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Review Paper

An Overview of Sample Preparation Methods for Bioanalytical Analysis: Introduction, Recent Development and Application

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ABSTRACT

The selection of sample preparation technique is one of the experimental parameters that affect the quality of the analytical results acquired for extensive and prolonged liquid chromatography mass spectroscopy based bioanalytical investigations. Because every biological matrix is different and complex, sample preparation is regarded as the bottleneck phase in bioanalysis. Protein precipitation, liquid-liquid extraction, solid phase extraction and microextraction techniques are among the sample preparation procedures frequently used in bioanalysis that are examined in this review article. The methods tenets, benefits and drawbacks are examined, as well as how well it works with various analyte and matrix types. Furthermore, new developments in sample preparation are emphasized, including cloud point extraction, automation etc. In order to help researchers, choose the best approach for particular analytical requirements, this article aims to give a thorough overview of sample preparation approaches in bioanalysis.

INTRODUCTION

Bioanalysis is a subdiscipline of analytical chemistry for the determination of xenobiotics (chemically synthesized or naturally extracted drug candidates and genetically produced biological molecules and/or their metabolites or post-translationally modified products) and biotics (macromolecules such as proteins and DNA, small-molecule endogenous metabolites) in biological systems.[1] Many techniques have been

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employed in bioanalysis, including liquid chromatography with ultraviolet/ fluorescence detection (LC-UV/FL), liquid chromatography–mass spectrometry (LC-MS), and gas chromatography–mass spectrometry (GC-MS).[2] Among these techniques, Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become the primary technique used for the quantitation of drugs and metabolites in various biological specimens in support of toxicokinetic (TK) and pharmacokinetic (PK) studies.[3] The development of bioanalytical sample preparation techniques has become challenging over the decades because of the need to constantly accomplish higher sensitivity, accuracy, and speed of analysis in complex biofluids (e.g., blood, serum, plasma, saliva, faces, and urine).[4] Matrix effects are system specific and depend on matrix type, detector, analyte properties and limit of detection requirements. Various strategies can be used to clean up the matrix and isolate and/or concentrate the target an analyte. Inappropriate sample preparation techniques will result in residual matrix build up in the analytical system, which will affect all analysed samples. The matrix effect on bioanalysis techniques and the solutions for its reducing are continuously reviewed.[5] The first and foremost important aspect of sample preparation in LC-MS bioanalysis is to understand and utilize the relevant physicochemical properties of the analyte(s) of interest. These include but are not limited to hydrophilicity, lipophilicity, and protolytic properties (logP, logD, and pKa, etc.). Understanding these properties can help select suitable sample preparation techniques and the associated experimental conditions for the analyte(s) of interest. Therefore, continuous improvement of novel sample preparation techniques is necessary to accelerate bioanalytical research.[6] This short review presents the main difficulties encountered during sample preparation for analysis of small molecules from biofluids by LC-MS/MS and summarizes several critical factors that particular attention should be paid to, followed by an overview of the latest developments in sample preparation techniques to overcome common difficulties with complex matrices.

2. Sample preparation techniques

The purpose of sample preparation is to remove interfering substances (including proteins, salts and lipids) and also enrich the analytes.[7] here are a few common techniques:

- Protein Precipitation (PP)
- Solid Phase Extraction (SPE)
- Liquid-Liquid Extraction (LLE)
- Filtration
- Phospholipid Depletion Techniques in LC-MS Bioanalysis: Supported Liquid Extraction
- Microextraction Techniques
- Ultrafiltration

2.1 Protein Precipitation (PP)

The simplest sample preparation approach for biofluids is protein removal. Proteins can be denatured using acids or heat, or removed by using ultrarational cut-off membranes. Another possibility is to use organic solvents for protein precipitation (PPT). PPT removes a part of the phospholipid content present in serum and plasma samples, depending on the organic solvent used. Studies have shown that methanol extracts contain 40% more phospholipids compared to acetonitrile, and are also less clean than tetrahydrofuran or ethanol extracts. Automated and semi-automated precipitation methods have been used by a number of studies.[8, 9]

