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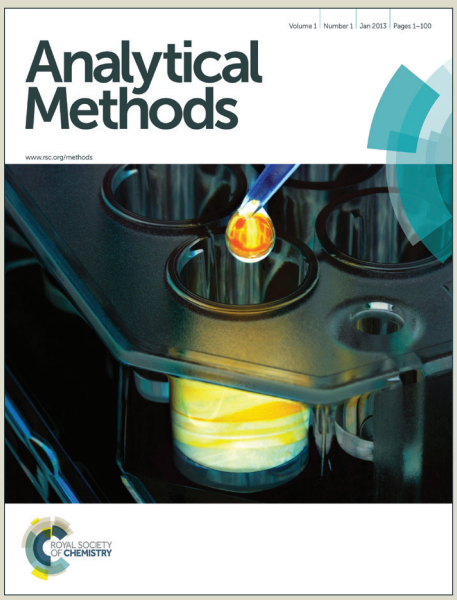

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A Review of Sample Preparation Methods for Quantitation of Small-molecule Analytes in Brain Tissue by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

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Abstract

Concentration measurements are one of the most important and fundamental approaches in preclinical and clinical studies of small-molecule drugs, metabolites and biomarkers, since information about the absorption (drug), synthesis (biomarker), distribution, metabolism and elimination can be obtained by determining the concentrations of target analytes in biological fluids or tissue samples. Among all the bioanalytical techniques, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been widely used, due to its high sensitivity, selectivity and reproducibility. Attention has been paid to the quantitation of small-molecule analytes in brain tissue samples by LC-MS/MS, because the important information about brain concentrations obtained via such studies can be used to interpret the distribution and function of target chemicals in the central nervous system (CNS). In order to be analyzed by LC-MS/MS, brain tissue samples need to be properly obtained and carefully prepared into an LC-MS/MS compatible form. The choice made here will which greatly influence the sensitivity and robustness of the method. As a result of the vital function and complex composition of brain tissue, sample collection and preparation can be very challenging. In this review, we summarize the current techniques for the collection and preparation of brain tissue samples, which can be used as a reference for future method development for quantitation of small-molecule analytes by LC-MS/MS.

Key words

Review, LC-MS/MS, brain tissue, sample preparation

1. Introduction

Bioanalysis, defined as the quantitative measurements of xenobiotics and biotics in biological matrices, has been of great importance in drug discovery, research and development, because of the crucial information it provides on drug absorption, distribution, metabolism and elimination (ADME). Though the development of large-molecule drugs and biomarkers has been rapidly growing recently, small-molecule drugs, which account for over 90% of FDA-approved drugs according to the DrugBank 3.0 database, as well as small-molecule drug metabolites and biomarkers are still the most studied analytes in bioanalysis. Different techniques have been used for the quantitative bioanalysis of small molecules, including liquid chromatography with ultraviolet detector (LC-UV), liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS), among which liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been the most widely used and most reliable tool, due to its high sensitivity, specificity, precision and accuracy. Typically, a high performance liquid chromatography (HPLC) or ultra high performance liquid chromatography (UHPLC) system is used to separate the analytes from the processed biological matrices based on the specific interactions between the analytes and the analytical LC column. The LC eluent is then directly introduced to a mass spectrometer for the detection and quantitation of the analytes, using a multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) function. During this process, collision-induced dissociation (CID) is often used to generate fragment ions from the precursor ion, which provides a specific precursor-product ion transition (or multiple ion transitions) for the instrument to record, integrate and quantify. With the specificity and sensitivity provided by both the LC column and the MRM function, LC-MS/MS can serve as one of the most suitable tools for small-molecule quantitation in most scenarios.

Quantitative bioanalysis of small-molecule drugs or biomarkers by LC-MS/MS has been studied and applied with different types of matrices, especially those related to drug ADME or organ-specific biomarkers, including different types of body fluid (*e.g.* plasma, serum, whole blood, saliva, tears and urine) and organ tissue (*e.g.* kidney, liver, lung and brain tissue). Unlike pure standard solutions or drug formulations, biological samples usually have much more complex biochemical compositions, containing various components like salts, organic small molecules, fibers, proteins and lipids, which may cause a series of issues including LC column degradation, mass spectrometer contamination, signal interference, and most importantly, matrix effects. Matrix effects represent a phenomena of enhancement or suppression of analyte ion intensity caused by coeluting matrix components.[1] Therefore, in order to improve the sensitivity, selectivity and reproducibility while measuring analytes in biological samples, sample preparation, also known as sample pretreatment or sample cleanup, is needed before LC-MS/MS analyses in most occasions. Sample preparation can be considered a pre-analytical separation process in bioanalysis, which mainly involves selective isolation of analytes of interest from the matrix, minimization or elimination of matrix components in processed samples and, if required, enrichment of analytes. Based on the analyte properties and matrix complexities, different sample preparation techniques have been developed, such as dilute-and-shoot, protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE), which are the most traditional and most commonly used ones.[2] Recently, to cope with the demand for improved selectivity, sensitivity and regulations, combinations of these techniques and other innovative sample preparation strategies are becoming increasingly used in bioanalytical practices. An ideal sample preparation method should be able to reduce biological matrices to minimal levels while maintaining the recovery of analytes above 80%. However, due to the

numerous factors affecting matrix removal and analyte extraction (e.g. choice of solvents, ion pairing agents, temperature and buffer pH), the developing work for sample preparation can be very difficult, tedious and labor-intensive, which makes it one of the most significant parts in the development of a whole analytical method. With optimized sample preparation methods and LC-MS/MS conditions, small molecules in biological samples can be measured sensitively, specifically, precisely and accurately.

Among all the common biological matrices studied in bioanalysis, brain tissue has been drawing significant attention from researchers over the last decade. As a specific organ of higher animals, the brain has multiple functions in different brain regions to control a variety of complicated behaviors in human beings and animals. Thus distributions of small-molecule drugs, metabolites or biomarkers in brain tissue are of great pharmacological or physiological importance, due to their direct impact on such brain functions as information processing, body movement control, homeostasis and memory.[3] Quantitative studies of small molecules in brain tissue can serve purposes such as: (1) to study the brain tissue distribution (pharmacodynamics) of drug candidates or different formulations; (2) to study the brain tissue pharmacokinetic profiles of drugs or metabolites; (3) to measure biomarker levels in the brain tissue; and (4) to evaluate the toxicity of chemicals on the brain.

As a special organ with unique functions and anatomy, the brain has a special matrix composition that is different from all other organs, which makes it more challenging for sample preparation development. The biggest challenge for brain tissue analysis is its high lipid composition, which constitutes about one-half of the dry weight of brain tissue. Like other tissues, the brain contains phospholipids, sterols and sphinggolipids; while many other complex lipids, including gangliosides, cerebrosides, sulfatides and phosphoinositides, are also highly

enriched compared to other tissues.[4, 5] Due to the nonpolar phosphate ester group and the long alkyl chain, lipid molecules are usually highly hydrophobic, and may be extracted together with the analytes when sample preparation methods based on hydrophobicity are used. These lipids have a chance of coeluting with the analytes of interest from the LC column and entering the MS. All of the lipids in the brain, most of which are permanently or easily charged in the ion source, generally cause serious matrix effects, especially when an electrospray ionization (ESI) ion source is used. Glycerophosphocholines, a class of lipid molecules with high abundance in the brain tissue, have been specifically believed to broadly cause serious matrix effects in LC-MS/MS analyses.[6] Meanwhile, other components like salts, proteins and carbohydrates in brain tissue can also introduce interferences to the detection of analytes by LC-MS/MS, not only by causing matrix effects, but also by causing other issues, including but not limited to low extraction efficiency, peak distortion and signal interferences in MRM detection. At last, just as all the tissue samples, brain tissue samples need to be properly collected and prepared into an operable physical state, solutions or homogenates in most occasions, in order to be processed in sample preparations and LC-MS/MS analyses. Brain homogenates are problematic due to blockages of the LC system and the irreversible adsorption of impurities on the stationary phase, resulting in elevation of the column backpressure or decreases in column performance, and therefore reduced robustness or even failure of the method.[7]

Due to the fact that LC-MS/MS is a type of off-line analytical technique that can only analyzed injected liquid samples, biological samples cannot be directly analyzed *in-situ* and, instead, must be acquired from the tested individual prior to the analysis. Solid brain tissue samples must be properly processed into a liquid state in order to be analyzed by LC-MS/MS instruments. Therefore, the general concept of sample preparation for bioanalysis of brain tissue

samples should include sample acquisition, pretreatment and the traditional concept of sample preparation. Traditional sample preparation in a narrow sense represents for one or a series of chemical or physical sample cleanup processes that may involve extraction, separation, derivatization, enrichment and many other techniques. In addition to obtain and prepare samples into an LC-MS/MS compatible form, sample preparation is of great important for brain tissue by removing impurities that may cause interference, ion suppression, column congestion and instrument contamination. In fact, the sensitivity, selectivity and reproducibility of an LC-MS/MS method are largely determined by the instrumentation, leaving the sample preparation even more critical in method development. Different techniques and strategies for sample preparation can be used by the analyst to gain more leverage in the optimization of the bioanalytical method.

Based on the significance and challenges faced in the bioanalysis of brain tissues, it is necessary to look into current techniques and strategies in sample preparation, which will be of great instructional value for future method development work. The goal of this review is to summarize published sample preparation methods in quantitation of small molecules in brain tissue by LC-MS/MS. All currently available methods and techniques for each step of sample preparation will be introduced and summarized in terms of their advantages and disadvantages.

2. Sample Collection

While samples from some biological fluids such as plasma, serum and CSF can sometimes be directly analyzed with LC-MS/MS, brain tissue samples, however, must be properly sampled and/or processed into a liquid form that can be analyzed by LC-MS/MS. In order to obtain a liquid sample from the brain tissue, homogenization, microdialysis, ultrafiltration and solid-phase micro extraction are the major techniques involved.

2.1 Homogenization

Since brain is a solid organ located in the skull of most tested animals, the most direct way of tissue sampling is to obtain the whole brain or a certain region of brain tissue by surgical disection after sacrifice, which is the most widely used approach in all published methods.[7-92] High throughput and low equipment cost are the most significant advantages of homogenization. The biggest disadvantage of homogenization is that the test animal has to be sacrificed to yield one data point, which means that multiple animals are need for experiments designed for multiple time points.

Several significant issues need to be highlighted in brain tissue homogenization. The excised brain tissue needs to be frozen at -80 °C or kept on ice in order to maintain a low temperature for the freshness of brain tissue as well as the stability of analytes. When the whole brain was excised from the tested animal, there is a small amount of blood left in the blood vessels in the brain. The analyte distributed in the circulatory system may exist at a high concentration in the dwelling blood in the brain tissue, which may raise the measured brain tissue concentration, especially when the analyte has poor blood-brain barrier (BBB) permeability and therefore a low brain tissue concentration. In order to remove the extraneous blood to achieve a more accurate measurement of the analyte concentration in the brain tissue, the tissue sample can be rinsed in cold physiological saline or PBS (phosphate buffered saline) buffer solutions.[28, 30, 40, 47, 56, 66, 78, 83] After the excess liquid is wiped off, the brain tissue sample can be weighed for quantitative purposes. However, when the analyte is very hydrophilic and water soluble, one should be extra careful with the rinsing step, because longer exposure to aqueous solutions may wash away analytes in the brain tissue and cause the measured concentrations to be lower than the actual values.

After being obtained from the test animal and the excess blood removed, solid brain samples will be homogenized into a liquid form. Homogenization is a process that uses mechanical power to disrupt the brain tissue and disperse it into a certain solvent or solution, so as to form a relatively stable suspension for further sample preparation. Also, spiked samples in the method validations are prepared by the addition of a known amount of analytes into blank matrices, so only liquid samples can be mixed well with the reference standards.

Based on the mechanism of action, tissue homogenization can be fulfilled by different types of techniques, including grinding, rotating blade, bead beating and ultrasonication.[93] Due to the softness of brain tissue, almost all the mechanical homogenization techniques mentioned here can be used for the preparation of brain tissue without extra treatment. Though it has a low throughput and high labor intensity, manual grinding with a Dounce homogenizer (a.k.a. Potter-type homogenizer) is still used in some methods, mainly due to its low cost and high availability.[17, 28, 44, 57, 60, 61, 66] Among all these homogenization methods, rotating blade is the most commonly used one for brain tissue samples, due to its low cost and easy operation.[19, 21, 22, 31, 36, 38, 40, 41, 43, 75, 77, 79, 81, 84-86, 90] However, extra attention needs to be paid to the cleanliness of the blades, otherwise contamination can be a possible issue with the traditional rotating blade homogenizer. Bead beating [15, 55] and ultrasonication [8, 24-26, 39] are both newer techniques that have the advantages of higher efficiency, higher throughput as well as lower chance of contamination when compared to the traditional grinding or rotating blade homogenization techniques, making these two new techniques more popular in recent studies. In addition, pulverization is another highly efficient homogenization method. In a paper published by Golovko et al. in 2008, brain samples were snap frozen in liquid nitrogen and then pulverized to a fine homogeneous powder.[17] The brain tissue powder could be extracted directly by organic solvents due to the

increased surface area of the brain tissue, making this homogenization protocol of great advantage with high efficiency. Though different techniques have been used for the homogenization of brain tissue samples, the purpose and the results are always to form a homogeneous suspension for further steps of sample preparation.

