Solid phase microextraction for *in vivo* determination of pharmaceuticals in fish and wastewater

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.

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Abstract

This thesis describes the development and application of solid phase microextraction (SPME) as a sample preparation technique for *in vivo* determination of pharmaceutical residues in fish tissue and wastewater. The occurrence, distribution and fate of pharmaceuticals in the environment are a subject of concern across the globe due to the impact they may have on human life and aquatic organisms. To address this challenge from an analytical perspective, a simplified and reliable analytical methodology is required to investigate and determine the concentration (bioconcentration factors) of trace pharmaceutical residue in fish tissue and environmental water samples (exposure). An improved SPME method, coupled with liquid chromatography with tandem mass spectrometry has been developed and applied to both controlled laboratory and field-caged fish exposed to wastewater effluent for quantitative determination of pharmaceutical residue in fish specific tissue.

A new SPME configuration based on C_{18} thin film (blade) was developed and optimized to improve SPME sensitivity for *in vivo* determinations of trace pharmaceuticals in live fish. The C_{18} thin film extraction phase successfully quantified bioconcentrated fluoxetine, venlafaxine, sertraline, paroxetine, and carbamazapine in the dorsal-epaxial muscle of living fish at concentrations ranging from 1.7 to 259 ng/g. The reproducibility of the method in spiked fish muscle was 9-18% RSD with limits of detection and quantification ranging from 0.08 - 0.21 ng/g and 0.09 - 0.64 ng/g (respectively) for the analytes examined. Fish were sampled by *in vivo* SPME for 30 min to detect pharmaceutical uptake and bioconcentration, with experimental extracts analyzed using liquid chromatography coupled with tandem mass spectrometry.

In addition, a simplified analytical methodology based on SPME was developed and optimized for determination and bioconcentration factor of different classes of pharmaceuticals residues in fish bile. The reproducibility of the method in spiked fish Rainbow Trout bile was 3-7% RSD with limits of detection (LOD) ranging from 0.3 – 1.4 ng/mL for the analytes examined. The field application of SPME sampling was further demonstrated in Fathead Minnow (*Pimephales promelas*), a small-bodied fish caged upstream and downstream of a local wastewater treatment plant where fluoxetine, atorvastatin, and sertraline were detected in fish bile at the downstream location. Also, a simple automated analytical method using high throughput robotic system was developed for the simultaneous extraction of pharmaceutical compounds detected in surface waters. The proposed method successfully determined concentrations of carbamazepine, fluoxetine, sertraline, and paroxetine in treated effluent at concentrations ranging from 240 - 3820 ng/L with a method detection limit of 2-13 ng/L, and a relative standard deviation of less than 16%. Application of the method was demonstrated using wastewater from pilot-scale municipal treatment plants and environmental water samples from wastewater-dominated reaches of the Grand River (Waterloo, ON).

Finally, 4 and 8-d laboratory exposures were carried out with Rainbow Trout exposed to wastewater effluent collected from pilot scale at Burlington, ON. Additionally, wild fish, White Sucker (*Catostomus commersonii)* were collected and sampled from Waterloo and Kitchener downstreams containing local municipal effluent. Bioconcentration factors of the selected compounds were determined in both fish muscle and bile samples. The results show that anti-depressant drugs such fluoxetine, sertraline and paroxetine were uptake in the fish

muscle and fish bile for both laboratory and field exposure. In summary, exposure of fish to micro-pollutants such as pharmaceuticals may be monitored through the analysis of bile, particularly at low concentration exposure of pharmaceuticals, where the sensitivity of analytical method may be challenged. SPME is a promising simple analytical tool which can potentially be used for monitoring of pharmaceuticals in fish tissue and wastewater.

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Dedication

I dedicate this thesis to God the Father, Son and the Holy Spirit for his love, support and encouragement throughout my program.

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List of Abbreviations

a- Time constant

A- Surface area of a SPME fiber

α- Effective volume fraction in a tissue

BCF- Bioconcentration factor

BAF- Bioaccumulation factor

C0- Initial analyte concentration

CE- Collision energy

CAS- conventional activated sludge

CAS-N conventional activated sludge with nitrification

CAS-BNR- conventional activated sludge with Biological Nitrifying Reactor

CID- Collision induced dissociation

CXP- Collision cell exit potential

CBZ- Carbamazepine

DP - Declusting potential

d - Days

D_s -Diffusion coefficient of analyte in the sample matrix

EP- Entrance potential

ESI- Electrospray ionization

ISTD- Internal standard

Fibre- extraction phase

GC -Gas chromatography

HPLC- High performance liquid chromatography

J- Mass flux

K- Equilibrium constant of the analyte between the solid fiber surface and the sample matrix

-Distribution coefficient of the analyte between the liquid fiber and the sample matrix

LC- Liquid chromatography

LOD- Limit of detection

Log Kow- log of octanol/water partition coefficient

LOQ- Limit of quantitation

LLE- Liquid-liquid extraction

MRM- Multiple reaction monitoring mode

MDL- Method detection limit

MS- Mass spectrometer/spectrometry

MW- Molecular weight

n- Amount of analyte extracted by SPME fibre at a specific time

ne -Amount of analyte extracted by SPME fibre at equilibrium

NA- Not available

ND- Not detected

PPCPs- Pharmaceuticals and Personal Care Products

PBS- Phosphate buffered saline, pH 7.4

PDMS- Polydimethylsiloxane

q0- Amount of calibrant preloaded on fibre

Q- Amount of calibrant remaining on the fibre after extraction

QC -Quality control

Rpm- Revolutions per minute

RSD- Relative standard deviation

SPE- Solid phase extraction

SRM- Selected reaction monitoring mode

SPME- Solid-phase microextraction

Z- Diffusion path length

t- Sampling time

V_f- Volume of fibre coating

V_s -Sample volume

δs- The thickness of the boundary layer

Chapter 1

Introduction

1.1 Emerging contaminants in environment

The near ubiquitous detection of emerging contaminants is a global issue raising concern among scientists, government agencies, and the general public, due to the potential impact of such contaminants on human life and aquatic organisms[1-8]. According to the United States Environmental Protection Agency (USEPA), "emerging contaminants" can be broadly defined as synthetic or naturally occurring chemicals or microorganisms that are not commonly monitored in the environment, but have the potential to enter the environment and cause known or suspected adverse ecological or human health effects[8]. These contaminants are predominantly unregulated despite their potential threat to environmental and human health. Many of these contaminants were unrecognized as persisting in environmental comportments until new, sensitive analytical methods were developed, hence use of the term "emerging". Classes of environmentally significant emerging contaminants include pharmaceutical and personal care products, persistent organic pollutants, perfluorinated and brominated compounds, and nanomaterials, among many others. One of the most prominent classes of emerging contaminants receiving widespread media and scientific attention are pharmaceutical and personal care products, which include chemicals that are used for health or cosmetic purposes[9]. The main sources of pharmaceuticals and their metabolites in the environment are either through discharges via sewage treatment plant effluents, or by run-off leaching over or through the soil draining to surface water [10-12].

1.2 Pharmaceuticals in aquatic environments

Pharmaceuticals are classified as a large and diverse group of medicinal compounds used for the diagnosis, cure, treatment or prevention of disease in humans and animals[9]. The persistence of pharmaceuticals and their active metabolites in the aquatic environment, as well as the possible effects they may cause on living organisms have recently raised concern, since these compounds are not completely removed during sewage treatment processes. Factors that may affect the bioavailability of pharmaceuticals and their respective metabolites include their physical/chemical properties as well as the conditions of the surrounding ecosystem. As well, the environmental distributions of pharmaceutical/metabolites are greatly influenced by their properties governing partitioning between different environmental phases. A number of studies have shown that the presence of sediments or particulates phases can dramatically affect bioavailability of pharmaceuticals and their metabolites in aquatic organisms [13-14].

1.3 Occurrence of pharmaceuticals and their impacts on aquatic organisms

The primary source of pharmaceuticals and their metabolites in our environment is municipal wastewater continually infused with receiving environments due to the inefficient removal of these compounds by municipal treatment plants. Studies indicate that these emerging contaminants also enter the environment via disposal of unused or expired pharmaceutical products, although this is likely a minor pathway [12, 15, 16]. In some cases, a significant portion of pharmaceuticals are excreted in unmetabolized form as the parent compound, or, if metabolized, often retaining biological activity as active metabolites[17]. Some of these compounds may escape the wastewater treatment plant with little discernible degradation, with the pharmaceutical pollutant entering the environment in the effluent at concentrations comparable to that of the

influent. Upon entering the environment, pharmaceutical pollutants are subject to a number of processes such as hydrolysis and photolysis which may, depending on the nature of each compound, elicit a chemical transformation of the pharmaceutical. Biodegradation and biotransformation are the most common mechanisms depleting drug residues, with seasonal variations in temperature and light intensity considered to be significant factors determining the fate of pharmaceuticals in surface waters [18, 19, 20]. Figure 1.1 illustrates the major pathways of pharmaceutical residues entering and distributing in aquatic environments. The specific distribution of pharmaceutical pollutants is dependent on human use, the physicochemical properties of the compounds (such as solubility, octanol-water partition coefficient), and compound-specific pharmacokinetic/metabolic transformation. In addition, the type of wastewater treatment process (es) employed for the removal of pharmaceuticals at a particular site can affect its distribution in surface waters. Upon entrance to surface water sources, the pharmaceutical is now available to interact with biological receptors, potentially impacting the health of human and aquatic organisms. Recently, it was reported environmental pharmaceutical exposure, particularly in drinking water, may result in abnormal physiological processes and increase potential for reproductive impairment in children, pregnant women[21, 22]. From an environmental perspective, the effects of synthetic estrogens used in birth-control pills have been well documented on fish, particularly with respect to the feminization of males, impaired reproductive capacity, and abnormal sexual development [23-25].

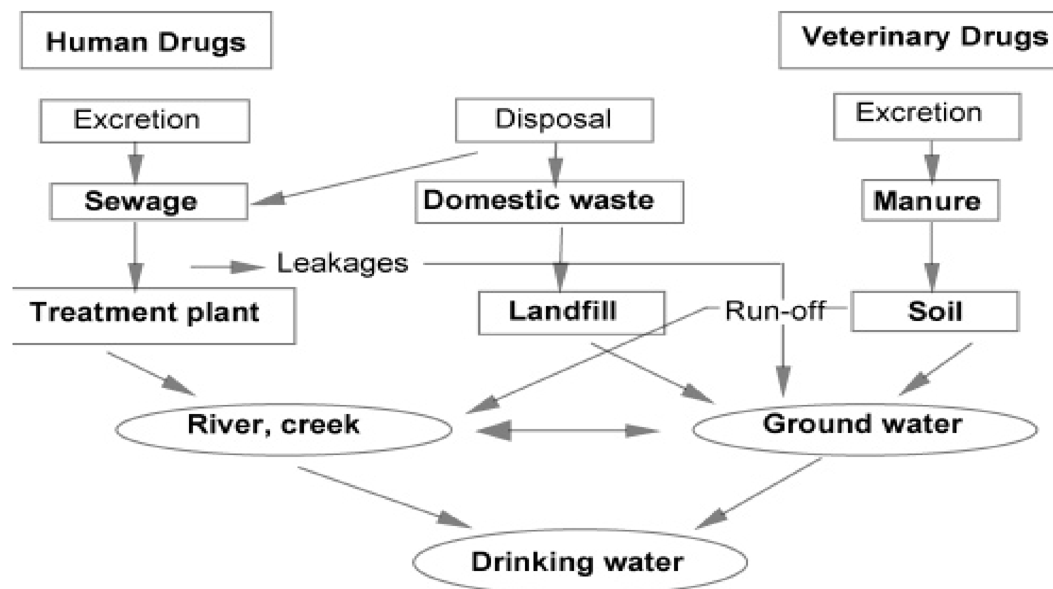

Figure 1:1 Sources and distribution of pharmaceuticals in the environment[15]

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1.4 Analytical methodology for determination of pharmaceuticals

Appropriate analytical methodologies and instrumentation (selectivity and sensitivity) are important tools for obtaining reliable data when determining pharmaceutical concentrations in environmental water and complex biological samples (*e.g.* fish tissues). Analytical procedures comprise the following steps: sampling, sample preparation, separation, detection, and data analysis. In general, each step is important to ensure accurate results, but sampling and sample preparation steps are critical steps affecting the success of the analysis, accounting for over 70-80% of the analysis time [26, 27]. The goal of sampling and sample preparation is to isolate and pre-concentrate analytes of interest from a complex matrix before instrumental analysis for quantification. Conversion of analytes in real matrix to suitable isolated states amenable for analysis requires an understanding of the interactions of analytes with their matrix based on their physical and chemical properties. Hence, the physicochemical properties (such as log K_{ow}, pKa)

of analytes can affect the applicability of sample preparation technique in terms of accuracy and precision[27]. Consequently, the choice of sample preparation technique for specific analytes in a given matrix is critical to ensure reliable analytical results. Table 1.1 summarizes the principle and application of sample preparation techniques which are commonly employed for pharmaceutical analysis in aquatic matrices.

