overcome to some extent by incubating an antibody-coated membrane in the sample and performing spray ionization directory from the membrane[31]. A large number of lipids have also been measured from a variety of matrices using PS-MS. Examples include homogenates of mouse kidney, liver, brain, spinal cord tissue[24] and non-homogenized porcine adrenal gland[20]. Analysis from complex matrices can often make identification of specific species difficult. This can be addressed in part through the use of high resolution MS, which was demonstrated for the analysis of lipids using paper spray on a Fourier transform ion cyclotron resonance (FT-ICR) MS from urine and calf lung surfactant extract[32].

Another interesting application involving biomolecule detection is the rapid analysis and discrimination of microorganisms. Oradu and Cooks studied polar lipids from green microalgae[33]. In this method a low resolution mass spectrometer was utilized to classify lipids while a high resolution orbitrap mass spectrometer was used for exact m/z measurements and identification. Further information on lipid species was obtained by integrating a cross stream of reactive ozone. By examining the products of the double bond cleavage it was possible get information on the position of points of unsaturation. In addition to microalgae, lipids from bacteria have also been analyzed. Rapid analysis of bacteria cultures for the purpose of bacterial species discrimination has been shown[34]. In this work the lipid profile of bacteria colonies grown on agar was used in combination with multivariate data analysis to identify species of gram positive bacteria with a 98% success rate and of gram negative bacteria with a success rate of 87%. In similar work by the same group, eight different species of Candida were differentiated at the species level based on their lipid profile with similar levels of success[35]. Applications related to microorganisms aren't limited to species identification. The ability to gain information on the metabolism of cells was demonstrated by monitoring glucose levels in a cell culture[36]. The glucose levels of cultures of human hepatic cells were monitored over time via an integrated paper spray system to monitor the effect of adding hormones such as insulin or epinephrine.

Technological Developments

A. Spray substrates

Paper

Chromatography paper has been used as a main substrate material in paper spray mass spectrometry[24]. Other paper substrates have been employed as discussed above. Print paper, which has a much smaller pore size, has been shown to provide improved sensitivity for the analysis of nicotine and its metabolites in wet blood samples[5]. Print paper also was used in forensic analysis of inks in documents[37]. Two series of "paper" that made from natural fibers (gampi paper, tengujou paper, glassine paper and cicada paper) and synthetic fibers (microarray membrane and nanofibers) were studied. The results indicated that paper substrate with the characteristics of thin, tough, and hydrophobic normally provided a lower LOD[38].

Some research has been performed examining coated or modified paper substrates. Silica coated paper was used in the analysis of therapeutic drugs in dried blood spots, including a notably improve in recovery and a higher sensitivity in comparison with chromatography papers[11]. Paper with a layer of graphene has been used to achieve an in-situ production and detection of intermediates at graphenic surfaces[39]. In another study, a facile vacuum filtration method for directly coating different commercially available

materials (e.g. oxides, polymers, metal powders and carbon nanotubes (CNTs)) onto the surface of filter paper substrate was described[40]. The coated paper improved the sensitivity of paper spray analysis, possibly by reducing of the interactions between free hydroxyl groups at the paper surface and target analytes. Carbon nanotube (CNT)-impregnated paper has also been used to achieve ambient ionization at very low voltages (>=3 V)[41]. The nanoscale features at the CNT paper surface were confirmed to be responsible for the high electric fields at paper tip. CNTs-modified paper has also been used for the intact proteins after separation on a polyacrylamide gel[29].

Leaf spray

Leaf spray mass spectrometry is a variant of paper spray in which the plant material itself is the spray substrate[42]. This method provided real-time information on sugars, amino acids, fatty acids, lipids, and alkaloids in intact plant material, demonstrated its possibility of studying plant metabolism. An electrical potential is applied to the plant resulting in ionization both with and without application of solvent (Figure 2a). To create a high electric field, the sharp tip is needed which could be either natural or cut in a leaf or other part of the plant. Tissues from animal and human also have been used for direct chemical analysis[43-46].

Leaf spray ionization methods have been used for performing direct analysis of steviol glycosides from stevia leaves[47], phenolic glycosides[48], rapid identification of molecular changes in tulsi (Ocimum sanctum Linn) upon ageing[49], rapid detection of urushiol allergens of toxicodendron genus[50], polyhydroxylated alkaloids in mulberry[51], and pesticide residues[52]. Leaf spray also be used for in situ chemical analysis of raw herbs[53], and distinction of coffee origin[54], phytochemicals in petals[55], and Chinese and Japanese star anise[56].