2.2 Solid Phase Extraction (SPE)

SPE was developed and applied for bioanalysis after LLE. The removal of both phospholipids and proteins was the key advantage of SPE which attracted more bioanalysts.[10] Solid-phase extraction has been used for more than 50 years and is beneficial for purifying and concentrating trace elements in biological samples. SPE is a

simple method that uses a cartridge with a volume of 50–200 mg to isolate desired analytes from a sample.[11] The mechanism of SPE is similar to that of liquid chromatography (Figure 1), which is based on the affinity or interaction between solutes (analyte of interest) dissolved in a liquid (mobile phase) and sorbent materials (stationary phase). Due to the difference in physicochemical properties, different components in the liquid sample have different affinities or interactions with the sorbents in the stationary phase of the SPE device.[12]

A typical protocol for an SPE method using C8 or C18 sorbent (25–50 mg) is as follows:

- Condition each well with methanol (1.0 ml);
- Equilibrate with water or ammonium acetate buffer (0.1 M, pH 6, 1.0 ml); n Load plasma sample (100 µl diluted 1:1 [v/v] with 0.1M ammonium acetate buffer, pH 6);
- Wash with water or 0.1 M ammonium acetate buffer (pH 6): methanol (95:5, v/v, 1.0 ml);
- Elute with MeOH/1.0 M ammonium acetate (99.5/0.5, v/v, 500 µl);
- Evaporate and reconstitute in the LC mobile phase (100–200 µl)

2.2.1 Common SPE Platforms in LC-MS Bioanalysis

According to respective retention mechanisms, SPE commonly used in LC-MS bioanalysis can be classified into three categories, i.e., reversed-phase SPE, ion- exchange SPE, and mixed-mode SPE. Normal-phase SPE is designed to extract analyte(s) from organic extracts, very nonpolar solvents, and fatty oils, etc.

A) Reversed-phase (RP)-SPE

The first type is reversed-phase (RP)-SPE, which involves a polar or moderately polar sample matrix and a nonpolar stationary phase. In RP-SPE analytes of interest are intermediate polar to non-polar. The retention mechanism here is due to hydrophobic interactions between analytes and functional groups (nonpolar– nonpolar interactions, van der Waals and dispersion interaction). Several SPE materials such as the alkyl- or aryl-bonded silica (Si-C4, Si-C8, Si-C18 and Si-Ph, with 60Å pore size and 40µm particle size) and polymer sorbent as polystyrene ENV can be used.

B) Ion-exchange SPE

It utilizes ionic functional groups (strong or weak organic acids and bases bonded to the supporting base) of the sorbents and can be further classified as strong/weak cation/anion ion- exchange SPE

i. Strong cation exchange (SCX) SPE
ii. Weak cation exchange (WCX) SPE
iii. Strong anion exchange (SAX) SPE
iv. Weak anion exchange (WAX) SPE

C) Mixed-mode SPE

Mixed-mode SPE is an extraction approach involving sorbents that exhibit two or more primary interactions for retaining the analyte(s) of interest. Commercially available mixed-mode sorbents can be silica- or polymer-based, and are typically produced by either bonding the sorbents concurrently with two different functional groups (e.g., C2, C8 vs. sulfate) or by blending discrete sorbent chemistries in appropriate ratios to create the combination of retention properties. The most commonly used mixed-mode sorbents have a hydrophobic functional group in combination with an ion-exchange functional group

D) High-throughput SPE

In the last decade, liquid-handling workstations became more widespread and characteristic in modern bioanalysis laboratories. A 96-well SPE setup is used for high-throughput applications in combination with liquid handling workstations for high-throughput bioanalysis. Today, more efficient and faster methods to work with a greater number of samples were applied using a 96-well SPE plate, and there are now many bioanalytical applications using a 96-well SPE plate in the pharmaceutical industry.[13, 14, 15]

