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Review Article

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RECENT ADVANCES IN SAMPLE COLLECTION, PREPARATION AND ANALYSIS IN PHARMACEUTICAL AND BIOANALYTICAL FIELD

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ABSTRACT

Liquid chromatography tandem mass spectrometry (LC-MS-MS), because of its high degree of sensitivity, selectivity and robustness, has come out as a great tool in pharmaceutical industry for many analytical needs. This review summarizes the state-of-art in liquid chromatography–mass spectrometry (LC–MS) and related techniques with the main focus on recent developments in the last decade. LC–MS records an enormous growth in recent years due to the application potential in analytical chemistry, biochemistry, pharmaceutical analysis, clinical analysis and many other fields, where the qualitative and quantitative characterization of complex organic, bioorganic and organometallic mixtures is needed. Current trends in sample collection and preparation is important for isolating desired components from complex matrices and greatly influences their reliable, accurate analysis and include miniaturization, automation, high-throughput performance, online coupling with analytical instruments and low-cost operation through extremely low or no solvent consumption are discussed as well.

KEY WORDS

Bioanlytical, Dry blood spotting (DBS), Dry matrix spotting (DMS), LC MS/MS, Microextraction.

1. INTRODUCTION

The use of high-performance liquid chromatography combined with mass spectrometry (HPLC-MS) or tandem mass spectrometry (HPLC-MS-MS) has proven to be the analytical technique of choice for most assays used in various stages of new drug discovery (1). LC–MS records an enormous growth in recent years due to the application potential in analytical chemistry, biochemistry, pharmaceutical analysis, clinical analysis and many other fields, where the qualitative and quantitative characterization of complex organic, bioorganic and organo metallic mixtures is needed (2).It can also analyze many samples in short period of time by multiple reactions monitoring mode. Liquid chromatography tandem mass spectrometry (LC-MS-MS), because of its high degree of sensitivity, selectivity and robustness, has come out as a great tool in pharmaceutical industry for many analytical needs. Current article reviews vital information about techniques which is used in bio analytical laboratories including an efficient sample preparation and also shows various spotting techniques like dry blood spotting (DBS), dry matrix spotting (DMS) and stripping technique like charcoal stripping (for endogenous substances).

Nowadays the most frequently applied extraction technique uses very small volume (some ml-s) of organic solvents or there is no solvent consumption either (so called solvent free extraction). New trends in sample

preparation have appeared, such as sample preparation in 96-well plates and the automation of conventional methods for solid phase extraction (SPE), protein precipitation (PP) and liquid-liquid extraction (LLE). Also more recent approaches are now available such as liquid-liquid microextraction (LLME), solid phase microextraction (SPME) and microextraction by packed sorbent (MEPS) etc**.** These new approaches both offer some advantages but also suffer from limitations, which explain why the conventional off-line sample preparation methods are still used in routine laboratories.

2. Sample collection

Dried blood spots (DBS) and dried matrix spots (DMS) are now widely used for the collection of samples for the quantitative determination of circulating exposures of pharmaceuticals in animal toxicokinetic (TK), clinical pharmacokinetic (PK), and therapeutic drug monitoring studies at physiologically relevant concentrations (3-6).

2.1. Dried blood spot sample collection

Recently, there has been increasing interest in the use of DBS to support pharmacokinetic and toxicokinetic studies in small-molecule drug discovery and development (7). Dried blood spot (DBS) sampling involves spotting blood from a heel- or finger-prick onto a card which is then air dried and sealed in bags with desiccant for storage and shipping (8). The technique offers many advantages compared to traditional liquid blood or plasma samples including: Low blood volume requirements are beneficial in pediatric studies and enable serial bleeding from one animal, so reducing the number of animals used in studies, improved data quality in preclinical toxicokinetic and pharmacokinetic studies, less invasive and more patient-friendly than venous cannulation, cards can be stored at ambient conditions, reducing sample storage and transport costs (9).

The standard sample preparation approach for DBS analysis consists of punching out a disk from the card that contains the DBS followed by extraction of the analyte. The punched sample disks typically range in diameter from 3 to 8 mm and are extracted with an organic solvent, or a mixture of aqueous and organic solvent. Internal standard (IS) is usually added to the extraction solvent. The extract is then analyzed by LC–MS/MS (10).