Recent advances in analytical instrument capability in combination with sample preparation techniques and clean-up procedures have made it possible for more accurate quantification of pharmaceuticals and their metabolites in complex matrices. Moreover, chromatographic resolution as well as detection sensitivity and specificity have increased considerably in recent years. Gas and liquid chromatographic techniques, in combination with mass spectrometry detectors, are two commonly used analytical methodologies for analysis of pharmaceuticals in environmental and biological samples[28, 29]. In the case of gas chromatography–mass spectrometry (GC–MS or GC-MS^2) methods, derivatizations of acidic compounds (polar compounds) are often required before analysis because pharmaceuticals have high polarity and ionizability. However, liquid chromatography–mass spectrometry (LC–MS) and LC–tandem MS do not require a derivatization step, thereby making the technique suitable for analysis of polar pharmaceuticals and their metabolites in aqueous environmental samples[30]. Table 1.2 summarizes the different sample preparation techniques and analytical methodologies used for analysis of pharmaceutical residuals in fish and water samples.

Table 0:1 Advantages and limitations of commonly used sample preparation techniques for pharmaceutical analysis in aquatic organisms and environmental samples[31-38]

Sample preparation methods	Principles of extraction	Advantages	Limitations
Liquid-liquid extraction (LLE)	Partitioning of analytes between two immiscible liquid (equilibrium partition) based on solubility and log Kow of the analytes. Applicable for aqueous samples.	-Exhaustive extraction of analytes through repeated extraction. -Cheap solvent for extraction of semi-volatile compounds.	-Labor intensive and time consuming, low/variable recovery. -Large usage of solvent and sample, disposal of solvent. Clean up step required. -Poor selectivity and co-extraction of matrix components.
Solid phase extraction (SPE)	Adsorption and partitioning of analytes to extraction phase based on affinity of analytes. Applicable for aqueous samples.	-Exhaustive extraction technique with good recovery. -Requires less solvent compared to LLE. -Automation possible for high throughput.	- Multiple steps can introduce larger uncertainty in results. -Labor intensive and time consuming. -Requires selection of right sorbent for efficient SPE recovery.
Pressurized Liquid Extraction (PLE) or Accelerated Solvent Extraction	Partitioning of analyte from solid matrix to solvent at elevated pressure and temperature. Analytes	-Fast extraction technique with minimal use of solvent. -Fully automated for high throughput.	- Initial equipment cost is high. -Large amount of samples may be required.

(ASE)	solubility increases and mass transfer is faster. Applicable for solid samples.	-Exhaustive extraction technique with good recovery.	- Multiple steps are involved including a clean-up step. -Poor selectivity and co-extraction of matrix components.
Matrix Solid Phase Dispersion (MSPD)	Partition, adsorption and interaction of analytes in solid matrix with solid support bonded-phase, eluting solvent using mechanical blending to produce complete sample disruption. Applicable for solid samples.	- Rapid and inexpensive technique, it uses a smaller size of sample combined with lower solvent consumption. - Exhaustive extraction technique with good recovery.	-Labor intensive and time consuming. - Multiple steps can introduce larger uncertainty in results. - Clean up steps may be required. - It cannot be completely automated.
Semi-permeable membrane device (SPMD)	Analyte partition into the extraction phase (triolein) by diffusion across a membrane. It is based on solubility of analytes in triolein (extraction phase) and diffusion co-efficient of analytes of interest. Applicable for aqueous samples.	-It is reproducible and determines time-weighted average concentration of analytes in water sample. -It is relatively less expensive. - Good for passive sampling of hydrophobic compounds.	-Labor intensive and time consuming. -Multiple steps can introduce larger uncertainty in results. -Clean up step may be required.

Polar Organic Chemical Integrative Sampler (POCIS)	Analyte partition into the extraction phase (sorbent) by diffusion across the membrane. It is based on affinity of analytes to the sorbent used. Applicable for aqueous samples.	-It is reproducible and determines time-weighted average concentrations of analytes in water samples. -It is relatively less expensive - Good for passive sampling of polar compounds	-Labor intensive and time consuming, -Multiple steps can introduce larger uncertainty in results. -Clean-up step may be required
Solid phase microextraction (SPME)	Adsorption and absorption of analytes to the extraction phase (sorbent) based on the affinity of analytes of interest. Equilibrium based extraction technique	-Simple, cheap and rapid extraction technique -Requires smallest sample volume and least solvent compared to other approaches. -Automation possible for high throughput - Applicable for in vivo sampling in living organism	-Susceptible to low recovery of analytes in biological samples. -Possibility of fibre fouling and competition effect.

Note: SPE and SPME are the most important and popular methods for pharmaceutical analysis

Table 0:2 Analytical methodologies for determination of pharmaceuticals in fish tissues[14, 39-51]

Analytes or pharmaceutical class	Sample matrix type	Type of sample preparation	Analytical methodology	Remarks	References
Anti-depressant: fluoxetine, sertraline, norfluoxetine, desmethylsertraline	Fish liver, brain, lateral fillet tissues	Homogenization and SPE	GC/MS	fluoxetine, sertraline, norfluoxetine, desmethylsertraline were detected in all fish tissues	14
Tetracycline antibiotics	Fish muscle	Solvent extraction and in tube-SPME	HPLC-UV (photodiode array detector)	tetracycline, oxytetracycline, chlortetracycline and doxycycline were detected	39
Anti-depressant: fluoxetine, paroxetine, norfluoxetine	Fish muscle	Pressurized Liquid Extraction (PLE)	HPLC-APCI-MS/MS	These selected pharmaceutical were determined in fish muscle	31
23 pharmaceuticals and 2 metabolites with differing physicochemical properties	Fish muscle	Solvent extraction and SPE	HPLC-ESI-MS/MS	Diphenhydramine, diltiazem, carbamazepine and norfluoxetine were determined in muscle	40

Analytes or pharmaceutical class	Sample matrix type	Type of sample preparation	Analytical methodology	Remarks	References
Non-steroidal anti-inflammatory drugs (NSAIDs): ibuprofen, naproxen, ketoprofen diclofenac and gemfibrozil	Fish plasma	SPE	GC/MS	ibuprofen, naproxen, diclofenac, gemfibrozil were detectable in the plasma	41
Anti-depressant fluoxetine	Fish muscle	Pressurized Liquid Extraction (PLE) and SPE	HPLC-APCI-MS/MS	Fluoxetine was detected in the muscle	42
Diltiazem, diphenhydramine, carbamazepine, fluoxetine, and norfluoxetine	Fish muscle	SPME	HPLC-ESI-MS/MS	Diphenhydramine and diltiazem were detected in the muscle of fish	43

Analytes or pharmaceutical class	Sample matrix type	Type of sample preparation	Analytical methodology	Remarks	References
pharmaceuticals of differing physicochemical properties	Fish liver and muscle	Solvent extraction	HPLC-ESI-MS/MS	Most of the target compounds were detected in fish muscle and liver	44
Diclofenac	Fish bile	SPE	HPLC-ESI-MS/MS and QTOF	Diclofenac and its metabolites were detected in fish bile	45
Antidepressant pharmaceuticals including fluoxetine, norfluoxetine, sertraline, norsertraline, paroxetine, citalopram, fluvoxamine, duloxetine, venlafaxine, and bupropion	Fish brain	Solvent extraction	HPLC-ESI-MS/MS	Fluoxetine, sertraline, and their degradation products were detected in the fish brain tissue	46
9 pharmaceuticals and 1 metabolite	Fish muscle and adipose tissue	Space resolved SPME	HPLC-ESI-MS/MS	gemfibrozil, carbamazepine, ibuprofen, and fluoxetine were detected in fish muscle and adipose tissue	47

Analytes or pharmaceutical class	Sample matrix type	Type of sample preparation	Analytical methodology	Remarks	References
Antidepressants and their metabolites	Fish muscle, brain and liver	Solvent extraction and SPE	HPLC-ESI-MS/MS	Sertraline and its metabolite desmethylsertraline were observed in all tissues in decreasing abundance: liver > brain > muscle.	48
Diclofenac, naproxen, ibuprofen, bisoprolol, and carbamazepine)	Fish blood plasma and bile	SPE	HPLC-ESI-MS/MS	Bioconcentration of pharmaceutical in plasma was low, Metabolites of diclofenac, naproxen, and ibuprofen were detected in fish bile	49
Naproxen and its metabolites	Fish bile	SPE	HPLC-ESI-MS/MS	naproxen and its metabolites were detected in fish bile	50
Ibuprofen	muscle, liver and plasma	Solvent extraction and SPE	GC/MS and LC/MS	Ibuprofen was detected in all the fish tissues	51

1.5 Solid phase microextraction: a sample preparation technique

Solid phase microextraction (SPME) was introduced in the early 1990s as a solvent-free sample preparation technique for analysis of volatile compounds using gas chromatography[52-54]. A convenient and simple technique, its applications have been demonstrated in the sample preparation of semi-volatile compounds and non-volatile compounds such as pharmaceutical products, drugs and pesticide, among many other compounds. Presently, SPME is increasingly

being used with liquid chromatography for determination of pharmaceutical compounds in complex biological and environmental samples[55-57]. The principle of SPME is based on the equilibrium partitioning of analytes between a sample matrix and the extraction phase (coating on a fibre). Figure 1.2 illustrates equilibrium partitioning of analytes between the SPME fibre coating and the sample matrix. According to SPME fundamental principles[52], the amount of analyte extracted by SPME is proportional to the volume of the extraction phase as shown in equation 1.1. The larger the partition coefficient of an analyte between the coating and the matrix, the greater the amount of analyte extracted.

$$n = \frac{K_{fs} V_f V_s C_0}{K_{fs} V_f + V_s} \qquad \text{.. 1.1}$$

where n is the amount of analyte extracted at equilibrium, K_{fs} is the partition coefficient between the extraction phase and the sample matrix, V_f is the volume of extraction phase, V_s is the volume of sample, and C_0 is the original concentration of the analyte. For large sample volumes, $K_{fs}V_f$ $<< V_s$, equation 1.1 can be simplified to equation 1.2

$$n = K_{fs} V_f C_0 \qquad \text{... 1.2}$$

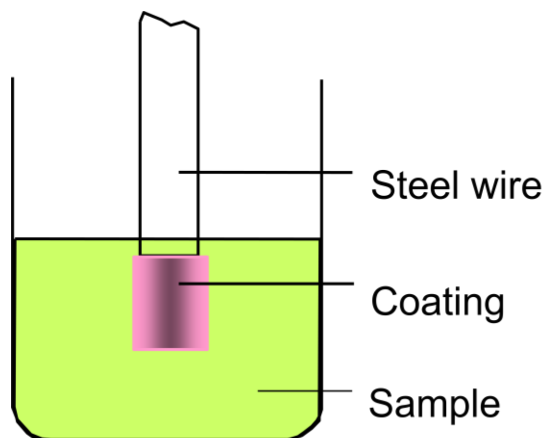


Figure 1:2 Schematic of equilibrium partitioning of analytes between the sample matrix and SPME extraction phase

The mechanism of analyte extraction from sample matrices is either by absorption or adsorption depending on the type of sorbent (extraction phase) used. Since different types of coatings provide different extraction mechanisms for different kinds of analytes, a careful selection of appropriate solvents is necessary during method development. Generally speaking, liquid coatings extract analytes from sample matrix via absorption mechanisms, while solid coatings extract via adsorption. Consequently, the extraction process for absorption- and adsorption-type SPME coatings is different[52, 58], highlighting the importance of SPME coating selection as a critical step to the success of such determinations. SPME methods have three main steps for determination of analytes using liquid chromatography. The first is to extract analytes from a sample matrix; the second is to desorb those analytes directly into solvent in a vial, while the third utilizes instrumental analysis for quantification.

1.6 *In vivo* sampling by SPME

An emerging and important application of SPME technique is its ability to sample analytes from living animals without the requisite destruction of the living system. Indeed, SPME technique can be considered a non-lethal sampling technique option, which, together with the development of biocompatible extraction phases has made possible *in vivo* sampling without major side-effects or toxic consequences to the living system. In fact, SPME engenders minimal disturbances to the investigated system, as no biological fluids and only small fractions of analytes are removed (by non-exhaustive extraction) from the investigated system[59-61]. This unique feature of SPME allows monitoring of chemical changes and partition equilibrium in a living system with minimal disturbance to the system. As a non-lethal sampling technique, SPME has been demonstrated as a viable option in the determination and monitoring of volatile emissions and non-volatile compounds (including pharmaceutical compounds) from plants and animals[62-64]. SPME is increasingly gaining favour for use in *in vivo* sampling due to its unique format and convenience of design. The miniaturization of the technique is a great advantage for *in vivo* sampling because of the ability to deploy and sample analytes in small animals with minimal or no invasiveness, including determinations of pharmaceutical bioconcentration factors[62]. SPME is an environmentally friendly sample preparation technique ideal for *in vivo* sampling technique due to its portability, as well as the integration of sampling and sample preparation into a single step[65].

1.7 Calibration methods in SPME

Calibration of SPME for quantitative analysis of compounds in biological and environmental samples is an important aspect of the methodology, since this technique uses non-exhaustive

extraction and only a small portion of the target analytes are extracted from the sample matrix [66-68]. For *in vitro* studies, traditional calibration methods and equilibrium extraction are the methods most frequently used for quantification analysis. Traditional calibration may utilize external, internal and standard addition methods. Equilibrium calibration is based on equation 1.1, where the amount extracted by the extraction phase is directly related to the initial concentration of analytes in the sample matrix under equilibrium extraction conditions. From equation 1.1, the amount of analyte extracted onto the coating (n) is linearly proportional to the analyte concentration in the sample (C_0), which is the analytical basis for quantification using SPME. In order to accurately determine analyte concentrations by equilibrium calibration, the partition coefficient of each analyte must first be determined experimentally in the appropriate sample matrix where the analyte is found.

