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Review Article

Recent trends in sample preparation strategies employed in efficient Bio-analytical methods -An overview

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Abstract
The most recent developments in sample preparation techniques for bioanalysis are examined in this article, offering a synopsis of the theory, potential applications, and automation, along with a comparative analysis of the advantages and disadvantages of each approach. Nowadays, the conventional methods of protein precipitation (PP), liquid-liquid extraction (LLE), and solid-phase extraction (SPE) are thought to be archaic. In the past ten years, new methods for bioanalysis sample preparation have advanced quickly. There has been discussion of advancements in for all separation procedure. SPE technology provides distinct benefits over bioanalytical methods and a range of extraction phases. Selective sorbents and the general SPE strategy, such as hybrid SPE, molecularly imprinted polymer SPE, and solid-phase micro-extraction, including its various Dispersive solid-phase extraction, pipette extraction, and packed-sorbent micro-extraction methods. Bioanalytical techniques in PPT included the use of PPT filter plates, 96well PPT plates, and hybrid SPE PPT plate techniques. For LLE, the methods used include Liquid-Liquid Micro Extraction (LPME) and Dispersive Liquid-Liquid Microextraction (DLLME). In bioanalytical applications, online SPE employing column-switching techniques is quickly gaining traction. Hollow fiber-based LPME possibly offers a wide range of potential applications in the field of drug analysis, since it can offer high drug enrichment and good sample clean-up. There are several benefits to using PP filter plates and tubes for sample preparation, including the elimination of proteins and phospholipids from plasma and serum.

Keywords: Microextraction, Liquid -Liquid extraction, Solid phase extraction, Bioanalytical method, protein precipitation

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Introduction

The development of analytical techniques for quantitative evaluations in food, environmental issues water, biological samples matrices, and dietary supplements using fewer hazardous solvents and substituting them with not dangerous ones without sacrificing extraction effectiveness is an important goal for modern researchers [1,2]. During the method development and validation process, these factors are highly valued across all domains [3].

Numerous technological advances in analytical methods and instruments have occurred throughout the past ten years. Modern analytical instrumentation has made bioanalytical technique development and validation faster and more affordable, in addition to offering enhanced selectivity and sensitivity[4]. Liquid chromatography in conjunction with mass spectrometry, which imparts speed, sensitivity, and specificity, is regarded as the premier method for quantitative and qualitative bioanalysis among these contemporary analytical techniques. Nevertheless, this highly sensitive and selective analytical method has drawbacks as well, including matrix effect, reduced selectivity, and decreased Sensitivity of the biological matrix's target analyte after processing[5].

A crucial element of the bioanalytical methodology is sample preparation, which is also known as sample treatment, sample extraction, or sample cleanup. In the biological matrix of a clinical setting, the drug, its metabolite, or the relevant biomarker is present[6]. This matrix is complex biochemically and is made up of many different components, comprising minerals, proteins, acids, bases, cells, and both endogenous and exogenous molecules.

Lipids and lipoproteins are organic compounds. Nevertheless, based on the type of matrix (tissue, whole blood, plasma/serum, urine, saliva, etc.), the biochemical complexity of the matrix may change. To put it simply, sample preparation involves extracting the target analyte from the matrix, reducing or removing matrix elements from the sample after processing, and, if required, concentrating the target analyte[7]. The key to a successful sample preparation process is guaranteeing the sample's quality. Controlling sample collection, storage, transportation from the sample collection location, and temperature are crucial because the bioanalytical laboratory is not in charge of maintaining these conditions. These factors guarantee sample integrity, which gives sample preparation accuracy, precision and robustness[8].

Up to 80% of the time needed for bioanalysis is devoted to skillfully preparing the sample Additionally, out of all the bioanalytical technique processes, it is the most labor-intensive and prone to error.

The most common approaches of sample preparation include dilution and shot, solid-phase extraction (SPE), protein precipitation (PP), and liquid-liquid extraction (LLE). nevertheless the search for novel and more effective techniques to remove the analyte from the biomatrix has always focused heavily on sample preparation. Recent advancements in robotics and material science, along with a deeper comprehension of biomatrices, have made it possible to meet the increasing demands for enhanced controls and selectivity[9]. This has led to the development of numerous novel approaches to sample preparation. Numerous of these recently developed methods for preparing samples have been put to the test, approved, and brought to market[10]. This study aims to provide an overview of these methods and inform bioanalytical experts about the range of resources available to them.

