# In vivo solid phase microextraction for brain tissue analysis

**by**

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## Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

# Abstract

In the last decade, a great deal of information has been revealed about chemical neurotransmission occurring within the brain. Methods used to monitor brain neurochemicals offer an exciting opportunity to enhance quest to understand neurodegenerative disease pathology. In addition, these new methods augment the discovery of new and innovative pharmacotherapies used for the treatment of neurodegenerative illnesses. Microdialysis has been routinely used to monitor the chemical constituents of the brain extracellular fluid in freely moving rats. However, there are still analytical challenges such as laborious and time consuming calibration methods associated with measurements and monitoring of these neurochemicals using microdialysis. In addition, the general complexity of the brain's chemical activity and the rapid nature some neurochemical concentrations change makes brain tissue analysis a challenging task.

Solid phase microextraction since its introduction has been successfully applied to both invasive and non-invasive biological tissue sampling. Advancement in the development of calibration methods, introduction of novel biocompatible coatings and the easy coupling to liquid chromatography tandem mass spectrometry have contributed to the overall success of the method for various *in vivo* applications. This thesis utilizes the potential of solid phase microextraction coupled to liquid chromatography tandem mass spectrometry for *in vivo* brain tissue analysis of freely moving animals.

Preliminary research in this thesis focused on the investigation of factors that can negatively affect post *in vivo* microextraction of a biological system. Subsequently, a multi-fiber semi-automated desorption device was developed on a 96-well plate format and the performance of the

device was evaluated. Results presented did not only show very good inter- and intra-well variations (% RSD ≤ 15) but also the device, by design, was capable of preventing any possible fiber contamination and/or damage.

The thesis also demonstrates the potential of using new mixed-mode coatings for simultaneous extraction of selected multiple endogenous neurochemicals with varying polarities. A new robust chromatographic separation method was introduced for the analysis of polar neurochemical substances (glutamic acid, gamma amino butyric acid, dopamine and serotonin) without the need for derivatization of the analytes. Chromatographic separation of the selected neurochemical substances was achieved on a pentafluorophenyl column with a 5 min total runtime with column pre-conditioning. Applying the proposed method to *in vitro* extractions of neurotransmitters from brain tissue samples and cerebrospinal fluid demonstrated the potential of *in vivo* analytical technique. Subsequently, an *in vivo* technique was developed to simultaneously monitor changes in the concentrations of multiple neurochemicals in the brain extracellular fluid. The solid phase microextraction method was validated against *in vivo* microdialysis, a well-known sampling tool for brain neurochemical measurements. The proposed solid phase microextraction method can be used not only for measurements of basal concentrations of neurochemical, but also changes in their concentrations after the application of an external stimulus (intraperitoneal administration of fluoxetine drug). Both solid phase microextraction and microdialysis recorded an approximately 3- to 4-fold increase in basal concentrations of 5-HT in extracellular fluid after the administration of the drug. In addition, solid phase microextraction was used successfully for global metabolomics studies; a novel sampling approach with the potential of improving overall metabolites coverage. Thus, improving identification of possible disease biomarkers. The new

sampling approach combines microdialysis and solid phase microextraction for extracting polar and non-polar chemical substances. Thus, for the first time microdialysis and solid phase microextraction have been combined in a single platform for untargeted metabolomics studies. In addition, as a proof of concept, solid phase microextraction can be used to spatially resolved concentration gradient of drugs and/or endogenous compounds within the brain extracellular fluid. This was demonstrated using solid phase microextraction to monitor changes in the concentration of drugs (carbamazepine and cimetidine) in both frontal cortex and striatum of the brain of rats.

Finally, but not least, the thesis demonstrates the potential application of *in vivo* solid phase microextraction to clinical studies. In this aspect of the thesis, solid phase microextraction was used to study to potential effect of deep brain stimulation on neurotransmitters. Among the four analytes monitored, the concentration of serotonin increased by 2 to 3x during deep brain simulation and remains constant as long as the stimulation was applied. The method linearity range was 0.01 pg/ml to 150 ng/mL for all selected neurotransmitters. A 30-min SPME extraction protocol was developed and applied for all *in vivo* experiments.

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# Dedication

This thesis is dedicated to the Almighty God for His protection and sustenance during the tough times of the program.

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# List of Abbreviations

| | |
|---|---|
| **5-HT** | 5-hydroxytryptophan |
| **5-HIAA** | 5-hydroxyindoleacetic acid |
| **aCSF** | artificial cerebrospinal fluid |
| **ACh** | acetylcholine |
| **AD** | Alzheimer's disease |
| **AP** | anteroposterior |
| $C_0$ | original concentration |
| **cLc** | capillary liquid chromatography |
| **CNS** | central nervous system |
| **CSF** | cerebral spinal fluid |
| **CV%** | percent coefficient of variation |
| **DA** | dopamine |
| **DANSYL** | 1-dimethylaminonaphthalenesulfonyl |
| **DBS** | deep brain stimulation |
| **DOPAC** | 3,4-dihydroxyphenylacetic acid |
| **DS** | Down syndrome |
| **DVB** | divinylbenzene |
| **ECD** | electrochemical detection |
| **ECF** | extracellular fluid |
| **EDCs** | endocrine disrupting compounds |

| | |
|---|---|
| **EDTA-Na** | disodium ethylenediaminetetraacetic acid |
| **ESI** | electrospray ionization |
| **ESI-LC-MS/MS** | electrospray ionization-liquid chromatography -tandem mass spectrometry |
| $f_c$ | fiber constant |
| **GA** | glutamic acid |
| **GABA** | gamma aminobutyric acid |
| **GC** | gas chromatography |
| **GC-MS** | gas chromatography mass spectrometry |
| **HESI** | heated electrospray ionization |
| **HILIC** | hydrophilic interaction chromatography |
| **HPLC** | high performance liquid chromatography |
| **ICP-MS** | inductively coupled plasma mass spectrometry |
| **i.p.** | intra-peritoneal |
| **L-DOPA** | 3,4-dihydroxyphenylalanine |
| **LC** | liquid chromatography |
| **LC-MS** | liquid chromatography-mass spectrometry |
| **LC-MS/MS** | liquid chromatography tandem mass spectrometry |
| **LLE** | liquid-liquid extraction |
| **LOD** | limit of detection |
| **MD** | microdialysis |
| **MS** | mass spectrometry |
| **NA** | noradrenaline |

| | |
|---|---|
| **NDA** | naphthalene-2,3-dicarboxaldehyde |
| **OPA** | ortho-phthalaldehyde |
| **PA** | polyacrylate |
| **PBS** | phosphate buffer solution |
| **PD** | Parkinson’s disease |
| **PD** | pharmacodynamic |
| **PDMS** | polydimethylsiloxane |
| **PDMS-DVB** | polydimethylsiloxane/divinylbenzene |
| **PFP** | pentafluorophenyl |
| **PK** | pharmacokinetic |
| **PPT** | protein precipitation |
| **PPY** | polypyrrole |
| **RPA** | reverse phase amide |
| **RSD** | relative standard deviation |
| **SPE** | solid phase extraction |
| **SPME** | solid phase microextraction |
| **SZ** | Schizophrenia |
| **UV** | ultraviolet |
| **vmPFC** | ventromedial prefrontal cortex |

# Chapter 1

# General Introduction

## 1.1 Brain Tissue Sampling: Relevance and Challenges

The brain arguably is the most complex and important organ in animals and with over 100 billion nerve cells in continuous communication.[1] This communication, which occurs across the synapse, is known to transmit important neurochemical information that is directly link to the function of the central nervous system (CNS), thus controlling our behavior, cognition, memory, etc. The interference or alteration of the brain neurotransmission process has been linked to various neurological disorders, such as Alzheimer's Disease (AD),[2] Parkinson's disease (PD),[3] and schizophrenia (SZ)[4] among others. The disturbing fact about some of these diseases is the predicted increase in the number of cases in the very near future.[5] Thus, it will be vital if further insights can be gained into the fundamental molecular/chemical information or mechanisms of the diseases pathogenesis. Successful brain metabolomics or chemical profiling using appropriate analytical tools certainly will offer appropriate platform for significant advancement in clinical, medical and research studies. In addition, enrich chemical information will facilitate the identification of disease biomarkers for clinical intervention for early prognosis, diagnosis and even treatment of these neurodegenerative disorders. Currently, disease-related changes in local and distributed neural circuits, synaptic, and molecular levels are detected through the direct study of the brain tissue. For example, through analysis of the brain tissue, it was established that the major cause of PD is due to significant reduction in dopamine concentrations in the basal ganglia of the brain,[6,7] a condition

in which the neurons in the substantia nigra gradually degenerate. Secondly, brain tissue analysis provides insights into the potential effects of drugs of abuse in neurological disorders. Issues related to drug toxicity can also be well addressed through quantitative brain tissue analysis. Direct studies of the brain tumor tissues will provide insight into the existing chemical information. Finally, *in vitro* postmortem studies of the brain tissue can be used to establish the mental state of an individual. However, to obtain appropriate and reliable chemical information from the brain, very good analytical methods and techniques will be required. In addition, the ability to obtain accurate information via effective sample preparation methods or tools has always been a challenge and often the method's effectiveness is debatable.

Sampling brain tissue for chemical information is fraught with difficulties. The heterogeneity of the brain anatomy itself poses difficulties to brain sampling. Typically, for *in vitro* experiments, sample handling, storage, sample pre-treatment, post mortem among others and their possible impact on data reliability and interpretation are very critical. The conventional homogenization methods like ultra-sonication, acid or base digestions, organic solvents, etc, are often faced with the challenge of data interpretation due to the complex heterogeneity of the brain structure and also the multiple roles of certain neurotransmitters found in the brain. For example, data interpretation can be difficult for *in vitro* glutamate analysis in brain tissue, which plays both metabolic and neurotransmitters functions in the brain.[8] In a typical *in vitro* animal postmortem studies, the high oxygen demand of the brain often lead to degradation of various substrates and loss of certain key metabolites such as glycogen and glucose shortly after death.[9] Complications associated with postmortem delays can often lead to misinterpretation of detected metabolite changes in the brain during

sampling.[10] In addition, most of these conventional approaches used for brain tissue analysis are often time-consuming and can be labor intensive. Issues of choosing the appropriate extraction method in order not to compromise analyte stability and data quality, the amount of representative sample required for the tissue sample preparation, effect of matrix especially for analytical techniques that require liquid chromatography coupled to mass spectrometric detection systems, and the use of appropriate internal standard among others can pose significant challenges. In the wake of the difficulties associated with conventional sample preparation methods coupled with the demands to obtain faster analysis and to reduce overall analytical cost, there is a gradual paradigm shift to *in vivo* brain tissue analysis. This may be due to obvious gains, such as obtaining richer analytical information that is more representative of the biological system under study, significant reduction in the number of animals sacrificed for studies, improving overall data quality by minimizing inter-animal variability, etc., associated with *in vivo* studies. In addition, there is a gradual shift from bioassays and assays of postmortem tissues to the use *in vivo* extracellular fluid sampling coupled to high performance liquid chromatography (HPLC) and other separation analytical systems. Generally, the continuous advancements of other technology have significantly improved the quest for understanding the dynamics of neurochemicals in the nervous system.

The role of separation science in tissue bioanalysis in general cannot be overemphasized. Separation science literally unlocks detail information of any biological sample often in a well-characterized and reproducible fashion, and creates the basis for accurate identification and quantitation of the components of the sample. In this regard, it is a common phenomenon to have analytical separation systems coupled to sample preparation.

Since most of the analytes of interests have relatively negligible volatility, HPLC systems have been a common analytical separation tool used in combination with brain tissue analysis. An even more powerful approach involves the use of sensitive and selective hyphenated analytical techniques, which augment analyte detection and reliable quantitation at ultra-low concentrations. In particular, HPLC coupled to mass spectrometry is now a well-accepted technique for tissue bioanalysis as well as an analytical technique of choice for both selective and sensitive detection of compounds in challenging biological matrices. Although other well developed separation and detection methods like capillary electrophoresis coupled with fluorescent and electrochemical detection systems have been applied to tissue bioanalysis, HPLC coupled to mass spectrometry (MS) continues to appeal to many researchers. Application of this hyphenated analytical technique has significantly facilitated studies of the chemical dynamics of the central nervous system (CNS) through monitoring of amino acid and biogenic monoamine neurotransmitters and neuropeptides among others.[11–19]

There is obviously no doubt that the coupling of *in vivo* analytical sampling tool to the appropriate selective and sensitive separation systems will continue to provide the necessary platform in overcoming aspects of the challenges associated with brain tissue analysis.

## 1.2 In vivo brain sampling methods: focus on neurotransmitters

### 1.2.1 Neurotransmitters

In recent years, measurements of neuronal chemical signals, neurotransmitters, have been accepted as a fundamental approach to understanding the chemical dynamics of the CNS. Accurate analysis of the brain tissue for neurotransmitters is by no means a trivial issue. This

is because the brain nervous tissue is full of nerve endings (neurons), which differ from other living tissues, which are in constant communication with each other. However, before proceeding to mention *in vivo* sampling methods for neurotransmitters, it is worthwhile to allude to the importance of brain neurotransmitters. Very briefly, neurotransmitters are low molecular weight endogenous compounds that play a significant role in brain function and are known to affect our behavior, cognition, mood, health, etc. The simplest and commonly used classification approach is based on their chemical structure. In terms of their chemical structures, neurotransmitters can be grouped as choline ester (acetylcholine), monoamine (dopamine, 5-hydroxytryptamine/serotonin, noradrenaline, adrenaline and histamine), amino acids (glutamate, gamma amino butyric acid and glycine), peptides (endorphins, enkephalins and cholesystokinins) and purines (adenosine and adenosine triphosphate). Most of these neurotransmitters are known to play major role in various biological activities.

Amino acid neurotransmitters are the most abundant in the brain although their acceptance as involved in neurotransmission was much later compared to monoamines.[1,20] Glutamic acid (GA) and gamma amino butyric acid (GABA) are respectively fast excitatory and inhibitory neurotransmitters with related metabolism. Formation of GABA is through the enzyme, glutamate decarboxylase from GA. GA is a known neurotoxin which is converted into a nontoxic glutamine within the glial cells. GA is also associated with metabolic regulation in the brain and often makes it challenging when studying the role of GA in neurotransmission. Neurological disorders such as epilepsy, cerebral ischaemia and hypoxia are associated with GA.[1] Disruptions in excretions of GABA are observed in pathophysiology of epilepsy, anxiety and schizophrenia. Dopamine (DA) is formed from 3,4-dihydroxyphyneylamine (L-DOPA)

with L-amino acid decarboxylase acting as a catalyst.[21] Serotonin (5-HT) is formed through hydroxylation of tryptophan by tryptophan hydroxylase and subsequently reacting with L-amino acid decarboxylase.[22] A portion of 5-HT is metabolized through aldehyde dehydrogenase to form hydroxyindoleacetic acid (5-HIAA) whereas DA forms dihydroxyphenylacetic acid (DOPAC) through a monoamine oxidase. Serotonin in the brain is associated with various pathological states like migraine, SZ, depression, etc.[22] Serotonin is also associated with feeding and cognition, sleep, thermoregulation, etc. in animals.[22] Serotonin also mediates in brain development, regulates the growth of serotonergic neurons and target tissues, and the likely cause of autism and Down Syndrome (DS) known in humans.[23] DA is known to be involved in PD[3], SZ[4], attention deficit hyperactivity disorder, as well as influencing cognition,[24] focusing,[24] and reward.[25] Figure 1.1 shows structures of some selected neurotransmitters (DA, 5-HT, GA and GABA) used in this project.

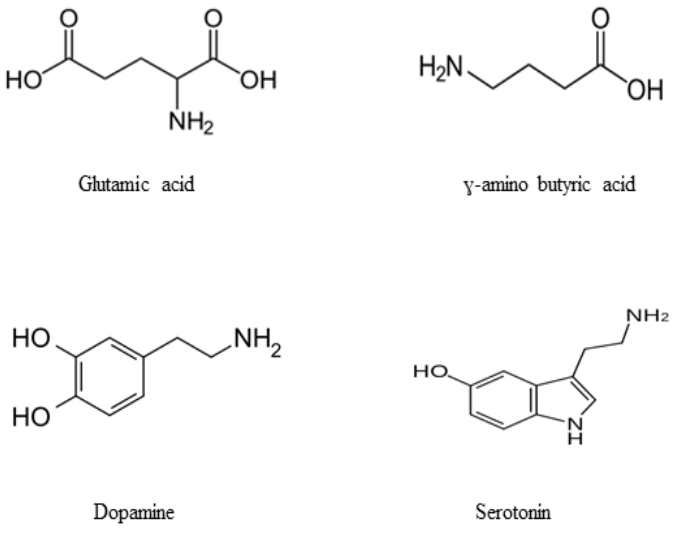


Figure 1.1 Structures of the selected amino acid (gamma amino butyric acid and glutamic acid) and monoamine (dopamine and serotonin) neurotransmitters studied in this thesis.

Some neurotransmitters and their metabolites are generally found in very lower µM concentrations in the brain. Thus, sampling and monitoring of these neurochemicals have been challenging, as it requires very sensitive, robust and specialized analytical methods and techniques. Although, conventional *in vitro* extraction methods like tissue slicing may be still relevant, recently *in vivo* brain sampling methods have gain significant interest in neurosciences and have been used to study neurotransmission within the extracellular fluid of the brain. This may be attributed to fact that *in vivo* methods are able to capture the dynamics of neurotransmitters in the ECF, which is a measure of the function of the biological system, compared to the static *in vitro* measurements of in brain tissues. Some of the commonly applied analytical methods for *in vivo* monitoring of neurotransmitters include voltammetry (electrochemical), microdialysis, biosensors, etc.

### 1.2.2 Voltammetry for monitoring neurotransmitters

*In vivo* electrochemical methods have been used for direct monitoring of some neurotransmitters within the ECF of brain since chemical neurotransmission is initiated by an electrical signal, an action potential. Electrochemical methods, which often involve the use of microelectrodes, require that the detected species be electroactive, i.e., voltammetry is based on oxidation and reduction reactions of the neurotransmitter(s) at the surface of an electrode. In principle, the technique involves the application of a controlled potential between two electrodes and the resultant current that flows is indicative of the amount of electroactive material in the solution. The unique advantages of the technique are observed with its sensitivity, spatial resolution, due to the small size of the carbon electrode (3 µm in diameter) and high temporal resolution (fast detection 100 ms or less)[26] properties. The technique is

commonly used for monitoring monoamines such as 5-HT, DA, noradrenaline (NA), etc. and their metabolites because they are readily oxidized at specific potentials on the surface of the electrodes within the ECF. Common electrodes used for direct *in vivo* electrochemical techniques are carbon-fibre[27] or chemically modified platinum electrodes.[28,29] Other electrodes used for voltammetric measurements include the graphite modified with nafion polymer,[30] for the determination of DA, NE and 5-HT. Electrodes made from nanoparticles[31,32] have been also explored in *in vivo* voltammetry applications.

Normally, for *in vivo* voltammetric measurements of the changes in concentrations of neurotransmitters in the ECF of, say the rat's brain, microelectrodes are implanted into the desired brain region for initial quantitative determination of baseline concentrations. Subsequently, the animal is presented with a form of stimulus and the changes in the electrochemical potential are correlated with the particular neuronal activity within the ECF. Very often the approach used during *in vivo* voltammetry is dependent on experimental objectives such as slow-scanning methods used in monitoring neurochemical behaviour may require longer time scale compared to the fast-scanning techniques often used for monitor dynamics of transmitters release on a millisecond scale. Most commonly used methods fall under three main techniques; cyclic voltammetry, chronoamperometry and differential pulse voltammetry.

Generally, in cyclic voltammetry, the voltage between the working and reference electrodes is applied linearly with time and the current is measured. The observed current increases with the applied potential until it reach a maximum. At this point, the concentration of the analyte at the surface of the working electrode decreases to zero and analyte's molecules

can diffuse to the electrode at the highest rate. The current reaches a steady-state value. The process can be repeated in a backward scan by reversing the potential between the electrodes to re-oxidize the product formed during the first reduction reaction for reversible reactions. Subsequently, a current of reverse polarity is also formed. Features of the voltammogram (plot of current versus potential) are used to obtain information concerning the chemical properties of the substance detected. For example, the ratio of the peak current during the forward and reverse scanning provides information that can be used to identify the chemical substance in addition to the measured voltage.[33] The detection limits of cyclic voltammetry is dependent on the magnitude of the charging and residual currents generated from other components that may be present in the ECF other than the current obtained from the electroanalyte of interest. Charging current occurs from migration of all other charge chemical species (ions) present at the electrode surface but do not form part of the redox reactions. It is important to note that the net current measured is always a sum of the current obtained from the redox reactions and the charging current, which contributes to the background current and substantively influence the method's detection limit. Due to the fact that the residual current also results from activities at the surface of the electrode, often detection limit is improved by employing very slow scan rates. This limits the potential of using cyclic voltammetry for monitoring rapid changes in the concentrations of neurotransmitters found in the brain.

In chronoamperometry, the potential of the working electrode is stepped and held constant for a period of time, and the resultant current from the faradaic process is measured as a function of time at the end of each applied voltage. At the beginning of the applied voltage an increase in current is observed, which later reduces as the electroactive species at the surface

of the electrodes is depleted. The advantage of chronoamperometry over cyclic voltammetry is that the magnitude of the charging current reduces exponentially compared to the faradaic current, as the voltage is held constant. In addition, since the measured current is taken at the end of the potential step, the interferences observed from the charging current is significantly minimal or negligible. In chronoamperometry, the recorded current during the potential step is proportional to the electroactive species concentration in the ECF. However, due to the lack of selectivity, as other species can be electrolyzed with the applied potential, the main species contributing to the observed current is not accurately known. The lack of selectivity therefore serves as a major disadvantage of the technique.

Differential pulse voltammetry may be viewed as a hybrid of cyclic voltammetry and chronoamperometry. With this technique the linearly applied voltage is superimposed with small amplitude pulses, constant voltage pulses. By this approach, interferences from other electroactive species within the potential range are reduced thereby producing minimal background noise as charging current. The current measured is the difference between the sampled current prior to the change in potential and that observed at the end of the pulse. The scan rate is usually low for improved sensitivity (compared to cyclic voltammetry and chronoamperometry methods) at the expense of time resolution. Another advantage of the technique is that it allows species electrolyzed at each pulse potential to be determined easily.

The first successful *in vivo* voltammetry work was reported by Clark, *et al.* in 1965 as cited by Adams, R. N. in his article "Probing brain chemistry with electroanalytical".[34] Subsequently, the technique has been well explored for *in vivo* monitoring of monoamine neurotransmitters. Marsden, *et al.* in 1979 in a drug-induced studies, successfully monitored

the effect of p-chloroamphetamine and fluoxetine on release of 5-HT in the brain striatum of freely moving rat using *in vivo* voltammetry with graphite working electrodes.[35] There are various applications of voltammetry for *in vivo* measurements of neurotransmitters.[36–42] The technique has evolved over the years with Crespi demonstrating wireless *in vivo* voltammetric monitoring of DA and 5-HT in the rat's pre-frontal cortex of the brain in drug-induced stimulus studies.[43]

#### *1.2.2.1 Challenges with in vivo voltammetry methods*

A major challenge encountered with *in vivo* voltammetry is the effect of other electroactive compounds present in the brain tissue, which are oxidized at similar potentials as the analyte. Due to the lack of selectivity from the probe, often voltammetry method is unable to resolve multi-component signals into separate electrochemical peaks. For example, ascorbic acid and DOPAC oxidize at similar electrode potentials as catecholamines and in addition both compounds have much higher basal concentrations in the ECF than catecholamines. Although this challenge can be overcome by separating compounds of similar oxidation potentials by HPLC, often it is a daunting process. Alternatively, selectivity can be improved by modifying the surface of the electrode.[44] An electrode coated with the negatively charged nafion polymer has increased selectivity for cations such as DA and NE relative to ascorbic acid and DOPAC that are negatively charged within the operating electrode potential.[30] With this approach the tendency of the anions migrating to the surface of the working electrode is minimized. Other methods to improve selectivity include the selective sampling of appropriate regions of the brain rich in the electroactive compound of interest. For example, monitoring DA in the striatum minimizes the potential influence from NE, which is almost negligible in that region.

It is also important to note that electrochemical measurements in the brain tissue is often more difficult than in the cerebrospinal fluid (CSF). Firstly, the size of the probe is very critical as the technique has the potential of causing tissue damage. In addition, it is also possible to have the surface of the electrode distorted or blocked from deposits of torn out from the brain tissue during probe insertion. Also, it is not clear whether electrode surface is exposed to the ECF or the brain tissue for that matter.

In all *in vivo* voltammetry techniques for monitoring neurotransmitters in the brain, it is important that the measured signal is due specifically to the electroactive compound of interest. The electroactive analyte when injected in close proximity to the *in vivo* electrode must demonstrate the redox reaction at the same potential as the *in vivo* electrochemical peak.

### 1.2.3 Microdialysis sampling and neurotransmitters

Microdialysis (MD) is an analytical sampling tool for the extraction of the free-concentration of small-molecular-weight substances from the interstitial space. This technique has gained exponential attention in the neurosciences since its introduction and is a widely accepted analytical method available that permits quantification of neurotransmitters. In recent times, it has been used for quantification of neurotransmitter release from ECF of the animal brain.

The first developed and implemented dialysis technique used for sampling in the brain was introduced in 1966 by Bito *et al*.[44] Small dialysis sacs were filled with 6% dextran in saline solution and placed into the brains and subcutaneous neck tissues of dogs and allowed to equilibrate with the extracellular surroundings for 10 weeks sampling and the contents were

later analyzed for amino acid and electrolyte content. The "dialytrode" was introduced later in 1972 as an improvement of the "compartment" design of Bito's work.[45] The dialytrode included two small stainless steel rods fused to provide a typical push-pull type cannula with a seven-electrode contact. This device was capable of providing electrical stimulation and recording, together with chemical injection and collection. Based on the dialytrode concept, Ungerstedt *et al.* introduced the thin dialysis tubes or hollow fibers into the brain.[45] Deuterated dopamine was perfused through the fibers to attain the baseline and subsequently amphetamine stimulated release of dopamine was measured. After these initial experiments, the use of microdialysis as analytical sampling tool in the neurosciences has expanded. Recent neurochemical research using microdialysis has ranged from studies of the effects of traumatic brain injuries,[46] neurodegenerative disorders[47] and the pharmacokinetics and pharmacodynamics of drugs.[48]

#### *1.2.3.1 Theory of Microdialysis Sampling*

Microdialysis (MD) sampling is a diffusion-based process in which compounds of interest migrate through a porous membrane into a gentle flow of perfusion fluid. The perfusion fluid, a physiological fluid, deficient in the analyte of interest, is perfused through the membrane or is pumped through the MD probe inlet tubing at typical flow rates of 0.5 – 5 μL/min. As the perfusion fluid (perfusate) traverses the probe, analytes from the surrounding fluid, often the ECF for brain sampling, diffuse through the porous membrane and swept along by the perfusate. The outflow from the microdialysis probe, termed the dialysate, is collected either on-line for real-time analysis or off-line for further analysis. The schematic sampling procedure for MD is depicted in Figure 1.2. The diffusion process is as a result of a

concentration gradient present between the perfusate and the surrounding ECF.[49,50] A typical characteristic of MD is the ability to measure only the unbound of free fraction of the analytes in the ECF. This is mainly due to the specificity of molecular weight cut-off of the semi-permeable membrane, which prevents large protein molecules from diffusing through the membrane. This implies that in the quantitation of exogenous compounds such as drugs, only the pharmacologically active portion of the drug will be measured. MD has therefore been used successfully in drug pharmacokinetic and pharmacodynamics studies.

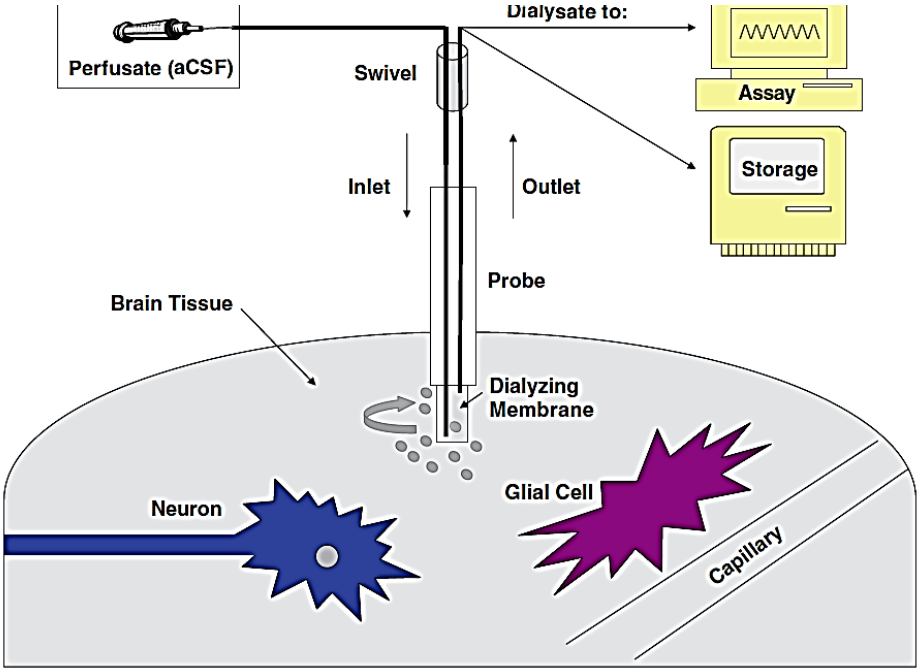


Figure 1.2 A schematic diagram of a typical in vivo microdialysis setup*

*Printed with permission from "Handbook of neurochemistry and molecular neurobiology; practical neurochemistry methods, Eds. Lajtha, A., Baker, G., Dunn, S. Holt, A., Plenum Press, New York , 2007, 219 – 256.

### *1.2.3.2 Microdialysis Probe Considerations*

As tissue samples may be heterogeneous/homogeneous so selecting the right probe is very a critical factor in any MD application. The most common MD probe for monitoring neurochemicals is the concentric cannula or rigid probe design.[51] Other probe designs include the linear, shunt and flexible probes. Often the type of tissue determines the probe type that is appropriate for sampling. The linear probe has been used in most applications that involve sampling of homogenous soft tissues such as heart, liver and muscles whereas the concentric cannula is used for the brain due to the latter's heterogeneity.

In addition to the probe design, another critical factor that can affect recovery of the analyte is the material used in the fabrication of the semi-permeable membrane and the tubes. This is mainly due to the fact that the physicochemical properties of the analyte and its interactions with the MD membrane, and thus affecting the analyte's diffusibility through the semipermeable membrane. Generally, the molecular weight cut-off and membrane hydrophobicity are among factors that may grossly influence the analyte's recovery. Some of the commercially available probes include polyacrylonitrile, polycarbonate, polyethersulfone, and cuprophan.[50]

### *1.2.3.3 Microdialysis Calibration methods*

Microdialysis sampling is a non-equilibrium diffusion-base process. Thus, at any point during the sampling process, the analyte concentration in the sample represents a fraction of its actual concentration in the immediate vicinity of the probe. In this regard, the extraction efficiency of the probe is a vital parameter that relates to the recovery of the analyte. The

extraction efficiency in most cases is expressed as the relative recovery (when the analyte concentration is higher in the ECF) or relative delivery (when the analyte concentration is higher in the dialysate). A mathematical representation of the relative recovery is given as,

$$Relative\ recovery = \frac{C_{dial}}{C_{perf}} \qquad (1.1)$$

i.e., the ratio of the analyte concentration in the dialysate ($C_{dial}$) to the concentration of the analyte in the perfusate ($C_{perf}$).

Parameters that influence the relative recovery include the perfusate flow rate, temperature, dialysis membrane and analyte properties, probe geometry and typical for *in vivo* applications, physiological processes such as metabolism, uptake, release, transport and binding. [51] In MD sampling, the relative recovery is a very critical parameter needed for calibration purposes and its determination usually precedes any *in vivo* sampling. Typically, *in vitro* calibration of the MD probe is carried out by perfusing the probe with artificial cerebrospinal fluid (aCSF) devoid of the analyte of interest under well-controlled agitation and temperature conditions (typically 37 $^{o}$C). Dialysate samples are later collected, analyzed, and the relative recovery can be calculated using equation 1.1. A major disadvantage of the *in vitro* calibration method is that effect of the matrix such as tissue vascularization, metabolism rate and analyte reuptake into cells, all of which may affect the calculated *in vitro* recovery, are not taken into account. Thus, *in vitro* probe calibration often leads to approximation of the analyte's recovery into the microdialysis probe.[52] To provide more accurate data *in vivo* calibration methods have been proposed.

The "no net flux" *in vivo* calibration method involves perfusing the probe with analyte concentration estimated to be below or above the expected extracellular concentration in the tissue in a random fashion, thus creating conditions in which the analyte molecules are either lost to or gained from the tissue.[52] In the event that the analyte concentration in the perfusate is greater than in the ECF of the brain, the analyte diffuses from the probe through the membrane into the brain. The reverse process is observed when the analyte concentration in the brain is greater than that in the perfusate. A regression analysis of the relationship between the concentration of the analyte in the perfusate and the difference between analyte perfusate and dialysate concentrations reveals a linear curve passing through zero. At this point the analyte concentration in the perfusate and dialysate is equal, i.e., the point of no net flux (no diffusion across the membrane occurs). Despite the reliability of the calibration method, the method is very time consuming due to the multiple changes in the perfusate solution and the corresponding equilibration time associated with each change.

An alternative to the no net flux method is calibration by delivery, or retrodialysis.[53] In this method, once the microdialysis probe has been implanted, a known concentration of the analyte is perfused through the probe. The dialysate is collected, analyzed, and compared to the perfusate.

$$Relative\ recovery = \frac{(C_{dial} - C_{perf})}{C_{perf}} \qquad (1.2)$$

Equation 1.2 represents the comparison of the two substances. However, this method does not consider the dynamic changes in analyte recovery of the microdialysis probe throughout the entirety of the experiment. In this method, an internal standard is added to the perfusate during

the course of the experiment.[54] The diffusion properties of the selected internal standard should match that of the analyte to facilitate comparison of the relative recovery of the internal standard to the relative recovery of the analyte. The advantage of using an internal standard is that probe performance over the course of an experiment does not affect the overall recovery results.