For *in vivo* sampling of analytes in living systems by SPME, the traditional calibration method is not relevant as there is no direct spiking of standard to the animal or plant tissues. Further, equilibrium sampling may not be feasible in a living system, particularly for compounds that have longer equilibration times. Therefore, a new kinetic calibration method was developed in order to accurately determine target analytes under pre-equilibrium conditions. Kinetic calibration methods are used under pre-equilibrium conditions to determine concentrations of target compounds in living systems. This calibration method is based on isotropic diffusion-controlled mass-transfer between absorption and desorption process during extraction in a sample matrix[69-72]. The procedure uses desorption of standards preloaded in the extraction phase to calibrate the extraction of analytes from the living system. The initial concentrations of target analytes in the sample matrix, C_0, can be calculated using equation 1.3

$$C_0 = \frac{q_0 n}{K_{es} V_e (q_0 - Q)} \qquad \text{.. } 1.3$$

where n is the amount of analytes extracted pre-equilibrium time, Q is the amount of preloaded standard remaining in the extraction phase (fibre) after sampling, q_0 is the initial amount of deuteurated standard preloaded on the extraction phase before extraction, V_e is the volume of the extraction phase and K_{es} is the distribution coefficient of the analyte between the extraction phase and the sample matrix. Utilizing this kinetic calibration approach, accurate and precise quantification of analytes in living organisms can be achieved with SPME technique.

1.8 Thesis objectives- analytical challenges of analyzing pharmaceuticals in fish and wastewater

The challenge of accurately quantitating active pharmaceutical ingredients (APIs) in the environment is a subject of great interest worldwide. As a result, improvements in analytical methodology (and capabilities), particularly with respect to sample preparation techniques, are important in order to reliably determine these pollutants in environmental and biological samples. Since these contaminants are frequently detected in water and fish samples, development of a simple and suitable analytical methodology to monitor pharmaceuticals in fish tissue and wastewater is paramount.

The main objective of this thesis is to develop improved analytical methods based on solid phase microextraction as the sample preparation method for in *vivo* determination of pharmaceuticals of diverse physicochemical properties in living fish and water. Figure 1.3 showsthe structures of the selected pharmaceuticals used in this study. The analytical methods developed in the course

of this thesis were utilized to investigate uptake and bioconcentration of pharmaceuticals in different fish tissues. In addition, the environmental relevance of this analytical methodology was assessed for determination of pharmaceuticals in fish collected from regional effluent-dominated streams.

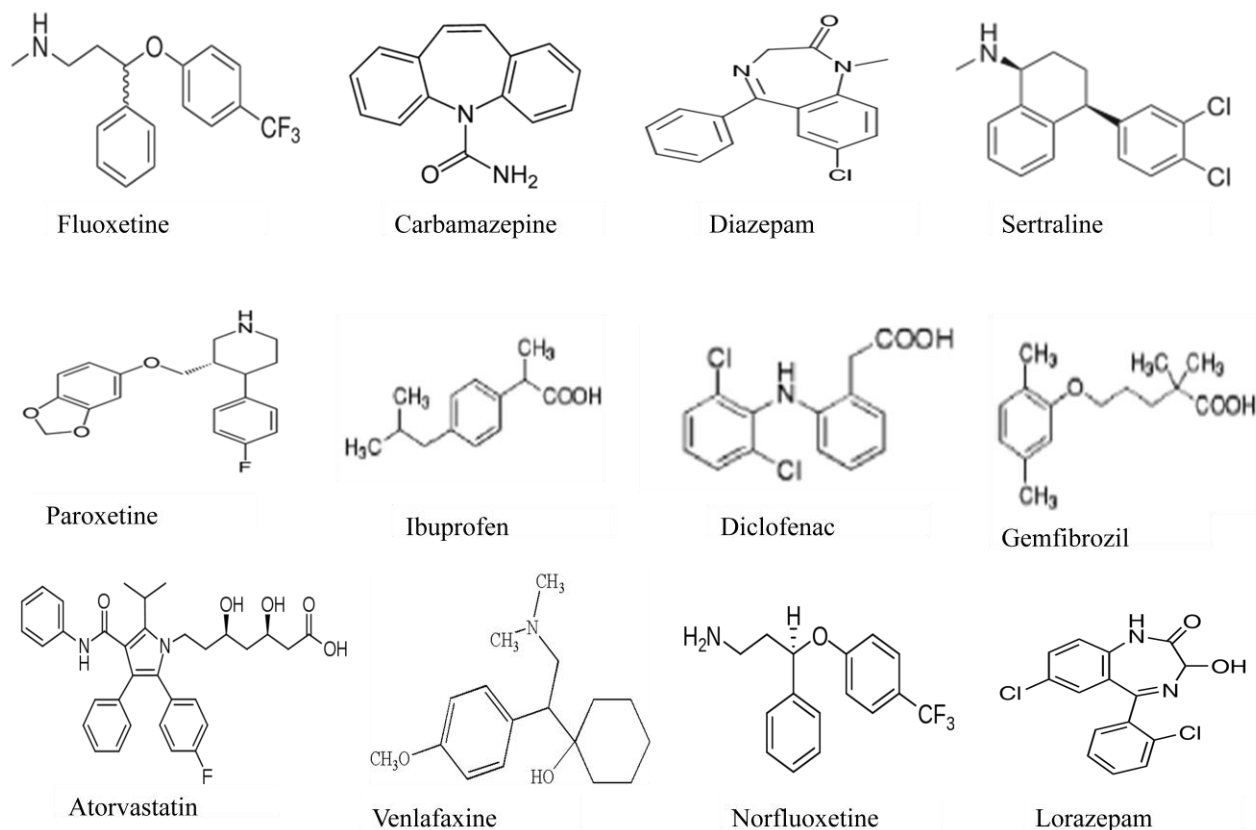


Figure 1:3 Structures of the selected pharmaceuticals utilized in the study

The thesis can be divided into five main parts: First, *in vitro* evaluations of the SPME method developed in fish tissue and gel (mimicking matrix) were carried out. Analytical performance and characterization of the method were also evaluated (Chapter 2). Secondly, the development and application of SPME with liquid chromatography-tandem mass spectrometry (LC-MS/MS) for determination of pharmaceuticals in fish bile is described in Chapter 3. Thirdly, *in vivo*

determination of pharmaceuticals in live fish by SPME with liquid chromatography-tandem mass spectrometry is described in Chapter 4. Fourthly, the development and optimization of automated SPME (based on blade geometry) with liquid chromatography-tandem mass spectrometry method for high throughput analysis of pharmaceuticals in wastewater was investigated and developed (Chapter 5). Fifth, development and evaluation of new *in vivo* SPME device for rapid determination of pharmaceuticals in fish is described in Chapter 6. Finally, the use of *in vivo* SPME method for determination of pharmaceuticals in fish exposed to municipal effluent and wild fish (White Sucker) collected adjacent to a municipal treatment plant outfall is described in Chapter 7. Conclusions of the research are summarized in Chapter 8, in addition to recommendations for future considerations.

Chapter 2

Study of kinetic desorption rate constants in fish muscle and agarose gel model using solid phase microextraction coupled with liquid chromatography with tandem mass spectrometry

2.1 Preamble

This chapter has been modified and published as a paper: O.P. Togunde, K. D. Oakes, M. R Servos, and J. Pawliszyn " Study of kinetic desorption rate constant in fish muscle and agarose gel model using solid phase microextraction coupled with liquid chromatography with tandem mass spectrometry", Oluranti Paul Togunde, Ken Oakes, Mark Servos, and Janusz Pawliszyn. *Anal. Chem. Acta* (2012), doi:10.1016/j.aca.2011.12.034. The figures and tables are reprinted from this manuscript with the permission of Elsevier (Copyright Elsevier 2012).

2.2 Introduction

Solid phase microextraction (SPME) is increasingly used for measuring freely dissolved concentration of compounds in biological and environmental samples due to its simplicity as a sample preparation method for drug analysis [73-75]. Understanding the underlying mechanism of transport (diffusion) of pharmaceuticals from aquatic environments (such as water) into fish muscle and subsequent distribution in fish tissue is very important so as to know the fate and bioavailability of drugs in fish muscle. Some studies have shown that pharmaceuticals in water can diffuse into fish muscle leading to bioaccumulation [14, 40].

In addition, movement (transport) of drugs from water to fish tissue is based on the diffusion pathway, such as partitioning processes between fish tissue and water. Hence, diffusion parameters such as mass-transfer coefficient (diffusion coefficient), absorption and desorption rates are important parameters needed in order to understand the partitioning process. Agarose

gel is being used to study partition process of the drugs because the diffusion of analytes in the agarose gel follows Fick's law of diffusion, in which free movement of drugs is allowed without significant obstruction of the diffusion of drugs in the gel [76-82]. As a result, an agarose gel of different viscosity was used to investigate the kinetic studies of selected drugs in order to find mass-transfer phenomena in both SPME polymer coating and semi-solid matrices such as gel and fish tissue. Previous studies have also shown that the unionised form of most drugs diffuse across the cell membrane readily [77, 83]. As a result, Fick's law of diffusion can be applied to study the diffusion of freely dissolved drugs in fish muscle [84]. Accumulation of drugs in *in vivo* fish muscle is a dynamic and complex process because it involves simultaneous drug uptake, metabolism, transport and excretion until equilibrium is reached between the tissue compartment and interstitial body fluid. Therefore, drug distribution rate can be affected by the rate of blood flow, tissue mass, and partition characteristics of the drugs between blood and tissue compartment [85]. In addition, the porosity of fish tissue and tortuosity (tissue hindrance to diffusion) play a significant role on the rate of diffusion in the tissue sample, thereby affecting distribution of drugs in the fish tissue. In the present study, agarose gel is considered as an alternative for *in vitro* approach of mass transfer phenomenon study.

The aim of this study is to investigate desorption rate constants of selected drugs in tissue samples (fish muscle) and agarose gel using solid phase microextraction as an investigative tool. In this study, kinetic of desorption of the target drugs was studied under specific conditions using different sample matrices. Also, desorption rate constants in gel and fish matrices were compared with the aim to determine the gel concentration which could be used to mimic the diffusion of drugs in fish muscle matrix. Based on free diffusion of drugs, estimation of the mass transfer phenomenon between polymer coating of fibre and gel matrix would provide insight into the

distribution and partitioning of the drugs in a specific matrix. Hence, determination of desorption rate constants of the selected drugs in gel and in fish muscle (*in vitro, in vivo*) are compared with a view of gaining insight into the mass transfer phenomenon in gel matrix and fish muscle.

SPME is a sampling and sample preparation technique which has demonstrated its usefulness for pharmacokinetic studies in biological samples and *in vivo* systems [43, 86]. SPME technique is an equilibrium extraction method in which analytes partition between the extraction phase and sample matrix until equilibrium is reached. Therefore, the amount of analytes extracted at equilibrium (n_e) is governed by distribution constant and volume of extraction phase as shown in equation (1.1) [38, 87]

The diffusion of analytes between fibre coating and sample matrix (fish muscle) follows Fick's law of diffusion, though the diffusion path length in the tissue matrix is greater due the porosity of the matrix (tortuosity). As a result, the kinetic of diffusion of analytes in fish muscle is a slow process which has been described elsewhere [88, 89] as shown in equation (2.1).

$$J \equiv \frac{1 \partial n}{A \partial t} = -D_f \frac{\partial C_f}{\partial x} = -\propto D_s \frac{\partial C_s}{\partial x} \qquad 2.1$$

where J is the mass flux of the analytes from fibre coating to the sample matrix, A is the surface area of the fibre, δn is the amount of analyte absorbed on the fibre surface at a specific time δt, D_f is the diffusion coefficient of analyte in the fibre coating, D_s is the diffusion coefficient of the analyte in the tissue. α is the effective volume fraction (V_0/V) which is usually lower that 1 for soft tissue. V_o is the interstitial space volume and V is the total volume. C_f and C_s are the concentrations of the analyte in the fibre and the sample matrix, respectively.

From equation 2.3, the extraction kinetic (absorption process) to the extraction phase is governed by equation (2.2) [71, 72, 90]

$$\frac{n}{n_e} = 1 - \exp(-at) \qquad 2.2$$

Also, the desorption process (desorption kinetic of preloaded standard) is mathematically expressed in equation (2.3) [90-92]

$$\frac{Q}{q_0} = \exp(-at) \qquad 2.3$$

where n is the amount of analytes in the extraction phase for a particular sampling time t, q_o is the amount of standard preloaded in the extraction phase and Q is the amount of standard remaining in the extraction phase after the extraction phase is exposed to the sample matrix for a particular sampling time t, a is a time constant that is used to describe how quickly equilibrium is reached. Based on the symmetry of absorption and desorption of compounds on the extraction phase, the time constant can be related to the diffusion coefficient in the sample matrix. The concept is based on diffusion controlled mass transfer between the extraction phase and the sample matrix, hence a is described as a parameter that depends on fibre parameters like fibre-sample distribution coefficient and diffusion coefficient of the analytes in the sample matrix [72] as described in equation (2.4)

$$a = \frac{D_s A}{\delta_s K_{fs} V_f} \qquad 2.4$$

where δs is the thickness of boundary layer which is affected by agitation condition, A is the surface area of the fibre coating. All other parameters have been previously defined.

