Current development in bioanalytical sample preparation technique in pharmaceuticals

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Abstract: **Biological samples represent analysis challenges due to the presence of interfering constituents within the sample matrix. contaminants in these complex samples may clog sampling and extraction devices, non-specifically interact with analyte, coextract with target compounds and/ or foul instrumentation through adsorption. Adding to the difficulty, the sample matrix often contains only trace levels of analyte that require a form of preconcentrating or purification in order to deliver a sufficient quantity of target compound to the analytical instruments. Because of the low preconcentration factors and extensive use of toxic organic solvents, there has been a growing need to displace them with alternative approaches. The bioanalytical sample preparation is step in the bioanalytical process typically consist of an extraction procedure that results in the isolation and enrichment of components (analyte / metabolite) of interest from a biological matrix (e.g., plasma, urine, skin, saliva etc).**

These feature article highlights a selection of sample preparation techniques and important recent advances that have significant impact in the life sciences and pharmaceutical industry.

Keywords: **Bioanalysis, sample preparation, Cloud point extraction, dried blood spot, air-assisted liquid–liquid microextraction, air-assisted dispersive liquid– liquid microextraction**

INTRODUCTION

Many scientific endeavors are dependent upon accurate quantification of drugs and endogenous substances in biological samples; the focus of bioanalysis in the pharmaceutical industry is to provide a quantitative measure of the active drug and/or its metabolite(s) for the purpose of pharmacokinetic, toxicokinetic, bioequivalence and exposure–response (pharmacokinetics/pharmacodynamics studies). Bioanalysis also applies to drugs used for illicit purposes, forensic investigations, anti-doping testing in sports, and environmental concerns.

Bioanalysis was traditionally thought of in terms of measuring small molecule drugs. However, the past twenty years has seen an increase in Biopharmaceutical (e.g., protein and peptides), which have been developed to address many of the same diseases as small molecules. These larger biomolecules have presented their own unique challenges to quantification.

History**:** The first studies measuring drugs in biological fluids were carried out to determine possible overdosing as part of the new science of forensic medicine/toxicology.

Initially, nonspecific assays were applied to measuring drugs in biological fluids. These were unable to discriminate between the drug and its metabolites; for example, aspirin (c. 1900) and sulphonamide (developed in the 1930s) were quantified by the use of colorimetric assays. Antibiotic were quantified by their ability to inhibit bacterial growth. The 1930s also saw the rise of pharmacokinetics, and as such the desire for more specific assays Modern drugs are more potent, which has required more sensitive bioanalytical assays to accurately and reliably determine these drugs at lower concentrations. This has driven improvements in technology and analytical methods [1]

Analysis of drug/metabolites/biomarkers (qualitative/ quantitative) in biological matrices such as plasma, serum, whole blood, urine, saliva, tissues, etc., is commonly termed ‘bioanalysis’. It is an imperative part of overall drug development process starting with in vitro/in situ testing, pre-clinical studies through to clinical studies. In today’s high-throughput drug discovery industry, bioanalytical laboratories usually operate under pressure to meet the demands and reduce development times. As the results of the bio analysis directly affect the clinical decision-making process, bioanalytical processes are part of regulatory filings. Thus, to improve and regulate these findings, regulatory agencies worldwide have issued guidelines and procedures to ensure the quality of bio analytical data. [2]

The development of bio analytical 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 bio fluids (e.g., blood, serum, plasma, saliva, feces, and urine). In addition, because of the minute concentration of analytes, samples are often required to be preconcentrated prior to analysis. However, this often increases the levels of interfering components, such as small molecules (e.g., drugs, salts, and metabolites) or large molecules (e.g., nucleic acids, proteins, and peptides). Consequently, highly specific sample cleanup actions are necessary for accurate and selective bioanalysis for regulatory purposes. Subsequently, these studies support regulatory filings such as investigational new drug application, new drug application, and abbreviated new drug application. Therefore, bioanalytical sample preparation techniques need to be thoroughly validated before they can be employed in actual sample analysis. In most biological samples, carbohydrates, proteins, lipids, salts, and other endogenous components are present in large amounts. They can hamper the preferred trace analytes via matrix effects, where their elimination is the primary purpose of sample preparation prior to analysis. In the present article, we review the current publications associated with sample preparation techniques in bioanalytic. [3]

Sample preparation: Sample preparation is a technique used to clean up a sample before analysis and/or to concentrate a sample to improve its detection. When samples are biological fl uids such as plasma, serum or urine, this technique is described as bioanalytical sample preparation. The aim of sample preparation is to clean up the sample before analysis. Material in biological samples that can affect with analysis, the chromatographic column or the detector includes endogenous macromolecules, proteins, salts, small molecules, and metabolic by products. The sample preparation the determination of drug concentrations in biological fl uids yields the data used to understand the time course of drug action, or PK, in animals and man and is an essential component of the drug discovery and development process. Most bioanalytical assays have a sample preparation step to remove the proteins from the sample. Protein precipitation, liquid–liquid extraction and solid phase extraction (SPE) are routinely used. [4] [5]

Bioanalytical Techniques**:** [6]

Some techniques commonly used in bioanalytical studies include:

Hyphenated techniques

LC–MS (liquid chromatography–mass spectrometry)

GC–MS (gas chromatography–mass spectrometry)

LC–DAD (liquid chromatography–diode array detection)

CE–MS (capillary electrophoresis–mass spectrometry)

Chromatographic methods

HPLC (high performance liquid chromatography)

GC (gas chromatography)

UPLC (ultra-performance liquid chromatography)

Supercritical fluid chromatography

Electrophoresis

Ligand binding assays

Dual polarization interferometry

ELISA (Enzyme-linked immunosorbent assay)

MIA (magnetic immunoassay)

RIA (radioimmunoassay)

Mass spectrometry

Nuclear magnetic resonance

advantages: *[7]*

Specificity, Improve Reproducibility, Improve Recovery, Improve Instrument Life, Improve Detection limit, reduce backpressure and LC system fouling, Improve Sensitivity

materials and methods

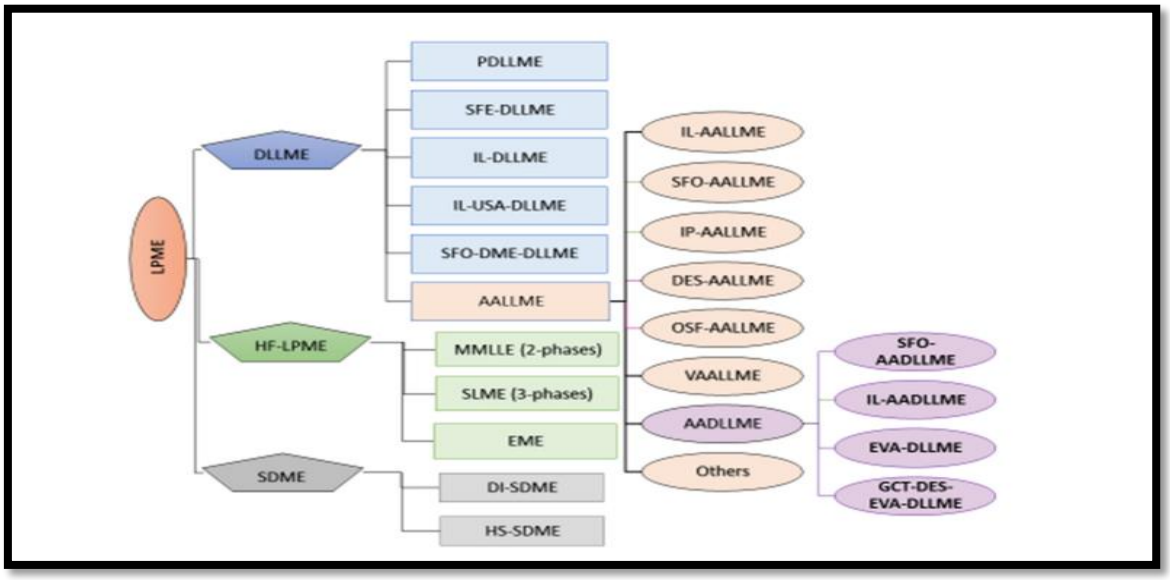

Figure 1: Classification of LPME and AALLME modes

Dispersive Liquid–Liquid Microextraction (DLLME)

DLLME was introduced in 2006. DLLME is a rapid, economical, and easy approach that has been used to determine a variety of chemicals in a variety of samples. However, methods have been employed to improve the classic DLLME. Because of its low solvent volumes and high effective parameters, it has been developed to remove organic molecules and metals from the matrix. Chloroform and dichloromethane are the most widely known extraction solvents in traditional DLLME, both of which are highly volatile and dangerous.