Another important factor affecting the outcome of homogenization is the media that a brain tissue is dispersed into, which can be pure water, organic acids, aqueous buffers, organic solvents or mixtures of more than one of these agents. Due to the simplicity of the method, water has been the most commonly used homogenization media.[13, 14, 19, 21, 22, 35, 40, 41, 43, 49, 50, 57, 70, 71, 74, 75, 77, 80, 81, 84-86, 88, 90] In these methods, to one unit weight of brain tissue, at least one to two volumes of ice-cold water is add for homogenization, forming a homogeneous suspension of diluted brain tissue. In some studies, more water is added to yield a more diluted brain homogenate, which can be treated similarly to plasma or even serum samples in sample preparation, making it possible to use the same method for multiple sample species.[71, 75] Other than the low cost and simplicity of preparation, another major advantage of using water as the homogenization media is the lysing effect resulting from the low osmotic pressure of pure water. When samples are lysed by water and homogenized by mechanical forces, it can be considered that the suspension is a uniform mixture of both the intracellular and extracellular contents of brain tissue.[40]

Instead of pure distilled water, there are also many methods using aqueous solutions as the homogenization media.[8, 16, 18, 20, 27, 29, 31, 33, 36, 44, 45, 47, 52, 54-56, 58, 59, 61, 63, 67, 69, 79, 82, 83, 87, 91, 92, 94] Organic acids, formic acid or acetic acid in most occasions, are common components of aqueous homogenization media, due to their ability to facilitate the breakdown of cell membranes as well as adjusting the pH of the resulting homogenate for the ionization/deionization or stability of the analyte.[18, 36, 58, 61, 82, 91] Inorganic acids such as trichloroacetic acid (TCA)[54, 79] or perchloric acid

(PCA)[52] solutions are also common aqueous homogenization media. In addition to lowering the pH, such strong acids can also irreversibly denature and therefore precipitate the proteins in the brain tissues samples. As a result, homogenization and protein precipitation of brain tissue samples can be combined together in one step, followed by centrifugation to finish the tissue sampling as well as the rough sample preparation. The supernatant can be neutralized and directly injected into the LC-MS/MS system or further processed by other sample preparation methods. Aqueous buffers made from salts and other additives are also common homogenization media used in brain tissue sample preparation.[8, 16, 20, 27, 29, 31, 33, 44, 45, 47, 55, 56, 59, 63, 67, 69, 83, 87, 92, 94] Buffered media are usually aqueous solutions of a weak acid and its conjugate base or a weak base and its conjugate acid, which can maintain the pH value of the solution at a certain level. By maintaining the osmolarity, ionic strength and pH value similar to the physiological conditions of biological samples, most buffer solutions used as homogenization media can dilute the tissue homogenate without changing the solution conditions or disrupting protein integrity, so that the distribution, charge state and solubility of the analytes are not drastically changed. Tris-HCl buffer is one of the most common homogenization buffers used in the current literature, with concentrations ranging from 5 mM to 100 mM.[8, 27, 33, 59, 87] The effective pH range of Tris-HCl buffer is between 7.07 and 9.07, so the physiological pH of 7.4 can be well maintained with the use of this buffering system, while a slightly basic pH can also be reached when needed. In a study about the determination of two endogenous isoprenoids, farnesyl-(FPP) and geranylgeranylpyrophosphate (GGPP), Tris-HCl buffer with pH 8.5 was used as the homogenization media together with phosphatase inhibitors, which was designed to neutralize the analytes for a hydrophobicity-based solid-phase extraction (SPE) process as well as to protect the analytes from hydrolysis. Normal saline or phosphate bufferred saline (PBS) solutions are

also common buffer solutions for brain tissue homogenization, because of their easy preparation, low cost, and, most importantly, being isotonic and non-toxic to cells.[20, 47, 56, 63, 67, 83, 95] However, non-volatile ions in Tris-HCl, saline, PBS and many other common buffers can cause a series of fatal issues for the mass spectrometry, including ion suppression, high base line levels and serious instrumental contamination. Therefore, if no further extraction is involved to separate the analytes from the ions, special attention needs to be paid to the LC separations of brain tissue homogenate made with such aqueous buffers, in order to make sure that the unwanted ions are identified, separated and diverted from the mass spectrometer. Considering this issue, aqueous buffer solutions containing volatile salts are widely used as tissue homogenization media, including ammonium formate and ammonium acetate as the most used ones.[16, 31, 92] These two buffer salts are composed of weak acid/base functional groups and, more importantly, are volatile under high temperature so that they can be evaporated, ionized and transported in the mass spectrometer without causing serious ion suppression or instrument contamination. Besides these common buffer systems used as homogenization media, there are many types of buffers used in brain tissue homogenization, which are more analyte specific.

In addition to pure water or aqueous solutions, organic solvents are also used in brain tissue homogenization in many studies.[9, 11, 15, 24-26, 28, 30, 32, 34, 38, 39, 42, 53, 68, 76] To be used as homogenization media, the organic solvent needs to be miscible with water and hydrophobic enough to compromise interactions between protein molecules. Due to their abilities to disrupt protein structures and therefore precipitate proteins, the use of organic solvents as homogenization media, which is similar to the use of TCA, can be combined with homogenization. Usually, at least two volumes of organic solvents are added to each unit weight of brain tissue; while the more added, the more thorough the precipitation will be. Methanol

(MeOH) and ethanol (EtOH) are the most commonly used organic solvents as the homogenization media of brain tissue, due to their low cost, and most importantly, their moderate hydrophobicity and precipitating strength.[11, 15, 34, 42, 76] When brain samples are homogenized with the addition of MeOH or EtOH, brain tissue will be dispersed into the media and form a suspension of flocculent structures, due to the protein precipitation. Acetonitrile (ACN) was another common organic solvent used as a homogenization media.[15, 34, 42, 76] It has a stronger precipitating ability and therefore can form precipitated pellets right after the homogenization. Differently from aqueous homogenization media that cannot precipitate brain tissue, brain homogenate prepared with precipitating organic solvents is unstable and thus hard to pipette with high volume accuracy. Therefore, the amount of brain tissue needs to be measured at the beginning of the experiment and the whole homogenate has to be used in all the following steps. Aliquots can only be taken after the homogenate is processed into a stable solution by centrifugation or extraction. In a study about the quantitation of dihydroetorphine in rat plasma and brain published by Ohmori in 2000, two volumes of MeOH was used in the homogenization of brain tissue weighed beforehand. After centrifugation, the supernatant was separated and used as the sample instead of the brain homogenate. In this case, the brain homogenate supernatant could be accurately transferred and further processed.

In order to utilize the properties of buffer systems as well as organic solvents, a mixture of more than one of these agents can be used as the homogenization media.[10, 23, 48, 60, 64, 66, 78] Instead of using pure organic solvents that have very strong precipitating abilities, a mixture of organic solvents (ACN or MeOH) and water (or aqueous solutions) was used as the homogenization media, so that the proteins in the brain tissue were not drastically precipitated and, therefore, the resulting homogenate was more stable. Moreover, the presence of water in the homogenization

media can make it possible to add ionic or polar additives, inorganic salts or acids for example, which otherwise are not soluble in pure organic solvents.

With the leverage of choosing proper solvents or solutions as the homogenization media, brain tissue can be uniformly dispersed to achieve ideal analyte recovery and method precision and accuracy. Nevertheless, low-concentration additives are also quite commonly used in the homogenization media to add unique effects to the homogenate. Since most extraction techniques are based on hydrophobic interactions or ion-exchange mechanisms, the charge states of the analytes are of great importance in the sample preparation process, which means that the pH value of the initial tissue homogenate needs to be well controlled in a small range to maintain the analytes in the intended charge states. As mentioned with the buffered homogenization media, acids and bases are common additives for adjusting the pH of the resulting homogenate. When hydrophobic extractions or reversed phase liquid chromatography (RPLC) are used in sample preparation, the analytes need to be neutralized; when ion-exchange extractions or hydrophilic interaction liquid chromatography (HILIC) are used, the analytes need to be positively or negatively charged, according to the properties of the analytes. To facilitate such chromatographic separations, the change of pH by the addition of acid or base can be used to alter the charge states of the analytes. In a study published by Wu and coworkers, an LC-MS/MS method was developed for the determination of rat plasma and tissue concentrations of melamine, a compound with multiple primary and tertiary amine groups.[79] The brain tissue was homogenized in 1% TCA, providing not only a mild protein precipitating environment, but also an acidic condition. Therefore the analyte was ionized by receiving protons and further separated by mixed-mode cation exchange (MCX) solid phase extraction (SPE). In addition to acid or base used to adjust pH, there are also many other additives used in the homogenization media mainly

for the stability of analytes. Enzyme inhibitors are common additives used to inhibit the activities of enzymes that can catalyze the decomposition or transformation of the analytes. Sodium fluoride (NaF) has been widely used as an esterase inhibitor. In a study for the determination of heroin in brain tissue, 4 mg/mL of NaF was added in the brain tissue homogenate to minimize the deacetylation of heroin, together with low pH and low temperature.[31] In another study for the determination of irinotecan and 7-ethyl-10-hydroxycamptothecin (SN-38), Goldwirt and coworkers added 100 mM zinc sulfate ($ZnSO_4$) to the brain tissue homogenate as a carboxylesterase inhibitor to preserve the analytes from degredation. In addition to enzymes, pH values and metal ions are also important for the stability of some analytes. In a study published by Najmanova et al., an LC-MS/MS method was developed for the determination of dopamine and serotonin in brain tissue, in which HCl and EDTA were added to increase the stability of the analytes. Dopamine and serotonin are unstable and oxidize rapidly, especially in a strong alkaline medium. Therefore the addition of HCl to the brain tissue led to the stabilization of dopamine and serotonin in their hydrochloride form. Furthermore, biogenic amines and their metabolites are sensitive to light. They are easily oxidized in the presence of transition-metal cations such as Fe^{2+}. Thus it was necessary to store the samples in the dark with the use of the chelating agent EDTA.

With the proper homogenizing instruments, media and additives, brain tissue can be processed into a relatively stable and homogeneous suspension, which should also fit the properties of the analytes as well as the following sample preparation methods. However, one of the biggest concerns with brain tissue homogenization was the difference between spiked samples and real biological samples, which may affect the credibility of the measured extraction recovery. Real biological samples are body fluids or organ tissue samples directly obtained from

the animal. If the analyte is a drug in its original form, its presence in the real biological is a refection of its natural absorption, distribution, metabolism and elimination (ADME); other analytes as drug metabolites or endogenous biomarkers are generated by the test animal and exist in the biological samples based on biosynthesis or metabolism. However, samples with known concentrations have to be used in the method development and validation experiments, so that recovery, stability and accuracy can be accessed against nominal concentration values. In this case, spiked samples are used as standards and quality control (QC) samples in method validations, which are made by adding a known amount of analytes to blank biological matrix to yield an artificial biological sample of a known concentration. When it comes to tissue samples, concerns arise that the recovery of spiked samples may not accurately reflect that of the real samples, since the distribution of spiked analytes could be different from that of the natural samples. Moreover, if the internal standards (IS) are added in the same approach to both spiked and real samples, the accuracy of the measurement may be affected, because the recovery of the IS may be different from that of the analytes. In order to prepare spiked samples as close to the real samples as possible, the homogenization step of the sample preparation needs to be thorough enough so that the resulting homogenate is a uniform system that has an even distribution of analytes. Meaning, the tissue cells need to be completely broken down, assuming that the analyte of interest can freely distribute in/out of tissue fragments and constituents in the tissue homogenate.[93] Due to the softness of brain tissue, this can be achieved with the use of a proper lysing buffer or organic solvent, together with powerful homogenizing equipment. By mixing analyte standards with a highly uniform brain homogenate, spiked samples are considered to be the same as real samples. Internal standards (IS) are usually added to the spiked samples or real samples as the first step of sample preparation. With adequate vortexing, ISs are considered to be

distributed in the same manner as the analytes. Nevertheless, IS can also be added before the homogenization. In a study published by Jantti et al. in 2010, ISs for the determination of steroids and their intact glucuronide conjugates were added to brain samples 1 h before homogenization to mimic the natural absorption of compounds into the brain matrix. In this case, the ISs are absorbed and distributed evenly in the brain tissue in a more similar way to the analytes. Even if the homogenization was not strong enough to yield a uniform homogenate, accurate recovery and concentration measurements can still be fulfilled, since the ISs can compensate for the loss of analytes due to incomplete homogenization or extraction.

When homogenization-based sample collection is finished, the brain tissue homogenate needs to be stored at -80 °C until use, so that the freshness of the homogenate and the stability of analytes can be protected. Though the storage of whole brain homogenate is the most common practice, there are methods using a different sample storage strategy with a simple sample pretreatment. After brain sample collection and the homogenization are completed, the homogenate is subject to one or two rounds of centrifugation to separate the cell residues. The resulting supernatant is then transferred to a new tube and stored frozen until use.[15, 37, 39, 55, 68, 77, 88] Instead of transferring brain homogenate that might stick to the pipette tips, one only needs to pipette the less viscous supernatant in all the following steps, which indirectly improves the precision and accuracy. One single concern about this method is non-specific binding. Only under the assumption that the analyte of interest is not largely bound to the cell residues can this method be considered effective without losing too much analyte in the centrifuged pellet.

2.2 Microdialysis

Microdialysis is a minimally-invasive sampling technique that can continuously collect free analytes directly from live animals. This technique originates from the principle of the “push-pull

cannulae", which was first published by Gaddum in 1961.[96] The push-pull technique was largely improved when the "continuously perfused dialytrode" was introduced in 1972.[97] The actual technique that was most close to today's microdialysis was invented by Ungerstedt in 1974, when the dialytrode was modified with a tubular semipermeable membrane with a diameter of approximately 200 to 300 μm to fulfill minimally-invasive sampling.[98] Due to the advantages of continuous *in situ* sampling, this technique is now widely used in tissue sample bioanalysis.[95, 99-124]

The principle of microdialysis is based on passive diffusion of the analytes between the two sides of a semipermeable membrane. Generally, a microdialysis probe was inserted into the brain tissue, which was composed of a semipermeable membrane surrounding two flowing channels (inlet and outlet). The probe is continuously perfused with an isotonic solution (perfusate) at a constant flow rate, during which the small-molecule analyte in the tissue can freely pass through the semipermeable membrane and diffuse into the dialysate traveling to the outlet. The dialysate can be collected at multiple time points for analysis. At a low flow rate, the diffusion of the analyte of interest across the semipermeable is considered to be a dynamic equilibrium, so that the analyte concentration in the dialysate can be a reflection of that in the extracellular fluid of the tissue analyzed. In summary, the working mechanism of a microdialysis probe can be described as an artificial blood capillary manually inserted into the tissue of interest to sample small molecule analytes from a live animal.