Wooden tips

Hu *et al.* first demonstrated electrospray ionization using disposable wooden tips (wooden toothpicks) in 2011[57] (Figure 4b). Samples were loaded by pipetting onto the tip or dipping the tip into the sample solutions. This technique have been used for analysis of pharmaceuticals[58], ketamine and norketamine in urine and oral fluid[59], and Chinese herbal medicine preparations[60-62]. It has also been shown to act as a chromatographic column for separation of sample components, such as salts and proteins[63]. Deng *et al.* modified the surface of sharp wooden tip to form a solid-phase microextraction (SPME) probe, which was used for selective enrichment of perfluorinated compounds from complex matrices[64].

Thin Layer Chromatography Plates

Thin layer chromatography (TLC) has been coupled directly to mass spectrometry using an approach similar to paper spray. In fact, this approach predates paper spray[65]. The TLC plates are cut to sharp point and an electrospray is induced from the tip. Aluminum backed TLC plates, which are easy to cut, have been the most widely used to date[65-68]. The TLC plate can be coupled directly to the MS in an online mode[65]. Depending on the size and properties of the TLC plate, elution times may be too long with the approach. Another option is to perform the TLC separation offline and cut the bands out in a triangle

shape. Extraction and ionization can then be done directly from the removed portion of the TLC plate[68].

Other solid substrates

The use of size exclusion membranes was investigated for the detection of proteins in biofluids[31](Figure 4C). The authors found that MS signals for molecules below the molecular weight cutoff were suppressed. They hypothesize that low molecular weight compounds diffused into the lumen of the membrane, while extraction occurred primarily from the surface of the hydrophobic membrane where the larger molecules had remained. As a result, interference from salts and other low molecular weight species was minimized for protein targets. In the same study, the sensitivity for protein detection was further improved by coating antibodies on the surface of microdialysis membrane. By incubating that membrane in a large volume of sample with a subsequent wash step, 10 ng/mL of a small protein could be detected from urine. In another work, a substrate coated with a solid phase microextraction (SPME) material was used to detect low pg/mL levels of small molecule drugs[69]. A large volume of sample was required, however, as well as preconditioning of the SPME substrate, an incubation step in the sample, and a wash step prior to analysis. Alternative substrates can also be of interest due to ease and convenience of sampling, rather than improved analytical performance. Medical swabs, for example, have been used for direct detection of strep throat causing bacterium[70] and direct analysis of drugs of abuse in oral fluid[71].

Any material that contains microchannels/pores may be suitable for containing a sample and acting as the support medium for solvent flow by capillary action and electrospray ionization. Other materials that have been explored include bamboo, fabrics, and sponge[63]. Publications have compared the analytical performance of various solid substrates, including limits of detection among paper-spray, wooden tip-spray, and nib spray[72], sensitivity and selectivity among hydrophobic/hydrophilic materials as spraying tips[73], and negative electrospray ionization performance on polyester, polyethylene and wooden wicks[74].

B. Paper spray cartridges

Cartridges for paper spray broadly perform two functions. First, they serve a practical role by enabling easier handling of the paper, permitting better automation, and providing some protection for the sample and the delicate sharp tip of the paper during transportation. As a second function, cartridges could also be designed to perform some of additional analytical tasks, such as sample preparation or the addition of the internal standard. The current commercially available paper spray cartridge is an example of the former. To enable convenient and automated analysis, the paper is precut and loaded into a plastic cartridge, which protects the paper during shipment and makes handling of the cartridge easier for the user. The cartridge is also necessary for automated analysis; the autosampler can more easily move around a sample cartridge compared to bare paper, the cartridge provides an integrated high voltage contact which prevents carryover, and the cartridge has a solvent reservoir behind the sample to provide a more consistent fluid path during sample application. The paper spray cartridge from Prosolia Inc. has been used in several studies[4, 10, 16]. The use of this cartridge was also demonstrated on a prototype

miniaturized mass spectrometer, raising the possibility for rapid MS analysis of blood samples in clinical settings[75] (Figure 5).