Recent developments include ultrasonic assisted with DLLME, vortex assisted with DLLME, microwave assisted with DLLME, and air-assisted liquid–liquid microextraction (AALLME). In this situation, the purpose was to diminish or remove DLLME's dispersive liquids. Vortexing or sonication improves the mass transfer rate of the analytes into the extractant by increasing the surface contact between them and the organic layer. The fundamental LPME and AALLME models are shown graphically in Figure

1. In 2012, air was used as a component to improve the microextraction procedure. This is a low-cost, environmentally friendly option that can considerably improve the extraction efficiency. The AALLME is simpler, greener, economical, and quicker to use when compared to traditional or modified DLLME approaches. This study aims to provide a recent review of the use of AALLME for the detection of organic and medicinal materials in various matrices such as biological fluids, food, and water. The reviewed analytical characteristics are highlighted, and the tables with current applications of conventional and modified AALLME modes are supplied. AALLME sample preparation foundations, practical limitations, and future developments are briefly discussed. The literature search was conducted using the terms "Air-Assisted liquid–liquid Micro-Extraction," "AAMLLE," and "Organic and Medical Compounds" as descriptors in the Scopus, Springer, and Science Direct indexes, and the analysis was limited to studies published in the last 10 years (from 2012). According to our information, it is the first overview of the modified AALLME modes. This involves the extraction of medicines and organic compounds from different samples.[8]

the general AALLME basics

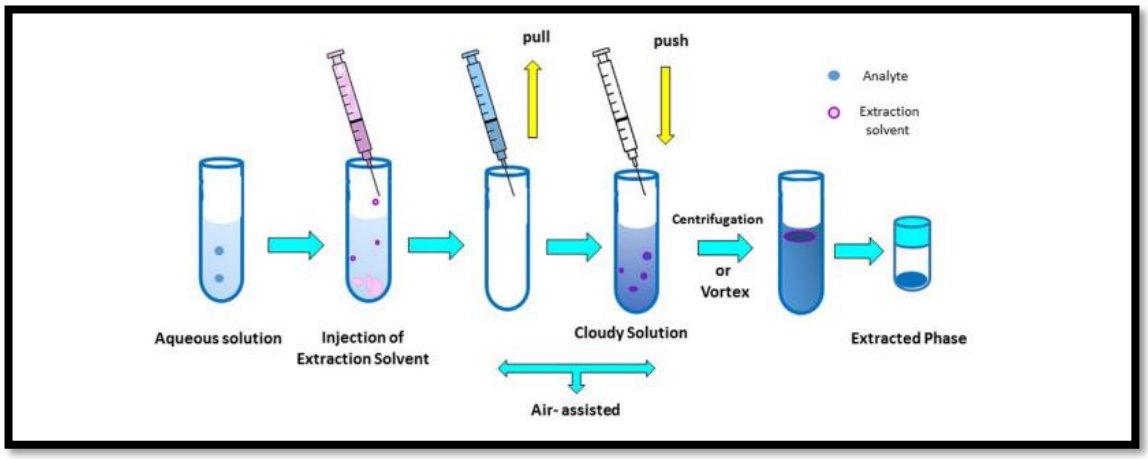

Figure 2: Basic AALLME schematic design

The general procedure of AALLME was explained in Figure 2.

In 2012, AALLME was initially used to analyze phthalate esters in aqueous solutions . This approach was analogous to DLLME in that it did not require the use of an organic layer to disperse an extracted liquid into the test solution. Using a syringe fitted with a needle, an extract phase at 1 mL concentration was dispersed into the test solution by conducting sucking and dispersing cycles repeatedly. Despite the absence of a dispersing solvent, this technique considerably enhanced the contact area of the extractant with the test solution. The viscosity and interfacial tension of the extractant were shown to be the two most important factors in LPME procedures. These factors regulate both the extractant droplet size and the mass transfer rate of the analytes at the same time. Aspirating dispersion cycles transform the extraction solvent into very small droplets, greatly increasing the contact area of the test solution with the extractant. The AALLME method works in a similar way to batch extraction. In two different hydrodynamic situations, the many-batched extraction procedure is applied. The extractant and the aqueous layer are not actively agitated in the first place, and the contact between the two phases is flattened, with diffusion determining the solutes' mobility ratio. In this case, the equilibrium condition is achieved after almost 2–3 h. Every second, the extractant and test solution are agitated for a predetermined amount of time, and diffusion-controlled mass transfer is supplanted by convective mass transfer. As a result, there is a large effective area. During the aspirating and dispersing cycles in AALLME, there is a lot of turbulence in the solutions, and the mass transfer of the solutes is mostly regulated by the convective process (Figure 2).

The rate law for convective mass transfer can be explained using equation (1):

$$N_A = K_C \Delta C_{A,} \qquad (1)$$

where NA denotes an analytes molar mass flux, KC is the convective mass transfer constants, and ΔCA is the variation in concentrations between the aqueous and organic phases.

The physicochemical characteristics of the organic solvent and aqueous solution are intricately connected to KC. The significant factors of KC, as determined by dimensional analysis and experiment, are viscosity, density, momentum, and the circular radius of the extractant. It is worth noting that KC and circular radius are inversely connected, with a large KC resulting from small spherical radii. Because AALLME creates very small droplets of extractant in the solution, it is only normal that KC is larger than in other approaches. The number of steps in which the combination of extractant and test solution is sucked into an injector and pumped out into the glass vial should be tuned to increase the mass transfer rate of the solutes and the procedure's recovery (R).

