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Basic Sample Preparation Techniques in LC-MS Bioanalysis

Protein Precipitation, Liquid–Liquid Extraction, and Solid-Phase Extraction

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1.1 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. The focus of bioanalysis in the human health industry is to provide a quantitative measurement of active drug and/or its metabolite(s) and/or biomarkers for the accurate assessment of pharmacokinetics, toxicokinetics, bioequivalence, bioavailability and/or exposure–response (e.g., pharmacokinetics/pharmacodynamics, toxicokinetics/toxicodynamics) relationships in support of drug discovery and development, and post-approval therapeutic drug monitoring (Unger et al. 2013).

Many techniques have been 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). Among these techniques, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been the most widely used and most reliable tool due to its high sensitivity and specificity. In LC-MS bioanalysis, a high-performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UHPLC) system is employed to separate the analyte(s) of interest from other unwanted matrix components in sample extracts based on the specific interactions between the analyte(s) of interest and the analytical LC column. The LC eluent is then introduced to a mass spectrometer for molecule-based separation via multiple reaction monitoring (MRM), also named as selected reaction monitoring (SRM). In MRM or SRM, the specific precursor ion(s) of analyte of interest is selected for collision-induced dissociation (CID) or collision-activated dissociation (CAD) to generate fragment ions that are also specific to the analyte(s) of interest, and usually the most abundant fragment ion is selected for MS detection. With the specificity and sensitivity provided by both the LC separation and the MRM of the MS system, LC-MS/MS has become one of the most suitable tools for quantitative bioanalysis (Unger et al. 2013).

The common matrix that is subjected to LC-MS bioanalysis includes various body fluids (e.g. plasma, serum, whole blood, saliva, tears, and urine) and organ tissues (e.g. kidneys, liver, lung, skin, and brain tissue). In general, these biological samples contain abundant various endogenous components like salts, small molecules, proteins, and lipids, or exogenous components such as formulation ingredients. In contrast, the analyte(s) of interest is often at very low concentration levels, typically in low $ng\,ml^{-1}$ concentration range and even at $pg\,ml^{-1}$ level for highly potent molecules. The presence of abundant (typically at $\mu g\,ml^{-1}$ to $mg\,ml^{-1}$ range) endogenous or exogenous components in the biological matrix, compounded by the very low concentration of the analyte(s) of interest, is definitely a challenge for bioanalytical scientists in the field in developing and validating a robust LC-MS bioanalytical assay (Unger et al. 2013).

In order to ensure adequate sensitivity, selectivity, and reproducibility of the LC-MS assay method for measuring analyte(s) of interest in biological samples, sample preparation, also known as sample pretreatment or sample cleanup, is a must step. Sample preparation in LC-MS bioanalysis is considered a pre-analytical separation process that involves selective isolation of analyte(s) of interest from the matrix, minimization or elimination of matrix components in the extracted samples, and, if necessary, enrichment of analyte(s) to ensure achievable assay sensitivity. An ideal sample preparation method should be able to reduce matrix effect to a minimal level

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while maintaining a reasonable and consistent extraction recovery (REC) (e.g. 80%). However, due to the many factors affecting matrix removal and analyte recovery, developing an optimal sample preparation procedure can be difficult, tedious, and labor-intensive, which makes it one of the most significant parts in the development of a robust LC-MS bioanalytical method. In this chapter, we describe the commonly used sample preparation techniques in LC-MS bioanalysis, namely protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE), with focus on the importance of understanding the physicochemical properties of the analyte(s) of interest that determines its extractability and the need of balancing REC and assay selectivity. There are a variety of techniques that are derived from these three basic sample preparation workflows as well as emerging methodologies that utilize advanced techniques for more selective and/or efficient extraction of analyte(s) of interest. They will be covered in detail in subsequent chapters of the book and therefore will not be discussed here. In addition, although dilution-and-shoot (DAS) of biological samples has been used in LC-MS bioanalysis, it has been primarily used in the area of drug discovery for the analysis of samples generated from *in vitro* system rather than *in vivo* studies, and therefore is not covered in this chapter.

1.2 Physicochemical Properties of Drugs and Their Metabolites

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 ($\log P$, $\log D$, and pK_a, etc.). Understanding these properties can help select suitable sample preparation techniques and the associated experimental conditions for the analyte(s) of interest.

1.2.1 Hydrophilicity vs. Lipophilicity of Analyte(s)

Hydrophilicity refers to the ability of a molecule to dissolve in water and other hydrophilic solvents. The interaction of a hydrophilic or polar molecule with water and other polar molecules is more thermodynamically favorable than its interactions with oil or other hydrophobic/lipophilic molecules. A hydrophilic molecule is typically charge-polarized and capable of hydrogen bonding. In contrast, lipophilicity refers to the ability of a molecule to dissolve in fats, oils, lipids, and nonpolar solvents such as hexane or toluene. Such nonpolar solvents are themselves lipophilic. In the field of bioanalysis, lipophilicity, hydrophobicity, polarity and nonpolarity may have been used to describe the same tendency of a molecule and these terms are often used interchangeably. There are two key parameters to help understand the hydrophilicity vs. lipophilicity of the analyte(s) of interest:

The partition coefficient, P, is the concentration ratio of a molecule, specifically for unionized molecule, in two immiscible solvents at equilibrium. When one of the solvents is water and the other is a nonpolar one (e.g. *n*-octanol), the $\log P$ value is the logarithm of the concentration ratio of the unionized form of the molecule between the two immiscible phases. $\text{Log}P$ is considered a measure of lipophilicity or hydrophobicity of a given molecule, for which the higher the $\log P$ value, the more lipophilic or hydrophobic the molecule is.

$$\log P = \log_{10}\left(\frac{C_{\text{organic}}}{C_{\text{aqueous}}}\right),$$

where C_{organic} is the concentration of the neutral form of the molecule in the water-immiscible solvent; C_{aqueous} is the concentration of the neutral form of the molecule in the aqueous phase.

The distribution coefficient, D, is the ratio of the sum of the concentrations of both ionized and unionized forms of the molecule between the two immiscible phases, i.e. aqueous and organic phases. Since ionization of a molecule in aqueous phase is pH dependent, the $\log D$ value is also pH dependent. The aqueous phase is adjusted to certain pH for $\log D$ measurement.

$$\log D = \log_{10}\left(\frac{C_{\text{organic}}}{C_{\text{aqueous(ionized)}} + C_{\text{aqueous(neutral)}}}\right),$$

where C_{organic} is the concentration of the molecule (ionized and neutral) in the water-immiscible solvent, $C_{\text{aqueous (ionized)}}$ is the concentration of the ionized form of the molecule in the aqueous phase, and $C_{\text{aqueous (neutral)}}$ is the concentration of the neutral form of the molecule in the aqueous phase. Accordingly, in LLE and to some extent SPE, the REC of a given molecule under given conditions (pH, organic solvent, volume ratio between aqueous and organic) can be predicted (Liu and Aubry 2013).

1.2.2 Protolytic Properties of Analyte(s)

Protolytic refers to a reaction or process in solution that involves transfer of a proton from one molecule to another (one of the molecules usually belonging to the solvent). Many molecules show protolytic properties and

are often present in both ionic/ionized (acidic [H^+ donor] or basic [H^+ acceptor]) and neutral forms in aqueous solution.

In chemistry, pH is the negative logarithm of the concentration of the hydrogen ion ($pH = -\log_{10}(H^+)$) and is used to specify the acidity or basicity of an aqueous solution. Solutions with a pH <7 are considered acidic while solutions with a pH >7 are considered basic. Pure water is neutral at pH 7 (25 °C). pK_a is the negative base-10 logarithm of the acid dissociation constant (K_a) of a solution ($pK_a = -\log_{10} K_a$) and is also defined as the pH where a molecule exists as 50% ionized and 50% unionized. pK_a is a property of a given molecule that tells us how acidic or basic it is. The lower the pK_a a molecule is, the stronger the acid it is. For example, the pK_a value of acetic acid is 4.8, while the pK_a value of a stronger acid, lactic acid is 3.8. The pK_a value of a given molecule can be related to its charge state in solution under a given pH. In this regard, the pH of an aqueous solvent has a great impact on degree of ionization of a given analyte and the choice of the sample preparation method to be employed in LC-MS bioanalysis. An acid analyte in an acid solution will generally not ionize. In contrast, it will ionize in a basic solution. Similarly, a basic analyte will generally not ionize in basic solution but will ionize in acidic solution. When pH is equal to pK_a, 50% of the analytes are in ionized form and 50% in unionized (neutral) form. However, when pH is $<<pK_a$, almost 100% of the acidic analytes are unionized but basic analytes are almost 100% ionized; when pH is $>>pK_a$, almost 100% of the acidic analytes are ionized but basic analytes are almost 100% unionized.

Accordingly, in LLE and SPE with reverse-phased (RP) stationary phase, the best REC can be obtained at a pH at which most of the analytes are not charged, i.e. neutral form. In contrast, in SPE with ion-exchange stationary phase, the best REC can be obtained when the analyte molecules are all ionized (charged) to interact with the charged stationary phase (Liu and Aubry 2013).

1.3 Pre-analytical Variables of Analyte(s) of Interest in Biological Matrix

LC-MS bioanalysis is dealing with a variety of biological matrices such as plasma, serum, whole blood, urine, CSF, tissue homogenates, etc. The composition and complexity (e.g. pH, nature, and concentration of proteins, lipids, and salts) of these matrices is significantly different from one to the other. Even for the same matrix, it can be largely different from one subject to the other, depending on the age, gender, disease stage, medication, and other factors of the subject. Understanding the above can generally be helpful in developing an overall sample preparation strategy. However, each analyte is considered a unique entity in LC-MS bioanalysis. In addition to understanding the physicochemical properties and others discussed in Section 1.2 and complexity of the matrix discussed above, understanding some analyte-specific variables, including stability, possible nonspecific binding, protein binding and/or blood-to-plasma ratio, and red blood cell partition can be pivotal in defining and/or optimizing a specific sample preparation method for LC-MS bioanalysis of the analyte(s) of interest.

1.3.1 Stability

Stability is an important pre-analytical variable for quantitative LC-MS bioanalysis of drug molecules and/or their metabolites in biological matrices. Instability of an analyte in any stage of the bioanalysis process, including sample collection, processing, storage, extraction, and LC-MS analysis, can result in under- or over-estimation of analyte exposure if an adequate preventive procedure is not in place (Li et al. 2011). Therefore, one should carefully examine the structural characteristics and the *in vivo* and/or *in vitro* biotransformation of any analyte of interest prior to developing a specific LC-MS bioanalysis method. Previous internal and/or external (i.e. literature) experiences with structurally similar compounds can be very useful for a general understanding of the potential instability alert. The possible instability of some analyte(s) that contains biologically or chemically labile moieties, e.g. thiol, ester, or catechol, etc., can be readily predicted and estimated for effectiveness of specific precautions throughout the entire method development, validation, and sample analysis process. However, instability may not be readily predictable for many analyte(s) unless necessary stability assessment has been conducted using QC and/or incurred samples against freshly prepared calibration standards. For example, an unstable conjugated metabolite could convert to parent drug leading to overestimation of parent drug concentration, a phenomenon that can only be estimated using incurred samples when the putative conjugated metabolite is not available. Instability of an unstable analyte may occur at one or several points of the bioanalytical process, i.e. sample collection, processing, storage, extraction, and LC-MS/MS analysis, etc. Some general strategies should be formulated with incorporation of one or more specific guidance (e.g. addition of enzyme inhibitors, pH modifiers, or antioxidants, etc.) in developing a robust LC-MS/MS quantitative bioanalytical method for the unstable molecules. For more details on stability strategies one can refer to Strategies in quantitative LC-MS/MS analysis of unstable small molecules in biological matrices (Li et al. 2011, 2013a).

1.3.2 Nonspecific Binding

Some matrix, e.g. urine or CSF, does not normally contain proteins and lipids that are present at ~8% in whole blood, plasma, or serum (Lentner 1981). The lack of protein and lipids in these samples can be associated with issues of nonspecific binding or container surface adsorption of drug molecules, especially those lipophilic/hydrophobic and those with high affinity to proteins (high protein binding), in LC-MS bioanalysis (Li et al. 2010). The nonspecific binding or container surface adsorption is often evidenced by the unusually low REC of the analyte(s) of interest and/or nonlinearity of the calibration curves or highly variable QC sample results. Unfortunately, the issue is often overlooked during the early stage of bioanalytical method development, especially when assay sensitivity is not an issue and/or both the calibration standards and QC samples are prepared in the same fashion, i.e. freshly spiked, daily prepared, or pre-pooled. In the latter cases, the unexpected low recovery of analyte would often be interpreted due to matrix effect or signal suppression because the problem, if any, may be masked by a similar degree of LC-MS signal loss of the analyte for both the calibration standards and QC samples that are prepared in the same fashion. In some cases, the issue might not be realized until after many failed feasibility runs. Failure to promptly assess and adequately address this issue would result in underestimated urine/CSF drug concentrations. Details on how to assess and address the issues due to nonspecific binding or container surface adsorption can be found in some good research and review articles (Fu et al. 2011; Ji 2013; Ji et al. 2010; Li et al. 2010). Some detailed strategies can be found in Chapter 18 of this book.