Liquid–Liquid Extraction (LLE)

The method widely used in LLE LC-MS bioanalysis for sample preparation. To clean samples, one must transfer them from one immiscible liquid phase (like a biological sample) to another (like an organic solvent) that contains the analyte(s) or interference elements that are not wanted. Additives such as buffers, acids, or bases are commonly used in LLE to ensure that biological samples (such as serum, plasma, blood in its entirety, urine, or tissue homogenate) effectively extract the target molecules. The next step is to combine the IS working solution with the extraction solvent, an organic solvent not soluble in water.

Afterwards, the sample and organic solvent are mixed in tubes or wells that already contain the two immiscible phases, and then left to incubate for a set amount of time. This procedure involves the transfer of target molecules between the organic and water phases, or vice versa. The next step is to use centrifugation to separate the phases. After centrifugation, you can store the phase that contains the target molecules for further processing and analysis[11].

Table 1. Bioanalytical method using Liquid Phase Extraction

Analyte	Matrix	Analytical technique	Column used	Reference
Morachalcone	Rabbit Plasma	HPLC	RP-C_{18}	[12]
Lurasidone	Rat Plasma	liquid chromatography–tandem mass spectrometry	RP-C_{18}	[13]
Pazopanib hydrochloride	Human Plasma	UV- Visible Spectrometry and RP- HPLC	LC C_{18}	[14]
Dexamethasone	human plasma	liquid chromatography coupled with triple quadrupole mass spectrometer		[15]
Zonisamide	Spiked Human Plasma	HPLC		[16]
Flutamide	Rat Plasma	HPLC	Cromasil C18	[17]
Alkaloids, terpenoids, and flavonoids	rat plasma	LC-MS/MS	EC-C_{18}	[18]
Nine mental drugs	Human Plasma	HPLC-MS/MS	Cromasil C18	[19]
Larotrectinib	Human Plasma	UPLC-MS	C18-Phenominex reverse phase	[20]
Oseltamivir	Human Plasma	HPLC-MS/MS	C18-Phenominex	[21]
Donepezil	Human Plasma	Liquid Chromatography–Tandem Mass Spectrometry	Thermo Hypersil Gold C18	[22]

Mechanism of LLE

The expression "like dissolves like" can be used to summarize the LLE mechanism[23]. The ideal solvent for dissolving a solute is one whose polarity is identical to the solute. In comparison to water, nonpolar molecules are more soluble in organic solvents. Ionic or polar substances, on the other hand, dissolve more readily in aqueous solutions compared to solvents made of organic materials. The solute dissolved in the less soluble solvent in a system of two immiscible solvents will diffuse into the more soluble solvent by way of the corresponding liquid-

liquid interphases. Interactions between solvent and analyte molecules occur in LLE whenever analyte molecules move from the water phase to the organic phase, or vice versa. The following are the main interactions in LLE [24].

Hydrophobic interactions: These occur when a polar solvent, typically water, interacts with a nonpolar analyte (or analytes) in a nonpolar organic solvent or solvent mixture. When hydrocarbon moieties interact with related nonpolar molecular components, the result is a nonpolar attractive interaction known as hydrophobic interaction.

Dipole interactions: When two molecules interact, their permanent dipole moment is caused by attractive electrostatic forces, which draw one molecules positive end toward the others negative end. High permanent dipole moments are present in both parts.

Dispersion Interaction: Interactions between a polar (or charged) molecule and a comparatively nonpolar electron-rich molecule are called dispersion interactions. Usually, the interaction is weakly attractive. The nonpolar molecules in the system undergo a change in electron density due to the minor polarization caused by the polar molecules. To rephrase, nonpolar molecules that are rich in electrons are induced to form a dipole moment by their polar counterparts.

H**ydrogen binding interactions:** Interactions between electronegative atoms with a single pair of electrons, like O and N, which are known as hydrogen bonding acceptors, and hydrogen atoms in polar bonds, which are known as hydrogen bonding donors. Another kind of dipole contact is the hydrogen bonding interaction.

General Procedures in LLE

Jian et al. (2010a) states that the LLE of biological specimens consists of three primary steps: (i) extracting by mixing the two incompatible phases; (ii) introducing solvents made from organic materials, additives, and/or IS; and (iii) transferring, evaporating, and reconstituting the two phases by themselves[25]. The last ten years have seen a critical evaluation of liquid-liquid extraction and its various adaptations because of their high toxicity, expensive disposal needs, and role in additional environmental pollution. This examination has resulted in the invention of liquid phase micro extraction (LPME). A variety of methods quickly developed, all aimed at reducing the amount of solvent used during the sample preparation procedure.