Recovery (R)

$R = C_{of} V_{of} / C_{aq} V_{aq}$,

where Cof is the final concentration of analytes in the organic solvent, and it was measured from an appropriate calibration graph achieved by directly injecting the standards into an experimental platform. Caqf and Caqi are the final and initial analytes' quantities in the water, respectively; Vo and Vaq are the volumes of two phases. [9]

recent modified AALLME applications for extraction of medicines and organic compounds [10]

The AALLME advanced microextraction method was used to determine analytes from aqueous and extractant phases by centrifuging them away from the standard solution. The separation solvent had to meet certain conditions, such as being low in solubility in water, being capable of extracting the substances, and creating a specific density that was different from the standard solution. Many kinds of solvents (ionic liquids [ILs], organic solvent free, ion-pair liquids, surfactants, chloroform, deep eutectic

solvents [DESs], and solidified floating organic solvents [SFO]) have been used as extractants in AALLME (Figure 3). DES has been the most used with the AALLME technique, followed by chloroform, 1-dodecanol, n-octanol, and chloromethylene (ChCl). Recently, the AALLME procedure has been used with different extractants due to its more sensitive modes. And the combination with other procedures produced 11 new modes, as follows:

1. The traditional air-assisted liquid–liquid microextraction (AALLME)
2. The air-assisted dispersive liquid–liquid microextraction (AADLLME) procedure
3. The air-assisted liquid–liquid microextraction coupled with solidification of floating organic droplets (AALLMESFO) procedure
4. Air-assisted liquid–liquid microextraction coupled with deep eutectic solvent (AA-LLME-DES) procedure
5. Vortex-assisted-air liquid–liquid microextraction procedure (VAALLME)
6. Ionic liquid-based air-assisted liquid–liquid microextraction (IL-AALLME) procedure
7. Ion-pair air-assisted liquid–liquid microextraction (IP-AALLME) procedure
8. One-step air-assisted liquid–liquid microextraction (OS-AALLME) procedure
9. Organic solvent-free air-assisted liquid–liquid microextraction (OS-FAALLME) procedure
10. Salt- and air-assisted liquid–liquid microextraction (SAALLME) procedure
11. Tandem air-agitated liquid–liquid microextraction (TAALLME)
12. Ultrasound air-assisted liquid–liquid microextraction (US-AALLME)

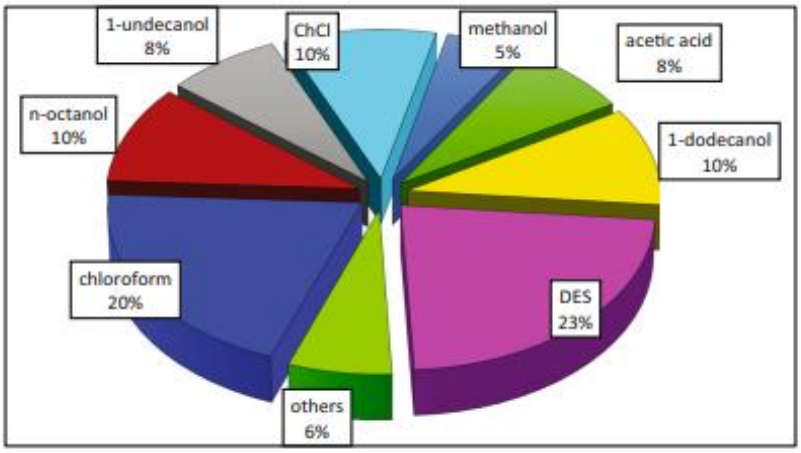

Figure 3: The most used extractants in the AALLME procedure.

This review shows the traditional AALLME has been used the most, followed by AALLME-SFO, AALLMEDES, and AADLLME. The different modes of the AALLME and their percentages are depicted in Figure 4.

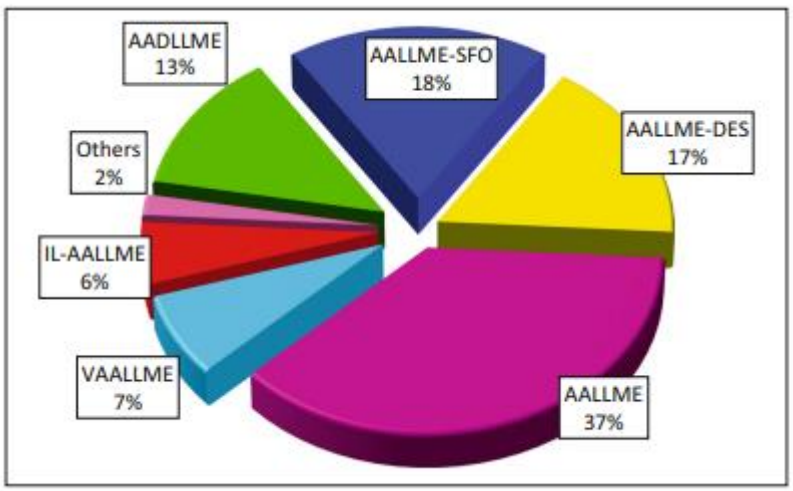

Figure 4: The various modes of the air-assisted liquid–liquid microextraction procedure.

Air-Assisted Dispersed Liquid–Liquid Microextraction (AADLLME)

In the DLLME technique, a different concept for dispersing tiny extractant drops throughout the test solution has recently been established. Air bubbles can assist you in doing this. The extract is withdrawn into the syringe with a little air and pumped out into the tubes for predefined cycles in the AA-LLME process, resulting in a turbid mixture with the extract scattered as tiny drops in the aqueous medium. Air was used as a component to improve the microextraction procedure. This is a low-cost, environmentally friendly option that can considerably improve the extraction efficiency. To create a turbid solution, air-assisted dispersion was employed instead of the dispersive liquid in a typical DLLME. The removal of the dispersive solvent can aid in the transfer of analytes into the extraction solvent's tiny droplets. Economic benefits, easiness, speed, high recoveries, and nontoxicity are all

features of using the AA-DLLME approach in the analysis of medications and organic compounds in real samples. By periodically extracting the mixture containing the aqueous sample and a few microliters of solvent, a stable dispersion is created injecting the solvent into the syringe and then forcing it down the tube. As a result, the extraction solvent may become dispersed without the use of a dispersant liquid. For the first time, an organic solvent-free AALLME approach was used to extract ortho-phthalic, meta-phthalic, and para-phthalic acids from edible oil. A basic solution and the oil test combination are repeatedly aspirated and injected into a funnel-bottom centrifuge tube to create the turbid solution. After centrifugation, the sediment layer is directly identified by HPLC-DAD. GCT–DES–EVA–DLLME is a novel EVA–DLLME form that has been created by using nitrogen gas. Bifenthrin, phenothrin, tetramethrin, cyhalothrin, permethrin, and cypermethrin were measured in fruit juices using this approach. The suggestion was easy, dependable, and effective. LODs were obtained at 9–21 ng L–1.[11]

Air-Assisted Liquid–Liquid Microextraction Coupled With Solidification Of Floating Organic Droplets (AALLME-SFO)

A tiny volume of organic phase is moved into the aqueous phase in most AALLME, and the mixture is then repetitively withdrawn into a needle and injected into a tube. Drug molecules transfer into the organic phase via the bolus flow created during the water-soluble sample withdrawal and ejection system. New technologies are overcoming the extraction solvent challenges. This method uses an organic phase with a lower density and a melting temperature of between 10 and 30°C. The extractants can be solidified after separation by exposing them to low temperatures. This allows them to be removed as droplets of floating solvent by the centrifugation method. The approach has the benefit of combining the dispersive and extraction procedures into a single glass syringe. As a result, no dispersive liquids are used, which cuts down on extraction time. Furthermore, after solidification, the organic layer may be easily removed from the aqueous solution. The procedure's suitability as a beneficial alternative for the evaluation of actual food and environmental samples is further supported by its great results in actual analysis. Researchers developed a technique that uses the solidification of float organic droplets (SFO-AALLME) to extract benzoylurea pesticides from water sources and honey samples. [P14,6,6,6] PF6 and 1-dodecanol were used as extraction solvents in this approach. Because the employed ILs had a lower density than water, solidification was performed to remove the foggy state. Another article that has been extracted is clozapine from biofluids by using surfactants in the SFO-AALLME technique. To speed up the mass transfer rate of the clozapine into the extractant, sodium dodecyl sulfate was added as a surfactant. [12]

Air-Assisted Liquid–Liquid Microextraction Using Deep Eutectic Solvent As Extractants (AALLME-DES)