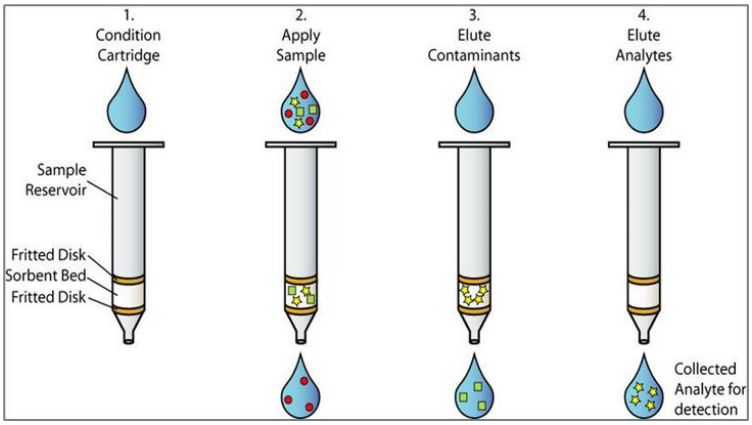

Figure 1: Solid Phase Extraction Cartridge Principle [16]

2.3 Liquid-Liquid Extraction (LLE)

LLE methods were developed later and were studied continuously in order to develop more productive methods. The method involves the extraction of the analyte(s) of interest or unwanted interference components from one liquid phase (e.g., biological samples) to another immiscible liquid phase (e.g., organic solvent), resulting in sample clean-up. In LLE, biological samples (plasma, serum, whole blood, and urine or tissue homogenate) are commonly mixed with additives (buffer, acids, or bases) to ensure efficient extraction of the target molecules. This is followed by addition of IS working solution and an organic solvent (extraction solvent), which is immiscible with water. Then the two-immiscible phase mixture in tubes/ wells is shaken or vortex-mixed for a certain period of time to mix the sample and the organic solvent, during which the target molecules are transferred from the aqueous phase to the organic phase or vice versa. This is followed by centrifugation for phase separation. After centrifugation, the phase containing the target molecules can be collected for further processing and analysis.

The predominant interactions in LLE are the following

i. Hydrophobic interactions
ii. Dispersion interactions
iii. Dipole interactions
iv. Hydrogen bonding interactions

The considerations of an organic solvent for LLE should include but are not limited to polarity (solubility/distribution for the analyte(s) of interest), density, viscosity, and water solubility. Examples of solvents with density less than water are n-hexane, methyl tert-butyl ether, n- butanol, butyl chloride, and ethyl acetate. In contrast, when solvent with density higher than water is used, the aqueous phase floats on top of the organic phase in LLE.[16]