1.3.3 Protein Binding

Depending on the affinity of drug molecules to plasma protein, a portion of the drug molecules may become bound to plasma proteins, with the remainder being unbound. Therefore, a given drug molecule generally exists in two forms in blood, plasma or serum: bound and unbound. If the protein binding is reversible, then a chemical equilibrium will exist between the bound and unbound states of the drug molecules, such that: Protein + drug ⇌ Protein–drug complex. Since albumin, a plasma protein, is alkalotic, acidic and neutral drug molecules will primarily bind to albumin. If albumin becomes saturated, then these molecules will bind to lipoprotein. In contrast, basic drug molecules will primarily bind to the acidic alpha-1 acid glycoprotein in whole blood, plasma, or serum.

In general, a LC-MS bioanalytical method is employed to measure the total analyte concentrations in the intended study sample matrix unless otherwise specified. Therefore, release of the analyte of interest from plasma proteins by interrupting the protein binding is considered a key step for good and consistent REC, regardless of choice of the sample preparation method and protein binding rate of the analyte. In this regard, the biological samples are generally treated with acid (e.g. acetic acid or formic acid), base (e.g. ammonium hydroxide), buffer, or organic to free the analyte from the plasma proteins prior to further processing (de Boer and Wieling 2013).

1.3.4 Blood-to-plasma Ratio and Red Blood Cell Partition

The blood to plasma ratio (Kb/p) of a drug is the ratio of its concentration in whole blood (containing both red blood cells, RBCs, and plasma) to the corresponding value in plasma, namely C_B/C_P. In contrast, the red blood cell partition coefficient (Ke/p) is the ratio of the drug concentration in the RBCs (i.e. not including plasma) to its concentration in plasma, namely C_{RBC}/C_P. In practice, when working with whole blood samples, the intended method of sample preparation should be capable of releasing the analyte from the RBCs (Brockman et al. 2007).

Temperature-dependent re-equilibration of drug molecules between RBCs and plasma is a known phenomenon (Dell 2004). Ideally, if plasma is the matrix of choice for bioanalysis, blood samples should be centrifuged to separate the plasma as soon as they are collected. Extended stay of the collected blood samples at 4 °C may be an issue if temperature-dependent re-equilibration occurs to the analyte(s) of interest. As a result of temperature-dependent re-equilibration, the analyte concentration in plasma obtained from blood that have been left on wet ice (~4 °C) for some time may no longer be the "original" one. For example, dehydronorketamine, a ketamine major metabolite, is stable in plasma under all normal conditions. Due to re-equilibration of compound into blood cells at 4 °C with time, a significant decrease in its plasma concentration was observed over time. In contrast, no change was seen in the concentrations of the plasma samples prepared from the blood that has been stored at ambient temperature. This phenomenon can be overcome by centrifuging the blood immediately after collection, so that the actual *in vivo* plasma analyte concentration can be maintained and determined (Hijazi et al. 2001). In the case where the collected blood samples have somehow stayed on wet ice for a period of time, the blood samples can be incubated at 37 °C for ~30 minutes to resume the equilibrium between RBCs and plasma for the analyte of interest (to mimic *in vivo* condition) prior to centrifugation of the blood for plasma (unpublished data).

1.4 Most Commonly Used Sample Preparation Methods in LC-MS Bioanalysis

The most commonly employed sample preparation methods in LC-MS bioanalysis generally include PPT, LLE, and SPE. Many other contemporary sample preparation methods, such as online extraction and column switching, phospholipids depletion, salting-out assisted LLE, supported liquid extraction (SLE), immunoextraction, microextraction, sample preparation via nanomaterials, molecularly imprinted polymers (MIP), aptamers or electromembranes, and stir-bar sorptive extraction, etc. are captured in other chapters of this book.

1.4.1 Protein Precipitation (PPT)

The common biological matrix, such as blood, plasma, or serum contains ~8% (w/w) of proteins as mentioned earlier. Direct injection of these samples onto the LC-MS system is generally not a good option for LC-MS bioanalysis. This is because the proteins in these samples could readily precipitate as a result of contact with organic solvents and/or buffers in the mobile phase. As a result of PPT in the LC system, the performance of the LC column will rapidly deteriorate unless the majority of the protein in the samples is removed.

Proteins are large biological molecules composed of amino acids that are linked to each other by peptide bonds. Under normal physiological conditions, a soluble protein has one or more peptide chains in a folded conformation. Within this conformation, the majority of hydrophobic amino acid residues are toward inside while the charged or hydrophilic amino acid residues are toward outside. In the inside of the conformation, the peptide chains are folded together mainly through hydrophobic interactions between hydrophobic amino acid residues; other interactions such as hydrogen bonds, salt bridges, or disulfide bonds also contribute to the folded structure of proteins. On the outside of the conformation, the charged or polar surface residues interact with the biological environment, where water forms a solvation layer that surrounds the protein. The formation of the solvation layer weakens the ionic interactions between proteins and decreases the likelihood of aggregation (Li and Bartlett 2014).

PPT is a simple, quick, and convenient sample preparation technique in LC-MS bioanalysis. In this process, a small volume of blood, plasma, serum, tissue homogenate, or other aqueous matrices is mixed with a certain volume of protein precipitant. When proteins in sample matrix/solution come in contact with precipitant, the conformation of the proteins is altered due to the interaction. This results in aggregation and precipitation of the proteins. As a result of conformation changes of the proteins, the analyte(s) of interest that are bound to the proteins are released into and stay in the solution. Upon centrifugation and/or filtration, the precipitated proteins are separated from the analyte(s) containing supernatant.

The equipment required for PPT is relatively simple. It includes low-cost and disposable centrifuge tubes or 96-well plates and a centrifuge. A bioanalytical scientist can find many published methods ready for use as the starting point. If method development and/or optimization are necessary, it is relatively simple and straightforward due to the simple nature of the technique. Briefly, optimization of a PPT method includes selection of precipitant and the amount of precipitant along with centrifugation and/or filtration.

A significant advantage of PPT is its high recovery as compared to other techniques, e.g. LLE and SPE. Since only proteins are hypothetically removed from the sample matrix by the method, small-molecule analyte(s) should remain in the solution and this yields a theoretical recovery of 100%. Such an advantage of PPT has made it very popular in the bioanalytical community.

Common protein precipitants include: (i) water-miscible organic solvents, (ii) acids, (iii) metal ions, or (iv) salts. Among these precipitants, the water-miscible organic solvents and acids are the most common ones.

1.4.1.1 Water-miscible Organic Solvents

Common water-miscible organic solvents include acetonitrile, acetone, ethanol, and methanol and they are 100% miscible in aqueous solution. When these solvents are added to blood, plasma, serum, or tissue homogenate, they quickly displace water molecules of the solvation layer of the proteins in the sample matrix. When the solvation layer becomes thinner and thinner, proteins get closer and closer to each other via attractive electrostatic or dipole interactions, leading to the aggregation. The protein aggregates grow by diffusive additions of other protein molecules and eventually reach a critical size for precipitation, forming protein sediments or floccules in the mixture of samples and precipitant (Li and Bartlett 2014).

Acetonitrile, acetone, ethanol, and methanol are all good precipitant with PPT efficiency in the order of acetonitrile > acetone > ethanol > methanol. Among these organic solvents, the most commonly used one is acetonitrile. It is worth noting that PPT efficiency via an organic solvent also depends on the volume of the organic solvent added. With acetonitrile as an example, when 0.2 ml of acetonitrile is added to 1.0 ml of plasma, only 13.4% of proteins are removed. With the volume of acetonitrile increased to 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.0, and

4.0 ml, the percentage (%) of protein removed is increased, respectively, to 14.8, 45.8, 88.1, 97.2, 99.4, 99.7, 99.8, and 99.8% (Blanchard 1981). It is apparent that by adding 2.0 ml of acetonitrile to 1.0 ml of plasma for PPT, the resulting supernatant is basically protein-free.

Although pure organic solvents can perfectly fit the purpose of PPT, it is a common practice to add a small volume of acids (e.g. formic acid, acetic acid) (Li et al. 2014) or bases (e.g. ammonium hydroxide) (Li et al. 2007b) to the sample matrix, followed by proper mixing to interrupt protein binding and/or change the charge states of the analyte by changing the pH of the sample matrix. The acids or bases can also be added to the organic solvent to prepare a PPT solution. The addition of a small volume of acids or bases can help improve REC. On the other hand, internal standards (IS) can be added to the organic protein precipitants. This allows for the addition of the IS at the same time as for protein precipitant. This practice not only improves the throughput by combining two steps together but also enhances the precision of the assay by pipetting a relatively large volume of IS working solution instead of spiking a relatively small volume. In addition, for certain analytes with high protein binding, it is recommended to add IS in aqueous solution to plasma sample first. This allows for additional time of equilibrium before addition of an organic precipitant. By doing so, the IS may better mimic the extraction of the analyte and therefore improve the assay accuracy and reproducibility (unpublished data).

As the strongest precipitant, use of acetonitrile can lead to the formation of very solid protein precipitates of large particle sizes. Being relatively weaker protein precipitants, the use of ethanol or methanol can lead to the formation of looser flocculent precipitates. However, there is a concern that using 100% acetonitrile as the protein precipitant may not give the highest recovery. Due to the very quick PPT using acetonitrile, some analyte(s) with high plasma protein binding might coprecipitate with plasma proteins if the analyte(s) are not disassociated with the proteins during the process of protein precipitation. In a study with no acids or base added to the sample matrix prior to PPT using acetonitrile, an increasing percentage of methanol (10, 20, 30, 40, 50, 60, and 70%) was added to acetonitrile for PPT prior to direct analysis via hydrophilic chromatography (HILIC)-MS/MS for atenolol in human plasma. The analysis of the samples showed that use of acetonitrile containing 10% methanol yielded a sample extract with LC-MS signal higher than that obtained using acetonitrile alone (Li et al. 2005).

1.4.1.2 Acids

The commonly used acids for PPT are trichloroacetic acid (TCA) (5–15%, TCA) and perchloric acid (PCA) (6%). Both reagents are highly efficient in precipitating proteins in the sample matrix (Hee et al. 2017). It is understood that protein denaturing is a key in PPT by TCA and PCA. Upon addition of the acids, the pH of the solution/sample matrix is greatly lowered and the protein conformation is drastically altered, resulting in the aggregation of proteins. Addition of 0.2 ml of TCA (10%) to 1 ml of the matrix is capable of removing >99% of the proteins in the plasma samples. One advantage of using acids for PPT is that the resulting supernatant (of filtrate) after centrifugation is still highly aqueous and the supernatant can be directly injected onto the LC-MS/MS system. The drawback of this approach is that the resulting supernatant is strongly acidic with pH < 2.0, which might be a concern of analyte instability in the sample extract (Li and Bartlett 2014; Pedersen-Bjergaard et al. 2015).

Other than the composition and pH of the organic protein precipitants discussed above, temperature is another factor that should be considered for efficient PPT. Ice-cold organic solvents are generally more efficient in precipitating proteins in the sample matrix. This approach is particularly useful when dealing with unstable analyte(s) (Li et al. 2011).

In the PPT process, upon addition of protein precipitant (discussed above), IS (in working solution or prepared in protein precipitant solution), and pH modifier (can be added to the protein precipitant), adequate vortex-mixing has to be applied to the sample mixture. Most proteins in the sample mixture are expected to precipitate. The next step is a physical process to separate precipitated proteins from the supernatant. Usually 5–10 minutes of centrifugation at $\sim 1500 \times g$ is sufficient to settle the protein precipitates. The higher centrifugation force and the longer centrifugation time are applied, the more solid the protein pellet will be formed in the bottom of the sample preparation device (individual tubes or 96-well plate). This makes it easier to transfer the supernatant without disturbing the pellet.

Since at least two volumes of organic solvents are commonly employed in PPT and the resulting supernatant generally has a high organic composition, direct LC-MS/MS analysis of the supernatant using reversed-phase columns may not be feasible. If assay sensitivity is permitted, dilution of the supernatant with aqueous solution (e.g. water) should be applied to lower the concentrations of organic solvents in the final sample extract prior to LC-MS/MS analysis. Otherwise, the resulting supernatant should be subjected to evaporation step, at which the organic solvents in the supernatant are evaporated under a stream of nitrogen and resulting sample residues are reconstituted in aqueous solutions (with proper pH, organic and/or acid concentrations) or starting mobile phase. The volume of the reconstitution solution used should be adjusted to ensure suitable assay sensitivity on

the intended LC-MS/MS platform. In contrast, depending on the polarity and other physicochemical properties of the analyte(s) of interest, the resulting supernatant obtained from PPT may be directly subjected to analysis on HILIC-MS/MS that employs mobile phases with high organic composition. This high organic composition is, in general, compatible with sample extract (supernatant) (Jian et al. 2010b; Li et al. 2005).

A representative protocol of PPT with an organic solvent is shown below.

A representative example of PPT protocol (for the analysis of a steroid 11β-hydroxylase inhibitor, in human plasma by Li et al. 2014).