2.1.1. Automation for DBS analysis

Automation of DBS analysis usually involves the robotic punching of DBS cards followed by adding solvent for extraction. The process can be fully or semi-automated. A barcode reader can be added for sample identification to streamline the automation process. A disk detection system ensures that the punched disk is placed in the correct well of the 96- or 384- well collection plate. In a fully automated system, a robotic arm can pick up the sample plates and place them onto a liquid- handling workstation for the downstream processing. The options for automation systems are likely to increase as DBS collection becomes more widely adopted for bio analysis (11).

2.1.2. Direct elution of DBS samples

The use of direct online elution of analytes from DBS samples was first reported by Deglon et al. [12]. However, this approach required the prepunching of DBS samples and the mounting of each punched disk in an online holder. This system had limited sample capacity and is not suitable for high through put analysis.

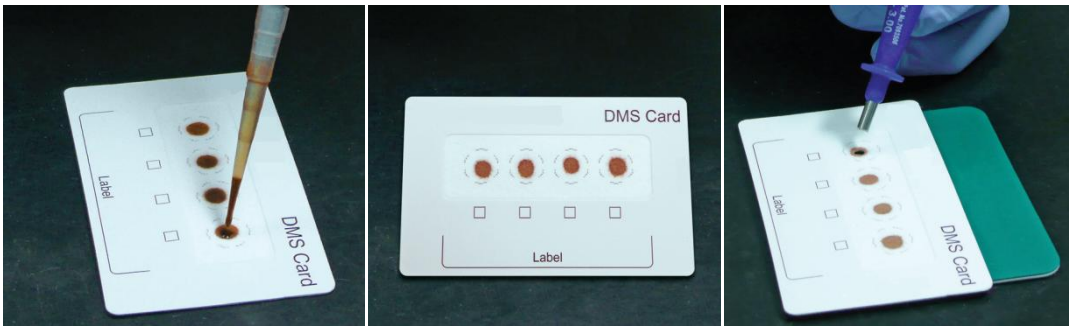
2.1.3. Direct desorption of DBS samples

Direct desorption of DBS samples can be achieved by ambient-ionization MS techniques (13, 14) such as desorption electro spray ionization (DESI) (15) and direct analysis in real time (DART®) (16). For analysis by DESI, DBS cards are cut into strips and secured onto a microscope glass slide whereas blood is spotted

directly on a glass slide for analysis by DART. The surface is moved in one direction at constant velocity so that an ion spray in a DESI source or meta stable gas phase species (helium) in a DART source desorbs analytes directly from the surface of the DBS without prior sample preparation. These direct desorption techniques greatly increase the throughput of DBS sample analyses by eliminating the extraction step and by utilizing short analysis times (from seconds to a minute). However, they also suffer from the absence of chromatography resulting in poor sensitivity due to ion suppression and possible interference from decomposition of metabolites. An important step in the DBS preparation for quantitative analysis is the introduction of an internal standard. A novel piezoelectric system was used to spray methanol solution with dissolved internal standard over the DBS.

2.2. Dried Matrix Spot Sample Collection

Dried Matrix Spot (DMS) sampling is a method of collecting biological matrices obtained from human or animal subject and spotting it on DMS card/paper. For detection of spotted portion, discshaped area of every spot is punched out from DMS card/paper, **Figure 1**. The punched circular discs are then extracted by using different techniques suitable for analysis. The extracts obtained are analysed by high sensitivity LC-MS/MS instruments (17).



Spot **Dry** **Punch**

Figure-1: Dried Matrix Spot (DMS) sampling method

Dried matrix spot (DMS) sampling is required small sampling volume and easy sample handling, the samples collected by DMS technique are easy to store and does not require deep freezer. Thus, it provides significant cost saving.

It can pose limitations in sensitivity due to small sample size. Bond Elute DMS is a non-cellulose card/paper and is not soaked into reagents. Due to absence of impregnation this avoids non-specific binding of analyte of interest. This results in increasing mass spectrometry response and also improves the signal-to-noise ratio. Bond Elute DMS is compatible with standard punching tools and automation, and it needs five times less punching force as compared to cellulose based cards. This causes faster workflow, less technician fatigue and smoother automation (18, 19).