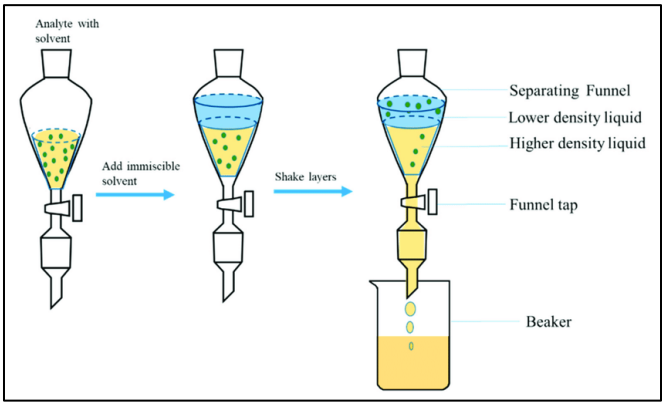

Figure 2. Liquid Liquid Extraction

In general, there are three types of application of LLE for sample preparation in LC-MS bioanalysis, i.e. (i) forward extraction, (ii) backward extraction (or back extraction), and (iii) double extraction (or two-stage forward extraction). The forward extraction and backward extraction may be alternatively staged in a complex LLE procedure for higher assay selectivity or specificity for the analyte(s) of interest in the intended sample matrix. Different from the alternatively staged forward and backward extraction, double extraction is to carry out LLE for the same aqueous sample matrix initially with highly nonpolar solvent (e.g., hexane) and then with moderately nonpolar solvent (e.g., methyl-tert butyl ether or ethyl acetate) for an improved assay selectivity and/or minimized matrix effect. With the recent developments in robotic equipment and extraction plates, most of the LLE procedures can be automated. There are two major alternatives of LLE, i.e., salting-out assisted LLE (SALLE) and supported LE (SLE) that have been commonly employed in LC-MS bioanalysis.[17]

2.4 Filtration

Filtration, either alone or in combination with other methods, is one of the most frequently applied sample preparation methods. Whenever it is used as the pre-treatment method, the selected membranes and possibility of automation can be considered as productivity-increasing parameters. Vacuum devices are needed for proper filtration and combination of filtration with a dilution process can facilitate filtration and enhances productivity (by decreasing the filtration fail). According to the porous size of the utilized filters, different applications of the method have been developed. Ultra-, micro- and nano-filtration are used for concentrate or clean-up of biological samples. Miniaturization and automation of these methods (e.g., in syringe filters, 96-well plate filters) reduced sample depletion during filtration as well as the required sample amount. Automation also decreased the sample-to-sample variation. Furthermore, in vivo sampling techniques were developed using ultrafiltration and microdialysis systems which enabled direct sampling and injection of the target analyte.[18]

2.5 Phospholipid Depletion Techniques in LC-MS Bioanalysis: Supported Liquid Extraction

Supported liquid extraction (SLE), a process that is mechanistically similar to LLE but requires less solvent use, involves using a porous solid material (usually diatomaceous earth) on which aqueous samples will adsorb, facilitating removal of PLs from the final sample during sample extraction. This process has been shown to be superior to PPT in removal of PLs from plasma and others have suggested that SLE performs as well as HybridSPE for removal of GPChos and SMs from plasma. SLE extraction uses an inert, high surface area support to serve as a stationary phase for interaction with aqueous samples. An aqueous sample is loaded onto a SLE cartridge or well and allowed time to adsorb to the surface of the solid support. Then, an immiscible organic solvent is passed over the inert material, eluting analytes of interest and leaving behind unwanted matrix like salts and polar interferences.

SLE cartridges differ from other cartridge-based extractions, like solid phase extraction (SPE), because the original sample is loaded onto the extraction bed and is not intended to flow through.[19, 20]

2.6 Microextraction techniques

With the efforts of researchers, non-exhaustive microextraction techniques have developed appreciably in terms of sensitivity and accuracy from complex biological matrices. Microextraction techniques are based on the principle of using low volumes of solvents.[21] Microextraction techniques are robust, versatile, solvent-free, and cost-effective. Automation of several microextraction techniques is appropriate

for regular laboratory analyses. The primary microextraction techniques developed over the past decade are as follows:

2.6.1 Air-assisted liquid-liquid microextraction (AALLME)

In 2012, AALLME was initially developed for the study of phthalates with high extraction recovery and low solvent consumption. Here, a non-polar organic solvent at a mL-concentration was dispersed in the sample solution. This technique has ample recognition among researchers because of its ease of handling, economical nature, and convenience in most bioanalytical laboratories. AALLME has been used to determine six fluoroquinolone compounds in milk powder and eggs, triazole pesticides (e.g., penconazole) in edible oils, warfarin in biological samples, therapeutic lectin, and multiclass pesticide residues in vegetable and fruit juice samples.