- A 0.100 ml aliquot of blank plasma (for matrix blanks, zero samples [blank matrix mixed with the IS only] or fresh calibration standards), QC samples, or unknown samples was added to a 2-ml, 96-well plate.
- To the standard samples, 0.100 ml of the respective standard working solution (in 50% aqueous methanol, v/v) was added.
- To the matrix blanks, zero samples, QC samples, and unknown samples, a 0.100 ml aliquot of 50% methanol in water (v/v) was added.
- This was followed by brief vortex-mixing.
- A 25 µl aliquot of the IS working solution in 50% methanol in water (v/v) was added to each well except for the matrix blank, to which a 25 µl aliquot of 50% methanol in water (v/v) was added.
- After adding 25 µl of 40% formic acid in water (v/v) to each well, the plate was vortex-mixed briefly.
- To each well, 0.500 ml of acetonitrile/ethanol/acetic acid (90/10/0.1, v/v/v) was added and the plate was covered and vortex-mixed for ~15 minutes on a pulse vortex-mixer with a motor speed setting of about 50 units.
- The sample plate was centrifuged at ~3500 rpm (~2000 × *g*) for ~10 minutes at ~10 °C.
- The supernatants (0.500 ml) were transferred via a TomTec Quadra 96 system (Hamden, CT, USA) to the corresponding wells in a 1-ml 96-well plate, evaporated to dryness under a stream of nitrogen at approximately 45 °C and the sample residues reconstituted with 100 µl of reconstitution solution (30% methanol in water, v/v).
- After brief vortex-mixing and centrifugation at ~3500 rpm (~2000 × *g*) for approximately five minutes remove potential un-dissolved contents in the sample, a 10 µl aliquot was injected onto the LC-MS/MS system.

Conventional PPT involves multisteps of manipulation, including centrifugation and transfer of the resulting supernatant. The overall throughput is low. Therefore, it is not ideal in support of analysis of a large number of study samples. One of the recent approaches to overcoming such a disadvantage is the use of membrane-based PPT filter plates, such as Unifilter® from Whatman, Strata® Impact™ from Phenomenex, Isolute® PPT+ from Biotage, and Sirocco™ from Waters. By using PPT plates, separation of the supernatant can be carried out by filtration without the need of supernatant transfer.

In practice, the 96-well PPT plate is placed on top of a 96-well collection plate. The PPT plate contains filters in the bottom of each well with a typical pore size of 0.2 µm. Upon centrifugation or application of vacuum, the supernatant from PPT flows through the filter into the collection plate, while the pellets are retained on the filter. By this approach, significant reduction in the sample preparation time (four-fold, from 4–5 hours to 1–1.5 hours) was reported as compared with the manual PPT method (96 samples each) (Kocan et al. 2006). Furthermore, the use of PPT plates yields cleaner sample extracts with a higher solvent recovery.

Issues related to solvent leaking with PPT plates have been reported. Among the PPT solvents tested, acetonitrile showed higher leaking than methanol. It has been also suggested that the precipitating solvent, filter material, pore size, centrifugation speed or vacuum strength, nonspecific binding of analyte to the plate, and matrix effects (ion suppression) should be concurrently considered when choosing a PPT plate. Table 1.1 summarizes commercially available PPT plates with respective technical details (Kole et al. 2011).

Matrix effect is considered a major issue related to PPT. This is because, upon PPT, all other matrix components, including phospholipids, are present in the sample extract. Phospholipids are an important class of biological compounds containing one or more phosphate groups. Their molecular structures have two major functional group regions, i.e. (i) a polar head group which includes an ionizable organic phosphate moiety as well as other polar groups of various types and (ii) one or two long-chain fatty acid ester groups which are hydrophobic. The highly ionic nature of phospholipids makes them responsible for influencing the ionization of the analyte(s) of interest and desolvation of the LC effluent droplets in the electrospray MS source, causing significant matrix effects.

Noticeably, some of the recently developed PPT plates are packed with materials that specifically retain phospholipids and, therefore, have been increasingly employed to remove the abundant phospholipids along with proteins in LC-MS bioanalysis. These plates include but are not limited to Captiva™ ND Lipids from Agilent, Ostro™ from Waters, Phree™ from Phenomenex, and

Table 1.1 Commercially available protein precipitation plate/tubes.

Company	Trade name	Format	Volume handling range (µl)	Membrane/depth filter	Phospholipids removal	Advantages offered
3M	Empore™	96-well	50–200	Polypropylene filter	—	98% removal of particles >10 µm
Biotage	Isolute PPT+	96-well	15–400	Depth filter (optimized porosity distribution)	—	Solvent First Technology
Glygen	Glysci	96-well	—	3 mm filter	Yes	Minimum sample loss, no leaking; the plate packed with $TiO_2 + ZrO_2$ can be used for the removal of phospholipids at the same time as the protein is precipitated
Millipore	MultiScreen® Deep well and multiscreen filter plates	96-well	50–200	Membrane; Durapore® (variety of membranes)	—	Low binding, low extractables, and high recoveries
Orochem Technologies	OC21PPT20	96-well	400	Variety of membranes	—	Solvent First Technology
Pall Corporation	AcroPrep™	96-well	350	PTFE membrane (variety of membranes)	—	Available in a variety of housing colors
Phenomenex	Phree	96-well	25–400	Membrane filter	Yes	The Phree sorbent selectively removes phospholipids from precipitated plasma samples
Phenomenex Inc.	Strata Impact	96-well	25–300	Membrane filter	—	Solvent Shielding Technology™
Sigma	HybridSPE	96-well	20–300	Upper 5 µM PFFE frit and Lower 0.2 µM hydrophobic filter, packed bed assembly acts as depth filter	Yes	The Zr atom coated on the silica surface acts as a Lewis acid which interacts with phosphate moiety of phospholipids, which is a strong Lewis base
ThermoFisher	HyperSep™	96-well	400	Dual frit	—	No wetting out, no leakage; nonspecific binding of compound to filter/plate
ThermoFisher	Pierce™	96-well	15–400	0.2 µM membrane	—	Leakfree, no dripping
Varian Inc.	Captiva ND^{Lipids}	96-well and single filter cartridges	50–200	Non-drip membrane	Yes	Proteins and surfactants removed
Waters	Ostro	96-well	5–200	Valve and cap mat	Yes	Effective for protein and phospholipids removal
Waters	Sirocco	96-well	200	Valve and cap mat	—	Sealing cap mat and patented valve technology
Whatman	Unifilter	96-well	200	Non-drip dual membrane (variety of membranes)	—	Available in two types: fast flow and standard

Source: Modified from Kole et al. (2011). Reproduced with permission of John Wiley & Sons.

HybridSPE® from Sigma. By using these plates, the resulting sample extracts are not only free of proteins, but also free of or with significantly reduced phospholipids. The sample extracts are readily available for the subsequent LC-MS analysis. In the case of using a HybridSPE PPT plate, once PPT occurs after the addition of acetonitrile (containing 1% formic acid), the sample extract passes through the HybridSPE packed bed. The packed bed consists of proprietary zirconia-coated silica particles. The zirconia sites exhibit Lewis acid (electron acceptor) properties that interact strongly with Lewis bases (electron donor). As mentioned above, phospholipids consist of zwitterionic phosphonate moieties (the polar head group) and a large hydrophobic tail (two fatty acyl groups that are hydrophobic). The phosphate group(s) on the phospholipids acts as a very strong Lewis base that interact strongly with zirconia atoms functionalized on the particle surface. Formic acid or other acids is a critical modifier in PPT via phospholipid removal PPT plate approach to improve the recovery of analytes of interest, particularly those acidic. The acid plays a critical role in preventing analyte retention without affecting phospholipids' retention on the packed bed (Ahmad et al. 2012).

Each of the commercially available PPT plates comes with standardized procedures recommended by the providers. The procedures include direct PPT on the plates (Pucci et al. 2009) or conventional PPT followed by loading the resulting supernatant onto the PPT plates (Asimakopoulos and Thomaidis 2015; Jiang et al. 2011; Zeng et al. 2010).

A representative example of PPT protocol using Sirocco PPT plate.

- A 200 µl aliquot of acetonitrile was added to each well of a Sirocco PPT plate with a 1-ml 96-well collection plate underneath.
- A 25 µl aliquot of IS working solution in acetonitrile : water (50/50, v/v) was added to each well except for the blanks, to which a 25 µl aliquot of acetonitrile : water (50/50, v/v) was added.
- A 25 µl aliquot of blank, zero, standard, or QC was added to the assigned well. The plate was covered and vortex-mixed for one minute on a pulse-vortex-mixer and then centrifuged for five minutes at ambient temperature.
- The filtrate in the collection plate was evaporated to dryness at a temperature of 45 °C under a flow of nitrogen. A 200 µl aliquot of reconstitution solution consisting of acetonitrile in water (10/90, v/v) with 0.1% formic acid was added to each well and the plate was vortex-mixed for five minutes.
- A 10 µl aliquot of the reconstituted extract was injected onto the LC-MS/MS system.

1.4.2 Liquid–Liquid Extraction (LLE)

LLE is another common sample preparation technique that has been widely used in LC-MS bioanalysis. 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 (Liu and Aubry 2013).

1.4.2.1 Mechanism of LLE and Extraction Recovery

The mechanism of LLE can be explained using a simple phrase "like dissolves like" (Li and Bartlett 2014). A solute can be dissolved best in a solvent that has a similar polarity to itself. Nonpolar compounds have higher solubility in organic solvents than in water. In contrast, ionic or polar compounds have higher solubility in aqueous solutions than in organic solvents. When two immiscible solvents are present in one system, the solute currently being dissolved in the solvent with less solubility will diffuse across the liquid–liquid interphase of the two immiscible solvents to enter the one in which the solute solubility is higher. When analyte molecules are extracted from the aqueous phase into the immiscible organic phase or vice versa in LLE, interactions take place between the analyte molecules and the solvent molecules. The predominant interactions in LLE are the following (Li and Bartlett 2014; Pedersen-Bjergaard et al. 2015):

- **Hydrophobic interactions:** The interactions between the nonpolar analyte(s) and nonpolar organic solvent or solvent mixture in a polar (aqueous, usually water) solvent. Hydrophobic interactions are nonpolar attractive interactions between hydrocarbon moieties and related nonpolar molecular elements.
- **Dispersion interactions:** The interactions between a relatively nonpolar electron-rich molecule and a polar (or charged) molecule. It is generally a weak attractive interaction. The polar molecules in the system cause

electron density change in the nonpolar molecules by making them slightly polar. In other words, the polar molecules induce dipole moment in the nonpolar but electron-rich molecules.

- **Dipole interactions:** The interactions between two molecules with permanent dipole moment due to attractive electrostatic forces, by which the positive end of one molecule is attracted by the negative end of another molecule. Both components have high permanent dipole moments.
- **Hydrogen bonding interactions:** Interactions between the hydrogen atom that is part of a polar bond (hydrogen-bonding donor) and an electronegative atom with a lone pair of electrons such as O and N (hydrogen-bonding acceptor). Hydrogen bonding interaction is another type of dipole interaction.

As discussed in Section 1.2.1, **partition ratio** (P) of a given analyte in two immiscible phases can be expressed as follows: $P = [C_{\text{Organic}}]/[C_{\text{Aqueous}}]$, where $[C_{\text{Organic}}]$ is the concentration of the un-ionized analyte in the organic phase (e.g., *n*-octanol) while $[C_{\text{Aqueous}}]$ is the concentration of the un-ionized analyte in the aqueous phase (e.g., water) at equilibrium.

The P value is constant for a given analyte under given conditions of the two-phase (aqueous vs. immiscible organic solvent) system. In LLE, equilibrium of the analyte(s) of interest between the two immiscible phases is normally established at the end of the extraction. The higher the P value of the analyte(s) of interest, the more efficiently it is extracted from the aqueous samples (e.g. plasma) into the organic solvent.

For neutral analyte(s), the REC can be maximized by optimizing the type of extraction solvent. The pH value of the sample matrix does not affect the extraction efficiency. However, for acidic or basic analyte(s), the situation is different. Both acidic and basic molecules tend to dissociate in aqueous solution. The degree of dissociation (or ionization) is largely dependent on the pH of the solution.

For acidic analyte(s) ($HA \leftrightarrow H^+ + A^-$), as only the uncharged fraction of the molecules can be extracted from the aqueous phase into the organic phase whereas the charged species will remain in the aqueous phase during LLE, the **distribution ratio** (D) can be re-defined according to the following equation:

$$D = \frac{P \times \left[H^+\right]}{\left[\left[H^+\right] + K_a\right]}$$

where K_a is the dissociation constant ($K_a = [H^+] \times [A^-]/[HA]$) and P is the partition ratio as discussed above. Apparently, the ionization of an acidic analyte increases with decreasing $[H^+]$ and increasing pH in the solution.

Similarly, the following equation can be applied for extraction of a basic analyte ($A + H^+ \leftrightarrow AH^+$), which partly dissociates (ionizes) in the aqueous sample:

$$D = \frac{P \times K_a}{\left[\left[H^+\right] + K_a\right]}$$

where K_a is the dissociation constant for the corresponding acid AH^+. Apparently, the ionization of basic analyte(s) increases with increasing $[H^+]$ and decreasing pH in the aqueous solution.

In summary, acidic analyte(s) are charged in a basic solution. Their charged form is generally soluble in water (aqueous samples) and, therefore, they will mainly remain in the basified aqueous samples. These molecules are less charged or become neutral in an acidic solution and, therefore, they can be easily extracted from acidified aqueous samples into organic solvent. In contrast, basic analyte(s) are charged in an acidic solution. Their charged form is generally soluble in water (aqueous samples) and, therefore, they will mainly remain in the acidified aqueous samples. These molecules are less charged or become neutral in basic solution and, therefore, they can be easily extracted from basified samples into organic solvent. Taking the above into consideration, biological samples have to be acidified or basified for good REC in LLE for acidic or basic analyte(s).