DESs are described as a fluid eutectic mixture containing two or more chemicals having a low melting temperature. The DESs are currently receiving a lot of interest as a possible replacement for traditional organic solvents. The DESs are made up of a H-bond acceptor (HBA) and a H-bond donor (HBD) that is suited for the environment. HBA is typically made up of quaternary ammonium salt and choline chloride (ChCl). There are major benefits, including volatility, excellent thermal stability, and conductivity. DESs are also inexpensive, readily available, and harmless. The DES synthetic procedure is also quite simple. More critically, because of the safe and environmentally friendly components, some of which can even be made with food-grade substances, DESs are deemed to be naturally nontoxic. Because of the wide range of HBA and HBD available, the physicochemical features of DESs can be tailored to meet the needs of a wide variety of industries. As a result, for the analysis of rare ginsenosides in KA injection, the AALLM-DSE process was created. The goal of this research is to extract ginsenosides from KA injections and quantify their levels. Magnetic nanoparticles were also used to swiftly and efficiently extract the DES droplets from the solution. The water-soluble extraction solvents in this study were DESs (ChCl and phenol). The polarity of the aqueous phase was adjusted with the emulsified solvent (tetrahydrofuran). A small amount of N2 was added to produce a rather thorough emulsification step. In other studies, the phthalates esters, amitriptyline and imipramine, and valproic acid and 3- heptanone have all been determined using AALLME paired with GC. Due to the use of DES, this approach did not require the use of a centrifuge and was also environmentally benign.[13]

Ionic Liquid Used In Air-Assisted Liquid–Liquid Microextraction (ILAALLME)

ILs are distinguished by their low vapor pressure, high thermal stability, ability to dissolve different chemical species, and low aqueous solubility. They are classified as environmentally benign and relatively safe solvents. Another significant benefit of ILs is the ability to mix and match cations and anions to get the necessary physicochemical properties. As a result, ILs are frequently referred to as modeling solvents. The IL-based AALLME approach has received a lot of interest because of these benefits. Also, ILs can act as both an extraction solvent and a surfactant during the separation process. They are reducing the interfacial tension between two immiscible liquids through adsorption at the liquid–liquid interface. The isolation of phthalic acid, iso-phthalic acid, and terephthalic acid in aqueous systems was very sensitive. This has been advanced by utilizing ion-pair air-assisted liquid–liquid microextraction (IP-AALLME) with a low-density extractant coupled with an HPLC-DAD detector. In this procedure, an aqueous test solution is mixed with tri-butyl amine (as an ion-pair agent) and toluene (as an extractant). By aspirating and spreading the mixture with a syringe needle, tiny organic-phase droplets are generated. After that, the generated ion-pairs are isolated into toluene, centrifuged, and the collected layer is transferred into a microtube and evaporated to dryness at 25°C below the nitrogen stream.[14]

Coupled Air-Assisted Liquid–Liquid Microextraction Procedure With Other Methods

The evaluation of B-naphthol, naphthalene, and anthracene in the wastewater sample was then done using a vortex assisted-air liquid–liquid microextraction (VAALLME) technique in a limited bore tube. The extraction liquid in this process was a lighter organic solvent than water. There was no need for centrifugation because the air bubbles increased the extraction solvent collection. Other studies were reported in Table 1. The illegal azo-based dyes and their major metabolites were extracted using the ultrasonic-enhanced air-assisted liquid–liquid microextraction (US-AALLME) process. The procedure was conducted without the use of organic solvents, and ILs were considered suitable extractants. The micro determination of naproxen, diclofenac, and ibuprofen medications from plasma and urine was reported using a combination of back extraction and air assisted liquid–liquid microextraction (AALLME-BE) techniques. Chloroform was used as an extractant. The analytes were separated into a hydrophobic layer and then back-extracted into an alkaline medium. A tandem TAALLME was utilized to extract diclofenac, ibuprofen, and mefenamic acid (NSAIDs), and a response surface approach was employed to optimize the factors. A second application of AALLME was employed to extract drugs by using 1,2-dichloroethane (37 µL) as extractant and coupled with back-extraction in

pH = 10.01 in 2 min. A novel supramolecular solvent (SUPRAS)-based AALLME for the separation and extraction of Morin in fruit and beverage tests that is simple, fast, and environmentally friendly. The use of an alkanol-based nanostructure of supramolecular (500 µL 1-dodecanol/THF) increases the effectiveness of the microextraction method. Additionally, the analytical time is significantly reduced. A new on-site sample preparation procedure for forest water tests has recently been introduced. A synthetic DES (1:2 of DL-menthol to citric acid) with excellent stability and extraction efficiency was produced and employed as an extractant. At first, AALLME was used to create an array device. Without the use of electricity, up to six tests were conducted on-site in 20 min. [15]

Hollow Fiber Liquid–Liquid Microextraction (HF-LPME)

Pedersen-Bjergaard and Rasmussen advanced HF-LPME to address SDME's drop instability. The analytes are isolated first into an assisting liquid membrane maintained in the holes of a hydrophobic fibrous HF and then into an acceptor solvent placed within the fiber canal. The extractor liquid is covered in microliters within the lumen of a fibrous HF under this method, so it is not in direct contact with an aqueous solution. The feature of this technique is that the sample can be vigorously stirred without losing the analyte because it is manually protected. Prior to analysis, the HF is drenched in a suitable immiscible liquid, causing the organic layer to become adsorbed in the HF pores. A small layer of appropriate solvent, approximately less than 20 µL, forms within the HF's wall. The HF is then loaded into a sample tube containing the desired aqueous solution. The test is stirred extensively to speed up the extraction. The analytes are subsequently extracted from the water system by passing them through the organic layer in the HF's pores and into an acceptor liquid within its lumen. The HF's single-use design removes the possibility of sample carry (15%). It was effectively implemented in the real vegetable samples. Long extraction times (0.5–1.5 h) are a drawback of HF-LPME, particularly for super molecules and biomolecules. [16]

Principles and Possible Modes Of The HF-LPME [17]

In HF-based LPME methods, the extraction phase is placed in the lumen of a porous hollow fiber. In such a way, the acceptor phase is protected by the SLM. To perform HF-LPME, a polypropylene hollow fiber membrane may first be sonicated in acetone to remove polymer impurities. Then the hollow fiber membrane is soaked in an impregnating solvent to fill the pores of the HF membrane by capillary forces, and excess solvent may be removed by washing with distilled water. After this step, the lumen of the hollow fiber is filled with the acceptor phase. As is thoroughly discussed below, the acceptor phase can be the same organic solvent as the SLM (the two-phase mode) or can be an aqueous solution (the three-phase mode). Finally, the hollow fiber is immersed in the sample solution and the analytes are extracted from the sample, through the SLM and into the acceptor phase. Four different arrangements for HF-LPME have been reported, i.e., rod like, u-shaped, hollow-fiber solvent bar, and knotted hollow-fiber. Considering the physicochemical characteristics of the desired analytes and levels of complexity of the sample, HF-LPME can be performed in a two- or three-phase mode.

Two-Phase HF-LPME

In two-phase HF-LPME, the pores in the wall of the hollow fiber and the lumen are filled with an organic solvent, which is immiscible with the aqueous sample solution. Solvents like 1-octanol and dihexyl ether are commonly used. Two-phase HF-LPME is applied for extraction and preconcentration of analytes with low polarity such as polycyclic aromatic hydrocarbons (PAHs). Since the analytes are extracted into an organic solvent, two-phase LPME is compatible with gas chromatography. Fig. 5 illustrates the principle of two-phase HF-LPME. According to this the SLM and the organic acceptor phase are the same in two-phase HF-LPME.