When fibre coating is preloaded {direct extraction from spiked water or Phosphate Buffer Salt solution, pH = 7.4 (PBS)} with analytes of interest and subsequently exposed to the sample

matrix (gel, fish muscle), the mass transfer of the analytes from the extraction phase to the sample matrix is based on free diffusion of analytes due to concentration gradient between the two phases [81, 93-94]. Therefore, the kinetic of desorption of the analytes from the extraction phase to the sample matrix can be described by Fick's laws of diffusion for free diffusion of compounds. As a result, kinetic of desorption, which is described in equation (2.3), can be used to compare desorption rate of analytes in fish tissue and agarose matrix based on free diffusion of analytes. Hence, comparison of the desorption time constant of fish muscle and different gel concentrations would provide valuable information on the mass transfer phenomenon of analytes under *in vitro* condition, thereby yielding information on the gel concentration, which could be used to mimic the rate of desorption in fish muscle. In addition, desorption rate of the compounds can be investigated in *in vitro* and *in vivo* fish muscle, thereby providing information on *in vivo* diffusion of compounds in fish muscle.

2.3 Experimental

2.3.1 Chemicals and materials

All chemicals used for the study were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. Fluoxetine, diazepam, nordiazepam, diazepam d_5 were purchased as certified standards from Cerilliant Corp. (Round Rock TX, USA) and carbamazepine was obtained from Sigma-Aldrich (Oakville ON Canada) while paroxetine and sertraline were purchased from Toronto Research Chemical (Toronto, ON, Canada). Diazepam, nordiazepam and lorazepam were purchased from Cerilliant (Round Rock, TX USA) as 1 mg/mL methanolic solution. Lorazepam was used as internal standard. Also, HPLC grade acetonitrile, methanol acetic acid were purchase from Fisher Scientific (Unionville, ON, Canada). Chemicals used for

the preparation of phosphate buffered saline (PBS, pH = 7.4) were purchased from Sigma Aldrich. PBS was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.20 g of potassium phosphate and 1.44 g of sodium phosphate in one litre of purified water and adjusting the pH to 7.4 as necessary. Also, distilled water used in this study was purified and deionised to 18.3 MΩ with a Barnstead Nanopure Diamond UV water purification system. For the preparation of biocompatible PDMS fibres (silicone tubing), the fibres were purchased from Helix medical silicone tubing (Carpenteria CA) with internal diameter of 0.31 mm and outer diameter of 0.64mm. This silicon tubing was used as extraction phase as described in section 3.2. Medical grade stainless steel wire (0.483 mm o.d.) was purchased from Small Parts Inc. (Miami Lakes FL., USA). The experimental procedures for *in vitro* and *in vivo* study were approved by local Care Committee at the University of Waterloo (AUP #'s 04-24, 08-08). Rainbow Trout (*Oncorhynchus mykiss*) were purchased from Silver creek Aquaculture (Erin, ON, Canada).

2.3.2 Preparation of fibre and SPME procedure

PDMS fibres were made in-house by inserting the silicon tubing (made of PDMS) onto a 4 cm stainless steel wire and conditioning in methanol/water (1:1 v/v) for a minimum of 30 min as described previously [72,85,88]. Following, the preconditioned fibres were loaded directly from a spiked PBS solution at two different concentrations (0.1 and 1 ug/mL) for 3 hours. Organic content (methanol) of all spiked PBS and standards which were used in this study were kept at 1% (v/v) in order to reduce partition of the drugs to organic phase. The rationale for the use of spiked PBS solution for loading the target compounds to the extraction phase is based on the fact that the solution is free of binding matrix, which can interfere with the extraction phase[81].

2.3.3 Desorption of compounds in gel model

Agarose was used as a matrix-free mimicking system (diffusion medium) for the kinetic studies of the modelled drug because it allows free diffusion of drugs in a semi-solid matrix. Hence, useful information such as rate of diffusion and diffusion coefficient can be estimated from mass-transfer kinetics of the drugs in the gel [71, 90, 91]

Kinetic desorption of model drugs were performed at different concentrations (viscosity) of gel matrix prepared in PBS (pH = 7.4).The concentration (viscosity) used ranged from 2.0 % (w/v) to 0.8 % (w/v). For example, 2.0 % (w/v) gel matrix was prepared by adding 2 g agarose gel (Agarose 15, BDH Chemical Ltd, Poole England) with 100 ml of PBS. Subsequently, the mixture heated for about 30 min at temperature 90°C until the agarose completely dissolved in PBS and a clear solution is observed. The solution of the agarose is stirred intermittently to ensure that the solution is homogeneous and the temperature is held at 70°C when dispensing the solution to vials. Having prepared the gel solution, 3 ml of the gel solution is dispensed to a 4 ml vial where the solution is allowed to cool to ambient temperature. Consequently, a semi-solid gel is obtained when the sample is allowed to stay at ambient temperature for about 3 h of stabilization.

Moreover, the amount of the drug "preloaded" (direct extraction from PBS, pH = 7.4) on the fibre can be determined by direct extraction of the compounds in a known spiked PBS solution (0.1 mg/L used previously). The fibre is introduced to a spiked PBS solution in a 2ml vial while the extraction is carried out at a predetermined time or until equilibrium is reached between the fibre and the sample matrix. The extraction is carried out under agitated condition at 1000 rpm on an oscillating shaker. Subsequently, the fibre was desorbed in a mixture of acetonitrile: water {ACN/H_20 (1:1 v/v)} under an agitated condition similar to the extraction process. Therefore, the amount preloaded on the fibre (q_0) is determined by LC/MS/MS quantitation of the sample

extract. The amount of compound remaining on fibre (Q) at different times of kinetic desorption was determined by preloading the fibre from a spiked PBS solution. After kinetic desorption in gel matrix, the fibre is desorbed in desorption solvent {ACN (acetonitrile) /Water 1:1)} in order to determine the amount of analytes remaining on the fibre. However, subsequent desorption of the preloaded fibre was done in a known gel sample for a specific time (10 -1600 min). Therefore, the amount of compounds remaining on the fibre for a specific time was determined by desorbing the fibre again in ACN/ water (1:1) immediately as the fibre was removed from the gel sample.

In the case of fish muscle (*in vitro* study), the muscle was collected from Rainbow Trout held in clean reference water. The fish muscle was immediately stored at -80°C until it was used for this study. The muscle was cut and weighed into centrifuge tube, then preloaded fibres (loaded with the target compounds) were desorbed in fish muscle (unhomogenized) in the centrifuge tube for specific time ranging from 10 – 300 min. This is similar to desorption kinetic study performed in gel matrix. The amount of the analyte remaining on the fibre after each timepoint was determined by desorbing the fibre in the desorption solution (ACN: Water, 1:1) after the preloaded fibre was removed from each centrifuge tube. After desorption for 90 min, 20 uL of the sample extract was injected to the LC/MS/MS for instrumental quantification.

2.3.4 *In vivo* desorption kinetic study in fish

In this case, Rainbow Trout were kept in a 34L aquarium tank where the fish were allowed to acclimatize to the laboratory conditions of the well water for 1 day. Subsequently, the preloaded compounds on the PDMS fibres were desorbed in the live fish for different times (0.5 – 50 hrs), in which the fibres were inserted in fish muscle (dorsal muscle of Rainbow Trout). After each of

the fish was anesthetized, a 12- gauge needle was used to pierce the fish muscle, and then the fibres were inserted in the muscle. After the insertion of the fibre (n = 3), the fish were returned to the tank, and, within 1 min, the fish were able to regain their consciousness. After a specified period of time, the fibres were removed from the fish muscle. Consequentely, the fibres were desorbed in a mixture of water/acetonitrile (1:1) containing internal standards and the amount of the target drugs remaining on the fibre at each time point was determined.

2.3.5 Evaluation of PDMS and C_{18} extraction phase

The performance of biocompatible coatings (C_{18} and PDMS) was evaluated and compared at equilibrium. Extraction of the selected compounds was carried out in spiked buffer at pH 7.4. PBS buffer solution was spiked with selected compounds at a concentration 50 ng/mL and subsequently, extraction was performed with the fibres for 3 h with agitation speed of 1000 rpm. After extraction, the analytes were desorbed from the fibre to the desorption solvent under the same agitation conditions for 90 min. The results obtained for the comparison of the two fibres are shown in Figure 2.1.

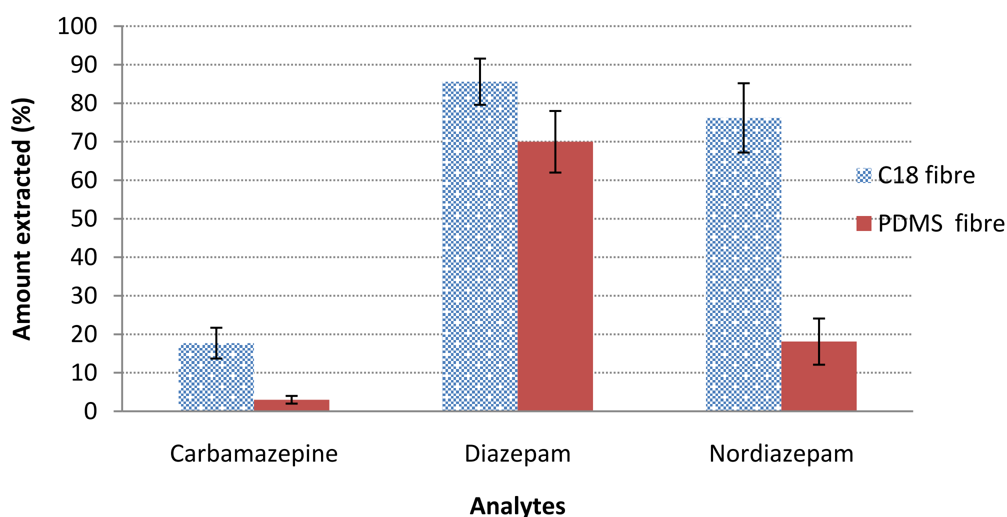


Figure 2:1 Extraction efficiency of PDMS and C_{18} fibres in phosphate buffer solution (PBS pH = 7.4)

2.3.6 Instrumental analysis – LC/MS/MS

Analysis of gel and fish samples was carried out using LC/MS/MS system that is made up of the following: Varian 500 LC-ion trap-MS, Prostar model 430 autosampler and Varian 212-LC pump (Walnut creek, CA USA). The mobile phase used was A: acetonitrile: water: acetic acid (100:90:0.1 v/v) while B contains acetonitrile: water: acetic acid (90:10:0.1 v/v). The total solvent A and B were gradually programmed as: 100% held for 0.5 min, subsequently, there was linear increase to 90% B in 2.5 min, held for 2 min. Then the column is re-equilibrated for 1 mins at 100% A. Therefore, the total chromatographic run for the separation of target compounds is 6 min. All the compounds were optimized on the MS by direct infusion of 1 µg/mL standards of each of the compound using electrospray ionization in positive ion mode; the dry gas temperature was set at 400°C. For the chromatographic separation of the compounds, a Supelco 3.5 µm C_{18}, analytical column was used, having dimensions of 50 mm by 2 mm. The flow rate used was 0.3mL/min while 20µL of the sample was injected to the MS in duplicate. After all data acquisition was obtained, the processing was done on Varian MS workstation version 6.6. The optimised parameters on the MS are summarized in Table 2.1a and 2.1b.

Table 2:1 a The retention time and MS parameters of the compound studied using Varian MS/MS.

Analytes	Retention time (mins)	Q1 Mass (amu)	Q3 Mass (amu)	Needle Voltage (V)	Capillary Voltage (V)	RF loading (%)	Excitation amplitude (V)
Carbamazepine	2.8	237	194	3920	67	70	1.00
Diazepam	3.4	285	257	3920	85	79	1.31
Nordiazepam	3.2	271	140	3920	85	77	1.06
Lorazepam (IS)	3.0	321	275	3920	62	88	2.02

RF- Radio frequency

Table 2.1b The MS parameters of the compounds studied using Sciex QTrap MS/MS

Compound	Precursor	Product	DP (volts)	EP (volts)	CEP (volts)	CE (volts)	CXP (volts)
Carbamazepine	237.1	193.3	55	4.9	14	51	3
Fluoxetine	310.3	44.3	48	2.9	12	44	7
Diazepam	285.5	154.2	71	4.0	37	4	10
Nordiazepam	271.1	140.2	176	7.0	39	4	20
Paroxetine	330.1	70.1	51	5.5	45	4	14
Sertraline	306.0	159.0	26	3.5	35	4	14
d_{10}-carbamazapine	247.2	204.4	61	4.3	17	28	3
d_5-fluoxetine	315.2	44.2	50	4.0	19	38	3
d_5-diazepam	290.0	198.3	66	2.0	12	39	3
d_4-paroxetine	334.0	74.0	106	7.0	14	51	12
d_3-sertraline	309.0	275.0	36	3.0	14	15	8

DP- Declustering potential, EP- Entrance potential, CEP- Collison entrance potential, CE- Collision energy, CXP- Collison exit potential.