REC is an important indicator in demonstrating the efficiency of a LLE method. As discussed above, under optimal pH conditions (i.e. low pH for acids and high pH for bases), the partition ratio of the analyte(s) of interest can be used to estimate its REC. For a given molecule to be extracted under optimal pH conditions (at which point it is in nearly 100% neutral form) from a fixed volume of biological sample (V_{Aqueous}) and into a fixed volume of organic solvent (V_{Organic}), the REC can be expressed by the following equation:

$$REC = 100 \times \frac{\left[P \times V_{\text{Organic}}\right]}{\left[P \times V_{\text{Organic}} + V_{\text{Aqueous}}\right]}.$$

Clearly, the REC in LLE can be increased by increasing the volume of extraction solvent and by selecting proper extraction solvent with a higher partition ratio (P) for the analyte(s) of interest. In practice, both the volume of extraction solvent and solvent selection have to be considered concurrently when developing a LLE method for sample extraction.

The partition ratio varies from a compound to the other in a given mixture of two immiscible solvents (i.e. aqueous vs. organic). In addition, it is also dependent on the experimental conditions, including temperature and matrix components. Although such information may not

be readily available for the analyte(s) of interest in the intended organic solvent, the log *P*-value of the analyte(s) of interest should be considered as a reference for the possible extraction efficiency assessment in LLE. Theoretically, a compound with a higher log *P*-value can be more easily extracted by LLE than a compound with a lower log *P*-value because of the high partition ratio of the former. As a general rule, compounds with log *P*-values below 0 are very polar and difficult to extract with any organic solvent in LLE. Compounds with log *P*-values between 0 and 1 are relatively polar and not suitable for LLE. Compounds with log *P*-values above 1 are more hydrophobic, and they can be easily extracted with organic solvent in LLE (Li and Bartlett 2014; Liu and Aubry 2013; Pedersen-Bjergaard et al. 2015).

1.4.2.2 Solvent in LLE

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. Listed in Table 1.2 are the physicochemical parameters of some organic solvents commonly used in LLE.

Generally, for a solvent selected for extraction, its solubility in water should be low to avoid substantial amount of the solvent being dissolved into the aqueous sample. In other words, the organic solvent selected for LLE has to be immiscible with the aqueous samples, so that a sharp interface can be formed between the organic phase and the aqueous phase at the equilibrium at the end of LLE. Only when the two phases are physically separated can one of them be removed to achieve separation of the analyte(s) of interest from the unwanted matrix components. As can be seen from the table, all the solvents have positive partition coefficient (log *P*) values, meaning that they are all nonpolar and immiscible with water (or have very low solubility in water).

The volatility and density of the solvent are also important factors to consider in LLE. Solvent with a lower boiling point is preferred because this makes evaporation of solvent after extraction much faster than the one with a higher boiling point. Solvents with a lower density than water float on top of the aqueous phase in LLE. This makes it easier to transfer the organic layer by either pipetting or via freezing the aqueous phase followed by pouring into new tubes/wells. Examples of solvents with density less than water are *n*-hexane, methyl *tert*-butyl ether, *n*-butanol, butyl chloride, and ethyl acetate (Table 1.2). In contrast, when solvent with density higher than water is used, the aqueous phase floats on top of the organic phase in LLE. This makes it difficult in sample manipulation. Examples of solvents with density higher than water are chloroform and dichloromethane (Table 1.2).

In addition to the above general rules, an important factor in solvent selection in LLE is the target analyte itself. Listed below are some general rules to consider when choosing solvent for LLE for the analyte(s) of interest (Li and Bartlett 2014; Liu and Aubry 2013; Pedersen-Bjergaard et al. 2015):

- **Analyte(s) with no or very little functional groups (highly hydrophobic):** The primary interaction between the analyte(s) and solvent appears to be hydrophobic interactions. Therefore, the best solvents should be very nonpolar solvents, such as *n*-hexane.
- **Analyte(s) with low to medium polarity (hydrophobic to slightly hydrophobic):** The primary interaction between the analyte(s) and solvent appears to be dispersion interactions and/or dipole interactions mixed with hydrophobic interactions and/or hydrogen bonding interactions. A slightly polar solvent should

Table 1.2 Commonly used organic solvents in LLE and their physicochemical parameters.

Solvent	Log *P*	Polarity index	Water solubility (w/w, %)	Boiling point (°C)	Density (g ml^{-1})	Viscosity (cp 20 °C)	Dipole moment
n-Hexane	3.9	0.1	0.014	68.7	0.655	0.31	0.08
Chloroform	2.3	4.1	0.795	61.2	1.483	0.58	1.15
Dichloromethane	1.5	3.1	0.132	39.8	1.325	0.45	1.14
Methyl *tert*-butyl ether	0.9	2.5	5	53–56	0.735	0.27	1.66
Diethyl ether	0.98	2.8	7.5	34.5	0.713	0.24	1.15
Heptane	4.274	0.1	0.01	98.4	0.684	0.42	0
Ethyl acetate	0.7	4.4	8.7	77.1	0.897	0.46	1.88
n-Butanol	0.839	4.0	6.3	117.7	0.810	2.95	1.66
n-Butyl chloride	2.39	4.0	0.05	78.5	0.88	0.0045	1.90

be considered, but the solvent should not be too polar. For slight to medium polar analyte(s), solvents with high dipole moments, such as dichloromethane, methyl-*tert* butyl ether, and ethyl acetate, are efficient in extraction of these molecules from aqueous phase. For extraction of basic analyte(s) containing nitrogen, solvents with hydrogen-bonding donor properties, e.g. chloroform, may be employed. However, the higher density of the solvent than water makes it less favorable. Other solvents like butyl chloride or methyl-*tert* butyl ether can be used as alternatives. For acidic analyte(s) with high hydrogen-bonding donor properties, hydrogen-bonding acceptor solvents, such as methyl-*tert* butyl ether, should preferably be considered.

- **Analyte(s) with medium to high polarity (slightly hydrophilic to hydrophilic):** In general, LLE is not recommended unless the analyte(s) of interest can be extracted after derivatization (Zeng and Cao 2018) or under modified extraction conditions. For example, tiotropium is a quaternary amine, which is extremely polar and positively self-charged. However, tiotropium is in the form of halogenated ion-pair salt, i.e. tiotropium bromide. Dichloromethane was found to be the solvent for selective extraction of tiotropium bromide from biological samples. It was also found that adding potassium iodide to the sample mixture until its saturation can facilitate LLE. Furthermore, the added potassium iodine increases the density of aqueous layer, causing the dichloromethane layer to stay in the top, which is convenient for solvent transfer (Chi et al. 2016).

Although LLE can be achieved by using a single solvent, the use of solvent mixtures has been often. By mixing solvents, the polarity of the extraction solvent can be modified for an improved REC with minimized matrix effect. Typical examples are the addition of alcohols (such as 1-propanol or 1-butanol) or acetonitrile to relatively nonpolar solvents like methyl-*tert* butyl ether (Xue et al. 2004), addition of isopropanol to *n*-hexane (Jian et al. 2010a), a mixture of diethyl ether and dichloromethane (Yin et al. 2015; Zhang et al. 2009a), a mixture of ethyl acetate and *n*-hexane (Jian et al. 2013), and a mixture of ethyl acetate, heptane, and dichloromethane (Oiestad et al. 2009).

1.4.2.3 General Procedures in LLE

Similar to PPT, LLE of biological samples involves three major steps: (i) addition of additives, organic solvents, and/or IS, (ii) mixing of the two immiscible phases for extraction, and (iii) separation of the two phases followed by transfer, evaporation, and reconstitution (Jian et al. 2010a).

1.4.2.3.1 Addition of Solvents, Additive(S), and/or Internal Standard

A general rule in LLE is to use a minimum two volumes of a preferred/selected organic solvent for extraction. In some cases, a mixture of two solvents is employed for better REC (Xue et al. 2006a) as discussed previously. Prior to this step, the aqueous biological sample needs to be treated with a certain amount of an acid (e.g. acetic acid, formic acid) (Cheruvu et al. 2018), a base (e.g. ammonium hydroxide) (Li et al. 2007a, 2007c, 2013a), or a buffer to adjust the pH of the samples (Li et al. 2006). As mentioned above, pH adjustment of the aqueous sample prior to LLE is important for acidic and basic analytes. A general recommendation is to adjust the pH value in the sample at least two units from the pK_a-value of the analyte(s) of interest. Thus, acidic analyte(s) should be preferably extracted from samples that are acidified to a pH value of two units below the pK_a-value of the analyte(s). For basic compounds, the pH in the sample should be adjusted two units higher than the pK_a-value of the analyte(s). However, under strongly acidic or alkaline conditions, certain analyte(s) might be subjected to degradation. In addition, precipitation of matrix components may occur in biological fluids under extreme pH conditions. Therefore, care should be taken when using extreme pH values in LLE.

1.4.2.3.2 Mixing

After addition of additives, organic solvents, and/or IS, the sample mixture needs to be mixed thoroughly to ensure the highest and consistent extraction efficiency. LLE is a thermokinetic process that involves diffusion of the analyte(s) of interest from the aqueous phase to the organic phase, which may take a certain period of time. Therefore, under a fixed LLE condition such as solvents, additives, pH, and volumes, the highest extraction efficiency can only be achieved after the distribution equilibrium of the analyte(s) of interest between the two immiscible phases has been reached, i.e. when the amount of analyte crossing the interphase does not increase any more. To facilitate the diffusion of the analyte(s) of interest to reach its equilibrium between the two phases, vortex-mixing is usually applied. Since the aqueous biological samples are immiscible with the organic solvents, turbulence due to high-speed vortex-mixing creates very small droplets of both phases. The formation of the droplets significantly increases the interface area between the two phases, thus facilitating the diffusion of the analyte(s) of interest across from one phase to the other, thus shortening the time that is needed to reach equilibrium. In general, a 5- to 10-minute of vortex-mixing is typically needed for efficient LLE of biological samples by organic solvents.

1.4.2.3.3 Phase Separation

After the distribution of the analyte(s) of interest has reached equilibrium between the two phases, separation of the organic and aqueous phases has to be carried out in order to isolate the analyte(s) of interest from those unwanted in the sample matrix or remove the impurities of interest from those in the remaining phase for further processing. Centrifugation is the most common technique for phase separation in LLE. In general, a 5- to 10-minute of centrifugation at ~2000 × *g* is sufficient to achieve sharp phase separation. In some cases, an extended centrifugation at a higher speed and/or longer centrifugation time might be necessary. The relative position of organic vs. aqueous phase is dictated by the density of both phases. Most commonly used organic solvents, such as *n*-hexane, methyl-*tert* butyl ether, *n*-butanol, butyl chloride, and ethyl acetate, have densities lower than that of water. They will become supernatant after phase separation. In contrast, chloroform and dichloromethane have densities higher than that of water and they stay in the bottom layer after centrifugation. However, in some cases, by adding salt(s), such as potassium iodide to the sample mixture until its saturation can significantly increase the density of aqueous layer, causing the dichloromethane layer to stay in the top, leading to a convenient solvent transfer (Chi et al. 2016).

Upon centrifugation, phase separation can be easily fulfilled by transferring the wanted phase or removing the unwanted phase. However, care has to be taken not to disturb the interphase between the two phases. In the case where the top layer is organic, the tubes/wells can be frozen in acetone containing dry ice. When the bottom aqueous layer becomes frozen, the top organic layer can be easily poured into new tubes or transferred to new wells. However, when the analyte(s) of interest stay in the bottom layer containing chloroform or dichloromethane, phase separation might be a challenge unless the density of the aqueous layer can be manipulated by adding salts but without interfering the outcome of the LLE (Chi et al. 2016).

1.4.2.3.4 Direct Analysis or Evaporation of Sample Extract Followed by Reconstitution Prior to LC-MS Analysis

With the organic and aqueous phases separated from each other, the extraction of the analyte(s) of interest from the aqueous samples can be considered finished. If the analyte(s) of interest are present in the top organic phase after LLE, upon transfer, direct LC-MS analysis using a HILIC column may be feasible as the solvent used for LLE is generally compatible with acetonitrile which is a common starting mobile phase in HILIC-MS bioanalysis (Jian et al. 2010b). However, assay sensitivity may be a challenge if the evaporation and reconstitution is not applied (Song and Naidong 2006; Xue et al. 2006a).

On the other hand, if the analyte(s) of interest stay in the top layer of the aqueous phase when an organic solvent such as chloroform or dichloromethane is employed in LLE to remove unwanted matrix components, the analyte-containing aqueous phase can be directly subjected to LC-MS analysis using RP column. Again, assay sensitivity may be a challenge in direct LC-MS bioanalysis. If needed, evaporation and reconstitution should be applied to the aqueous sample extracts to achieve the needed assay sensitivity (Onorato et al. 2010).