2.6.2 Solid-Phase Microextraction

SPME was introduced in 1989 by Arthur and Pawliszyn in an attempt to reduce the operation time and reagent consumption associated with the traditional SPE methods. SPME is currently roughly divided into two types, static in-vessel microextraction (sample stir microextraction) and dynamic in-flow microextraction the mostly commonly used formats of SPME are fibers (fiber SPME) and capillary tubes (in-tube SPME) coated with an appropriate stationary phase. SPME was first introduced in rod fiber geometry (fiber SPME) and it was invented to extract organic compounds in combination with gas chromatography (GC) analysis. Indeed, the most widely used technique of SPME is fiber SPME, in which a fiber-coated polymeric stationary phase is used as an extraction device, and the absorption or adsorption of analytes occurs on the outer surface of the fiber.

The following improvements in SPME compared with SPE have led to the higher productivity of SPME methods:

• Reducing solvent consumption (it could be regarded as a solvent-free method);
• Reducing sorbent and other reagents consumption;
• Reducing time of extraction;
• Reducing sample amount

2.6.3 Conventional Fiber SPME

The fiber SPME is a sample preparation technique in which fused-silica fibers coated on the surface of an appropriate stationary phase are employed. More specifically, two types of fiber SPME techniques have been used to extract analytes: headspace (HS) for the analysis of highly volatile compounds and direct immersion (DI) for the analysis of compounds with lower volatility.[22, 23]

2.7 Ultrafiltration

UF separates free from protein-bound drugs according to molecular weight and size using low-adsorptive hydrophilic membranes with a specified MWCO value and a centrifugal force. Compared to ED, UF is much more suitable for high-throughput analysis.[24]

The advantages and disadvantages are discussed in Table 1.

Table 1. Advantages and disadvantages of the UF technique

Advantages	Disadvantages
Highly accurate. High-throughput potential (96-well format). Relatively fast and easily implemented. Can be used for unstable compounds. Inexpensive and easy to perform.	Uses external forces (pressure). Susceptible to experimental artifacts, e.g., protein leakage. Temperature and pH cannot be easily controlled. Nonspecific binding affects the unbound fraction. Possible drug insolubility in ultrafiltrate.

3. Innovative Sample Preparation Techniques

During recent years, substantial efforts have been directed towards miniaturization of sample preparation to reduce sample preparation time, reduce the usage of hazardous organic solvents,

facilitate automation and online coupling, and to obtain selectivity and enrichment from small sample volumes.[25]

3.1 Solid-phase nano-extraction (SPNE)

SPNE is another approach that depends on the sound affinity of analytes to adsorbents consisting of nanoparticles (NPs). SPNE retains the merits of SPME and shows novel advantages, such as a better adsorption area, several active sites for recognition of the analyte(s), modificative solid NP surfaces, and controllable surface states. Due to several applications, SPNE is set as a green extraction practice. The SPNE technique has been used to determine polychlorinated biphenyls, carbaryl, and mercury ions in environmental water and polycyclic aromatic hydrocarbons in drinking water. In addition, magnetic NPs have been recommended primarily for blood, serum, plasma, milk, tissue extracts, and urine sample preparations.[26]

3.2 Cloud point extraction (CPE)

In 1976, Miura et al. established a CPE technique. CPE is based on surfactant solutions that become cloudy and separate into two isotropic phases. CPE has several merits over SPE, LLE, and SPME. CPE does not require the utilization of organic solvents; hence, the samples needed for the analysis were lower in amounts. The surfactants used in CPE are harmless and cheap, and have been utilized for the estimation of metals and several chemical moieties (e.g., drugs, vitamins, and pesticides) in diverse biological matrices using green surfactants. CPE is a method of choice for saliva analysis in bioanalytics. CPE has advantages such as simplicity, low cost, higher extraction kinetics, and eco-friendly profile over other sample preparation techniques.[27]