To carry out microdialysis on the brain tissue, the first step starts with a minimally-invasive surgery on the test animal. Since microdialysis can be used to study the analyte concentration in the certain region of the brain, the cannulation site needs to be determined beforehand. After the test animal is immobilized and anesthetized, a small hole is drilled in the skull at the region of

interest for cannulation. A cannula is stereotaxically implanted into the brain to a certain depth from the pre-drilled hole, according to the anatomy of the animal species. In most studies using rats, the stereotaxic atlas of Paxinos and Watson is used to determine the coordinates of cannulation.[125] At this point, a replaceable dummy probe is inserted into the cannula. Then the test animals are put back into the cages with free food and water access for at least 1 to 7 days to recover from the trauma of the surgery. At the beginning of the experiment, the dummy probe is replaced with a microdialysis probe, which is made of a 3-4 mm-long semipermeable membrane with a certain molecular weigh cut-off (MWCO) from 1 to 100 kDa. The inlet and the outlet of the microdialysis probe are connected to a microdialysis pump, by which the perfusate is pumped through at a constant flow rate of approximately 0.1-5 μL/min. The perfusate is usually an isotonic solution that can mimic the physiological environment of the cerebrospinal fluid (CSF). Ringer's solution is one of the most widely used perfusates in microdialysis, which is an aqueous solution containing 149 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 0.8 mM $MgCl_2$ with the pH adjusted to 7.4.[99, 100, 102, 103, 108, 109, 113, 115, 116, 118, 119, 121-123] Artificial cerebrospinal fluid (aCSF) is another commonly used perfusate in brain tissue microdialysis.[101, 104, 107, 110, 114, 117, 120, 124] aCSF may have different formulas in different studies, but in general it is an isotonic aqueous solution that is close to the composition and pH of physiological CSF. Other solutions used as perfusates in microdialysis include 2 mM ammonium acetate or Dulbecco's phosphate-buffered saline.[111, 112] Besides inorganic salts added in the perfusate to maintain the osmotic pressure, additives can also be added for different purposes. Internals standards are commonly added in the perfusate as calibrators to calculate the microdialysis recovery.[103, 116] These internal standards are either structural analogues or stable isotope-labeled analogues of the analytes of interest. Small amounts of analyte stabilizer can also be added in the perfusate to protect the

analyte of interest from degradation, as long the osmotic pressure and the pH of the perfusate is not drastically changed. In a study concerning the quantitation of remoxipride in brain tissue, an anti-oxidant was used in the microdialysate to prevent the oxidation of analytes.[95] After starting the perfusion, the test animal is put back into the cage for at least 1 to 24 hours to let the microdialysis system reach equilibrium, which also allows the animal to recover from the insertion of the probe. At this point, dialysate can be collected as blank samples to describe the baseline concentration of the analyte of interest. Then the experiment can begin by dosing the animal with the studied drug. If the study is of endogenous compounds in the brain, which do not require extra dosing of the animals, samples can be collected directly after the equilibrium. To collect the microdialysis samples, the dialysate coming out from the probe is collected either manually or, in most occasions, by an automatic fraction collector. Multiple samples can be collected at different time points to describe the change in analyte concentration over time.

If the microdialysis membrane and the perfusate are correctly chosen, the resulting samples should only contain the analytes of interest, inorganic salts, small-molecule endogenous compounds and minimal levels of small proteins or peptides. In this case, microdialysis samples are clean enough to be directly analyzed by LC-MS/MS. Since the microdialysate contains high concentrations of buffer salts, desalting is needed to protect the mass spectrometer from being affected by salt ions. The easiest approach to separate the analytes from the salts is online desalting using a solvent delay.[104, 121] When a reversed phase LC column is used, the retention of the analytes is based on hydrophobic interactions. Therefore the salt ions would not be retained on the column and will elute at the dead time of the LC run. By using a divert valve to divert the LC eluent in the first several minutes to waste, the salts will not enter the inlet of the mass spectrometer. Meanwhile, the analytes of interest can be loaded into the mass spectrometer when

the divert valve is switched back to the main pass position. Another way to fulfill online desalting is by using column-switching, which will be discussed in the LC chapter.[77, 102, 103, 108, 116, 123] When instrumentation is limited, desalting can also be fulfilled by offline techniques. In a study involving the quantitation of dopamine and its metabolites in brain microdialysates, Syslová et al. developed an offline desalting method using lyophilization.[117] After sample collection, microdialysis samples were freeze-dried and reconstituted in methanol. Since the inorganic salts were not soluble in pure organic solvent, centrifugation was used to separate the analyte dissolved in the supernatant from the precipitated salts. In most studies, the sample preparation methods for microdialysis samples are facile. Usually the samples only need to be mixed with internal standards and then can be directly analyzed by LC-MS/MS, which is the most common sample preparation method for microdialysis samples.[95, 99-104, 107-110, 112, 114, 115, 117, 121-124] In studies that require cleaner sample preparation, protein precipitation has also been used for microdialysis sample clean-up.[102, 113] In a study involving the determination of three opioidmimetics in rat brain dialysates, Igarashi and coworkers used protein precipitation as the sample preparation. To each 60 μL of microdialysate sample, 60 μL of methanol was added and mixed well. The supernatant obtained from the following centrifugal filtration was injected into the LC-MS/MS system for quantitative purposes.

Microdialysis is a technique that extracts analytes from the brain tissue of a live animal, yielding analyte concentrations that may not accurately reflect the actual concentration. To describe the extraction efficiency of microdialysis, one needs to consider the dialysis recovery, which is defined as the ratio of analyte concentrations between the dialysate and the CSF. All the factors that may affect the passive diffusion of analytes between the inside and outside of the semipermeable membrane can contribute to the final recovery and therefore, need to be carefully

considered in microdialysis. These factors include the membrane MWCO and the flow rate. The MWCO of the semipermeable membrane has to be bigger than the molecular weight of the analyte but not so large as to allow impurities to pass through. The bigger the MWCO is, the easier it is for diffusion to reach equilibrium, resulting in a higher recovery and higher dialysate concentration. The flow rate of perfusion is usually 0.1-5 μL/min, which is considered to be enough for most small-molecule analytes to reach diffusion equilibrium between the two sides of the probe membrane. The lower the flow rate is, the more analytes in the brain tissue can diffuse into the perfusate, resulting in a higher recovery and therefore a higher dialysate concentration. With properly chosen membrane MWCO and flow rate, microdialysis can be conducted with high recovery and reproducibility.

Microdialysis can be considered as a semi-quantitative technique, since the real analyte concentration in the CSF can only be calculated with the measured concentration and the dialysis recovery. The dialysis recovery can be determined by several different techniques, among which retrodialysis is the most commonly used one, due to its simplicity of operations as well as high accuracy.[103, 107, 111, 115, 116, 118, 119, 121-124] To conduct *in vivo* retrodialysis, a probe that is the same as that used in the microdialysis is inserted into the brain of a live test animal. Instead of blank isotonic solutions, the probe is perfused with analyte solution of a known concentration at the same flow rate as the microdialysis experiment. During this process, the analyte in the perfusate will pass through the semipermeable membrane and enter the CSF outside the probe. After the diffusion reaches the equilibrium, it is assumed that the distribution of analyte at both sides of the membrane is the same as the equilibrium reached by normal microdialysis. With the perfusate concentration (C_p) and dialysate concentration (C_d) measured by LC-MS/MS, the relative recovery of microdialysis (R) can be calculated using this equation: $R = (C_p - C_d) / C_d$.

Therefore retrodialysis can be considered as the reverse process of microdialysis, yielding a relative recovery as the ratio of analyte passing through the membrane to that remaining on the original side. Instead of using real animals, retrodialysis can also be conducted *in vitro*. The calibrator used in retrodialysis can be the analyte itself, stable isotope-labeled (SIL) analogues or structural analogues. It is the most common practice to use the analytes as the calibrator, because of high accuracy and easy access.[107, 111, 115, 118, 119, 122-124] Retrodialysis with analyte calibrators must be conducted separately on a blank test animal, since the analyte calibrators will interfere with the analytes in real biological samples. SIL analogues can be a good choice as the retrodialysis calibrator, because they closely resemble both the physiochemical and the biological properties of the analyte.[116, 121] The most significant advantage of using SIL analogues as the retrodialysis calibrator is that they will not interfere with the analyte of interest, which means that the retrodialysis experiment can be conducted simultaneously with the microdialysis, simply by adding SIL analogues of the analyte in the perfusate. When SIL analogues are not available, structural analogues can also be used as the retrodialysis calibrator for simultaneous probe calibration.[103] Both SIL analogues and structural analogues can be used for the retrodialysis of either endogenous or exogenous analytes, while the analyte itself can only be used for exogenous analytes. In addition to retrodialysis, *in vitro* calibration is another method to determine the microdialysis recovery.[109, 113, 118] By immersing the microdialysis probe into an isotonic solution containing the analyte at a known concentration (C_s) and perfusing the perfusate through the probe under the same conditions as the microdialysis experiment, this method can be considered to mimic microdialysis. Dialysate samples are collected at different time points and the steady-state concentration (C_d) is determined by LC-MS/MS. Assuming that the small amount of analyte entering the dialysate will not change the analyte concentration in the large

volume of solution, the recovery (R) can be calculated by the equation: $R = C_d / C_s$. This method is a simple and direct way to measure microdialysis recovery. But the measured recovery may not sufficiently reflect the actual *in vivo* recovery, which can be affected by other factors, such as the possible interactions of tissue components with the analyte or the membrane materials.[118] After microdialysis recovery (R) is determined, the measured analyte concentration (C_m) can be converted to actual *in vivo* concentration (C_r) by the equation: $C_r = C_m / R$.

The microdialysis technique has multiple advantages over traditional homogenization-based sampling techniques. The most significant advantage of microdialysis is the capability of continuous sampling from a live animal. Unlike homogenization-based sampling methods that require the sacrifice of the animal for each sample, microdialysis can continue sampling at multiple time points from the live animal, which is of great convenience for pharmacokinetic (PK) studies. By sampling from the same animal, it can help improve the accuracy and credibility of the PK profile, since all the data points are obtained from the same animal and therefore inter-individual differences are avoided. Microdialysis also reduces the workload and cost by using fewer animals and less surgical operations. The throughput can also be greatly improved by the use of automated fraction collectors. Last but not least, microdialysis makes it possible to obtain samples from different functional regions of the brain simultaneously by inserting multiple dialysis probes into the brain, which otherwise would be much more difficult to fulfill by homogenization.

However, microdialysis also has some limitations and disadvantages compared to homogenization. One of the major differences between homogenization and microdialysis is the analyte coverage. Since homogenization disperses everything in the brain tissue into the media, it does not require any specific properties of the analytes and can be considered a “lossless”

sampling technique for most analytes. These characteristics make homogenization a widely compatible technique with nonspecific analyte coverage. Microdialysis, on the other hand, involves the diffusion of analytes from the brain tissue into the dialysate, and therefore limits its application to some analytes. Usually microdialysis can only be applied to small-molecule analytes, since it is hard for large-molecule analytes to diffuse into the dialysate. Another concern with microdialysis is the recovery, which is often much lower than that of traditional extractions following homogenization. Usually one would expect the microdialysis recovery to be in the range of 10 – 30%, which may compromise the sensitivity of the analytical method. In all microdialysis-based methods, the recovery needs to be determined to accurately measure the analyte concentrations in the targeted tissue, requiring extra experiments and labor. Unlike homogenization-based methods that can measure the total analyte concentrations in the targeted tissue, microdialysis can only measure the unbound analyte concentrations, since only unbound analytes can penetrate the semipermeable membrane. This will be a problem when total analyte concentrations are needed, especially for analytes with a high protein binding affinity. In addition, only analytes in the extracellular fluids of the targeted tissue can enter the dialysis probe, which means that microdialysis cannot measure analytes remaining in the tissue cells. In contrast, homogenization-based method can yield a total analyte concentration in the targeted tissue, since all bound or unbound, intracellular or extracellular analytes are collected in the final extract. Finally, the temporal resolution of microdialysis is lower than that of homogenization. Unlike homogenization samples that can be obtained at an exact time point, it takes some time to collect microdialysis samples for each time point. Usually the middle point of the collecting period is used to plot the concentration curve over time. Therefore the temporal resolution of

microdialysis is determined by the sample volume and the flow rate, which can be a potential issue for analytes with low stability or short half-lives.

2.3 Ultrafiltration

Ultrafiltration is another sampling technique that can be applied to sample collection from brain tissue. It can be considered as an altered technique that is analogues to microdialysis. Similar to microdialysis, ultrafiltration is also an *in vivo* sampling technique that can directly extract samples from the brain tissue of live animals. Due its convenience and, more importantly, the capability of continuous sampling, ultrafiltration has gained more attention for the sample collection of brain tissue for quantitative LC-MS/MS analysis.

The principle the ultrafiltration sampling technique is the extraction of biological samples through a semi-permeable membrane under the driving force of negative pressure. Like microdialysis, *in vivo* ultrafiltration also relies on the use of an implanted semi-permeable membrane, which only allows molecules with the molecular weight below the molecular weight cut-off (MWCO) of the membrane material to pass through. By applying a negative pressure on the collecting side of the membrane, biological samples can be drawn from the tissue side to the collecting side. Due to the filtering property of the semi-permeable membrane based on molecular weight, tissue components, blood cells and other large-molecule impurities are blocked in the tissue side of the membrane, while small molecule analytes, together with the extracellular fluid in the target tissue (CSF in the brain), will pass through the membrane and enter the collecting side. Samples collected by ultrafiltration are real extracellular fluid from the target tissue, which can be further processed or directly analyzed by LC-MS/MS. In summary, *in vivo* ultrafiltration can be described as a sampling technique that can physically pull the

biological samples from the target tissue and, in the mean time, filter the samples with a semi-permeable membrane.