In most applications, direct RPLC-MS bioanalysis of the organic extract obtained from LLE is not feasible. This is because organic solvents are strong eluents in reversed-phase liquid chromatography and their presence in the sample extract to be injected will dramatically weaken the analyte retention on the column. Furthermore, analyte concentrations in the final extract are generally lower than those in the original samples as a large volume of organic solvents, e.g. more than twofold of the aqueous sample size, are usually used in LLE. Without proper concentration of the analyte(s) of interest, the needed assay sensitivity may not be achievable. Therefore, evaporation of organic solvent of the resulting supernatant and reconstitution of the resulting sample residues are commonly employed in LC-MS bioanalysis following LLE. In this step, the organic solvent in the sample extract is evaporated for complete dryness under a steam of nitrogen or by vacuumed centrifugation. The resulting sample residues are reconstituted in the reconstitution solution, which can be the starting mobile phase or a solution with polarity and pH similar to the starting mobile phase. The volume of the reconstitution solution added should be adjusted based on the needed assay sensitivity and the available instrument platform. Analyte enrichment may be achieved by decreasing the volume of the reconstitution solution in this step. Upon vortex-mixing, followed by centrifugation to push the possible insoluble residues to the bottom of the assay vial/well, the reconstituted sample extracts can be subjected to LC-MS analysis.

1.4.2.4 Application of LLE in LC-MS Bioanalysis

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.

1.4.2.4.1 Forward Extraction

The **forward extraction** generally refers to a LLE procedure by which the analyte(s) of interest stay in the organic layer after extraction. After discarding the aqueous phase, the resulting organic phase is subjected to direct HILIC-MS analysis or further processing (evaporation and reconstitution) as discussed above prior to LC-MS analysis. This approach has been widely used in many applications for nonpolar analyte(s).

A representative LLE protocol (for the analysis of NVP-1 by Li et al. 2013b)

- An aliquot of 100 µl of blank plasma (for matrix blanks and zero samples), calibration standard, QC sample, and study sample was added to the appropriate well of a 96-well assay plate.
- A 25 µl aliquot of the IS working solution in 50% aqueous methanol (v/v) was added to each well except to the matrix blanks, where a 25 µl aliquot of 50% aqueous methanol (v/v) was added.
- A 100 µl aliquot of a sodium carbonate (100 mM) solution in water was added to each individual well, and the plate was vortex-mixed for about 0.5 minute on a pulse-vortex-mixer with a motor speed setting of ~65 units.
- A 500 µl aliquot of methyl *tert*-butyl ether was added to each well, and the plate was covered and vortex-mixed for ~10 minutes using the same vortex-mixing setting as above.
- The sample plate was centrifuged at ~4000 × *g* for ~10 minutes at 4 °C. The supernatant (400 µl) from each well was transferred via a TomTec Quadra 96 system (Hamden, CT, USA) to the corresponding well in a 1 ml, 96-well plate. This was followed by evaporation of the supernatants to dryness under a stream of nitrogen at 45 °C. The sample residues were reconstituted with a 100 µl volume of reconstitution solution (50% aqueous methanol, v/v).
- After a brief vortex-mixing and centrifugation, a 10 µl volume of the reconstituted sample extracts was injected onto the LC-MS/MS system.

1.4.2.4.2 Backward Extraction

In situations that require higher assay selectivity or specificity for the analyte(s) of interest in the intended sample matrix, a single forward extraction may not be sufficient as interfering matrix compounds can also be extracted into the organic solvent. In such cases, it may be necessary to perform a backward extraction, by which the organic extract from the first extraction (done by forward extraction) is extracted with a new aqueous solvent with suitable pH. Although the use of backward extraction is less common as compared to forward extraction, it can be very useful and practical in leaving the unwanted hydrophobic interference molecules in organic layer while extracting the analyte(s) of interest into the aqueous phase for LC-MS analysis (Bolden et al. 2002; Constanzer et al. 1997) or extracting the interfering polar compounds into the aqueous phase while leaving the analyte(s) of interest in the organic phase for further processing prior to LC-MS analysis (Buhrman et al. 1996).

In a study for the analysis of dextromethorphan (DEX) and dextrorphan (DOR) using LC-MS, Bolden et al. (2002) reported a semiautomated backward extraction method. The procedure started by extracting DEX and DOR using diethyl ether in a 96-well rack of plastic tubes via the Microlab liquid handling system. Following extraction, the tubes in the 96-well rack were placed in a dry ice–acetone bath to freeze the plasma layer. After the plasma layer was frozen, a portion of the ether layer (400 µl out of 600 µl) was transferred (using the Microlab) to a 96-well rack of clean polypropylene tubes that already contained 200 µl of 1% formic acid. The DEX and DOR in the samples were back-extracted into the acidified water. Finally, the Microlab was used to transfer a portion (150 µl) of the acidified extract to clean autosampler vials prior to LC-MS analysis (Bolden et al. 2002).

In a separate study published by Buhrman et al. (1996), a combination of multiple steps of forward and backward extraction was developed and validated for the analysis of SR27417, a platelet-activating factor receptor antagonist, in human plasma. By this procedure, 1.5 ml of 0.1 M sodium carbonate and 7 ml of ethyl acetate were added to a 1 ml plasma sample, which was followed by rotary extraction and centrifugation. The resulting organic layer was mixed with 1 ml of 0.025 M sulfuric acid, followed by rotary extraction and centrifugation. The organic layer was removed and discarded. To the aqueous layer, 1.5 ml of 0.1 M sodium carbonate and 5 ml of ethyl acetate were added, followed by rotary extraction and centrifugation. The resulting organic layer was collected and evaporated. The resulting residues were reconstituted in 200 µl of 50/50 acetonitrile/water prior to LC-MS analysis. The final ethyl acetate extract, which

contained the fewest co-eluting components, had the highest ion intensity for the analyte, with curve range of 5–2000 pg ml^{-1} and accuracy ranging from −11.6 to 2.61% of the nominal concentrations. In contrast, the liquid–liquid method using hexane alone had poor accuracy and precision below 20 pg ml^{-1} (Buhrman et al. 1996).

A representative backward LLE protocol (for the analysis of dorzolamide and its de-ethylated metabolite by Constanzer et al. 1997).

- To 1.0 ml of samples, a 0.5 ml of 10% TCA (for PPT) was added, followed by vortex-mixing for one minute.
- The samples were then buffered to pH 8.0 with 5 ml of 0.2 M phosphate buffer followed by LLE with 10 ml of ethyl acetate : toluene : isopropanol at 50/40/10 (v/v/v).
- The tubes were capped, rotate-mixed for 20 minutes, and centrifuged for 5 minutes.
- The organic layer was transferred to a 15-ml centrifuge tube containing 0.2 ml of phosphoric acid, followed by rotate-mixing for 15 minutes and centrifugation for 5 minutes.
- The organic layer was aspirated and discarded.
- The acidic layer containing the analyte(s) was additionally washed with 2 ml of hexane, followed by rotate-mixing for 10 minutes and centrifugation for 5 minutes. The organic layer was aspirated and discarded.
- 50 µl of the acidic layer was injected onto the LC-MS/MS system.

1.4.2.4.3 Double Extraction

This approach is often referred to as "two-stage forward extraction." In the first stage of extraction, a very nonpolar solvent, e.g. hexane, is employed to extract hydrophobic interfering compounds from the sample matrix and the extract is discarded. This is followed by extraction using a moderately hydrophobic solvent, e.g. acetyl acetate, to extract the analyte(s) of interest. In a study published by Hou et al. (2012), a double LLE method was employed to prepare brain tissue homogenate samples for the quantitative determination of a novel anti-Parkinson's disease candidate drug FLZ. Endogenous hydrophobic interferences were initially extracted by *n*-hexane, while the analyte remained in the sample matrix. With the hexane layer removed and discarded, the aqueous phase was further extracted by using ethyl acetate, yielding a clean sample extract with high purity and low matrix effects (Hou et al. 2012).

A typical example of double LLE protocol (for the analysis of an anti-Parkinson's disease drug candidate drug FLZ by Hou et al. 2012).

- To 0.5 ml of the brain homogenate (tissue : methanol 1 : 5) were added 20 µl of the IS working solution.
- The mixture was vortex-mixed for 20 seconds and evaporated to dryness with a gentle stream of nitrogen gas at 36 °C and the sample was reconstituted with 100 µl of 80% methanol.
- Then, 400 µl of water was added to the reconstitution (to facilitate LLE).
- After adding 1.5 ml of *n*-hexane, the mixture was vortex-mixed for 90 seconds and centrifuged at 1721 × *g* for 10 minutes, then the *n*-hexane layer was removed and discarded.
- The remainder was extracted with 1.5 ml ethyl acetate by vortex-mixing for 90 seconds. After centrifuging at 1721 × *g* for 10 minutes, the organic layer was transferred into another test tube and evaporated to dryness again.
- The residue was reconstituted in 50 µl 80% methanol by the ultrasonic method for 180 seconds and centrifuged at 16 654 × *g* for 10 minutes.
- Finally, 5 µl supernatant was injected onto the LC-MS/MS system.

As a sample preparation method with moderate selectivity, LLE is ideal for nonpolar to moderately polar analyte(s) with favorable distribution in water-immiscible organic solvent. However, when developing a bioanalytical assay for more than one analyte, particularly a drug plus its metabolites, a fine-tuning of the LLE conditions may be difficult to achieve acceptable recoveries for both molecules as the metabolite tends to be more polar than the parent molecule. In this case, other sample preparation procedures such as PPT or SPE should be considered from the very beginning of the assay development.

Depending on the assay needs, efforts should be made to balance REC and selectivity (matrix background removal) in LLE. When matrix interference is a concern, REC may be sacrificed for selectivity. In this case, use of a stable isotope labeled IS should be preferably used to compensate for the variation in sample preparation under nonoptimal REC. Nevertheless, efforts should be made to ensure a consistent REC across the low, middle, and high concentration levels.

1.4.2.5 Other Formats of LLE

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 (Tang and Weng 2013; Valente and Rodrigues 2015).

SALLE employs water-miscible organic solvent (such as acetonitrile) and high concentration of salts, such as magnesium sulfate, potassium carbonate, sodium chloride, or ammonium acetate, to induce phase separation of the water-miscible organic phase (Zhang et al. 2009b). Compared to conventional LLE, SALLE has a broader application, covering molecules from low to high lipophilicity with a better analyte recovery. One apparent drawback of SALLE is the higher matrix effect as compared to conventional LLE. This is because SALLE extracts tend to contain more endogenous compounds. More details of SALLE can be found in Chapter 5.

SLE is an alternative LLE that is carried out on the high-surface interface of an inert solid support in a 96-well format. Briefly, biological samples are mixed with aqueous buffer and then loaded onto the solid support. Subsequently, water-immiscible solvent is added, passing through the solid support to extract the analyte(s) of interest. The extraction mechanism involved is mainly a partition of the analyte(s) between the organic solvent and the absorbed aqueous phase on the solid support. The advantages of SLE include no emulsion formation, easy automation, and high extraction efficiency (Wu et al. 2010). More details of SLE can be found in Chapter 6.

1.4.3 Solid-phase Extraction (SPE)

SPE is a powerful sample preparation technique that has been used for decades for the selective extraction and enrichment of trace analyte(s) of interest in various biological samples. The mechanism of SPE is similar to that of liquid chromatography, 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. By SPE, an liquid sample, after a proper treatment (dilution, pH adjustment, and/or addition of the IS), is loaded onto preconditioned/equilibrated column/cartridge/plate packed with appropriate sorbent materials; the analyte(s) of interest is retained by interacting with the sorbent materials (stationary phase) through different interaction mechanisms; the interfering matrix components either directly pass through the column/cartridge/plate during the loading step or are washed away during the wash steps with a proper solvent; the analyte(s) of interest is subsequently eluted from the column/cartridge/plate with a suitable elution solvent. The collected eluate is either directly subjected to LC-MS analysis or subjected to drying process to evaporate organic solvent. This is followed by reconstitution or starting mobile phase with a proper solvent prior to LC-MS analysis (Li and Bartlett 2014; Liu and Aubry 2013; Pedersen-Bjergaard et al. 2015).

With SPE, some issues associated with PPT and/or LLE can be prevented or minimized. These issues include but are not limited to (i) matrix effect in PPT and (ii) poor recovery due to incomplete phase separations in LLE. SPE products are commercially available from a variety of vendors in a wide variety of chemistries, sorbents, sizes, and formats to accommodate different sample sizes and bioanalytical applications.

1.4.3.1 SPE Stationary Phases (Sorbents)

SPE sorbent materials are commonly irregular-shaped rigid particles, with nominal sizes ranging from 8 to 70 µm (most commonly 40–60 µm), which allows for reasonable flow rates of the samples or solutions through the sorbent bed during loading, washing, and elution. Smaller particles of the SPE sorbent materials require higher pressure for processing. The exception is for the sorbent materials in plate formats. Because of the very short bed paths of such formats, the total resistance due to the small particle sizes is less than those of the typical sorbent bed formatted into column or cartridge.

Most SPE materials are fully porous in nature. The smaller porosity of the sorbent materials is associated with a higher total surface area and, consequently, a higher capacity per mass of the sorbent materials for active adsorption. Typically, the capacity of a given sorbent material in SPE is about a few percent (%) retained compounds per mass of sorbent (for example, 5 mg of compounds are retained by 100 mg of sorbents).

SPE sorbents can be broadly divided into two major classes, i.e. silica-based sorbents and polymer-based sorbents.