2.3. Charcoal Stripping

Charcoal stripping (adsorption) is a process used to remove hormones and steroids (endogenous substances) from serum or plasma, required for bio analytical study. It has also been utilized for improving immunoassay systems. The active binding sites on the charcoal bind with hormones or steroids, charcoal is removed by centrifugation. Filtration will efficiently eradicate remainder charcoal in serum or plasma (20). Charcoal: dextran stripping decreases the serum

concentration of many hormones and certain growth factors, such as estradiol, cortisol, corticosterone, vitamin B, T3, T4 and prostaglandins. This serum is useful where these endogenous molecules may interfere with experimental work. It is made by exposing the serum to a mixture of dextran coated activated charcoal for a certain period of time (21).

3. Sample Separation techniques

3.1. On-Line SPE-LC

The on-line SPE technique offers speed, high sensitivity by the pre-concentration factor, and low extraction cost per sample, but typically requires the use of program controlled switch valves and column re configuration. However, the on-line techniques can be fully automated and several generic approaches have recently been developed for on-line sample extraction coupled to LC-MS (22). The most commonly method used for on-line coupling of SPE with LC is achieved through column switching. For this purpose, a small, typically 2–15 mm long and 1–4.6 mm i.d. precolumn used as SPE column is connected to a conventional LC analytical column via a switching valve. Column switching configurations can contain various numbers of precolumns, switching valves, and pumps. Firstly, on-line SPE technique reduces the sample preparation time and, thus, increases the sample throughput. Conditioning, washing, and elution steps can be performed automatically (23). Secondly, the technique can decrease the risk of sample contamination, eliminate the analyte loss by evaporation used in off-line mode, improve the accuracy, and reduce the risk for the operator (24). In addition, on-line SPE can decrease the solvent consumption and the costs for organic solvents waste disposal (25). In on-line mode, the whole extract is transferred to the LC analytical column, and in off-line mode, only a small aliquot is injected, so the sensitivity of the on-line method is increased. However, this easily leads to overloading of the analytical column, especially for the complex samples. In on-line mode, efficient cleaning of the extraction system is necessary in order to avoid memory effects.

Advantages of this technique is time savings and higher throughput, improved precision and accuracy, safety, the extraction and analytes take place in a closed system, which decreased the exposure to hazardous samples or solvent in mass spectrometry detector, reusable SPE column, minimal loss or degradation of analytes, no evaporation steps and direct elution of the analytes after preconcentration into LC column, minimal consumption of organic solvent.

Disadvantages of this technique are limited portability, expensive equipment. One switching valve and one high pressure pump are needed at least and matrix effects; ionic suppression or enhancement.

The on-line SPE-LC method is mainly used for the determination of liquid samples. If the samples are solid, the analytes in the sample matrix were usually extracted into solvent first. Then, the extract was introduced into the on-line system for further treatment and analysis. Alternatively, the SPE-LC method also can be further coupled on-line to other sample preparation techniques, such as supercritical fluid extraction (SFE), subcritical water extraction (SWE), microwave-assisted extraction (MAE), or ultrasound-assisted extraction (UAE) for direct analyzing solid samples.

3.2. 96-Well SPE Plates –

The new format of SPE is 96-Well SPE Plates. The 96-well plate format is based on the standard 96-well microtiter plate format. Parallel sample processing allows 96 samples to be extracted in approximately one hour or less. Processing 96 samples simultaneously reduces handling errors and limits labour-input. Each of the 96 wells has a small 1 or 2 ml SPE column with 3-10 mg of

packing material. The packing material in an SPE cartridge is placed between the bottom frit ormembrane and the top frit. Types of 96-well SPE plates exist: fixed and flexible. The plates are called fixed because most of them have a fixed volume and fixed amount of sorbent. Compared with typical SPE cartridges, these small packed bed wells have different flow characteristics, masses and volumes, so they require some adjustment of the SPE conditions [26, 27]. These plates are rather costly and, given that a test may use only a few of the wells, laboratories must incur considerable expense in method development experiments.

The modification of Flexible 96-well SPE plates. The flexible well plates have removable small round or square plastic SPE cartridges that fit tightly into separate 96-hole base plates. Each individual cartridge has top and bottom frits and a stationary-phase packing, as used in the fixed 96-well SPE plate design. Users can place individual cartridges in as many positions as needed. The individual cartridges can contain different stationary phases or different amounts of the same phase. In some instances, different cartridge volumes (1 or 2 ml) are available and can be used for larger samples. A vacuum manifold is often used to pull liquids through a flexible well plate, and users can plug the unused holes of the base plate with plug strips if only a portion of the 96 wells is needed [28].