To conduct ultrafiltration for brain tissue sampling, the preparation of test animals is similar to microdialysis, which starts with the implantation of a sampling probe. After the site of cannulation is determined based on the need of the analysis. A small hole is drilled in the skull at the region of interest of the pre-immobilized and anesthetized test animal, according to the stereotaxic atlas of Paxinos and Watson.[125] Similar to the surgical operations in microdialysis, a replaceable dummy probe is inserted into the cannula and the test animal is allowed back to normal diet to recover from the trauma. At the beginning of the sampling, the dummy probe is replaced with an ultrafiltration probe, which is a hollow fiber made of a semi-permeable membrane. The semi-permeable membrane should have a specific MWCO (usually from 1 to 100 kDa) that is slightly greater than that of the analyte of interest, so that the analyte and the CSF can pass through, while other impurities are excluded. To start the ultrafiltration sample collection, the sampling probe is connected to a vacutainer or a peristaltic pump, which applies a negative pressure to the probe and pulls the analyte-containing CSF through the probe membrane pores and into the collecting vial. The collected samples are ready for additional sample preparation or direct quantitative analysis.[126]

To develop an effective ultrafiltration method for the extraction of CSF samples from brain tissue, several factors need to be carefully considered. First, the membrane should be chosen with the proper material, and more importantly, the proper MWCO. The most basic rule is that the MWCO of the membrane should be greater than that of the analyte of interest, so as to achieve satisfactory analyte recovery. Under this condition, lower MWCO can provide cleaner CSF samples, but will increase the pressure and require lower flow rates. A higher MWCO, on

the other hand, is more technically favored but provide samples with more large-molecule impurities, so that further sample preparation will be needed. Usually, an ultrafiltration membrane with a MWCO of 3,000 will be suitable for most small-molecule sample collection from brain tissue. Second, the size of the probe and the depth of the insertion should be considered, which contributes to the surface area of the tissue-membrane interface. The bigger the surface area, the lower the pressure difference that is needed to pull the CSF samples out, which means that higher flow rates can be used. Meanwhile, bigger surface areas may also lower the temporal resolution of the sampling site, especially when the CSF sample from a specific functional region of the brain is needed. Typically, the diameter of an ultrafiltration fiber ranges from 0.2 to 3 mm and the insertion is 0.5 to 5 mm into the brain tissue.[127] Third, the negative pressure needs to be controlled to maintain a proper flow rate. Higher flow can lead to higher throughput and therefore higher time point resolution. More importantly, higher flow rates can enhance the sweeping effect across the membrane surface and prevent deposition of impurities. However, high negative pressure and higher flows may lead to rapid loss of CSF and cause serious brain damage to the test animal. It also requires more expensive instrumentation including membranes, tubing and a pump to achieve high flow rates. Compared to microdialysis, the sampling rate of ultrafiltration is slower (0.5 to 2 μL/hour/cm of membrane length) and can not exceed the rate at which the extracellular fluid is replaced by the blood vessels within the tissue

According to the basic principle and practical characteristics of ultrafiltration, ultrafiltration has several advantages over other sample collection techniques. Similar to microdialysis, ultrafiltration is capable of continuous *in vivo* sampling from the live animals, which allows for a better description of the analyte concentration change in the live animals over a certain period of

time. Also, the CSF samples obtained by ultrafiltration are free of tissue components, blood cells or large-molecule impurities, and can be directly forwarded to LC-MS/MS analysis with none or minimum sample preparation in most occasions. In addition, ultrafiltration has a significant advantage over microdialysis, in that it has high accuracy. Unlike microdialysis that only extracts sample solutes into the dialysate via diffusion, ultrafiltration pulls the real CSF from the brain tissue, which includes the analyte of interest, small-molecule solutes and the biological fluids. Therefore, the analyte concentrations in the samples obtained by ultrafiltration are a true reflection of the *in vivo* analyte concentrations in the brain tissue, and provides better accuracy than the semi-quantitative microdialysis technique. Finally, ultrafiltration can also be fully automated to achieve higher throughput and reduce labor.

Ultrafiltration also has some drawbacks and limitations in the *in vivo* sampling from the brain tissue. The most significant issue with ultrafiltration is membrane fouling. Since all the particles, cells and large-molecule impurities are blocked at the tissue side of the ultrafiltration membrane, they may accumulate over the sampling process and form a solid deposit on the membrane surface, leading to clogging of the membrane. Optimization of membrane pore size and flow rate is needed when membrane fouling is affecting ultrafiltration. Another big limitation of ultrafiltration on the brain sample collection is the limited quantity of CSF. Only a very limited volume of CSF can be continuously drawn from test animals before causing serious damage. Therefore, the flow rate of microdialysis and the total volume of samples are strictly limited for *in vivo* ultrafiltration on brain tissue. Another possible issue with ultrafiltration is the nonspecific binding of the analytes on the membrane, which may lead to lower recovery. At last, the cost of instrumentation and consumable materials is relatively high for ultrafiltration, especially when high pressure, high flow rate or some specific conditions are needed.

2.4 Solid-phase Microextraction

Solid-phase microextraction (SPME) is another sampling technique that can be applied to sample collection from the brain tissue. In terms of instrumentation and principle, SPME is also analogous to microdialysis and ultrafiltration. Generally, SPME can be described as a sample collection and preparation technique that involves the use of a fiber coated with an extracting phase, which is capable of extracting the analyte of interest from the live animals or biological samples.[128]

The principle of SPME is similar to that of solid-phase extraction (SPE). By immersing a porous fiber that is coated with a specific extracting stationary phase into the target tissue of a live animal or collected biological samples, sample collection, preparation an enrichment can be fulfilled during sampling.[129] Different coating materials can be chosen for the sampling probe, which is based on reversed-phase or hydrophobic interactions between the analytes and the hydrocarbon-based stationary phase (C4, C8, C18 or HLB).[130-132] After the analytes of interest are absorbed onto the surface of the coating material, the sample probe is pulled out and washed with a strong solvent, resulting in a sample solution that can be analyzed with LC-MS/MS with no or minimal sample preparation. This technique can be applied to collected biological fluids or tissue homogenates as a sample preparation technique.[132] However, what attracts more attention to the SPME technique is its capability for *in vivo* sampling from a live animal.[133] By implanting a sampling probe in the brain or other organs of the test animal, sample collection can be fulfilled from the live animal over a certain period of time, which is especially suitable for pharmacokinetic studies or other studies that require samples from the same animal at multiple time points.

To conduct *in vivo* SPME, the procedure include animal pretreatment, surgical implantation of the sampling probe and sample collection, which is similar to the procedure of microdialysis and ultrafiltration. What is different in SPME is that the sample collection process is not assisted with any liquid or gas, and is simply based on the free movement of analytes in the target organ and the unassisted adsorption onto the sampling probe. After a fixed period of time, the probe is removed from the target tissue. Unlike microdialysis and ultrafiltration that yield a liquid sample after the collection process, SPME only extracts solutes from the target tissue or biological samples, including the analyte(s) of interest together with other impurities that can be adsorbed under the same conditions. Therefore, the analytes need to be washed from the probe with a strong solvent (e.g. MeOH, ACN), resulting in a liquid sample that can be forwarded to LC-MS/MS analysis or further sample preparation. Since samples containing high concentrations of organic solvents will not run well in LC-MS/MS, a round of evaporation and reconstitution may be needed after SPME.[133]

To effectively conduct an SPME-based sample collection method, several important factors need to be optimized. First, the coating material on the sampling probe needs to be properly selected based on the properties of the analytes. In addition to the hydrocarbon materials that can provide affinity to non-polar analytes based on hydrophobic interactions, ion exchange interactions can also be utilized when ionic analytes are measured.[134, 135] By selecting the proper sorbent, SPME-based sample collection method can provide very high recovery and specificity, yielding clean samples with high analyte concentrations. Second, all the parameters determining the amount of extracted analytes need to be well controlled. The amount of analytes adsorbed onto the sampling probe is proportional to the radius of the sampling probe, depth of the insertion into the tissue and the length of the sampling time, which need to be optimized and then

fixed in the whole experiments to reach satisfactory sensitivity, precision and accuracy. However, one should be careful about maximizing these parameters, which may lead to the adsorption of large amounts of unwanted impurities.

Based on the principle and practical properties of SPME, this sampling technology has several significant advantages that made it increasingly popular for LC-MS/MS analysis. First, SPME combined the sample collection, sample preparation and sample enrichment are all in one procedure, which is very convenient and greatly improves throughput. Second, SPME can provide very high specificity towards the analytes of interest. By using the proper sorbents coated on the sampling probe, SPME can selectively extract analyte molecules without also recovering other impurities, which not only provides a cleaner sample, but also improves sensitivity. Samples collected by SPME usually only require minimal preparation before LC-MS/MS analysis. Third, SPME can provide high sensitivity. Since SPME is a solvent-free sample collection method, there is no dilution of analytes due to the addition of solvents. Moreover, the sampling process is also a sample enrichment process, so that the final concentration of analytes can be much higher than in the biological samples, which is a big advantage for methods measuring trace-levels of analytes. Finally, SPME can also be fully automated to reduce labor.

SPME also has some disadvantages and limitations that one needs to consider before choosing this technique. First, the accuracy of SPME greatly relies on the similarity between the calibration standards and the real tissue, when online *in vivo* SPME is used. Because the sample concentration measured with SPME is only a proportional reflection of the original concentrations in the tissue, the exact concentration values cannot be measured directly. Calibration curves are built by calibration standards (spiked samples with known analyte

concentrations), which have to be as close to the original tissue as possible. Second, when measuring analytes with very high *in vivo* concentrations, SPME probes may be saturated, yielding nonlinear recovery and therefore low precision and accuracy. Third, as adsorption of analytes on the probe is the primary mechanism of SPME, nonspecific binding of unwanted impurities can also be potential problem. Fourth, SPME only extracts and measures the fraction of free analytes in the target tissue, leaving the intracellular and protein-bound analytes hard to assess. Fifth, it takes from several to 30 minutes to finish the sample collection of each data point by SPME, which limits the density of data points in a continuous sampling experiment. Finally, the cost of SPME can be higher than other traditional sample collection techniques.

3. Sample preparation

After brain tissue samples are obtained from test animals, there are large quantities of impurities remaining in the samples, especially for brain tissue homogenates that maintain all of the tissue components. Impurities such as lipids, proteins and salts can cause a series of problems for LC-MS/MS analysis, including but not limited to matrix effects, peak shape distortion, column congestion and instrument contamination. Therefore, to achieve satisfactory sensitivity, and selectivity of the analytical methods as well as to maintain the performance of the instruments, further sample preparations of brain tissue samples are needed before the injection into the LC-MS/MS system. As mentioned in the last section, microdialysis samples are usually clean enough for direct LC-MS/MS analysis. Sample preparations discussed in this section are mainly focused on brain tissue homogenate. Due to their low cost and ease of development, the most common sample preparation methods are protein precipitation (PPT), liquid-liquid extraction (LLE), or solid phase extraction (SPE). There are also other minor sample preparation methods available for the clean-up of brain tissue homogenate, including online SPE and

enzymatic digestion. One can choose the proper technique or combinations of multiple techniques for the preparation of brain homogenate samples, on the basis of the analytical goal, sensitivity, cost or efficiency.

3.1 Protein Precipitation-based Sample Preparation

Protein is one of the major components of brain tissue homogenate, which may cause matrix effects and column congestion. It is important to remove proteins from the biological samples before LC-MS/MS analysis or further finer sample preparation operations. Since insoluble proteins can be easily removed by filtration or centrifugation, the major targets of sample preparation are soluble proteins. In order to remove the soluble proteins from the brain tissue homogenate, the proteins dissolved in the buffer or water need to first be precipitated. Protein precipitation is a traditional sample preparation technique designed for such purposes. It is capable of simple and fast treatment of biological samples, and can be considered as the most widely used sample preparation method. [9, 10, 13, 15, 16, 19-22, 24, 25, 29-31, 34-39, 41, 42, 46, 48, 49, 51-53, 55, 57, 58, 60-63, 66-68, 72, 74, 76-80, 87, 88, 90, 92, 94]

Proteins are large biological molecules that are composed of amino acids linked by peptide bonds. Under physiological conditions, a soluble protein has one or multiple peptide chains assembled into a folded conformation, with most hydrophobic amino acid residues facing the inside and charged or hydrophilic amino acid residues exposed on the surface. On the inside of the protein, the peptide chains are folded together mainly by hydrophobic interactions between hydrophobic amino acid residues; while other interactions including hydrogen bonds, salt bridges or disulfide bonds are also contributors to the folded structure. On the outside of the protein, charged or polar surface residues can interact with the environment and increase the solubility of a protein. In a brain tissue homogenate, repulsive electrostatic forces exist among soluble

proteins to prevent aggregation and facilitate dissolution. When a soluble protein is dissolved in water or an electrolyte buffer during tissue homogenization, water forms a solvation layer surrounding the protein and establishes a concentration gradient with the highest concentration at the protein surface. This weakens the ionic interactions between proteins and decreases the likelihood of aggregation. Therefore, to precipitate or induce the accumulation of proteins from the solution or suspension, proper agents can be added to reduce the hydration layer.

Protein precipitation usually involves three steps: addition of precipitants, mixing and removal of the precipitated proteins. First, a proper amount of precipitating agents are added to the brain homogenate. Organic solvents and inorganic acids are the most often used protein precipitants for small-molecule analytes in brain tissue homogenate, though other agents like neutral salts, non-ionic hydrophilic polymers and polyelectrolytes have also been used for protein precipitation. When miscible organic solvents are added to the brain tissue homogenate, the solvation layer around the protein will decrease as the organic solvent molecules displace water molecules from the protein surface. With a smaller solvation layer, proteins can get closer and form interactions with each other via attractive electrostatic or dipole interactions, leading to the aggregation of proteins. Commonly used organic solvents for protein precipitation are acetonitrile (ACN) and methanol (MeOH). ACN is the most common organic precipitant for the pretreatment of brain tissue samples, due to its strong precipitating ability.[13, 15, 19-21, 34, 37, 42, 49, 55, 57, 58, 62, 63, 67, 72, 77, 80, 87, 88] Unlike ACN that can yield more rigid protein pellets, MeOH is a milder organic precipitant, yielding flocculent protein sediments. It is also widely used in brain tissue sample preparation, due to its lower cost.[9, 14, 22, 24, 39, 51, 53, 61, 68, 90, 136] To each unit weight of brain tissue, at least two volumes of organic solvents are needed for efficient protein precipitation; while the more organic added, the more thorough the precipitation will be. Though pure organic

solvents can perfectly fit the purpose of precipitating proteins, other additives or solutes are commonly added for other purposes. It is a common practice to dissolve internal standards (IS) in the organic protein precipitants, which allows for the addition of the IS at the same time as protein precipitation.[20, 42, 51, 55, 61] This practice not only improves the throughput by combining two steps together, but also enhances the precision by pipetting larger volumes of IS solutions instead of a small-volume spike. Organic acids, formic acid and acetic acid for example, are also often added in the organic protein precipitants to lower the pH for the adjustment of analyte charge states.[15, 19, 24, 62, 72] The protein precipitation can also be facilitated by the addition of such acids, which can help with the reduction of the hydration layer around proteins. Other than the composition and pH of organic protein precipitants, the temperature is another factor to consider during protein precipitation. Usually ice-cold organic solvents are used to guarantee the stability of analytes, especially for those that are temperature sensitive.[15, 37, 39, 55, 68, 77, 88]

Similar to the choice of homogenization media, inorganic acids can also be used as the protein precipitant for the preparation of brain homogenate. Trichloroacetic acid (TCA) and perchloric acid (PCA) are the most commonly used acids for protein precipitation.[25, 41, 52, 54, 66, 79] Though the actual mechanism is not well know, it has been believed that the precipitation by TCA and PCA involves the denaturing of proteins.[137] Upon addition of these acids, the pH of the solution is greatly lowered and the protein conformations are drastically changed, leading to the exposure of hydrophobic amino acid residues resulting in the aggregation of proteins.[138, 139] The ideal concentration of TCA for protein precipitation is approximately 15%, while either lower or higher concentrations generally result in lower efficiency. Similar to the organic protein precipitants, low temperature and the addition of IS can also be featured when inorganic acids are used as precipitants. In a study published by Onorato et al. in 2010, pre-chilled 10% TCA

with supplemented IS was used for the protein precipitation of pulverized brain tissue samples.[54] Another important feature is that the lowered pH by the addition of acids can also be utilized for further sample preparations. In a study about the quantitation of cotinine and metabolites published by Li et al. in 2012, TCA is used for the protein precipitation of brain tissue homogenate.[41] Since all the analytes have a tertiary amine structure, they were ionized at lower pH caused by the addition of TCA, which facilitated the following sample preparation by solid-phase extraction (SPE). The retention of the protonated analytes on the mixed-mode cation exchange (MCX) SPE cartridges was enhanced at the lower pH.