Liquid-Liquid Micro Extraction (LPME)

Following the principles of "Green Analytical Chemistry," the major objective of research is to create analytical methods that increase the efficiency of extraction operations while decreasing or eliminating the usage of hazardous solvents[26]. For trace analytes in a variety of matrices, liquid phase micro extraction technologies (LPME) have shown notable improvements over solid phase (micro) extraction. The food, environmental, and biological industries are just a few of the many that LPME serves by processing organic molecules and inorganic trace elements.

The three different LPME procedure modes are headspace LPME (HS-LPME), direct-immersed LPME (DI-LPME), and hollow fibre LPME (HF-LPME). At the tip of a microsyringe needle in HS-LPME, an extraction solvent drop which could be either an organic or water solution is exposed to the headspace of the sample. For the study of volatile chemicals, this approach is perfect. The only notable differences between DI-LPME and other methods are the extraction solvent's direct immersion in a stirred sample solution and its requirement to be immiscible with aqueous solutions. Large molecules and particles cannot interfere with the small fibre pore size, but HF-LPME uses a hollow fibre to stabilise and shield the extraction solvent. This might result in the sample needing to be cleaned more thoroughly during the extraction procedure. The main uses for these LPME approaches were recently discussed by Sharifid coworkers [27].

Dispersive Liquid-Liquid Micro extraction (DLLME)

Some dispersive liquid-liquid extraction (DLLME) variants have special advantages that are compatible with nearly all analytical measurement methods, have a high pre-concentration factor for the target analytes, and are inexpensive. These variations include ionic liquid-based DLLME (IL-DLLME), sugaring-out assisted liquid-liquid extraction (SULLE), deep eutectic solvent-based DLLME (DES-DLLME), and ultrasound-assisted DLLME (UA-DLLME)[28,29]. Among the several solvent microextraction techniques that have been recorded are liquid- phase microextraction (LPME), drop-by-drop microextraction, and differentially-labeled microextraction (DLLME). Compared to HF-LPME, this extraction process provides better analytical performance, according to Xiong and Hu[30]. Target analyte(s) in the organic phase can be measured using a variety of instrument configurations. LPME is typically used to examine aqueous solutions or water samples. Solid sample analysis is often performed in two stages: first, an appropriate pretreatment method is used to turn the solid sample into an aqueous solution, and then LPME is used.

Solid phase Extraction (SPE)

For many years, target trace analyte(s) have been selectively extracted and enriched from a range of biological materials using SPE, an efficient sample preparation technique. Both solid phase extraction (SPE) and liquid chromatography rely on the particular relationship or attraction between sorbent materials, which make up the stationary phase, and solutes, which are dissolved in a liquid and serve as the analyte of interest. Due to their distinct physicochemical characteristics, the various liquid sample components interact with the sorbents in the stationary phase of the SPE device in distinct ways.

To do SPE, a liquid sample is first subjected to the necessary treatments (pH adjustment, dilution, and/or IS addition) before being loaded into a preconditioned or adjusted column, cartridges, or plate containing the required sorbent materials. While washing, the matrix components that are causing interference are either dissolved with the right solvent or sent straight through the loading process, whether it's a column, cartridge, or plate. The next step is to use the correct elution solvent to remove the target analyte(s) from the columns, cartridge, or plate. Various interaction mechanisms involving

the sorbent materials (stationary phase) are employed to retain the analyte(s) of interest. The collected elute is promptly sent for LC-MS analysis or dried to eliminate organic solvent.

Before doing an LC-MS study, reconstitution or beginning the mobile phase with the appropriate solvent comes following. Using SPE can help you prevent or minimise several issues that are associated to PPT and/or LLE. These problems include (i) incomplete phase separations in LLE leading to poor recovery and (ii) matrix effect in PPT. Many different chemicals, absorbents, sizes, and formats of SPE products are available from commercial manufacturers to accommodate a broad range of size requirements and bioanalytical applications.

Table 2. Bioanalytical method using Solid Phase extraction

Analyte	Matrix	Analytical technique	Column used	Reference
Fluoxetine	Human Plasma	LC-MS/MS	C_{18}	[31]
Milnacipran hydrochloride	Rabbit Plasma	HPLC	C_{18}	[32]
Lorcaserin	Human Plasma	HPLC	Phenomenex Luna C18	[33]
Ilaprazole and Glimepiride in Rat Plasma	Rat Plasma	HPLC method for simultaneous estimation	Kinetex C_{18}	[34]
Rivaroxaban	Human Plasma	HPLC	C_{18}	[35]
Pantoprazole	Human Plasma	HPLC	LiChrospher 60 RP	[36]
Glyphosate	human urine	HPLC with tandem mass spectrometry	SCX and NH2	[37]
Olanzapine and Samidorphan	Human Plasma	RP-HPLC	Zorbax SB-C_{18} column	[38]
Tranexamic acid	Human Plasma	UPLC-MS	C_{18}	[39]
Paliperidone	Human Plasma	LC–MS	C_{18}	[40]
Pentoxifylline	Human Plasma	Liquid Chromatography–Tandem Mass Spectrometry	C_{18}	[41]
Nine Hallucinogenic nbome Derivatives	Human Plasma	Liquid Chromatography-Tandem Mass Spectrometry	C_{18}	[42]