The final but not the least method used for *in vivo* calibration is the flow rate variation.[55] With this approach, the flow rate of the perfusate is varied throughout the probe calibration process. The concentration of the recovered analyte is plotted against the flow rate. An extrapolation to zero provides an estimation of the analyte concentration and the relative recovery of the probe can be measured. The main disadvantage of this method is that the longer sampling times are required for the low flow rates leading to poor temporal resolution.

### 1.3 *In vivo* microdialysis for sampling brain ECF

Since its introduction many important and successful neurochemical studies have been carried out using MD coupled with various analytical systems. MD is currently a well-established technique for studying physiological, pathological and pharmacological changes of a range of low molecular weight compounds within the brain ECF. Major applications involve quantitative determination of primarily neurotransmitters, [56–63] and also MD as a tool for drug infusions/delivery.[64–67] *In vivo* MD in freely moving animals is commonly used to study the relationship between changes in neurotransmitters at discrete regions of the brain and behavioral changes. In addition to continuously studying extracellular neurotransmitter levels in discrete regions of the brain, *in vivo* MD studies have successfully been used to studies of

physiological processes in humans.[68] In 2001, MD was used to demonstrate how dopamine neurotransmission relates to cognitive processing in humans.[69,70] A typical challenge in *in vivo* neurochemical monitoring is the detection of neuropeptides. This is because most of these neuropeptides have very low concentrations. However with the combination of tandem MS, dialysate fractions were found to contain endogenous met-enkephalin[71] and neurotesin,[72] and other neuropeptides[73] from the brain ECF. MD has also been applied in clinical studies to determine specific neurotransmitters in patients with traumatic brain lesions.[74]

### 1.3.1 Analysis of dialysates

MD, unlike voltammetry, requires an additional analytical equipment for the analysis of dialysates. Since the dialysates are often in small sizes, contains high concentrations of inorganic salts and the fact that neurochemicals are in very low concentrations in the brain extracellular fluid, detection and quantitation of brain neurotransmitters still remain an analytical challenge. Coupling of MD to chromatography has significantly facilitated separation and measurements of individual neurotransmitters in dialysates. Gas chromatography (GC) however, has not been a method of choice due to the fact that most neurotransmitters are non-volatile. Therefore, further analytical step like chemical derivatization of the analytes to improve volatilization must be introduced into GC assays for neurotransmitters. For example, analysis of choline and acetylcholine by GC was achieved through demethylation of the quaternary nitrogen atom with benzenethiolate.[75] Although that study was successful, the process of derivatization in itself introduces potential analytical errors to the entire technique. Simple, reliable and robust sample preparation methods are

always the key elements for any new analytical method. Understandably HPLC systems are better alternatives and provide the needed separation stability for the analysis of dialysates.

Apart from the fact that most of the components captured in dialysates are none volatile, HPLC separation is suitable for analyzing dialysates because they are devoid of proteins, which reduces fouling. In addition, the development in capillary liquid chromatography (CLC) has further enhanced interest in chromatographic separation of dialysates. Despite the advantage of HPLC over GC for the analysis of non-volatile compounds, it is important to note that neurotransmitters such as, DA, GABA, GA, 5-HT, Ach, NA, etc., are small low molecular weight polar compounds. Thus, their retention and separation is not easily attained on a typical reverse phase HPLC system. If required, ion-pairing agents are employed in order to achieve good retention of neurotransmitters on a standard reverse phase HPLC column. In addition to ion pairing, different stationary phases like the pentafluorophenyl (PFP) and chemically modified reverse phase stationary phases may be employed to enhance interactions with the polar neurotransmitter molecules. Alternatively, hydrophilic interaction chromatography (HILIC) can be explored for separation of neurotransmitters.[76]

Like any other analytical technique, stable, accurate and reliable detection are paramount for attaining excellent data quality. Common methods such as electrochemical detection (ECD),[56] fluorescence[57] and mass spectrometry (MS)[73] have been coupled to HPLC for routine measurements of neurotransmitters.

Electrochemical detection (ECD) is primarily used for ionic compounds or compounds that are easily oxidized or reduced. Electrochemical detectors are very sensitive with detection

limits typically in the nanomolar region and thus very applicable to the analysis of neurotransmitters in brain ECF. The selectivity of ECD is derived from the oxidation/reduction potential of the neurotransmitters. Despite these advantages, a typical limitation of ECD is fouling at the surface of the electrodes, which subsequently masks its overall performance. Fluorescence detection is applicable to analytes that contain fluorophoric moieties within its core structure and thus able to fluoresce. An advantage of this technique is that very few organic compounds are able to fluoresce and therefore enhancing its selectivity. On the contrary, this poses analyte detection technique challenge since most neurochemical compounds do not have fluorophores. In order to improve detection, fluorophoric groups can be introduced to enhance detection. Notable for its specificity and sensitivity, the MS compared to the other detection systems, provides further molecular information of the analytes. As a result, LC-MS platforms have generally been accepted for bioanalysis.

For LC-MS applications, the common atmospheric pressure ionization (API) processes are electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI). In ESI, ions are generated in the ionization source, which can affect the sensitivity of the analytical signal. Typically, the LC eluent is nebulized in source in the presence of a strong electrostatic field and heated drying gas. The electrostatic field enhances dissociation of the analyte with the heated drying gas causing solvent evaporation. This leads to reduction in the size of droplets containing a cluster of ions. Finally, the generated repulsive forces between ions of similar charges surpass the cohesive forces within the droplets leading to ejection of ions into the gaseous state. The ions are further attracted to the mass analyzer through an orifice. The APCI can be considered as a gas phase chemical ionization (CI) process where the

mobile phase solvent acts as the CI to assist in the ionization. In APCI, the LC eluent is sprayed through a heated vaporizer, and electrons discharged by a corona needle ionize the resultant gas-phase solvent molecules. The formed gas phase ions transfer charge to the analyte and the resultant ions passed through an orifice into the mass analyzer. In the mass analyzer the generated ions are separated and identified according to their mass-to-charge (m/z) ratios. LC-MS has extensively been applied to a wide range of thermally labile analytes, typically high molecular weight biomolecules. On a LC coupled to tandem mass spectrometry (LC-MS/MS) platform, further ion fragmentation is generated in the analyzer for enhanced elucidation of analyte molecular information. Commonly applied techniques for MS-MS are the triple quadrupole and the ion trap mass spectrometers. The triple quadrupole instruments are very rugged and sensitive whereas the ion trap MS systems have the capability of conducting $MS^n$ experiments to provide clearly define precursor-product ion relationships. In addition, hybrid geometries of triple quadrupole and ion trap MS, such as linear quadrupole ion trap systems are also available for various bioapplications. The LC-MS/MS is the commonly used method for fast and sensitive quantitation of small molecules such as neurotransmitters, peptides and drugs from various biological complex matrices including plasma, blood and tissue. This is because MS/MS is more sensitive and significantly specific than other LC detectors. In addition, the MS/MS can analyze compounds with no suitable chromophore and also components in unresolved chromatographic peaks, thus reducing the need for absolutely resolved chromatographic peaks. In a triple quadrupole MS, the first quadrupole (Q1) is used to select a precursor ion and collision induced dissociation (CID) occurs in the second quadrupole (collision cell). For analyte specificity, ion fragmentation is effected through collision with neutral molecules. Subsequently, the third quadrupole (Q3) generates a spectrum

of the resulting product ions. A major advantage of the LC-MS/MS platform is the ability to use Q1 to filter unwanted ions for enhanced sensitivity. The technique has thus been applied successfully to targeted analysis of compounds in various biological matrices where the analyte concentrations are very low, such as quantitative analysis of neurotransmitters in brain tissue. For global untargeted metabolomics studies using LC-MS, where the targeted analytes are unknown, reliable confirmatory assays are required. This additional requirement is obtained by using mass spectrometers with high accurate mass, i.e., the higher the accurate mass of the analyzer; the easier it is to confirm the identity of the chemical substance. The orbitrap is an example of a typical mass analyzer with higher resolving power and mass accuracy that has been applied for global metabolomics studies.

Thus, for LC-MS assays, the neurotransmitters will be identified by both retention time and molecular weight. Although fluorescence detection is also known for its remarkable sensitivity, the LC-MS/MS assay has been reported as the most sensitive.[73–78] However, a critical concern in LC-MS or LC-MS/MS assays for neurotransmitters is the potential ion suppression or enhancement typically observed with the ESI interface. This phenomenon is more pronounced in dialysates due to the higher inorganic salt contents (higher ionic strength) and therefore generates high background noise and ionization suppression leading to considerable loss of sensitivity. It is vital therefore to attain good separation of the analytes from co-eluting components in the dialysates in order to minimize ion suppression from the sample matrix. Alternatively, the greatly reduced column bore in capillary liquid chromatography (CLC) and the lower flow rates can be explored since its introduction has augmented the overall effect of mass sensitivity[79] and further improved matrix compatibility

to MS while reducing also mobile phase consumption. Another advantage of CLC-MS is the overall improvement in temporal resolution as noted for ultra-high performance chromatography (UPLC). Kennedy *et al.* reported of 2 – 4 min temporal resolution for measurements of acetylcholine in dialysates[80] compared to previously reported 20-min temporal resolution for LC-MS method.[72] Despite these achievements, instrumentation setup require specialized parts often due to the associated backpressure, the overall instrument cost and sample introduction can be very challenging and thus require careful manipulation.

## 1.4 Comparison of *In vivo* Microdialysis and Voltammetry

Since its introduction in the mid-1960s, voltammetry has been primarily used for the analysis of biogenic monoamine neurotransmitters and their metabolites in the brain. Despite the broad acceptance of voltammetry as a neurochemical technique, *in vivo* MD has become the most widely used sampling tool of the brain ECF, CSF sampling through lumbar puncture in humans. The two analytical methods are currently applied to extensive studies of the neurochemical composition of the brain. However, due to the differences in their sampling principles, voltammetry and MD provide very specific information on the composition of the brain ECF. *In vivo* MD provides further advantage of chemical specificity when coupled to HPLC-MS but usually characterized by low temporal resolution in the range of minutes. On the contrary, *in vivo* voltammetry is typically used to measure rapid changes in analyte concentration within the ECF and the samples are analyzed over millisecond to a minute interval. Thus, by combining these two techniques, simultaneous measurement of slow changes in neurotransmitters concentrations, basal levels of neurotransmitters and their metabolites, and the dynamic information of neurochemical kinetics can be obtained. Lastly,

*in vivo* voltammetry utilizes extremely small size recording electrodes and thus facilitates spatial monitoring of different regions of the brain simultaneously whereas the relatively bigger size of MD probe integrates chemical changes from various surrounding tissues. It's worth mentioning that both methods over the years have evolved significantly with the introduction of microelectrodes in voltammetric techniques, improvement of its selectivity and the attainment of 1s temporal resolution with *in vivo* MD have been reported.[81]

### 1.5 Electrochemical biosensors for brain neurotransmitters

Neuronal activities occurring within the mammalian brain typically involve rapid changes of some neurotransmitters. As an example L-glutamic acid is known for its fast excitatory release during normal neuronal activity. Current analytical techniques used for measurements of such rapid changing processes lack the needed fast response to measure accurately changes in neurotransmitter concentrations within the rain ECF. For example, MD is the primary method for measurements of endogenous neurotransmitters; however it still does not have the required temporal resolution for direct measurements of fast changes in the concentration of neurotransmitters in the brain ECF. In addition, MD coupled to other detection schemes does not always capture the true extracellular concentrations of the analytes. Electrochemical techniques, which have been employed extensively, are primarily meant for measurements of electroactive neurotransmitters like monoamines and their metabolites within the CNS. The technique has shown significant temporal resolution in capturing fast transient changes in neurotransmission over most existing conventional methods. However, not all endogenous neurotransmitters exhibit electroactive properties for possible ECD measurements. A typical example is L-glutamic acid, which does not show electroactivity.[82] In

order to study the rapid changes in the concentration of neurotransmitters during brain activity, there is a growing demand for sensitive, reliable and most importantly rapid analytical techniques. In this regard, enzymatic biosensors have also emerged.

The major motivation for the development of electrochemical biosensors is due to the fact that most enzymatic reactions associated with neurotransmission produce, for example, hydrogen peroxide as a product, which can be determined via electrochemical measurements. Thus, a biosensor can be viewed as any sensor that employs a biological component, such as antibody or enzyme, to bind a specific analyte of interest to provide a signal in the form optical or amperometric that relates directly to the amount of analyte in a given matrix.[83] Among the numerous biosensor designs available, enzymatic biosensors form the relatively larger portion applied to the brain research study. The principle of enzymatic biosensors is based on the reaction between the analyte and a specific biological substrate to produce a measureable product and the amount of the product formed in the biosensor reflects the concentration of the analyte. The product formed is usually measured through amperometric detection methods for most brain research.

Common techniques used in the fabrication of biosensors include electropolymers, electrodeposition paints, sol-gel and hydrogel methods.[83] With electropolymer method, monomers such as pyrrole, thiophen or tyramine are used to form a continuous network of polymer on a polarized electrode surface. Subsequently, the enzyme(s) can be embedded into to the polymer via two possibilities. The first process entails physically entrapping the enzymes within the core structure of the polymer. In the second process, the enzymes are cross-linked to the previously formed electropolymer that are functionalized for covalent interaction or a

non-covalent linkage.[83] Due to small size of the electrodes (3 - 25 μm), the previous process often leads to formation of a highly hydrophobic electrode surface making the entire device less sensitive. This is explain the low density of trapped enzyme with a subsequently lower enzyme activity. Electrodepostion methods involve the use of long chain polymers with charged groups like amines or carboxylic acids. Through careful manipulation of the pH surrounding the electrodes, precipitation of the polymer can occur either through protonation of the carboxylic or deprotonation of the amine functional groups. In the process, enzymes present in the mixture are also trapped in the insoluble polymer formed on the surface of the electrode. Sol-gel methods largely involve the encapsulation of the enzyme into through conventional sol-gel production processes. The encapsulation of the enzyme can be done during the slow curing procedure for the sol-gel formation. Lastly, redox hydrogels can be fabricated by crosslinking water-soluble polymers such as polyvinyl imidazole with other polymers resulting in the formation of new high water content polymer in the form of hydrogels. The presence of the hydrogels provides the needed excellent aqueous environment for most enzymes.[83]

### 1.5.1 *In vivo* measurements and challenges using electrochemical biosensors

A major challenge associated with electrochemical biosensors is interference from other non-electroactive species that are present in the brain ECF. The interference occurs normally when interactions with the non-electroactive species at the surface of the electrode often leads to the generation of charging current and thus masking accurate determination of the analyte of interest. Another challenge often encountered with amperometric biosensors is the loss potential of sensitivity with time during its operation. This may be due to fouling at

the surface of the electrode, fouling of the surface of the outer biolayer, which often hinders analyte access to the electrode and lastly the loss of enzyme activity due to involvement in breakdown of protein.[83] Nonetheless, it is worth mentioning that biosensors are very useful analytical devices and provide the opportunity for direct measurements of neurotransmitters in the brain.

In 1988, Crespi, *et al*. successfully developed a voltammetric biosensor capable of *in vivo* measurements of the basal concentrations of 5-HT stimulations in the brain.[84] In a more recent publication, Wahono, *et al*., explored the possibility of using amperometric glutamate biosensor for *in vivo* measurements of glutamate after recording a 35-fold increase in extracellular glutamate through *in vitro* experiemnts.[85] In addition to *in vivo* glutamate monitoring in the brain ECF, electrochemical biosensors have be applied to measurements of neurotransmitters such as DA, NA, 5-HT, among others,[86–88] and even by combining with on-line MD to monitor dynamic changes in brain metabolism.[89]

### 1.6 *In vivo* brain tissue sampling: the need for alternative approaches

It is clear from the foregoing sections that despite the significant success attained in the development of *in vivo* analytical sampling methods to measure changes in the concentrations of endogenous compounds within the brain ECF, there are still intrinsic challenges. Although, this thesis focused on mainly commonly used *in vivo* analytical methods for brain tissue sampling, there is no doubt that introduction new analytical sampling approaches will augment the quest to monitor chemical activity within the brain ECF. MD to date remains the most accepted analytical tool for brain tissue sampling, especially in neuroscience. However, till

date issues with probe calibration for accurate quantitation still pose a challenge as most calibration methods are carried out through *in vitro* experiments. These *in vitro* experiments, which provide the relative probe recovery does not take into account factors such as tissue tortuosity, possible analyte reuptake and metabolism. Menacherry, *et al*. reported an *in vivo* calibration method for quantitation of cocaine in the brain. Unfortunately, the approach itself was very time-consuming and challenging.[90] Other *in vivo* calibration methods have also been reported elsewhere for measurements of other endogenous compounds.[52,91,92] However, these methods are rarely used because they are also very time consuming and laborious.

Another important issue is influence of the matrix component on the quality of data. From the previous sections, it was obvious that all the *in vivo* methods like most bioanalytical assays were significantly susceptible to matrix effect. This may primarily be due to the poor method selectivity, and thus compromise sensitivity especially when coupled to ESI-MS, a popular technique used for bioanalysis.

Given the variety of challenges described above, it is even more paramount that new approaches are introduced to boost the efforts made to gain insight in the mammalian brain through acquisition of molecular/chemical information within the brain ECF. This can be achieved either by the introduction of a new brain tissue sampling approach or by integrating new methods with existing systems so as to improve overall data quality. One such method worth exploring is solid phase microextraction (SPME).

## 1.7 Introduction to Solid Phase Microextraction Method

Solid Phase Microextraction (SPME), originally introduced in the early 1990's[93] as a non-exhaustive equilibrium extraction method, integrates sampling, sample preparation, analyte enrichment and sample introduction in a single step. The general concept was to utilize a small extraction phase volume to extract the analytes, typically volatile and semi-volatile compounds, from a given matrix. The introduction of the method and subsequent coupling to

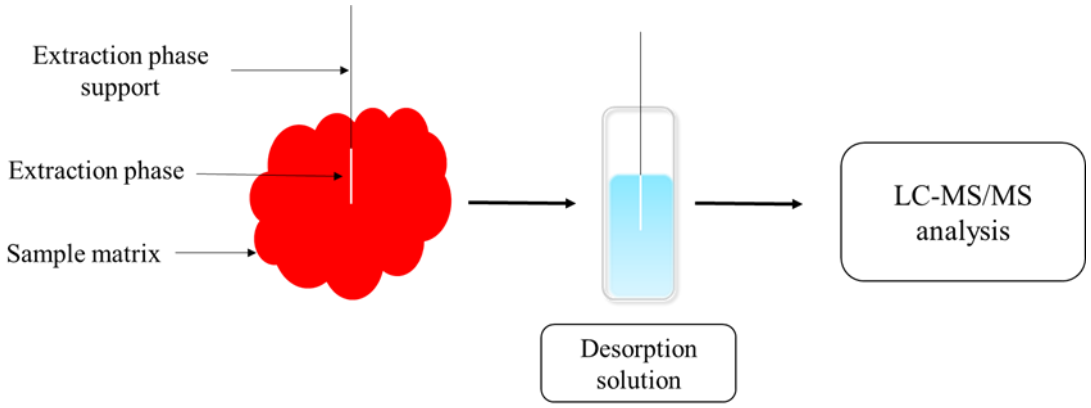


Figure 1.3 Schematic representation of a typical direct SPME in vivo microextraction workflow from a given biological matrix coupled to LC-MC/MS

gas chromatography, thus served as an essential analytical technique for the analysis of environmental samples.[94,95] Extraction phases such as polydimethylsiloxane (PDMS) and polyacrylate (PA), to mention a few, were among the first commercially available SPME coatings used for analysis of non-polar and polar compounds respectively.[96,97] SPME has subsequently evolved and has been applied to the analysis of thermally labile compounds amenable to liquid chromatography and liquid chromatography hyphenated to other detection systems, in various matrices such as food,[98–101] urine,[102–105] and plasma[106–108] to mention a few.

In terms of LC-MS applications, some of the interesting features include the development of the open-bed SPME configuration for automated parallel extraction of biological samples on a 96-well plate format[109–111] and the development and evaluation of new "in-house" biocompatible coatings for biological applications.[112] The development of biocompatible SPME coatings facilitated *in vivo* SPME bioapplications because the issue with fouling was addressed. SPME has therefore been a very useful sample preparation tool for the analysis of biological samples[113,114] and even *in vivo* applications.[115,116] In addition to overcoming issues associated with fouling, SPME also provides efficient sample cleanup due to the smaller extraction phase volume, which restricts potential matrix interferences that can mask the extraction of the analyte of interest. This important feature about SPME makes it very compatible with LC-MS/MS and thus suitable for the analysis of biological samples, urine, whole blood, tissue, etc. Figure 1.3 shows a schematic representation of a typical direct SPME *in vivo* microextraction workflow from a biological sample when coupled LC-MS/MS. The analyte amount extracted from any given matrix by SPME depends on the exposure time between the extraction phase and the sample. For sufficiently long extraction, equilibrium between extraction phase and sample is established. Under such conditions, the extracted amount is given by the following.

$$n_e = \frac{K_{fs} V_f V_s C_0}{K_{fs} V_f + V_s} \qquad (1.3)$$

where $n_e$ is the amount of analyte extracted at equilibrium, $C_0$ is the initial analyte concentration in the sample, $V_s$ and $V_f$ are the volume of sample and fiber coating, respectively, and $K_{fs}$ is the distribution constant between SPME extraction phase and sample matrix.

The $K_{fs}$ value is dependent on the nature of analyte and the extraction phase selected for the analysis. But is also affected by properties of sample matrix such as temperature, pH, ionic strength, etc., and therefore it is paramount to maintain these factors constant during sample analysis. It is important to note that at equilibrium, no further extraction of the analyte occurs and thus the microextraction process may be deemed as complete unlike MD where the extraction process is continues as long as the probe remains in contact with the sample. Thus, for SPME the probability for local depletion of the analyte from the matrix is low, making it suitable for *in vivo* applications since it does not disrupt the internal equilibrium of the living system.

From Equation 1.1, under conditions on negligible depletion of the analyte, when $V_f K_{fs} <<< V_s$, the amount extracted can be calculated from the Equation 1.4.

$$n_e = C_0 K_{fs} V_f \qquad (1.4)$$

where all factors remain the same as previously defined.

The microextraction process in SPME may be an absorptive or adsorptive mechanism depending on the type extraction phase selected. Absorptive processes are applicable to Equation 1.3 where the analyte diffuses within the complete volume of the extraction phase during the extraction process. A characteristic extraction phase is the PDMS liquid coating. In

the case of adsorptive process, which often occurs for solid porous extraction phases such as polypyrrole, the amount of analyte extracted is dependent on the number of surface active sites and diffusion within the entire volume of the extraction phase does not occur. For adsorptive process, the extracted amount can be obtained using Equation 1.5.[117]

$$n_e = \frac{C_0 K_{Afs} V_f V_s (C_{fmax} - C_f^{\infty})}{V_s + K_{Afs} V_f (C_{fmax} - C_f^{\infty})} \qquad (1.5)$$

where $C_{f\max}$ is the maximum concentration of active sites on the extraction phase, $K_{Afs}$ is the analyte's adsorption equilibrium constant and $C_f^{\infty}$ is the equilibrium concentration of analyte on the fiber. Due to the fact the extracted amount is dependent on the number of active sites, at sufficiently high analyte concentrations, surface saturation results, leading to nonlinear adsorption isotherms with characteristic shorter linear quantitative range.[117] Details of such characteristics associated with adsorption processes in SPME are well discussed elsewhere.[117,118]Displacement effects are therefore a common characteristic for solid porous extraction phase as analytes compete for the limited active sites. This phenomenon partly depends on the $K_{fs}$ values of the analytes, type and concentrations of components within the given matrix.

SPME is largely perceived as an equilibrium microextraction method; however pre-equilibrium processes can be carried out by terminating the microextraction prior to equilibrium being attained. In such cases, the amount extracted is less and if sensitivity is critical, often pre-concentration of the analyte in smaller desorption solvent volumes (often < 100 µL) enhances detection when coupled to LC-MS/MS. It is important to note that for pre-

equilibrium extractions, the analyte amount extracted by the extraction phase is based on its exposure time to the sample matrix. The amount extracted can therefore be calculated using the proposed equation below.[119]

$$n = (1 - e^{-at}) \frac{K_{fs} V_f V_s C_0}{K_{fs} V_f + V_s} = n_e \, (1 - e^{-at}) \qquad (1.6)$$

where *t* is the extraction time, *a* is a factor known as the time constant and its magnitude is dependent on the sample volume, volume and extraction phase surface area, mass transfer coefficients and the distribution constant. The main advantage of pre-equilibrium microextraction is the shorter sampling time and thus increases sample preparation throughput. Thus, in certain applications like PK or PD studies where temporal resolution is critical, kinetic calibration method can be employed.

A kinetic calibration method commonly applied for quantitative *in vivo* studies is the on-fiber standardization method.[120,121] The approach involves pre-loading the extraction phase with a known amount of the calibrant and then using it to calibrate the extraction process through simultaneous desorption during extraction process. However, this approach is applied only when the extraction and desorption processes are known to be symmetrical, i.e., the calibrant has the same time constant as the analyte. This is usually achieved by using the deuterated analogue of the analyte[122] or by using other compounds with similar diffusion properties or kinetics in a given matrix, as shown elsewhere.[123] Quantitative determination of the amount extracted can be obtained from the equation below.

$$\frac{n}{n_e} + \frac{Q}{q_0} = 1 \qquad (1.7)$$

where $Q$ is the amount of calibrant remaining on the fibre at time $t$, and $q_0$ is the initial amount of the calibrant preloaded on the fibre and $n$ and $n_e$ are the same as described previously. By combining Equations 1.3 and 1.7, the original amount of the analyte in the given matrix can be obtained using Equation 1.8.

$$\frac{nq_0}{q_0 - Q} \times \frac{1}{K_{fs}V_f} = C_0 \qquad (1.8)$$

From Equation 1.6, knowing the $K_{fs}V_f$ value the initial analyte concentration can be obtained. The $K_{fs}V_f$ value is obtained from equilibrium extractions of known calibration standards using the appropriate matrix. An advantage of on-fibre standardization method is that it also compensates well for unknown agitation conditions within the living system for *in vivo* applications since that cannot be simulated well using simple *in vitro* calibration methods.

From Equations 1.3, 1.4, 1.6 and 1.8, it is obvious that the amount of the analyte extracted at equilibrium is directly related to its original amount in the given biological matrix. In the case of Equation 1.6 (pre-equilibrium conditions), it is critical that the extraction time and agitation conditions remain constant. Equation 1.4 also demonstrates the fact that a defined sample volume is not critical for quantitative analysis. Therefore, sampling can be performed directly from a living system, like the brain tissue. This feature of SPME makes it suitable for *in vivo* applications while demonstrating the ability to integrate sampling and sample preparation in a single extraction process.

## 1.8 *In vivo* Solid Phase Microextraction and Solid Biological Tissue Sampling: An Overview

*In vivo* SPME due to its simplicity and suppleness has been applied for sampling of various biological tissues. In a human study, using a commercially available fibre (PDMS-DVB) for extractions, Zhang, et al. investigated volatile emissions from the surface of the skin on the arm.[124] The fibre was analyzed using GC-MS and over 100 compounds were identified. In a similar study, Gallagher et al. combined SPME and solid phase extraction (SPE) in a comparative study to demonstrate variability in volatile compounds emitted from the different parts of the body by sampling both the fore and upper back arm of various individuals.[125]

*In vivo* SPME has also been applied to the study of emerging contaminants in the rainbow trout and greenside darter.[126] In this study, authors were able to demonstrate the potential of *in vivo* SPME in determining bioaccumulation of selected pharmaceuticals in the fish muscles. Simon *et al*. was also able to determine the amount of drugs in the muscle of the live fish and in environmental water using *in vivo* SPME with pre-equilibrium kinetic calibration method.[127] In addition to quantitation drugs as contaminants in fish, elsewhere, in vivo SPME has also be used for the determination of organo-mercury compounds in fish by GC-MS[128] and also by ICP-MS.[129] Lord, et al., were able to quantify triazine herbicides (atrazine, simatryn and prometryn) in plants using a *in viv*o SPME-LC-MS.[130] In this communication, the authors demonstrated the potential of monitoring in real time the movement of the herbicides through the plant and therefore provide a tool for accurate assessment of both herbicide mode of action and the plant's physiological response. In another interesting development, space-resolved SPME was used to determine the distribution of

chemicals in different parts of a various biological matrices (onion bulb, fish muscle and adipose fish tissue).[131,132] In this study, segmented PDMS material placed on stainless steel wire was separately inserted into the plant (onion) and fish (coating placed in both muscle and adipose tissue). Authors were able to demonstrate the potential of *in vivo* SPME to spatially distinguish differences in the accumulation of chemical substance in the muscle and the adipose tissue of the fish, and in addition the chemical distribution in the onion.

## 1.9 Objectives of the research

Currently, a lot of effort is directed toward the acquisition of chemical/molecular information within the brain ECF in order to gain better understanding of the human brain. This has led to development various analytical sampling methods and techniques with a focus on sample preparation. As a result, improvements of current analytical methods, especially *in vivo* sampling approaches form a very critical part to reliably determine and/or monitor chemicals within the brain extracellular fluid. The main objective of this thesis was to develop an *in vivo* SPME method coupled to LC-MS that can be applied as a sampling tool for monitoring endogenous and exogenous chemical substances within the brain ECF. Thus the breakdown of the thesis is as follows.

In Chapter 2, the focus was on optimization of improve post *in vivo* SPME sampling throughput while maintaining sample integrity and stability for further analysis. This led to the development and evaluation of a semi-automated desorption device for *in vivo* SPME probes on a 96-well plate format. The evaluation was the carried out using selected benzodiazepines (diazepam, oxazepam, lorazepam and nordiazepam). Since most *in vivo* SPME methods

require offsite analysis of the probes, a critical part of the device is maintenance of analyte integrity by preventing possible contamination after sampling. Chapter 3 describes initial work carried out to identify the appropriate extraction phase/coating that can be used to extract chemical substances with wide range of polarities. Since part of the objective is to be able to capture small polar endogenous compounds within the brain extracellular fluid, selected coatings were evaluated for their ability to extract various neurotransmitters (DA, GA/GLU, 5-HT and GABA) with wide range polarities. The next chapter (4) describes the development of the *in vivo* SPME sampling method for the monitoring changes in the concentration of the selected neurotransmitters (targeted analysis) from the brain ECF. MD is a known sampling tool for brain endogenous compounds within the brain ECF that typically extracts more polar compounds. In this regard, the results obtained from the newly developed *in vivo* SPME method were compared with that of MD. The chapter also describes the complimentary nature of SPME and MD in a typical untargeted metabolites analysis from the brain ECF. Within the same chapter, in addition to the analysis of endogenous chemical substances, an *in vivo* SPME method for quantitation of exogenous drugs has been described and the results compared with that for *in vivo* MD, also carried out concurrently. Chapter 5 describes an initial application of *in vivo* SPME in a clinical study on the effect of deep brain stimulation on selected neurotransmitters. The study was extended to investigate the possible involvement of other compounds affected by deep brain stimulation in a global metabolomics untargeted analysis. Conclusions of the research are summarized in Chapter 6 and highlights proposals for future directions and challenges associated with this type of studies.

# Chapter 2

# Optimizing *in vivo* solid phase microextraction coupled to liquid chromatography–mass spectrometry applications

## 2.1 Preface and Introduction

### 2.1.1 Preface

This chapter of the thesis is already published as an article under the title "A multi-fiber handling device for *in vivo* solid phase microextraction–liquid chromatography–mass spectrometry applications" by Erasmus Cudjoe and Janusz Pawliszyn., *J. Chromatogr. A.* 1232. (2012) 77-83. All tables and figures were reprinted from this publication with permission from Elsevier Copyright.

### 2.1.2 Introduction

Lately, understanding sample complexity, the quest for improvement of sample throughput and data quality has influenced the noticeable paradigm shift in analytical procedures. Apart from the conventional factors like high accuracy/precision and robustness/reliability, which still remain valid and critical, very often an ideal sample preparation method must have high throughput, environmentally safe, simple, and cost-effective, and in some cases, be amenable to automation.

Sample preparation continues to form a critical stage and often the bottleneck in any quantitative chemical analysis process especially in when dealing complex matrices like biological samples. This is because notwithstanding the sophisticated resource of available analytical techniques, it is often literally impossible to obtain every bit of accurate information without a well-developed sample preparation step. A very good sample preparation procedure often enhances analyte sensitivity by either removing or minimizing matrix/contaminants influence, which will hitherto impact negatively on the data quality. As a result, significant amount of time and money is often expended for maintaining sample integrity and effective preparation procedures.