Protein precipitants with more than one component are also used in multiple published studies.[10, 16, 30, 31, 38, 48, 76, 78] In a recent study about the quantitation of loxapine, amoxapine and their hydroxylated metabolites, combinations of MeOH and PCA at different relative ratios were tested in the sample preparation of brain tissue homogenate, suggesting that one volume of MeOH and 7 volumes of PCA yielded the best analyte recovery.[78] In another method published by Karinen et al. in 2009, a mixture of ACN and MeOH was used for the protein precipitation of brain homogenate.[31] Advantages of different agents can be taken at the same time when multiple protein precipitants are used in a single protocol.

Besides the choice of correct protein precipitants, the time of the addition of precipitants can also be different. As mentioned in the section of homogenization-based sample collection, protein precipitation can be combined with the step of homogenization, by using protein precipitants as the homogenization media.[9, 11, 15, 24-26, 28, 30, 32, 34, 38, 39, 42, 52-54, 68, 76, 79] If added after the homogenization, the addition of precipitant can be stepwise, which can yield higher precipitation efficiency by allowing for more complete interactions between the solvent molecules and the proteins. In a paper published by Hatziieremia et al. in 2007, 2 × 300 μL of

ACN was added to each 100 μL 20% brain homogenate to achieve complete protein precipitation.[21] In another study about the disposition of cannabichromene, cannabidiol, and Δ^9-tetrahydrocannabinol and its metabolites in mouse brain, Poklis and coworkers developed a protein precipitation method for mouse brain homogenate by adding ice-cold ACN drop by drop while vortex mixing, which maximized the ACN-protein interactions and therefore fully utilized the precipitating effects of added ACN.[57]

After the addition of protein precipitants, the mixture of brain tissue homogenate and the protein precipitants needs to be fully mixed by vortexing. Usually at least 1 to 10 min of vortexing is needed, depending on the volume of samples. During vortexing, the precipitant molecules are evenly distributed across the fluid eddies and interact with proteins. The solvation layers around the protein molecules are greatly reduced by precipitants, making it possible for proteins to get closer to each other. Protein molecules will collide into each other and form submicroscopic sized protein aggregates, under the influence of attractive electrostatic forces. These protein aggregates keep growing by diffusive additions of other protein molecules and eventually reach a critical size for precipitation, forming protein sediments or flocculus. In all the common protein precipitants, ACN and TCA have the strongest precipitating abilities and can form very solid protein precipitates of larger particle sizes. Being a weaker protein precipitant, MeOH forms looser flocculent precipitates. When proteins are precipitated by the addition of precipitants, small-molecule analytes will still remain in the solution. Meanwhile, analytes bound to proteins will be released into the solution, since the protein conformations are changed by the precipitants and therefore cannot hold on to the analytes any more.

When adequate vortexing has been applied to the mixture, most proteins in the homogenate have already been precipitated. The next step of protein precipitation-based sample preparation is

a physical process to separate precipitated proteins from the supernatant, in which the small-molecule analytes remain dissolved. Usually a round of centrifugation at 3,000 to 12,000 × *g* is sufficient to settle the protein precipitates and form a solid pellet at the bottom of the container. The higher speed of centrifugations is used, the more solid the pellet will be, making it easier to obtain the supernatant without disturbing the integrity of the pellet. Multiple rounds of centrifugations can be used for better results.[60]

Theoretically the supernatant obtain from protein precipitation is free of most proteins and can be directly analyzed by LC-MS/MS. However, additional processes are involved before LC-MS/MS analysis in many studies. Considering centrifugation is not efficient enough to remove insoluble impurities, especially those with lower densities than that of the supernatant, centrifugal filtration has been widely used to further purify the supernatant.[15, 21, 48, 52, 61, 92] By loading the supernatant onto a filter tube with submicrometer-sized (0.2 or 0.45 μm in most applications) membrane, small, low-density and insoluble impurities can be removed under centrifugation. Since at least two volumes of organic solvents or 15% TCA are needed for protein precipitation, the resulting supernatant usually has a high organic composition or very low pH, neither of which is favorable for LC-MS/MS analysis or further sample preparation. Therefore, either dilution or evaporation can be used to lower the concentrations of organic solvents or acids. By diluting the supernatant with aqueous solutions, the relative organic or acid concentrations can be effectively lowered. Since the dilution method also lowers the analyte concentrations inevitably, it is not used as much as evaporation in current methods. By evaporating the supernatant to complete dryness and reconstituting in aqueous solutions with proper pH values, organic or acid concentrations can be greatly reduced for further extractions or direct LC-MS/MS analysis.[9, 20, 21, 24, 25, 31, 58, 60, 68, 72, 87, 88] Moreover, sample enrichment can be

achieved by reconstituting the residue in a reduced volume relative to the original sample volume, which improves the method sensitivity at the same time. Due to the fact that protein precipitation is a rough sample preparation method that may not be enough to remove interfering impurities in some situations, it is often coupled with other sample preparation methods, including liquid-liquid extraction and solid-phase extraction.[10, 13, 16, 24, 25, 29, 30, 36, 39, 41, 53, 58, 60, 68, 72, 74, 76, 78-80]

In addition to the traditional protein precipitation by the addition of chemical agents, microwave fixation is another alternative sample preparation technique that can denature proteins in brain samples, and has been used in some studies for this specific purpose.[140-143] By applying a rapid high-energy microwave fixation to the brain samples collected by surgical dislocation, proteins in the brain tissue can be denatured. Unlike other traditional protein precipitation techniques that are mostly focused on the removal of undesired protein, microwave fixation is focused on the denaturing of the enzymes in the brain tissue. Brain concentrations of some analytes, including endogenous neurotransmitters, prostanoids and lipid-mediators, can be changed in response to conditions like ischemia or post-mortem delay, which are similar to the effects of decapitation. Therefore, after the brain tissue is removed from the decapitated animal, concentrations of these analytes in the brain tissue may be changed, leading to inaccurate concentration measurements. Accordingly, a rapid, head-focused and high-energy microwave radiation can be introduced to the brain sample right after the decapitation, so that all of the enzymes are denatured and the analyte concentrations are "frozen" at the original levels. In a study published by Bazinet *et al.* in 2005, an LC-MS/MS method was developed for the determination of anandamide (N-arachidonoylethanolamine, AEA) in rat brain. Since the brain AEA concentrations were reported to increase in response to ischemia and decapitation, brain samples were subject to head-focused microwave irradiation (5.5 kW, 3.4 s) following

decapitation to limit brain metabolism. Similarly, such techniques can be applied to studies that involve endogenous analytes in the brain that can be altered by enzymatic metabolism associated with decapitation.

Protein precipitation is a fast and low-cost sample preparation method that has been widely used for the pretreatment of brain tissue homogenate samples for LC-MS/MS studies. The easy three-step operation is simple to conduct, leaving less chance for error resulting from inter-batch or inter-operator differences. Cost of reagents and equipment for protein precipitation are very low, since only common precipitants and centrifuges are needed in most occasions. Protein precipitation is capable of removing most proteins and cell residue from samples, yielding a relatively clean solution for further sample preparation or direct LC-MS/MS analysis. With the option of evaporation and reconstitution in different solutions, samples prepared by protein precipitation are compatible with most other sample preparation techniques. Another significant advantage of protein precipitation is its high recovery. Since only proteins are hypothetically removed by this sample preparation method, small-molecule analytes should remain in the solution and yield a theoretical recovery of 100%. Such advantages of protein precipitation have made it very popular in bioanalytical applications.

However, there are also some disadvantages of protein precipitation, among which low selectivity is the most significant one. Without specific selectivity for the analytes of interest, protein precipitation is just a general sample cleanup technique to remove proteins from biological samples. The types and quantities of impurities removed are mostly determined by the types of precipitants chosen for the protein precipitation, leaving less leverage for the analyst to have analyte-specific sample preparation to prepare cleaner samples and achieve higher selectivity. Another significant disadvantage of protein precipitation is nonspecific binding.

Analytes can be adsorbed on the brain tissue residue or even the surface of the containers. In a study published by Zhou *et al.* in 2010, an LC-MS/MS method was developed for the quantification of sunitinib in mouse plasma, brain tumor and normal brain. When protein precipitation was used for sample preparation, peak shape and recovery were not satisfactory, due to the non-specific binding of sunitinib to the brain tissue. This issue is more common with lipophilic analytes, which have higher affinities for the fats, lipids and proteins in brain tissue. In some other occasions, protein precipitation is not efficient enough to yield samples that are clean enough for direct LC-MS/MS analysis, especially for brain tissue homogenate samples with large amounts of proteins and lipids. Direct analysis of tissue samples prepared by protein precipitation will cause a series of issues, including matrix effects, column congestion and instrument contamination. Therefore additional sample preparation techniques are often needed following protein precipitation.

3.2 Liquid-liquid Extraction-based Sample Preparation

Liquid-liquid extraction (LLE) is a common sample preparation technique that has been widely used in the bioanalysis of brain tissue samples by LC-MS/MS. It involves the extraction of solutes from one liquid solution to another immiscible liquid, usually biological samples and organic solvents are the scenario for bioanalytical sample preparation. The mechanism of LLE is based on the simple principle “like dissolves like”, suggesting that a solute will dissolve best in a solvent that has a similar polarity to itself. Due to hydrophobic interactions between solutes and organic solvents, nonpolar compounds have higher solubility in organic solvents. Ionic or polar compounds, in contrast, have higher solubility in aqueous solutions, which is facilitated by multiple interactions including ion-dipole interactions, ion-induced dipole interactions and hydrogen bonding. When more than one immiscible liquid phase is present, solutes will prefer to

be dissolve in the phase with similar polarity. If one solute is currently dissolved in the solution with less similar polarity, it will diffuse across the liquid-liquid interphase and enter the liquid phase with more similar polarity, which is a thermodynamically driven process. In the case of LLE-based sample preparation for brain tissue samples, nonpolar analytes or impurities in the aqueous phase can be extracted when an immiscible organic solvent is added, so that the separation of multiple solutes (analytes and impurities) is fulfilled by the physical separation of these two immiscible phases.

Based on the mechanism of LLE, two basic strategies have been widely used in the preparation of brain homogenate samples, "forward extraction" and "backward extraction". The "forward extraction" here refers to the direct extraction of the analytes of interest. By applying the proper organic solvents and pH conditions, the analytes of interest are extracted into the organic phase. Then the aqueous phase is discarded and the organic phase is forwarded to further preparation and finally LC-MS/MS analysis. This is the most direct practice of LLE and has been widely used in many studies, especially for highly hydrophobic analytes.[11, 13, 17, 28, 33, 39, 40, 47, 50, 71, 81, 83-86, 89, 91, 95, 136] However, large amounts of hydrophobic lipids and proteins present in the brain tissue can also be extracted by organic solvents, which may cause serious interference in LC-MS/MS analysis. In this case, the opposite strategy "backward extraction" can be employed to avoid the interference of hydrophobic impurities. The "backward extraction" refers to the practice of extracting unwanted hydrophobic impurities by organic solvents and leaving the analytes of interest in the aqueous phase. After the organic phase is removed, the aqueous phase containing the analytes can either be further processed or directly analyzed by LC-MS/MS.[8, 17, 28, 54, 68] This strategy is more suitable for ionic and polar analytes, which are hard to extract by organic solvents without extracting other interfering impurities. In a study concerning the

determination of 5-hydroxytryptamine, norepinephrine, dopamine and their metabolites, Su and coworkers developed a "backward extraction" method, extracting hydrophobic impurities from the brain tissue sample by using the mixture of chloroform and isopropanol (10:3, v/v).[68] Since all the analytes are highly polar compounds, they were not extracted by the organic solvent and remained in the aqueous phase for direct LC-MS/MS analysis. In situations that require higher selectivity for the analytes, both strategies can be utilized to yield cleaner samples, which is referred to as "double extraction" or "back-and-forth extraction". In a study published by Hou et al., a double LLE method was used to prepare brain tissue homogenate samples for the quantitative determination of a novel anti-Parkinson's disease candidate drug FLZ. Endogenous hydrophobic impurities were first extracted by *n*-hexane, while the analyte was not extracted. With the organic phase removed, the aqueous phase was further extracted by ethyl acetate, yielding a clean sample with high purity and low matrix effects.[28] However, recovery and throughput might be compromised by using such complex and selective methods.

Similar to protein precipitation, LLE-based sample preparation involves three major steps: addition of organic solvents, mixing and separation. To achieve efficient LLE as the sample preparation of brain tissue homogenate samples, several important factors need to be considered for the choice of organic solvents. First, based on the fundamental mechanism of LLE, the organic solvent chosen must have a proper polarity based on the solute of interest. The analyte or impurity to be extracted must have higher solubility in the solvent than in the biological samples. Second, the organic solvent chosen for LLE has to be immiscible with the brain tissue homogenate, so that a sharp interface can form between the organic solvent and the aqueous phase. Only when the two phases are physically separated can one of them be removed to achieve separation of certain solutes from the samples. Though not being mandatory, volatility is

another important property for LLE solvents. In "forward extractions" when organic phases including analytes are obtained, the samples need to be evaporated and reconstituted in LC friendly solvents. Volatile organic solvents are usually preferred in LLE, due to the fact that they can shorten the time needed for evaporation. The last property of an LLE solvent that needs to be considered is the density. One needs to know the density of the solvent compared to that of the water, so that the organic phase can be recognized after extraction.