The MS response in terms of sensitivity and linearity was monitored for each set of samples injected by running a series of standards for each of the compounds (0.5 – 1000 µg/L) before and

after each set of samples. Also, the quality of the data obtained from the MS was monitored during each run of the sample by injecting a quality control sample of 50µg/L for every 12 injections. Good precision was obtained with an external calibration curve (RSD < 7%), and linearity ($R^2 > 0.99$) was good as well. *In vivo* study of the selected compounds was carried out using ABS-Sciex 3200 QTrap Mass Spectrometry equipped with a Turbo Ion Spray source (Applied Biosystems Sciex, Foster City, CA, USA). Liquid chromatography (LC) was performed on a HP1100 HPLC system (Agilent Technologies) equipped with a degasser, a binary pump, an autosampler and a column oven. Chromatographic separation was performed on a Zorbax Eclipse XDB C_{18} (150 mm × 21 mm, 3.5µm) column which was preceded by a C_{18} guard column at a flow rate of 0.8 mL/min with a mobile phase A (95% water, 5% methanol, 0.1 % acetic acid) and B (95% MeOH, 5 % water and 0.1% acetic acid). The compounds of interest were separated with a gradient elution program and the injection volume was set at 20 uL. The gradient elution was programmed as follows: mobile phase B was increased from 10% to 50% in 0.5 min and 50% to 100% in 7.5 min, held at 100% for 2 min and then reduced to 10% in 1 min, thereby bringing it back to initial chromatographic conditions within 5 min. Detection and quantification was done with a 3200 Qtrap triple quadrupole-linear ion trap mass spectrometer. The limit of detection (LOD) and Limit of Quantitation (LOQ) were determined using a statistical approach in which the standard deviation of the background signal of the blank sample were calculated and divided by the slope of calibration ($LOD = 3*SD_{blank}/slope$ and $LOQ = 10*SD_{blank}/slope$) where SD is the standard deviation of seven blank samples.

2.4 Results and discussion

2.4.1 Characterization of SPME extraction phase

The performance of two biocompatible coatings, namely PDMS and Supelco C_{18} fibres, were evaluated in PBS (pH =7.4) under the same condition in order to compare the amount of analytes extracted at equilibrium for the selected drugs. In general, the C_{18} fibres have higher amounts of extracted analytes compared to PDMS fibres. As expected theoretically, the results demonstrate that the C_{18} fibre has a higher amount of extracted analytes in a simple or clean matrix such as PBS solution since the fibre behaves as a porous extraction phase (coating) [58]. In addition, hydrophobicity of each of the compounds will affect the extraction efficiency of the fibre. Since detection (sensitivity) of the selected drugs is good, any of the two fibres can be used for this study. In this study, PDMS fibre is used since it behaves as a biocompatible liquid polymer coating, which has been used for study of pharmaceuticals in fish and it is not prone to displacement or competitive effects [43, 58]. The detection and quantitation limit of SPME method determined in gel and fish matrices are shown in Table 2.2.The detection limit in gel ranged between 0.01 – 0.07 ng/ml, while in fish muscle it is between 0.07 - 0.34 ng/g.

Table 2:2 Limits of detection and quantification in gel and fish muscle

	Gel		Fish	
Compounds	LOD (ng/mL)	LOQ (ng/mL)	LOD (ng/g)	LOQ (ng/g)
Carbamazepine	0.01	0.10	0.07	0.48
Diazepam	0.02	0.19	0.30	0.97
Nordiazepam	0.07	0.20	0.34	0.40

2.4.2 Absorption time profile in gel

The kinetics of absorption of the selected pharmaceuticals was investigated in the gel model (1 % w/v) and PBS solution in order to determine the equilibration time between fibre coating and gel matrix (or PBS solution). Also, useful information such as absorption rate can be extrapolated from the study. Therefore, the diffusion rate and partition processes of the selected drugs can be determined in a simple sample matrix such as gel. It can be deduced from observing the extraction time profile that the results obtained for static extraction in gel matrix clearly demonstrate that equilibrium is reached at 40 hours for all compounds, except for diazepam (Figure 2.2). As a result, a longer extraction time is required for diazepam to reach equilibrium because of its high distribution constant. The diffusion of the selected pharmaceuticals in gel matrix is generally slow due to the nature of the sample matrix. Essentially, gel samples are more viscous than water or PBS, and as a result, the diffusion of the drugs is slower; consequently the diffusion coefficient would be small due to slow mass transfer rate. In addition, the molecular weight and partition constant of each drug may also affect the partition and diffusion process.

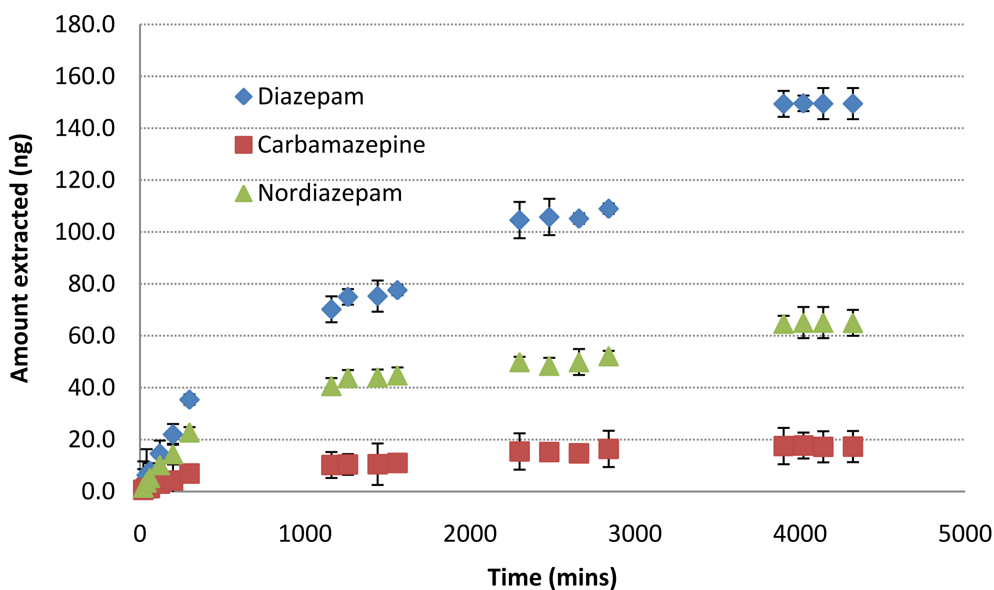


Figure 2:2 Extraction (absorption) time profiles of carbamazepine, diazepam, and nordiazepam in gel sample (0.9% w/v) at room temperature (25 °C).

2.4.3 Absorption rate constant in gel

The extraction time profiles of the selected drugs under study was obtained by analyzing freshly spiked gel samples that were extracted under static condition (without agitation) between 0.5 to 70 h at a constant room temperature (25 °C). The kinetics of extraction and partition of organic compounds to SPME fibre has been described in the literature in detail [95-97]. In this study, the extraction time profile of selected drugs in gel samples (0.9% w/v) under static conditions is shown in Figure 2.2. A consistent pattern of diffusion was observed for the extraction time profile in the gel sample without agitation, although the extraction rate is much slower compared to the aqueous sample. Therefore, the results suggest that the extraction process in gel samples is governed by Fick's law of diffusion. However, the diffusion coefficient in the gel sample is smaller due to obstructive and tortuosity effects in gel sample [78,89].

In addition, the absorption time (rate) constant is determined from the extraction profile data by using a linear regression for the plot of natural logarithm of $(1-n_t/n_e)$ against the extraction time, where n_t is the amount of analyte extracted at a specific time. The slopes of the regression are extrapolated as the absorption rate constant, as can be seen in Table 2.3.

Table 2:3 Absorption rate constant in gel sample (0.9 % w/v)

Compounds	Rate constant (a, min^{-1}) ± SE	R^2
Carbamazepine	$17.4 \times 10^{-4} \pm 7 \times 10^{-4}$	0.9813
Diazepam	$11.9 \times 10^{-4} \pm 5 \times 10^{-4}$	0.9883
Nordiazepam	$18.7 \times 10^{-4} \pm 5 \times 10^{-4}$	0.989

SE – Standard error

The absorption rate of carbamazepine and nordiazepam has a similar rate of diffusion in the gel, while the diazepam rate of diffusion is slower based on desorption time constant (Figure 2.3).

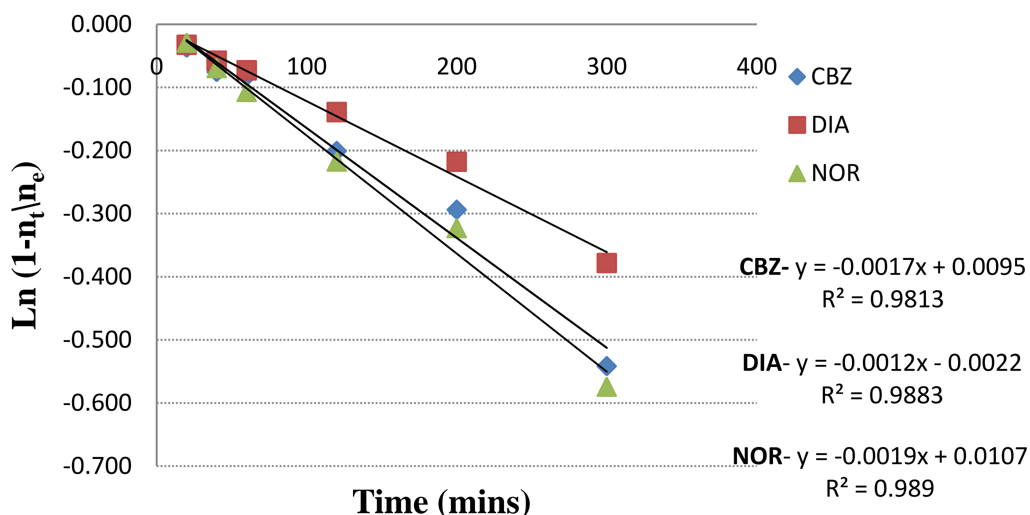


Figure 2:3Absorption time profiles of carbamazepine (♦), diazepam (■), and nordizepam (▲) in gel; sample (0.9% w/v) at room temperature (25 °C).

The time constant rate of each compound being investigated reflects how fast the equilibrium is reached between SPME and sample matrix, since time constant is concentration independent [89]. The rate of diffusion of each drug can be determined by its corresponding time constant. As a result, the diffusion rate (time) constant provides insight and useful information (diffusion coefficient) on the diffusion rate of the compounds in different sample matrices[72]. This study demonstrates one of the applications of SPME as a simple investigative tool to study the diffusion and partitioning process of compounds in a semi-solid sample matrix such as gel. The mass transfer kinetic of absorption (extraction) and desorption of drugs on SPME fibre are similar, since both are diffusion-controlled mass transfer processes based on Fick's first law of diffusion [72, 87]. Also, the diffusion of drugs in and out of the extraction phase (fibre) is controlled by a number of factors, such as concentration gradient, distribution coefficient of the analytes and boundary layer in the sample matrix. In the case of absorption, there is an upward mass

transfer of analytes from the sample matrix to the fibre due to the concentration gradient and partition process until equilibrium is established between the two phases. However, the desorption process involves mass transfer of analytes from the pre-loaded fibre to the sample matrix such as fish muscle, where a partition process is established, as previously discussed. Hence, the concept of standard on the extraction phase is a very useful technique for *in vivo* studies where the direct spiking of sample is not feasible.

2.4.4 Equilibrium partition constant in gel and PBS (pH = 7.4)

The equilibrium partitioning process was studied in gel model (0.9 % w/v) and compared to a matrix-free sample (PBS) in order to investigate if there is potential binding in the gel samples with respect to the compounds being studied. As shown in Figure 2.4, the amount extracted at equilibrium in PBS and gel samples were the same.

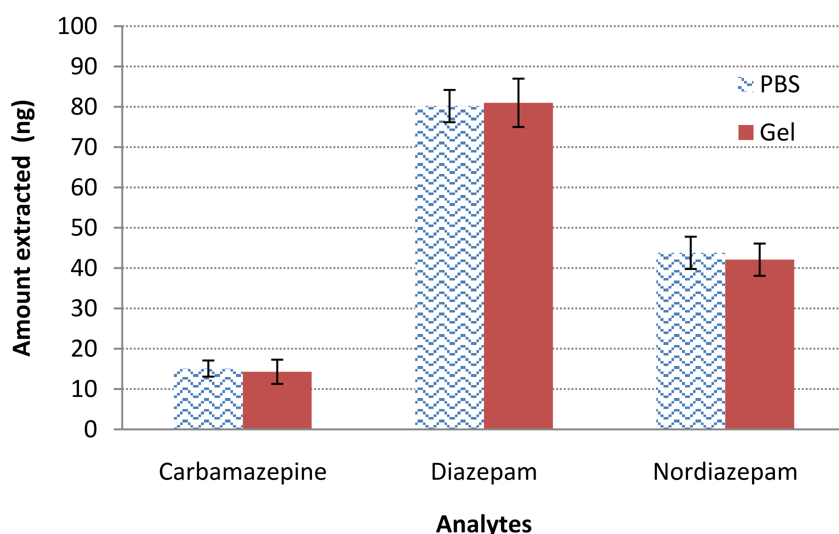


Figure 2:4 Equilibrium recovery studies of carbamazepine, diazepam, and nordizepam in gel sample (0.9% w/v) and phosphate buffer solution (PBS).