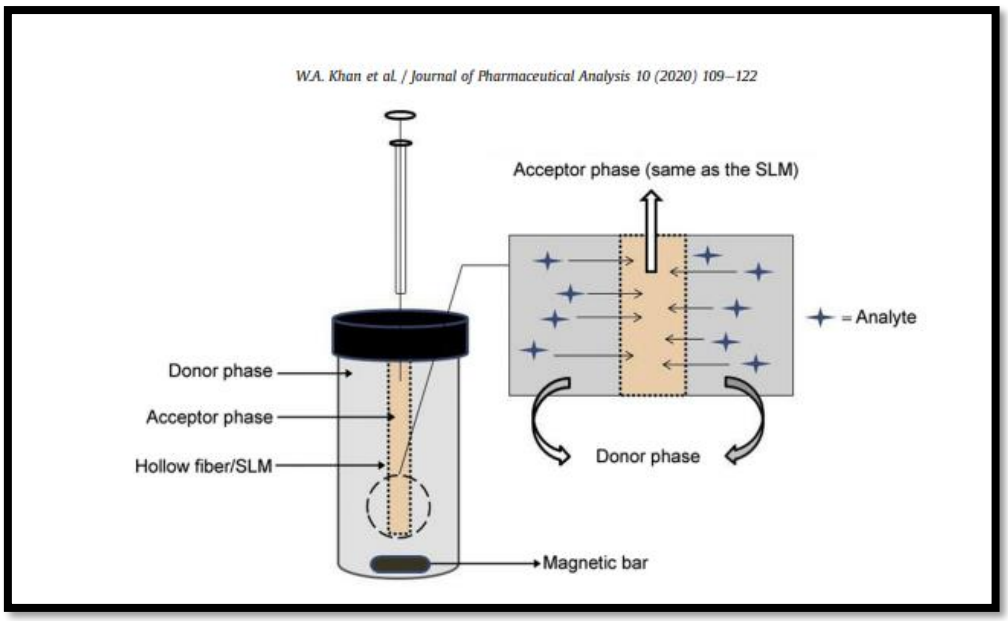

Fig. 5. A schematic mechanism of the two-phase HF-LPME

Three-phase HF-LPME

In three-phase LPME, the pores in the wall of the hollow fiber are filled with an organic solvent immiscible with water, while the lumen is filled with aqueous solution (acceptor solution). 1-Octanol and Dihexylether are common solvents. The mechanism of extraction is based on pH adjustment of the sample solution and the aqueous acceptor phase. For instance, extraction of acidic

compounds is achieved by acidification of the sample to suppress ionization of the target analytes. This results in successful transfer of analytes towards the acceptor phase through the organic SLM. pH of the aqueous acceptor solution should be adjusted to a pH value 2e3 units above pKa of the analyte. Carrier mediated three-phase HF-LPME was introduced where a hydrophobic carrier was dissolved in the organic SLM before the impregnation of the HF pores. The applied carrier is an organic compound capable of ion-pairing with analytes of interest. Hence, at the contact region between the sample solution and the SLM, the desired ion-pair complexes are formed, leading to successful extraction of target analytes. At the contact region between the SLM and the aqueous acceptor phase, the analyte is exchanged with a suitable inorganic counter ion dissolved in the acceptor solution, and is released into the acceptor solution. As mentioned, three-phase HF-LPME can be applied for ionizable compounds (acids and bases). Two-phase HF-LPME offers high PFs and extraction efficiencies for non-polar analytes. However, because the SLM and the acceptor phase are the same organic solvent, there is no phase boundary. Therefore, cleanup is limited. Interestingly, Ghambarian et al. introduced a variant where dodecane was used as the organic SLM, while organic solvents like methanol, ethanol, or acetonitrile, which are all immiscible with ndodecane, were used as acceptor phase. This strategy provided improved sample clean-up, while extraction efficiency was not sacrificed. Fig. 6 illustrates the principles of three-phase HF-LPME. HF-LPME can be carried out through static, dynamic, and even fully automated strategies. One of the most interesting approaches for dynamic HF-LPME was introduced by Esrafili et al. called TTextraction. Between two T connectors, a stainless-steel tube was mounted, housing the hollow fiber membrane. The sample solution was pumped into the TT-extractor through the two T connectors, while the acceptor phase was injected into the hollow fiber membrane via a syringe pump. This configuration was capable of carrying out two-phase HF-LPME. Fig. 7 illustrates this innovative approach with all the details. In another strategy offered by the same research group, a fully automated HF-LPME with capability for two- and three-phase HF-LPME procedures was developed. In this setup, a syringe pump was used for washing, filling, and ejection of different solvents. Four containers were used for washing solvent, SLM, extraction solvent, and elution solvent. The extraction system was successfully connected with liquid chromatography. Fig. 8 shows the graphical design of this HF-LPME instrument.