SPE Stationary Phases (Sorbents)

Because of their very nature, most SPE materials are totally porous. Sorbent materials with lower porosity have a larger total surface area and, thus, a greater active adsorption capacity per mass.There are two main types of sorbents: those based on silica and those based on polymers. When talking about SPE, one typical approach to describe sorbent capacity is in terms of percentage of chemicals retained per mass of sorbents.

Silica based sorbents:

As the name suggests, silica-based sorbents are created by adding different functional groups to silica; however, certain SPE processes also actively use base silica. A lot of silica-based sorbents that are sold commercially include acronyms in their names. The primary properties of the corresponding functional group on the silica are explained by these acronyms, which include C2, C4, C6, C8, C18, phenyl, cyclohexyl, cyanopropyl, aminopropyl, diethyl amine, diol, propylsulfonic acid, propyl carboxylic acid, and propyltrimethyl amine. The most widely used and hydrophobic SPE columns, cartridges, and plates are those based on C18. Many chemicals are strongly retained by them due to their great hydrophobicity. It might not, however, provide the finest selectivity. Higher choice in SPE can be achieved by using SPE columns, cartridges, or plates with less hydrophobic phases, like the widely used C8 columns.

Steric considerations during functional group binding cause a considerable amount of unreacted silanol species to remain on the surface of silica-based SPE sorbents, regardless of the bonding chemistry employed to create them. These silanol compounds can interact with the molecules of the analyte, which often leads to poor extraction efficiency and unexpected retention effects. There is no text provided. It is widely established that around 50% of the silanol species present on the surface of silica undergo ionisation at a pH of 4. As such, the solution's pH can be changed to guarantee that the silanol species are either fully ionised ($pH \geq 6$) or suppressed ($pH \leq 2$). It should be mentioned that prolonged exposure to extremely high pH can cause silica sorbents to hydrolyze[43] **.**

Polymer-based sorbents: A broad spectrum of polarities and chemistries are covered by the commercially available polymer-based sorbents. The most widely used polymer sorbents are based on copolymers of styrene and divinyl benzene. Ion-exchange polymer sorbents are made possible by further changes such as amination or sulfonation. The sorbents become water-wettable when certain polar functional groups are included, providing more options for retention mechanisms.

A noteworthy advantage of stationary phases based on polymers is that, unlike silica-based SPE columns, cartridges, or plates, they may not require the first conditioning step because their chemical properties remain unchanged even if they dry out during the SPE process.

Common SPE Platforms in LC-MS Bioanalysis

SPE, a commonly used technique in LC-MS bioanalysis, can be classified into three classes based on the retention mechanisms it utilises: mixed-mode, ion-exchange, and reversed-phase SPE. Through the utilisation of normal-phase solid-phase extraction (SPE), it is possible to extract the analyte(s) from fatty oils, organic extracts, and other solvents that lack polarity. The current debate does not address this topic as it is infrequently employed in the extraction of samples from biological matrices, such as plasma and urine.

Reversed-phase SPE: This process makes use of nonpolar stationary phases of sorbents based on polymers or silica, which cling to the majority of hydrophobic compounds. Among the common functional groups present in silica-based nonpolar sorbents are phenyl, cyclohexyl, C1, C2, C4, C6, C8, C18, and cyanopropyl. The long-chain (like C18) and the short-chain (like C1, C2) of these groups are more and less retentive, respectively. Reversed-phase solid-phase extraction (SPE) is considered to have the least selective retention mechanism compared to other types of SPE. Consequently, it is extremely beneficial for isolating analytes with significantly distinct structures from a single sample.

Ion-exchange SPE: This SPE method utilises the ionic functional groups, which are either strong or weak organic acids and bases that are attached to the supporting base, present in the sorbents. It can alternatively be described as a strong or weak cation or anion solid-phase extraction (SPE).

Mixed-mode solid phase extraction (SPE): It's an analyte extraction method that uses sorbents with two or more principal interactions to preserve the target analyte or analytes. The majority of commercially available mixed-mode sorbents are composed of polymers or silica. Sorbents can be manufactured by either bonding them simultaneously with two distinct functional groups (such as C2, C8, and sulphate) or by combining multiple sorbent chemistries in specific proportions to get the desired combination of retention qualities. The predominant mixed-mode sorbents are those that possess both an ion-exchange and a hydrophobic functional group[44.]