3D-printing is a promising technique to making functional paper spray cartridges. A 3D-printed paper spray cartridge made of polylactic acid[76] is shown in Figure 6. The replenishment of solvent from reservoir to the paper tip allowed for prolonged analysis (>10 minutes). The disadvantage of most 3D printed materials is that solvent compatibility is limited and the materials themselves are porous, meaning that the spray solvent will be absorbed by both the paper and the 3D printed cartridge material. On the other hand, higher end 3D printers offer materials with better solvent compatibility. Currently, 3D printing in this area is primarily of interest during research and development as a prototyping tool. With improvements in the technology and reductions in cost, 3D printing may become a viable option for laboratories to manufacture their own paper spray cartridges for routine use.

In order to improve the analytical performance of paper spray, such as signal intensity and detection limits, we recently reported a paper spray cartridge with integrated solid phase extraction[19], as shown in Figure 7. The "all-in-one" disposable cartridge performed sample extraction, pre-concentration, as well as ionization from complex samples such as plasma. Compared to direct paper spray, this approach required larger samples volumes, but improved the MS signal intensity and detection limits significantly. For the five analytes examined, detection limits improved by a factor of 14 to 70. The improvement was primarily due to the capability of preconcentrating the analyte from larger sample volumes.

C. Miniature Mass Spectrometers

There has been interest in coupling paper spray MS to portable or miniature mass spectrometers. On the one hand, paper spray is a good fit for such instruments because of the simplicity of the technology and the fact only small volumes of solvent and no nebulizing gas are needed for analysis. On the other hand, achieving sufficiently low detection limits and selectivity is likely to be even more challenging. There are several reports on the use of paper spray with miniature and portable mass spectrometers, primarily from the Cooks research group at Purdue University. Some applications on portable instruments have included synthetic cannabinoid detection from drug samples and biofluids[77], agrichemicals[78] and household chemicals[79], and detection of corrosion inhibitors[80]. A miniature, though not portable, mass spectrometer that used paper spray for analysis of drugs in biofluids was also reported as a proof of concept demonstration of a point of care analyzer[75].

D. Alternative sampling strategies

Analytes from a surface have been collected onto paper following by paper spray analysis[81]. This swabbing strategy facilitated the paper spray analysis of agrochemical residues on fruit[78], pesticides in agricultural products[82], and cocaine residues on various surfaces[83]. To achieve online monitoring of analyte by paper spray MS, some continuous sampling methods were employed to transfer the sample solution onto the paper, including drop-casting[84], gravity-driven microchip[85], online microfluidic chip[86], and homemade microdialysis module[36].

Challenges/Limitations

A. Selectivity

Paper spray MS, like other ambient or direct MS methods, does not employ chromatographic separations prior to analysis. While removing this step speeds up sample analysis and eases sample preparation constraints, it also hurts selectivity. Chemical discrimination in direct MS analysis methods occurs by mass spectrometry alone. Selectivity is often enhanced by performing tandem mass spectrometry (MS/MS) or by accurate mass measurement on high resolution instruments. Isomers cannot be distinguished by high resolution MS, however. In many cases, MS/MS without prior separation can distinguish structural isomers and even quantitate them simultaneously as long as there are unique fragment ions formed upon collisional activation. Closely related structural isomers, however, frequently fragment so similarly that no unique fragment ions exist. Diastereomers are even more challenging; they often have different physical properties and can normally be resolved by a well-designed HPLC method. The MS/MS spectra are typically identical, however, so direct methods such as paper spray cannot distinguish them. Finally, fragile metabolites that decompose in the source such as acyl glucuronides may lead to overestimation of the parent drug in paper spray MS and other ambient ionization methods because the fragile metabolite is not separated from the parent drug prior to ionization. Several approaches have been proposed in the literature for improving the selectivity of paper spray MS without significantly increasing the complexity and time of the analysis.

Reactive paper spray involves the use of derivatizing agents to chemically alter the analyte prior to ionization. Use of the term implies that the derivatizing agent is incorporated into the workflow without adding an extra sample preparation step; the reagent may be dried on the spray substrate prior to sample spotting, for example, or be dissolved in the extraction/spray solvent. Traditional off-line derivatization would not be considered reactive paper spray. The idea is similar to and inspired by reactive DESI[87-93]. Several papers describing reactive paper spray methods have been published, most of them dealing with derivatization of carbonyl groups[94-96]. In all of these cases, the motivation was primarily to increase the ionization efficiency (and therefore improve detection limits) of the targets. Reactive paper spray could also be utilized to resolve interferences due to closely related structural isomers. For example, a derivatizing reagent that reacts selectively with carbonyl groups could be used to distinguish a carbonyl containing compound from an isomer that has, for example, a hydroxyl group and a double bond (such as hydromorphone and morphine).