Arguably, environmentally safe sample preparation methods like dried blood spot,[133] SPE[134] and SPME,[135] to mention a few, are replacing the labour intensive conventional methods such as Soxhlet extraction[136,137] and LLE,[138–140] which require the use of large volumes of toxic solvents. In addition to the increase in relatively environmentally safe methods, automation of analytical procedures has also gained remarkable interest. This could be due to the improved precision and accuracy resulting from minimal or no human interventions and in certain instances, their cost effectiveness. Developments of hyphenated techniques such as gas and liquid chromatography coupled to tandem mass spectrometry have also boosted major advancements in quantitative bioanalysis. Although in certain cases, GC and LC applications would require analyte derivatization for improved volatility and/or chromatographic separation, general chromatography still remains the common analytical separation method. For example, the combination of chromatographic separation with the sensitivity and specificity of the mass spectrometer have been used in various applications

including clinical diagnostics,[141–143] environmental application,[144–147]toxicological studies[148–152]and food analysis.[98,153–156]

Fully automated sample preparation units coupled to hyphenated techniques (GC-MS, LC-MS, etc.) are common now because of the added advantage of reduced analytical labour and costs, reduction in probable analytical errors and the improvement of accuracy. In quantitative bioanalysis for example, using automated high throughput sample preparation (performing parallel extractions and/or dissolutions) units on a 96-well plate format have significantly improved overall analysis time. Jemal *et al*. in a comparative study of manual LLE, automated SPE and LLE for quantitation of determination of carboxylic acid in human plasma, demonstrated some clear advantages of automated methods; automation decreased sample analysis time by almost 3x more than the comparative manual method.[157] Subsequently, the technique has been used for the determination of insulin, drugs in human plasma, [158] and other metabolites in biological matrices.[159,160] Similarly, parallel extraction methods using the 96-well format has been applied to the analysis of drugs and their metabolites in biological matrices.[161–164] Recently also, SPME method coupled to LC-MS/MS on a 96-well plate format was also introduced for the first time.[165] In this particular case, the fiber geometry and the effectiveness of the 96-well plate open-bed SPME configuration was evaluated with selected benzodiazepines. The method has subsequently been applied to the analyses of drugs in urine[111] and whole blood without prior sample treatment.[110]

SPME being a two-stage (extraction and desorption process) sample preparation method was primarily introduced for the analysis of volatile compounds in the environmental samples. Subsequently, the method has evolved and successfully been coupled to HPLC and

other hyphenated methods for the analysis non-volatiles in various matrices. Various bioanalytical applications[166–175] available in literature demonstrate the practicality of SPME as a sample preparation method. By coupling to LC-MS/MS, the method has been applied to chemical analysis of biological matrices in both *ex vivo* and *in vivo* applications. For example, Zhang et al. developed a quantitative *in situ* method for ochratoxin A in cheese by direct insertion of the SPME probe.[123] Recent metabolomics studies, also showed that short-lived metabolites could be captured in freely moving rats with an *in vivo* SPME method.[176] Despite the fact that *in vivo* methods and for that matter *in vivo* SPME, offer enriched information of the biological system under study than its *in vitro* counterpart, most effort focused on improving and automating *in vitro* extraction process. Definitely at the moment, automation of the extraction process for *in vivo* SPME may be impractical, but little or no effort has been made to improve its desorption process. An *in vivo* SPME method till date is characterized by conventional/manual desorption process, which is tedious with extended analysis time. Briefly, after an *in vivo* extraction of any biological system, the SPME probes are immediately transferred in vials and capped in order to prevent any possible damage or contamination. In certain instances, they are placed in vial inserts depending on the volume of desorption solution to be used. Depending on the analyte(s) stability, the probes may be stored on dry ice and transported to the lab if the sampling location is different from the laboratory for subsequent desorption in appropriate solvent system or cannot be analyzed onsite. At the laboratory, the vials with the probes are loaded onto a tray, placed on a commercial shaker/agitator for desorption and subsequent LC-MS analysis. This post *in vivo* SPME extraction process, leads to low sample throughput often increasing overall analysis time. With the increasing *in vivo* SPME applications, desire for shortened analysis time and to obtain reliable and accurate

analytical information, it will therefore be expedient to improve the overall process. In this regard, this portion of the thesis shows the design and evaluation of a new tool for handling *in vivo* SPME probes for post extraction sample preparation.

The new tool, a multi-purpose SPME fiber handling device for *in vivo* and *in vitro* LC–MS was subsequently designed to improve desorption of *in vivo* SPME probes, prevent any possible external contamination during transport, enhance safe handling of probes and also improve overall analysis.

### 2.1.3 The multi-fiber handling desorption device

The multiple-fiber handling device was mainly intended as a portable tool for simultaneous/parallel desorption of multiple *in vivo* SPME samplers used for bioapplications on a regular 96-deep well plate. In addition, the entire unit was designed to permit easy packing and setting of probes to fixed positions inside each individual well of the 96-well plate. Thirdly, all loaded probes should be rigid once loaded to prevent damage during transport and agitation. In view of the fact that the device will be placed on commercial agitator during desorption, the weight of the device should not affect effectiveness of mass transfer inside any of the wells. Finally, the design should augment prevention of any external contamination during the experiment and/or transportation.

As a brief description, the device consists of a base, which supports and allows *in vivo* probes to be fitted directly into each well of a 96-deep well-plate immediately following an extraction. A flat plate with 96 holes aligned with the wells of the plate is placed on top of the base to serve as the guide for the SPME probes. A stopper is in place to ensure each fiber is at

the same distance inside the well. Figure 2.1-a shows the guide on the base part of the device while Figure 2.1-b is the unit place on a commercially available 96-deep well plate loaded with the *in vivo* SPME fibers. To ensure that the loaded *in vivo* probes are well protected against damage and possible contamination after the extraction process, the entire device is subsequently placed in a protective case or cover serving as housing (Figure 2.1-c). The protective cover with four clips is used to secure the device with the well-plate together for easier transportation to the laboratory for further analysis as shown in Figure 2.1-d.

To establish that the device works as expected, it was evaluated using commercially available SPME *in vivo* blood probes, which comprised a hypodermic needle and a medical grade stainless steel wire as a plunger with one end coated with the extraction phase. The other end of the plunger is fitted into a cylindrical rubber-like material. Depressing the plunger exposes the extraction phase for subsequent extraction and desorption processes in a given matrix.

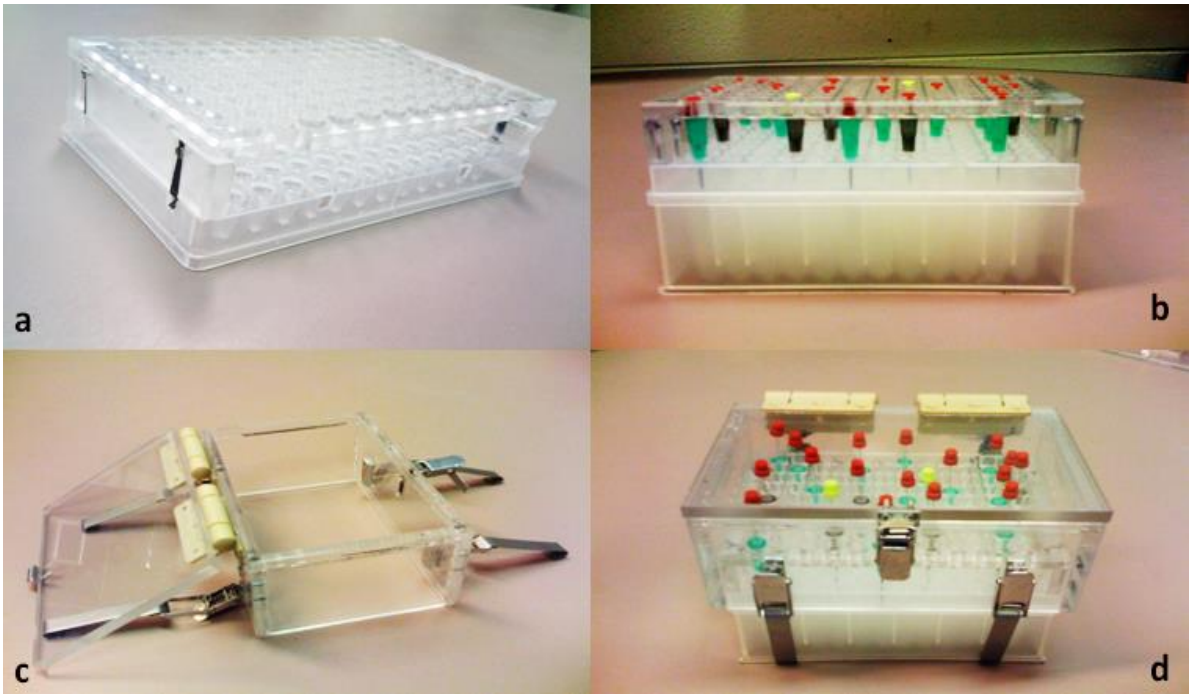


Figure 2.1 Prototype multiple SPME probe desorption device: (a) in vivo SPME sampler guide placed on the base part of the device, (b) device holding in vivo SPME samplers fitted on a regular 96-deep well plate, (c) protective casing with 4 clips as lockers, (d) entire unit is placed on a 96-deep well plate with clips locking the various parts as a single unit.

## 2.2 Experimental Section

### 2.2.1 Reagents and materials

HPLC grade solvents were used for chromatographic separation. Acetonitrile solvent were obtained from EMD Chemicals Inc. (Darmstadt, Germany) and HPLC grade acetic acid was obtained from Supelco (Bellefonte, PA, U.S.A.). Benzodiazepines (diazepam; nordiazepam; oxazepam; and lorazepam) were obtained from Radian International (Austin, TX, U.S.A.) as 1 mg/mL standard in methanol with the exception of lorazepam, which was in

acetonitrile. The drugs were stored at 4 $^{0}$C in a refrigerator. A mixed standard (100 ng/mL) of the drugs was prepared in 1:1 (v/v) acetonitrile-water mixture and always stored in the fridge. The mixed standard was used as the stock solution for all subsequent experiments. Phosphate buffer solutions (PBS) were prepared in the laboratory using analytical grade chemicals by mixing 8.0 g of NaCl, 0.2 g of KCl, 144 g of $Na_2HPO_4$, and 0.24 g of $KH_2PO_4$ in deionized water and the pH adjusted to 7.4. In vivo SPME probes with 5 µm C18 particles as extraction phase used in this study were obtained from Supelco (Bellefonte, PA, U.S.A.). Deionized water used in part for dilution of stock solutions was from a Barnstead/Thermolyne NANO-pure water system (Dubuque, IA, U.S.A.). The VWR DVX-2500 Multi-tube vortexer was used for vial agitations for all extractions while the PAS Concept 96 was used during desorption experiments. Figure 2.2 shows the structures of the benzodiazepines.

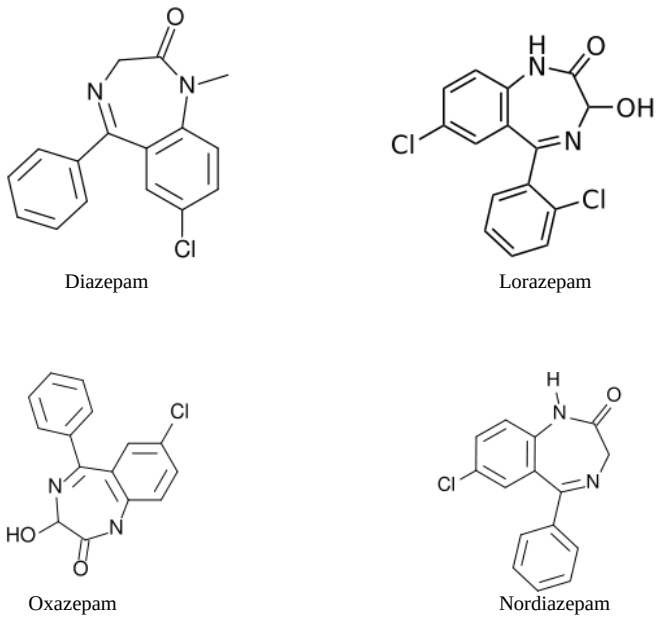


Figure 2.2 Molecular structures of selected benzodiazepines used in the evaluation of the multi-probe desorption device.

### 2.2.2 LC and mass spectrometry conditions

LC analyses were performed on the Accela™ instrument from Thermo Scientific® equipped with a binary pump. Chromatographic separation of analytes was achieved with a Waters® reverse phase $C_{18}$ column (Symmetry Shield; 5 µm, 2.1 x 50 mm) in 5 min using gradient elution at a flow rate of 0.5 mL/min. Mobile phase A consisted of 90% aqueous and 10% acetonitrile and mobile phase B was 90% acetonitrile and 10% aqueous. Both mobile phases contained 0.1% acetic acid to enhance ionization in the ion source. The LC method started with 100% of mobile phase A, which was held for 0.5 min. Mobile phase B was increased gradually to 3.0 min and held constant for half a minute. The initial column condition was subsequently restored for column re-conditioning till 5 min. The Accela™ autosampler from Thermo Scientific was used for sample introduction into the HPLC system. A sample volume of 10 µL was injected and analyzed by a triple quadrupole mass spectrometer (TSQ Vantage™).

The TSQ Vantage™ had the HESI™ probe installed for nebulization and ionization. All ions were monitored in the positive ionization mode. The mass ion transitions monitored were 271.1→140.1, 285.1→193.1, 287.1→241.1 and 321.0→275.1 for nordiazepam, diazepam, oxazepam and lorazepam respectively. The source voltage, vapourizer and capillary temperature were 2000 V, 350 $^0$C and 350 $^0$C respectively. The optimized sheath and auxiliary gases were set at 55 and 25 (arbitrary units) respectively. All data analyses were performed with the Xcalibur® software version 2.0.7.

### 2.2.3 Optimization of SPME procedure

Prior to evaluation of the multiple *in vivo* probe desorption device, optimization of the extraction and desorption processes were completed. The extraction process was optimized by generating an extraction time profile for each analyte and from the plots their equilibration times were determined. The equilibration times for all the analytes during the extraction process were determined with the VWR DVX-2500 multi-tube vortexer using preset agitation speed of 1200 rpm. For the extraction process, selected probes were placed in a 2.0 mL amber vial containing 100 ng/mL working solution of the drugs dissolved in PBS. The working solutions were prepared freshly in PBS while maintaining the same organic content. Extractions were carried out at different preset times (5, 10, 15, 30, 45 and 60 min) under the same agitation conditions. After the extraction process, the analytes were desorbed from the probes using 70% acetonitrile-water solution.

In SPME method development for liquid chromatographic applications, optimization of the desorption process involves selecting an appropriate solvent system for which the analytes have better affinity and also requires the least time to effectively remove almost all or all of the analytes from the probe. The purpose is to decrease the amount of carryover to negligible limits for accurate quantitation of analytes in the sample. Desorption of analytes from the SPME probe was achieved by placing the probes in a 1000 µL desorption solvent inside the well of a 96-well plate. The 1000 µL desorption volume was selected because it maximum solvent that could be contained in each well without any spilling at during agitation. Lower desorption volumes can be used also in cases where sensitivity is paramount. However, it is critical to ensure that all probes remain fully immersed in the desorption solution during

agitation to prevent any variability. The Concept 96® was used to provide effective agitation for enhanced desorption kinetics. The amount of carryover was determined to be less than 0.4% for all four analytes. Details of the optimized parameters are presented in Table 2.1. Subsequent experiments were all carried out using the optimized experimental conditions.

Table 2.1 Optimized SPME conditions for analysis of benzodiazepines

| ***Parameter*** | ***Condition*** |
|---|---|
| **Agitation speed** | 1200 rpm |
| **Desorption solvent** | 70% acetonitrile solution |
| **Equilibration time** | 25 min |
| **Desorption time** | 25 min |

The evaluation was carried out focusing on factors likely to affect data reproducibility during parallel desorption of the probes on a 96-well format. The results were also compared with data obtained for conventional desorption approach. All extractions were carried out with $C_{18}$ coated surface probes. Prior to the extraction process, probes were pre-conditioning in 50% methanol-water solution and subsequently washed PBS solution briefly to reduce the organic content. Each probe was placed in a 1.8 mL sample volume in a vial through the septum screw cap. All extractions were done using the optimized method conditions described in Table 2.1. After the extraction process, all the probes were loaded into the desorption device and placed directly unto a 96-deep well plate containing 1000 µL desorption solution. The SPME probe guide was subsequently removed while the base part with the deep-well plate was later transferred to a commercial agitator (Figure 2.3) for simultaneous desorption of the analytes from all the fibers using the optimized desorption conditions.

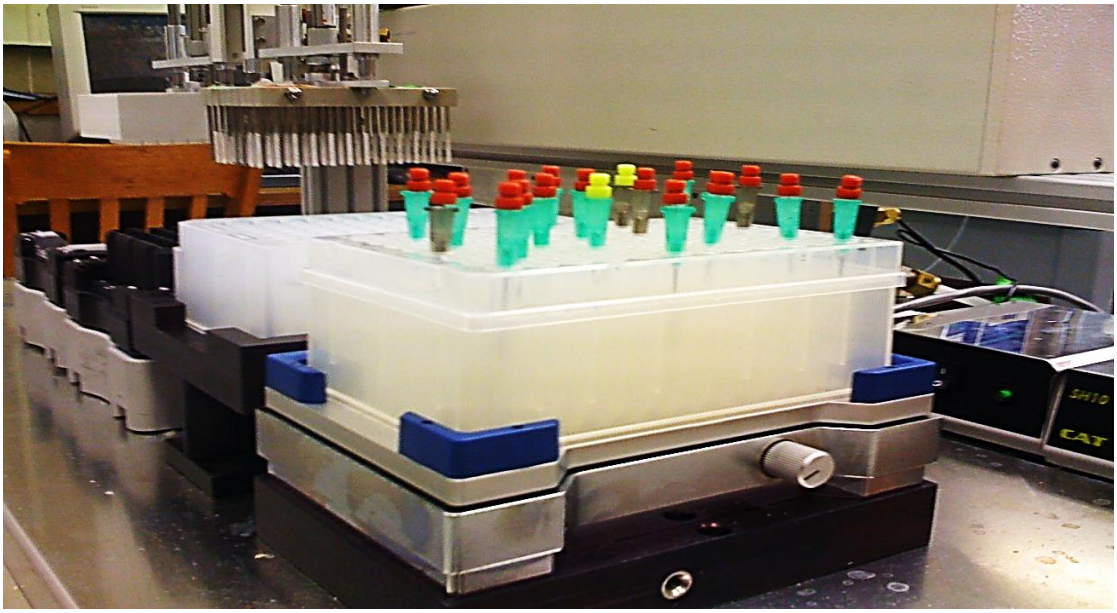

Figure 2.3 Prototype multi-fiber device used for desorption of SPME fibers on a regular commercial agitator for a 96-deep well plate

## 2.3 Results and discussion

*In vivo* sampling though is often challenging and complicated as the biosystem under study undergoes continual dynamic chemical changes, data acquired provide better indication of the biosystem. Therefore, any *in vivo* sampling that requires off-line sample treatment should preserve sample integrity especially in cases when the samples could not be analyzed immediately and must be transported to the lab. This will ensure that the data provide accurate information about the biosystem. SPME as a portable sampling technique that integrates sampling and sample preparation has seen remarkable application to various *in vivo* studies in recent times. However, all *in vivo* SPME-LC-MS applications require off-line probe analysis and in most cases off-site analysis. Thus, critical measures are required to prevent probe

contamination and protection from damage after sampling and during transport. Typically, these probes are kept in small vials and trays, which sometimes present handling challenges to the researcher. This part of the thesis focuses on a handling device, which not only provides a solution to possible probe contamination and damage, but also provides an alternative high throughput approach for parallel desorption of all *in vivo* SPME probes without compromising data integrity. For desorption processes, the handling device houses a maximum of 96 probes, directly fits into a deep well plate and can be placed on a commercial agitator for enhanced analyte desorption. To confirm its sturdiness, efficacy and reliability of the device for parallel desorption of multiple SPME probes, evaluation was carried out, by investigating the agitation uniformity during desorption process. Intra- and inter-well variations, effect of device weight on the agitation process and comparison of performance with conventional desorption approach were among the parameters considered.

### 2.3.1 Investigating effect of the device on the uniformity of agitation during fiber desorption

The device was designed to be placed on a commercial agitator after the probe-loading process for agitation. Thus, it was paramount that each probe was kept in steady position during agitation to prevent movements resulting in the change of the original positions. Typically, for open-bed configuration of SPME-LC-MS applications on a multi-well plate, probes were kept steady inside each well, which ensures effective desorption and data reproducibility. This is because probes movements inside the well result exposure of the coating out of the desorption solution and thus leading to poor data reproducibility. In addition, in this study it is possible that the weight of the device could also impact overall agitation and mass transport properties

in each well, because the entire device weight would be brought to bear on the agitator. The weight in this case included a regular 96-deep well plate containing the desorption solution, the total number of *in vivo* SPME probes, and part of the device holding the probes in place during the agitation process (Figure 2.3). The overall force exerted on the agitator during desorption could therefore be significant and affect the uniformity of the agitation inside each well. If the agitation and therefore mass transport properties within the wells were not uniform, this leads to errors in the amount of analyte desorbed in each well, and affected overall data precision/reproducibility. The effect of the device weight was consequently evaluated by comparing the amount of each drug extracted/desorbed in each of the selected well positions for 5 independent desorption processes with/without the device. To accomplish this, 100 ppb of benzodiazepines was prepared in PBS buffer and then extracted using 20 selected probes using the optimized extraction conditions. The selected probes were placed in various positions in the 96-well plate. After the extraction process, desorption of the analytes was carried out on multi-tube vortexer by placing the probes in 1 mL solution for an hour. Similar experiment was carried out using the same probes. However, in this case desorption was carried out using the multi-probe desorption device, which was placed directly on the agitator (Figure 2.3). The amount of analyte extracted by each probe was determined from an extraction calibration curve generated from standards prepared in PBS.

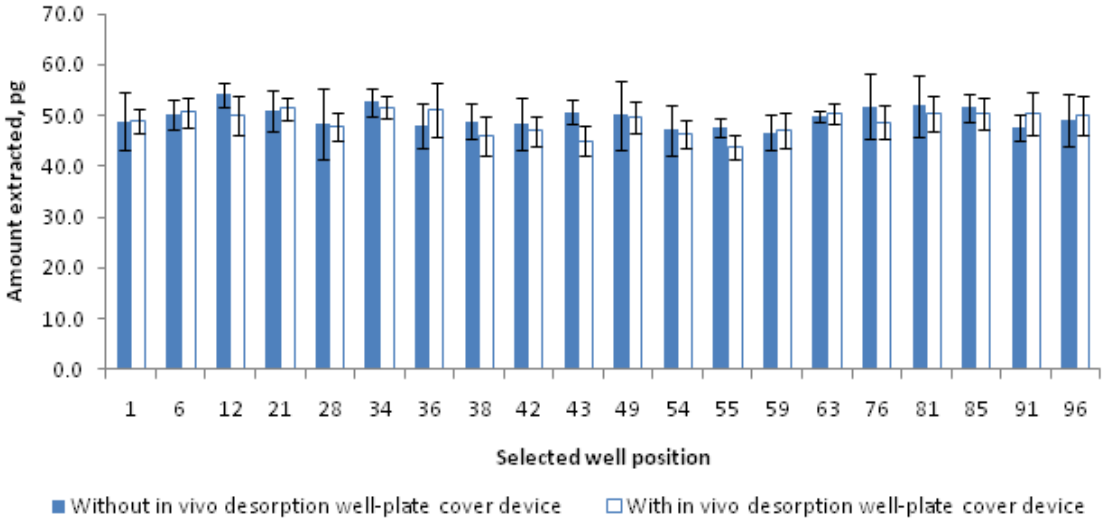


Figure 2.4 Comparison of the amount of diazepam extracted/desorbed from the fibers at selected well positions with/without the desorption device (n = 5). The results are expressed as the mean ± standard errors for 5 extractions.

For desorption of SPME probes in a 96 well-plate format, inter-well agitation differences could lead to non-uniformity in analyte mass transfer from each probe into the desorption solution, thus leading to poor data reproducibility. On the contrary, results shown in (Figure 2.4), suggest that the amount of analyte extracted from each well were not significantly different for both experiments. Thus, it could be concluded that the weight of the multi-probe desorption device does not affect the efficiency of well agitation for the individual wells. In addition, it can be inferred from the results that any relative movement of the probes during the agitation process does not affect the amount of the analyte extracted from the well.

Another important factor for parallel desorption of multiple probes in 96-well plate format is the agitation speed. As a proof of concept, lower agitation speeds are characterized by agitation non-uniformity and slow mass transfer from the probes into the solution. As a

result, the variation in the amount desorbed from each probe as a function of the agitation speed was monitored. This was achieved by determining the amount desorbed at two different agitation speeds, 500 rpm and 1200 rpm respectively, for five successive experiments. The percent relative standard deviations (RSD%) at higher agitation speed were lower (RSD% $\leq$ 7.0; n=5) compared to that obtained (RSD% $\geq$ 15; n=5) for lower speed for the selected wells. Thus, for excellent precision, reproducible and reliable data higher agitation speed (~1200 rpm) of the 96-well plate was necessary provided it does not lead to a possible cross-well contamination. The relatively higher RSD% observed at lower agitation could be attributed to incomplete desorption of analytes from all the wells. This phenomenon could be corrected though with longer desorption times. From the results obtained using the optimized experimental conditions, any differences in the amount extracted from the selected wells result from factors other than the probe positions, weight of the device and speed of agitation.

### 2.3.2 Investigating well variations during multiple probe desorption process

In addition to inter-probe variations, a critical factor in SPME-LC-MS applications with open-bed configurations that could affect data reproducibility is the inconsistencies in the bulk well conditions during agitation of the well-plate. Irreproducible well agitation conditions affect the extraction/desorption rate and thus change the amount of analyte extracted by or desorbed from the probe in different wells for same batch experiments. This leads to poor data reproducibility, and often inaccuracies in the data among batches. So for a 96-well plate where individual well conditions could vary from well to well, it is worthwhile to maintain same bulk movement of desorption solution. Thus, the overall inter- and intra-well variations for selected wells were investigated. This was carried out using a 100 ng/mL solution of benzodiazepines

in a physiological fluid (PBS) as sample. One mL of sample was placed in each selected well, and extraction and desorption processes were carried out from the same well positions for 5 independent experiments. The amount extracted/desorbed from each of the selected wells was determined and variations among and within wells were determined from a standard calibration curve using a neat solution of benzodiazepines.

The calculated inter-well variation (RSD%) for 20 selected wells (n = 20) for all benzodiazepines ranged from 8 – 12% with oxazepam and nordiazepam being the least and highest respectively. It is important to indicate that although variability from the probes may also contribute to the overall well variations, the reproducibility in general was very good. Intra-well variations were investigated by considering reproducibility of the amount extracted from the same well using the same set of probes for five successive experiments, while pre-conditioning each probe in 1:1 v/v methanol-water solution prior to the experiments. The pre-conditioning step was necessary for accurate quantitation of the amount extracted, as carryover effect will be negligible. The contributions to the variations obtained from the same well for each probe at specific well locations were each $\leq$ 10% RSD. This implies that any possible significant variations could be attributed to the differences in probe coatings and not the bulk sample movement within the wells.

To investigate contributions from differences in the coatings, a single probe was initially placed at different well positions for five independent extractions and desorptions for the same concentration of benzodiazepines in PBS. The experiment was also repeated using a single probe for 5 extractions and desorptions from the same well. As shown in Figure 2.5, results obtained were not significantly different for extractions from different well positions

and at the same well position for all the analytes. The RSD% calculated for all benzodiazepines was ≤ 9% in both cases. Thus, in a typical *in vivo* SPME-LC-MS/MS bioanalytical application using the 96-well format, poor data reproducibility may be due to other factors other than the multi-probe desorption device.

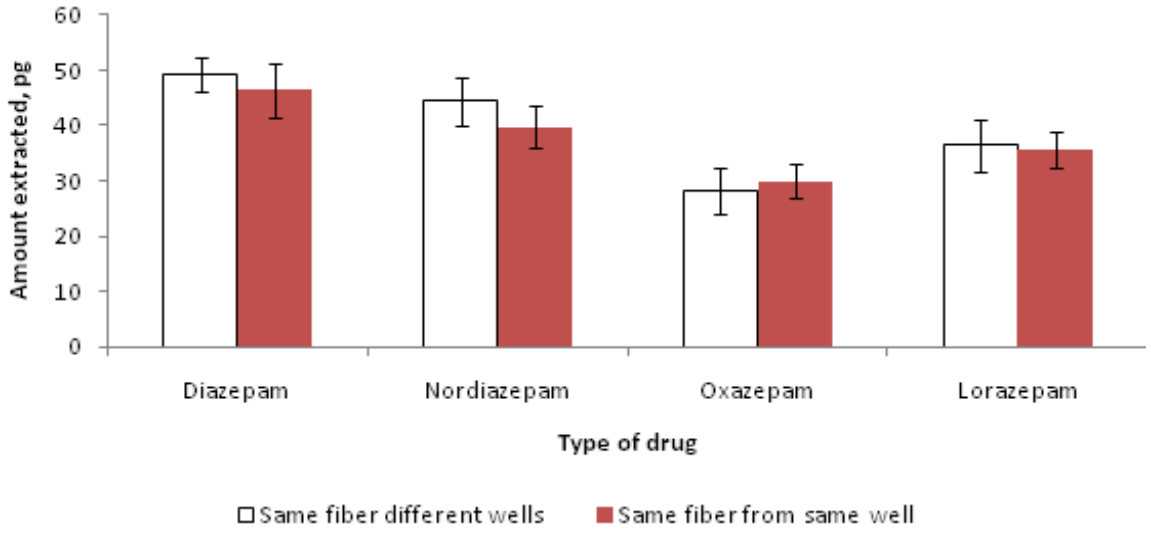


Figure 2.5 Comparison of variations obtained from same well using same fiber with same fiber at different well positions (n=5). The results are expressed as the mean ± standard errors for 5 extractions.

### 2.3.3 Investigating inter-probe variations for parallel desorptions on 96-well plate

After establishing the performance of the device on 96-well plate format during agitation, it was important to assess the variation associated with the use of different probes. This is because during *in vivo* SPME experiments, different probes are employed and therefore it will be important to establish at least the extent variability introduced. To investigate this, extractions and desorptions of 100 ng/mL benzodiazepines were carried out from the same

well using 5 different probes. The experiments were carried out in this manner so as to avoid any variability from the use of different wells. The RSD% for 5 independent experiments for each analyte was then calculated. The calculated RSD% for the benzodiazepines ranged from 10 – 15% with oxazepam and nordiazepam having the highest and least variations respectively.

Comparing results obtained from inter-well and inter-probe variations, reproducibility of the data was largely influenced by the variability in the extraction phase and not from the well. This implies that the handling device does not in any way significantly affect the data precision, reproducibility and reliability.

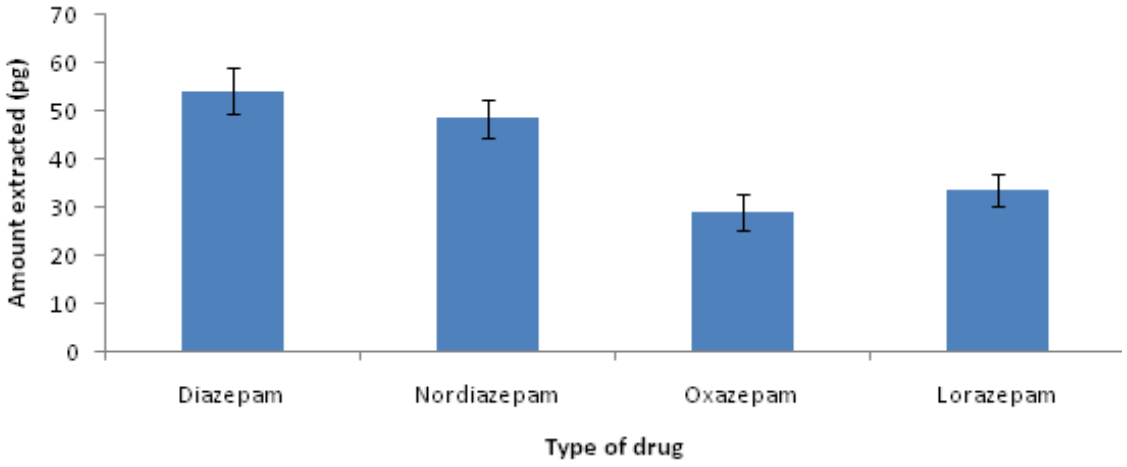


Figure 2.6 Monitoring inter fiber variability for multiple desorptions of benzodiazepines from different numbers of fibers (n = 7). The results are expressed as the mean ± standard errors for 7 extractions.

### 2.3.4 Comparison of device with conventional *in vivo* SPME probe desorption method

After careful evaluation of the device, the performance of the device was compared against the conventional desorption method for *in vivo* SPME applications. This was carried out to demonstrate the efficiency and advantage of the device as a tool for parallel desorption of multiple *in vivo* SPME probes in comparison with already existing method. Similar experiments using the optimized conditions as indicated in the previous were thus carried out with 50 ng/mL benzodiazepines. To facilitate good comparison, same optimized conditions were used for both approaches and the total analyses time in addition to the overall method reproducibility were determined after extraction of the drugs from PBS solution. In these experiments, a different set of 20 SPME probes were used.

Table 2.2 Comparison of the performance of the multiple fiber desorption device with conventional SPME desorption process

| ***Parameter*** | ***Multi-probe handling desorption device method*** | ***Conventional fiber desorption method*** |
|---|---|---|
| Total analysis time after sampling (min) | 30 min | 65 min |
| Reproducibility (CV%); n = 20 | 6 – 9% | 11 – 14% |

Table 2.2 shows an overall analysis time of 30 min for the multiple probe desorption device while the conventional SPME desorption approach took 65 min. Thus, providing an improved sample throughput with the multi-probe desorption device. In addition, lower RSD%

(6 – 9%) compared to the conventional SPME approach was observed. The significant difference in time between the two methods was due to the time taken for handling and preparing probes prior to desorption process. The new approach reduces human contact time of physically handling the probes and thus minimizes any possible contamination during preparation. In addition, results obtained were similar to previous work reported elsewhere[165] using the automated robotic autosamplers for parallel desorption of SPME blades on a 96-well plate format.

## 2.4 Summary

In this section, a multi-probe desorption device tailored for *in vivo* SPME applications using the LC-MS/MS platform for analysis has been described. The device, which fits into a 96-well plate, offers fast alternative approach to handling, preparing and desorption of *in vivo* SPME probes after extraction process. The results outlined the advantage of using the multi-probe device and its potential to enhance desorption process in SPME applications to bioanalysis, especially for *in vivo* analysis. From the evaluation, data recorded from all experiments had ≤ 15% RSD. Generally, successful *in vivo* SPME experiments require a lot of careful planning and effort to obtain meaningful and reliable, thus the need for prevention and protection of the probes cannot be overemphasized. The new device from the design effectively minimizes any possible external contamination of SPME *in vivo* probes during post sampling processes as there is no need for direct contact with the probes. Although the device was primarily introduced for *in vivo* SPME-LC-MS bioanalytical applications, it can also be applied effectively for batch analyses of contaminants in environmental water samples and biological matrices such as urine, plasma and whole blood for situations where the automated

robotic systems are not available for parallel analyses on a 96-well plate format. It is important to indicate that no direct bioanalytical application was considered during the evaluation process as this may introduce its own errors and thus prevent accurate assessment of the performance of the device.

With the completion of a device for handling and desorption of *in vivo* SPME probes primarily for bioanalytical applications, the thesis was further advanced into development of extraction phase to be applied for *in vivo* sampling of neurotransmitters from the rat brain for LC-MS/MS analysis.

# Chapter 3

# Development of new solid phase microextraction coatings for sampling of polar neurotransmitters

## 3.1 Preface and Introduction

### 3.1.1 Preface

This chapter of the thesis is already published online (In Press) as an article under the title "Optimization of Solid Phase Microextraction Coatings for Liquid Chromatography Mass Spectrometry Determination of Neurotransmitters" by Erasmus Cudjoe and Janusz Pawliszyn., *J. Chromatogr. A.* (http://dx.doi.org/10.1016/j.chroma.2014.03.035). All tables and figures were reprinted from this publication with permission from Elsevier Copyright.