Different organic solvents have been used in LLE-based sample preparations for brain tissue homogenate, including chloroform [8, 17], methyl *tert*-butyl ether (MTBE) [13, 40, 71, 95], *n*-hexane [17, 28], dichloromethane (DCM) [13, 91], ethyl acetate (EA) [13, 28, 33, 47, 81, 83, 136] and diisopropyl ether (DIPE) [84, 85]. Their physical properties are listed in Table 1. All the solvents have a positive partition coefficient (LogP) values, meaning that they are all nonpolar and immiscible with water (or have very low solubility in water). According to the "like dissolves like" principle, solvents with a higher LogP value are more capable of extracting more hydrophobic solutes. Generally, chloroform and hexane are usually used for the extraction of highly nonpolar analytes or phospholipids in the brain tissue homogenate. In a study published by Golovko and Murphy in 2008, chloroform was used to extract prostanoids, a series of highly nonpolar analytes, from the brain tissue samples.[17] DCM and DIPE are moderately nonpolar solvents that have a wider compatibility with most analytes. In a study concerning the quantitation of several different drug analytes, olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone, DIPE was used to extract all the analytes simultaneously from brain tissue homogenate.[84] EA and MTBE are relatively polar solvents that are commonly used for the extraction of slightly hydrophobic analytes. In a study for the determination of the brain concentration of remoxipride (LogP 2.1), MTBE was used in the LLE of the analyte from brain homogenate. To achieve finer

extraction aiming at a specific analyte, multiple solvents can be combined in LLE.[11, 39, 50, 54, 68, 86, 89] The mixture of chloroform and methanol (2:1, v/v) has been used by multiple studies, due to its moderate polarity and high extraction efficiency. A study involving the quantitation of cocaine and its metabolites in the brain tissue successfully used this combination of LLE solvents.[11]

Similar to homogenization media and protein precipitants, additives are also common in LLE for different purposes. One of the most important factors affecting LLE recovery is the pH, which can be adjusted by the addition of acids, bases or salt buffers.[11, 17, 33, 50, 54, 71, 84-86, 91, 95] The basic principle is that charged analytes have higher solubility in the aqueous phase and will not be extracted by organic solvents. If the analyte of interest is the target of LLE, the pH of the sample needs to be adjusted to maintain analyte molecules in their uncharged state to the largest extent, so that a high extraction recovery can be achieved. For example, Zimmer and coworkers published a study for the quantitation of metrifonate, an acidic compound that is charged at medium to high pH. In this study, orthophosphoric acid was added to the brain homogenate to lower the pH, so that the analyte was uncharged and therefore able to be extracted by DCM.[91] Sometimes the pH needs to be very finely adjusted to achieve satisfactory recovery of the analytes. In a paper published by Zhang and coworkers, an LLE-based sample preparation method required the pH to be adjusted to exactly 10.69 by disodium phosphate (Na_2HPO_4), so that the analytes olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone could be neutralized and maximumly extracted by DIPE.[84] If the extraction is aimed at the hydrophobic impurities, the analyte of interest needs to be maintained in its charged states to minimize the amount extracted by the solvent, which will be discarded in the separation step. In the study published by Bystrowska and coworkers in 2012, TCA was used for protein

precipitation, which lowered the pH and put a positive charge to cocaine and its metabolites as the analytes. The following LLE by the organic solvent only extracted unwanted hydrophobic impurities, leaving the analytes in the aqueous phase, which will be further processed and injected in the LC-MS/MS system.[11] Internal standards (ISs) should also be added with the solvents at the beginning of LLE, since they are needed to compensate for the extraction recovery. ISs can also be dissolved in the organic solvents and added to the samples in one step. This practice allows for higher throughput and precision, because ISs are added in a large volume of solvents in a single step.[81, 136] Last but not least, additives that stabilize the analytes can be dissolved in the organic solvents and added at the same time. In the study published by Golovko and Murphy, 0.005% butylated hydroxytoluene (BHT) was dissolved in the extraction solvent chloroform and added to the samples to prevent the oxidation of a series of air sensitive prostanoids.[17]

Brain tissue homogenate samples can be processed by LLE either directly or after some additional pretreatment, depending on the sample collection and other sample preparation processes. Brain tissue homogenate prepared with aqueous homogenization media can be directly processed by LLE, since the aqueous homogenate is immiscible with organic solvents. This has been the most widely used practice, due to convenience and high throughput.[13, 33, 40, 47, 50, 71, 81, 83-86, 89, 91, 95, 136] Brain tissue samples pretreated by PPT using inorganic acids can also be treated in this manner, since the samples after PPT are still aqueous solutions. In a study concerning the quantitation of malonyl-CoA in rat brain tissue, Onorato *et al.* added the LLE solvent chloroform/methanol (2:1, v/v) directly to the supernatant obtained from PPT by TCA.[54] One needs to make sure that the analytes of interest are not charged under such low pH conditions, otherwise addition of base is needed to raise the pH before LLE. However, brain

tissue samples homogenized or precipitated by organic solvents usually need some extra processing, since the organic-based samples are miscible with LLE solvents.[8, 11, 17, 28, 39, 68] One can change the solution composition of organic-based brain homogenate by evaporation of the organic solvents and reconstitution in aqueous solutions. In the study published by Su et al., brain tissue samples homogenized and precipitated by methanol were evaporated to dryness by vacuum freeze-drying. The residue was then reconstituted in water, which will be further extracted by the mixture of chloroform and isopropanol (10:3, v/v).[68] The reconstituted biological samples were purely aqueous and immiscible with the organic solvents. Another approach to facilitate the phase separation between the organic-based brain homogenate samples and organic LLE solvents is by dilution with water. By adding water to the system to adjust the organic/aqueous ratio, phase separation can also be achieved. In the study published by Hou *et al.*, brain tissue samples dissolved in methanol were diluted by a 4-fold volume of water before the extraction by *n*-hexane.[28] Similar approaches can also involve the LLE system composed of chloroform, methanol and water, with the volume ratio subject to adjustment to reach sharp phase separation.[8, 11, 39]

With properly pretreated brain homogenate samples and correctly chosen organic solvents, LLE can be initiated by the addition of solvents into the samples. In the "forward extraction", the extraction efficiency is primarily determined by the partition coefficient of the analyte of interest between the aqueous and organic phase, which is defined as the concentration ratio between the two phases when partition equilibrium has been reached. Therefore, when such conditions as pH and temperature are fixed, the amount of analytes extracted into the organic phase is determined by the volume ratio between the organic solvent and the biological sample. In the LLE of analytes from brain tissue homogenate, the more organic solvent that is used, the higher the

extraction efficiency that can be reached. Usually, at least 1 to 2 volumes of organic solvent are added to each unit volume of brain homogenate sample.[33] When the intended extraction efficiency cannot be achieved, one can increase the volume of organic solvent to extract more analytes. However, with more organic solvent added, more hydrophobic impurities are also extracted, which may cause matrix effects or instrument contamination in the later LC-MS/MS analysis. Therefore the volume of organic solvents needs to be optimized to reach maximum recovery with the least matrix effects. Similar principles can also be applied to "backward extraction" techniques. With more organic solvent added, more hydrophobic impurities can be extracted to yield a cleaner sample, which also increases the chance of compromising recovery by extracting more analytes. The addition of organic solvents can be finished in one step or multiple steps. Multiple-step extraction has been a commonly used strategy to increase extraction recovery.[39, 71, 84, 85, 91, 136] In a study for the determination of 5-fluorouracil and methotrexate in mouse brain, the LLE of analytes by 10-fold volume of fresh EA was conducted twice, with the resulting supernatant combined for further processing.[136] Compared to adding 20-fold volume of fresh EA at once, extraction with 10-fold volume of EA twice can further improve the recovery by extracting more analytes.

After the addition of organic solvents, the extraction system needs to be well mixed. LLE is a thermokinetic process that involves the diffusion of analytes (or impurities) from the aqueous phase to the organic phase, which takes a certain period of time. Therefore, the extraction efficiency is highest after the distribution equilibrium of the analyte (or impurity) of interest has been reached, when the amount of analyte (or impurity) crossing the interphase will not increase any more. To facilitate the diffusion of the analyte (or impurity) of interest to reach its equilibrium, vortexing is usually used as the mixing technique. Since the aqueous biological

samples are immiscible with the organic LLE solvents, turbulence from the vortexing will create small droplets of both phases and mix them together at a high speed. The formation of droplets can largely increase the interface area between the aqueous phase and the organic phase, facilitating the diffusion of solutes and shortening the time needed to reach equilibrium. Considering the requirement of time and mixing, 10 min of vortexing is typically needed for efficient LLE of biological samples by organic solvents.

After the distribution of the analyte or impurities of interest has reached equilibrium, separation of the organic and aqueous phases is needed to isolate the analyte of interest from unwanted impurities. Centrifugation is the most commonly used technique to fulfill the phase separation. Usually, at least 10 min of centrifugation above 2,000× *g* is needed to achieve sharp phase separation. If the brain homogenate has not been pretreated by PPT or the precipitates from PPT have not been removed, extended centrifugation at higher speed might be needed to turn the insoluble impurities into solid pellets. Then the phase separation can be easily fulfilled by transferring the wanted phase or removing the unwanted phase, while attention needs to be paid to the pipetting operations not to disturb the pellets. The relative positions of organic and aqueous phases are determined by the densities of both phases. Most common organic solvents used in LLE, n-hexane, DIPE, MTBE and EA for example, are lower than that of water, meaning that they will be the supernatant after the phase separation, which is easy to transfer or remove. Chloroform and DCM, however, have densities higher than that of water and are usually the bottom layer in LLE. If they are used in "backward extraction", which means the aqueous phase is the one to be retained, phase separation is easier to fulfill by transferring the analyte-containing aqueous supernatant into a new tube.[8, 54, 68] If chloroform or DCM is used in "forward extraction" applications, the bottom organic layer needs to be transferred into a new tube, which

might be hard to achieve, especially when PPT residues are still in the system. Different strategies have been employed to facilitate the phase separation without losing sensitivity or selectivity. One approach addressing this issue is multi-step transfer. In a study concerning the determination of metrifonate enantiomers in brain samples, Zimmer and coworkers developed an LLE method involving multiple liquid transferring steps. After vortexing and centrifugation following the addition of DCM, the organic layer was transferred to a centrifuge tube (A) and afterwards transferred in a second step to another centrifuge tube (B). The intermediate transfer of the extract to tube A was to remove small amounts of aqueous phase transferred together with the organic extract in the first step.[91] Another approach commonly used to facilitate phase separation is freezing, which utilizes the fact that the melting points of organic solvents are usually lower than that of aqueous solutions. In the study published by Golovko *et al.* concerning the quantitation of brain prostanoids, brain homogenate samples were extracted by chloroform. After being transferred to a new tube, the organic phase was cooled at -20 °C for at least 2 h. This step allowed the separation of any residual upper phase, which was frozen and easily removed before analysis.[17]

With the organic and aqueous phases separated from each other, the extraction of the analytes of interest or unwanted impurities can be considered finished. However, some additional steps may be needed before LC-MS/MS analysis, depending on the extraction strategies involved. If the last step of extraction is “forward extraction”, the analyte of interest will be present in the organic phase. Since pure organic solvents are strong eluents in reversed phase liquid chromatography (RPLC) and will largely weaken the analyte retention in the column, the analyte-containing organic extract cannot be directly injected into a reversed phase column. Also, large volumes of organic solvents are usually used in LLE, which means that analyte

concentrations in the final extract are lower than those in the original samples. Direct analysis of such low-concentration extracts will greatly lower the method sensitivity. Considering both the LC separation and method sensitivity, evaporation and reconstitution are commonly used following LLE to prepare samples for LC-MS/MS conditions.[11, 13, 17, 28, 33, 39, 40, 47, 50, 71, 81, 83-86, 89, 91, 95, 136] After being separated from the aqueous sample residues, the organic extract is evaporated to complete dryness by nitrogen flow, vacuum centrifugation or lyophilization. The evaporated samples will be reconstituted using the initial mobile phase from the LC separation, which can be facilitated by vortexing or ultrasonication. Sample enrichment can be achieved by decreasing the volume of mobile phase added in this step, which can improve the method sensitivity. If there are any insoluble residues left after the evaporation, an extra centrifugation step can be added after the reconstitution to yield clean samples for LC-MS/MS analysis.[13, 28, 33, 39, 40, 54, 83-86, 95] If the last step of extraction is "backward extraction", the analyte-containing aqueous phase can be directly forwarded to using RPLC-MS/MS analysis, since water is a weak eluent and will not interfere the retention of analytes.[8, 68] However, evaporation and reconstitution can also be applied to the aqueous samples to achieve sample enrichment or the removal of insoluble impurities. In the study published by Onorato *et al.*, 1 mL of brain homogenate was used in the sample preparation by LLE. After the "backward extraction" by 10 mL of chloroform/methanol (2:1. v/v), the aqueous samples were blow-dried, reconstituted into 150 μL of 0.1% formic acid in 10 mM ammonium acetate/methanol (80:20, v/v) and centrifuged before LC-MS/MS analysis. These post-extraction steps not only removed insoluble impurities, but also improved the method sensitivity by approximately 7-fold.

Conventional LLE is widely used in the sample preparation of brain homogenate samples for quantitative LC-MS/MS studies, due to several significant advantages. First, it has higher

specificity and selectivity compared to PPT. Based on the difference in hydrophobicity, analytes of interest can be separated from endogenous impurities in the biological samples. With the leverage of altering extraction solvents, pH and the choice of "backward" or "forward" extraction, the selectivity of LLE can be even improved. Theoretically, the only impurities that will remain in the extracts are compounds that have very similar hydrophobicity as that of the analytes at the specific pH used in LLE. Second, the cost of LLE is relatively low, since only common agents and instrumentations are needed. Third, the throughput of LLE can be increased when multiple samples are processed at the same time.

However, LLE also has several disadvantages preventing it from being used in all studies. First, the selectivity of LLE can only be considered as moderate, compared to PPT and SPE. If high recovery and throughput are pursued, the selectivity of LLE has to be compromised. Usually the final samples injected into the LC-MS/MS system still contain large amounts of impurities from the biological matrices. Second, LLE is a relatively labor-intensive sample preparation technique with repeated manual operations, including liquid transfer, capping, centrifugation and so on. Third, LLE often involves the addition of large volumes of organic solvents, leading to the dilution of analyte concentration. If the organic solvent is compatible with the LC-MS/MS method, the sensitivity of the method is compromised when directly analyzed by LC-MS/MS; if evaporation and reconstitution are employed, extra time is needed and the throughput is lowered. Fourth, the separation of two liquid phases can be difficult when an emulsion forms during vortexing. Solutions such as protein precipitation pretreatment, choice of different solvents, or the addition of emulsion inhibitors can be used to avoid this issue. Considering all the advantages and disadvantages, LLE can be considered a fast sample preparation method with moderate recovery, selectivity and throughput.