Ion mobility, which separates gas phase analyte ions on the basis of collision cross section by measuring their mobility as they are passed through a buffer gas, is another way to increase selectivity. There are several different types of ion mobility, including drift tube ion mobility, traveling wave ion mobility, and differential mobility spectrometry (DMS)/field asymmetric ion mobility spectrometry (FAIMS). The various ion mobility approaches are attractive because they can be incorporated into the analysis without increasing analysis time or requiring additional sample cleanup. The selectivity of ion mobility is not as good as liquid chromatography because the potential for manipulating the stationary phase and mobile phase to change analyte retention does not exist in the gas phase. Nevertheless, resolution of closely related structural isomers and even diastereomers has been widely reported in the literature. Several MS manufacturers offer ion mobility as an option in their mass spectrometers, including Waters (Synapt line - traveling wave ion

mobility), Sciex (Selexion – planar DMS), Thermo (FAIMS), and Agilent (drift tube IMS). Paper spray has been coupled to stand-alone drift tube ion mobility(no MS detector)[83, 97]. Paper spray-FAIMS-MS/MS was also reported, and the separation of the structurally similar isomers of morphine, norcodeine, and hydromorphone was demonstrated[98].

B. Matrix effects

Matrix effects are known to be present in the paper spray analysis of blood and other biofluids[4, 7, 18, 19]. Matrix effects normally manifest as lower analyte signal in dirty matrices such as dried blood when compared to drying the same quantity of analyte on matrix-free paper. The lower analyte signal is caused by a combination of lower analyte recovery and ionization suppression. The magnitude of the matrix effect varies with the chemical and physical properties of the analyte and also the matrix. Recovery, for example, was found to be lower in blood than in urine. Ion suppression, on the other hand, was generally higher in urine than in blood[7]. Despite the presence of matrix effects, good quantitative performance has been widely reported in paper spray. Provided that matrix matched calibrators and internal standardization with SIL analogs are employed, matrix effects do not affect quantitative capabilities of the technique (although they do increase the limit of detection). For example, paper spray MS showed good correlation with HPLC-MS for incurred patient samples for a tacrolimus assay[4], and no effect from hemolysis, lipemia, icterus, or high concentrations of 50 different steroids, vitamins, diuretics, and immunosuppressive compounds was found for paper spray assays of tacrolimus, cyclosporine, or sirolimus[4, 16]. Assay robustness when using SIL internal standards was also demonstrated by constructing calibration curves in 5 different lots of biofluids[8]. Nevertheless, the presence of matrix effects can be problematic for the reasons already described: SIL analogs are preferred to maintain quantitative performance and the detection limits are higher compared to analysis of matrix-free samples.

C. Detection limits

As shown in Table 1, detection limits in the single digit ng/mL or pg/mL range from dried matrix spots range have been reported. Some potent drugs and small molecules have therapeutic and even toxic concentrations at the pg/mL level. Paper spray MS, without sample preparation, will not be able to detect those levels in many cases. Additionally, detection limits in low or sub ng/mL range are obtainable only in favorable cases: hydrophobic drugs (logP > ~2) with aliphatic amines analyzed in SRM mode on triple quadrupole mass spectrometers. Detection limits will tend to be higher for hydrophilic analytes and those that lack aliphatic amines. There are exceptions. Tacrolimus and cyclosporine, for example, do not form protonated ions well, but both form sodium and ammonium adducts efficiently and can be detected at sub ng/mL levels from dried blood spots. Methods for improving detection limits in paper spray are being explored. As discussed in previous sections, a paper spray cartridge with integrated solid phase extraction has been developed and alternative spray substrates have demonstrated improved detections limits by increasing analyte recovery or improving ionization efficiency.

Conclusion

Paper spray MS shows good potential for analysis of small molecule drugs from dried biofluids. It is a simple approach for analyzing dried blood spots by mass spectrometry

with surprisingly good detection limits. As with any direct or ambient ionization method, careful attention must be made to selectivity during method development, however. A particularly interesting direction for paper spray development is the potential for creating disposable cartridges that contain all of the elements required for sample preparation and ionization by mass spectrometry. Such "smart" cartridges have the potential to dramatically simplify MS analyses in clinical laboratories and pharmaceutical research and development while maintaining a high level of performance.