The results show that there is no significant difference in terms of the equilibrium partition constant between the gel and PBS for the selected pharmaceuticals. In addition, it was observed that the drugs under investigation reached equilibrium faster in PBS than in gel. One possible explanation for this observation is that PBS is an aqueous sample while the gel is a semi-solid, whose diffusive mass transfer rate is slower because of the high viscosity of the gel sample compared to simple aqueous PBS. As a result, faster distribution of drug and equilibration might have happened in the PBS sample. Hence, the diffusion and partitioning processes in PBS and gel are presumed to be largely controlled by the diffusion coefficients of the drugs in the sample matrix, although the thickness of the boundary layer may play a significant role as well during diffusion of the drugs in sample. In addition, as shown in Table 2.4, physico-chemical properties of the drugs such as their particular molecular weight, solubility and partition coefficient can also affect the diffusion coefficient of the drugs in sample matrix [78, 80, 94]

Table 2:4 Physico-chemical properties of the compounds [98, 99]

Analytes	pKa	Estimated Log Kow	Molecular weight
Carbamazepine	7	2.45	236.3
Diazepam	3.4	2.82	284.8
Nordiazepam	N.A	2.93	270.7

2.4.5 Desorption rate constant in gel models

Desorption rate constant of the selected drugs was studied in different concentrations (viscosity) of gel sample ranging from high concentration (highly viscous) of gel to very low concentration (less viscous), as shown in Table 2.5.

Table 2:5 Desorption rate constant of selected drugs in different concentration of gel and unhomogenized fish tissue

		Fish tissue		
Compounds	Time constant (a, min^{-1})	Standard Error	Estimated RSD (%)	R^2
Carbamazepine	0.00076	8 E-05	11	0.9742
Diazepam	0.00051	2 E-05	4	0.9781
Nordiazepam	0.00077	4 E-05	5	0.9792
		0.8 % (w/v) gel matrix		
	Time constant (a, min^{-1})	Standard Error	Estimated RSD (%)	R^2
Carbamazepine	0.00083	1 E-04	12	0.9002
Diazepam	0.00055	1 E-04	14	0.9004
Nordiazepam	0.00076	1 E-04	9	0.9698
		1.0 % (w/v) gel matrix		
	Time constant (a, min^{-1})	Standard Error	Estimated RSD (%)	R^2
Carbamazepine	0.00057	5 E-05	9	0.9185
Diazepam	0.0004	8 E-05	15	0.7482
Nordiazepam	0.00066	8 E-05	14	0.8721
		1.5 % (w/v) gel matrix		
	Time constant (a, min^{-1})	Standard Error	Estimated RSD (%)	R^2
Carbamazepine	0.00022	3 E-05	15	0.8480
Diazepam	0.00012	1 E-05	12	0.8990
Nordiazepam	0.00019	2 E-05	14	0.8660

	2.0 % (w/v) gel matrix			
	Time constant (a, min^{-1})	Standard Error	Estimated RSD (%)	R^2
Carbamazepine	0.000154	1 E-05	7	0.9617
Diazepam	0.000167	4 E-05	15	0.9000
Nordiazepam	0.000163	1 E-05	8	0.9491

The results suggest that as the concentration of gel increases from a less viscous to a high viscous gel matrix, desorption rate constant of the drug decreases. This observation can be explained in line with the viscosity of the gel. As the viscosity of the gel reduces, the diffusion of the drugs in the gel becomes faster. However, as the viscosity of the gel increases, the diffusion of the drugs becomes slow. Consequently, the mass transfer coefficient would be small, leading to a reduction in desorption rate constant in the gel. In addition, an increase in gel concentration would result in increased tortuosity and lead to a decrease in desorption rate constant [76]. Figure 2.5 shows the schematic experimental model for the study of mass transfer rate constant in agarose gel and *in vitro* fish samples. Also, desorption rate constant in unhomogenized fish muscle (Rainbow Trout) studied using the same SPME procedure is summarized in Table 2.5. Hence, a comparison of desorption rate constant in fish muscle under investigation suggests that the rates of desorption kinetics of the model drugs in the unhomogenized fish muscle and 0.8% (w/v) are similar, based on the similar values obtained for desorption rate constants for both sample matrices.

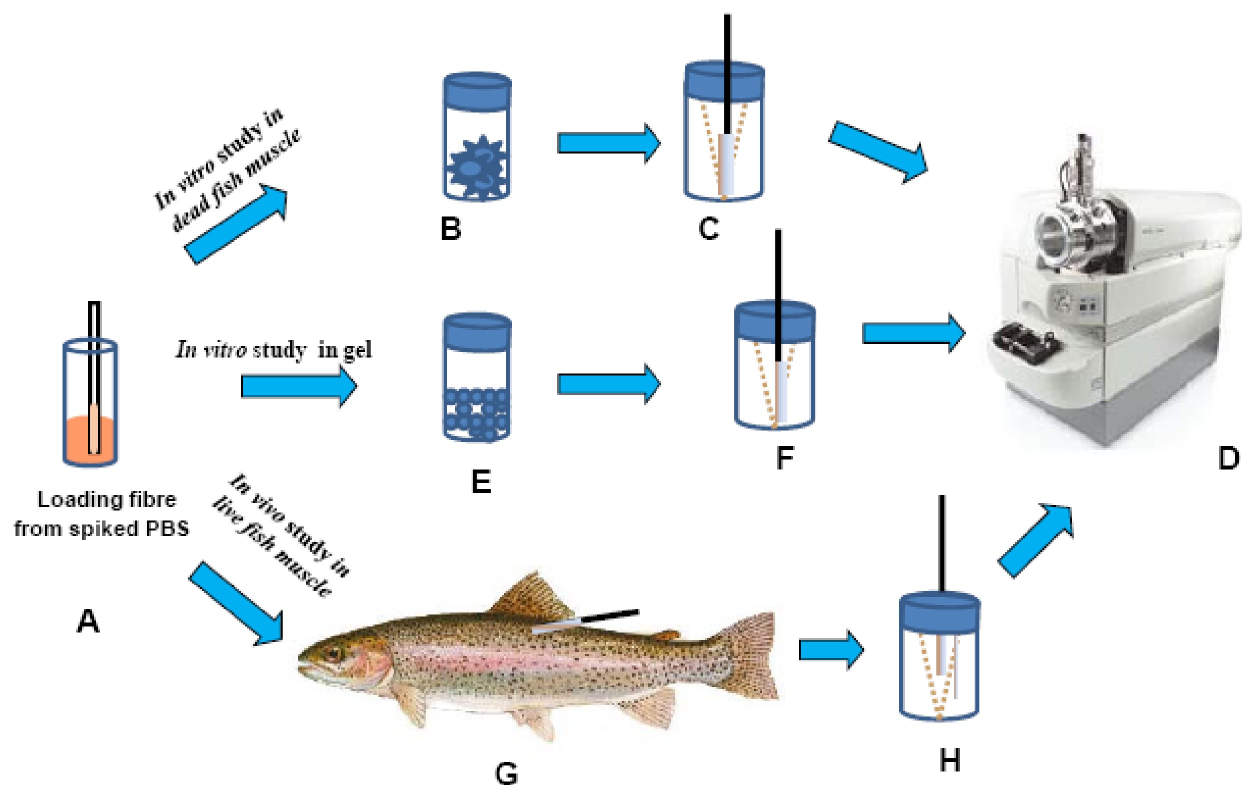


Figure 2:5 Schematic diagram of experimental model for the study of mass transfer rate constant in agarose gel, dead and live fish muscle

A- Preloading of standard to the fibre from spiked PBS (pH = 7.4)
B- *In vitro* desorption of preloaded fibre in dead fish muscle at different time
C- Desorption of fibre in acetonitrile: water (1:1) for 90 min with agitation speed of 1000 rpm
D- Instrumental analysis and quantification
E- *In vitro* desorption of preloaded fibre in the agarose gel at different time
F- Same as C
G- *In vivo* desorption of preloaded fibre in live fish muscle at different time
H- Same as C

2.4.6 *In vivo* SPME application in fish muscle

Desorption rate constants of the selected drugs was determined in live fish (Figure 2.5). As shown in Table 2.6a, carbamazepine displays faster diffusion in both gel and fish samples when compared to diazepam and nordiazepam, based on their desorption rate constants. In addition, faster diffusion and equilibration was observed for *in vivo* desorption kinetics of the drugs compared to the *in vitro* desorption study in fish muscle, as shown in Table 2.6a and b, although carbamazepine, nordiazepam and sertraline showed a different pattern. For example, there was no significant difference in terms of the desorption rate of carbamazepine for *in vitro* and *in vivo* fish muscle.

Table 2:6 a *In vivo* desorption time constant in Rainbow Trout muscle

Compounds	Time constant (a, min^{-1})	Standard error	Estimated RSD (%)	R^2
Carbamazepine	0.00150	1.6E-04	11	0.9654
Diazepam	0.00015	1.9E-05	13	0.9238
Nordiazepam	0.00032	3.1E-05	10	0.9627
Fluoxetine	0.00039	3.3E-05	9	0.9710
Sertraline	0.00041	6.1E-05	15	0.9176
Paroxetine	0.00036	2.0E-05	6	0.9880

Table 2.6b: ***In vitro* desorption time constant in Rainbow Trout muscle**

Compounds	Time constant (a, min^{-1})	Standard error	Estimated RSD (%)	R^2
Carbamazepine	0.00142	6.9E-05	5	0.9908
Diazepam	0.00003	5.9E-06	17	0.9196
Nordiazepam	0.00039	2.7E-05	7	0.9756
Fluoxetine	0.00012	6.0E-06	5	0.9907
Sertraline	0.00062	5.1E-05	8	0.9863
Paroxetine	0.00016	1.7E-05	10	0.9686

RSD- Relative standard deviation

In the case of sertraline and nordiazepam, the *in vitro* desorption rate constant is greater than the desorption rate constant determined in the *in vivo* fish muscle. One possible explanation for the observed difference between the two systems (*in vivo* and *in vitro*) under study is that the diffusion of compounds and its subsequent desorption in a complex and dynamic system such as live fish would be aided by potential agitation, coupled with the flow of the blood and biological fluids. As a result, there is facilitated diffusion/ transport of drugs in a dynamic system whose time constant would be affected compared to non-dynamic systems (dead fish muscle), where potential agitation does not occur. Therefore, the kinetics of diffusion of drugs in dead fish is likely to be different live fish, considering that the latter is a dynamic system. In conclusion, there is a need for careful extrapolation of rate constant or diffusion constant when comparing the kinetic of drugs in an *in vitro* and *in vivo* system (dead and live fish muscle).

2.5 Conclusion

The study demonstrates that desorption rate constant in 0.8 % w/v gel matrix and unhomogenized fish tissue are similar based on the free diffusion of drugs in the matrices. Therefore, this gel composition can be used as a template to study the kinetic of diffusion as well as its partition processes, in order to understand diffusion of drugs in fish tissue. In addition, it can be deduced that desorption rate constant of selected pharmaceuticals in a dynamic system (such as live fish) is different from a static system (such as dead fish). Also, SPME method has been demonstrated as a simple investigative tool to study the diffusion rate constant of organic compounds (pharmaceuticals) in stable model systems such as gels as well as a dynamic systems (live fish tissue). Hence, the SPME method is a promising analytical tool which can be used for the study of diffusive mass transfer in an *in vivo* or dynamic system such as fish. Also, the bioavailability of target compounds in fish tissue may be investigated through the use of SPME technique. Lastly, these studies demonstrate that the diffusion and equilibrium partitioning processes can be studied and determined in a dynamic system.

Chapter 3

Determination of Pharmaceutical Residues in Fish Bile by Solid-Phase Microextraction Coupled with Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS)

3.1 Preamble

This chapter has been modified and published as a paper: Oluranti P. Togunde, Ken. D. Oakes, Mark. R Servos, and Janusz Pawliszyn. 2012. "Determination of Pharmaceutical Residues in Fish Bile by Solid-Phase Microextraction Couple with Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS)". Environmental Science and Technology 46 (10):5302-5309. The figures and tables are reprinted from this manuscript with the permission of American Chemical Society (ACS 2012).

3.2 Introduction

The frequent detection of pharmaceuticals in aquatic environments has elicited concern in both public and scientific arenas due to the potential endocrine modulating impacts of these compounds on exposed aquatic organisms[100-102].The presence of pharmaceuticals in surface waters has been largely attributed to low removal efficiencies for many of these compounds in municipal wastewater treatment plants, which were not designed to remove these trace contaminants from their effluents[103-105.] Consequently, human pharmaceutical residues have been recognized as "emerging" environmental pollutants due to their near ubiquitous detection adjacent urban areas at trace or ultra-trace levels; a phenomenon accelerated by rapid urban growth and aging population demographics[43, 106-107.] Continuous release of these compounds and their bioactive metabolites and degradation products within municipal wastewater discharges often results in constant low-level exposure for organisms inhabiting such receiving

environments[108-110]. Consequently, the presence, uptake and bioconcentration of these pollutants by aquatic organisms should be monitored to characterize the environmental persistence and potential impact on exposed organisms.

Fish bile can be useful in assessing the exposure and uptake of xenobiotic compounds from water as biliary excretion is an important removal mechanism for many environmental contaminants, and considerable bioaccumulation of analytes may occur in this complex matrix.[111-113] Water insoluble contaminants are eliminated from organisms by either hydroxylating or metabolizing to more water-soluble forms, often with further conjugation to larger water soluble molecules to facilitate excretion. Glucuronidation is often the main conjugation process leading to the formation of concentrated environmental contaminants in the bile. Enzyme-assisted hydrolysis can cleave conjugated compounds and facilitate the detection of parent pharmaceuticals in fish bile[114-116]. Consequently, the detection of unmodified parent compounds or their metabolites in bile can be used as biomarkers of pharmaceutical uptake by fish exposed to these emerging contaminants in aquatic receiving environments.