384-well SPE plates also exist and have the same external dimensions as the current 96-well SPE plates, so SPE wells would be very tightly sandwiched together.

An advantage of automated method development is that analysts can expect improved method precision and accuracy over manual SPE methods because they generally have better control over the sample and solvent manipulation in an automated environment.

New formats for SPE provide reduced bed masses, high-throughput capabilities and greater convenience for method development. A small mass of the bed allows faster method development, reduced solvent volume and shorter sample preparation times. The transfer of manual methods to automated methods has been improved by the advent of removable, flexible cartridges that can be used manually or in an automated environment.

3.3. Micro extraction In Packed Syringe

MEPS are a new technique and represent an attempt of SPE miniaturisation using much reduced sorbent. It is based on using a syringe with a special needle as an extraction device, sample preparation takes place on the packed bed which can be packed or coated to provide selective sampling (29).

In MEPS the sorbent is inserted directly into the needle. The MEPS sorbent cartridge can be used for over 100 injections depending on the sample matrix (30). Another important feature is that MEPS can handle sample volumes starting from 10 µL and up to 1000 µL. MEPS can be used both manually and fully automated (the sampling, extraction and injection are on-line). The biological fluid is diluted before sample loading. For plasma the dilution factor is typically 5, while for whole blood it is typically 25. Thereafter, the sample is loaded by withdrawing and ejecting in the sample vial or ejecting to the waste. The loading step can be repeated many times to increase recovery of the analyte. Step two is the washing of the sorbent, to remove proteins and other unwanted material. The third step is the elution with organic solvent directly to the LC or GC injector, step four. Step five is washing A and B and takes place after elution to eliminate the carry-over and conditioning the material for further use. Wash A is usually the elution solution and wash B is the washing solution used

after sample loading, for a schematic presentation of the MEPS procedure.

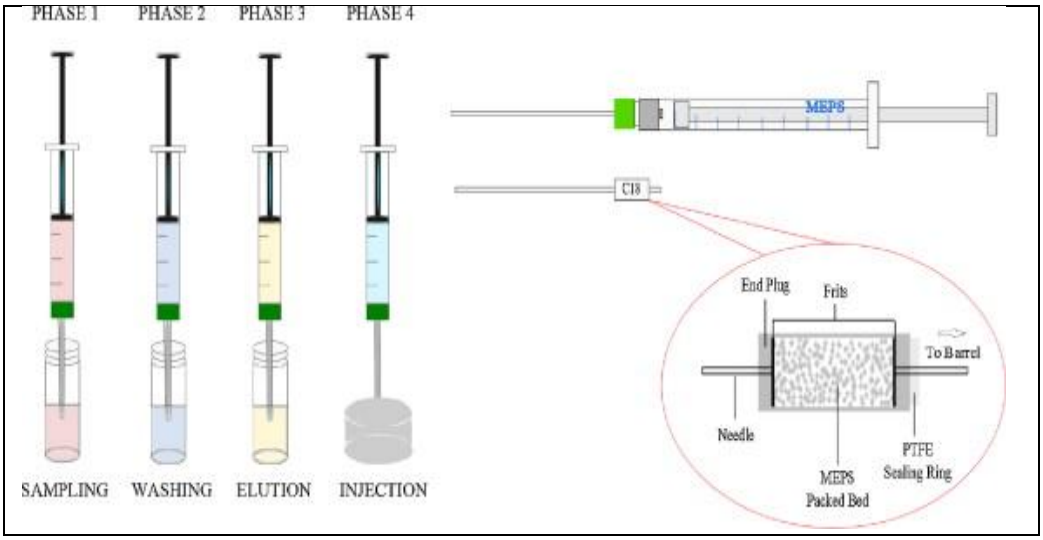


Figure 2. Schematic of the MEPS BIN in the syringe needle.

Application of MEPS

This technique has been successfully used to extract wide range of drugs and their metabolites from different biological matrices such as plasma, urine and blood. Other applications have been investigated like polycyclic aromatic hydrocarbons in water, wine and hair. MEPS have been connected on-line to LC and GC mass spectrometry (31). The technique can replace most existing SPE methods by scaling the reagents and sample volume. Some review articles are available about MEPS technique discussing the factors affecting the performance of MEPS and the recent advances in MEPS.