Based on the essential principles of LLE, there are also different improved LLE-based techniques used in quantitative LC-MS/MS analysis for brain homogenate samples. Due to the fact that LLE is a relatively labor-intensive sample preparation technique, automated LLE has been developed to reduce the human labor and improve throughput. By the adoption of 96-well formatted microliter plates and, more importantly, robotic liquid-handling systems, LLE can be conducted with minimum manual operations. Up to 96 samples can be processed simultaneously, which largely increases the method throughput. In a study for the quantitation of reboxetine enantiomers in rat plasma and brain by LC-MS/MS, Turnpenny and Fraier used the automated LLE technique for sample preparation.[71] Pressurized liquid extraction (PLE) is another improved LLE technique that has been used in current studies. PLE is a new LLE-based sample preparation technique that employs elevated temperatures and pressures during extractions. The elevated temperature allows the sample to become more soluble and achieve a higher diffusion rate while the elevated pressure keeps the solvent below its boiling point. Therefore, the new technique PLE has the advantages of high extraction efficiency, less solvent usage and shorter extraction time. In the method published by Zhou *et al.* in 2012, PLE was conducted at 140 °C and 10.34 MPa to achieve high extraction efficiency.[89] They also used a dispersion agent in the PLE, which enhanced solute exchange and reduced the solvent volume. However, PLE also has its significant disadvantages. Since the extraction of each sample has to be done individually in a pressurized extractor, the overall throughput of PLE is actually much lower than conventional LLE, though the extraction time for each sample is slightly shorter. Solid-supported liquid-liquid extraction or matrix-assisted liquid-liquid extraction has been reported as a new sample preparation technique based on LLE, which can be described as LLE with the assistance of inert sorbents.[59] Solid sorbents provide a surface for the aqueous sample to interact with the organic

solvents, improving the extraction efficiency. More importantly, better recoveries and precision can be achieved by reducing the emulsions that are often formed in conventional LLE.

3.3 Solid-phase Extraction-based Sample Preparation

Solid-phase extraction (SPE) is a powerful sample preparation technique that can be used for the enrichment and purification of biological samples before LC-MS/MS analysis. The mechanism of SPE is similar to that of column liquid chromatography, which is based on the affinity between solutes dissolved in a liquid (mobile phase) and solid materials (stationary phase). Due to different physical and chemical properties, different components in the liquid sample have different affinities with the stationary phase in the SPE devices. Therefore, by passing the liquid sample through the stationary phase, either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase, so that separation of desired and undesired components can be achieved. The general strategy of SPE is based on the retention and elution of analytes of interest. If the mobile phase passing through the stationary phase contains the analytes of interest, it is collected for further preparation or LC-MS/MS analysis. If the mobile phase passing through the stationary phase contains only undesired impurities, which means the analytes of interest are retained on the column, the mobile phase passing through the stationary phase is discarded. Then the analytes can be eluted from the stationary phase when rinsed with a proper eluent. By separating the analytes of interest from undesired impurities in different column eluents, sample preparation can be achieved with high selectivity.

Though different devices, both the stationary phase and mobile phase can be optimized in SPE to achieve different selectivity. The general procedures of SPE for brain homogenate samples are similar in most studies. Since brain homogenate samples contain large amounts of

insoluble cell debris that may cause congestion of the SPE stationary phase, centrifugation is commonly applied to brain homogenate before SPE.[16, 36, 45, 56, 60, 65, 70, 74, 82] Endogenous proteins can cause serious issues including stationary phase congestion, nonspecific binding and matrix effects. Therefore, PPT is also used frequently as a sample pretreatment before SPE.[23, 24, 26, 30, 41, 53, 58, 64, 78-80] Depending on the different SPE stationary phases and the option of evaporation/reconstitution, either organic solvents or inorganic acids can be used as the PPT agents. After the centrifugation (evaporation and reconstitution in some studies), the supernatant can be forwarded to SPE. In most occasions, the SPE stationary phase needs to be properly prepared before the biological samples are loaded. The SPE stationary phase first needs to be rinsed with pure organic solvents, ACN and MeOH for example, as the activation step. Due to the low surface tension, organic solvents can quickly soak the stationary phase and pass through, dissolving and removing impurities trapped in the stationary phase during manufacturing. This step is also important for removing air in the packing material to maximize the extraction efficiency of SPE. Then the SPE stationary phase is subjected to the equilibrium step, during which a weak eluting agent is flushed through to prepare the stationary phase into proper conditions for the retention of analytes. After the stationary phase is activated and conditioned, biological samples are loaded and allowed to pass through the stationary phase slowly by gravity. Typically in conventional SPE, the analytes of interest need to be retained on the stationary phase, which is then subjected to one or two rounds of washing steps. Weak eluting agents are applied to remove unretained impurities from the stationary phase, while the retention of the analytes of interest should be minimally affected. During all the activation, condition, sample loading and washing steps, the flow rate of liquid passing through the stationary phase should be maintained dropwise. The flow must be stopped when the liquid level reaches the surface of the

packing material to prevent the stationary phase from drying, except for the last washing step after which the stationary phase is completely dried. Finally, the analytes are eluted by applying a strong eluting agent, which is optimized to maximally elute the analytes of interest but minimally elute hydrophobic impurities retained on the stationary phase. If the SPE protocol is properly optimized, the analytes of interest are retained on the stationary phase as a short plug. By eluting the analytes with small amounts of eluting agents, sample enrichment can be achieved to improve sample sensitivity and fulfill trace level detection. Multiple eluting steps with small amounts of eluting agents can be used to achieve higher analyte recovery. If the solution condition is compatible with the LC method, the eluent containing the analytes of interest can be directly analyzed by LC-MS/MS.[64, 80] In most occasions, however, a round of evaporation and reconstitution is employed.[16, 24, 26, 27, 30, 36, 41, 43, 45, 53, 56, 58, 60, 65, 70, 74, 78, 79, 82, 117] Since the eluent condition is optimized to minimize the retention of analytes, direct injections of such solutions into the LC-MS/MS system may result in poor separation, if the retention mechanism of the SPE method is similar to that of the LC method. By evaporating the eluent and reconstituting in an LC-compatible solution, better LC separation can be achieved. In addition, the adjustment of reconstituting volume can be utilized to improve the method sensitivity, especially when the eluent volume is bigger than the original samples. An extra centrifugation step can be added here to further eliminate insoluble impurities for the protection of the LC column and instruments.[78, 82]

Many types of SPE have been developed based on different chemistry. Physical and chemical properties of the analytes of interest, including hydrophobicity, hydrogen bonding and pKa, should be considered during method development when using SPE-based sample preparation. The type of chemistry and all the solutions or solvents used in SPE can be optimized to achieve

high recovery and selectivity. Normal phase, reversed phase and ion exchange are commonly used SPE chemistries.

Due to the fact that most drugs, metabolites or endogenous compounds found in brain tissue are hydrophobic compounds, reversed phase is the most commonly used SPE chemistry in the sample preparation of brain homogenate samples.[16, 23, 24, 26, 36, 45, 58, 70, 80, 82, 117] Reversed phase SPE separates analytes and impurities based on their polarities, providing selectivity for nonpolar analytes. Nonpolar analytes or impurities will be retained on the stationary phase, while polar impurities will pass through. The affinity between the analytes and the stationary phase is based on pi-pi bonding and hydrophobic interactions. The stationary phase of reversed phase SPE is usually made of a silica or polymeric sorbent derivatized with varying length hydrocarbon chains. Similar to LC columns, C4, C8 and C18 are common stationary phases for reversed phase SPE. Longer hydrocarbon chains can provider better selectivity for more nonpolar analytes. Based on the mechanism of reversed phase SPE, water is the weak solvent, which should be used in the conditioning, sample and washing solutions to achieve better analyte retention on the stationary phase. Brain tissue samples homogenized or precipitated by organic solvents often need to be centrifuged, evaporated and reconstituted in an aqueous solution.[58, 80] Dilution with water or aqueous solutions can also be used to lower the percentage of organic solvents.[26] In contrast, organic solvents, ACN and MeOH in most applications, are the strong eluting solvents. Therefore, organic solvents are the strong solvents and should be used in the final eluting solutions, which can maximize the amounts of analytes eluted from the stationary phase and maximize recovery. In a study published by Liu *et al.* in 2011, a reversed SPE-based sample preparation method was developed. Since the analyte hydralazine was a hydrophobic compound under neutral pH, an ODS-C18 SPE cartridge was chosen. After the cartridge was activated with

3 mL of MeOH and balanced with 3 mL of water, the biological sample in an aqueous solution was loaded. Following a washing step with 1 mL of 10% MeOH, the analyte was eluted with 1 mL of 80% MeOH. In this method, pure water and low percentage organic solvents were used to maintain the retention of the analyte. Unretained hydrophilic impurities were washed off by the weak wash (10% MeOH), while the strong wash (80% MeOH) was used for the elution of analytes.[45] Besides the polarity of solvents, pH is another important condition to adjust in reversed phase SPE. The general principle is that uncharged analytes (or impurities) can be retained on the reversed stationary phase, while charged analytes (or impurities) have high polarity and therefore cannot be retained. Accordingly, retention of the analytes of interest or undesired impurities can be adjusted by altering the charge states under different pH conditions. In a study publish by Goldwirt *et al.* in 2012, a reversed phase SPE sample preparation method was used to prepare brain homogenate samples for LC-MS/MS analysis. Both analytes, irinotecan and 7-ethyl-10-hydroxycamptothecin (SN-38), are hydrophobic compounds that are uncharged at low pH. Besides the selectivity provided by the adjustment of solvent polarity, ammonium acetate buffer (10mM, pH 3.5) was used for both conditioning and washing, in which the analytes are uncharged and well retained on the stationary phase.[16] Reversed phase SPE has been widely used in the sample preparation of biological samples due to its multiple advantages. Its selectivity for nonpolar analytes is suitable for most drugs, metabolites and endogenous compounds in brain tissue. Because hydrophobic interactions are the major driving force in reversed phase SPE, method development is relatively simple, which is primarily fulfilled by use of different types and percentages of organic solvents. However, it also has some significant disadvantages, which need to be considered during the method development. Brain tissue has large amounts of hydrophobic lipids, proteins and other endogenous compounds that may co-

elute with the analytes, causing matrix effects and instrument contamination in LC-MS/MS analysis. These impurities sometimes cannot be fully removed by method optimization. Another issue with reversed phase SPE is its incompatibility with organic PPT and reversed phase LC, which requires additional evaporation and reconstitution steps before and after the SPE. Throughput of the method will be compromised when such extra operations are added.

In addition to the conventional reversed phase SPE, hydrophilic-lipophilic-balanced (HLB) SPE is a newer technique based on reversed phase SPE. By using sorbents that have both hydrophobic and hydrophilic function groups, such SPE stationary phases can be used for the separation of a wide range of acids, bases and neutrals. In a study published by Hooff and colleagues in 2010, SPE using HLB cartridges was used as the sample preparation of brain homogenate for the quantitative LC-MS/MS analysis of farnesyl-(FPP) and geranylgeranylpyrophosphate (GGPP). The extract was finally eluted with an ammonium hydroxide/propanol/n-hexane mixture (1:7:12, v/v/v), which lowered the pH and increased the organic solvent composition for the maximum elution of analytes.[27] HLB SPE is a powerful sample preparation technique, due to its dual selectivity and wide compatibility. High recovery and sensitivity can be achieved by using HLB SPE, especially when simultaneous measurement of multiple analytes with different properties is needed.

Normal phase SPE can provide selectivity for polar compounds, which is opposite to that of reversed phase SPE. A stationary phase of underivatized silica or functionally bonded silica with short carbons chains is commonly used in normal phase SPE. The retention of analytes on the stationary phase is on the basis of dipole-dipole interactions and hydrogen bonding, creating affinity between polar analytes and the stationary phase. In typical normal phase SPE, polar analytes and impurities are retained on the stationary phase, while nonpolar impurities can pass

through with little or zero retention. In contrast with reversed phase SPE, nonpolar organic solvents are the weak solvents in normal phase SPE, which can be used for conditioning, sample loading and washing; water and polar solvents are the strong solvents, which are used for the elution of analytes in the final step. In a study published by Richardson *et al.* in 2007, a normal phase SPE method was used to prepare brain homogenate samples for LC-MS/MS analysis.[60] The analytes, endocannabinoids and related compounds, are a series of nonpolar compounds that have only weak retention in normal phase SPE, so that silica SPE cartridges were used for the cleanup of polar impurities. After samples were loaded onto the cartridges in chloroform, 3 × 1 mL of chloroform was used to wash unretained nonpolar lipids. Then the analytes were eluted in three steps: 2% methanol in chloroform (2 × 1 mL), 2% methanol and 0.2% TEA in chloroform (4 × 1 mL), and 2% methanol and 0.05% TFA in chloroform (4 × 1 mL), which altered the solvent polarity and pH to achieve maximum analyte recovery.

Ion exchange SPE is another commonly used SPE technique that provides selectivity for charged analytes. The separation of the analytes of interest from the undesired impurities is based on electrostatic interactions between the solute ions and the charged sorbents. At a pH where the stationary phase and the analytes of interest are oppositely charged, the analyte ions are retained on the stationary phase via ion exchange mechanisms, while uncharged impurities will pass through the stationary phase with little or zero retention. To elute the analytes of interest from either the sorbents, the stationary phase is washed with a solvent that neutralizes the charge of the analyte, the stationary phase, or both. If charged functional groups are mixed with hydrophobic carbon chains and hydrophilic function groups of the sorbent, hydrophobic selectivity can also be added to the stationary phase. This results in mixed-mode ion exchange stationary phases, which are now widely used in the majority of ion exchange SPE techniques.