Future Perspective

Interest in paper spray MS has been growing steadily since its initial description in 2010. The method has the potential to impact chemical measurements in fields requiring rapid, affordable and facile analytical techniques such as therapeutic and illicit drug monitoring. Major innovations likely to occur in the near future include optimization of spray substrates to minimize ion suppression while maximizing analyte recovery. Another important area could be the development of disposable cartridges that perform additional analytical functions, such as analyte preconcentration or incorporation of internal standards. Improving selectivity without increasing the complexity of the method will be critical for paper spray and ambient ionization in general. Improvements in ion mobility technology as well as development of rapid chemical tagging approaches may play a role in selectivity improvement.

Table 1. Alphabetical listing of the xenobiotics that have been analyzed by paper spray MS from biological samples. Separate entries for are shown for analytes appearing in multiple references or analyzed with different conditions in the same paper. LOD and LLOQ (without regard for how they were determined) are included only if it is stated explicitly in the publication. Range is included only when a calibration curve is reported. Other important experimental variables omitted from this table include spray substrate, solvent, and sample volume.

Analyte	Instrument	Matrix	Range (ng/mL)	LOD (ng/mL)	LLOQ (ng/mL)
4-chloroamphetamine[22]	Finnigan LCQ Classic	Saliva		100	
4-fluoroamphetamine[22]	Finnigan LCQ Classic	Saliva		100	
6-acetylmorphine[9]	TSQ Quantum Access Max	bovine blood	5-800		5
7-ethooxycoumarin[8]	TSQ Quantum Access Max	Bovine Blood		1	
Acetaminophen[8]	TSQ Quantum Access Max	Bovine Blood		250	
alprazolam[19]	LTQ-XL	Bovine Plasma	1.3-1000	1.3	4
Amitriptyline[75]	Mini 12	Bovine Blood	15-510		7.5
Amitriptyline[8]	TSQ Quantum Access Max	Bovine Blood	0.9-443		
amitriptyline[11]	TSQ Quantum Access Max	Bovine Blood	0.1 -10000		0.1
amphetamine[10]	TSQ Quantum Access Max	Human Blood	10-4000	1	
anabasine[5]	TSQ Quantum Access Max	bovine blood	0.1-100		1
Atenolol[12]	Thermo Fisher LTQ	Bovine Blood		50	
Atenolol[19]	LTQ-XL mass spectrometer	Bovine Plasma	2.2-500	2.2	7
Benzethonium[8]	TSQ Quantum Access Max	Bovine Blood		0.02	

Benzethonium[25]	LTQ	Human and mouse plasma	5-500		5
benzoylecgonine[9]	TSQ Quantum Access Max	bovine blood	1-800		1
buprenorphine[9]	TSQ Quantum Access Max	bovine blood	1.6-500		1.6
carbamazepine[19]	LTQ-XL	Bovine Plasma	0.3-1000	0.3	1
chlorpromazine[21]	Shimadzu model 2010	Urine	30-500 ng	1.5 ng	
Citalopram[8]	TSQ Quantum Access Max	Human Blood	1-500		1
citalopram[11]	TSQ Quantum Access Max	Bovine Blood	0.1 -10000		0.1
clenbuterol[17]	TSQ Quantum Access Max	Beef Homog enate			1 ng/g
cocaine[10]	TSQ Quantum Access Max	Human Blood	10-1000	0.05	
cocaine[9]	TSQ Quantum Access Max	bovine blood	0.5-1000		0.5
Cotinine[5]	TSQ Quantum Access Max	bovine blood	0.8-200		3
Cotinine[5]	TSQ Quantum Access Max	Liquid Saliva	0.8-200		2
Cotinine[5]	TSQ Quantum Access Max	Liquid Urine	0.1-100		5
Cyclophosphamide[6]	TSQ Quantum Access Max	Bovine Blood		11	
cyclosporine[16]	TSQ Vantage	Human Blood	35-1200	5	35
Dextrorphan[8]	TSQ Quantum Access Max	Bovine Blood		0.6	