Advantages: Reduce the time to prepare and inject samples from hours to minutes, eliminate all extra steps between sample preparation and sample injection, reduce buffer and solvent volume from Milliliters to Micro liters, reduce the sample volume needed to as little as 3.6 Ml.

Disadvantages: Recovery from MEPS can be affected by different factors, mainly the used sorbent and the influence of number of extraction cycles, carry-over is an expected problem when using MEPS.

Liquid chromatography linked to tandem mass spectrometry (LC–MS/MS) has played an important role in pharmacokinetics and metabolism studies at various drug development stages since its introduction to the pharmaceutical industry. Newly introduced techniques such as ultra-performance liquid chromatography with small particles (sub-2 µm) offer improvements in speed, resolution and sensitivity compared to conventional chromatographic techniques. it can be successfully applied even if you have only a tiny quantity available for analysis—as little as 10-12 g, 10-15 moles for a compound of mass 1000 Daltons(Da). Compounds can be identified through mass spectrometry at very low concentrations (one part in 1012) in chemically complex mixtures. The basic mass spectrometry processes of instrumentation are consisted of (a) introduction of sample; a sample which can be a solid, liquid, or vapor is loaded onto a mass spectrometry device and is vaporized, (b) ionization; sample components are ionizedby

one of several available methods to create ions, (c) analyzer sorting; the ions are sorted in an analyzer according to their *m/z* ratios through the use of electromagnetic fields, (d) detector; the ions then pass through a detector where the ion flux is converted into a proportional electrical current and (e) date conversion; the magnitude of the ion/electrical signals is converted into a mass spectrum MS instruments consist of three modules: an ion source, which can convert gas phase sample molecules into ions (or, in the case of ESI, move ions that exist in solution into the gas phase); a mass analyzer, which sorts the ions by their masses by applying electromagnetic fields; and *a* detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present.

3.4. Solid Phase Micro extraction

Solid-phase micro-extraction (SPME), initially introduced for the analysis of environmental samples, has been used increasingly for the extraction of drugs and other lipophilic analytes from biological matrices. In its simplest form, a spring-loaded solid probe (originally a fused silica fibre) coated with a polymer film is inserted into the sample containing the analyte(s) (usually aqueous liquid or headspace), and after an appropriate period the fibre is retracted (32). Analyte partitions between the sample and the liquid phase supported on the fibre, usually poly (dimethylsiloxane) (PDMS), poly (dimethylsiloxane-divinylbenzene) or polymethacrylate. Unlike LLE, the aim is not to extract all the analyte from the sample as SPME is an equilibrium extraction (33); indeed the analyte concentration of the sample may be little affected if only a very small proportion of analyte is removed. . Subsequently, extracted analytes are either thermally desorbed in the case of GC/GC-MS, or injected via a sample loop in the case of HPLC (34, 35). Obvious attractions of SPME are that no solvents are required and all the material that is extracted by the probe may be analyzed directly.

The advantages claimed for SPME were no use of solvents, easy handling, little equipment necessary, fast method, ease of automation, good linearity and high sensitivity

Disadvantages are extraction is very slow in contrast to LLE and SPE with bed columns, the recovery was very low, and the occurrence of a huge number of interferences in the chromatogram which come from endogenous trace substances in biological fluids prevents the analysis of target analyte at low concentrations.

3.5. Liquid-Phase Micro-Extraction

In liquid-phase micro-extraction (LPME), analytes are extracted from biological samples such as whole blood or plasma (0.1–4 mL) into an acceptor solution contained in the lumen of a disposable porous hollow fibre (polypropylene tubing crimped at one end) supported in a sealed glass vial. Internal standardization can be used as with LLE, and direct GC, HPLC, or CE analysis of the acceptor solution eliminates further extract handling (36). Advantage of LPME over SPME is that the small pore size ensures microfiltration, thus yielding very clean extracts. When the SPME is used in complex matrices, a sample-pre-treatment step (such as filtration), high salt concentration and pH adjustment are applied to the sample solution can damage the SPME fiber. This is not the case for LPME, where the ionic strength of the sample solution and high or low pH values do not influence the repeatability of the method or the condition of the hollow fibre.