SPE Workflows in general

SPE, or low-pressure chromatography, can be performed offline or online using an LC-MS system. The following should be taken into account When creating a sample preparation technique for LC-MS bioanalysis based on SPE: (i) The sorbent type and quantity; (ii) The sample volume (considering the volume needed for the initial, repeated, and incurred reanalysis of the sample); and (iii) The loading, washing, and elution conditions (time, volume, and composition). Comparable LC columns: A variety of SPE products are offered for sale in various configurations.

Solid phase micro-extraction (SPME): It is a novel sample preparation method developed by Arthur and Pawliszyn in 1990, involves coating a fused silica fiber exterior with a suitable stationary phase. According to Augusto and Valente (2002), it is a physically altered syringe with stainless steel microtubing inside the syringe needle. This microtubing features an organic polymer coating on the 1 cm fused silica fiber tip. The syringe's plunger can be used to move this coated silica fiber forward and backward. Sample preparation time, the requirement for organic solvents, and disposal expenses are all decreased by the integration of all stages into a single phase made possible by the design of the SPME syringe assembly. Compared to this, packed-bed cartridge-based conventional SPE is used [45].

For SPME, there are two different extraction methods: in 2004, Colon and Dimandja introduced direct DI-SPME. It involves merely immersing SPME fibre (DI) in a liquid sample matrix. Head-Space (HS) extraction involves positioning the fibre directly above the sample matrix. This is done after heating the sample of liquid matrix in a vial to vaporise the analytes [46-47].

Two essential steps in a typical SPME technique are extraction and desorption for a given analyte. Adjusting a number of parameters is necessary for the analyte extraction onto SPME fibre, including pH, agitation/stirring speed, sample volume, temperature, and length of extraction time (length) in order to achieve the salting out effect. Analyte extraction in both DI and HS-SPME can be affected by the headspace in the sample vial [48- 49]. There are several options for analyte consumption offered by SPME. The process of extracting analytes from the fibre is simple when GC and SPME are linked. When the septum-piercing needle of the SPME device is placed into the GC injector, the fibre is exposed to the heated chamber. This exposure causes the generation of the thermally desorbed analytes[50]. The solvent-free sample preparation method (SPME) is regarded as ecologically beneficial due to the absence of any solvent in the technique[51].

When using the LC method, SPME fiber can be dissolved in an appropriate volume of the method's selected solvent or mobile phase. Thus, The HPLC can receive the analytes that were transferred to the desorbent solvent. By changing the injection port, this procedure can also be automated[52] stirring speed, Temperature and desorption duration are a few examples of factors that are discussed in the extraction process and should be improved as they can impact the desorption efficiency in SPME[53]. In bioanalytical procedures, SPME has been widely employed for the estimation of many analytes in various modes. The literature on bioanalytical techniques and SPME is extensive.

Sorptive Extraction of Stir Bars

A novel sample preparation technique called stir bar sorptive extraction (SBSE) was first presented in 1999 [54] and was founded on a concept that was comparable to SPME. Unlike SPME, which employs fibre, SBSE uses magnetic stir bars to coat the extraction phase (such as PDMS). A stir bar usually consists of a magnetic rod that is covered in glass. A specific thickness of polymer covers this glass jacket. The analytes will separate between the extraction phase and the sample matrix when this stir bar is added to the ready sample[55] . As its name suggests, the basis of SBSE is sorptive extraction, which is an

equilibrium approach by definition[56]. The solute is extracted from the aqueous phase into the extraction phase in SBSE according to its partitioning coefficient between the extraction phase and the aqueous phase.

Similar to SPME, SBSE offers two extraction modes: head space (HS, where a specialized stir bar holding mechanism is available) and direct immersion (DI)[57]. Optimisation is needed for analyte extraction from the matrix to stir bars.

Sample extraction can be done repeatedly with various stir bars or with multiple stir bars working at once. One glass tube can hold all the stir bars in either of these scenarios, and it can be fully desorbed with heat or, in the case of LD, a small amount of solvent.

This method improves the system's sensitivity multiple times [58,59]. Similar to SPME, SBSE is a very affordable method of sample preparation because the stir bars may be easily reused (with the proper cleaning and phase activation) (Pedrouzo et al., 2010)[60].It is clear from the discussion above that the SBSE sample preparation methodology has the potential to become a standard procedure in bioanalytical procedures, is automatable, and offers a number of advantages.