diazepam[19]	LTQ-XL mass spectrometer	Bovine Plasma	6.1-1000	6.1	20
Docetaxel[6]	TSQ Quantum Access Max	Bovine Blood		13	
Heroin[12]	LTQ	Urine		125	
Heroin[9]	TSQ Quantum Access Max	bovine blood	5-800		5
Hydralazine[5]	TSQ Quantum Access Max	rat tissues	16-2000		
Ibuprofen[8]	TSQ Quantum Access Max	Bovine Blood		500	
Imatinib[6]	TSQ Quantum Access Max	Bovine Blood		9	
Imatinib[15]	TSQ quantum max	Blood	10-4000		
imatinib[1]	LTQ	Bovine Blood	62.5 -4000		
Irinotecan[6]	TSQ Quantum Access Max	Bovine Blood		13	
lidocaine[20]	TSQ quantum access max	Blood	250-1000	4	
lidocaine[11]	TSQ Quantum Access Max	Bovine Blood	0.1 -10000		0.1
MDA[10]	TSQ Quantum Access Max	Human Blood	10-4000	2	
MDEA[10]	TSQ Quantum Access Max	Human Blood	10-1000	0.3	
MDMA[10]	TSQ Quantum Access Max	Human Blood	10-1000	0.04	
methamphetamine[10]	TSQ Quantum Access Max	Human Blood	10-1000	0.3	
methamphetamine[9]	TSQ	bovine	5-500		5

	Quantum Access Max	blood			
morphine[10]	TSQ Quantum Access Max	Human Blood	40-4000	12	
morphine[9]	TSQ Quantum Access Max	bovine blood	5-800		5
nicotine[5]	TSQ Quantum Access Max	Bovine Blood	1-100		1
Nicotine[5]	TSQ Quantum Access Max	bovine blood	1-100		1
Nicotine[5]	TSQ Quantum Access Max	Wet Bovine Blood	0.1-100		0.1
o-, m-, p-chloroamphetamine[23]	LCQ Classic	Saliva			100
o-, m-, p-fluoroamphetamine[23]	LCQ Classic	Saliva			100
oxycodone[9]	TSQ Quantum Access Max	bovine blood	16-1000		16
Paclitaxel[6]	TSQ Quantum Access Max	Bovine Blood		12	
Paclitaxel[8]	TSQ Quantum Access Max	Bovine Blood		15	
Pazopanib[6]	TSQ Quantum Access Max	Bovine Blood	10-1000	0.5	
Proguanil[8]	TSQ Quantum Access Max	Bovine Blood		0.08	
Propranolol[20]	TSQ quantum access max	Blood	250-1000	106	
ractopamine[17]	TSQ Quantum Access Max	Beef Homogenate			10 ng/g
Simvastatin[8]	TSQ Quantum Access Max	Bovine Blood		50	
Sirolimus[16]	TSQ Vantage	Human Blood	2-60	0.5	2

Sitamaquine[8]	TSQ Quantum Access Max	Rat Blood	5-1000		5
sulfamethazine[19]	LTQ-XL	Bovine Plasma	0.08-1000	0.08	0.2
Sunitinib[8]	TSQ Quantum Access Max	Bovine Blood	1-500	0.25	
sunitinib[17]	TSQ Quantum Access Max	Bovine Blood	1-100		1
Sunitinib[25]	LTQ	Human and mouse plasma	1-500		1
Sunitinib[11]	TSQ Quantum Access Max	Bovine Blood	0.1 -10000		0.1
Tacrolimus[4]	TSQ Vantage	Human Blood	1.5-30	0.2	1.5
Tamoxifen[6]	TSQ Quantum Access Max	Bovine Blood		8	
Telmisartan[8]	TSQ Quantum Access Max	Bovine Blood		0.3	
terabutaline[17]	TSQ Quantum Access Max	Beef Homogenate			10 ng/g
THC[10]	TSQ Quantum Access Max	Human Blood	10-1000	4	
Topotecan[6]	TSQ Quantum Access Max	Bovine Blood		17	
Trans-3'-hydroxycotinine[5]	TSQ Quantum Access Max	bovine blood	0.8-200		2
Verapamil[20]	TSQ quantum access max	Blood	250-1000	3	
Verapamil[8]	TSQ Quantum Access Max	Bovine Blood		0.75	
verapamil[11]	TSQ Quantum Access Max	Bovine Blood	0.1 -10000		0.1