### 3.1.2 Introduction

Neurotransmitters as used in this chapter of the thesis refer to small endogenous polar molecules or chemical substances found in the brain, which are used to communicate information throughout the brain and body. Information about their classification, functionality and involvement in various neurological diseases had been mentioned in the preceding chapter. DA, a monoamine, is an important neurotransmitter with changes in its function linked to, for example, Parkinson's disease and schizophrenia.[177] Amino acid transmitters unlike monoamines are the most abundant transmitters in the brain and the CNS. Their acceptance as neurotransmitters in the mammalian brain came much later than the monoamines probably

because they were involved in intermediate metabolism and constitute important building blocks for proteins.[178] Currently, GABA and GA are putative major neurotransmitters for inhibitory and fast excitatory synaptic transmission respectively. Due to their large abundance and utilization, GA and GABA are usually involved in many functions of the CNS as well as associated with various neurological diseases. Many clinical conditions including psychiatric disorders appear to involve an imbalance in excitation and inhibition.[179] Basically, abnormal neurotransmission has been linked to a wide range of conditions, including depression, drug dependence and degenerative diseases among many others. Consequently, over the years numerous analytical methods like MD, voltammetry, biosensors, etc., to mention a few, have been developed for measurements of brain neurotransmitters. Measurements of these compounds have undeniably improved understanding of the relationship between the chemistry in the CNS and the behavioral, cognitive and emotional state of an organism.[180] However, being endogenous polar compounds coupled with the dynamics of neurotransmission in the extracellular space, development of quantitative sample preparation methods is a challenge.

Developing analytical methods for quantitation of very polar compounds is critical for pharmaceuticals as most of these drugs have polar characteristics. Polar compounds are also often intermediates of various biological processes, and thus can provide insight into some of the mechanisms of these processes. In addition, they form also the metabolites or end products of certain biological reactions. In clinical applications, there can be more than one polar compound that may assist in disease diagnoses or as biomarkers. Last but not least, polar

compounds, for example melamine[181,182] and folic acid[183,184] can also be used as indicators in food safety and nutrition.

Microdialysis is an analytical tool used for sampling of drugs and metabolites, and other endogenous substances from various body fluids or the interstitial cell fluid of selected tissues. The technique is known for its selectivity towards polar hydrophilic compounds notwithstanding the challenge with very complicated calibration methods, especially for *in vivo* bioanalysis of endogenous compounds. Despite its effectiveness in sampling small polar substances, MD is often characterized by low recoveries at higher perfusion rate, the challenge handling efficiently smaller dialysate volumes when the probe is perfused at lower flow rates, and the potential matrix influence when coupled to ESI-LC-MS/MS technique.

Often analysis of highly hydrophilic molecules in biological matrices is challenging due to low extraction recovery. For most *in vitro* applications, protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE) are typically used sampling tools. For higher throughput, PPT has been the commonly used sample preparation method. Nonetheless, matrix contributions due to the large presence of extracted phospholipids and the competition between aqueous and organic phases may result in lower recoveries. With LLE, apart from not being environmentally safe due to the larger solvent volumes involved, the analyst is often confronted with the challenge of choosing the appropriate organic solvent for the extraction of the polar hydrophilic compounds. Sorptive methods like SPE have been often applied to the extraction of polar analytes in various matrices including *in vitro* bio-applications. The primary interest in sorptive methods is due to the ability to modify the characteristics of the sorbent material to enhance selectivity for target compounds.[185]

Conventional polymeric sorbents can be modified to improve their hydrophilicity by incorporating polar substrates into their structure. Various hydrophilic sorbents with affinity for polar analytes have been prepared either by copolymerization of appropriate functional monomers or by chemically modifying the hydrophobic polymer with a polar moiety.[185] The Oasis HLB™ for example; a divinylbenzene-based copolymer used in SPE method has been shown for its potential to extract highly polar compounds. An online SPE method coupled to liquid chromatography and tandem mass spectrometry (LC-MS/MS) was used for the extraction of antibiotics in aqueous matrix.[186] Also off-line SPE-LC-MS methods have been developed for the analysis of herbicides[187] and endocrine disrupting compounds (EDCs)[188] with the Oasis HLB sorbents. Smith *et al.* used commercially available sorbent (Porapak RDX) from Waters® for the determination of polar nitroaromatic compounds in aqueous matrix.[189] Despite the vast availability for sorbents, SPE techniques are characterized with clogging of the cartridge complex matrices, especially biological samples. This limitation invariably requires additional step(s) of sample pre-treatment prior to SPE extraction, which not only makes the method time consuming but may also lead to loss of some chemical information resulting from matrix modifications.

Another sorptive method that has gain considerable success for the analysis of polar hydrophilic compounds in various matrices is SPME. Unlike the column-like packing for SPE cartridges, where wider range of sorbents can be easily applied, SPME coatings are usually immobilized on a rigid support. This limits SPME applicability to sorbents having appropriate morphology, which allow direct deposition unto a rigid support. Despite this challenge various types of commercially available SPME coatings such as polydimethylsiloxane (PDMS),

polyacrylate (PA), polypyrrole (PPY), divinylbenzene (DVB), etc, to mention a few, and in-house silica-based octadecyl ($C_{18}$) and reverse phase amide (RPA) have been used for the extraction of analytes from complex matrices. For example PPY, a fiber fabricated through electro-deposition has been used for the determination of β-blockers,[190,191] phenols,[192] and aliphatic alcohols.[193] Since SPME was initially introduced for gas chromatography applications, analysis of polar analytes required derivatization. Derivatization can be performed either during or post analyte extraction for enhanced efficiency and to prevent destruction of thermally labile compounds in the GC injector. Online in-tube SPME-LC, [194–196] which involved the use of the stationary phase of GC capillary columns as extraction material provided the platform for the analysis of thermally labile polar and apolar analytes in various matrices using LC for separation. This on-line method can be performed without the need for derivatization, provided good retention and was achieved on the LC separation column. Subsequently, recently developed "in-house" coating methods pioneered the fabrication of new SPME extraction phases for LC-MS applications to both *in vitro* and *in vivo* complex systems.[197,198]

Another form of challenge encountered by researchers during the analyses of small polar organic molecules is effective retention in LC separation without the need for derivatization. The C8 and C18 stationary phases are the most commonly used for reverse phase LC and by using the appropriate solvent composition, temperature, pH, etc, separation has been attained for many analytes. However, chromatographers sometimes encounter challenging separations for which selectivity, ruggedness or reproducibility is not easily obtained using traditional C8 and C18 phases. Generally, conventional reverse phase

chromatography does not have good retention for polar compounds and thus make it unreliable for quantitative analysis. Thus, the use of reverse phase columns often require ion-pairing agents as one of the approaches to improve retention factor for polar compounds.[199] Derivatization agents like *ortho*-phthalaldehyde (OPA),[200] naphthalene-2,3-dicarboxaldehyde (NDA),[201] and 1-dimethylaminonaphthalenesulfonyl (DANSYL),[202] to mention a few, have been used for the analysis of neurotransmitter molecules. Understandably, apart from being time consuming, derivatization introduces another analytical step to the entire procedure, which makes the method more susceptible to errors. The hydrophilic interaction chromatography (HILIC) column revolutionized separation of various polar analytes without the need for derivatization. HILIC since its introduction has established itself as the separation mode of choice for uncharged highly hydrophilic compounds that are too polar to be well retained in reverse phase chromatography. HILIC separation still continues to attract a lot of interest because it solves various hitherto difficult separation problems, such as the separation of small organic acids, basic drugs, and many other neutral and charged substances.[27] However, the characteristics of the stationary phase may affect and in some cases limit the choices of mobile phase composition, ion strength or buffer pH value available.[203] The technique often requires careful manipulation of the mobile phase pH and buffer salt concentration. The resultant effect is often signal or ion suppression depending on the analyte type when hyphenated to mass spectrometry. Alternatively the pentafluorophenyl (PFP) bonded to silica stationary phase can be used for retention of polar compounds. PFP stationary phases have demonstrated unique retention for small polar analytes. PFP stationary phase separate compounds based upon selective interactions such as steric recognition, charge transfer or by $\pi$-$\pi$ interactions. By using a novel selective phase, like PFP, it is often possible to improve

separation and elution of difficult polar compounds in LC for easier quantitation. The PFP stationary phases also offer the flexibility of using simpler mobile phases thus avoiding ion-pairing reagents, concentrated buffer systems, strong pH conditions and complex mobile phase preparations. Since most of the mobile phases used with PFP stationary phase do not require strong buffers as observed in the case of HILIC column, enhanced MS signals with improved sensitivities can be observed.

This study therefore investigates the potential of using silica- or polymer-based mixed-mode as new sorbents for quantitative SPME-LC-MS/MS analysis of selected polar neurotransmitters with wide range of polarities without the need for derivatization. The selected neurotransmitters encompass polar organic molecules of varying polarities. In order to improve extraction efficiency, coatings with higher surface to volume ratio were prepared using the flat blade/thin film configuration. The study compares the extraction efficiencies of various "in-house" mixed-mode SPME coatings for the analysis of neurotransmitters. The mixed mode sorbents where chosen due to their ability to offer multiple modes of interaction. In addition to exploring the new mixed-mode SPME coatings, this chapter demonstrates LC separation of polar neurotransmitters without the need for analyte derivatization or use of buffers. The approach offers a robust LC separation using very simple chromatographic solvents compatible with the mass spectrometer. Chromatographic optimization and retention of the polar neurotransmitters were performed using the HILIC and PFP stationary phases. The PFP stationary phase was finally chosen for retention and separation of both amino acid (glutamic acid and γ-aminobutyric acid) and monoamine (dopamine and serotonin) neurotransmitters due to higher signal-to-noise ratio.

## 3.2 Experimental section

### 3.2.1 Materials and reagents

HPLC grade acetonitrile (ACN) was purchased from EMD Chemicals Inc., Ontario. All mixed-mode sorbent particles were obtained through the assistance of Chromatographic Specialties®, Ontario as research samples. The Loctite 349 impruv™ (R. S. Hughes Company, Plymouth, MI) and Kasil 1®, (PQ Corporation, Valley Forge, PA) were used as adhesives. Medical grade stainless steel tubes used for making flat surface blades with the assistance of University of Waterloo science machine shop were purchased from Small Parts® Inc., Miami, FL. Glutamic acid (GA), γ-aminobutyric acid (GABA), dopamine (DA) and serotonin (5-HT) were purchased from Supelco®, Oakville, Ontario. Artificial cerebrospinal fluid (aCSF) used for preparing samples and method development was obtained from Harvard Apparatus (Holliston, MA). HPLC grade formic acid was also purchased from Supelco®, Oakville, Ontario. Whole rat brain samples were obtained from a certified and qualified animal facility, NoAb BioDiscoveries, Mississauga, ON. Deionized water for preparation of standards and LC mobile phases were from Barnstead/Thermolyne NANO-pure water system (Dubuque, IA, U.S.A.) and the Thermolyne® Maxi mix *plus* vortexer was also from Barnstead/Thermolyne (Dubuque, IA, U.S.A.). The HPLC columns for chromatographic separation were obtained from Phenomenex (Torrance, CA). Sprague Dawley rat cerebrospinal fluid (CSF) was purchased from Bioreclamation, Hicksville, NY.

### 3.2.2 Preparation of standards

The selected neurotransmitters represent polar organic compounds with wide range of pKa values: 2.13, 4.03, 8.9 and 9.8 for GA, GABA, DA and 5-HT respectively. Individual stock standard solutions (1 mg/mL) were all prepared in a final solution of acetonitrile/water/formic acid in amber vials and kept refrigerated for a maximum of four weeks until discarded. With the exception of GA, which was initially dissolved in acidified water (0.1% formic acid), all other standards were directly prepared in acetonitrile/water 2:3 (v/v) mixture with 0.1% formic acid. Instrument calibration standard solutions were freshly prepared by serial dilution of 1 μg/mL solution prepared from the stock to cover a concentration range of 0.006 – 200 ng/mL. All samples and working calibration standards were prepared in physiological fluid (aCSF) while maintaining an organic content of less than 1%.

### 3.2.3 Chromatographic procedure and mass spectrometry conditions

Three main types of LC columns (reverse phase $C_{18}$, HILIC and PFP) were examined for their effectiveness in separating the polar neurotransmitters (GABA, GLU, DA and 5-HT) as reported in this chapter. Other polar-embedded alkyl phases did not yield any better results in providing good retention and separation of the neurotransmitters. A 25-ng/mL standard containing all the analytes was used for the chromatographic method optimization. As part of the objectives, chromatographic separation of the compounds was attained in their non-derivatized form in order to reduce the number of analytical steps and subsequently improve throughput. Various mobile phase compositions appropriate for a specific column type/stationary phase were used to determine the efficiency in retaining and separating the

analytes. Initially, separate isocratic runs (reverse and normal phase method) using mobile phases A (95 % water, 5 % acetonitrile and 0.1 % formic acid) and B (5 % water, 95 % acetonitrile and 0.1 % formic acid) at 300 μL/min flow rate were used for the $C_{18}$ column (100 mm x 4.6 mm x 5 μm). The Accela™ autosampler (Thermo Scientific, USA) equipped with temperature controlled tray chamber was used to introduce 10 μL sample for chromatographic separation using the Accela™ HPLC Pump with a dual piston pump. In addition to evaluating the $C_{18}$ column, the PFP and HILIC columns were also assessed using acetonitrile/water mobile phase systems with 0.1 % formic acid and acetonitrile/ammonium formate buffer respectively.

The TSQ Vantage™ triple quadrupole mass spectrometer (Thermo Scientific, USA) in a positive mode was used to identify and quantify all analytes. The source voltage was optimized and set at 1000 V, while the capillary and vapourizer temperatures were 250 $^0$C each. Optimized sheath and auxiliary gases were respectively, 55 and 15 arbitrary units. The parent and daughter ions monitored for each compound were as follows: GABA (104.1; 69.1), GA (148.1; 84.1), DA (154.1; 91.2) and 5-HT (177.1; 115.1). All data analyses were performed with the Xcalibur® software version 2.0.7.

### 3.2.4 Preparation of SPME coatings

Two different coating preparation methods were firstly evaluated in this project. The first coating method involved the use of Loctite 349 impruv™ glue as an adhesive to support particles unto a stainless steel metal blade. With the second coating approach, Kasil 1® was used as adhesive to immobilize a thin film of extraction phase on the flat stainless steel blade.

Prior to the coating process, the metal blades were pre-treated in concentrated nitric acid for about an hour to etch the surface. Subsequently, the blades were washed thoroughly with tap water followed by deionized water. After drying the surface of the metal for few minutes the metal blades were then placed into acetone in a beaker and then agitated for 30 min to remove any possible organic contaminants introduced during the washing process. Later, the blades were thoroughly dried under a gentle stream of nitrogen gas.

Using the Loctite 349 impruv™, the first set of metal blades were dipped into the adhesive inside a 2 mL vial covered with a cap and a pre-cut septum. To ensure that each blade was exposed to the same length of adhesive inside the vial, a small piece of Teflon material was placed underneath the adhesive. Thus, the metal blade will be pushed through the pre-cut septum and into the adhesive until it touches the top of the Teflon material. The blade was then withdrawn from the vial with the septum ensuring that a fine layer of the adhesive was deposited on the surface of the metal. After covering surface of the metal with the adhesive, the blade was subsequently rotated several times in a pile of particles on a clean paper. The prepared coating was then placed under a UV lamp for an hour while rotating the blade every 10 min. In the case of the Kasil 1™, the treated metal blade was dipped into the adhesive for about 15 sec followed by rotating the adhesive coated blade in the pile of particles. The particle-coated blade was subsequently passed over fumes of concentrated nitric acid for a few seconds. Later the particles were kept inside a desiccator overnight.

Sorbents used for this study were categorized into two main groups: silica- and polymer-based support (Table 3.1). For the purpose of evaluation, 3 replicates of each coating were selected, initially evaluated and later assessed for their extraction efficiency prior to use

for further extractions. This was done to minimize variations between coatings. Prior to SPME extractions, all coatings were pre-conditioned overnight in 1:1 (v/v) methanol/water with agitation on at 150 rpm on a SK-300 mechanical shaker (JEIO TECH, Korea). Subsequently, the coatings were placed in aCSF, diluted 10x with deionized water, for less than 2 minutes to reduce the organic content prior to extraction. As part of the objective, the reusability of each coating type was also monitored to ascertain its robustness.

Table 3.1 Types of SPE sorbents used for SPME coatings

| ***Sorbent*** | ***Support*** | ***Type of interaction*** |
|---|---|---|
| ***Chromabond SA*** | Silica | strong ion exchange |
| ***DPA-6S*** | n/a | polyamide resin |
| ***$C_{18}$ particles*** | Silica | reverse phase |
| ***Clean screen DAU*** | Silica | reverse phase and strong ion exchange |
| ***Clean screen GHB*** | Silica | n/a |
| ***SSBCX*** | Silica | strong ion exchange |
| ***$C_{18}$+B*** | Silica | reverse phase with mixed-mode strong ion exchange |
| ***$C_8$+B*** | Silica | reverse phase with mixed-mode strong ion exchange |
| ***MCX*** | Polymer | reverse phase with strong mixed-mode ion exchange |
| ***MAX*** | Polymer | reverse phase with strong mixed-mode ion exchange |
| ***WCX*** | Polymer | reverse phase with weak mixed-mode ion exchange |
| ***WAX*** | Polymer | reverse phase with weak mixed-mode ion exchange |

### 3.2.5 SPME extraction procedure

All extractions and desorptions were carried out in aCSF and water/acetonitrile 3:2 (v/v) with 0.1 % formic acid respectively. An hour extraction of 50 ng/mL solution of the

analytes was carried out under static conditions with each sorbent type and subsequently the neurotransmitters were desorbed in 180 μL desorption solution in a 300 μL amber vial. During the one-hour desorption process, the blades were agitated at 800 rpm on the SK-300 mechanical shaker. After the desorption process, the samples were further subjected to liquid chromatographic separation and tandem MS analysis using the conditions stipulated in the previous section. Details on chromatographic method are presented in the following section.

### 3.2.6 Extraction of neurotransmitters from CSF and rat brain samples

Approximately 2.0 g of brain samples were weighed into previously cleaned 20 mL vials and subjected to high speed vortex for about 3 – 5 min at approximately 30 sec intervals to avoid heating of the samples beyond laboratory room temperature. All the brain samples were later pooled into a laboratory petri-dish, covered and vortexed again. After this process, approximately 1.0 g of the macerated brain tissue sample was transferred into 3 separate vials and distinctly spiked at different concentration levels (50 ng/mL and 500 ng/mL) and then 6 vials spiked at 5 ng/mL and vortexed for about 2 min. Each of the spiked samples was prepared in 3 replicates and extracted with the $C_{18}$+B SPME blade coating for 30 min with agitation at 750 rpm. In addition, unspiked macerated brain tissue samples were also extracted and treated as blank correction. After the extraction process, the SPME blades were wiped with Kimwipes™ to physically remove the deposits of brain tissue from the surface of the coatings, dipped into a deionized water for about 2 sec and then desorbed in 300 μL of desorption solution containing 5 ng/mL diazepam as an internal standard. The same set of SPME blades were used for all extractions at a particular concentration level. Prior to the extraction process, preliminary extractions from aCSF under steady state conditions revealed that all the analytes

reach equilibrium within 20 min. A relatively longer extraction time was chosen, despite the agitation, to ensure that all analytes reaches equilibrium due to the tortuosity of the brain tissue samples. The average area response of each analyte for the blank samples (unspiked samples) was subtracted from their respective responses at each concentration level. A 6-point calibration curve was prepared by extracting spiked samples of aCSF with neurotransmitters at concentrations ranging from 1 ng/mL to 200 ng/mL. A plot of the area ratio of each analyte to the internal standard (diazepam) versus the nominal concentration of the standard was used for the quantification.

Extractions were also carried out using the rat CSF obtained from Bioreclamation®. The sample and desorption solution volumes used in this experiment were 750 μL and 150 μL respectively. However, all CSF experiments were carried out without agitation and spiking, and the experiments were carried out in triplicate.

## 3.3 Results and discussions

### 3.3.1 Chromatographic method optimization

A major challenge that most chromatographers encounter during separation of small polar compounds, in their non-derivatized form, is the ability to develop a robust bioanalytical method with good retention. This is because these small polar compounds, and in this case neurotransmitters, elute in the void volume of a conventional reverse phase separation on alkyl hydrophobic chains on silica. In order to improve their retention, the analytes are usually derivatized and in certain cases improve analytical signal when coupled to MS. The derivatization process not only introduces additional step(s) to the analytical process, but also

makes it susceptible to more analytical errors. Ion-paring agents have also been employed for chromatographic analysis of very polar organic compounds. However, the major limitation of this technique is the lack of method specificity and therefore cannot be applied to the analysis of group of compounds with widely different functionalities. In addition, some of the ion-pairing agents are not compatible with MS detection systems. To avoid the difficulties and the laborious characteristics of a derivatization procedure and use of ion-pairing agents, in this thesis, a robust chromatographic separation method coupled to the MS for the analysis of the non-derivatized neurotransmitters with wide range of polarities has been demonstrated.

As a proof of concept, a 300 μL/min flow rate with the $C_{18}$ column (150 mm × 2.1 mm × 3 μm) did not provide any retention of the analytes for isocratic reverse phase separation method with an acetonitrile/water mixture spiked with 0.1 % formic acid. As shown in Figure 3.1, the non-derivatized polar analytes were not retained on the hydrophobic reverse phase $C_{18}$ column. This was because the polar neurotransmitters did not have strong interactions with the relatively hydrophobic C18 stationary phase.

Subsequently, two types (Supelco Discovery® HS F5; 100 mm x 2.1 mm x 3 μm and Phenomenex Kinetex® core shell; 100 mm x 2.1 mm x 2.6 μm) of PFP stationary phase columns were also evaluated for their effectiveness in separating and retaining the polar molecules using mobile phases previously indicated. A maximum flow rate of 300 μL/min in a reverse phase chromatographic separation was used for the Supelco Discovery HS F5.

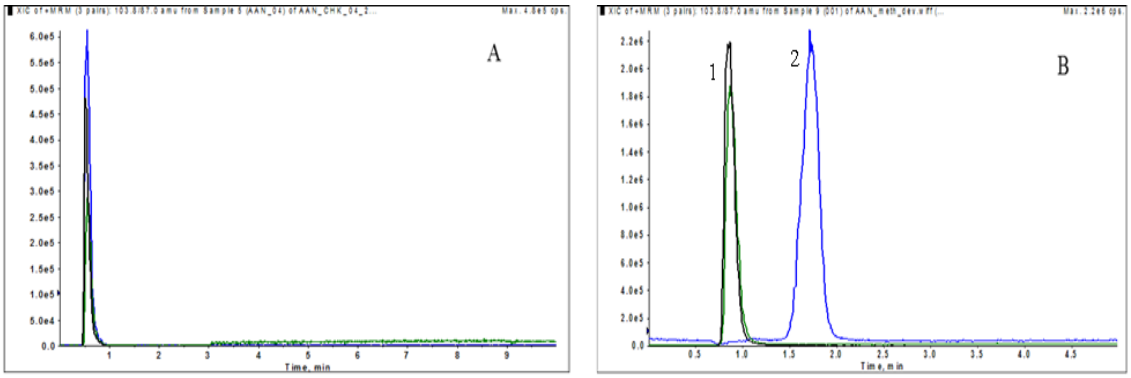


Figure 3.1 Chromatograms of polar neurotransmitters eluting on a $C_{18}$ column. (A) Unretained polar neurotransmitter peaks eluting within a minute of chromatographic runtime under high aqueous content. (B). GABA and GA peaks (1) eluted under a minute at the same retention time and DA (2) eluted at 1.7 min under high organic content mobile phase.

The chosen flow rate resulted from the huge backpressure experienced with this column at higher flow rates. This resulted in a longer run time of 15 min. DA, which eluted at about 11.5 min, had a characteristic broader peak. In order to improve the observed broad peak for DA at lower flow rate, the Kinetex® core shell PFP column (100 mm × 2.1 mm × 2.6 μm) was used. This column had relatively smaller particle sizes and with the core shell technology it was able to accommodate higher flow rates with relatively low backpressure. The overall effect was narrower peak shapes with an improvement in the signal-to-noise ratio for each analyte.

Mobile phase used for the Kinetex® column consisted of A (90 % water, 5 % methanol, 5 % acetonitrile and 0.1 % formic acid) and B (10 % water, 90 % acetonitrile and 0.1 % formic acid) at 450 μL/min flow rate for both normal and reverse phase methods. In the typical reverse

phase gradient separation mode, most of the analytes were tailing and eluted with the void volume as shown in Figure 3.2.

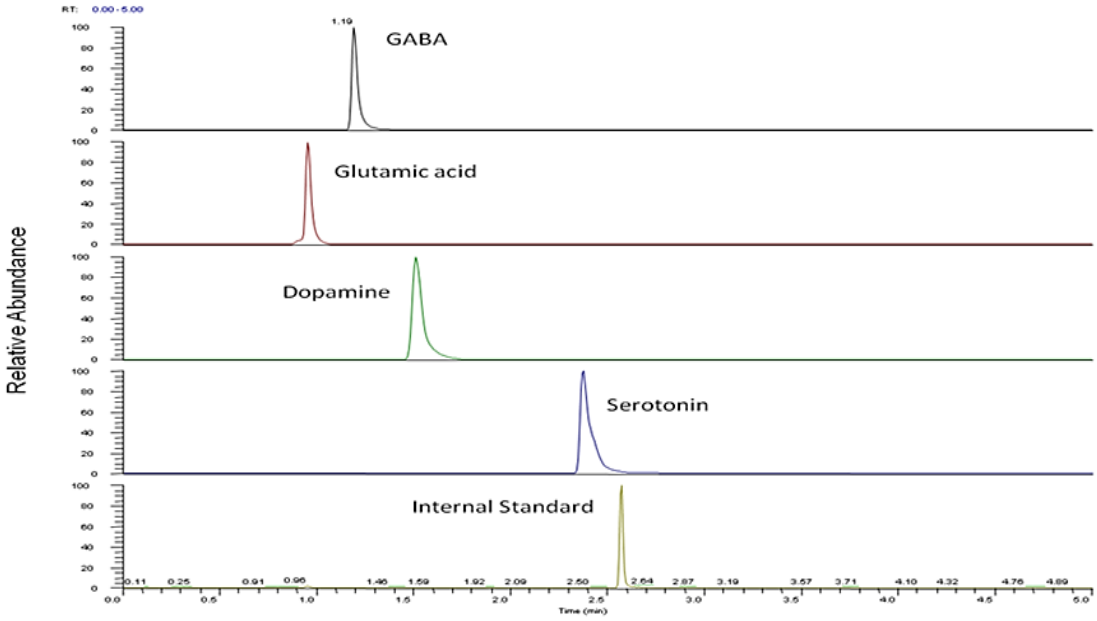


Figure 3.2 Separation of underivatized polar neurotransmitters in a reverse phase chromatography mode using the PFP column. GABA and Glutamic acid eluted under a minute.

Alternatively, the chromatographic separation was started with 100 % mobile phase B phase followed by gradual increase in the aqueous content. Retention and separation was achieved within a 5-min runtime starting with 0 % mobile phase A, held for one minute and then gradually increased to 100 % A by 3.5 min. Mobile phase A (100 %) was maintained for half a minute before re-conditioning of the column for another minute. Diazepam used as an internal standard for correction of any potential injection errors eluted earlier due to its characteristic higher hydrophobicity compared to the neurotransmitters.

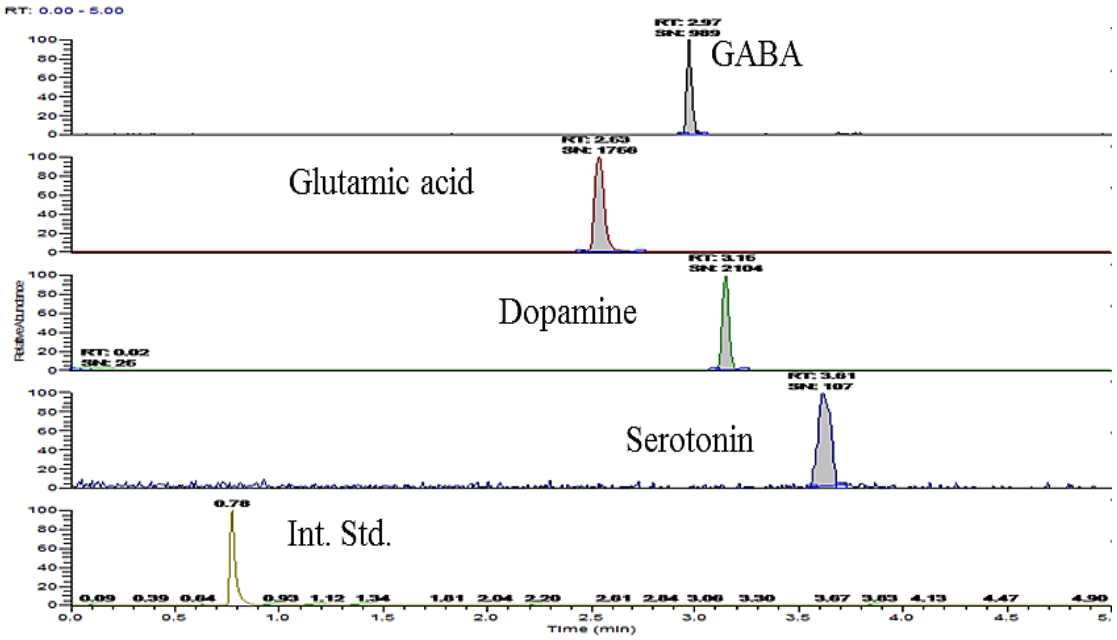


Figure 3.3 Robust chromatographic separation of underivatized polar neurotransmitters in a typical normal phase chromatography without the need for buffers

In the case of the HILIC column, (Phenomenex Kinetex® HILIC; 50 mm × 2.1 mm × 5 µm) mobile phase A consisted of 1:1 (v/v) water and ammonium formate buffer and B contained 95 % acetonitrile and 5 % ammonium formate with pH adjusted to 3.5. The chromatographic method started with 0 % mobile phase A, held for one minute, gradually increased to 95 % A by 4 min and then the column was re-conditioned for the next minute. Although retention and separation of all the neurotransmitters was attained within the 5 min runtime on the HILIC column, there was about 50-fold decrease in sensitivity compared to the response observed with the PFP column. The reduction in sensitivity most likely resulted from signal suppression at the MS ion source due to the presence of the high concentration of buffer ions in the mobile phase, which might have affected effective ionization of the neurotransmitters in their non-derivatized forms.

### 3.3.2 SPME coatings evaluation

Evaluation of SPME coatings was based on the overall extraction efficiency for the analyte from aCSF solution by each coating type. The pH of the aCSF was maintained at physiological conditions to mimic a typical biological system. The aCSF pH was maintained so that the optimized method can be applied to *in vivo* biological systems where the physiological pH cannot be adjusted. With the exception of the Chromabond™, Clean Screen™ sorbents, DPA-6S™ and $C_{18}$ particles, the rest were all mixed-mode sorbents on either a silica- or polymer-based support (Table 3.1). The mixed-mode sorbents were chosen because of their characteristic multi-interactions of hydrophobic and hydrophilic mechanisms. All the analytes were typically polar with pKa ranging from 2.13 for GLU to 9.8 for 5-HT in aqueous media.

### 3.3.3 Evaluation of coating procedures

To compare the two coating procedures, the extraction efficiencies, robustness, inter- and intra-coating reproducibility of the selected fibers were compared. Each coating was prepared in triplicate and was used for 5 extractions of GA, DA and GABA from aCSF. Figure 3.4 shows percentage of GA that was extracted by each sorbent for the two different coating approaches. However, of the two methods, coatings made from the Kasil 1™ adhesive extracted higher amount of GA. The difference in the amount extracted could be attributed to the difference in the particle sizes. Smaller particle sizes ($\leq$ 10 μm) were used with Kasil 1™ adhesive method, while larger particle sizes (30 μm $\leq$ particle size $\leq$ 60 μm) were used with the Loctite adhesive method. It is important to note that with the smaller particle sizes, a larger surface area of the coating would be obtained and thus improve the extraction efficiency. The

smaller error bars may also be due to the uniformity of the particle sizes used with the Kasil 1® adhesive compared to the greater variation in the particle sizes used with the Loctite 349 impruv™ adhesive. The %RSD for 3 replicates of coatings were respectively 15% and 9% for the Loctite 349 impruv™ and the Kasil 1™ adhesive. Subsequently, all analyses were performed using the Kasil 1™ adhesive coating procedure.

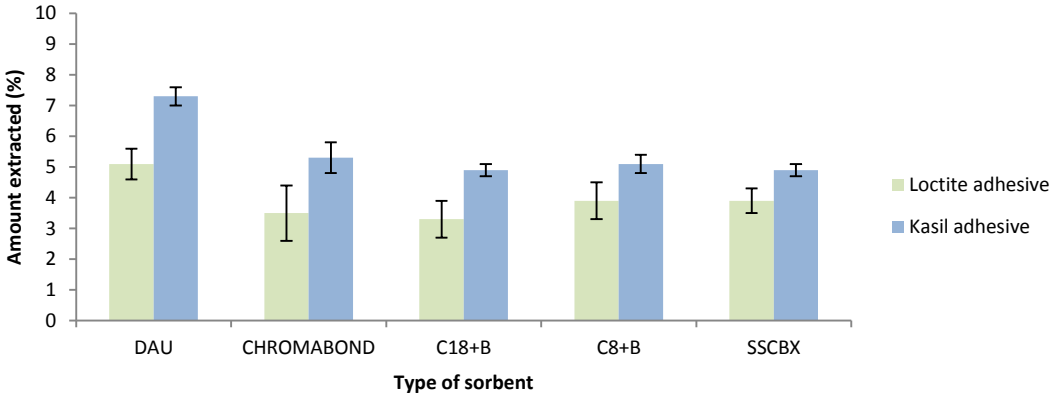


Figure 3.4 Comparison of the efficiencies of SPME coating methods for the extraction of glutamic acid from artificial cerebrospinal fluid for selected sorbents (n=5). The results are expressed as the mean ± standard errors for 5 extractions.