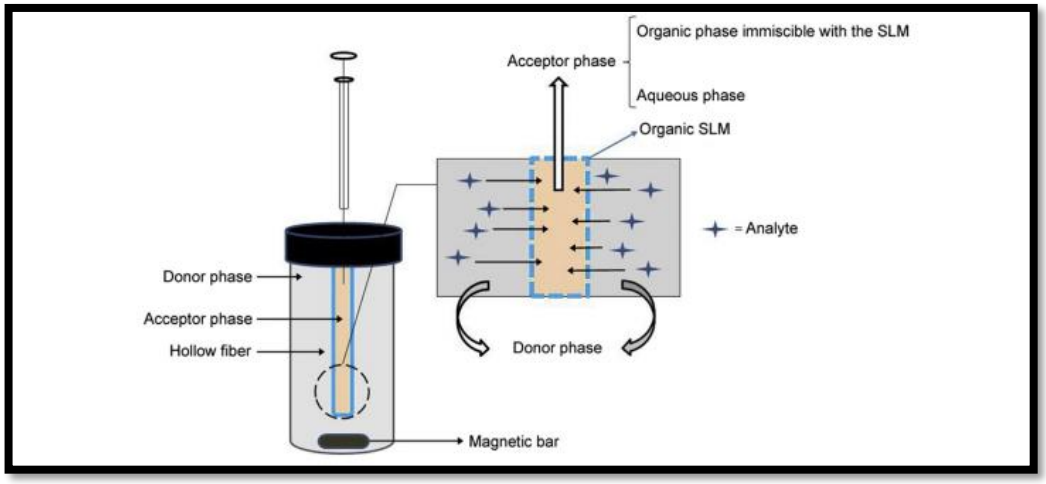

Fig. 6. Schematic mechanisms of two possible modes of three-phase HF-LPME

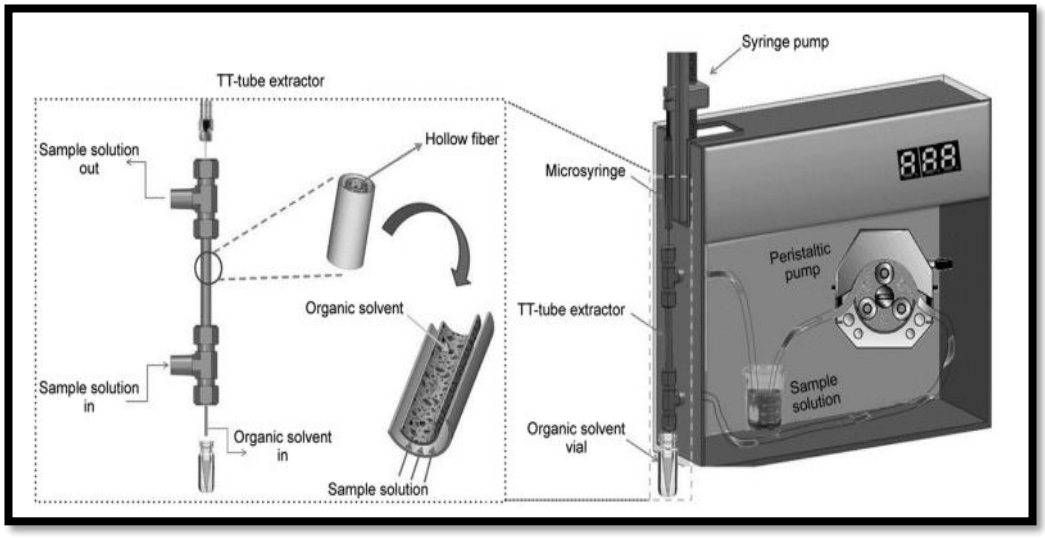

Fig. 7. Schematic configuration of the dynamic HF-LPME

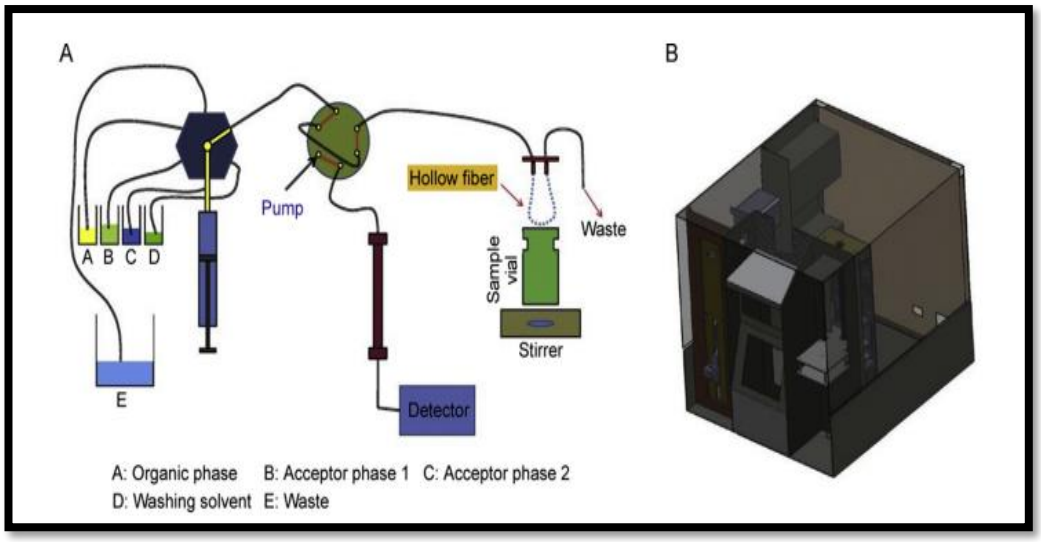

Fig. 8. (A) Illustrative configuration and (B) schematic structure of the automated HF-LPME

Electromembrane Extraction [18]

In HF-LPME, mass transfer is by passive diffusion and this results in relatively slow extraction kinetics. Alternatively, ionizable compounds can be extracted efficiently and with outstanding sample cleanup by electromembrane extraction (EME). The principle of EME is similar to that of HF-LPME. However, in EME the analytes are extracted selectively in their charged form under the influence of an electrical field. Thus, mass transfer is by electrokinetic migration across the SLM, and 2-nitrophenyl octyl ether (NPOE) is often used as SLM. The setup for EME is very similar to three-phase HF-LPME, but in EME two electrodes (usually platinum wires) are utilized. One electrode is placed in the acceptor phase into the hollow fiber lumen, while the second electrode is inserted into the sample solution. Fig. 6 shows the principle of EME. For extraction of basic analytes, the cathode (-) is placed inside the acceptor phase, while for acidic analytes the direction of the electrical field is reversed. After extraction, the acceptor phase is collected with a micro-syringe and injected into an analytical instrument for quantitative analysis. Due to the electrical field across the SLM, bubble formation, Joule heating, and even punctuation of SLM may occur in EME systems operated under nonoptimal conditions. These phenomena occur in longer extraction times as a result of excessive migration of ions across SLM. Also, when the electrical field is applied, electrical double layers and local pH gradients are formed in the interfaces between the SLM and the aqueous solutions on both side. These may impact the mass transfer. To reduce the impact of these phenomena, application of constant direct electrical current and application of pulsed voltage have been reported. Although the utilization of a stabilized and constant direct current improved the efficiency and stability of EME, this approach is still not common in EME publications. Pulsed electromembrane extraction (PEME) was introduced as a strategy to enhance the efficiency of EME using a simple and inexpensive extraction setup. Pulsed voltage was found to improve extraction efficiency and system stability.

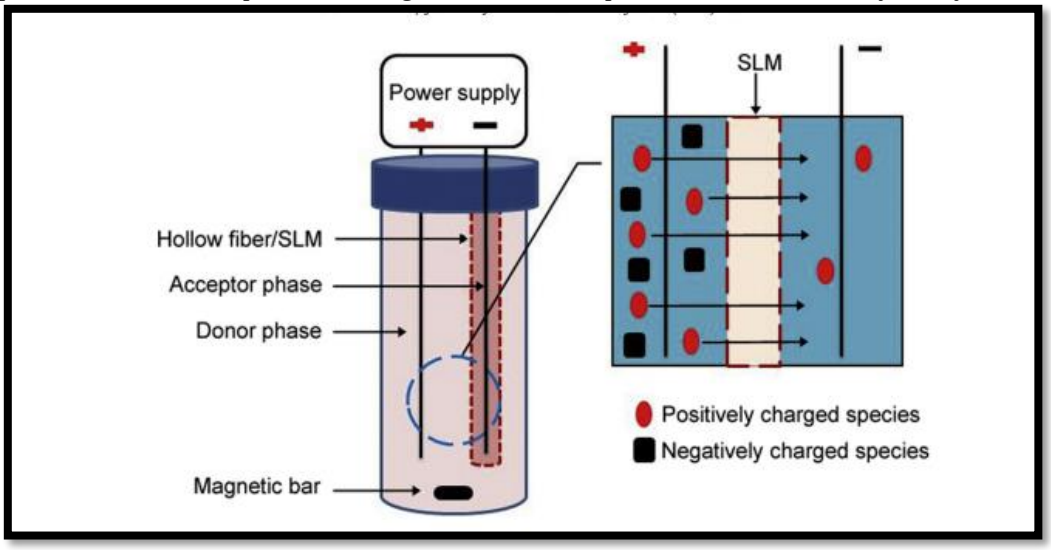

Fig. 9. A schematic mechanism for electromembrane extraction

Dried Blood Spot (DBS)

This simple method was originally developed for large-scale clinical screening of phenylketonuria in neonates and has also been used for the detection of sickle cell disorders and human immunodeficiency virus infection. A fingerpicks using a lancet releases a few drops of blood that is absorbed onto a sample collection card for drying and storage, prior to analysis. Samples are most suitable for the bioanalysis of small molecules using liquid chromatography/mass spectrometry (LC/MS) platforms, including an internal control to account for sample prep variation. One problem with DBS is that hematocrit (HCT; volume fraction of red blood cells)

can affect the results of quantitative tests. The DBS method has been used in the determination of levels of therapeutic antibodies in blood samples by different LBA methods (1,2) including the microfluidic flow-through Gyro lab® technology.[19]

Volumetric Absorptive Micro Sampling (VAMS)

A Mitra® device with a VAMS® tip sampler absorbs a fixed volume of blood (~10 µL) in a few seconds with low volume variation and reduces the hematocrit effects seen with DBS (4). VAMS enables easy, minimally invasive sampling of small volumes, can be done at home, and samples can be stored and transported at room temperature. Mitra devices with VAMS have been used in for example, sampling for COVID-19 tests and are suitable for preparing samples to be analyzed by LBAs.[20] **Capillary Micro Sampling (CMS)[21]**

This sampling method involves taking up an exact microliter-scale volume of blood or other biological fluid in a capillary tube and diluting with a washout liquid. Plasma can be prepared by centrifuging the tube. CMS samples are suitable for analysis by LBAs and is the micro sampling technique that has been used most widely in animal studies to address the 3Rs (replacement, reduction, and refinement) initiative for ethical animal research.