- **Silica-based sorbents:** By definition, silica-based sorbents were made by bonding different functional groups to silica, although base silica also plays an active role in some SPE workflows. Many commercially available silica-based sorbents are named using acronyms. Those acronyms describe the primary character of the respective functional group on the silica, including C18, C8, C6, C4, C2, phenyl, cyclohexyl, cyanopropyl, aminopropyl, diethyl amine, diol, propylsulfonic acid, phenylsulfonic acid, propyl carboxylic acid, and propyltrimethyl amine. C18-based SPE columns/cartridges/plates are very popular and most hydrophobic. Because of their strong hydrophobicity, they give strong retention to many compounds. However, it may not give the best selectivity. In order to obtain a better selectivity in SPE, one can use SPE column/cartridge/plate with less hydrophobic phases, such as C8-columns, which are also very popular.

Regardless of which bonding chemistry is used in making silica-based SPE sorbents, the number of unreacted (or residual) silanol species that remain on the sorbent surface is still high (>50% for a typical C18-bonded phase) due to the steric factors involved in binding the functional groups. These silanol species are capable of interacting with analyte molecules, often leading to unexpected retention effects and poor extraction efficiency. It is also well known that at pH ~4 of a solution, about 50% of the silanol species on the silica surface are ionized. Therefore, the solution pH can be adjusted to ensure ion suppression (pH ≤ 2) or full ionization (pH ≥ 6) of the silanol species. It should be noted that silica sorbents may be subjected to hydrolysis when exposed to extreme pH for an extended period of time. At pH ≤ 2, the bonded surface species are susceptible to hydrolysis and efficiency of the sorbent can be drastically reduced. At pH ≥ 8, the silica itself is susceptible to hydrolysis and the sorbent will rapidly deteriorate upon extended exposure (Li and Bartlett 2014; Liu and Aubry 2013; Pedersen-Bjergaard et al. 2015).

- **Polymer-based sorbents:** Various polymer-based sorbents are commercially available, covering a wide range of polarities and chemistries. The most common polymer sorbents are based on styrene–divinyl benzene copolymers. Further modifications via amination or sulfonation help create ion-exchange polymer sorbents. Incorporation of some polar functional groups makes the sorbents water-wettable, which offers additional possibilities for retention mechanisms. An important advantage of the polymer-based stationary phases is that their chemical properties do not change even if they dry out during the SPE procedure, and the initial conditioning step used for silica-based SPE column/cartridge/plate may not be required. Furthermore, compared to silica-based sorbents, most polymeric sorbents are stable over the entire pH range (Li and Bartlett 2014; Liu and Aubry 2013; Pedersen-Bjergaard et al. 2015).

1.4.3.2 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. It is not included in the current discussion as it is rarely applied in sample extraction for biological matrix such as plasma and urine.

- **Reversed-phase SPE:** It employs nonpolar stationary phases of the silica- or polymer-based sorbents, which retain most molecules with any hydrophobic character. The common functional groups of silica-based nonpolar sorbents include but are not limited to C18, C8, C6, C4, C2, C1, phenyl, cyclohexyl, and cyanopropyl. Among these groups, the short-chain one (e.g. C1, C2) is less retentive, but the long-chain one (e.g. C18) is highly retentive. Reversed-phase SPE is considered the least selective retention mechanism when compared to other type SPEs. Because of this, it is very useful for extracting analytes that are very diverse in structure within the same sample.
- **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:
  - **Strong cation exchange (SCX) SPE:** The sorbents typically contain sulfonic acids as the ionic groups for ion-exchange. The sulfonic acids have very low pK_a values and are negatively charged almost in the entire pH range.
  - **Weak cation exchange (WCX) SPE:** The sorbents often contain carboxylic acids as the ionic groups. The carboxylic acids are weak acids with pK_a values around 4.5–5.0 and are ionized only in parts of the pH range. If pH in the SPE column/cartridge/plate is adjusted to above pH 3, the ion exchanger is gradually turned "on." In contrast, if pH is reduced well below pH 3, the ion exchanger is turned "off."
  - **Strong anion exchange (SAX) SPE:** The sorbents typically contain quaternary amine moieties as the functional group. The quaternary amines are positively charged in the entire pH range.
  - **Weak anion exchange (WAX) SPE:** The sorbents often contain secondary or tertiary amines as the functional groups for ion-exchange. The secondary or tertiary amines are weak bases with pK_a values of ~10. Therefore, by adjusting pH > 10, the ion-exchangers are gradually turned "off." In contrast, if pH is adjusted to well below pH 10, i.e. pH 8 or lower, the ion-exchangers is turned "on."
- **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. The hydrophobic groups can be short chain (e.g. C2) which is less retentive or long chain (e.g. C18)

which is highly retentive. Ion-exchangers can be either cation- or anion-oriented. Mixed-mode SPE allows for compound retention by both the ionic and hydrophobic interactions.

1.4.3.3 General SPE Workflows

Technically, SPE is a form of low-pressure chromatography that can be carried out either off-line or online with the LC-MS system. When developing a SPE-based sample preparation method for LC-MS bioanalysis, in addition to physicochemical properties of the analyte(s) of interest, parameters that need to be considered include (i) the type and amount of sorbent, (ii) the sample volume (considering the required volume for initial analysis, repeat analysis, and incurred sample reanalysis), and (iii) loading, washing, and elution conditions (time, volume, and composition).

Similar to LC columns, a variety of SPE products are commercially available in various formats. These include but are not limited to single-use cartridge, multiple-well plate (96-well, 384-well), and online SPE column from some major vendors, including Waters (www.waters.com), ThermoFisher Scientific (www.thermofisher.com), Agilent (www.agilent.com), Biotage (www.biotage.com), Phenomenex (www.phenomenex.com), Sigma-Aldrich (https://www.sigma-aldrich.com). In general, each specific SPE product is supplied with a recommended procedure or method protocol from the vendor. These procedures or protocols should be considered a starting point to method development of the intended SPE method for the analyte(s) of interest. The overview, detailed mechanisms, and associated workflows of various SPE in sample preparation can also be found from CHROMacademy website (www.chromacademy.com).

1.4.3.3.1 Reversed-phase SPE

Reversed-phase SPE is a common approach for extracting relative nonpolar analyte(s) from aqueous sample solutions.

- **Mechanism of extraction:** Hydrophobic interactions between the analyte and the stationary phase. Retention of the analyte(s) of interest increases with increasing hydrophobicity of the analyte via van der Waals forces, which is primarily between hydrogen–carbon bonds in the stationary phase and hydrogen–carbon bonds in the analyte molecules. This interaction is promoted in the aqueous phase but suppressed or disrupted in the organic phase.
- **Applicable analyte(s):** Compounds with log*P* values >1.0, most commercially available silica-based or polymer-based reversed-phase SPE can be considered. However, for compounds with log*P* values ranging from −1.0 to 1.0, modified polymer-based reversed-phase SPE should be preferably considered as the polar modifications on polymer-based SPE sorbent materials allow for increased polar selectivity as compared to the silica-based C18 or C8 materials; for compounds with log*P* values <−0.1, their retention on reversed-phase SPE is expected to be poor, reversed-phase SPE is generally not recommended.
- **Sample pretreatment:** Samples need to be diluted with a proper buffer or water (for neutral compound). For ionizable compound, pH adjustment is important as the ionization state of the analyte(s) of interest can drastically change its retention and elution characteristics on a given RP SPE sorbent. A general rule is to adjust the sample pH to 2 pH units above (for basic compound) or below (for acidic compound) the pK_a value of the compound to ensure the compound is neutralized prior to being loaded onto SPE column/cartridge/plate. In addition to commonly used buffers, some common reagents, such as 2% formic, 2% TCA, 2% acetic acid, TFA, phosphoric acid, or ammonium hydroxide, $NaHCO_3$, Na_2CO_3, can be employed for sample treatment.
- **Sorbent conditioning and equilibration:** Sorbent conditioning is to "activate" or "wet" the SPE sorbent to ensure consistent interaction between the analyte(s) of interest and the functional groups of the SPE sorbents. The most common conditioning method involves application of one to two volumes of water-miscible organic solvent (typically methanol or acetonitrile) to reversed-phase sorbents. This is followed by equilibration of the SPE sorbent by introducing a solution similar to the sample loading in terms of solvent strength and pH in order to maximize retention of the analyte(s) of interest to the SPE sorbents. One to two volume of buffer (the same as used in sample pretreatment) or water (for neutral compound) is a common choice for equilibration of the reversed-phase sorbents.
- **Sample loading:** The key element of sample loading is the linear velocity when the sample passes through the SPE sorbents to ensure optimal retention. For off-line application, the samples are often allowed to flow through by natural gravity to ensure sample contact time for the analyte(s) of interest with the stationary phase. The aqueous sample can be loaded directly onto the equilibrated reversed-phase SPE column/cartridge/plate. The nonpolar nature of the analyte results in strong retention by hydrophobic interactions with the stationary phase of the SPE sorbents.
- **Wash:** Interference compounds in the biological sample matrix are often co-retained on reversed-phase SPE column/cartridge/plate with analyte(s) of interest after sample loading. The goal of washing is to selectively remove unwanted matrix components, while leaving the analyte(s) of interest retained on the SPE sorbents. During the washing step, pure water or buffer is generally used first to remove water-soluble interferences (e.g. polar compounds or salts) that have

no affinity for the SPE sorbents. This is followed by the use of a solvent, typically aqueous methanol with higher elution strength than water or buffer to clean the sample as much as possible but without washing away the analyte(s) of interest. This will require some level of evaluation and/or optimization, during which the strength of wash solvent is increased stepwise by increasing the percentage of organic solvent (e.g. methanol) in the washing solution and the analyte(s) of interest, if any, in the washings is analyzed to identify and/or confirm the optimal strength of the wash solvent. In general, the higher log*P* value of the analyte(s) of interest, the stronger its interaction with the reversed-phase SPE sorbent materials and the stronger wash solvent should be employed. A good estimate is that every unit increase in log*P* value of the analyte(s) of interest will loosely correspond to an increase in 10% in methanol (in water) in the wash solution. For example, testosterone has a log*P* value of 3.37, use of ~35% of methanol in wash solvent can lead to an optimal recovery of the analyte with minimum background interference. For an efficient optimization of wash solution, a solvent with its strength around the estimated percentage (e.g. 35% methanol) can be a good starting point by bracketing it with ±10% (i.e. 25, 35, and 45%) of methanol in water (Rummel 2012).

- **Elution:** Elution is to disrupt all of the hydrophobic interactions between the analyte(s) of interest and the functional groups of the reversed-phase SPE sorbents with an organic solvent (e.g. methanol or acetonitrile) or solvent combination. Otherwise, partial elution may occur. Partial elution is often associated with irreproducible LC-MS results and poor and inconsistent REC of the analyte(s) of interest. In general, a higher log*P* value compound requires a stronger solvent or solvent combination in elution in order to obtain the highest REC with the lowest elution volume. Therefore, in reversed-phase SPE, the solvent with lower polarity is a stronger elution solvent. In other words, choosing a more nonpolar solvent leads to better results. If the analyte(s) are basic or acidic compounds, pH of the elution solvent should be adjusted to charge the analyte(s), which further reduces the hydrophobic interactions. In this case, use of weaker elution solvents and/or reduced elution volumes may be feasible. In practice, a consistent and low flow rate during elution is commonly associated with an improved recovery and reproducibility. The elution step is considered an additional opportunity for sample cleanup. Use of a very strong elution solvent often results in elution of interferences along with the analyte(s) of interest. Thus, elution step should be performed with the weakest solvent possible that provides complete elution of the analyte(s) of interest but with no or minimum interfering compound in the final eluate. In this regard, optimization of the eluent solution should be carried out by decreasing the strength of the elution solvent stepwise from high (e.g. 100% methanol) to low and monitoring both recovery and matrix effect in the eluate (Rummel 2012).
- **Direct LC-MS analysis of eluate or evaporation of the eluate followed by reconstitution of the resulting residues prior to LC-MS analysis:** In reversed-phase SPE, the resulting eluate may be subjected to direct LC-MS analysis if the eluate has less organic than the starting mobile phase in reversed-phase LC-MS or has more organic than the starting mobile phase in HILIC-MS analysis. However, in many applications, post-treatment of the eluate is necessary to evaporate the organic solvent and reconstitute the resulting residues using the starting mobile phase prior to reversed-phase LC-MS analysis.**

A typical sample preparation protocol using a RP SPE plate (for the analysis of goserelin, an agonists that stimulate the pituitary gland, using LC-MS by Kim et al. (2010)).

- A 500 µl volume of rabbit plasma sample was placed in a 2-ml Eppendorf tube, and 50 µl of IS working solution and 50 µl 3 M hydrochloric acid were added, and then the mixture was vortexed for 30 seconds.
- The SPE cartridge was pretreated with 1 ml methanol, followed by 1 ml deionized water.
- The sample mixture was gently loaded onto the SPE cartridge and left for 30 seconds. The SPE cartridge was washed with 1 ml deionized water and then dried for 60 seconds before elution with 1 ml methanol.
- The eluate was collected in a clean microtube and was evaporated to dryness at 40 °C under a stream of nitrogen.
- The dry residue was reconstituted with 100 µl mobile phase A (0.05% acetic acid in deionized water/acetonitrile 85/15, v/v) and centrifuged at 13 000 rpm for 10 minutes.
- The clear supernatant was transferred to an autosampler vial, and 20 µl of the supernatant was injected onto the LC-MS/MS system.