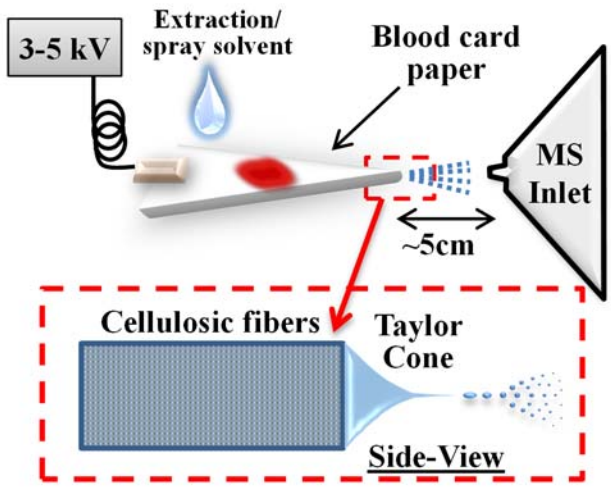

Figure 1. Analysis of a dried blood spot by paper spray. A drop of whole blood is applied directly to a triangular section of chromatography paper. A DC voltage is applied to the paper wetted with solvent, which acts to both extract the analyte and form a Taylor cone for electrospray ionization. Figure credit: Ryan D. Espy.

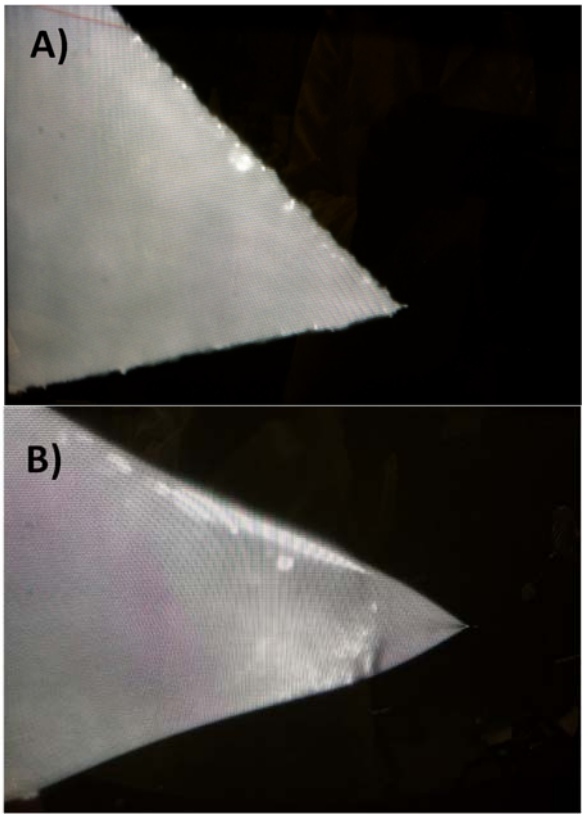

Figure 2. Picture of the paper substrate during MS analysis using a long focal length microscope. A) paper wetted with spray solvent but without high voltage applied. B) 3.5 kV is applied to the wet paper, initiating Taylor cone formation at the tip of the paper. Photo credit: Rachel Potter.

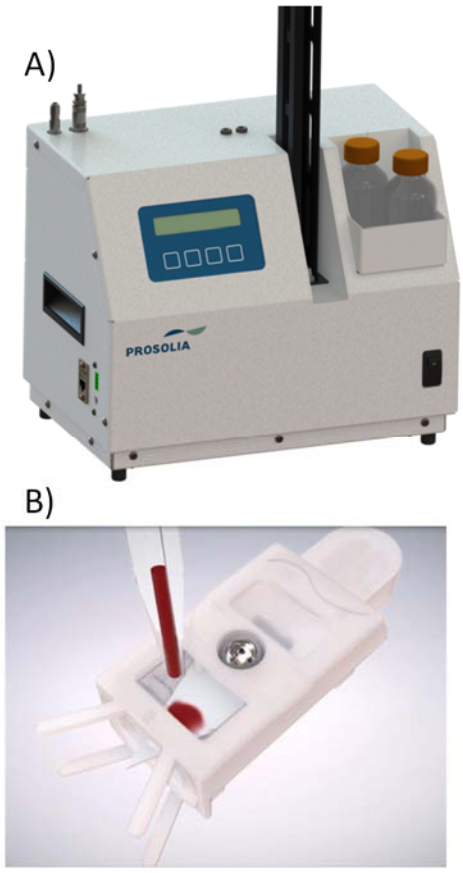

Figure 3. Renderings of A) the paper spray autosampler and B) the disposable paper spray cartridge, which is about 4.5 cm long.