Recently, polydimethylsiloxane (PDMS) as SPME liquid fibre coating (extraction phase), was evaluated in several complex matrices, where it demonstrated its suitability for extraction of pharmaceuticals in fish tissue without significant biofouling and with sorptive properties unaffected by the matrix composition.[47,117-118] SPME is advantageous as an analytical tool within complex matrices such as biological tissues because it incorporates extraction and clean-up into a single step and often has no significantly matrix effects, particularly when internal standard is used.[119] The aim of this study is to develop and validate an analytical method based on SPME to investigate uptake and bioconcentration of pharmaceuticals in fish bile. Recently C_{18} extraction phase (fibre) has also been successfully used for bioanalysis of moderately polar or non-polar

compounds, and may extract a greater range of analytes than are extractable by PDMS fibre coatings, particularly polar compounds.[120] In the case of hydrophobic compounds such as atorvastatin, the PDMS extraction phase will provide higher recovery. PDMS and C_{18} fibres were used as the extraction phases for SPME method to determine concentration of target pharmaceuticals using small volumes of fish bile samples, collected from fish exposed to selected pharmaceuticals in the laboratory and caged fish in the field near a municipal wastewater treatment plant effluent outfall. The development of SPME protocol for assessing exposure and uptake of environmental pharmaceutical residues would be of significant advantage for both fundamental research and biomonitoring purposes. One of the major advantages of SPME is that it is a simple, solvent-free, environmentally friendly extraction technique that integrates sampling and sample preparation into a single step.

Based on the basic fundamentals of the method, the amount of analyte extracted by SPME at equilibrium is governed by the distribution constant between the extraction phase and samples matrix as shown in equation (1.1). When equilibrium partition of analytes between the extraction phase and the sample matrix is achieved, the concentration of analyte in the sample can be determined, since the amount of target compound on the extraction phase is directly proportional to the concentration of the analyte in the sample using equilibrium calibration approach. During the method development, important parameters such as partition constant (K_{fs}) are determined in the real samples (bile). The ability of SPME technique to determine free or total concentration of analytes from a single biofluid sample is dependent on using appropriate calibration strategies. In order to determine the total concentration of analytes in bile samples, matrix-matched calibration is used by performing the calibration using standards prepared in blank bile samples or biological fluid of interest. Then, the amount of analyte extracted by SPME at equilibrium is

plotted against the total analyte concentration (analyte concentration spiked in the biological fluid). Based on this equilibrium calibration approach, the total concentration of analytes can be determined in the real samples. In addition, measurement of free concentration of compounds in biological samples by SPME can provide important information about the bioavailability of a chemical and binding affinity or partition coefficient of a compound.[121, 122]

3.3 Experimental section

3.3.1 Chemicals and supplies

All chemicals used in this study were obtained at the highest available purity and used without further refinement. Fluoxetine, diazepam, nordiazepam, and diazepam-d_5 were purchased as certified standards from Cerilliant Corp (Round Rock, TX, USA). Gemfibrozil, atorvastatin, ibuprofen, atrazine, carbamazepine, diclofenac, naproxen, and bisphenol-A (BPA) were obtained from Sigma-Aldrich (Oakville, ON, Canada) while paroxetine and sertraline were purchased from Toronto Research Chemical (ON, Canada). Isotopically-labeled atorvastatin-d_5, atrazine-d_5, BPA-d_{16}, carbamazepine-d_{10}, diclofenac-d_4, fluoxetine-d_5, gemfibrozil-d_6, ibuprofen-d_3 and ^{13}C-naproxen-d_3 were purchased from CND Isotope Inc (Point-Claire, QC, Canada) while sertraline-d_3 and paroxetine-d_4 were purchased from Toronto Research Chemical. Chemical stock solutions were prepared in methanol and stored at -20°C while working solutions were diluted aliquots of these stock solutions. Dilution water was obtained from a Barnstead Nanopure diamond UV water purification system deionized to 18 Ω. Deconjugating enzymes (β-glucosidase, β-glucoronidase and sulfatase) as well as methanol (HPLC grade) were obtained from Sigma-Aldrich. All animal experimental procedures were approved by the local Animal Care Committee at the University of Waterloo (AUP #'s 04-24, 08-08). Rainbow Trout

(*Oncorhynchus mykiss*) were purchased from Silver creek Aquaculture (Erin, ON, Canada) while Fathead Minnow were obtained from Silhanek Baitfish (Bobcaygeon, Ontario).

3.3.2 Fish exposures

Laboratory exposure to carbamazapine and fluoxetine. The juvenile Rainbow Trout (*Oncorhynchus mykiss*) used in the carbamazepine (CBZ) exposure (14.4±0.34 cm; 25.4±1.46 g, mean ±SE, *n*=22) and the fluoxetine (FLX) exposure (15.4±0.26 cm; 29.3±1.18, *n*=30) were purchased from Silver Creek Aquaculture (Erin, ON Canada). Fish were acclimatized to laboratory conditions in continuously flowing non-chlorinated well water and fed every other day with 2.0 Pt floating commercial trout ration (Martin's Feed Mill, Ontario) until 4 d prior to the onset of the experiment. Fish were not fed during the exposure to prevent the release of bile from the gallbladder to the duodenum, a response stimulated by feeding. These static exposures were conducted in 34 L glass aquaria with three fish per tank, renewed every 48 h with an array of concentrations (0, 3.2, 32 and 320 μg/L) of fluoxetine or carbamazepine to accumulate a range of each analyte in fish bile. The actual concentration of these compounds was determined using traditional solid phase extraction (SPE) method. Exposure water quality was checked daily and maintained at conditions considered optimal for trout (12.5 ± 0.05 ^{o}C; pH 8.17 ± 0.06; ammonia 23.5 ± 1.5 μg/L). At the conclusion of the 7-day exposure, fish were anaesthetized with 0.1% ethyl 3-amino benzoate methanesulfonate and killed by spinal severance according to Animal Care Protocols (#04-24; 08-08) approved by the University of Waterloo Animal Care Committee. Bile was collected from the gallbladder, immediately frozen in liquid nitrogen and stored at -80°C prior to analysis.

3.3.3 Laboratory exposure to a mixture of pharmaceuticals

In a second study, juvenile Rainbow Trout were also used, with length and weight of 23.1 ± 1.1 cm and 108.1 ± 15.9g respectively, and *n* = 20. Fish were again acclimatized for 3 days in 34 L exposure aquaria containing municipal dechloraminated water prior to the onset of an 8 d exposure to a 3 µg/L mixture of pharmaceuticals (fluoxetine, gemfibrozil, atorvastatin, ibuprofen, carbamazepine, atrazine, diclofenac, naproxen, and bisphenol-A) spiked to the dechloraminated reference water in 100 µL of ethanol as the carrier solvent and renewed daily. Water quality remained optimal (12.4 ± 0.05 ^{o}C; pH 7.79 ± 0.04; ammonia 54.9 ± 3.6 µg/L, *n* = 20) for this species throughout the exposure. At the conclusion of the exposure, fish were sampled as described previously.

3.3.4 Field exposure to municipal wastewater effluents

Fathead Minnow (*Pimephales promelas*), a small bodied fish (5.58±0.03 cm; 1.43±0.02 g; *n* =300) were caged in the Grand River watershed (southern Ontario, Canada) adjacent the Doon Wastewater Treatment Plant (43° 24’03.29”N; 80°25’ 12.04”W) at 2 upstream and 3 downstream sites (Figure 3.1) for 14 d in October 2010. The most upstream of the two reference sites is located 1.2 km upstream of the Doon municipal effluent outfall, but 19.45 riverine km downstream of the municipal wastewater discharge from the City of Waterloo. The second reference was 0.5 km upstream of the Doon outfall. The three downstream stations were 0.5, 1.7, and 5.6 km below the Doon wastewater effluent release. At each site, two cages (Rubbermaid™ containers) were deployed, each containing two commercial baitfish buckets (FlowTroll®, Frabill Inc, Jackson, WI) holding 15 fish each (30 fish/cage). The Rubbermaid containers were

perforated on all surfaces with 2 cm holes and contained a 60 x 60 cm concrete patio stone beneath the bait buckets for weighting.

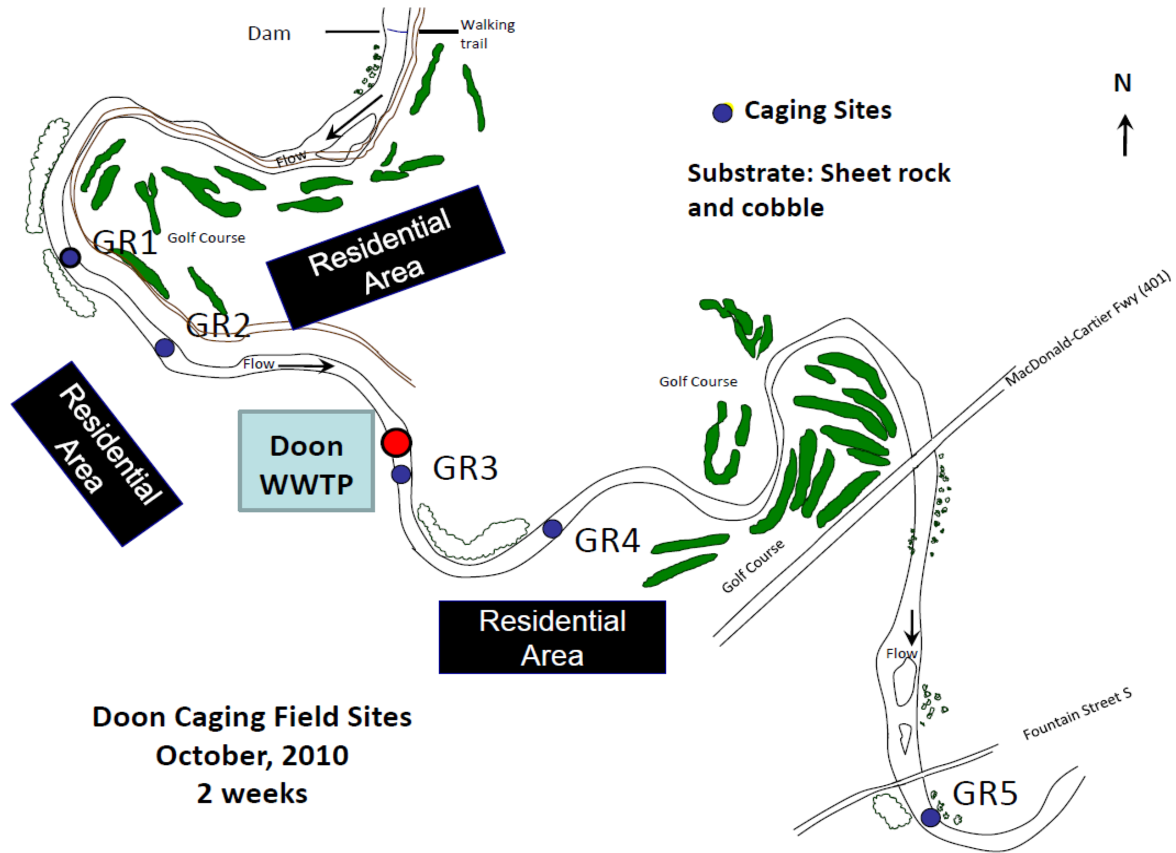


Figure 3:1 Description of field caging of Fathead Minnow (*Pimephales promelas*) along the Grand River at different locations for 2 weeks (Doon WWTP - Kitchener wastewater treatment plant)

The cages were anchored to the substrate with t-posts fastened to ¼" cable running through a homemade pipe frame. This caging design exposed fish to minimal current, thereby reducing exposure stress from constantly swimming, yet allowed water to pass freely through the enclosure. After 2 weeks of exposure, fish were anesthetized and sampled as described for the laboratory-based Rainbow Trout exposures. As the amount of bile collected from each fish was less than 10 µL, a variable number of fish bile samples were pooled to obtain 50 µL, which was the experimental unit extracted by SPME for the investigation. The workflow procedure of sampling is shown in Figure 3.2