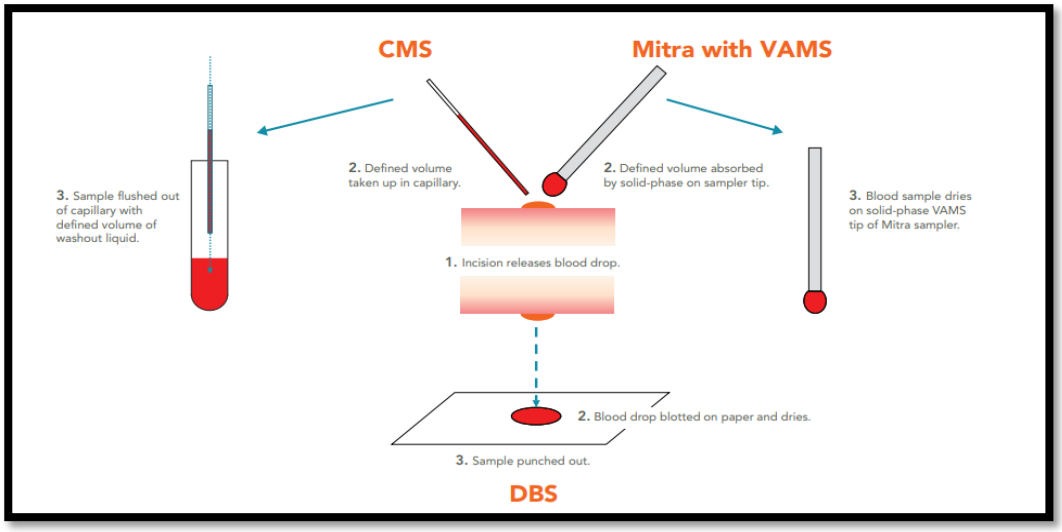

Figure 10. The principles of DBS, CMS and VAMS.

The samples from all methods must be further processed (e.g., extraction and/or purification) before analysis. CMS, Capillary Microsampling; VAMS, Volumetric Absorptive Microsampling; DBS, Dried Blood Spot.

Dried Urine Testing[22]

Dried urine is a form of collection where patients saturate a filter card with a urine sample. Orice dry, urine cards are extremely stable for shipment and storage, and this method eliminates the need for jure collection.

Easiest Method for Urine Testing

MyDiagnostic's dried urine method offers a discreet, at home testing alternative and eliminates the hassles of all-day jug urine collection Patients collect urine on a filter strip up to four times during the day Dried strips are shelf stable for 30 days and easy to mail back to the lab for analysis. Unlike other labs, patients do not need to stop hormone supplementation to use MyDiagnostic's testing because we have ranges adjusted for age, menstrual status and supplementation types. This is the ideal method for tracking the effectiveness of hormone treatments.

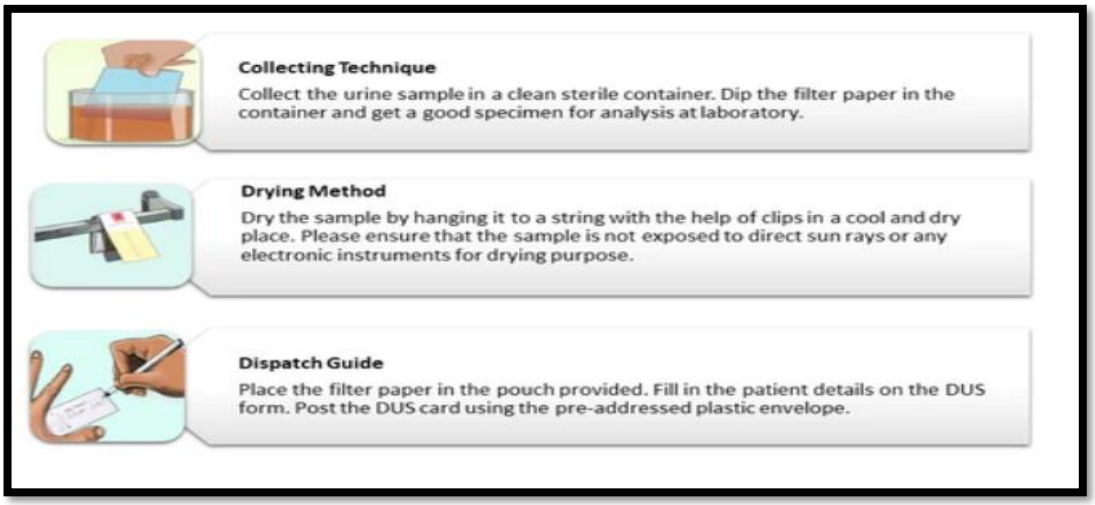

Fig 11 Steps to carry out Dried Urine Spot Testing

Dried Saliva Spot (DSS) technique

DSS is a well-known sampling technique for collecting saliva samples by spotting salivary 85 specimens on filter paper. Saliva sample collection is inherently noninvasive and painless; 86 furthermore, collection of saliva samples does not require specialized training, can be 87 performed at home, has practically no risk of infection, is readily accepted by patients, and has 88 a considerable advantage in terms of sample collection from special groups, including children 89 or persons with disabilities or those with anxiety disorders [23]

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. Recently, DPS was shown to be suitable for the determination of amikacin, lithium, abiraterone, D(4)-abiraterone, lamotrigine, ceftolozane, fluoroquinolones, gabapentin, and caffeine in biological matrices. DPS has proven to be a prominent technique when applied to PK studies, where plasma sample preparation is rapid and requires negligible plasma volumes.[24]

cloud point extraction [25]