1.4.3.3.2 Ion-exchange SPE

Ion-exchange SPE is a common approach for extracting ionizable analyte(s) from aqueous sample solutions.

- **Mechanism:** Electrostatic (ionic) interactions of charged functional groups of the analyte(s) to oppositely charged functional groups on the SPE sorbents. Compared to hydrophobic interactions employed in

the reversed-phase SPE, ion exchange via electrostatic (ionic) interactions is a much stronger retention mechanism that allows for the use of more aggressive wash steps for sample cleanup. It is especially ideal for removing hydrophobic interfering compounds with a strong organic wash. If the analyte(s) of interest are retained by cation or anion exchange, 100% methanol, acetonitrile, or other solvent can be used as the organic wash without eluting the analyte(s) of interest. **Note:** Because the kinetic exchange processes between the functional groups of the analyte(s) of interest and the functional groups of the SPE sorbents in ion-exchange SPE are considerably slower than that for reversed-phase SPE, flow rates should be generally low.

- **Applicable analyte(s):** Compounds that can be positively or negatively charged. Strong cation exchangers (e.g. aliphatic sulphonic acids) are permanently negatively charged in aqueous solution and can be used to extract bases with a wide range of pK_a values. In contrast, strong anion exchangers (e.g. aliphatic quaternary amines) are permanently positively charged in aqueous solution and can be used to extract acids with a wide range of pK_a values. Weak cation exchangers (e.g. aliphatic carboxylic acids, pK_a typically 4.5–5) can be used for the extraction of bases (pK_a 9 and above) as long as there are 4 pH unit difference between bonded phase and analyte pK_a values. Similarly, weak anion exchangers (e.g. secondary or tertiary amines) can be used for extraction of acids. The 4 pH rule is an important factor that is often overlooked when developing an ion-exchange SPE method. As discussed earlier, for an analyte with an ionic group, in order to ensure it is either totally neutral or charged, the pH of the sample matrix/solution must be adjusted to a minimum of 2 pH units away from the pK_a value of the ionic group. This might be an issue with ion-exchange SPE. In the case where an anionic sorbent (cation exchange SPE) with a pK_a value of 5.0 is used to extract an analyte with a pK_a value of 7.0. Based on the 2 pH unit rule, in order to ensure that the analyte is fully charged, the pH of the sample matrix must be adjusted to 5.0 or lower. However, in order to ensure the SPE sorbents are also fully charged, the pH of the sorbent environment must be adjusted to pH 7 or higher. Apparently, the two requirements cannot be met at the same time unless the difference in the pK_a values between anion (sorbents or analyte) and cation (analyte or sorbents) counterparts are 4 or greater in ion-exchange SPE. Furthermore, the cation (sorbent or analyte) must have pK_a values higher than the anion (analyte or sorbents). Taking the above into account, understanding the pK_a of both the analyte(s) of interest and the sorbents will definitely help to decide on what kind of ion-exchange SPE sorbent should be chosen for the intended analyte(s).
- **Sample pretreatment:** Based on the pK_a values of the analyte(s) of interest, sample can be diluted with a buffer of appropriate pH to ensure the functional groups of the analyte(s) are positively or negatively charged so that the analyte(s) can be retained by cation or anion exchange. For basic compounds, the pH of the sample needs to be adjusted to 2 pH units below the pK_a. This turns the positive charge of the analyte "on" so that they can be retained by cation exchange. In contrast, for acidic compounds, the pH of the samples needs to be adjusted to 2 units above the pK_a to negatively charge the molecules. This turns the negatively charge of the analyte "on" so that they can be retained by anion exchange. It is recommended to adjust the pH of the sample matrix with a strong acid or base or strong buffer. By doing so, the possible increase in the ionic strength of the sample matrix can be minimum. For example, it is most likely to add a much smaller quantity of hydrochloric acid than acetic acid to drop the pH of a sample when an amine analyte is to be extracted using cation ion-exchange SPE. Of course, using a strong acid or base can be a concern of the stability of the analyte(s) of interest during sample processing. Therefore, necessary evaluation of the analyte stability under such an environment should be performed prior to implementation. In addition, some samples (e.g. urine) contain high concentration of salts, a large-scale dilution may be necessary to reduce the ionic strength prior to application of an ion-exchange SPE. Alternatively, a reversed-phase SPE should be applied first to remove the salts prior to the ion-exchange SPE procedure.
- **Sorbent conditioning and equilibration:** Condition the ion exchange SPE cartridge/plate/plate with one to two sorbent bed volumes of methanol or acetonitrile. This is followed by a few bed volumes of pure water prior to equilibration, which is carried out with a buffer or a salt solution containing a counter ion with affinity for the SPE sorbents lower than the analyte(s) of interest. For pH adjustment of the sorbents, it is necessary to raise the pH to a minimum of 2 pH units above the pK_a value for the cation sorbents or lower the pH to a minimum of 2 pH units below the pK_a of the anion sorbents. It is also important that the chemistry environment of the sorbents (solvent/ionic strength) should be similar to that of the sample as much as possible to ensure the extraction environment does not alter during sample loading.
- **Sample loading:** It is important that both the ion exchanger and the analyte(s) remain charged in the loading process to promote ionic interactions and retention. In the loading step, the pretreated samples can be loaded to the pretreated column/cartridge/plate slowly to ensure optimal retention without breakthrough. A

longer residence time is necessary to achieve a reasonable analyte orientation relative to the sorbent surface.

- **Wash:** Ion exchange sorbent can be washed using solvents with certain pH, ionic strength, or an alternative sorbent counter ion to remove interfering species. The initial wash can be the same solution used for sample pretreatment. This can be followed by 100% organic solvent to remove more hydrophobic interferences. The pH of the wash solvent can be adjusted to remove more interfering components by neutralizing the charge of these molecules. However, adequate control of pH and ionic strength is necessary to ensure the analyte(s) of interest and the sorbent surface remain charged to prevent premature elution. Therefore, washing step should be optimized by washing with several solvents at various pH values and analyzing the washings for evidence of premature elution.
- **Elution:** Elution is carried out through interrupting the ionic interactions between the analyte(s) of interest and the sorbents by (i) neutralizing either the analyte(s) or sorbents, (ii) using high ionic strength, or (iii) using a strong counter ion. The most common elution strategy is by pH manipulation, i.e. eluting with basic organic solvent at least pH 2 units above the pK_a of basic analyte or eluting with acidic organic solvent at least pH 2 units below the pK_a of acidic analyte(s). This turns the charge of the molecules "off," i.e. neutralization, and disrupts the ion exchange retention, releasing the compounds from the SPE sorbents. It is worth noting that neutralization of the analyte(s) of interest is more desirable than neutralization of the sorbents. Neutralization of SPE sorbents might release other unwanted compounds that are retained during the ion-exchange SPE process and these compounds might interfere with LC-MS. In addition, using high ionic strength or a strong counter ion for SPE elution can be a concern of interference with LC-MS detection in some cases.
- **Eluate:** Post-treatment of the eluate is often necessary to evaporate the organic solvent and reconstitute the resulting residues in the mobile phase prior to LC-MS analysis.

A typical sample preparation protocol using strong cation-exchange (SCX) SPE plate (for the determination of dopamine in human plasma by Zhang et al. 2016).

- To each sample, 10 µl of IS working solution was spiked before extraction.
- 200 µl of plasma sample was diluted with 400 µl of 0.1 N HCl and then loaded onto a previously conditioned SCX cartridge.
- The cartridge was washed with 0.4 ml of 0.1 N HCl and 1 ml of acetonitrile.
- The analyte was then eluted using 1 ml of acetonitrile–propionic anhydride (90/10, v/v) with 2% (v/v) pyridine.
- The eluent was incubated in an oven at 60 °C for 20 minutes, evaporated to dryness under nitrogen at 60 °C, and reconstituted with 100 µl of acetonitrile–water (10/90, v/v).
- Each sample was centrifuged at 18 000 × *g* for 15 minutes before injection.

A typical sample preparation protocol using strong anion-exchange (SAX) SPE plate (for the determination of methylmalonic acid [MMA] in human plasma by Schmedes and Brandslund 2006).

- SPE was performed automatically on a Gilson Aspec XL4 liquid handler.
- 750 µl of serum (calibrator, control, or sample) was mixed with 375 µl of IS working solution in water.
- The SAX columns were conditioned with 1 ml of methanol and 1 ml of water before 750 µl of the mixed sample (corresponding to 500 µl of serum) was loaded.
- The columns were washed sequentially with 1 ml each of water, methanol, and butanol before elution of the MMA with 300 µl of a 10 : 90 mixture of concentrated (37%) HCl and butanol.
- Conical vials were placed directly in the collection racks of the Gilson Aspec. After sample collection, 80 µl of formic acid (1 g l^{-1}) was added to each vial.

1.4.3.3.3 Mixed-mode SPE

The mixed-mode SPE has been designed to utilize two or more primary interaction mechanisms for the extraction of the analyte(s) of interest from biological samples with improved selectivity and recovery. Compared to reversed-phase or ion-exchange SPE alone, mixed-mode SPE offers some unique advantages in terms of applicability as most of the analyte(s) of interest may exhibit both ion-exchange and reversed-phase retention for SPE.

- **Mechanism:** In mixed-mode SPE, both hydrophobic interactions and ionic interactions are used for analyte retention.
- **Applicable analyte(s):** The use of mixed-mode sorbent is to extract an analyte that exhibits functional groups capable of interacting with both of the hydrophobic and ionic groups of the SPE sorbents. For example, basic analyte with certain degree of hydrophobicity may work well with a mixed-mode sorbent containing a cation exchanger and a hydrophobic

chemistry. When the analyte is loaded, the cation exchanger interacts with the basic functional group of the analyte, while the hydrophobic sorbent group interacts with the hydrophobic group(s) of the analyte. Mixed-mode SPE can be used to simultaneously extract acidic and basic compounds, for which two separate extractions must be carried out for the same sample by conventional SPE. With mixed-mode cation-exchange sorbents, the acidic and neutral molecules can be retained via the reversed-phase mechanism and basic compounds can be retained by the mixed-mode mechanism at low pH.

- **Sample pretreatment:** Depending on which mechanism is to be employed as the primary retention mechanism in mixed-mode SPE, sample pretreatment can be different. In other words, the samples must be treated according to the primary mechanism of the mixed-mode SPE to be employed in sample processing prior to LC-MS. If ion-exchange is considered the primary retention mechanism, similar approach as for ion-exchange mode SPE as above should be used for sample pretreatment. Based on the pK_a values of the analyte(s) of interest, dilute the sample with a buffer or a solvent of appropriate pH to ensure ionic functional groups of the analyte(s) are positively or negatively charged.
- **Sorbent conditioning and equilibration:** Typically the surface of the sorbents is first conditioned with methanol or acetonitrile. This is followed by equilibration of the SPE sorbents with buffer similar/identical in pH to buffer used in the sample pretreatment. This buffer may also be employed in ionizing the ion-exchange groups if the ion-exchange mechanism is the primary one for compound retention.
- **Sample loading:** Same as reversed-phase or ion-exchange SPE, the pretreated sample should be loaded slowly to ensure optimal retention. In the sample loading step, hydrophobic interactions are normally used as the primary source of retention. This is because hydrophobic interactions are less affected by variations in the sample matrix than ionic interactions.
- **Wash:** As the target analyte(s) are retained by both retention mechanisms, interferences that are retained only by a single mechanism can be easily washed off by suppression of such mechanism. Therefore, in a mixed-mode SPE, two separate wash steps are commonly employed. For basic and hydrophobic analyte(s) that are retained on a mixed-mode SPE sorbents via both cation-exchange and hydrophobic interaction mechanism, a ionic buffer is commonly used in the first wash step, by which ionic matrix components (e.g. salts) are generally removed. Such a wash step should have no influence on the retention of the analyte(s) via a hydrophobic mechanism. In the second wash, a pure organic solvent is used, which will remove matrix components that have only hydrophobic character but without affecting the ionic retention of the analyte(s). By using this double-wash approach, many interfering components can be very effectively removed from the mixed-mode sorbents without affecting the analyte(s). The same as for other retention mechanisms, maximum extract purity can be achieved by washing with the strongest solvent possible but without eluting the analyte(s) of interest.
- **Elution:** Elution can be carried out through suppressing both hydrophobic and ionic retention mechanisms simultaneously by using an elution solution that contains organic solvent (such as methanol, acetonitrile) and a strong acid or base. The organic solvent in the elution solution is employed to suppress the hydrophobic interactions while the bases (e.g. ammonium hydroxide) or acids (e.g. hydrochloric acid) are used to suppress the ionic interactions. Attention should be made to check the stability of the analyte of interest under such a strong acidic or basic condition. Different from the washing step above, the weakest solvent possible should be employed in elution that gives the highest REC for the analyte(s) of interest but without eluting the interfering compounds.
- **Eluate:** Post-treatment of the eluate is often necessary to evaporate the organic solvent and reconstitute the resulting residues in the mobile phase prior to LC-MS analysis.

A typical sample preparation protocol using a mixed-mode cation-exchange (MCX) SPE plate (for quantification of 5-azacytidine (5Ac) in plasma Zhao et al. 2004).