On-Line Solid Phase Extraction (OLSPE)

Combined with enhanced selectivity and sensitivity, tandem mass spectrometry (MS/MS) and HPLC has completely changed the quantitative bioanalysis process. Common techniques for optimising extraction involve assessing the pH and aqueous phase of the sample, determining the phase ratio (b), adjusting the stirring speed, controlling the sample temperature, regulating the extraction period, and evaluating the impact of salting out.[61,62]. In order to optimise the extraction efficiency, these factors can be individually modified or manipulated using a design of experiments approach[63,64]. Thermal desorption (TD) or liquid desorption (LD), also known as back-extraction, can be used to remove the solute from the stir bar. In the case of liquid desorption, a tiny volume of liquid solvent is used.

Liquid-liquid extraction can be utilised for both gas chromatography (GC) and liquid chromatography (LC). Historically, SBSE has been utilised for conducting assessments of soil, food (Ridgway et al., 2010), and the environment (Huang et al., 2010). Recently, there has been a surge of interest in utilising solid-phase microextraction (SPME) in the field of bioanalysis. Table 3 compiles the latest applications of solid-phase microextraction (SBSE) in the field of bioanalysis, namely for estimating drugs, metabolites, and biomarkers in various biological matrices. When there are several analytes of interest, such as a medicine and its related metabolites, sample preparation becomes challenging due to their structural similarities but differences in chemical nature, Ko/w, pKa, etc. This is especially true when the sample volume is limited. In this scenario, a sequential SBSE approach can be utilised.

Methods for online solid phase extraction (OLSPE) are simpler and quicker when compared to analytical column transfer. The RAM column [65-67] and the turbulent flow chromatography (TFC) column are the two types of online SPE columns that are commercially available [68] .

It has been reported that TFC, a high flow chromatographic technique, is used to accomplish biological fluid automated online clean-up. The fundamental method of TFC techniques involves injecting biological samples directly, without extraction or treatment, into a column that is packed with large particles (20–60 mm) and has a small internal diameter (1 mm or 0.18 mm). To further improve selectivity, stationary phase chemistry is employed in this process. According to Chassaing and Robinson (2009), TFC has the benefit of unique flow dynamics that occur inside the column at high flow rates in the 1.5–5 ml/min range[69]. The linear velocities that are produced by the mobile phase passing through a TFC can be up to 100 times higher than those that are usually observed in HPLC columns.

When the mobile phase velocity is very high and there are a lot of empty spaces in the column, it might generate turbulence. Since small molecules diffuse more rapidly than big molecules, the tiny compounds cling to the internal surfaces of the stationary phase particles as they pass through their pores. Proteins and other large molecular weight molecules are quickly washed out of the column by the mobile phase's turbulent flow before they can diffuse into the particle pores. Commercially available TFC columns incorporate a range of chemistries to handle various analytes. The benefits of TFC for in vitro drug metabolism and in vivo pharmacokinetics (PK) studies have been highlighted in recent work, with a focus on high-throughput optimisation. (Verdirame et al., 2010). TFC is more sensitive than PP, according to a comparison between the two (based on the S/N ratio and the quantification limit).

Direct biomatrix injection throughput has increased and sample preparation has become simpler with the use of RAM and the TFC method. When contrasted with more conventional offline sample clean-up techniques like PPT, SPE, or LLE, matrix effects such ion suppression are either totally abolished or drastically reduced. Generic approaches are appropriate for most bioanalytical applications and reduce the amount of time needed to develop new methods.

Protein precipitation Technique

As previously stated, the standard biological matrix, comprising blood, plasma, and serum, contains approximately 8% (w/w) of proteins. Injecting these samples directly onto the instrument is generally not advisable for LC-MS bioanalysis. This is due to the possibility of protein precipitation in these samples when they come into contact with buffers and/or organic solvents in the mobile phase. If the bulk of the protein in the samples is not removed, the PPT in the LC system will quickly lead to the LC column's performance declining.

Table 3. Bioanalytical method using Protein precipitation technique

Analyte	Matrix	Analytical technique	Column used	Reference
Nitrated Fatty Acids	Plasma	LC-MS/MS		[70]
Xanthohumol	Rat Plasma	HPLC		[71]
curcumin and quercetin	Rat Plasma	RP-HPLC	SPE Coulmns	[72]
Kashmiri Saffron	Rat Plasma	Ultra-Fast Liquid Chromatography-Tandem Mass Spectrometry (UFLC-MS/MS)		[73]
Metformin, Amlodipine, Glibenclamide and Atorvastatin	Human Plasma	HPLC-UV	RAM-Column TFC	[75]
Fexofenadine	human serum	liquid chromatography with tandem mass spectrometry		[76]
Tacrolimus	Human Plasma	liquid chromatography with tandem mass spectrometry		[77]
Brivaracetam	Human Plasma	RP-HPLC		[78]
riluzole	Human Plasma	liquid chromatography with tandem mass spectrometry		[79]
Esomeprazole	Human Plasma	HPLC		[80]
Aceclofenac	Human Serum	RP-HPLC		[81]
Sulfasalazine	Rabbit Plasma	HPLC-UV		[82]