Therefore, both pH and percentage of organic solvents in the mobile phase can be adjusted to achieve optimal sample preparation performance. Based on the different selectivity, ion exchange SPE can be divided into two major categories, anion exchange SPE and cation exchange SPE. Anion exchange SPE uses a positively charged stationary phase, which can provide selectivity for negatively charged ions, such as acids. Strong anion exchange sorbents contains permanently charged quaternary ammonium groups, providing affinity for moderate to weak bases; weak anion exchange sorbents have amine groups which are charged at pH below 9, providing affinity for strong acids. The washing and eluting conditions are summarized in **Table 2**. In a study published by Shah *et al.* in 2008, mixed-mode strong anion exchange SPE was used for the preparation of brain slice superfusion samples. Because all the analytes, N-acetylaspartate, N-acetylaspartylglutamate and glutamate, were weak acids, mixed-mode strong anion exchange was chosen as the SPE chemistry. After the stationary phase was balanced with 1M phosphate buffer (pH 6.5), superfusion samples in 0.1 M ammonium hydroxide were loaded, followed by a two-step wash with 2 mM phosphate buffer (pH 6.0) and deionized water. The high pH of the sample solution and the slightly acidic pH in the washing solutions were both well above the pKa of the analytes, meaning that the analytes were negatively charged and therefore retained on the stationary phase. The bound analytes were eluted from the SPE column using a mixture of 0.3 M HCl and methanol (80:20, v/v).[64] The pH of the eluting solution was low enough to change the analytes into their uncharged state, disrupting the ionic exchange interactions. Together with the increase of organic solvent composition, the analytes of interest were eluted from the stationary phase to the largest extent. In contrast to anion exchange SPE, cation exchange SPE uses stationary phases bearing negatively charged functional groups, which can provide specificity for positively charged analytes, such as bases and amines. When loaded on the stationary phase,

positively charged analytes and impurities can be retained via electrostatic interactions, while uncharged impurities will pass through with little or no retention. Strong cation exchange sorbents contain aliphatic sulfonic acid groups that are always negatively charged, providing affinity with moderate to weak bases. The retention or elution is adjusted by the change of charge states of the analytes. In contrast, weak cation exchange sorbents contain aliphatic carboxylic acids that are charged when the pH is above 5, which is more specific for strong bases or quaternary amines. The retention or elution is adjusted by the change of charge states of the stationary phase, shown in **Table 2**. In a study concerning the determination of dihydroetorphine in rat plasma and brain samples, Ohmori and colleagues develop an SPE-based method to prepare biological sample for LC-MS/MS analysis. Since the analyte of interest has a tertiary amine group and is positively charged at low pH, mixed-mode strong cation exchange SPE was used. Considering organic solvents can weaken the retention of analytes on the mixed-mode SPE sorbent, brain homogenate samples pretreated by MeOH PPT were diluted with 6-fold volume of 50 mM phosphate buffer (pH 6.0). After the SPE cartridges were washed sequentially with 3 mL of methanol and 3 mL of 50 mM phosphate buffer (pH 6.0), the diluted samples were loaded. Then the sorbent was washed with 3 mL of 100 mM acetic acid, which removed neutral, negatively charged and hydrophilic impurities that were not retained. The low pH maintained the analyte in its positively charged state and did affect its retention. This was followed by another wash with 2 mL of methanol, which removed neutral or negatively charged impurities that were retained solely by hydrophobic interactions. Finally, the analyte was eluted using 4 mL of 2% (v/v) ammonium hydroxide in ethyl acetate.[53] At high pH, the positive charge on the analyte was neutralized and the ion exchange interactions no longer existed. Together with the hydrophobic interactions compromised by the organic solvent, complete elution of the analyte was achieved

for high extraction recovery. Ion exchange SPE has been widely used to prepare brain homogenate samples for the LC-MS/MS analysis of charged analytes. Especially for strong acids and bases, which cannot be well extracted by reversed phase or normal phase SPE, ion exchange SPE can provide good selectivity as well as high analyte recovery. Moreover, the additional selectivity based on hydrophobicity makes mixed-mode ion exchange SPE more powerful in terms of removing undesired impurities.

SPE can also be integrated with other sample preparation techniques. In a study for the determination of phosphodiesterase-5 inhibitors and their main metabolites in rat brain tissue, Unceta *et al.* used HybridSPE-PPT cartridges (Supelco, Bellefonte, USA) in the sample preparation.[72] After adding brain homogenate and protein precipitant into the cartridges, the sample preparation was finished by vortexing and vacuum eluting. The SPE sorbent worked as a filter, removing precipitated proteins. It also worked in a manner of reversed phase SPE, strongly binding and removing hydrophobic lipids from the brain homogenate. By integrating PPT and SPE in one step, endogenous proteins and lipids can be effectively removed with high throughput.

Besides the different chemistries that can be used to achieve analyte-specific sample preparation, there are also different formats of SPE that can be chosen for the consideration of cost, throughput and performance. Cartridges and manifolds are the most commonly used SPE devices.[16, 24, 26, 27, 30, 36, 41, 43, 45, 53, 56, 58, 60, 65, 70, 74, 78-80, 82] The sorbents are packed in syringe-shaped cartridges that are available in different sizes and chemistry. These disposable cartridges can be mounted on the ports of a manifold, which can be connected to vacuum via a control valve to facilitate the flow of mobile phase. Alternatively, positive pressure can also be used for the same purpose.[60] Waste and samples are collected by test tubes located in the manifold. The

biggest advantage of such configurations is the low cost, due to the low prices of cartridges and manifolds. However, the disadvantage of low throughput is also very significant. Multiple steps of liquid transfer and replacement of collecting test tubes require intensive labor. In addition, the size of the manifold, commonly 24-port at most, largely limit the number samples that can be simultaneously processed and therefore decreases throughput. To improve the throughput of SPE, 96-well plate and automatic liquid handling robots have been introduced in recent years. However, the high cost of such configurations prevents it from being widely used. SPE spin-columns are another format of SPE devices, which are made by packing sorbents into small columns with a collection chamber. By applying centrifugation instead of vacuum, the SPE processing time can be reduced. Simultaneous processing of multiple samples also contributes to its high throughput. Moreover, smaller amounts of solvents are needed for SPE spin-columns, which can enrich the analytes of interest and therefore improve the method sensitivity.[64] Similar improvements have also been made by the invention of SPE tips, which put SPE sorbents in the pipette tips. SPE in a smaller scale with higher sensitivity can be achieved using SPE tips. Throughput, however, is lowered, since each sample has to be processed individually. [117] Online SPE can be considered as the most advanced SPE technique, due to its capability of complete automation. The column balancing, sample loading, washing and analyte eluting procedures are all completed automatically with the use of an online SPE column, and more importantly, a column switching system equipped with multiple solvent pumps. In a study published by Heinig and Bucheli in 2008, an online SPE system was used for the sample preparation of biological samples, which is directly coupled with the LC-MS/MS system.[23] Manual operations are minimized in online SPE, greatly improving the method throughput. Nevertheless, the method development can be very difficult, which involves not only condition optimization but also

instrument engineering. Moreover, high cost of instrumentation is another significant limitation of online SPE.

SPE is a commonly used technique for the sample preparation of brain homogenate for quantitative LC-MS/MS analysis. Several advantages have made it a very powerful sample preparation technique. First, SPE is compatible with a very wide range of analytes by using different SPE chemistries. Specific and efficient analyte extraction can be achieved by SPE to purify most compounds, whether the analytes of interest are polar, nonpolar, basic or acidic. Second, SPE can be optimized to be very specific for the analytes of interest. By optimizing proper washing and eluting conditions, the analytes of interest can be separated from the biological sample with minimal amounts of co-eluting impurities. Such issues as matrix effects, LC peak distortion, column congestion and instrument contamination can be minimized when employing a specific SPE method, which is of great importance for complex biological samples like brain homogenate. Third, high recovery can be achieved by SPE, thanks to its wide compatibility and high specificity. Fourth, sample enrichment can be fulfilled by SPE, which is hard to perform by PPT or LLE. Fifth, SPE can be automated to largely decrease manual operations. However, SPE also has some significant disadvantages. The most crucial disadvantage of SPE is its low throughput, compared to PPT and LLE. Unless automated SPE systems are used, multiple steps of liquid transfer, evaporation and reconstitution are often involved, making conventional SPE a labor-intensive and low-throughput sample preparation technique. Another disadvantage of SPE is its complexity. Since there is no universal SPE protocol available, large amounts of extraction conditions and parameters need to be optimized during the method development, which can be very costly and time-consuming. Last but not least, SPE has a much higher cost than that of PPT or LLE, due to the cost of the consumable materials

and equipment of SPE. With all the advantages and disadvantages, SPE can be considered a highly specific but more complex technique in sample preparation method development. Due to its high performance in recovery, specificity and compatibility, SPE can be used when a satisfactory sample preparation cannot be achieved by simpler techniques like PPT and LLE. SPE can be an ideal sample preparation technique in situations that require simultaneous measurement of multiple very different analytes, thorough cleanup of highly complex biological samples or fully automatic sample analysis.

4. Summary

Research on the ADME and mechanism of action of small-molecule drugs, metabolites and biomarkers in the CNS are largely dependent on the distribution and dynamics of such chemicals in the brain tissue. Methods to accomplish this must be capable of quantitating of concentrations of one or a series of analytes. Among all the current techniques, LC-MS/MS has been widely used for the concentration measurements of small-molecule analytes in brain tissue samples. In order to effectively measure analytes in brain tissue using LC-MS/MS, brain tissue samples need to be obtained from the test individual and prepared into an LC-MS/MS compatible status, making sample collection and sample preparation two essential steps in bioanalysis.

Traditional sample collection involves sacrifice of the test individual, surgical removal of the brain tissue and physical homogenization in a proper media, generating a homogeneous brain tissue suspension. This is a low-cost, convenient but rough sample collection technique, yielding a brain homogenate that is extremely complex. In contrast, microdialysis, ultrafiltration and SPME are more advanced sample collection techniques, which involve the *in situ* extraction of analytes from the brain of a live test animal. Though these techniques can yield very clean samples, they all have some significant disadvantages limiting them from being widely used in

all studies. Therefore, it is suggested that homogenization is the primary sample collection technique to be considered in most occasions. Only when the large quantities of interferences introduced by brain homogenate severely affect the method performance, should one consider microdialysis, ultrafiltration or SPME for sample collection.

Sample preparation is of crucial importance for brain sample analysis by LC-MS/MS, not only due to its capability of preparing the tissue sample into an injectable liquid that is LC-MS/MS compatible, but also because of its capacity of removing impurities that may cause column congestion, matrix effects, signal interference and instrument contamination. Protein precipitation (PPT), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are common sample preparation techniques, which are listed in their order of ascending selectivity. However, cost, labor and processing time of these three techniques also increase in the same order. When a new sample preparation method needs to be developed for the extraction of a particular analyte from brain tissue samples, one needs to consider the choice of sample preparation technique or combinations of multiple techniques based on the properties of the analytes, biological matrices and the expectations of the method. All the advantages and disadvantages of these available sample preparation techniques need to be taken into account. Different materials and conditions can be adjusted to achieve the optimal balance between the analyte recovery and the selectivity. A proper sample preparation method should maximally remove the impurities under the prerequisite condition of losing minimal amounts of the analytes of interest.

Different sample collection and sample preparation techniques have different characteristics, allowing the analysts to choose or combine the optimal techniques during method development. Disadvantages and disadvantages of all sample collection and preparation techniques discussed in this paper are summarized in **Table 3**. By using proper sample collection and sample

preparation techniques, clean, enriched and representative liquid samples can be obtained for LC-MS/MS analysis, contributing to a sensitive, selective and reproducible method for the quantitation of small-molecule analytes in the brain tissue. With no universal technique that can fit every bioanalytical scenario, one should always carefully choose the most proper sample preparation strategies for the analytes of interest, by comprehensively considering analyte properties, recovery, selectivity, throughput, cost and complexity during method development.

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Table 1. Partition coefficient (LogP3), density and boiling point values of common LLE solvents at 25 °C, 1 atm.

	LogP3	Density (g/mL)	Boiling point (°C)
Ethyl acetate	0.7	0.897	77.1
Methyl *tert*-butyl ether	0.9	0.735	55.2
Dichloromethane	1.5	1.326	39.8
Diisopropyl ether	1.5	0.725	68.5
Chloroform	2.3	1.483	61.2
n-Hexane	3.9	0.655	68.7

Table 2. Summary of four commonly used ion exchange SPE techniques.

Chemistry	Stationary Phase	Analyte Selectivity	Washing Conditions	Eluting Conditions
Strong Anion Exchange	Quaternary Ammonium	Acids (Weak)	High pH Low Organic	Low pH High Organic
Strong Cation Exchange	Aliphatic Sulfonic Acid	Bases (Weak)	Low pH Low Organic	High pH High Organic
Weak Anion Exchange	Amine	Strong Acids	Low pH Low Organic	High pH High Organic
Weak Cation Exchange	Aliphatic Carboxylic Acids	Strong Bases Quaternary Amines	High pH Low Organic	Low pH High Organic

Table 3. Summary of advantages and disadvantages of all the sample collection (A) and sample preparation (B) methods discussed in this paper.

A

	Advantages	Disadvantages
Homogenization	Wide compatibility; Low material and instrument cost; Loss-less sampling technique; High precision and accuracy.	Higher chance of contamination; Spiked samples different from real samples; Single data point from one test animal; Extensive sample preparation needed;
Microdialysis	Direct *in vivo* sampling; Continuous sampling from live animals; None or minimum sample preparation; Fewer animals and less surgical operations; Automation capability.	Small-molecule analytes only; Semi-quantitative technique; Only measures free drug fraction; Low precision and accuracy; Low temporal resolution. High cost.
Ultrafiltration	High precision and accuracy; Direct *in vivo* sampling; Continuous sampling from live animals; None or minimum sample preparation; Fewer animals and less surgical operations; Automation capability.	More suitable for small-molecule analytes; Membrane fouling; Only measures free drug fraction; Limited sample volume and data points; Nonspecific binding; Low temporal resolution. High cost.
Solid-phase microextraction	Direct *in vivo* sampling; Sample enrichment; Continuous sampling from live animals; None or minimum sample preparation; Fewer animals and less surgical operations; Automation capability.	Semi-quantitative technique; Saturation; Nonspecific binding; Only measures free drug fraction; Limited data point density; High cost.

B

	Advantages	Disadvantages
Protein precipitation	Low cost; High throughput and low labor intensity; High precision and accuracy; High recovery due to minimum loss of analytes.	Further sample preparation often needed; Sample dilution due to addition of agents; Non-specific adsorption; Matrix effects caused by remaining impurities.
Liquid-liquid extraction	Higher selectivity over protein precipitation; Low cost; Multiple adjustable factors to achieve specificity; Automation capability.	Cannot remove all the impurities; Labor-intensive; Evaporation and reconstitution often needed; Emulsion; Matrix effects caused by remaining impurities.
Solid-phase extraction	Wide analyte coverage; High specificity; High recovery achievable with proper conditions; Automation capability.	High cost; Low throughput; Labor-intensive if not automated;. Extensive method development needed