### 3.3.4 Evaluation of sorbent extraction efficiency

The initial criterion used in the evaluation process involved the selection of sorbent(s) that is/are able to extract quantitative amounts of all four neurotransmitters, i.e., the ability of the developed SPME coating to extract analytes with wide range of pKa values. Thus, the sorbents were screened for their ability to extract all 4 neurotransmitters using spiked samples of aCSF containing 50 ng/mL of each analyte. Table 3.2 shows that most of the mixed-mode

sorbents were able to extract all 4 neurotransmitters. Chromabond SA, Clean screen GHB, SSBCX, DPA-6S and $C_{18}$ sorbents extracted $\leq 3$ analytes with DPA-6S and $C_{18}$ sorbent extracting only glutamic acid and dopamine, respectively. This observation may be due to the fact that DPS-6S and the $C_{18}$ do not exhibit multiple interaction modes (hydrophobic and hydrophilic interactions) with the analytes and therefore the observed low extraction efficiency. The remaining sorbents extracted quantitative amounts of all 4 neurotransmitters.

Table 3.2 Screening of sorbents used for SPME coatings for their ability to extract Neurotransmitters

| ***Sorbent type*** | ***Neurotransmitter*** | | | |
|---|---|---|---|---|
| | ***GA*** | ***GABA*** | ***DA*** | ***5HT*** |
| ***Chromabond SA*** | + | + | - | n/a |
| ***DPA-6S*** | + | - | - | n/a |
| ***Clean screen DAU*** | + | + | + | n/a |
| ***Clean screen GHB*** | + | + | + | - |
| ***SSBCX*** | + | + | + | - |
| ***$C_{18}$+B*** | + | + | + | + |
| ***$C_8$ + B*** | + | + | + | + |
| ***MCX*** | + | + | + | + |
| ***MAX*** | + | + | + | + |
| ***WCX*** | + | + | + | + |
| ***WAX*** | + | + | + | + |
| ***$C_{18}$ particles*** | - | - | + | n/a |

n/a: not available at the time of experiment
(+): quantitative amount extracted
(-): analyte detected but cannot be quantified

Subsequent to the initial sorbent screening process, the amounts extracted by each of the selected mixed-mode SPME coatings ($C_{18}$+B; $C_8$ + B; MCX; MAX; WCX; WAX) were determined and compared in a separate set of extractions. The comparison process entailed triplicate one hour SPME extractions of 50 ng/mL neurotransmitters in aCSF samples with subsequent desorption for another hour. A second desorption of the same SPME coating was

carried out to ascertain any carryover amounts after the initial desorption. The percent amount of each extracted analyte was determined for the selected coatings. Results showed that $C_{18}$ with benzenesulphonic acid group ($C_{18}$+B) extracted equal amounts of GABA, DA and 5-HT with GA being the highest. Among the analytes, GA was extracted the most by all the sorbents with DA being the least extracted. With the exception of $C_{18}$+B, there were no significant differences in the amounts of GABA and GA extracted by the MAX, $C_{18}$+B and $C_8$+B sorbents. MCX sorbent showed higher extraction efficiency for 5-HT however, there was no significant difference in the amount when compared to that of $C_{18}$+B sorbent. Whereas WCX sorbent did not show any significant difference in its extraction efficiency for all the analytes, WAX showed higher extraction efficiency for GABA and GA only. Finally, in terms of base support (silica- or polymer-based sorbents), there were no observable patterns in the extraction efficiencies of the sorbents for these analytes. This implies that the extraction efficiency of the sorbent was not necessarily dependent on the base support. In addition, the strength of the ion exchange properties of the sorbents did not show any remarkable influence on the extraction efficiencies.

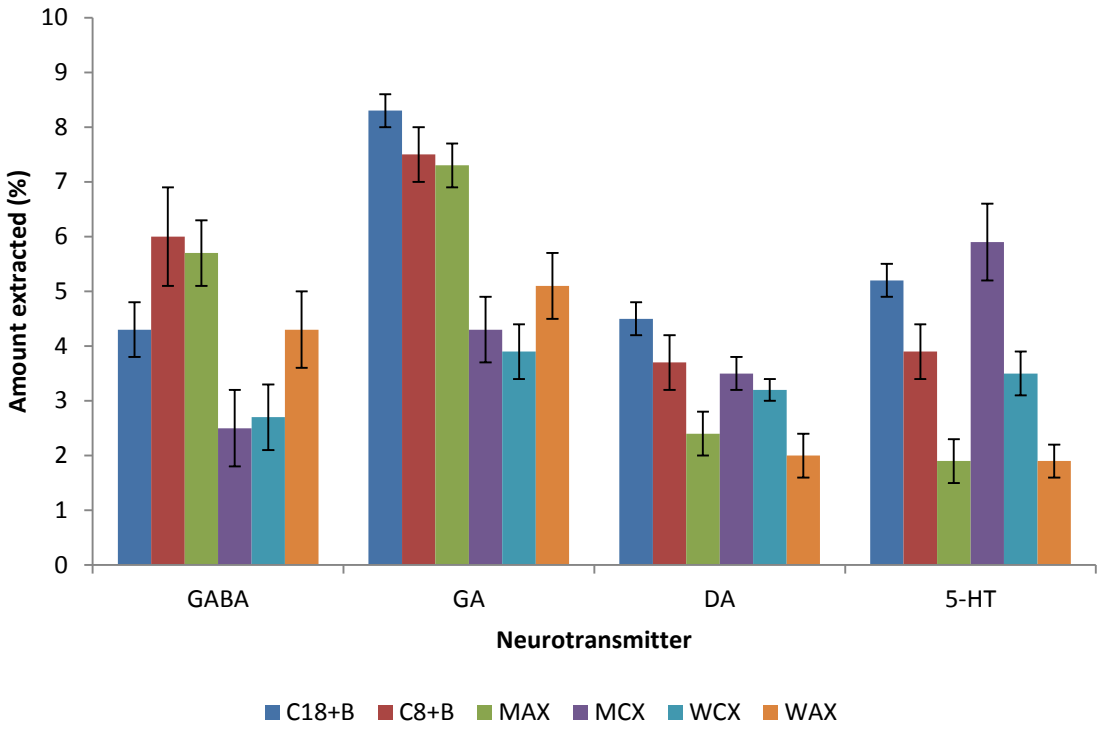


Figure 3.5 Comparison of the extraction efficiency of selected mixed-mode sorbents as SPME coatings for neurotransmitters spiked in artificial cerebrospinal fluid (n=5). The results are expressed as the mean ± standard errors for 5 extractions.

### 3.3.5 Extraction of neurotransmitters from CSF and rat brain samples

Results obtained for the extraction of neurotransmitters from CSF and rat brain samples are shown in Table 3.3

Table 3.3 Results obtained for the extraction of spiked rat brain tissue sample and CSF

| | | Neurotransmitter (ng/mL) | | | |
|---|---|---|---|---|---|
| | | GA | GABA | DA | 5-HT |
| **Spiked brain samples** | **50 ng/mL (n=3)** | 48.0 (4) | 45.0 (6) | 52.0 (5) | 43.0 (4) |
| | **500 ng/mL (n=3)** | 510 (11) | 493 (9) | 505 (8) | 496 (10) |
| | **5 ng/mL (n=6)** | 5.50 (1.2) | 4.80 (1.0) | 5.10 (0.7) | 4.50 (1.0) |
| | | GA | GABA | DA | 5-HT |
| **CSF Sample** | | 0.92 (0.1) | 0.53 (0.06) | nd | nd |

nd: not detected

The precision (%RSD) for each analyte at each concentration level determined was less than 12%. The analytical figures of merit determined were the limit of detection and quantitation, and the linear range using neat solutions of standards prepared in desorption solution and method robustness. The RDS% obtained for 6 replicates of lowest concentration (5 ng/mL) spiked brain sample was used to measure method robustness. The linear range was determined from to be between 0.01 – 150 ng/mL for all the analytes except for GABA, which was from 0.1 – 100 ng/mL. The limit of detection range from 6 pg/mL to 10 pg/mL for all the neurotransmitters and the limit of quantitation were in the range of 20 pg/mL to 35 pg/mL.

## 3.4 Summary

This chapter demonstrated the use of simple solid phase microextraction method coupled to liquid chromatography mass spectrometry for the analysis of four neurotransmitters with a wide range of aties in physiological fluid. A chromatographic method was developed without pre-column derivatization of analytes by considering the retention capacity of various types of liquid chromatography columns. Among three column types investigated, the pentafluorophenyl core shell and hydrophilic interaction chromatographic columns showed significant retention of the polar compounds. A normal phase chromatography method on the pentafluorophenyl column was optimized to separate the neurotransmitters with wide range of acidities on a triple quadrupole mass spectrometer: glutamic acid (pKa 2.13), gamma aminobutyric acid (pKa 4.03), dopamine (pKa 8.9) and serotonin (pKa 9.8). New solid phase microextraction method using "in house" coatings were prepared for extraction and pre-concentration of the analytes using Loctite® 349 impruv and Kasil 1® as adhesives. The performance of both coating procedures was evaluated and the latter was adopted for this study. Coating(s) selection was based on the ability of the commercially available sorbent(s) to extract quantitative amount of all the analytes. Among the silica-base sorbents, reverse phase with mixed-mode strong ion-exchange properties proved superior for the extraction of all analytes within the range of polarities investigated. Clean screen DAU showed the highest efficiency, followed by $C_{18}$ and $C_8$ with benzenesulphonic ion exchange and SSBCX sorbents, which were of comparable extraction efficiencies. The polymer-based reverse phase mixed-mode sorbents with strong ion exchange properties also had higher extraction efficiencies compared to similar sorbents but with weak ion exchange properties. Generally, there were no significant differences in the extraction efficiencies of the silica-base mixed-mode reverse phase coatings

and their polymer-based counter parts. Clean screen gamma hydroxy butyric acid showed good affinity for compounds with lower pKa. The method limit of quantitation was 20 pg/mL. Inter-coating variation was ≤ 15 % and repeatability was ≤ 10 %.

# Chapter 4

# *In vivo* solid phase microextraction method for monitoring endogenous and exogenous chemical substances in the brain of freely moving rats

## 4.1 Preface and Introduction

### 4.1.1 Preface

Major portions of this chapter of the thesis is already published as an article under the title "Solid phase microextraction: A complementary *in vivo* sampling method to microdialysis" by Erasmus Cudjoe, Barbara Bojko, Inés Delannoy, Victor Saldivia & Janusz Pawliszyn. J., *Angwandte Chemie*. Vol. 52, 46, 12124 - 12126. Some of the tables and figures were reprinted from this publication with permission from Wiley VCH.

In this chapter of the thesis, the contributions from the author, Erasmus Cudjoe, are indicated as follows:

- Author developed and optimized SPME method for *in vivo* extractions of neurotransmitters and drugs from the rat brain
- Authored performed *in vivo* SPME and MD extraction experiments, which were carried out at NoAb BioDiscoveries® animal facility
- With the exception of a portion of MD samples, which were analyzed for 5-HT and DA at NoAb BioDiscoveries®, author carried out all LC-MS/MS analysis of

SPME and MD samples for neurotransmitters and drugs using the HPLC and TSQ Vantage® instruments. Subsequently analyzed all data.

- Author carried out LC-MS analysis of SPME and MD samples in a global metabolomics studies using the HPLC and (Orbitrap) Exactive® instruments. Subsequently participated in the statistical evaluation of metabolomics data.

## 4.2 Introduction

Effective brain tissue sampling is critical for clinical diagnosis and therapeutic treatment of neurological diseases. *In vivo* analysis of brain tissue compartments facilitates brain disposition studies aimed at understanding drug uptake in specific brain regions and also obtains evidence on the concentration of physiologically important endogenous compounds and their metabolites. Conventional brain tissue sampling methods, such as brain excisions, are labour intensive, and often result in the loss of vital chemical information.[1] Often the approach also requires the sacrifice of a large number of animals.[204] The advantages of continuous *in vivo* measurements in the same animal over time, therefore, cannot be overemphasized. Apart from providing a more comprehensive view of the dynamic biological system under study, *in vivo* measurements also avoid the complications associated with data interpretation typical of brain isolation studies and reduce variations resulting from the use of multiple animals. In view of this, analytical methods that offer an opportunity for *in vivo* measurements have been very much embraced for brain studies.

Microdialysis, since its early application in the early 1970s, has continued to receive extensive use for *in vivo* analysis, especially for the analysis of neurotransmitters in the

brain.[205] Although *in vivo* MD has had relatively poor temporal resolution compared to electrochemical methods due to lower flow rates for improved recoveries, it is still applied considerably for measurements of brain neurotransmitters. This may be due to that fact that sampling occurs over a continuum and it is possible to measure multiple neurotransmitters in a sample, and thus facilitates studies of potential neurotransmitter interactions.[204] Coupling of the method to other hyphenated analytical techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS) has significantly improved its applications to bioanalysis. *In vivo* MD is a microextraction sampling process, which is able to extract small amounts of the bulk analyte at any given time *via* a concentration gradient created between the biological matrix and the MD probe. This characteristic can be critical for *in vivo* sampling of brain neurotransmitters, as the issue with local depletion of the analyte(s) at any given time during sampling may be avoided. MD generally has been applied extensively to animal studies of brain neurotransmitters[206–215] and also in other forms of applications.[77,216–222] Details on the principles underlying *in vivo* MD method, calibration methods and technical considerations have previously been discussed in Chapter 1.

Another emerging *in vivo* microextraction method that has successfully been applied to drug bioanalysis in dogs,[223,224] rats,[172,225] mice and fish[226–228] is SPME. SPME coupled to LC-MS/MS has been successfully used in various pharmacokinetic (PK) studies in conscious animals.[225,229,230] Like MD, the SPME sampling process is driven by the concentration gradient of the analyte in the extraction phase and in the bulk matrix system. However, unlike MD that depends on the molecular weight cut-off of the pores in the dialysis membrane to screen analytes, the selectivity of SPME extraction is primarily dependent on the type of extraction phase used for the analyte enrichment process. Recent use of commercially available solid

phase extraction particles (mixed-mode phases) as new *in vivo* SPME biocompatible coatings has facilitated the ability to extract analytes of a wide range of chemical properties, giving improved compound coverage.[231] This may be attributed to the multiple interactions of the mixed-mode particles with various analytes. Thus, by utilizing the right mixed-mode coating, multiple endogenous neurotransmitters in a targeted analysis may be monitored by exposing an SPME fiber to the brain ECF of a specific region of interest. The new biocompatible SPME fiber for *in vivo* extractions also prevents extraction of proteins and other bio-interferences due to the coatings small pores and subsequently minimizes matrix effect significantly. Thus, *in vivo* SPME has the potential to provide enriched chemical information for tissue analysis when coupled to analytical techniques such as LC-MS/MS.

As mentioned earlier, SPME derives its selectivity from the type of extraction phase selected for the analysis. This allows the analyst the flexibility to skew the investigations to particular biologically hydrophilic/hydrophobic compounds. However, for global untargeted analysis of the metabolome the extraction phase must have relatively lower selectivity so as to extract simultaneously hydrophilic and hydrophobic endogenous compounds.

Herein, an in vivo SPME and MD coupled to LC-MS/MS have been used to study the chemical components of the brain extracellular fluid in freely moving rat. Owing to their characteristic ability to interact with both hydrophobic and hydrophilic compounds, the mixed-mode *in vivo* fibers was used. The present study aims to provide an alternative *in vivo* microextraction method capitalizing on the selectivity and sensitivity of LC-MS for a targeted and untargeted analysis of the brain extracellular fluid, and also in a demonstrate the ability to use SPME to monitor exogenous drug concentration profile in the brain.

The targeted analysis focused on monitoring multiple neurotransmitters (GABA, GA, DA and 5-HT) with varying polarities in the rat brain ECF. Basal concentrations of these neurotransmitters were measured in the striatum and compared to concentrations measured following a single intra-peritoneal (*i.p.*) injection of vehicle (fluoxetine drug). The results obtained for 5-HT and DA were compared to those obtained from a microdialysis probe implanted in the striatum in the opposite brain hemisphere, sampling concurrently.

The untargeted chemical analysis focused using simultaneously SPME and MD for improved metabolites coverage. SPME and MD typically have stronger affinity for hydrophobic and hydrophilic chemical substances respectively. Thus, by combining these two methods, it is expected that compounds with wider range of polarities will be extracted and thus improve metabolites coverage for potential biomarker discovery.

Finally, till date no true quantitative measurements of drugs in the brain ECF have been carried out using SPME. Therefore, as part of the objectives, *in vivo* SPME method was used to determine the unbound concentration of exogenous drugs (carbamazepine and cimetidine) in discrete regions of brain. *In vivo* MD was used concurrently to validate the results obtained by SPME.

## 4.3 Experimental section

### 4.3.1 Reagents and Materials

Chromatographic solvents (Optima® grade acetonitrile, water and formic acid) were obtained from Fisher Scientific, (Ontario, Canada). GA, dopamine hydrogen chloride, GABA,

5-HT, carbamazepine (CBZ), carbamazepine-$d_{10}$, and cimetidine were obtained from Supelco (Bellefonte, PA. U.S.A.). Stock standard solutions were all prepared in a final solution of acetonitrile/water/formic acid in amber vials and kept refrigerated for a maximum of two weeks and then discarded. Artificial cerebrospinal fluid (aCSF) was purchased from Harvard Apparatus, (Holliston, MA, U.S.A.). Diazepam and lorazepam standards for SPME experiments was obtained in the form of 1 mg/mL methanolic solution and purchased from Cerilliant (Round Rock, TX, U.S.A). Prototype biocompatible *in vivo* SPME mixed-mode fibers ($C_{18}$ with benzenesulphonic acid group) and $C_{18}$ fibers were obtained from Supelco (Bellefonte, PA, U.S.A.). The biocompatible fiber coating thickness was 45 µm and the length was 7 mm. For the targeted analysis study, the coating length was reduced to 4 mm using a special precision tool manufactured at the machine shop at the University of Waterloo. This was necessary to minimize variability in coating length. Ultra-pure deionized water was obtained from a Barnstead/Thermolyne NANO-pure water system (Dubuque, IA, U.S.A.). Guide cannulae and microdialysis probes (CMA-12; 4 mm) were obtained from CMA Microdialysis® (Stockholm, Sweden).

### 4.3.2 Targeted Analysis

#### *4.3.2.1 HPLC and mass spectrometry conditions for SPME sample analysis*

HPLC and mass spectrometry analyses were performed using Thermo Scientific Accela™ and TSQ Vantage™ instruments, respectively. Chromatographic separation of the neurotransmitters (GABA, GA, DA and 5-HT) was possible using a Phenomenex® pentafluorophenyl (PFP) kinetex core shell column (2.6 µm, 2.1 x 150 mm) within a 5 min

run-time. The mobile phase flow rate was maintained at 450 μL/min using gradient elution program. Mobile phase A contained 90% water and 10% acetonitrile while mobile phase B consisted of 90% acetonitrile and 10% water. Both mobile phases contained 0.1% formic acid to enhance ionization in the ion source. The Accela™ autosampler from Thermo Scientific was used to introduce a 10 μL-sample into the HPLC system coupled to the TSQ Vantage™ triple quadrupole mass spectrometer. MS conditions were as follows: sheath and auxiliary gases were 55 and 15 psi, respectively. The spray voltage was set at 1000 V, while the capillary and vapourizer temperatures were set at 250 $^{0}$C. All ions were monitored in the positive ionization mode. The mass ion transitions monitored were 104.1→69.1, 148.1→84.1, 154.1→91.2 and 177.1→115.1 for GABA, GA, DA and 5-HT, respectively. The mass ion transition for diazepam used as an internal standard 285.1→193.1 All data analyses were performed with the Xcalibur® software version 2.0.7.

#### *4.3.2.2 HPLC-electrochemical detection conditions for microdialysis samples*

DA and 5-HT in dialysate samples were analyzed using a high-performance liquid chromatography method with an electrochemical detector system (EiCOM HTEC-700). DA and 5-HT were separated using an EiCOM PP-ODS column (4.6 x 30 mm) at 25°C with a mobile phase containing 0.1 M sodium phosphate buffer (pH 6.0), 500 mg/L of sodium decanesulfonate, 50 mg/L of EDTA disodium salt and 1% methanol at a flow rate of 0.5 mL/min. A graphite electrode maintained at +450 mV relative to the Ag/AgCl reference electrode was used for the detection. The retention time for DA and 5-HT was 2.0 min and 5.0 min respectively, and the total run time was 5.5 min per injection. Calibration standards

ranging from 10 to 1000 pg/mL were used for quantitative determination of DA and 5-HT in each dialysate sample. The calibration curve for each analyte based on peak height was generated using GraphPad Prism™ software and utilized for calculating the concentration of DA and 5-HT in dialysate sample.

### 4.3.3 Non-Targeted Analysis

#### *4.3.3.1 HPLC and mass spectrometry conditions for in vivo sample analysis*

The Accela™ autosampler, HPLC system and Orbitrap mass spectrometer (Exactive™) were obtained from Thermo Fisher Scientific and used for the analysis. A typical reverse phase chromatographic separation was carried out in positive mode only using a PFP (Discovery HS F5; 2.1 mm × 100 mm; 3 µm) from Supelco™. The total run time was 40 min at a constant flow rate of 300 µL/min. Mobile phase A consisted of water/formic acid (99.9/0.1, v/v) and mobile phase B was acetonitrile/formic acid (99.9/0.1, v/v). Due to the fact that the PFP column could handle 100 % aqueous conditions, the initial condition of 100 % A was held for 3 min followed by a linear gradient to 90 % B to 25 min. An isocratic condition maintained for further 9 min and then finally the column was re-equilibrated for the next 6 min. The volume of sample injected was 10 µL. Mass spectrometer conditions: The AGC was maintained at 100000 ions and the injection time into the C-trap was 100 ms. Sheath gas and auxiliary gas were set at 40 and 25 (arbitrary units) respectively. The ionization voltage, capillary voltage, tube lens voltage and capillary temperature were respectively 4.0 kV, 27.5 V, 100 V and 275 $^{0}$C.

Data processing was performed with the SIEVE® software version 1.2.0 (Thermo Scientific). A total of 20,000 features were generated with a 0.005 mass window and the

minimum signal intensity was set at 5,000. The first minute of the chromatographic run time was considered as the void volume of the column while the last 5.0 min was for re-equilibration purposes, and therefore were omitted in the data processing. All the 20,000 frames generated were manually and singly evaluated to exclude all frames that were not true chromatographic peaks. In addition, also peaks found at the same level in the blank samples were exempted. In terms of compound identification, putative identification is based on accurate mass (5 ppm mass window). Subsequent to the determination of the accurate mass, data was compared with an open access database (Human Metabolomics Database). Generally, the data generated (accurate masses), is compared with a list of accurate masses of various compounds and adducts are provided from the database with particular attention to the possible adducts that can form based on the LC mobile phase.

### 4.3.4 Measurements of unbound drug concentrations in extracellular fluid of the rat brain

#### *4.3.4.1 HPLC and mass spectrometry conditions*

Two types of drugs (carbamazepine and cimetidine) were considered for this project. Chromatographic separation of carbamazepine was achieved on the Symmetry Shield™ reverse phase $C_{18}$ column from Waters Corporation. Mobile phase A consists of 90% deionized water and 10% acetonitrile and mobile phase B had 10% deionized water with 90% acetonitrile. Both mobile phases contained 0.1% formic acid for enhanced ionization in the ESI ionization source. The chromatographic separation was attained in 5 min at a constant flow rate of 500 µL/min. The separation started with 100% of mobile phase A, held for 0.5 min and the gradually

decreased to 10%. This was maintained for another 0.5 min and then immediately increased to the initial conditions till 5 min. The Thermo Scientific® autosampler, HPLC system and tandem MS were used for the analysis. The parent/daughter ion masses monitored for lorazepam, CBZ and CBZ-$d_{10}$ were respectively 321.0/275.1, 237.1/194.1 and 247.1/204.2. The MS conditions were as follows: sheath and auxiliary gases were 50 and 25 arbitrary units, respectively. The spray voltage was set at 2200 V, while the capillary and vapourizer temperatures were both set at 300 $^0$C. All ions were monitored in the positive mode. CBZ-$d_{10}$ was used as an internal standard whereas lorazepam was used to monitor any injections errors.

In the case of cimetidine, the Kinetex ™ PFP column (50 mm × 2.1 mm; 2.6 μm) from Phenomenex® was used for chromatographic separation. Mobile phase A consisted of 90 % aqueous and 10 % acetonitrile while mobile phase B contained 60 % acetonitrile, 30 % methanol and 10 % aqueous. Both mobile phases were spiked with formic acid to make a final concentration of 0.1 %. The chromatographic separation was attained within 5 min at a flow rate of 500 μL/min in a gradient elution. The chromatographic separation method started with 90 % of mobile phase A and was held constant for 1 min. Mobile phase B was gradually increased to 100 % by 3 min, held for 0.5 min and immediately decreased to the starting condition for column equilibration. The mass ion transition for cimetidine was 253.2 to 159.1 in a positive ionization mode. The MS conditions were as follows: sheath and auxilliary gases were 55 and 25 arbitrary units, respectively. The spray voltage was set at 2500 V, while the capillary and vapourizer temperatures were 320 $^0$C and 300 $^0$C, respectively. Lorazepam was used as an internal standard.

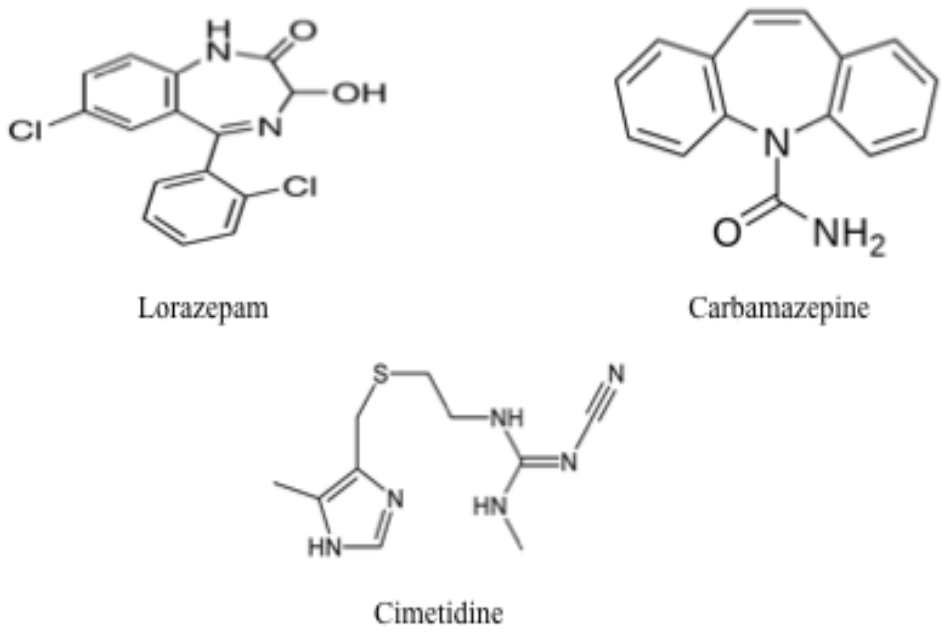


Figure 4.1 Chemical structures of lorazepam, carbamazepine and cimetidine

### 4.3.5 Sampling Procedure

#### *4.3.5.1 In vivo brain sampling for targeted and non-targeted analysis of endogenous chemical substances*

In these experiments MD and SPME were used to sample the brain tissue simultaneously. Their respective probes were placed in the opposing striatum of the two hemispheres of the brain. Male Sprague Dawley rats (Charles River Labs, St. Constant, QC) weighing 250 – 300 g were used. The rats were kept in the vivarium at a certified and qualified animal facility, NoAb BioDiscoveries®, maintained on a 12 h light-dark cycle with free access to food and water, and allowed to acclimatize for at least 5 days prior to surgery. Two guide cannulae were surgically implanted into the striatum (co-ordinates were AP (0.20 mm), DL (±

2.8 mm) and DV (-3.6 mm)) of the left and right hemispheres of the brain at least 2 days prior to the *in vivo* experiments.

#### *4.3.5.2 In vivo microdialysis sampling from the striatum of rat brain*

A 4 mm microdialysis probe with a molecular weight cut-off of 6 kDa was inserted into one hemisphere of the rat brain a day prior to the experiment. Immediately the probe was perfused overnight at a flow rate of 0.2 µL/min with aCSF, which was supplemented with freshly prepared 250 µM ascorbic acid. At least 1.5 hours prior to sampling, the flow rate was adjusted to 1 µL/min and the system was allowed to equilibrate. The rats were kept in a Raturn™ (BASi®), an automated sampling system where they were allowed to freely move throughout the study having access to food and water, except for SPME sampling times points when the probes were inserted and replaced. After the MD system has equilibrated, the dialysate was collected at 30 min intervals for a 3-hour period to determine baseline concentrations 5-HT and DA. Subsequently, a single dose of 10 mg/kg fluoxetine, which was prepared by dissolving fluoxetine in saline with the pH adjusted to 3.5 using 0.1 N HCl or a vehicle control was administered *i.p.* Dialysate samples were collected at 30 min intervals over another 3-hour period. In order to prevent any possible degradation of the neurotransmitters through oxidation reactions, samples were collected into vials already containing a 30-µL solution of 20 mM phosphate buffer (pH 3.5) and 25 mM disodium ethylenediamminetetraacetic acid (EDTA-Na). The MD samples were divided into two portions and a portion was analyzed by liquid chromatography coupled with electrochemical detection for 5-HT and DA at NoAb BioDiscoveries® Inc. The second portion of the MD samples was analyzed at the University of Waterloo for GA and GABA. MD samples transported to the university laboratory were

each diluted 6-fold using the desorption solution. Samples were vortexed for about 30 sec and then later analyzed by LC-MS/MS.

#### *4.3.5.3 In vivo solid phase microextraction from the striatum of rat brain*

With respect to the *in vivo* SPME sampling experiments, mixed-mode particles immobilized on a stainless steel fine wire by means of a biocompatible adhesive material was used as the extraction phase. The extraction phase (4 mm) was selected after initial thorough assessment of its applicability to measuring multiple neurotransmitters. The SPME probes were designed such that after insertion, only the extraction phase/coating was exposed at the end of the guide cannula similar to the MD probe. The same sampling time was used for both MD and SPME, except that a new probe was inserted into the opposing hemisphere at the beginning of each 30 min sampling. During the insertion and removal of each SPME probe, the rat was physically restricted from moving. After the extraction, the probe was removed from the brain, wiped clean with a tissue, briefly (~ 2 sec) exposed to deionized water and immediately placed inside an insert containing 60 µL desorption solution (3:2 water-acetonitrile mixture with 0.1 % formic acid) at an approximate pH of 3.5. Each insert was stored in a 2 mL amber vial, capped and immediately placed on dry ice inside a cooler until further LC-MS/MS analysis. All samples were transported to the laboratory and the *in vivo* probes were later desorbed for 60 min on a vortexer at 750 rpm. After the desorption process, the extracted samples were analyzed for 5-HT, DA, GA and GABA.

#### *4.3.5.3.1 In vivo brain sampling for non-targeted analysis of endogenous compounds*

For the non-targeted (global) analysis, all the *in vivo* MD and SPME samples were diluted a 100-fold in 3:2 deionized water/acetonitrile with 0.1% formic acid and then vortexed for about a minute. A quality control sample was generated for SPME and MD respectively by taking 30 µL of each individual fraction of all the *in vivo* samples collected. In this analysis, desorption solution transferred into 300 µL inserts was used as blank samples. Figure 4.2 shows a typical *in vivo* sampling of the rat brain using both MD and SPME probes.

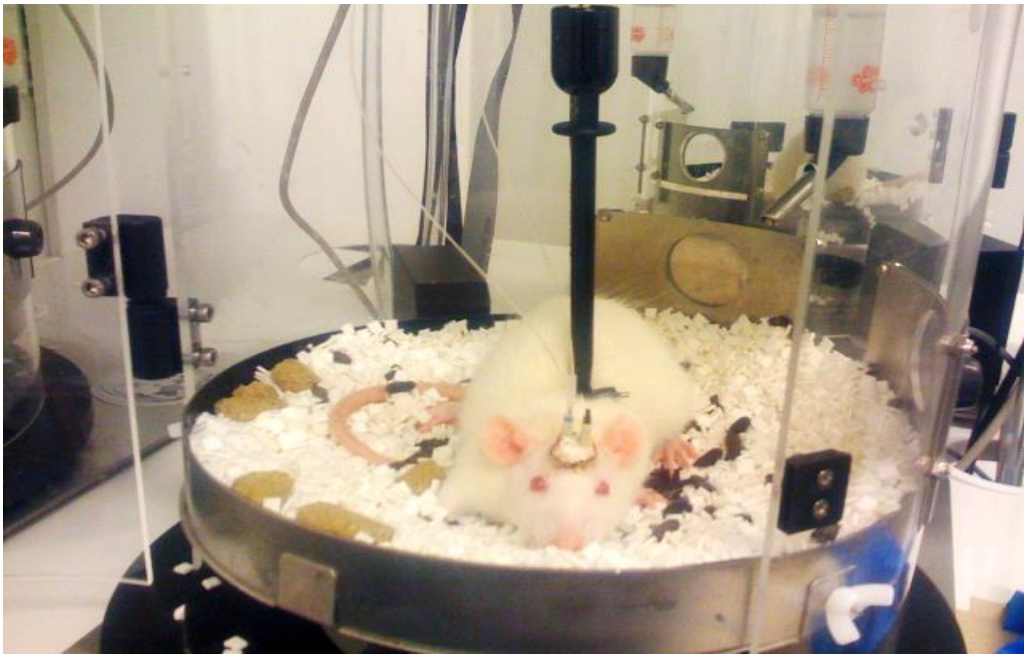

Figure 4.2 Simultaneous *in vivo* sampling of the left and right brain extracellular fluids of a Sprague Dawley rat using SPME and MD. The *in vivo* biocompatible SPME probe is to the right in the picture without any connecting tubes whereas MD tube is to the left showing connecting tubes to an external pumping device delivering constant flow perfusate.