Mechanism of PPT

The formation of proteins, crucial biological molecules, results from the peptide bonding of amino acids. Soluble proteins have one or more peptide chains folded in an orientation under normal physiological conditions. The majority of hydrophobic amino acid residues are positioned internally inside this shape, whereas charged or hydrophilic amino acid residues are situated externally. The primary process by which peptide chains fold together inside the conformation is through interactions between hydrophobic amino acid residues. Additionally, disulfide bonds, hydrogen bonds, and salt bridges are other types of interactions that also contribute to the folded structure of proteins. The presence of charged or polar surface residues on the protein leads to the formation of a solvation layer, which is created by interactions with the surrounding biological environment. Protein ionic connections are weakened and the chance of aggregation is reduced when the solvation layer forms.

Advantage

PPT is an easy, quick, and practical method to get samples ready for LC-MS bioanalysis. A fixed volume of protein precipitant is combined with a small amount of tissue homogenate, blood, plasma, serum, or other aqueous matrices in this procedure. The precipitant interacts with the proteins in the sample matrix or solution, causing a change in their structure. This leads to the proteins undergoing precipitation and aggregation. Protein conformational modifications lead to the liberation and preservation of the analyte(s) of interest that are attached to the proteins in the solution. The high recovery rate of PPT, in contrast to other procedures like LLE and SPE, is a significant advantage. The solution should retain any small-molecule analyte(s) since the approach just aims to extract proteins from the sample matrix in theory. A theoretical recovery of 100% results from this.PPT has gained a lot of popularity in the bioanalytical world because of this benefit.

Organic Water-miscible Solvents

In an aqueous solution, common organic solvents such as methanol, acetone, ethanol, and cotonitrile mix well. When these solvents are added to plasma, blood, serum, or tissue homogenate, the water molecules in the protein solvation layer within the sample matrix are quickly replaced. Dipole or attractive electrostatic interactions bring proteins closer together, causing them to aggregate as the solvation layer gets thinner.

With PPT efficiency, acetone, acetonitrile, ethanol, and methanol are the best precipitants, with acetonitrile being the most effective followed by acetone, ethanol, and methanol. The most often utilized of these organic solvents is acetonitrile. It is important to remember that the volume of organic solvent injected affects PPT efficiency when using an organic solvent.

Although pure organic solvents can serve PPT's objectives perfectly, it is customary to add a small volume of bases (such as ammonium hydroxide) or acids (such as formic acid, acetic acid) to the sample matrix and then mix the mixture well to

disrupt protein binding and/or alter the analyte's charge states by altering the sample matrix's pH. A PPT solution can also be created by adding the acids or bases to the organic solvent. Enhancing REC may require a tiny addition of bases or acids. However, it is possible to combine organic protein precipitants with internal standards (IS).

Acids

Perchloric acid (PCA; 6% TCA) and trichloroacetic acid (TCA; 5–15% TCA) are the two acids that are most commonly used for PPT. The precipitation of proteins within the sample matrix is the efficient use of both reagents. Protein denaturing is a critical component of PPT, according to TCA and PCA.

PPT filter plates

Using membrane-based PPT filter plates, such Whatman's Unifilter, Strata Impact™ from Phenomenex's, Isolute® PPT from Biotage's ⁺ and Sirocco™ from Waters, is one current method to get around this drawback. Supernatant transfer is not necessary when separating the supernatant through filtration utilizing PPT plates.

96 well PPT plates

Usually, a 96-well PPT plate is layered with a 96-well collecting plate. Filters with an average pore size of 0.2 µm are located at the bottom of every well on the PPT plate. The pellets remain on the filter as the PPT supernatant passes through it and onto the collection plate when centrifugation or vacuum is used. The sample preparation time was greatly decreased by using this method as opposed to the manual PPT method (96 samples per method), which took 4-5 hours instead of 1-1.5 hours. Additionally, using PPT plates results in sample extracts that are cleaner and have a higher solvent recovery. There have been reports of PPT plate solvent leakage issues. Comparing the PPT solvents that were tested, acetonitrile leaked more than methanol. Additionally, when selecting a PPT plate, it is advisable to take into account the precipitating solvent, filter material, pore size, vacuum strength or speed, matrix effects (ion suppression), and nonspecific binding of the analyte to the plate simultaneously. The matrix effect is thought to be a significant problem with PPT. The reason for this is that upon PPT, the sample extract includes all other matrix elements, including phospholipids. A significant class of biological materials with one or more phosphate groups is called phospholipids. Two primary functional group areas make up their molecular structures: (i) one or more ester groups of hydrophobic long-chain fatty acids; and (ii) a polar head group that consists of an ionisable organic phosphate moiety along with several other polar groups[83].