4. Recent development of in high-throughput quantitative bio analysis by liquid chromatography (LC) coupled with mass spectrometry (MS or MS/MS)

In the MS method, in contrast, analytes are detected directly from the molecular characteristics as molecular mass and molecular

disintegration patterns. Thus, MS techniques are very attractive for quantification of biomarkers or xenobiotics in the context of diagnostic procedures, since those techniques can enable analyses of much higher specificity compared to standard technologies such as photometry or ligand binding tests. Introduction of API techniques about 20 years ago made practically all potential bio-medical analytes amenable for MS. Furthermore, powerful new technologies of ion-analyses (tandem MS, TOF-MS, ion-trap MS) substantially increased the capabilities of MS analyzers with respect to specificity and to the extent of data read-out in the 1990s. These developments suggested a widespread use of MS methods in routine laboratory medicine. In particular tandem MS instruments hyphenated to LC systems used for sample introduction and prefractionation have been implemented in a constanly growing number of clinical laboratories worldwide now (37). LC-MS/MS is attractive for laboratory medicine for three main reasons. (a) The development of new methods is in general straightforward and independent from the diagnostic industry, without the need e.g. to develop analytical antibodies. (b) The highly multiplexed analyses are feasible with very low current costs; the range of potential analytes is practically unlimited; individual“meta bolomic analyses” addressing hundreds of analytes from different biochemical pathways and from different chemical classes are possible, as well as a comprehensive and individual description of xenobiotics (“xenobiom”). (c) When applying the principle of isotope dilution internal standardization, analyses on a reference method-level of accuracy can be performed in a routine laboratory setting (38). LC-MS/MS today holds enormous potentials for improvements in the pharmaceutical fields and laboratory medicine mainly including TDM, endocrinology, toxicology and meta bolomic analyses, therefore, hyphenated techniques are examples of new tools that adopted for developing fast and cost-effective analytical methods. One of the most prevalent hyphenated techniques, LC-MS/MS, has led to major breakthroughs in the field of quantitative bio analysis since the 1990s due to its inherent specificity, sensitivity, and speed. It is now generally accepted as the preferred technology for quantitating small molecule drugs, metabolites, and other xenobiotic bio molecules in biological matrices as like plasma, blood, serum, urine, and tissue (39). Because samples from biological matrices are usually not directly suitable with LC-MS/MS analysis, sample preparation is inevitable and has traditionally been done using protein precipitation (PPT), liquid-liquid extraction (LLE), or solid-phase extraction (SPE) processes. Manual operations associated with sample preparation are very laborious and time consuming. Parallel sample processing in 96-well format using robotic liquid handlers and direct injection of plasma using an on-line extraction method have significantly shortened the time for analysis and generated a lot of interests in recent years. A major advantage of on-line SPE over off-line extraction techniques is that the sample preparation step is included into the chromatographic separation and thus eliminates most of the sample preparation time classically performed at the bench-side. Fast gradients and short columns were first utilized in early applications of high-throughput LC-MS/MS assays to reduce run times. Better understanding of how matrix effects can compromise the integrity of bio analytical methods has reemphasized the need for adequate chromatographic separation of analytes from endogenous biological components in quantitative bio analysis using LCMS/ MS method (40). New developments from chromatographic techniques such as ultra-performance LC with sub-2 mm particles and

monolithic chromatography are showing promise in delivering higher speed, better resolution and sensitivity for high-throughput analysis while minimizing matrix effects. On the other hand, automation in LC-MS/MS is great advance in instrumentation field and has to address a number of different processes in laboratory medicine application of LC-MS/MS that includes management of primary samples, assay specific work-up of samples prior to actual MS analysis, integrated control of the subunits of the LC-MS/MS systems, processing of primary read-outs, and further handling of result data.

5. CONCLUSION

Until now, together with advancement including automation in the LC-MS/MS instrumentations along with parallel sample processing, column switching, and usage of more efficient supports for SPE, they drive the trend towards less sample clean-up times and total run times–high-throughput methodology-in today's quantitative bioanalysis area. Analysis with DBS, DMS have unique features that can markedly reduce blood sample volumes and simplify sampling logistics for both preclinical and clinical studies. However, current approaches are not optimal for processing the large numbers of samples generated in pharmacokinetic studies, and improvements in efficiency are required to fully exploit the benefits of DBS. An improvement required is the automation of manual sample-handling steps so that extracted analytes can be analyzed by standard analytical methods such as LC–MS/MS. These methods have the advantage of being familiar to scientists and widely accepted by regulatory authorities. On-line SPE utilizing column-switching techniques is rapidly gaining acceptance in bioanalytical applications to reduce both time and labor required producing bioanalytical results.

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