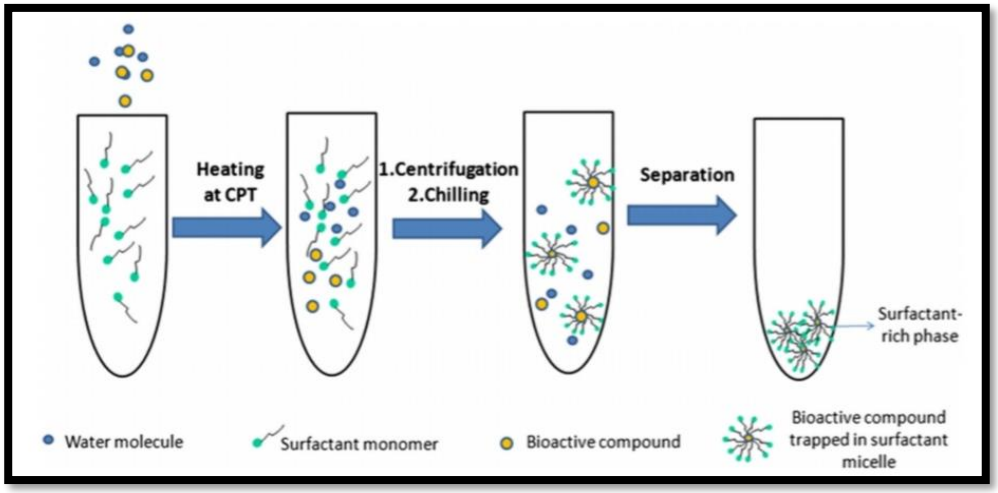

Fig 12 Mechanistic overview of cloud point extraction

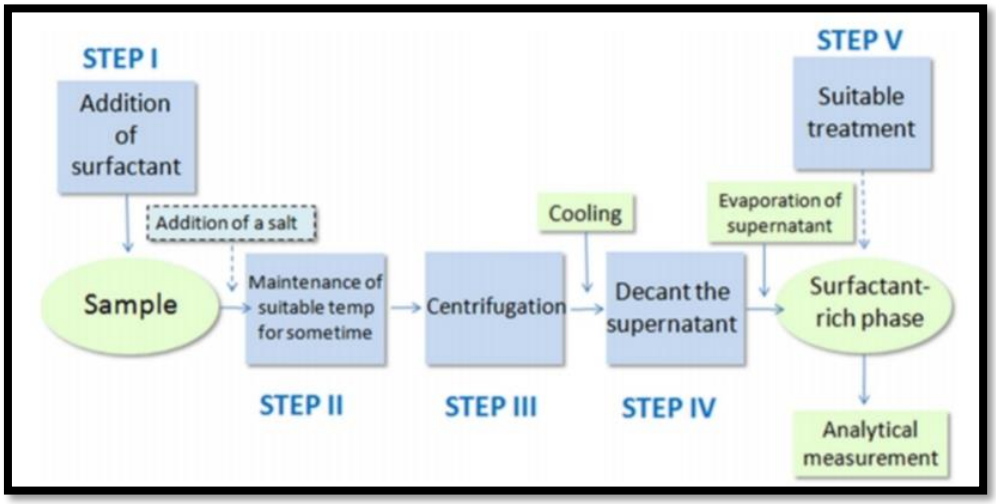

Fig 13 Key steps in cloud point extraction

Initially, in CPE extraction, a micellar (surfactant-rich) phase is added to the sample which is originated from homogeneous surfactant solution. A non-polar core is developed due to its hydrocarbon tails towards the centre by a micelle. Then the separation of bioactive compounds occurs in the hydrophobic core of micelles. During heating, cloud is generated due to nonionic surfactants. These clouds then form two coexisting isotropic phases. At a specific temperature, also called as clouding point temperature; surfactant-rich and a surfactant-lean phases are formed due to physical change in the homogeneous solutions of amphiphilic substances. Because of the attraction a cluster is formed. The mechanism by which this separation occurs is attributed due to the rapid increase in aggregation number of the surfactant's micelles, as a result of the increase in temperature, or any other critical phenomena (Fig. 12). This effect causes a decrease in the effective area occupied by the polar group on the micelle surface, increasing the size of the micelle that can be considered to become infinite at the cloud point, resulting in phase separation. Since, high temperatures cannot be applied to thermally sensitive compounds like food bioactive vitamins, pigments, polyphenols,

tocopherols, antioxidant compounds etc., cloud point extraction which is usually performed at mild or low temperature and do not use harmful and toxic chemicals is most preferred technique. Therefore, this extraction technique recently has been widely used

Key steps in CPE

The key steps in cloud point extraction are depicted in Fig. 13. It is very difficult to remove aqueous surfactant phase from micellar phase which contained isolated analytes. However, the separation is followed by cooling in which viscosity of the micelles increases and then supernatant decanted. Complete removal of traces of water can be achieved by evaporation under a stream of neutral gas (for example, nitrogen).

CONCLUSIONS

Bioanalysis is a key component of the discovery and development of pharmaceuticals. To combat the rising cost of drug development and increased sensitivity and specificity, new sample preparation and analyte detection techniques are being adopted. Moreover, many researchers are making the modification and improvement of classical techniques globally. Currently, laboratory automation is a key feature of easy, rapid, and eco-friendly methods. These newer techniques render miniaturization and rapid automatic high-throughput analysis possible. It is projected that these techniques for sample preparation will become mainstream in the near future. The current manuscript reveals dozens of promising bioanalytical sample preparation techniques, including LPME, AALLME, DLLME, CPE. The key features of several sample preparation techniques are summarized. A few sample preparation techniques (e.g., LPME, and DLLME) are easy, rapid, and economical but difficult to automate. The DBS technique is well known and adopted for patients undergoing clinical trials, which require preparation of a large amount of sample from participating volunteers. Newer sample preparation techniques, such as capillary microsample preparation, VAMS, , DSS, DUS, and dual-CPE have been found to be more precise, popular, and useful than classical techniques. Recently, the rapid increase in the application of ILs, aptamers, NPs, and microfluidic-based devices in bioanalysis has simplified analysis. Various available methodologies for sample preparation, especially when coupled with sophisticated analytical techniques, will greatly assist in the establishment of future drug metabolism and PK, pharmacodynamics, toxicokinetic, and bioequivalence studies of pharmaceutical discovery and development.

ABBREVIATIONS

[C6MIM] [PF6] -1-hexyl-3-methylimidazolium hexafluorophosphate
[P14,6,6,6]- PF6 tri-hexyl (tetradecyl) phosphonium hexafluorophosphate
AA-DLLME-OPS- air-assisted dispersive liquid–liquid microextraction procedure with organic-phase solidification
AA-LDSLLME-SFOD -air-assisted, low-density solvent-based liquid–liquid microextraction and solidified floating organic droplets
AA-LLME-SFDES- air-assisted liquid–liquid microextraction based on solidification of floating deep eutectic solvent
ASEME-SFO -air-assisted surfactant-enhanced emulsification liquid–liquid microextraction/solidification of floating organic droplets
BE- back extraction
ChCl -chloromethylene
DESs- deep eutectic solvents
DLLME- dispersive liquid–liquid microextraction
EMAC- ethyl methyl ammonium chloride/ pivalic acid
EVA-DLLME gas-controlled deep eutectic solvent-based evaporation-assisted dispersive liquid–liquid microextraction
GC-ECD gas chromatography with electron capture detector
GC-FID gas chromatography with flame ionization detection
GC-FPD gas chromatography-flame photometric detection
GC-MS gas chromatography–mass spectrometry
GCT–DES–EVA– DLLME gas-controlled deep eutectic solvent-based evaporation-assisted dispersive liquid–liquid microextraction
HMIMNTF2 1-hexyl-3-methylimidazolium bis (trifluoromethanesulfonyl)imide
HPLC-MS/MS high-performance liquid chromatography–tandem mass spectrometry
IL-AALLME ionic liquid-based air-assisted liquid–liquid microextraction
IP-AALLME ion-pair air-assisted liquid–liquid microextraction
LC-MS/MS- liquid chromatography tandem mass spectrometry
LPME- liquid phase microextraction
OS-AALLME- one-step air-assisted liquid–liquid microextraction
OS-FAALLME- organic solvent-free air-assisted liquid–liquid microextraction
SAALLME -salt- and air-assisted liquid–liquid microextraction
SDME- single-drop microextraction SPME solid phase microextraction
TAALLME- tandem air-agitated liquid–liquid microextraction
TNO -5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-ol
UHPLC-PDA ultra-high-performance liquid chromatographic with PDA detection developed and validated
USE-AALLME- ultrasound-enhanced air-assisted liquid–liquid microextraction
VAALLME- vortex-assisted-air liquid–liquid microextraction
VALLE-AALLME- vortex-assisted liquid–liquid extraction/air-assisted liquid–liquid microextraction
CPE- Cloud Point Extraction

DPS- Dried Plasma Spot
DSS-Dried Saliva Spot
DBS-Dried Blood Spot
DUS-Dried Urine Spot

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