#### *4.3.5.4 Extraction of unbound exogenous drugs from the rat brain*

Two CMA-12 guide cannulae were surgically implanted into the right and left striatum and frontal cortex of the rat brain hemisphere (coordinates: 0.2 mm anteroposterior, ±3.0 mm lateral and 1.0 mm dorsoventral relative to the bregma) respectively. The femoral vein and artery were catheterized. The rats were allowed to recover for at least two days after surgery. A day prior to the study, a microdialysis probe (2 mm) was placed into the frontal cortex and perfused with aCSF at 0.2μl/min overnight. The flow rate of the perfusate was later adjusted to 0.5 μl/min and allowed to equilibrate for at least an hour. The rat was then given an *i.v.* bolus (1.5 mg/kg for Carbamazepine or 13 mg/kg for Cimetidine) and subsequently an *i.v.* infusion (1 mg/kg/hr for Carbamazepine or 24 mg/kg/hr Cimetidine) for 5.5 hr (steady state). The *i.v.* infusion was later discontinued (dynamic state) and samples were collected for another 1.5 hours. Blood samples (150 μL) were withdrawn from the femoral artery cannula also collected at 30 min. The collected blood samples were centrifuged immediately and the resultant plasma was stored in the freezer at -80 °C. All the samples were stored at -80 °C until being assayed. MD dialysates were collected every 30 min in both steady and dynamic states. For the plasma samples, 15 μL of the plasma samples were carefully pipetted in a small centrifuge tube. Approximately, 85 μL of acetonitrile were added, centrifuged for 5 min and then finally 5 μL of the supernatant was pipetted and 55 μL of the desorption solution (80 % acetonitrile and 20 % deionized water containing 10 ng/mL lorazepam) was added. In the case of MD, 5 μL of the dialysate were pipetted and later diluted 10x with the desorption solution.

In the case of SPME sampling, the extraction phase was prepared from $C_{18}$ particles and was obtained from Supelco®. The length of the extraction phase was 7 mm. However, this

was later modified by carefully removing the middle portion (3 mm) of the extraction phase to obtain a segmented fiber. The final length of each segmented coating was 2 mm. *In vivo* SPME sampling was carried out by strategically inserting the probes in the frontal cortex and striatum of the rat brain. Each probe was exposed to the brain tissue for 10 min, carefully removed, wiped with Kimwipe® and then placed in a 100 μL desorption solution (80 % acetonitrile and 20 % deionized water containing 10 ng/mL lorazepam). A new SPME probe was introduced at 30 min interval into the brain few minutes prior to the collection of the MD dialysates.

### 4.3.6 SPME Calibration Procedure

#### *4.3.6.1 Calibration procedure for targeted endogenous compounds*

Working stock solutions were prepared in the desorption solution in 2 mL amber vials and kept refrigerated for a maximum of two weeks. Calibration standards for the instrument were freshly prepared by serial dilution of the 1 μg/mL stock solution to cover a concentration range of 0.006 – 200 ng/mL.

For the neurotransmitters, external calibration standards were prepared by extracting known concentrations (0.01 – 200 ng/mL) of the analytes spiked in aCSF while maintaining the total amount of organic content in each standard to be ≤ 0.5 %. Each calibration standard in aCSF was prepared in triplicate and extraction was carried out under static conditions to mimic as closely as possible the *in vivo* sampling procedure. A 1.8 mL volume of each calibration standard was extracted for 30 min in 2 mL amber vials and the analytes were later desorbed from the fibers in 60 μL desorption solution for 1 hour at 250 rpm. Calibration curves of the amount extracted *versus* nominal concentration were used to quantify each analyte.

Thus, an external equilibrium method was used for quantitative analysis of the neurotransmitters.

However, for quantitative analysis of CBZ and cimetidine, a pre-equilibrium kinetic calibration method was used. The fiber was pre-loaded with a known concentration of the deuterated analogue before the sampling commenced. Therefore, during sampling the deuterated desorbed while the analytes are extracted simultaneously from the brain extracellular fluid.

Prior to *in vivo* sampling, *in vitro* experiments utilizing spiked aCSF solutions were performed to pre-screen the fibers by comparing and grouping those with similar extraction efficiencies for DA and 5-HT.

#### *4.3.6.2 Developing an in vitro SPME external calibration method*

Two of the most commonly used SPME calibration methods are pre-equilibrium on-fiber kinetic and external equilibrium calibration methods. Briefly, pre-equilibrium calibration requires a calibrant for the analyte to be previously loaded onto the fiber. By means of a concentration gradient, the calibrant will be simultaneously desorbed from the coating into the matrix during extraction process while the opposite process occurs for the analyte. Details of calibration method have been mentioned in previous chapter. The major concern for the pre-equilibrium calibration method is that smaller amounts of the analyte will be extracted and therefore further detection can be very challenging depending on the type of coating and analyte used. The technique, however, has been applied successfully for other studies

elsewhere.[227] To improve sensitivity and detection, the external equilibrium calibration method was used.

For the external equilibrium calibration method, the initial concentration of each analyte is directly related to the amount extracted at equilibrium. Equation 4.1 shows the relationship between the original concentration ($C_0$) of the analyte that will be in the brain ECF, volume of extracellular fluid ($V_s$), fiber constant ($f_c$) and amount extracted at equilibrium ($n_e$) of the extracted analyte.

$$C_0 = \left(\frac{V_s + f_c}{V_s f_c}\right) n_e \qquad (4.1)$$

Under conditions of negligible depletion of the analytes from the matrix, in which case the fiber constant is far lower than the volume of brain ECF, the equation can be simplified as follows:

$$C_0 = f_c n_e \qquad (4.2)$$

From equation 4.2, the amount extracted at equilibrium ($n_e$) will be directly dependent on the original concentration of the analyte within the brain ECF. By using the appropriate matrix for external calibration standards the original concentration can be easily determined from the slope of the regression line, which will be equal to the fiber constant. In this study, all calibration standards were generated by extracting, under static conditions, known concentrations of the neurotransmitters spiked in aCSF.

Prior to obtaining the calibration standards, the effect of tortuosity of the brain tissue on the diffusive property of each analyte was investigated using an aCSF-agar gel matrix. This is because the diffusion-based extraction process conforms to Fick's diffusion law and, therefore, the path length for diffusion of the analytes in the brain tissue to the coating may significantly influence the equilibrium time as a result of the tortuosity, among other factors. Subsequently, the equilibration time for each analyte in aCSF under static conditions with 1% aCSF-agar gel matrices was determined and compared. Results obtained from the extractions show that the equilibrium times where similar and ≤ 20 min for all of the analytes in both matrices.

The mathematical representation of the extraction process is controlled by Equation 4.3:

$$\frac{n}{n_e} = 1 - e^{-at} \qquad (4.3)$$

where *n* is the amount of neurotransmitter extracted by the coating at a specific time *t* and *a* is the time constant, which is a measure of the rate of diffusion of the analyte in the coating. Therefore, similar time constants for each analyte in the brain tissue, aCSF and 1 % aCSF-agar gel matrices, implies that the extraction rate will not be significantly influenced by the tortuosity of the brain tissue. To determine the time constant, 100 ng/mL samples were separately prepared in aCSF and 1 % aCSF-agar gel and the analytes were extracted for 10 min from 3 replicate samples without agitation (static condition) under controlled temperatures (35 – 37 $^0$C). In order to avoid any possible inter-fiber variability a single fiber was used for each replicate, while ensuring that the fiber was appropriately pre-conditioned in a 1:1 (v/v)

methanol-water solution and then in water, before each extraction. Although, the current approach does not take into consideration the influence of other matrix components such as analyte re-uptake in a living system, it is a very good alternative approach to measurements of neurotransmitters in the brain.

#### *4.3.6.3 Developing an in vitro SPME pre-equilibrium on-fiber kinetic calibration method*

As previously indicated, a pre-equilibrium on-fiber calibration method was used for quantitation of the exogenous drugs in the brain ECF. Prior to the *in vivo* sampling, kinetics of the diffusion of the drugs in an agar gel was determined as shown in Figure 4.3 3 replicate extractions. The experiment was carried out at about 37 $^{0}$C on a hotplate. From the extraction profile, even at 180 min CBZ has not reached equilibrium yet, as a result the pre-equilibrium approach was adopted. The 10 min extraction time was chosen because quantitative amounts of the analyte could be extracted and analyzed by LC-MS/MS.

Thus, each SPME fiber was pre-loaded with CBZ-$d_{10}$ from a spiked solution of aCSF containing 100 ng/mL of the deuterated analogue. An overnight extraction with agitation was carried out on a mechanical shaker set at 250 rpm.

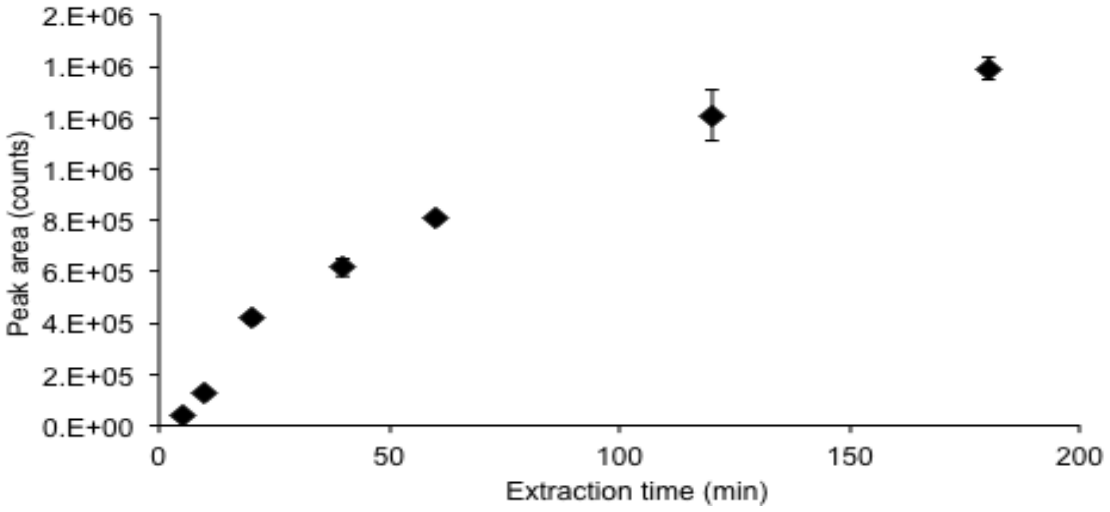


Figure 4.3 Extraction time profile for carbamazepine in a 1 % aCSF gel matrix (n=3). The results are expressed as the mean ± standard errors for 3 rats.

### 4.3.7 Results and Discussions

#### *4.3.7.1 Quantitative measurements of targeted endogenous compounds*

##### *4.3.7.1.1 In vitro external equilibrium SPME method for targeted analysis*

An external equilibrium calibration method requires that the *in vitro* conditions replicate to a greater extent the *in vivo* conditions within the living system. To this effect, the equilibrium times of each analyte in aCSF and 1 % aCSF-gel mixture were determined. Both experiments were carried out on a hotplate at a preset temperature (37 $^{0}$C) and the extractions from aCSF were carried out under static conditions. The observed equilibration times for the analytes in both systems were similar (≤ 20 min). This indicated that the kinetics of diffusion under both conditions was similar and thus the volume of brain ECF may not a rate limiting

step for the amount of analytes extracted. In order to ascertain the above observation, the time constant or rate constant (a), as shown in Equation 4.3 was determined for each of the analyte in both systems. Separate 10 min extractions in triplicates were carried out using spiked samples of aCSF only and aCSF-gel mixtures containing 100 ng/mL of each analyte. Results are shown in Table 4.1.

Table 4.1 Extraction rate constant of selected neurotransmitters in aCSF and aCSF-gel mixture

| **Compounds** | **Time constant (a) $min^{-1}$** | | **Standard error (n=3)** | |
|---|---|---|---|---|
| | aCSF | aCSF-gel | aCSF | aCSF-gel |
| Serotonin | 0.0014 | 0.0012 | 0.0003 | 0.0004 |
| Dopamine | 0.0011 | 0.0099 | 0.0002 | 0.0003 |
| Glutamic acid | 0.0021 | 0.0018 | 0.0002 | 0.0004 |
| γ-amino butyric acid | 0.0009 | 0.0085 | 0.0003 | 0.0004 |

The calculated time constants for each analyte from the aCSF and the aCSF-gel matrix were not statistically different. In this regard, it would be logical to conclude that the rate-limiting step of the absorption process will not be dependent on the tortuosity of the brain tissue in the absence of all other matrix influence. Rather the amount of analyte extracted onto the fiber will be dependent on the analyte's concentration in the brain ECF within the immediate vicinity of the fiber. This implies that calibration standards obtained from aCSF in a static mode could be used for quantitative measurements of the concentration of neurotransmitters in the brain ECF. It is worthwhile mentioning that possible matrix

interactions with the analytes within the brain ECF were not accounted for in the *in vitro* gel experiments.

#### *4.3.7.1.2 Effect of matrix on the amount extracted*

The influence of the matrix on the analytical method is very critical to obtaining reliable results when quantifying target analytes in complex biological matrices. Most analytical methods thus use internal standardization or a standard addition method to compensate for the effect of the matrix for quantification. For *in vivo* SPME involving analysis of exogenous molecules, using an appropriate internal standard, such as a deuterated analogue of the analyte, pre-loaded onto the fiber often compensates for the effect of the matrix. However, the use of an internal standard is not always possible for measuring endogenous compounds due to the non-availability of an appropriate deuterated analog or due to the significant expense. An alternative external approach would be to evaluate the effect of the matrix on the amount extracted using the same sample matrix, if possible. This would make it possible to determine the relative recovery in order to compensate for the influence of the matrix on analyte extraction. In this study, no internal standard was used for quantitation of neurotransmitters in the brain ECF. Therefore, the effect of the matrix was evaluated using homogenized brains, obtained from naive rats.

Five replicate extractions of the analytes from brain homogenate samples were performed using the optimized SPME method conditions determined above and then the analytes were subsequently desorbed from the fibers for LC-MS/MS analysis. Similar experiments were performed with blank solutions of aCSF. Both desorption solutions from the

brain tissue homogenate and aCSF extracts were later spiked with 50 ng/mL 5-HT and the samples were analyzed by LC-MS/MS. The peak area ratio (analyte/diazepam) obtained for each extract was compared to that obtained from a 50 ng/mL neat standard solution. By this approach, it is possible to ascertain the effect of the matrix and fiber coating on the recovery of the analytes.

The area ratio obtained for all brain homogenates and aCSF extracts were comparable as there were no statistically significant differences in the percent relative standard deviation (RSD %), which ranged from 4 - 12 % for 5 replicate extractions (Figure 4.4).

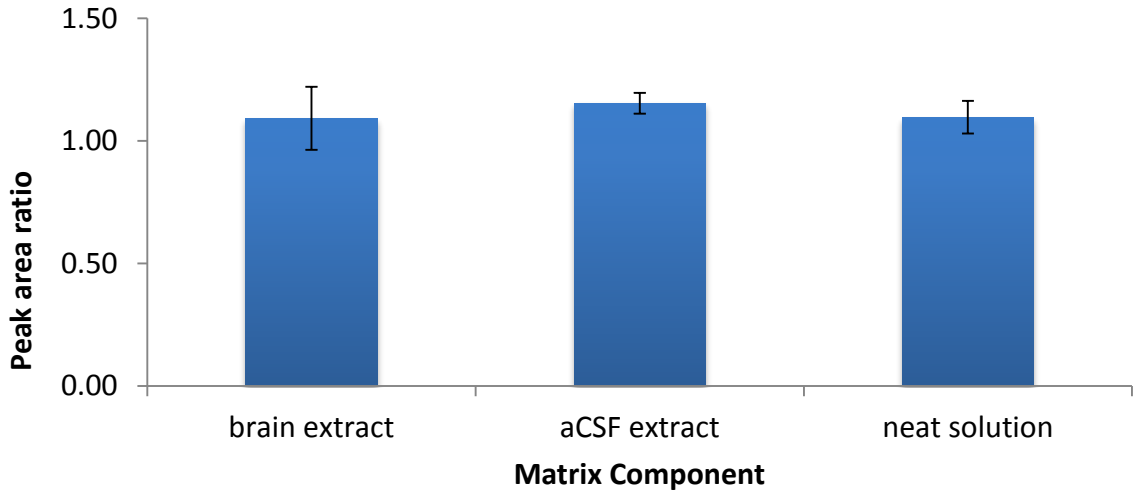


Figure 4.4 Evaluation of the effect of brain tissue matrix on the SPME extraction method (n=5 replicate determinations.). The results are expressed as the mean ± standard errors for 5 rats.

Since the analytes are endogenous and, therefore, it is impossible to obtain blank brain tissue samples, the basal concentration of the serotonin already present will contribute to the

analytical signal determined for the brain tissue sample. However, the concentrations of 5-HT typically found present in the homogenized rat brain were very low (pg/mL), therefore, the latter contribution to the overall analytical signal compared to the 50 ng/mL 5-HT added were negligible.

#### *4.3.7.1.3 In vivo monitoring of dopamine and serotonin by SPME and MD*

To facilitate the comparison of data obtained by SPME and MD, the concentrations of the neurotransmitters following fluoxetine or vehicle control administration were expressed as percentages of the average basal (pre-dose) concentration in the brain ECF. This was, in part, necessary as the dialysate samples were analyzed at NoAb BioDiscoveries, whereas the SPME samples were analyzed at the University of Waterloo.

#### *4.3.7.1.4 Monitoring serotonin and dopamine in both brain hemispheres*

Concentrations of 5-HT and DA were measured over a 2 hour period to determine the mean basal concentrations. Subsequently, a single *i.p*. injection of vehicle control or 10 mg/kg fluoxetine was administered and the neurotransmitter concentrations were measured over 4 hours by MD and SPME.

The average results, as shown in Figure 4.5, indicate a sustained increase in the concentration of 5-HT, which may be attributed to the inhibition of the serotonin re-uptake transporter in both brain hemispheres over the 4-hour period post-fluoxetine administration. The increase in the concentration of 5-HT was consistent with results obtained in the

literature,[232] which demonstrate that systemic administration of fluoxetine elevates extracellular 5-HT concentrations significantly by selectively inhibiting its re-uptake.

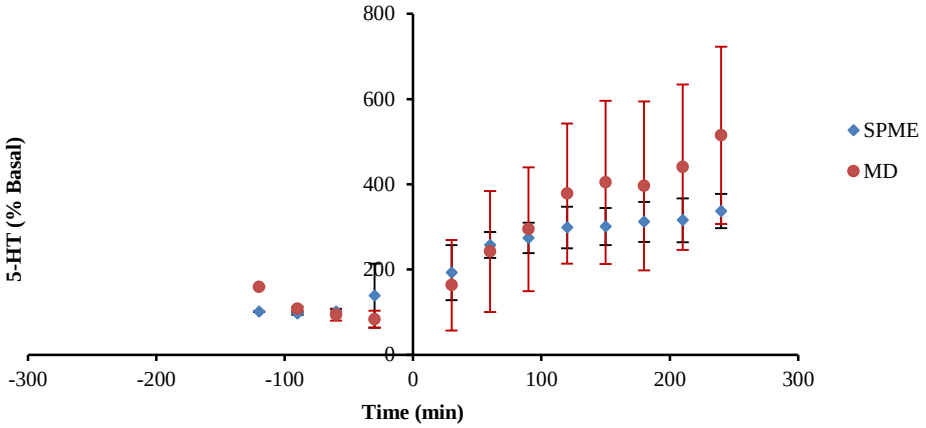


Figure 4.5 Percent changes in serotonin concentrations (relative to the mean basal concentration) in the rat brain extracellular fluid determined by SPME and microdialysis (MD) methods following an i.p. injection of fluoxetine (10 mg/kg) at time t = 0. The results are expressed as the mean ± standard errors for 12 rats.

Both SPME and MD exhibited similar increases in 5-HT concentrations following fluoxetine administration. Statistical analysis (paired t-test at 95% CI assuming unequal variance) of data obtained by both methods, showed no significant difference in the data for the average of each time point. The concentrations of 5-HT determined by SPME appeared to be less variable than those measured by MD. The increase in 5-HT corroborates results obtained in a fluoxetine-induced studies reported elsewhere in literature.[24]

As expected, DA concentrations (Figure 4.6) did not exhibit any significant changes in the ECF after a single *i.p.* injection of fluoxetine, when determined by MD and the SPME

methods. These results confirm that fluoxetine inhibits the re-uptake of 5-HT and not of DA. In addition, the effectiveness of SPME as a method for the *in vivo* monitoring of neurotransmitters is demonstrated relative to that of MD.

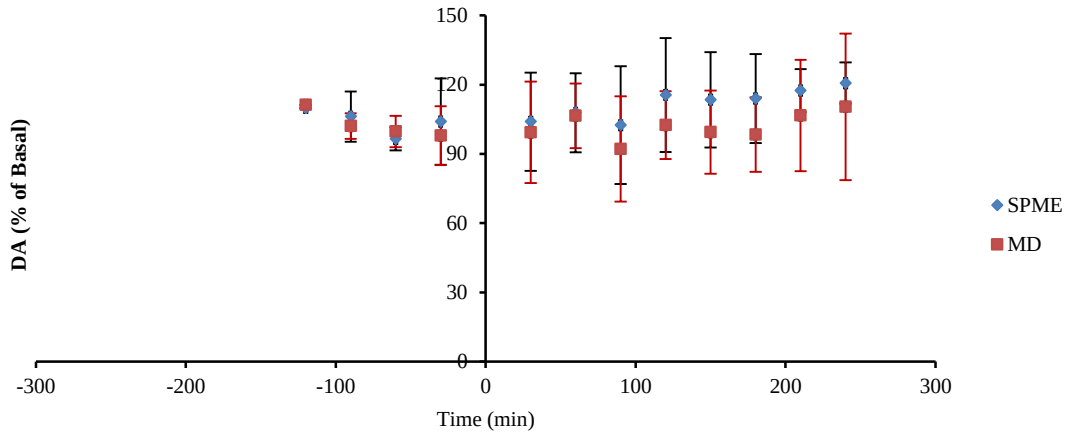


Figure 4.6 Percent changes in dopamine concentrations (relative to the mean basal concentration) in the rat brain extracellular fluid determined by SPME and microdialysis (MD) methods following an i.p. injection of fluoxetine (10 mg/kg) at time t = 0. The results are expressed as the mean ± standard errors for 12 rats.

Results obtained for monitoring 5-HT and DA in the ECF of the rat brain for the various sets of experiments are presented in Figure 4.7. With the exception of Figure 4.7 (a), which showed relatively higher concentrations of 5-HT after the administration of fluoxetine drug, results obtained for the other plots showed very similar concentration patterns for both DA and 5-HT. However, a paired t-test analysis of the results by MD and SPME revealed that there were no significant differences for the mean concentrations of 5-HT at any particular time point as recorded by both methods.

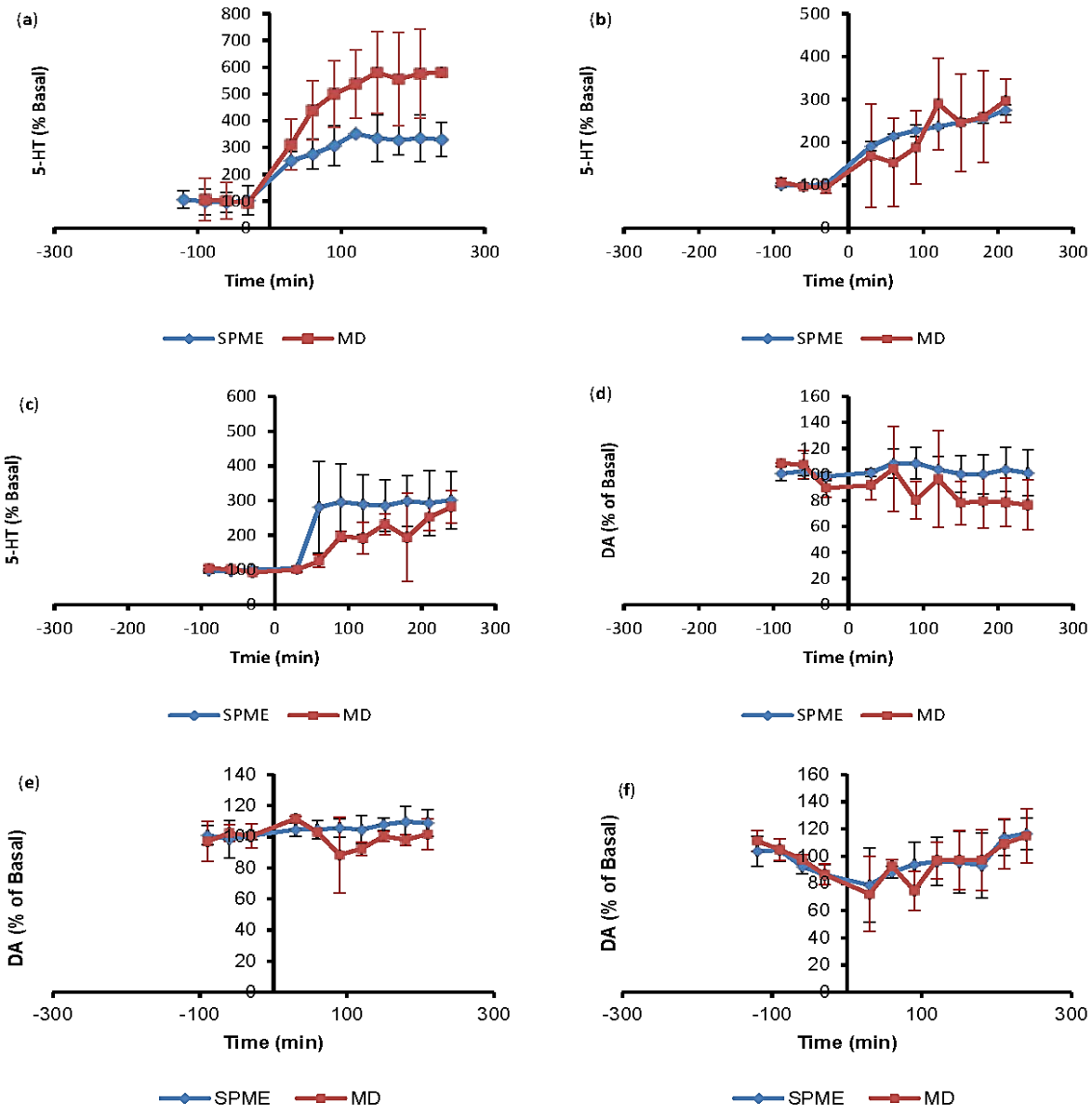


Figure 4.7 Results for *in vivo* SPME and MD study of the effect of single dose fluoxetine on selected neurotransmitters. Changes in serotonin (**a - c**) and dopamine (**d - e**) concentrations (relative to the mean basal concentration) in the rat brain extracellular fluid determined by SPME and MD methods following an *i.p.* injection of fluoxetine (10 mg/kg) at time t = 0. The results are expressed as the mean ± standard error for 3 rats (Figure 4.7a – f).

As depicted in Figure 4.8, a sharp increase (~ 4x increase relative to % basal) in the concentration of 5-HT in rat SPME-R04 as recorded by SPME compared to MD, which showed a gradual increasing response. This observation was attributed to a severe pain inflicted on the tail of the animal at the 30 min point sampling. Evidence of monoamine neurotransmitters, serotonin and norepinephrine, associated with pain has been reported elsewhere in literature.[233,234] This observation supports the notion that SPME is able to capture rapid changes in analyte concentration that can occur within the brain extracellular fluid. Similar observation was reported elsewhere by using SPME coating coupled to LC tandem mass spectrometry to capture elusive metabolites in metabolomics studies.[116] Microdialysis on the other showed a gradual increase in 5-HT with time. The relative gradual increase in 5-HT may be due to the slower response time of MD, which may be due to interference from hydrophobic components that often bind to the surface of the MD probe; a phenomenon of MD known to slow down diffusion of analytes through the membrane into the dialysate. It is important however to note that the sharp increase in 5-HT observed with SPME data phenomenon was not be fully substantiated due to the lack of adequate data to support the phenomenon irrespective of its corroboration with other reported cases in literature. In this regard, the result was treated as an outlier and was not used in determination of the average concentration of each analyte.

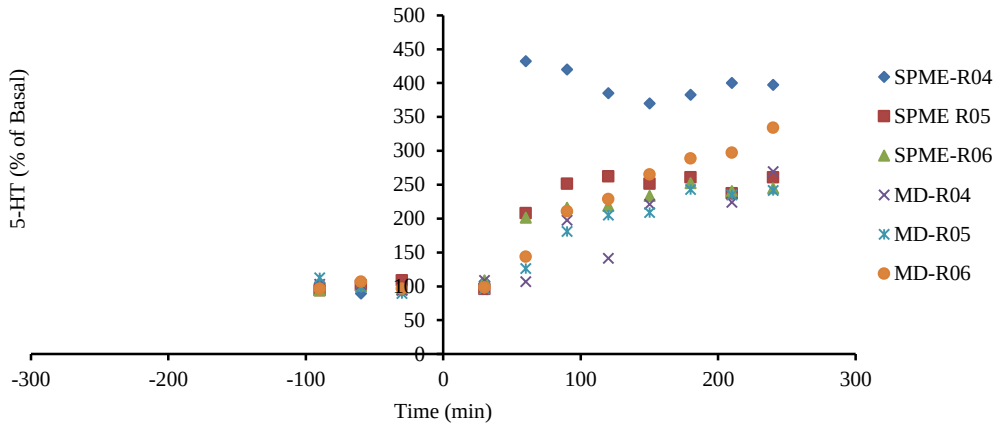


Figure 4.8 Effective and rapid response of SPME probe to capture sudden changes in concentration of 5-HT in rat R04 while MD showed an overall gradual increase in concentration

*4.3.7.1.5 Control Experiments*

In view of the fact that SPME method involved introduction of a new fiber at each sampling time point, possible tissue damage that may result from the multiple physical insertions and withdrawals of the fiber on the brain ECF on neurotransmissions was of primary concern. In addition, this experiment will confirm the fact that any change in the basal concentrations of the neurotransmitters was due to the fluoxetine drug and not from the mechanical insertions of the SPME fiber. Control experiments were therefore performed using 2 rats administering only the dosing vehicle. Figure 4.9 shows results obtained from control experiments carried out using SPME and MD.

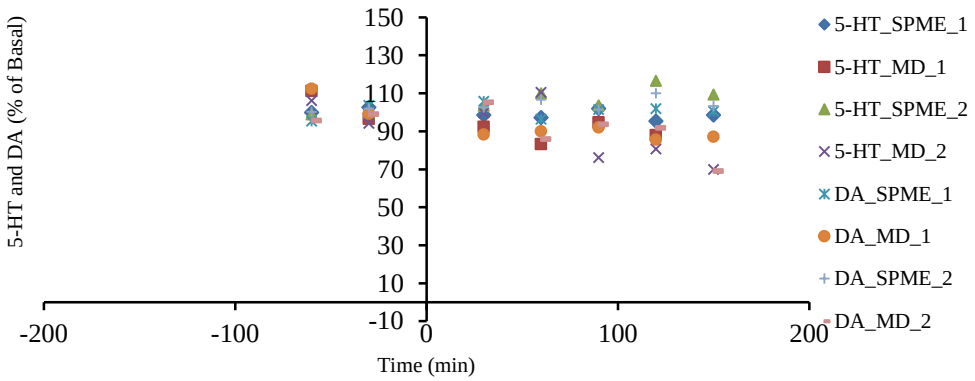


Figure 4.9 Comparison of SPME and MD methods for monitoring changes in serotonin (5-HT) concentration in the rat brain extracellular fluid following administration of the dosing vehicle for fluoxetine.

The data in Figure 4.9 indicate that relative to the single insertion of a MD probe and multiple insertions of the SPME fibers via a guide cannula into the same location in the brain do not have any effect on the concentrations of 5-HT and DA in the ECF space over the 4 hour sampling period. Therefore, the previously recorded increase in 5-HT was very likely caused by the fluoxetine drug.

*4.3.7.1.6 Monitoring GABA and Glutamic acid by SPME*

A major challenge associated with monitoring of neurotransmitters is the use of a single sample to measure analytes of varying physicochemical properties. Although MD facilitates the detection of multiple analytes and thus could be used to study interactions between neurotransmitters, the approach is often faced with critical methodological challenges. A notable disadvantage of MD is the low recovery for some analytes, which often poses serious challenge to the sensitivity of the analytical method. Apart from being time consuming and

often tedious due to the difficulty in the handling of very small dialysate volumes, issues with matrix influence due to the presence of salts in the dialysate tend to affect the general effectiveness of the method when coupled to LC-MS/MS. On the other hand, SPME is an analyte enrichment sample preparation method with its selectivity primarily dependent on the type of coating employed. This part of the study seeks to demonstrate the use of a single mixed-mode SPME fiber to extract multiple neurotransmitters, including GABA and glutamic acid in, addition to 5-HT and DA, in a single sample.

As depicted in Figure 4.10, the single dose injection of the fluoxetine drug did not significantly change the basal concentrations of GABA and GA in the brain ECF.

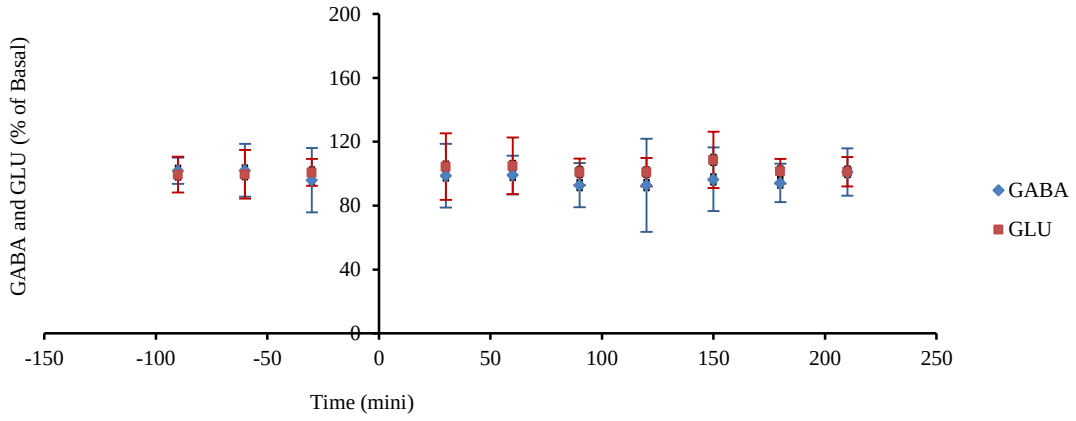


Figure 4.10 Percent changes in GABA and GA concentrations (relative to the mean basal concentration) in the rat brain extracellular fluid determined by SPME method following an *i.p.* injection of fluoxetine (10 mg/kg) at time t = 0. The results are expressed as the mean ± standard errors for 9 rats.

#### *4.3.7.2 Non-targeted chemical profiling of the striatum of rat brain*

As a result of their respective tendencies to extract hydrophilic and hydrophobic compounds, a combination of MD and SPME methodologies in global non-targeted metabolomics studies will significantly enhance the chance to identify any disease biomarkers. Subsequently, all MD and SPME samples were analyzed in a positive mode only on an LC-MS analysis platform.