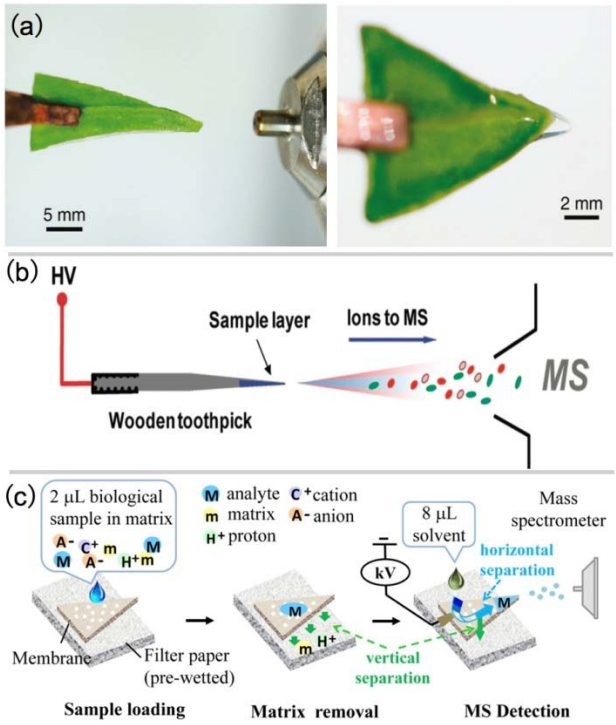

Figure 4. (a) Left: Photograph of leaf spray ionization of green onion leaf cut to a point and held by a high voltage connector in front of the atmospheric inlet of a mass spectrometer. Right: Photograph of leaf spray ionization of spinach leaf in negative ion mode. The spinach leaf was cut into a triangle, and methanol was applied on the leaf to achieve leaf spray ionization. Reproduced from [42] (b) Experimental setup of ESI using a wooden tip. Adapted from [57] (c) On-membrane matrix removal and analyte ionization in MESI. Adapted from [31].

Figure 5. The point-of-care analysis using a paper spray cartridge on the Mini 12 mass spectrometer. Adapted from [75].

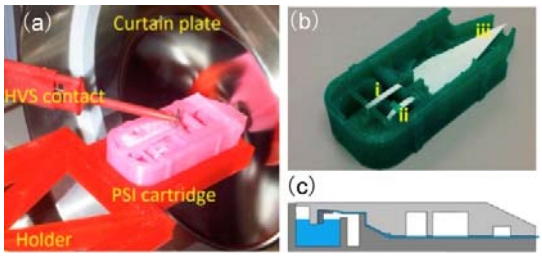

Figure 6 a) 3D-printed cartridge setup. (b) Bottom part of the 3D-printed cartridge with paper substrate. (c) When the reservoir of cartridge is filled, fluid will slowly move through the paper wick by capillary action and swiftly between the paper and the polylactic acid guide structures. Reproduced from [76].

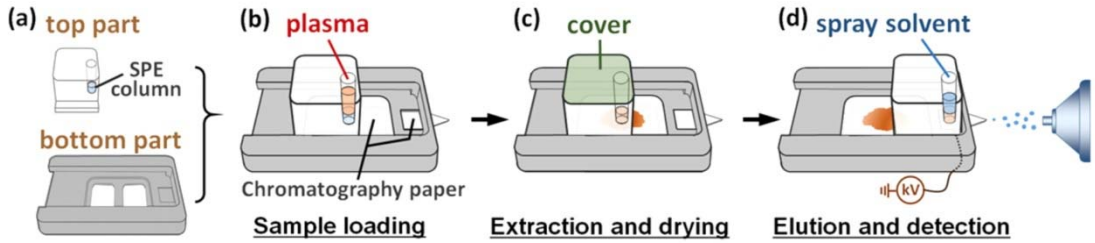

Figure 7. Diagram depicting the workflow for paper spray analysis with integrated on-cartridge solid phase extraction. The cartridge consisted of two parts: the top part containing an SPE column and a bottom part with an absorbent waste pad and the spray substrate. Adapted from [19].

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