Because phospholipids are very ionic, they have a considerable matrix effect via altering the LC effluent droplets' desolvation and the ionisation of the target analyte(s) in the electrospray MS source.

Hybrid SPE PPT

More and more LC-MS bioanalysts are turning to newly designed PPT plates that are filled with chemicals that bind to phospholipids, allowing them to extract not just proteins but also the abundant phospholipids. Among the plates are, to mention a few, Agilents' Captiva ND Lipids, Waters' Ostro, Phenomenex's Phree, and Sigma's Hybrid SPE.

These plates allow for the production of sample extracts with drastically decreased levels of phospholipids or ones that are entirely protein-free. The sample extracts needed for the LC-MS analysis that follows are readily available. A combination of HybridSPE PPT plates and acetonitrile, which includes 1% formic acid, enables the sample extract to be passed through the packed bed upon occurrence of PPT. The packed bed is composed of unique silica particles coated in zirconia.

Lewis bases (electron donors) and zirconia sites interact substantially due to the characteristics of Lewis acids (electron acceptors). As previously stated, phospholipids are made up of two hydrophobic fatty acyl groups that form a long hydrophobic tail and zwitterionic phosphonate moieties, which make up the polar head group.

Zirconia atoms functionalized on the surface of the particles are strongly interacted with by the phosphate group(s) of the phospholipids, which function as an extraordinarily strong Lewis base. Incorporating formic acid or other acids into the phospholipid removal PPT plate approach improves the recovery of target analytes, particularly acidic ones. In order to stop analyte retention on the packed bed without influencing phospholipid retention, acid is required.

PPT, LLE, and/or SPE in combination for LC-MS bioanalysis

There are benefits and drawbacks to the three technologies PPT, LLE, and SPE that were previously covered. None of them can meet the demands of contemporary LC-MS bioanalysis while maintaining low matrix effect and excellent REC. In fact, in certain unique situations, more advanced techniques are required to attain the required high sensitivity or eliminate enduring interferences. The physicochemical characteristics of the actual problematic matrix interferences are probably comparable to those of the targeted analyte or analytes. This means that single sample extraction methods will likely include their extraction together with the analyte(s) of interest. It is recommended to address these issues by utilising orthogonal or hybrid sample preparation techniques, such as PPT/LLE, PPT/SPE, and LLE/SPE.

Summary

There has been discussion of the three main sample preparation platforms: LLE, SPE and PPT. Every technique offers a special benefit over the others as well as drawbacks. In addition to being less expensive than LLE and SPE, the approach also has the speed and throughput that are required for regular analysis of a high volume of research materials. By carefully eliminating interference-causing matrix effects and selectively extracting the desired analyte or analytes, LLE offers the most efficient sample cleanup. The use of automated LLE platforms is becoming increasingly common, and for good reason: they can make LLE sample preparation much more efficient and accurate.

SPE technology will go further. There will always be sorbents on the market to suit the demands of extracting various kinds of chemicals from biological matrices. Additionally, there is a tendency toward the development of SPE plates suitable for

processing small amounts of biological material. Without a doubt, PPT is the most straightforward, rapid, and practical method among the three, and it is frequently used in LC-MS bioanalysis. As a result, PPT will continue to be the recommended approach for LC-MS bioanalysis sample preparation.

Conclusion and Future Advancements

In the realm of bioanalytical techniques, the selection and implementation of appropriate sample preparation methods play a pivotal role in achieving accurate and reliable results. This conclusion reflects on the significance of Liquid-Liquid Extraction (LLE), Solid-Phase Extraction (SPE), and Protein Precipitation (PPT) as key sample preparation techniques and their impact on the broader field of bioanalysis. The judicious selection and application of LLE, SPE, and PPT are pivotal in the success of bioanalytical endeavors. As bioanalytical challenges evolve, future advancements in sample preparation techniques will likely focus on enhancing automation, miniaturization, and selectivity. The continual refinement of these methods will undoubtedly contribute to the ongoing success and advancement of bioanalytical techniques, ensuring the robustness and reliability of analytical results in the ever-expanding field of life sciences.

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