- Plasma samples (200 µl) were initially deproteinized with 1 ml of acetonitrile containing 5 ng ml^{-1} IS in a borosilicate glass tube.
- The mixture was then centrifuged at 2000 × *g* for 10 minutes at ambient temperature. The supernatant was dried with a gentle nitrogen stream at 30 °C. The residue was dissolved in 0.5 ml of 0.1 N HCl and transferred to an activated Oasis MCX 1 cc (30 mg) ion exchange SPE cartridge (the SPE was conditioned and equilibrated by washing with 1 ml of methanol and then 1 ml of Milli-Q water).
- The samples were cleaned using two wash steps: first by 1 ml twice of 0.1 N HCl and then with 1 ml of methanol.
- The analyte was eluted with 2 ml total (1 ml twice) of ammonium hydroxide in acetonitrile (5 : 95, v/v).
- The sample extracts were evaporated to dryness under nitrogen at 30 °C. The residue was reconstituted in 100 µl of methanol : water (1 : 9, v/v) by vortex-mixing (30 seconds). The reconstituted sample extract was

transferred to a 250 µl polypropylene autosampler vial, and a volume of 20 µl was injected onto LC-MS/MS (**Note:** Due to the instability of 5AC in plasma, all processing and handling of 5AC samples were performed on ice until the samples were dried and reconstituted).

A typical sample preparation protocol using a mixed-mode anion-exchange (MAX) SPE plate (for the simultaneous analysis of glucocorticosteroid fluticasone propionate and its metabolite fluticasone propionate 17β-carboxylic acid in human plasma by Nair et al. 2017).

- To an aliquot of 500 µl of sample, 25 µl of IS working solution was added. After vortex-mixing for 30 seconds, the samples were centrifuged at 13 148 × *g* for 5 minutes at 10 °C.
- Plasma samples were then loaded on Oasis MAX (30 mg, 1 ml) cartridges, which were preconditioned with 1.0 ml of methanol and 1.0 ml of water.
- The samples were washed sequentially with 1.0 ml of *n*-hexane, 1.0 ml of water, and 1 ml of 5% methanol in water.
- The cartridges were dried for 1.0 minute by applying nitrogen (1.72 × 105 Pa) at 2.4 l min^{-1} flow rate.
- Elution was performed using 1.0 ml of 1.0 mM TFA in methanol.
- The eluent was evaporated to dryness in pre-labeled vials under a gentle stream of nitrogen and then reconstituted with 200 µl of the mobile phase.
- The resulting solution was briefly vortexed and 10 µl was used for injection.

A typical sample preparation protocol using Waters Oasis WCX plate, a mixed-mode weak cation-exchange SPE plate (for the analysis of pasireotide in human plasma by Fu et al. 2016).

- A volume of 0.1 ml of blank plasma or freshly prepared calibration standards, QC samples, or unknown samples was added to a 1 ml 96-well plate.
- A 25 µl aliquot of the IS working solution in 50% acetonitrile in water (v/v) was added to all wells except for the matrix blanks, to which a 25 µl aliquot of 50% acetonitrile in water (v/v) was added.
- After adding 100 µl of 2% formic acid in water (v/v) to each well, the plate was vortex-mixed briefly and centrifuged.
- The sample mixtures in the above plate were loaded onto the corresponding wells of a Waters Oasis WCX µElution plate (96-well format, 30 mg, Milford, MA, USA), which were pre-equilibrated with 200 µl of methanol followed by 200 µl of water.
- The plate was washed with 200 µl of 5% ammonium hydroxide in water (v/v) followed by 200 µl of methanol. Finally, the µElution plate was eluted with 100 µl of 5% formic acid in methanol (v/v).
- The eluent was diluted with 50 µl of water. After brief vortex-mixing and centrifugation at ~3000 × *g* for approximately five minutes, a volume of 15 µl of the reconstituted sample extract was injected onto the LC-MS/MS system for analysis.

A typical sample preparation protocol using Waters Oasis WAX plate, a mixed-mode weak anion-exchange SPE plate (for the analysis of carbazochrome sodium sulfonate in human plasma by Hu et al. 2014).

- 20 µl of IS working solution and 100 µl of water (supplementary volume) were added to 1 ml plasma sample and vortex-mixed for 30 seconds.
- The Oasis WAX (30 mg, 1 ml) cartridges were conditioned with 1 ml of methanol and 1 ml of water.
- The plasma samples were applied to the cartridges. Following sample application, the cartridges were washed with 1 ml of 2% formic acid water solution, and subsequently washed with 1 ml of methanol.
- After drying, the analyte(s) were eluted with 1 ml of methanol containing 5% ammonium hydroxide.
- The eluate was evaporated to dryness at 40 °C using a gentle stream of nitrogen.
- The residue was reconstituted in 100 µl of the mobile phase, then vortex-mixed and centrifuged at 10 500 rpm for 10 minutes.
- A 5 µl aliquot of the resulting solution was injected onto the LC-MS/MS system for analysis.

Some general considerations when developing a SPE method (Li and Bartlett 2014; Liu and Aubry 2013; Pedersen-Bjergaard et al. 2015):

- **Capacity:** The capacity of silica-based SPE phases is around 5–10% of the sorbent mass per analyte and around 20–25% for polymer-based SPE phases. This information can be useful in judging the sorbent mass needed for the intended bioanalytical assay.
- **Speed:** Hydrogen bonding and electrostatic (ionic) interactions are "point to point" mechanism. A low speed in loading, washing, and/or elution is necessary to ensure good analyte retention and recovery.
- **Soaking:** Under certain circumstances, it is necessary to include soaking steps in a SPE procedure. During a soaking step, the solvent that is applied to the SPE column/cartridge/plate is allowed to remain

stationary (~30 seconds to 2 minutes for a typical 100 mg sorbent bed mass) without application of vacuum or pressure. This allows for establishment of chemical and physicochemical equilibrium between the solvent and sorbent materials during conditioning and sorbent surface activation steps. During elution step, soaking allows the analyte(s) of interest diffuse into the elution solvent and reach equilibrium prior to application of vacuum or pressure to facilitate elution. In general, soaking can help improve retention and elution efficiency and reduce the volume of elution solvent.

- **Drying step:** Often a drying step is included after the wash step. This can be particularly important when water-immiscible solvent is employed in the elution step. Any water that remains on the surface of the sorbents may exclude the immiscible elution solvent and cause low recovery of the analyte(s) of interest.

1.4.3.4 Other Formats of SPE

Other formats of SPE are available with various packing materials. They include but are not limited to: immunoaffinity SPE, MIP SPE, and restricted access material (RAM) SPE, dispersive SPE, disposable pipette extraction (DPX), solid-phase microextraction (SPME), microextraction by packed sorbent (MEPS), and stir-bar sorptive extraction (SBSE). The sample extraction mechanism of some of these SPE techniques is captured in separate chapters of this book (Liu and Aubry 2013; Pedersen-Bjergaard et al. 2015).

1.4.4 Combination of PPT, LLE, and/or SPE in LC-MS Bioanalysis

PPT, LLE, and SPE each discussed above has advantages and disadvantages over each other. None of them can fit all requirements of modern LC-MS bioanalysis with optimal REC and minimal matrix effect. Indeed, some special cases require more sophisticated approaches to achieve the needed high sensitivity or remove persistent interferences. The real troubling matrix interferences most likely have similar physicochemical properties to those of the analyte(s) of interest. Therefore, they are very likely to be co-extracted with the analyte(s) of interest using the single sample extraction methods. To address these issues, orthogonal or hybrid sample preparation techniques, such as PPT/LLE, PPT/SPE, and LLE/SPE, should be considered and implemented.

1.4.4.1 Combination of PPT and LLE

While PPT is considered a plug-and-play method in LC-MS bioanalysis, sometimes persistent endogenous interferences may be encountered in the retention time window for the analyte(s) of interest. A simple LLE step added after PPT may be helpful in mitigating such interference as reported by Wang et al. (2015), who identified a large interfering peak eluted close to the retention time of octreotide in LC-MS. After screening extraction solvent among diethyl ether, ethyl acetate, trichloromethane, dichloromethane, etc. 2.5-fold of dichloromethane was found to be able to completely eliminate the impurity peak with no apparent sacrifice in recovery for the analyte. Mixing 200 µl of supernatant from PPT with 200 µl of water for LLE with 500 µl dichloromethane yielded the best extraction efficiency and lowest background noise for octreotide in LC-MS/MS analysis (Wang et al. 2015).

1.4.4.2 Combination of PPT and SPE

There have been many applications where biological samples were subjected to PPT followed by the SPE process. Depending on the physicochemical properties and assay sensitivity needs, the supernatants from PPT may be treated with proper solvents prior to being loaded onto SPE with suitable sorbents, such as phospholipid removal SPE plates (Nilsson et al. 2014), reversed-phase sorbents (Zhang et al. 2014), or mixed-mode sorbents (Guo et al. 2015) for further processing prior to LC-MS analysis.

PPT with acetonitrile and methanol along with formic acid or ammonium hydroxide can effectively remove plasma proteins and other endogenous components, keeping the basic or acidic compounds protonated or deprotonated even in a high percentage of organic solvents. The supernatants from the PPT can be directly loaded onto an unconditioned 96-well mixed-mode cation or anion-exchange SPE plate without analyte breakthrough and cartridge blockage during the loading step. The loading step is considered a rinsing step to remove lipid-soluble components; therefore, wash steps may not be necessary. This simplified PPT/SPE procedure was reported to effectively eliminate time-consuming sorbent conditioning and wash steps required by conventional SPE (Xue et al. 2006b).

1.4.4.3 Combination of LLE and SPE

A single-step SPE may be sufficient for the extraction of the analyte(s) of interest with a variety of physicochemical properties. However, blockage of the SPE cartridges/discs often occur during the sample loading step because of viscosity of study samples, particularly urine samples. Depending on the polarity of the analyte(s) of interest, a simple treatment with LLE prior to SPE can lead to a significantly reduced baseline in the followed-up LC-MS bioanalysis (Shin et al. 2014).

A typical sample preparation protocol using combined PPT and SPE procedure (for the analysis of peptide glucagon in human plasma by Howard et al. 2017).

- 400 µl of plasma sample and 20 µl of IS working solution were added to the wells of 2 ml 96-well plate. The plate was vortex-mixed.
- PPT was conducted by adding 1.1 ml of acetonitrile : water : ammonium hydroxide (75 : 25 : 0.1, v/v/v). The plate was vortex-mixed, sonicated for 5 minutes, and then centrifuged for 10 minutes at 2300 × *g*.
- A total of 1.2 ml of supernatant was transferred using an automated liquid handler system (Quadra Tower, Tomtec, CT, USA) to a 2 ml plate and evaporated to dryness at 40 °C under nitrogen (~90 minutes).
- Samples were reconstituted in 800 µl of 2% ammonium hydroxide in water and then vortex-mixed before being extracted using SPE.
- A Bond Elut Plexa SPE plate (96-roundwell, 30 mg; Agilent Technologies, CA, USA) was conditioned using 1 ml of methanol and 1 ml of water.
- The samples were loaded, washed with 1 ml of 5% methanol in water, eluted with 2 × 225 µl of acetonitrile : water : formic acid (75 : 25 : 0.1, v/v/v) into a 1 ml Lo-bind plate and then evaporated under nitrogen at 40 °C, before being reconstituted in 200 µl of 0.2% formic acid in water.
- The plate was centrifuged for 10 minutes at 2300 × *g*, and 40 µl of sample was injected onto the LC-MS/MS system for analysis.

1.4.5 Summary

Physicochemical properties of the analyte(s), assay dynamic range, and available MS instrumentation platform should be jointly considered when developing a sample preparation procedure for the intended LC-MS bioanalysis. Although not discussed extensively, pre-analytical parameters, such as stability in the intended sample matrix, protein binding, possible nonspecific adsorption, and blood-to-plasma distribution of the analyte(s) of interest should be assessed, as part of assay development.

Three major platforms of sample preparation, i.e. PPT, LLE, and SPE have been discussed. Each technique has its unique advantage and also disadvantages over the other.

There is no doubt that among the three, PPT is the simplest, quickest, and most convenient technique and has been widely employed in LC-MS bioanalysis. The method is not only associated with less cost as compared to LLE and SPE but also associated with the speed and throughput, which is needed in routine analysis of a large number of study samples. In authors' view, with the advancement of MS instruments, less sample volume, as in the current trend of microsampling, will be needed for the intended assay sensitivity. Less sample volume is associated with less matrix effect, which, if any, should be compensated for by the stable labeled IS that is being increasingly used in nowadays' bioanalysis practice. Therefore, PPT will continue to be the primary choice of sample preparation in LC-MS bioanalysis. In comparison to PPT and SPE, LLE provides the most-effective sample cleanup by selectively extracting analyte(s) of interest and removing matrix effects-causing interferences. There is a clear trend of automated LLE platform which can significantly improve the throughout and efficiency of LLE sample preparation. Obviously, SPE technology will continue evolving. New sorbents will continue to be available to meet the needs of extracting different types of molecules from biological matrices. There is also a trend of development of SPE plate that fits for processing of small volume of biological samples. Finally, the combination of different platforms, i.e. PPT/LLE, PPT/SPE, or LLE/SPE, has been demonstrated to be very useful in dealing with challenging assay development.

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