This was in agreement with the results obtained by Wibom et al.[235] and Hrydziuszko et al.[236] who used MD for a metabolomics study in Glioblastoma and liver transplants, respectively. However, the SPME data was biased generally to less polar/hydrophobic compounds. Some of the metabolites detected by SPME included arachdonyl carnitine, gangliosides, fatty acids and lysophospholipids including lysophosphatidic acid and lysophosphatidylethanolamine, etc. Carnitines have been reported for multifactorial functions in brain metabolism. Their neuroprotective, neurotropic and neuromodulatory properties have various medical implications.[237] For example, a malfunction of biochemical pathways involving carnitines was found in Alzheimer's, Parkinson's, Huntington's disease and attention deficit hyperactivity disorder (ADHD), as well as other neuropathies.[237] Thus, detection of these compounds also expresses the suitability of *in vivo* SPME for reliable analysis of brain lipids, especially when current *in vitro* methods such as brain tissue slicing, homogenization and extraction with organic solvents could be very challenging and laborious.[238] A lipid mediator, lysophosphatidic acid, involved in brain development was recently also cited as been likely involved in changing blood-brain barrier permeability.[239] The fatty acids and polyunsaturated fatty acids (PUFA) are main components of cellular membrane

and precursors for eicosanoid biosynthesis. Recently, reports have linked neurodegenerative diseases to changes in the profile of the PUFA membrane with subsequent change in fluidity. Additionally, modifications of arachidonic acid, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) could affect intra- and extra-cellular signal transduction.[238,240] Lastly but not least, impaired metabolism of gangliosides may result in pathological states.[241,242] These results thus create the opportunity to extend *in vivo* SPME to lipidomics studies and other clinical applications.

Table 4.2 Tentatively identified endogenous compounds present in SPME extract but absent in dialysate after sampling of the striatum of live Sprague Dawley rats

| **Name**[#] | **m/z** | **LogP** |
|---|---|---|
| Triglyceride | 529.41022 | 9.34 |
| Arachidonyl carnitine | 546.43671 | 3.47 |
| Ganglioside NeuAcalpha-2-3-Galbeta-Cer (d18:1/24:1(15Z)) | 551.39227 | 9.77 |
| Glycerophosphocholines | 530.41370 | n/a |
| Ganglioside GD3 (d18:0/23:0) | 530.41370 | 2.99 |
| Lysophosphatidyl ethanolamine (LysoPE (0:0/18:1(11Z)) or (0:0/18:1(9Z)) or (18:1(11Z)/0:0) or (18:1(9Z)/0:0)) | 480.30848 | 4.64 |
| Lysophosphatidic acid LPA(0:0/18:0) or LPA(18:0/0:0) | 480.30848 | 4.91 |
| Triglyceride | 311.29465 | 10.71 |
| Fatty acids/Fatty Acyls | 329.30530 | n/a |
| diglyceride DG(15:0/14:1(9Z)/0:0) or DG(14:1(9Z)/15:0/0:0) | 547.44000 | 9.25 |
| 18Z,21Z,24Z,27Z,30Z,33Z-hexatriacontahexaenoic acid | 547.44000 | 10.57 |
| 1,2-ditetradecyl-sn-glycero-3-phosphate | 547.44000 | n/a |
| *trans*-retinyl linolate | 547.44000 | 10.80 |

[#] identification based on the comparison of the experimental data against Human metabolome database

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Table 4.3 Provisionally identified endogenous compounds present in dialysate samples but not in SPME extracts after sampling of the striatum of live Sprague Dawley rats

| **Name**[#] | **m/z** | **LogP** |
|---|---|---|
| Dihydrouracil | 132.07671 | -1.28 |
| Creatine | 132.07671 | -1.59 |
| β-Guanidinopropionic acid (β-GPA) | 132.07671 | -1.7 |
| Glutamyl valine | 132.07671 | -2.49 |
| Valyl glutamate | 132.07671 | -2.6 |
| Norsalsolinol | 166.08627 | -0.13 |
| D-Aspartic acid | 178.00870 | -3.52 |
| Iminodiacetic acid (IDA) | 178.00870 | -2.5 |

[#] identification based on the comparison of the experimental data against Human metabolome and Metlin databases

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In addition to the above, principal component analysis of the data, clearly distinguished components obtained by *in vivo* MD from SPME. Figure 4.11 demonstrates that the results obtained by SPME complement the MD data and vice versa and thus supports the hypothesis that a combination of the two sampling methods will enhance the discovery of potential biomarkers with the larger metabolite coverage.

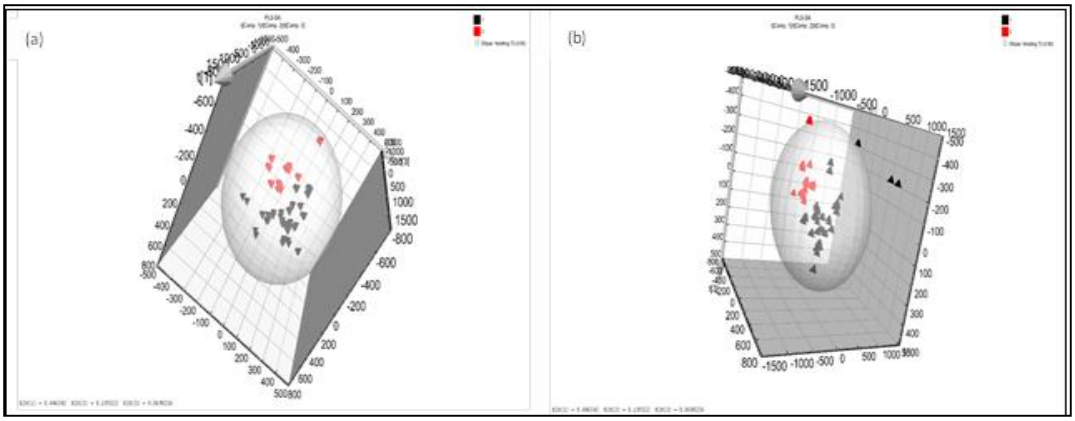


Figure 4.11 A principal component analysis (PCA) of data showed the complementary aspects of MD and SPME data in a global non-targeted chemical profiling of the brain extracellular fluid; (a) A 3-D PCA plot of data without any outliers (b) A 3-D PCA plot of data with outliers

#### *4.3.7.3 Quantitation of exogenous unbound drugs concentrations in brain extracellular fluid*

A typical characteristic of both MD and SPME methodologies is that they both measure the free/unbound concentration of the drug, which is pharmacologically active concentration, in the living system. For this reason, it is easier to compare data obtained from both methods.

Carbamazepine is generally an anticonvulsant, which is used for the treatment of epileptic seizures and other types of neurological disorders.[243] Although, there are various PK studies of the drug and its main metabolite in the rat brain, for the first time SPME is used to quantitatively measure the amounts of the free drug concentration in the brain. By using the on-fiber calibration method, issues with matrix match, which is typically observed in external

SPME equilibrium calibration method is avoided completely since both calibrant and analyte are treated in the same manner during sample preparation.

Prior to the simultaneous monitoring of CBZ in the brain ECF using SPME and MD, the concentration of CBZ was closely observed by taking dialysates at 30 min intervals for over 3.5 hours. Figure 4.12 shows concentration of CBZ in the brain ECF in the dialysates during *i.v.* infusion to attain a steady state. Subsequently, both SPME and MD samples were collected for another two hours in the steady state and later when the drug infusion was discontinued for 1.5 hr (dynamic state).

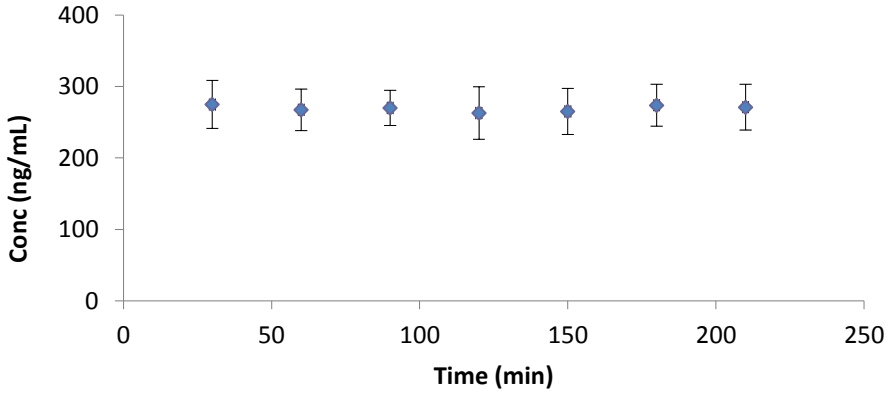


Figure 4.12 Monitoring steady state concentration of CBZ in the brain using microdialysis (n=6). The results are expressed as the mean ± standard errors for 6 extractions.

Figure 4.13 shows results obtained for SPME and MD samples both steady and dynamic states concentrations of CBZ in the frontal cortex of the brain. The comparable results indicate that SPME can be used for quantitative measurements of exogenous unbound drug concentrations in specific regions of the brain.

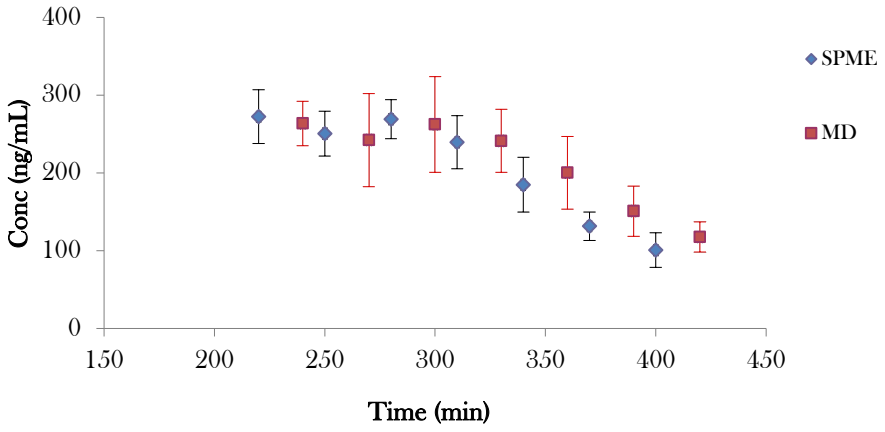


Figure 4.13 Measurements of free concentration of CBZ in the frontal cortex of the rat brain using *in vivo* microdialysis and solid phase microextraction (n=6). The results are expressed as the mean ± standard errors for 6 rats.

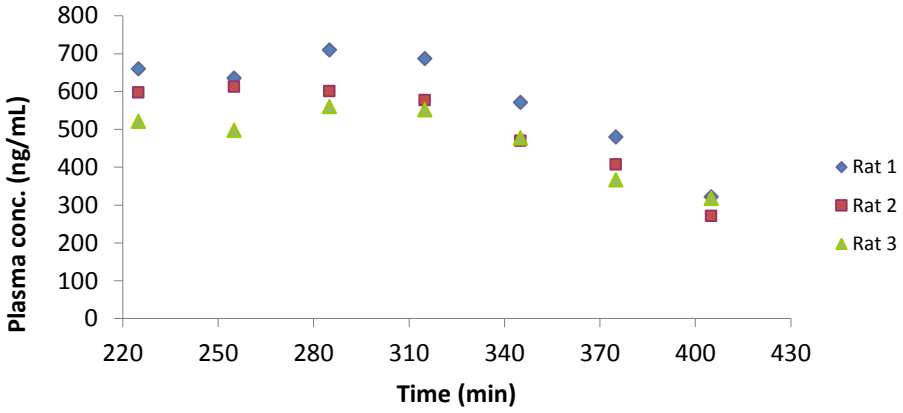


Figure 4.14 Measurements of the concentration of CBZ in the rat plasma samples.

The concentrations of CBZ found in the plasma samples were higher than the calculated concentrations in the frontal cortex of the rat brain. The difference in concentration may be attributed to the fact that the protein precipitation sample treatment method provides

information on the total drug concentration rather than the unbound or free concentration. Similar results were obtained for cimetidine concentrations in plasma samples compared to MD dialysates and SPME.

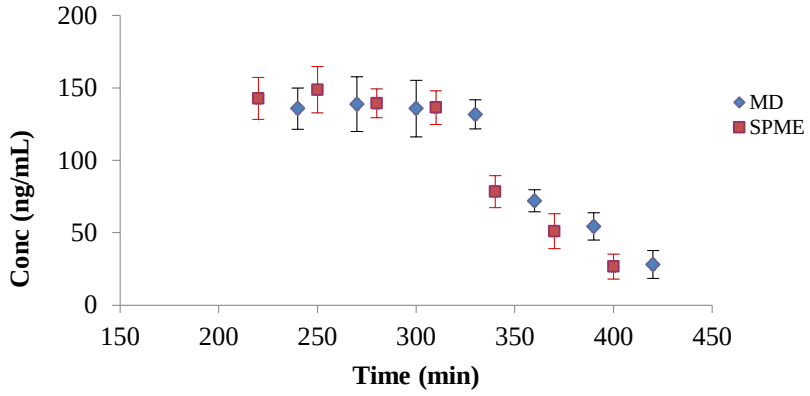


Figure 4.15 Measurements of free concentration of Cimetidine in the frontal cortex of the rat brain using *in vivo* microdialysis and solid phase microextraction (n=3). The results are expressed as the mean ± standard errors for 3 rats.

Cimetidine is a histamine $H_2$-receptor antagonist usually used for the treatment of peptic ulcers and heartburn. It is known for its low permeability across the blood-brain barrier into the brain. Owing to the fact that *in vivo* MD and SPME are relatively invasive sampling methods and that cimetidine has low permeability into the brain, assessing the concentration of cimetidine in the brain can be used as to gain insight into the disruption of the blood-brain barrier during sampling. From Figure 4.15, it can be observed that both SPME and MD detected cimetidine in the brain ECF. By comparing with the calculated concentrations of cimetidine in the plasma samples, a higher concentration of the drug was detected from the brain by both methods. The plasma samples were found to be about 40 % higher in

concentration than in the MD and SPME samples. However, a comparable ratio of CBZ concentration in the plasma and MD/SPME samples could not be obtained. This observation suggests that there are other factors contributing to the permeability of the drug through the blood-brain barrier to the brain.

Finally, results obtained for space-resolved concentration analysis of the cortex and striatum suggest no differences in the drug concentrations within this region. Table 4.4 shows the amount of CBZ determined by each method from the specific regions of the brain.

Table 4.4 Free concentration of carbamazepine from brain specific regions by MD and SPME

| | **Concentration of Carbamazepine (ng/mL)** | | | | | |
|---|---|---|---|---|---|---|
| | **R01** | | **R02** | | **R03** | |
| | **Cortex** | **Striatum** | **Cortex** | **Striatum** | **Cortex** | **Striatum** |
| **MD** | 208 | nd | 308 | nd | 219 | nd |
| **Mean (n=3)** | 245 (55) | | | | | |
| **SPME** | 215 | 212 | 250 | 271 | 221 | 240 |
| **Mean (n=3)** | Cortex: 229 (19) | | | Striatum: 241 (30) | | |

From the results there was no significant difference between the free drug concentration of CBZ in the frontal cortex by both MD and SPME. This confirms the fact that SPME is a potential *in vivo* tool that can be used for quantitative measurements of exogenous drugs in specific brain regions. Carbamazepine concentrations obtained by SPME in both the frontal cortex and striatum also showed no significant difference. This can be attributed to the fact that there is no concentration gradient in the distribution of the drug with these two regions.

#### *4.3.7.4 Histology studies*

##### *4.3.7.4.1 Protocol for histology study*

The Harris' hematoxylin and eosin staining protocol was used for this study. In brief, staining was achieved with Harris hematoxylin solution for 8 min and then rinsed following initial tissue preparation. Blue staining was carried out with 0.2 % ammonia solution for about 30 sec and a contrasting counterstaining procedure was performed in eosin-phloxine solution for about a min. Figure 4.16 shows results obtained for histological studies performed on some selected rat brain tissue.

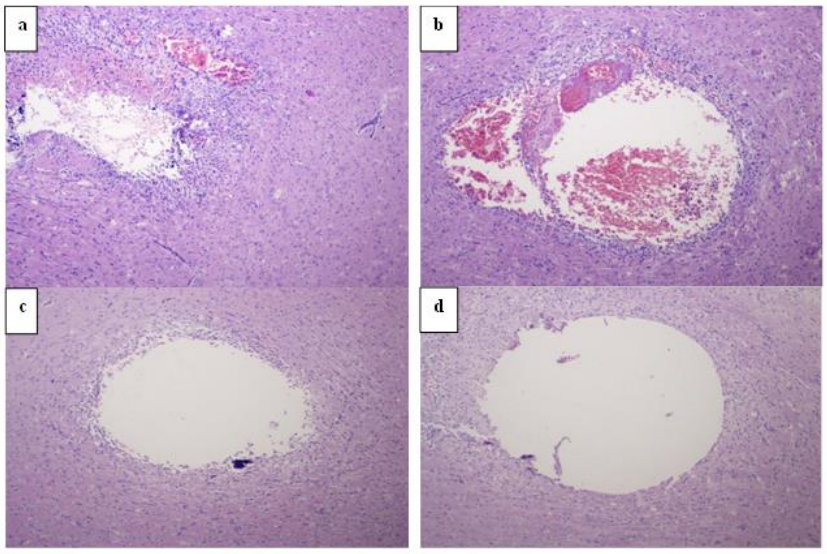


Figure 4.16 Results of histology studies of rat brain tissues; images (a & b) reflect tissue damage caused by SPME probes (magnification x1000) after multiple sampling from the same site using a probe for each sampling point for two different rat brain tissue; images (c & d) reflect tissue damage caused by single microdialysis probe (magnification x500) after sampling for two different rat brain tissue.

In general, data obtained from SPME method showed variations or inconsistencies in the extent of damage caused by the probe, and in some cases, less damage was observed compared to MD (Figure 4.16). This implies that reducing the size of the probe could further decrease the extent of tissue damage caused by SPME significantly. These results thus provide an avenue for introducing SPME microprobes using submicron/nano particle sizes immobilized on relatively smaller diameter wires for *in vivo* tissue bioanalysis. The approach will further augment overall extraction efficiency due to the increase in the effective surface area of the extraction phase.

## 4.4 Summary

An *in vivo* SPME method coupled with liquid chromatography tandem mass spectrometry was developed for monitoring changes in the concentrations of multiple neurotransmitters (5-HT, DA, GABA and GA) in the rat brain extracellular fluid (ECF). A SPME mixed-mode biocompatible coating was utilized. Intracerebral MD was performed to validate the method for the quantitation of 5-HT and DA. SPME and microdialysis probes (both 4 mm) were placed in the striatum of opposing brain hemispheres through implanted guide cannulae for simultaneous monitoring of the neurotransmitters after intraperitoneal administration of 10 mg/kg of the 5-HT reuptake inhibitor, fluoxetine. Dialysate was collected over 30 min intervals from the microdialysis probe, whereas a new SPME probe was introduced at successive 30 min extraction intervals. As expected, brain ECF 5-HT concentrations increased rapidly (~3 – 4-fold) relative to basal concentrations, whereas DA concentrations remained unchanged when quantified by both methods. GABA and GA concentrations, quantified by the SPME method, were also not affected by fluoxetine

administration. Control experiments carried out using dosing vehicle only did not show any change relative to the basal concentrations of the neurotransmitters. Statistical evaluation (one-tail paired t-test at 95% confidence interval) of the 5-HT and DA data confirmed there were no significant differences between the microdialysis and SPME methods.

An untargeted global chemical profiling of the rat brain striatum was successfully carried out using the MD and SPME coupled to LC-MS system. Overall data analysis of PCA plots of identified compounds revealed that both method produce complementary results. It can therefore be inferred that the combination of MD and SPME methodologies in global untargeted metabolomics studies will certainly augment the chances of discovery new disease biomarkers.

In addition to quantitatively measuring endogenous compounds, *in vivo* SPME can also be used to determine free concentrations of exogenous drugs in the brain tissue. Histological studies also showed disruption of the brain tissue as a result of the invasiveness of the methods.

In a nutshell, the results demonstrate that *in vivo* SPME method can be effectively utilized as a brain sampling tool to monitor multiple endogenous neurotransmitters, has the potential to efficiently distinguish rapid changes neurotransmitters and also applied to measurements of exogenous drug concentrations in the brain.

# Chapter 5

# Application of solid phase microextraction for monitoring neurotransmitters during deep brain stimulation in freely moving rats

## 5.1 Introduction

Tissue sampling is very critical in bioanalysis owing to its significant contribution to the pharmaceutical industry, forensics, and determination of drug/chemical toxicity, food science and molecular biology.[244] As an example, proper quantitative methods assist in obtaining drug and/or metabolite concentrations at their active sites in an animal tissue (brain, liver, lungs, etc.) and also improve data on research toxicity. In this regard, it is paramount that appropriate sample preparation methods are developed to obtain actual chemical information in a particular tissue. However, bioanalysis of solid biological tissues, pose significant often pose significant analytical challenges. Conventional analysis of tissues, typically tissue homogenization, introduces additional step(s) to the already often complex and tedious analytical procedure.[244,245] Issues with obtaining a representative biological control sample cannot be avoided. In addition, traditional on *ex-vivo* sample preparation methods for tissues are appropriate for situations where real monitoring of actual changes in the concentrations of drug/chemical and endogenous substances in the tissue are required.

MD is a sampling technique that has successfully been used to monitor concentration changes of molecules in biological matrices. Although primarily applied to research direct brain tissue analysis for neuroscience, microdialysis probes have been used for studies

involving various tissues/organs including the stomach,[246,247] skin,[248] liver,[249,250] and the ear,[251] to mention a few. Microdialysis despite its invasive characteristics is continually used sampling in the brain. This may be due to the fact that microdialysis offers high degree of selectivity and also facilitates the detection of multiple analytes in one sample, where other devices such as microsensors have largely failed to demonstrate high selectivity[252]. Zhang and Beyer, observed that microdialysis shows significant advantage in its ability to measure neurochemicals in discrete regions of the brain by employing multiple probes.[253] Despite the advantages offered by microdialysis for *in vivo* measurements of neurochemicals, the method is characterized with some major challenges. Some of these shortfalls include limited sample volume due to required low perfusion rates (0.2 – 3 μL), which often poses significant sensitivity challenges in cases where further dilution is needed to reduce the impact of matrix effect. In addition, the very small sizes of the dialysates make sample handling very difficult and often results in losses due to sample evaporation from longer sampling times. Issues related to adsorption of hydrophobic components to the membrane always pose greater challenge. The technique is relatively expensive and the use of tubing occasionally restricts the free movement of animals in cases where real-time monitoring of neurochemical changes is required.

In addition to microdialysis, microelectrodes and biosensors have been used not just for sampling, but also as integrated analysis device to measure directly the dynamics of neurochemicals.[254,255] Although these methods often exhibit high temporal and spatial resolution, detection of analytes is based on direct redox activity at the electrode, for which most neurotransmitters do not have such properties.[254] Other major setbacks characterized with microelectrodes or biosensors are the lack of selectivity (inability of electrode to detect more than one ion at a time) and gross interferences from relatively high concentration of other

electroactive neurochemicals. Solid phase microextraction, an equilibrium-based sampling method has in recent times also, gained significant attraction for *in vivo* measurements of chemicals from biological matrices.

SPME, which combines sampling and analyte-enrichment method, has been applied successfully for *in vivo* quantitative pharmacokinetic studies in dogs[223] and very small animals like mice,[229] measurement of real-time drug concentrations in a dynamic system,[256] determination of pharmaceuticals in fish,[257] and the space-resolving capability of solid phase microextraction was applied to the determination of pharmaceuticals using a segmented-sorbent fiber.[258] One of the unique advantages of solid phase microextraction that has contributed to its successful application to the measurements of chemicals *in vivo* biological systems is that a single experiment can be used to determine both free- and total-analyte concentrations. This is achieved, as stated in a review by Vuckovic *et al*., by using appropriate external calibration curves in which, (a) one curve is obtained from physiological buffer or artificial cerebrospinal fluid devoid of any binding component and used to calculate free analyte concentration, and (b) another curve obtained using suitable matrix matching sample which includes the binding components of the biological system under study is used to determine total concentration.[259] Another advantage offered by solid phase microextraction is that selectivity is dependent on the analyte-fiber partition coefficient. This implies that depending on the type of extraction phase, analytes of varying degrees of polarities can be selectively extracted from a given biological system. Finally, the method offers an efficient sample clean-up with minimal or no matrix interferences, making it easy to be coupled to liquid chromatography mass spectrometry.

In this section of the thesis, the potential of equilibrium-based solid phase microextraction as a sampling and analyte-enrichment method for simultaneous monitoring of neurotransmitters (GABA, GA, DA and 5-HT) in freely moving rats. This simple and relatively inexpensive approach employed a mixed-mode extraction phase fiber, which is capable of extracting compounds with a wide range of polarities. The fibre, supplied by Supelco®, also was made from biocompatible material and thus prevented fouling or adverse tissue reaction. To demonstrate that solid phase microextraction can successfully monitor changes in neurochemical concentrations in the brain, the animals were subjected to deep brain stimulation of the pre-frontal cortex. Deep brain stimulation (DBS), when used as a surgical treatment technique has been shown to provide major therapeutic advantages for neurological disorders like Parkinson's disease[260,261] and epilepsy.[262] Despite these achievements, the fundamental neural and chemical mechanisms associated with deep brain stimulation are still very much unclear. A recent report by Hamani *et al.* using MD demonstrated that DBS in the ventromedial prefrontal cortex (vmPFC) induces a significant increase in serotonin hippocampal levels.[263]

In the present study, for the first time, solid phase microextraction probes were implanted in the hippocampus of freely moving animals to assess the potential of the method to detect the neurotransmitter changes that occur after vmPFC DBS.

## 5.2 Experimental section

### 5.2.1 Reagents and materials

All chromatographic solvents were HPLC grade. Optima® grade acetonitrile solvents were obtained from Fisher Scientific (Ontario, Canada) and HPLC grade formic acid was obtained from Supelco (Bellefonte, PA, U.S.A.). Glutamic acid, δ-aminobutyric acid, dopamine hydrogenchloride and 5-hydroxytryptamine (Serotonin) were also obtained from Supelco (Bellefonte, PA, U.S.A.). The serotonin stock was stored at 4 $^{0}$C in a refrigerator. Diazepam was purchased from Cerilliant (Round Rock, TX) as a 1 mg/mL methanolic solution. Artificial cerebrospinal fluid (aCSF) was obtained from Harvard Apparatus, Holliston, MA. U.S.A. Solid phase microextraction fibers, which had mixed mode particles (C18-benzenesulphonic acid group) as extraction phase, used for this study, were obtained from Supelco (Bellefonte, PA, U.S.A.). Deionized water used for the preparation of standards was from a Barnstead/Thermolyne NANO-pure water system (Dubuque, IA, U.S.A.). Guide cannulae were obtained from CMA Microdialysis®, Stockholm, Sweden.

### 5.2.2 Liquid chromatography and mass spectrometry conditions

All liquid chromatographic separations were performed on a Thermo Scientific Accela™ instrument equipped with a binary pump. Chromatographic separation of analytes was achieved with a Phenomenex® kinetex™ core shell pentafluorophenyl column (2.6 µm, 2.1 mm x 150 mm) in 5 min using gradient elution at a flow rate of 0.45 mL/min. Mobile phase A consisted 90% aqueous, 10% acetonitrile and mobile phase B was 90% acetonitrile and 10% aqueous. Both mobile phases contained 0.1% formic acid to enhance ionization in the ion

source. An Accela™ autosampler from Thermo Scientific was used for sample introduction into the HPLC system. A sample volume of 10 μL was injected and analyzed by a triple quadrupole mass spectrometer.

The TSQ Vantage™ triple quadrupole mass spectrometer from Thermo Scientific had the heated electrospray ionization (HESI) probe installed for effective nebulization and ionization. All ions were monitored in the positive ionization mode. The mass ion transitions monitored were 104.1→69.1, 148.1→84.1, 154.1→91.2 and 177.1→115.1 for δ-aminobutyric acid (GABA), glutamic acid (GA), dopamine (DA) and serotonin (5-HT) respectively. The source voltage, vapourizer and capillary temperatures were 1000 V, 250 $^{o}$C and 250 $^{o}$C respectively. The lower source voltage (1000 V) was due to the fact that higher voltage settings may result in arcing at the tip of the nebulizer needle. This occurred mainly because the nebulizer needle was placed relatively close to the orifice of the metal ion transfer tube for improved sensitivity. Although higher voltages could be used with the nebulizer tip withdrawn farther away from the orifice, the sensitivities for all the analytes were lower compared to the optimized conditions used for this study. The optimized sheath and auxiliary gases were set at 55 and 15 respectively. All data analyses were performed with the Xcalibur® software version 2.0.7.

### 5.2.3 SPME brain probe for brain tissue sampling

In order to perform *in vivo* sampling from the brain tissue, the SPME sampler was designed such that the extraction phase can be exposed through a CMA guide cannula. The sampler consists of an approximately 200 μm diameter wire coated on one end with a 4 mm

length of mixed mode extraction phase and a thickness of 45 µm. The non-coated end of the wire was supported by a small piece of rubber septum material, cut appropriately to fit directly into the CMA guide cannula. Figure 5.1shows a schematic representation of the SPME brain sampler.

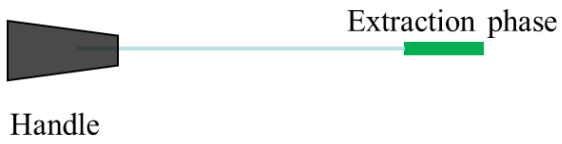


Figure 5.1 Schematic representation of SPME brain tissue sampler

### 5.2.4 Surgical Procedures

All protocols were approved by the Animal Care committee of the Centre for Addiction and Mental Health, Toronto. The surgical procedure was similar to that previously described.[22] Briefly, male Sprague-Dawley rats (250 to 300 g) were anesthetized with ketamine/xylazine (100/7.5 mg/kg intraperitoneal) and had bilateral cathode electrodes implanted in the vmPFC at the following stereotaxic coordinates: anteroposterior (AP) _ 3.0, lateral (L) _0.4, and depth (D) 5.6 mm. Stainless steel electrodes implanted over the somatosensory cortex were used as anodes. During the same procedure, guide cannulae were bilaterally implanted the dorsal hippocampus (AP 3.7, L 2.4, D 5.1). A total of eight hemispheres were studied after the surgical procedures of 4 rats.

### 5.2.5 In vivo brain SPME

After surgery, the animals, separated from each other were left a week for recovery before SPME sampling and were allowed to move freely in a plastic cage with access to food and water supply. Figure 5.2 shows the *in vivo* SPME extraction with the electrical probe connected to the frontal cortex.

Figure 5.2 *In vivo* SPME sampling of neurotransmitters from the pre-frontal cortex of a freely moving rat

SPME sampling was performed by exposing the 4 mm mixed-mode coating (extraction phase) to the pre-frontal cortex of the brain through each microdialysis guide cannula. The extraction was carried out for a period of 30 min. The 30 min extraction time was chosen based on *in vitro* experiments carried out in both aCSF and agar gel matrices to establish the equilibration time for each analyte. Details have been discussed in Chapter 4. The basal levels of the neurotransmitters were obtained over a 2-hr period simultaneously from both right and

left brain hemispheres and defined as the average of 4 samples for each brain hemisphere. SPME sampling was then stopped and the animals received electrical stimulation for a one-hour period. Afterward, SPME sampling was re-started for another 2-hr period during deep brain stimulation. All fibers were immediately placed into a 100 µL insert containing 60 µL desorption solution of water - acetonitrile mixture with 0.1 % formic acid (pH approximately 3.5). The inserts were subsequently placed in sealed amber vials and stored immediately on dry ice for later analysis.

## 5.3 Results and discussions

### 5.3.1 HPLC Analysis

Optimization of chromatographic separation has been widely illustrated in Chapter 3 (Paragraph 3.3.1). Owing to the advantage of the pentaflurophenyl column discussed in the paragraph mentioned above, the retention and separation was achieved in this study by a gradient elution, which starts with a high organic content while gradually increasing the aqueous content. Diazepam which has less hydrophilic properties eluted first at 1 min followed by separation of the neurotransmitters with 5-HT having the greatest retention factor under these conditions. The active particle size of 1.7 µm of the core shell column used for this study enhanced signal-noise ratios to ensure separation of both amino acid and monoamine neurotransmitters without the need for derivatization. This offers greater potential for sample preparation methods like SPME capable of selectively extracting these compounds without the need for multiple derivatization methods for the different classes of neurotransmitters.

### 5.3.2 Determination of SPME equilibration time

Equilibrium calibration method was used for this study as longer extraction time enhances the analyte-enrichment in the extraction phase. Therefore various *in vitro* experiments were carried out in artificial cerebrospinal fluid (aCSF), a physiological fluid and agar gel-aCSF matrices to establish the time within which all analytes would be expected to have reached equilibrium with the fiber.

To estimate the equilibration time, aCSF experiments were performed under static conditions as this will provide the slowest diffusion of the molecules similar to the molecular movement within the extracellular fluidic space in the biological matrix. The gel-aCSF experiments were carried out to investigate any possible effect of tortuosity on the diffusion of analytes to the fiber and thus affect the equilibration time. In addition, the effect of the sample volume was also investigated to establish whether the extracted amount will be independent of the sample volume as a proof of concept. If this deduction is true, then it is possible that the amount of analyte extracted from the brain extracellular space will not be significantly dependent on the fluid volume provided there is no analyte depletion from multiple extractions at the same sampling site.