3.3 Dried plasma spot (DPS)

Similar to DBS, DPS is a new emerging technique for the early diagnosis of neurodegenerative disorders. The DPS is a unique two filter-paper-based remote blood collection tool. It offers numerous benefits compared to conventional plasma collection methods. Dried spot collection on filter paper is easy, has no requirement for refrigeration, and can be transported with the least biohazard risk. These benefits offer significant flexibility to DPSs with respect to the classical methods of sample preparation. DPS has been used to determine fosfomycin, ritonavir, trimethoprim, and sulfamethoxazole in biological matrices.[28]

3.4 Disposable-pipette extraction

Disposable-pipette extraction (DPX) was introduced by Brewer in 2003 as a technique for sample preparation, and is based on conventional SPE, allowing online coupling with GC or LC. This extraction tip is sold by Gerstel. The solid-phase sorbent is loosely packed into a disposable-pipette tip, which is fritted at both the top and bottom of the pipette. After drawing the sample into the pipette tip, the analyte is adsorbed and concentrated on the sorbent by mixing the sorbent suspension with the sample by air bubbling. Dynamic mixing uses less sorbent and provides faster extractions compared with classical SPE. The analyte is eluted into a vial with an organic solvent after washing the sorbent if needed. Reversed-phase (RP) and cation-exchanged materials are commercially available. The sorbent particles in the DPX tips are small (10–20 µm in diameter), providing high surface areas. The adsorption mechanism of DPX-RP involves hydrophobic (similar to RPLC) and π–π interactions with the styrene–divinylbenzene sorbent. The combinations of these mechanisms provide a high potential for selective enrichment of most pesticides and removal of polar matrix interferences.[29]

3.5 electro membrane extraction (EME)

The recent societal requirements to explore more environmentaly friendly solutions in the field of sample preparation have gained increasing focus during recent years. A reduction in the consumption of hazardous organic solvent owing

to environmental and cost perspectives, small amounts of sample available and time reduction, have been major incentives for scientists to miniaturize existing sample preparation methods. Some of these challenges were addressed by the introduction of electromembrane extraction (EME), a totally new extraction principle where a potential difference is applied across a thin organic membrane immobilized in the pores in the wall of a porous polypropylene membrane.[30]

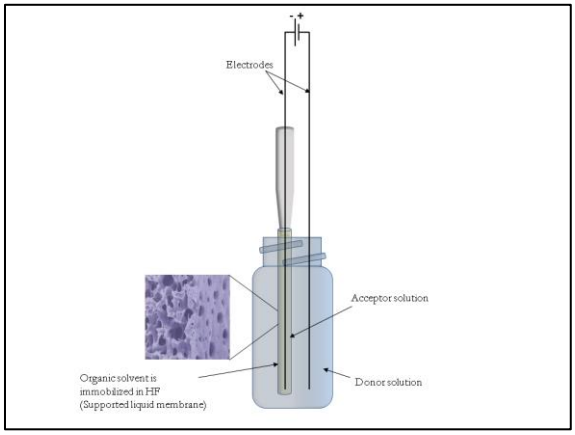

Figure 3. Electro membrane extraction (EME)

4. CONCLUSION

Increasing productivity is one of the main aims of developing novel sample pre-treatment methods. Attempts at reducing the cost of the developed method by reducing the size and automation of the processes along with the development of alternative materials and direct sampling methods that eliminate the need for sample manipulation are among the most popular strategies for increasing productivity. Previously, no systematic methodology was used to determine the productivity increasing aspects of the developed methods. Summarizing the reviewed results in this communication indicate that exact ways to improve productivity cannot be determined. It seems that developing reliable metrics for measuring reliability of developed methods is essential, and international organizations i.e., ICH, OECD need to be more concerned with this issue.

5. Future perspective

The trend in preclinical bioanalysis is towards an increasing number of samples, smaller sample volumes and, thus, lower concentrations of the analytes of interest. This requires high throughput and fully automated sample preparation methods and more sensitive and robust analytical instruments. The coupling of high-throughput sample preparation techniques with multiplexed LC–MS/MS will lead to even more selective and faster analysis.

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