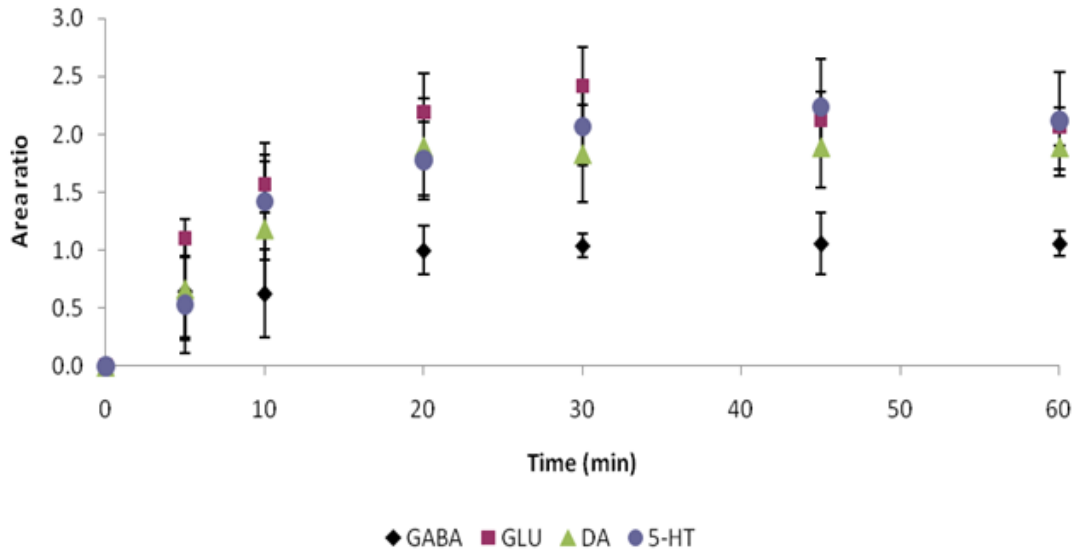


Figure 5.3 Equilibrium-time profiles for neurotransmitters in aCSF under static conditions. The results are expressed as the mean ± standard errors for 3 extractions.

The equilibration time for all the analytes in aCSF was determined using a 100 ng/mL solution. In these experiments, extractions were carried out using the 4 mm mixed mode fiber for different time points (5, 10, 20, 30, 45, 60 min) in a 2 mL vial. Volume of sample was 1.8 mL of the aCSF. After the extraction process, the fibers were desorbed in 60 µL desorption solution containing 3:2 ratio of water to acetonitrile with a 0.1% formic acid in 150 µL inserts. To enhance desorption of the analytes from the fiber, the vials were agitated at 1000 rpm on a vortexer. Carryover experiments performed by second desorption of the same fiber in new solution, showed no detectable amount of any of the analytes after LC-MS/MS analyses. A total of 3 replicates were obtained for each time point. The equilibration time for each analyte was estimated from plots of the area ratio of the analyte to internal standard (diazepam) versus time.

As shown in Figure 5.3, the equilibrium time for all analytes was reached within 20 min of extraction under static conditions (without agitation). This simply that implied that in the absence of any solid matrix component that could affect the diffusion kinetics of the analyte, a maximum of 20 min extraction was required by each analyte to equilibrate with the extraction phase. In an independent equilibration time, determined under agitation mode, a much shorter time of approximately 3 min was observed when the sample matrix was agitated at 250 rpm. The much shorter equilibrium time under agitated conditions showed that mass transport of analytes within the extracellular space could be significantly improved with fluid movement.

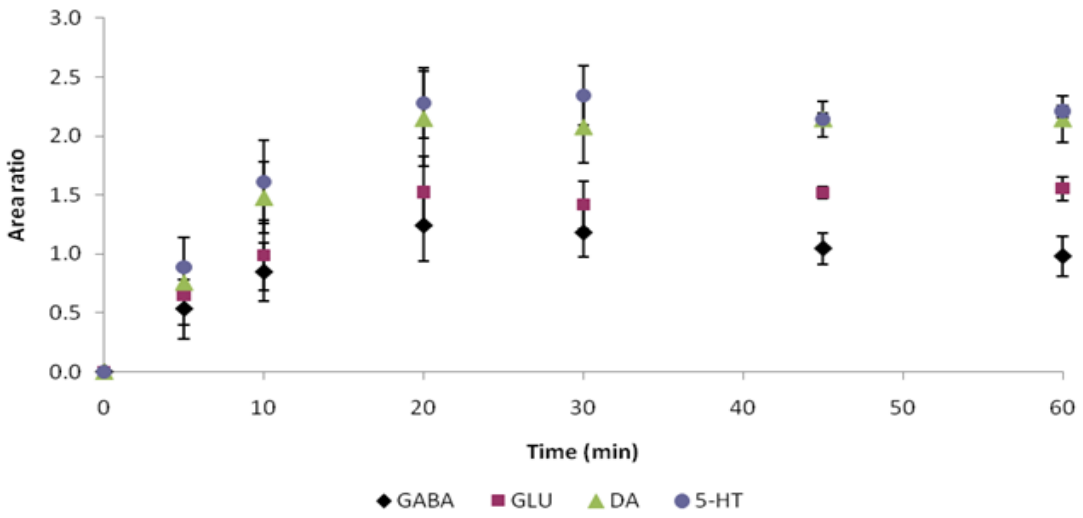


Figure 5.4 Equilibrium-time profiles for neurotransmitters in 1.5 % gel-aCSF mixture. The results are expressed as the mean ± standard errors for 3 extractions.

To investigate the possible effect of tortuosity of the brain tissue on the diffusion rates of the analytes to the extraction phase and subsequently, equilibrium time, equilibration time profiles were generated with different percent (1% and 1.5%) agar gel matrices prepared by

mixing aCSF with varying amount of agar gel. Gel composition ≥ 1 % was also chosen because 1 % gel composition was commonly used to model brain tissue and therefore the higher gel percentage the greater the tortuosity. The time required for the analytes to reach equilibrium were similar both 1 % and 1.5 % (Figure 5.4) gel composition and also for extractions from aCSF only. On the basis of this data, it was concluded that tortuosity of the brain tissue may not be the rate limiting step for the analytes to reach equilibrium within the brain extracellular space provided there is minimal or no depletion at the extraction site. Secondly, the amount extracted at equilibrium was so small and therefore was independent of the volume of fluid within the extracellular space. This was verified by investigating the effect of the sample volume on the extracted amount at equilibrium. Extractions were carried out thus carried using different sample volumes (100 µL, 500 µL and 1000 µL). Each extraction was done in triplicates. All fibers were initially pre-conditioned in 50% methanolic solution overnight with agitation at 250 rpm. As shown in Figure 5.5, there were no significant differences in the amounts of each analyte from the 100 µL, 500 µL and 1000 µL sample volumes. The implication of this result is that the analyte-fiber partition coefficient is very small and therefore the amount of each neurotransmitter extracted from the extracellular space by the extraction phase will not lead to depletion of any of the analytes.

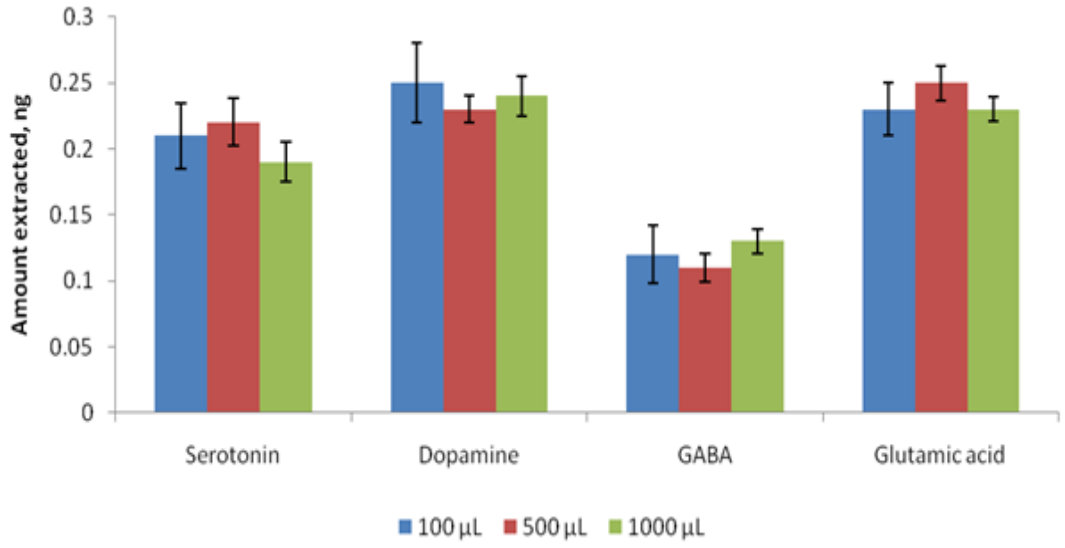


Figure 5.5 Comparison of the extracted amount of neurotransmitters from different volume of samples. The results are expressed as the mean ± standard errors for 3 extractions.

### 5.3.3 *In vivo* SPME analysis of neurotransmitters

The optimized *in vitro* method was used to monitor changes in the neurotransmitters brain extracellular space prior and during deep brain stimulation of the pre-frontal cortex (infra limbic region). Calibration curves used to calculate the amount of each analyte extracted for *in vivo* extractions were also obtained from aCSF. With this method, it implies that the calculated amounts will be the free analyte concentration within the extracellular space of the brain tissue. Limits of detection for all analytes ranged from 0.009 to 0.024 ng/mL whereas limit of quantitation was computed based on 3x signal-noise ratio (0.030 to 0.08 ng/mL) with GABA having the highest LOD value. The percentage relative standard deviation (RSD %) obtained for the basal levels ranged from 3–20 % for both left and right brain hemispheres for all neurotransmitters in all 4 rats (Figure 5.6).

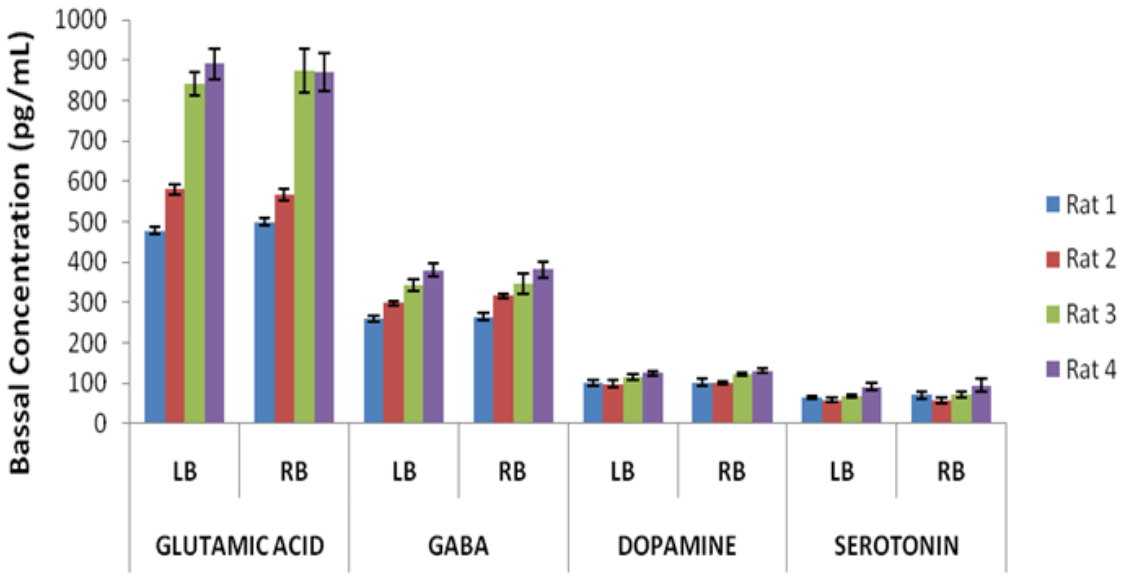


Figure 5.6 Basal concentrations of selected amino acid and monoamine transmitters in rat brain

Figure 5.6 shows the bilateral agreement of the concentrations of all the analytes between the left and right brain hemispheres. This is indicative of the fact that the multiple introductions of the solid phase microextraction sampler in the brain do not have any possible influence on the concentrations of the analytes in the extracellular space. Secondly, the 4-mm mixed mode fiber with the biocompatible material did not produce any fouling effect during the extraction process, which often characterized by very poor data. This observation has been confirmed elsewhere in an independent study, in which the same neurotransmitters were monitored over a 3.5 hours using both microdialysis and solid phase microextraction simultaneously[248].

With the exception of 5-HT which showed an increase of about 300% higher than the basal concentration, there were no observable induced increases in any of the amino acid transmitters and dopamine (Table 5.1).

Table 5.1 Monitoring changes in concentrations of glutamic acid, δ-aminobutyric acid and dopamine in both left and right brain hemispheres of rat 4

| | Time (min) | GABA (pg/mL) | | GLUTAMIC ACID (pg/mL) | | DOPAMINE (pg/mL) | |
|---|---|---|---|---|---|---|---|
| | | LB | RB | LB | RB | LB | LB |
| Pre-deep brain stimulation | -120 | 340 | 327 | 815 | 857 | 121 | 131 |
| | -90 | 326 | 382 | 873 | 923 | 136 | 139 |
| | -60 | 362 | 341 | 862 | 909 | 123 | 128 |
| | -30 | 344 | 330 | 823 | 806 | 119 | 128 |
| During deep brain stimulation | 30 | 326 | 349 | 893 | 913 | 126 | 130 |
| | 60 | 350 | 349 | 821 | 845 | 134 | 130 |
| | 90 | 328 | 344 | 853 | 909 | 125 | 127 |
| | 120 | 318 | 321 | 869 | 880 | 122 | 128 |

LB represents left brain hemisphere RB represents right brain hemisphere

This implies that vmPFC deep brain stimulation effects possibly do not have any effect on the selected amino acid transmitters and dopamine. However, the increased amount of 5-HT in the extracellular space of the infra limbic region could be attributed to the deep brain stimulation (Figure 5.7).

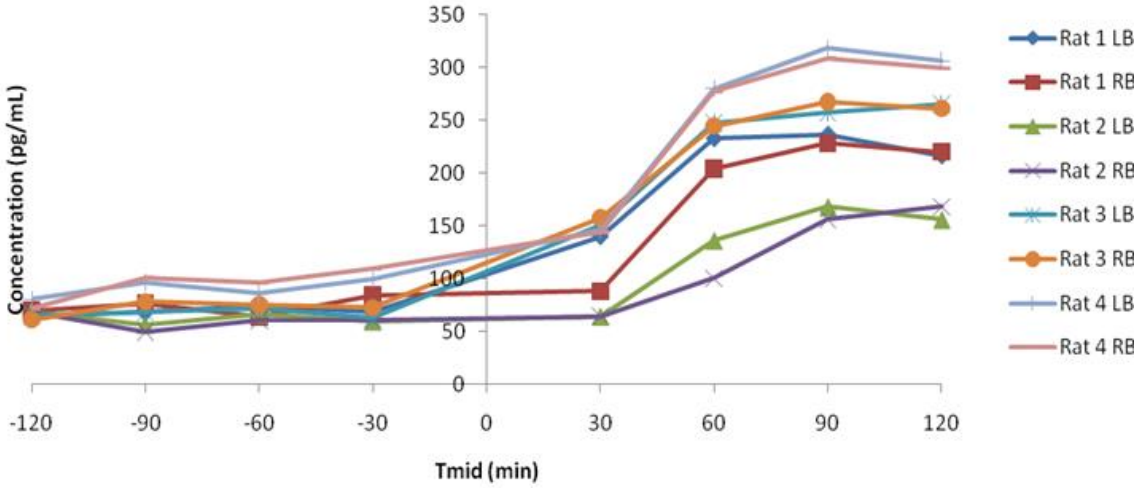


Figure 5.7 Monitoring changes in concentration levels of 5-HT in left and right hemispheres of the rat brain before and during deep brain stimulation

Increase in serotonin after DBS in the present study was similar in magnitude compared to previous results obtained by Hamani *et al.* using microdialysis.[248] This suggests that SPME as a tissue sampling tool may be a valid alternative approach to measure neurotransmitter changes after the application of an external stimulus.

In separate studies, portions of the *in vivo* samples were subjected to global untargeted chemical profiling. Samples were treated the same way as described in previous chapter. A quality control sample was generated from a pool of the samples and desorption solution was used as the blank. Details of the sample treatment, LC-MS and data analysis were well described in Chapter 4. The rational of the studies was to establish whether there are other compounds that might be affected by the DBS in addition to the neurotransmitters.

Table 5.2 shows some of the identified compounds from the analysis of the data when compared to the HMDB database. Subsequently, the peak intensities of the identified

compounds prior to and during DBS were compared. It was observed that compounds labeled as M1 to M8 (Refer to Table 5.2) seems to show changes in peak intensities before and during DBS. Most of these compounds were identified to be fatty acids.

Table 5.2 Some identified compounds from the analysis of *in vivo* SPME samples collected prior to and during deep brain stimulation of the rat brain

| ID | Qualitative identification | m/z | LogP |
|---|---|---|---|
| **M1** | Triglyceride | 529.41022 | 9.34 |
| **M2** | Lysophosphatidylcholine | 551.39227 | - |
| **M3** | Arachinonyl carnitine | 546.43671 | 3.47 |
| **M4** | Ganglioside | 530.41370 | 2.99 |
| **M5** | Lysophosphatidylcholine | 552.39575 | 3.14 |
| **M6** | Eicosanoid acid | 311.29465 | 8.4 |
| **M7** | nonadeca-10(2)-enoic acid | 329.30530 | 8.06 |
| **M8** | diglyceride | 547.44000 | 9.25 |
| **M9** | estradiol derivative | 363.25357 | 3.9 |
| **M10** | N-acetyl-D-mannosamine-6-phosphate/dimethyl heptanoyl carnitine | 116.14370 | -3.6 |
| **M11** | Unknown | 153.13900 | - |
| **M12** | glutamine | 147.07677 | -3.6 |
| **M13** | leukotriene/phytyl diphosphate | 913.47864 | 4.8 |
| **M14** | palmitaldehyde | 132.07704 | 5.1 |
| **M15** | Phosphate | 158.96431 | -3.6 |
| **M16** | leucine/aminobutyric acid | 164.12859 | -2.5 |
| **M17** | methylglutaconic acid | 223.06416 | 0.29 |

[#] identification based on the comparison of the experimental data against Human metabolome database

Figure 5.8 and

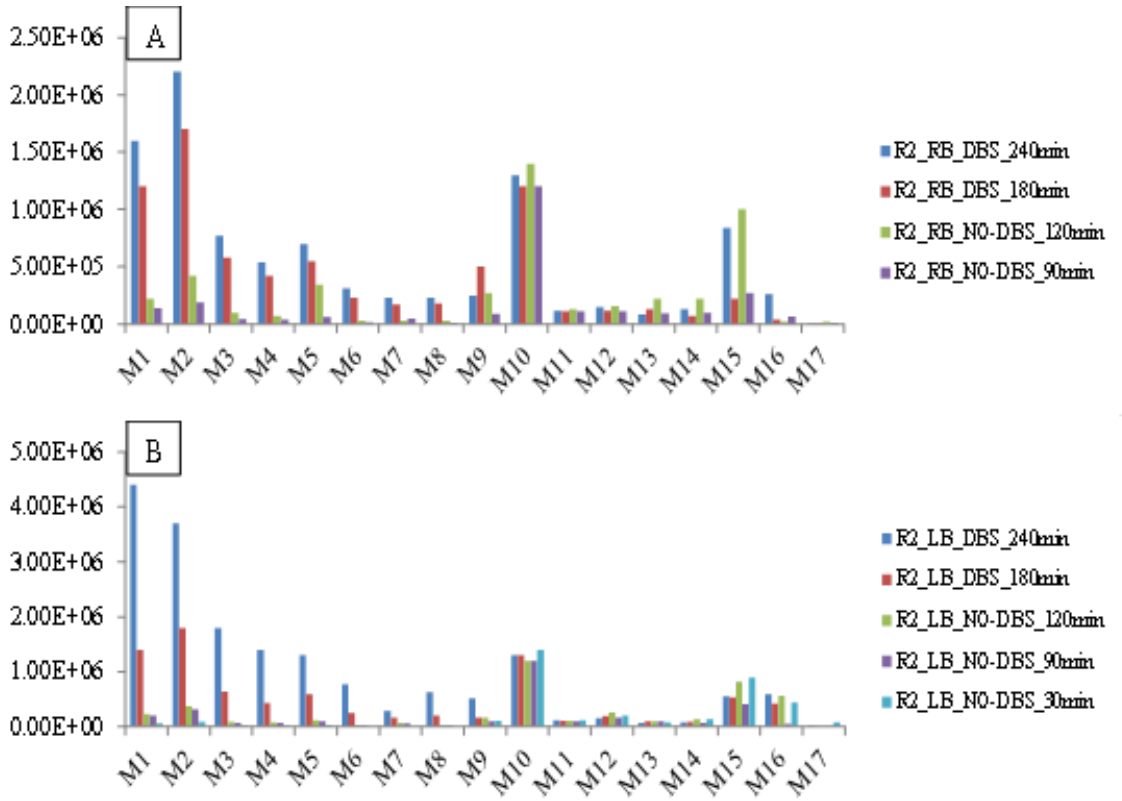


Figure 5.9 show the trend of the peak intensities for each of the identified compound prior to and during deep brain stimulation.

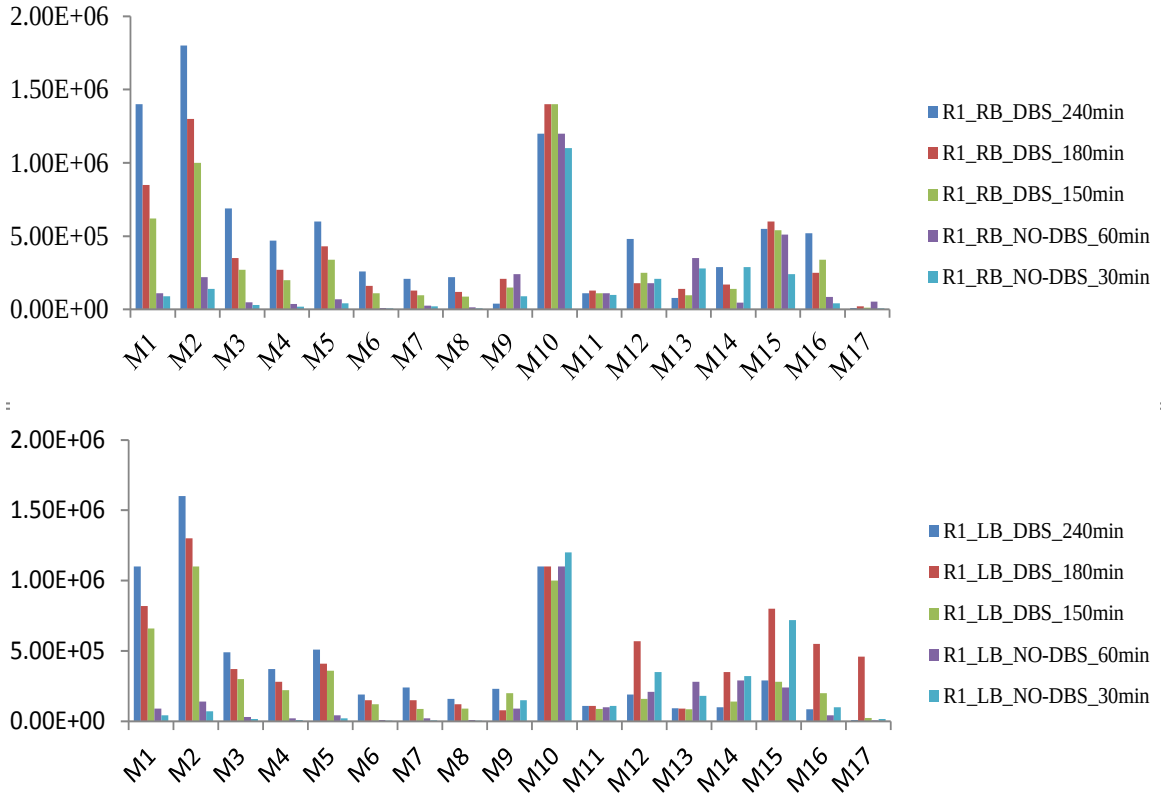


Figure 5.8 Effect of deep brain stimulation on intensity of peak areas of selected compounds in both right (top) and left (bottom) hemispheres of the frontal cortex of freely moving rats

From both figures, preliminary results showed that both the left and right hemispheres of the rat brain showed similar patterns of higher peak intensities during DBS whereas the peaks areas were very lower prior to the stimulation. Generally, the remaining compounds did not show changes in their peak areas during DBS with the exception of M15, M16 and M17. In addition, the peak areas of these compounds in both left and right hemispheres were not comparable.

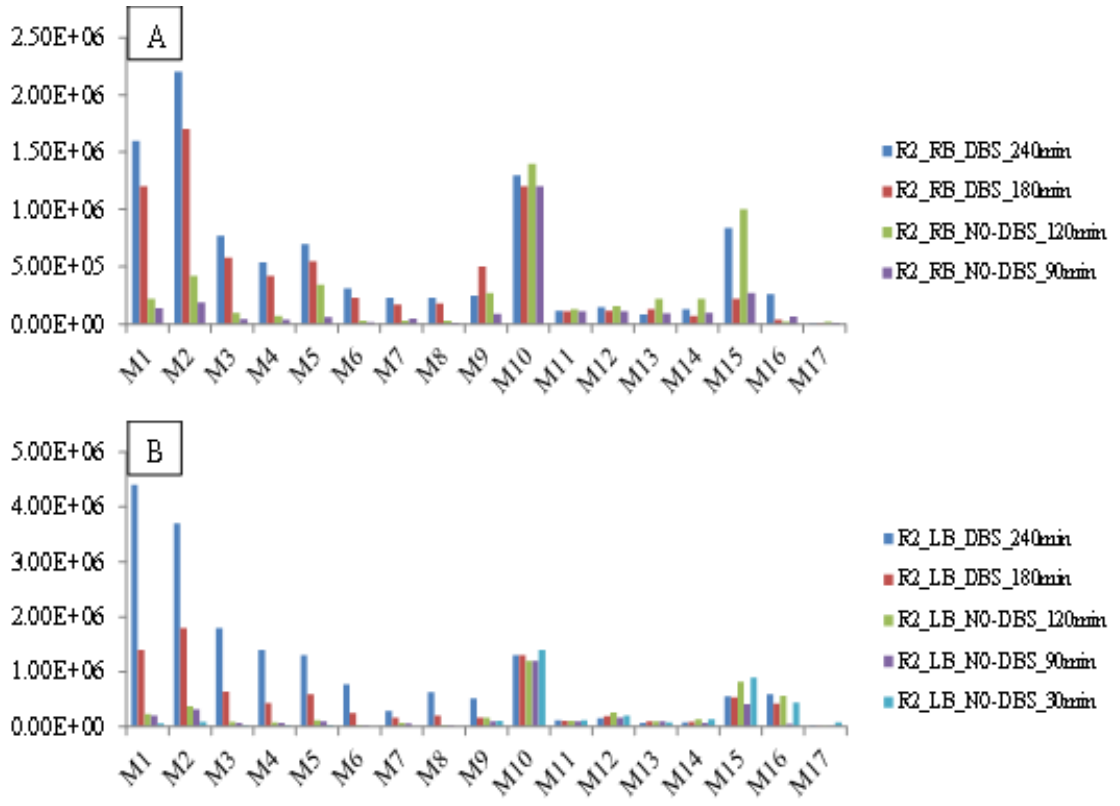


Figure 5.9 Effect of deep brain stimulation on the intensity of the peak areas of selected compounds in both right (top) and left (bottom) hemispheres of the frontal cortex of freely moving rats

Although, this study is in its preliminary stage, some of the metabolites identified such as the fatty acids and carnitines are known to be involved in multifactorial functions in brain metabolism and their neuromodulatory, neuroprotective and neurotropic properties have various medical implications. From the results it is obvious that a better alternative that can be used to gain insight into the therapeutic mechanism of DBS treatment is by global untargeted metabolomics studies. By this approach and with the appropriate statistical tool, the effects of DBS on the chemical activity of the brain can be better understood. That said, there is no doubt

that neurotransmitters may be significantly involved in the deep brain stimulation treatments for various neurological disorders.

## 5.4 Summary

In this study an equilibrium quantitative SPME method for *in vivo* monitoring of changes in neurotransmitters in the extracellular space of the pre-frontal cortex of the rat brain without prior derivatization. The excellent clean-up method of SPME also ensured that the huge signal suppression caused by the presence of matrix components in the sample extract, as observed for microdialysis when coupled with LC-MS/MS, was absent. This added advantage of solid phase microextraction, and also its analyte-enrichment (pre-concentration) characteristics enhanced the detection of analytes with improved sensitivity.

Analytes used in this study were serotonin, dopamine, gamma-aminobutyric acid and glutamic acid. A 4 mm long solid phase extraction mixed mode coating immobilized on a stainless steel wire with a biocompatible material was successfully used for the extraction of analytes from both hemispheres of the rat brain. The possible influence of brain tissue tortuosity and fluid volume in the extracellular space were investigated through *in vitro* experiments, and used to estimate the time required for analytes to reach equilibrium with the SPME fiber in the rat brain. A 30 min extraction time was found to be adequate to ensure equilibration of the analytes in the sample matrix with the fiber. Chromatographic separation without prior derivatization was achieved within 5 min run time using a pentafluorophenyl core shell column for separation and a triple quadrupole mass spectrometer was used for detection of analytes. To be able to effectively monitor changes in neurotransmitters, for the

first time, SPME extractions were carried out prior to and during deep brain stimulation (DBS) at the ventromedial pre-frontal cortex. Results show that SPME has the potential to detect changes in the neurotransmitters in the brain of freely moving rats. No significant changes were observed for all the analytes except for serotonin, which showed about 2 - 3x (~ 50 pg/mL - 400 pg/mL) increase during DBS. Results corroborate similar studies carried out independently using microdialysis elsewhere. The increased serotonin amounts obtained by SPME corroborated independent study using microdialysis. Relative standard deviation for serotonin at basal concentrations ranged from 3 % to 20 %.

A global untargeted chemical profiling of the brain showed that DBS of the frontal cortex of the brain could affect the levels of certain fatty acids and carnitines. Thus, *in vivo* SPME could be utilized as a potential tool that for various medical/clinical applications of such nature.

# Chapter 6

# Conclusions and Future Directions

## 6.1 Conclusions

Solid tissue bioanalysis is one of the most challenging, time-consuming and laborious bioanalytical tasks compared to the analysis of other biological samples such as blood, plasma, urine, etc. Despite its importance to clinical, medical, toxicological, toxicity applications, etc., there are currently no existing guidelines for sampling solid biological tissues. Nonetheless, the significance of quantitative analytical methods for measurements of exogenous and/or endogenous compounds in solid biological tissue cannot be over-emphasized. For sure, the development of simple, robust, reliable and appropriate bioanalytical methods for brain tissue analysis for endogenous and exogenous chemical substances, will contribute immensely to understanding various health related diseases affecting humans, physiological processes and metabolisms within a living system, to mention a few. Without doubt, *in vivo* research is more appropriate to monitor the overall effect in a living biological system than *in vitro* research by providing a better indication of the effect in real time. For this reason among others, there is rising interest in *in vivo* methods and techniques for bioanalysis, tissue bioanalysis for that matter.

SPME methods have been broadly applied for invasive and non-invasive *in vivo* studies. The development of biocompatible extraction phases has overall improved the sensitivity, selectivity and compatibility of SPME to *in vivo* applications. The continuous

advancement in SPME calibration methods in recent years has also facilitated accurate quantitation, especially for *in vivo* applications even involving pre-equilibrium extractions, which significantly improves throughput. However, pre-equilibrium SPME extractions are characterized by lower analytes extraction amounts and thus translate into generally lower analytical sensitivity. Despite this seemingly limitation, the easy coupling of the method to LC-MS/MS has proven its robustness as a quantitative analytical technique since the results obtained by SPME are comparable to conventional methods like MD as shown in this thesis.

Undoubtedly, results shown in this thesis demonstrate the ability of using *in vivo* SPME for brain tissue sampling when coupled to LC-MS/MS. The introduction of new the biocompatible mixed-mode SPME coatings showed the potential of the technique for simultaneous monitoring of changes in the concentrations of multiple neurotransmitters within the brain ECF. The results obviously provide the opportunity to measure and monitor interactions among endogenous chemical substances such as neurotransmitters and also interactions with drugs within the brain ECF. A critical factor to monitoring neurotransmitters within the brain ECF is the ability of the method to measure basal concentrations of the analyte. From the results obtained, *in vivo* SPME clearly has the potential of determining the basal concentrations of brain extracellular neurotransmitters. The quantitative determination of drugs within the brain as shown in Chapter 4, clearly demonstrates the potential of using *in vivo* SPME for monitoring and measuring neuroactive drugs in toxicity studies from specific regions of the brain. Currently, the initial results obtained from simultaneous monitoring of different brain regions (cortex and striatum) is a proof of concept for space-resolved SPME

with the advantage of using a single fibre compared to *in vivo* MD where multiple probes will be required.[264]

In general, untargeted tissue metabolomics is particularly interesting for the study of damaged tissues in search of novel biomarkers since the concentration of such biomarkers is often expected to be higher in such tissue. A fundamental concept of untargeted metabolomics is to develop a method that is capable to provide larger metabolites coverage in addition to been able to capture the true metabolome during the sampling process. This thesis has clearly demonstrated that a good sampling approach to untargeted brain tissue metabolomics analyses is through the simultaneous use of multiple analytical sampling methods. Preliminary results obtained in Chapter 4 for untargeted metabolomics studies demonstrate this phenomenon by combining *in vivo* SPME and MD for brain tissue sampling.

Despite the success of *in vivo* SPME for brain tissue sampling, there are inherent challenges especially if the method is intended for monitoring very fast changes in neurotransmission. The current sampling time of 30 min, though synonymous to conventional *in vivo* MD, it may not be an appropriate tool for monitoring rapid changes extracellular changes in the concentration of neurotransmitters. This will require further studies in order to improve the method. A possible approach will be exploring new mixed-mode coatings, include more neurochemicals and to use pre-equilibrium extraction approach for improved time resolution. Another challenge will be the extent of tissue damage due to multiple microextractions from the same site.

## 6.2 Future directions

Brain tissue metabolomics study offers exciting prospects for in-depth study of brain. Currently, there are very few brain tissue analyses that explore more simultaneous use of multiple bioanalytical methods. Owing to the fact that *in vivo* SPME and MD preferably extract non-polar and polar analytes, the combination of these methods will certainly advance the quest for biomarker discovery in a global metabolomics study. This thesis has clearly laid the foundation for further development of appropriate workflows for the two methods while capturing the sensitivity and selectivity of electrospray LC-MS/MS.

The issue of tissue damage and subsequent rupture of the blood brain barrier during *in vivo* brain tissue sampling has been of primary concern in brain research. This calls for minimizing the size of existing *in vivo* SPME probes used for brain sampling. Ultra small *in vivo* SPME probes can be developed using smaller particle sizes (nanoparticles). The advantage of such approach will be overall improvement in sensitivity due to the increased surface area to volume ratio. Though invasive, the method will provide an opportunity to carry out in vivo brain tissue sampling while the blood brain barrier remains intact. This obviously will improve pharmacokinetic and pharmacodynamics of exogenous drugs and their xenobiotics in the brain extracellular fluid.

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