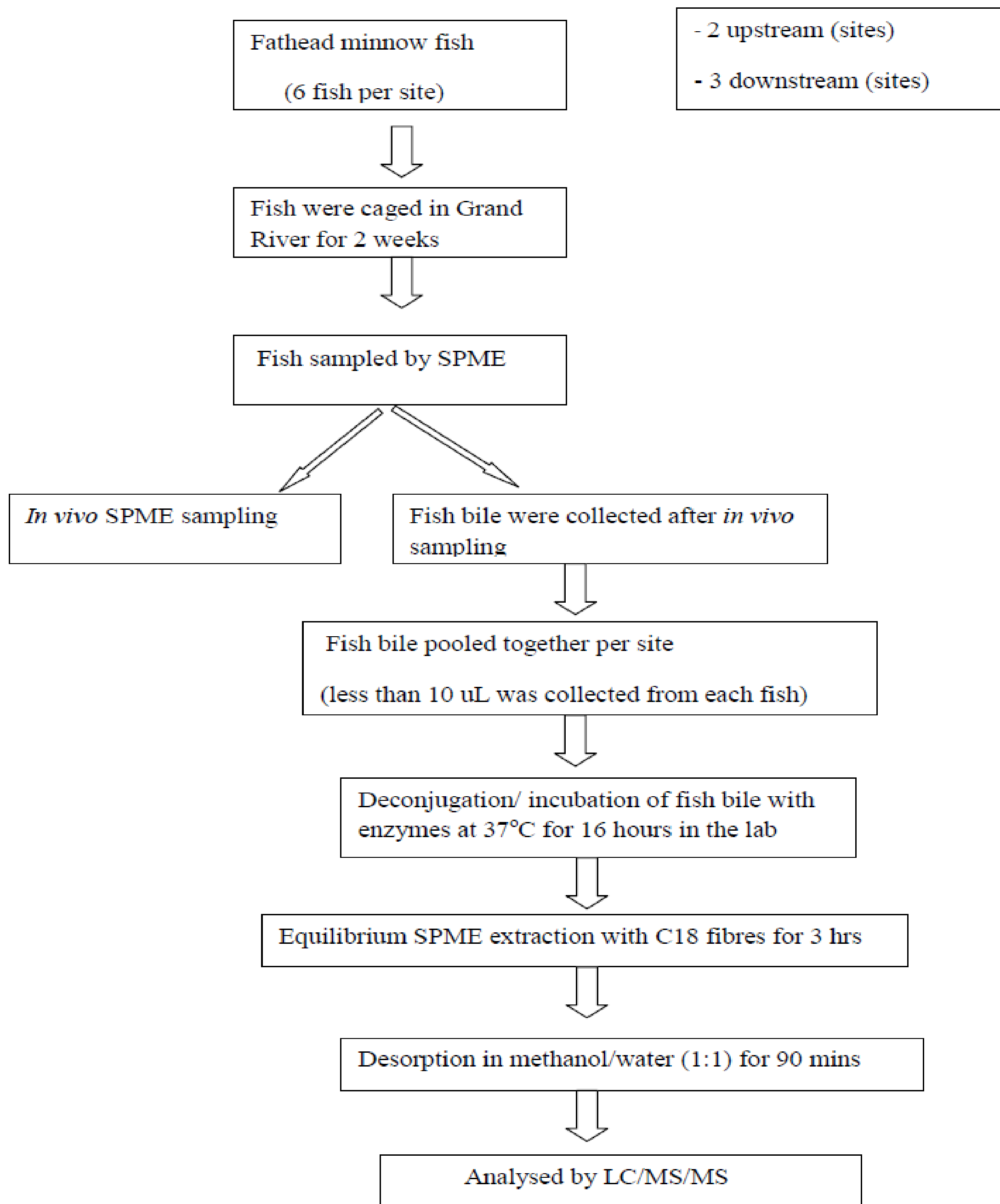


Figure 3:2 Workflow of field exposure of fathead minnow in the Grand River near wastewater fall. This figure shows the experimental step by step approach for the caging and sampling of fathead minnow fish tissue by SPME technique.

3.3.5 Enzymatic hydrolysis of bile samples

Deconjugation of bile samples was performed to determine the total concentration of the target analytes using the following hydrolytic enzymes: β-glucoronidase (1000 units/mL) sulfatase (2 units /mL) and β-glucosidase (20 units /mL). Deconjugation by these hydrolytic enzymes has been shown to be specific (with no cross reactivity among the three enzymes) when employed according to standardized procedures as described elsewhere.[115, 123] Briefly, 200 µL of each deconjugating enzyme was added to 100 µL of bile sample, followed by 750 µL of phosphate buffer solution (PBS, pH 6.0) and 400 µL of ultrapure water prior to a 16 h incubation at 37°C. The same identical procedure, but lacking enzyme hydrolysis, was used to determine the free (un-conjugated) fraction of pharmaceuticals in the bile samples. Extraction in bile samples, with and without enzyme hydrolysis, using both SPME extraction phases and solvent, enabled the determination of both relative method sensitivities, as well as free and total conjugated drug concentrations in bile samples. In order to confirm the presence or absence of the parent drug in fish bile, target metabolites such as norfluoxetine and carbamazepine10, 11-epoxide were investigated in the bile sample extract. When the sample was injected to the LC/MS/MS system, the target ion mass of the metabolites were monitored along with the parent compounds as well.

3.3.6 SPME method optimization

Polydimethylsiloxane (PDMS) fibre coating, supported internally by a 4 cm long stainless steel wire (0.483 mm diameter from medical grade; Small Parts Inc., Miami Lakes, FL), was one of the SPME extraction phases used in this study. Custom made PDMS hollow fibre membrane tubing (Helixmark, Carpinteria, CA) was cut into 1.5-cm portions. Each PDMS portion (165 µm thickness) was placed over one stainless steel wire before it was used. An additional C_{18} fibre

coating (1.5 cm coating length, 45 µm thickness supplied by Supelco Bellefonte, PA, USA) was used to monitor the metabolites (norfluoxetine and carbamazepine-10, 11-epoxide) of selected compounds while PDMS was used to determine the parent compounds. All SPME fibre coatings were pre-conditioned for at least 30 min in a mixture of water/ methanol (1:1) prior to use. At the onset of method development, extraction and desorption time was optimized for each extraction phase in a spiked PBS buffer (pH = 6.0). Subsequently, optimal conditions were further evaluated in spiked reference (clean) bile samples at different concentration levels (2.5, 5.0, 20 ng/mL) to evaluate the precision and accuracy of the SPME method. Method repeatability was checked by analyzing replicate aliquots of bile samples at different times and concentration ranges, and is expressed as a relative standard deviation (RSD). The method detection limit and limit of quantification were determined from 200 µl reference Rainbow Trout bile samples spiked at different concentrations and incubated for 30 min at 4°C prior to dilution with PBS (pH = 6) and ultrapure water as described previously. A 1.95 mL aliquot of the spiked bile sample was transferred to a 2 mL HPLC vial for extraction. PDMS fibres were inserted into the vial for equilibrium extraction at under 1200 rpm continual vortex agitation (model DVX-2500, VWR International, Mississauga ON, Canada) followed by a 1.5 h, 1200 rpm desorption of extracted analytes into 0.2 mL methanol/water (1:1) containing 20 ng/ml deuterated analogue of the target compounds as internal standards.

3.3.7 Liquid (solvent) extraction

Liquid extraction was used to determine the un-conjugated fraction of selected pharmaceuticals in bile. In addition, the total (free and bound) analytes were utilized to validate SPME-derived pharmaceutical concentrations extracted from fish bile. To determine free concentrations of

fluoxetine and carbamazepine in bile, 430 µL of MeOH was added to 100 µL of bile, followed by 20 µL of 500 ng/mL deuterated analogues (d_5-fluoxetine or d_{10}-carbamazepine). Following the addition of the internal standard, the resulting mixture was homogenized and agitated for 3 h using a digital vortex at 1000 rpm. After agitation, the sample was centrifuged at 3000 rpm for 5 min at 4°C. Then, the supernatant was syringe-filtered (0.2 µm Acrodisc 13 mm, Pall Corporation USA) into a glass vial. Subsequently, 20 µL of the sample extract was injected to the LC/MS/MS system for quantification analysis.

3.3.8 Instrumental analysis

Liquid chromatography (LC) was performed on a HP1100 HPLC system (Agilent Technologies) equipped with a degasser, a binary pump, an autosampler and a column oven. Chromatographic separation was performed on a Zorbax Eclipse XDB C_{18} (150 mm × 21 mm, 3.5µm) column preceded by a C_{18} guard column at a flow rate of 0.8 mL/min with a mobile phase A (95% water, 5% methanol, 0.1 % acetic acid) and B (95% MeOH, 5% water and 0.1% acetic acid). The injection volume was 20 µl and the gradient elution program was as follows: mobile phase B was ramped from 10% to 50% over 0.5 min, from 50% to 100% over 7.5 min, held at 100% for 2 min, then reduced to 10% over 1 min, thereby bringing back the initial chromatographic condition within 15 min. Detection was done with a 3200 QTrap triple quadrupole-linear ion trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems Sciex, Foster City, CA, USA). The target compounds in the bile samples were analyzed in positive and negative modes. Optimized MS/MS parameters were determined by manual infusion of a standards solution using syringe-pump with the automatic optimization function within the MS software. Method detection limit (MDL) was determined with seven replicate of bile samples

spiked at 2.5 ng/mL, the average recovery and standard deviation of the seven replicates were calculated. Hence, MDL was calculated as three times standard deviation at 95% confidence interval (MDL = (standard deviation) times (student t-value), t = 3 for seven replicates). The LOQ was determined as 10 times the standard deviation obtained from the seven replicate runs. Also, the linearity of the target compounds was studied by injecting different concentrations of standard solution in the range of 0.9 - 500 ng/mL. In addition, the precision of the method was examined at different concentration levels which were run at different days.

3.3.9 Statistical Analysis

A comparison of SPME and liquid (solvent) extraction analytical methods was performed using a linear regression on the log-transformed data. The relationship between the exposure concentration and amount of drugs detected in the fish bile was described using a linear regression at the 95% confidence level. Assessments of the effects of enzyme hydrolysis on bile analytes were performed by comparing slope and correlation coefficients from the linear regression.

3.3.10 Selection of target pharmaceuticals

The pharmaceuticals selected for investigation in this study (carbamazepine, fluoxetine, ibuprofen, gemfibrozil, paroxetine, sertraline, and diazepam) are frequently detected in both wastewater treatment effluents and surface waters adjacent urban areas at trace (ng/L –µg/L) concentrations.[14,23,124] All the drugs except ibuprofen and gemfibrozil (non-steroidal anti-inflammatory and hypolipidemic therapeutics, respectively), are prescription anti-depressives that are increasingly detected in wastewater effluent and environmental matrices.[100] The environmental persistence and bioaccumulation in fish has led to carbamazepine being classified

as one of the pharmaceuticals requiring heightened environmental monitoring for its fate in biotic and abiotic compartments.[108,125]

3.4 Results and discussion

3.4.1 Method Performance

The relative extraction efficiencies of both PDMS and C_{18} extraction phases were evaluated in phosphate buffer saline - PBS, (pH 7.4), and generally, more analyte was extracted by C_{18} than PDMS, although both fibres have shown affinity towards the pharmaceuticals of interest (Figure 3.3).

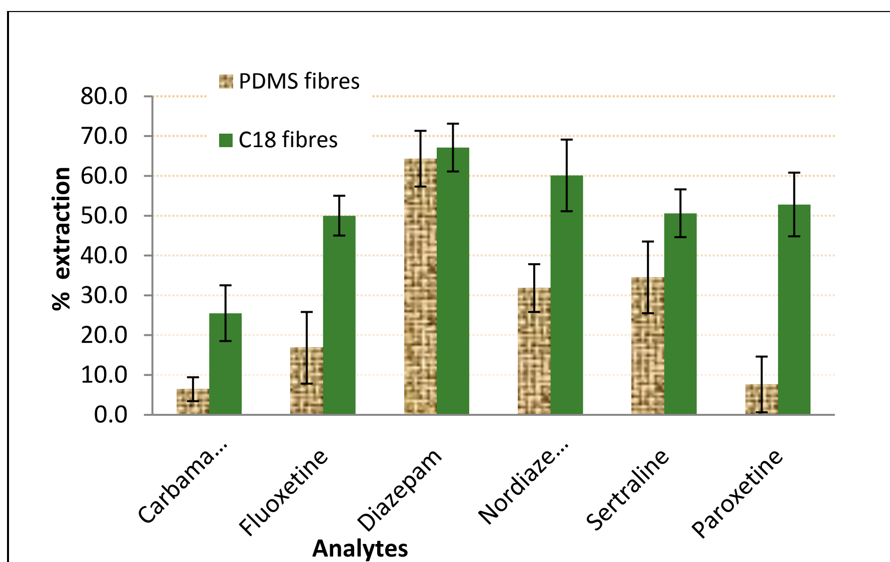


Figure 3:3 Comparison of extraction efficiency (recovery) of two extraction phases (Polydimethylsiloxane- PDMS and C18)

Both extraction phases had good extraction efficiency for the selected pharmaceuticals in this study. Method sensitivity was evaluated by determining the method detection limit (MDL) and Limit of Quantitation (LOQ) as reported in Table 3.1.

Table 3:1 Extraction efficiency (%) and precision of SPME method in spiked bile at two different concentration levels using PDMS fibre

	QC Low		QC Medium	
Compounds	Amount extracted (%)	RSD (%) n = 7	Amount extracted (%)	RSD (%) n = 8
Carbamazepine	9.0	7	8.4	6
Fluoxetine	35.2	5	30.9	3
Diazepam	64.6	3	72.8	4
Nordiazepam	36.0	8	36.4	6
Sertraline	66.6	5	37.0	8
Paroxetine	19.0	7	16.2	4

QC low = 2.5 ng/ml QC medium = 20 ng/ml (QC – Quality control spiked concentration in fish bile

A good linearity was observed over the specified concentration range with correlation coefficient $R^2 > 0.995$. Precision was expressed as relative standard deviation and summarized in supporting information Table 3.1 with RSDs within acceptable range (RSD < 9 %). Extraction recovery of the selected compounds in spiked bile samples ranged from 8 – 72 % depending on the affinity of each of the compounds for the extraction phase. Recovery in this study was calculated as the mean percentage extraction of the SPME at equilibrium in spiked bile samples. The comparison of SPME and Liquid (solvent) methods for bile samples collected from laboratory exposed fish using fluoxetine showed good agreement, with correlation co-efficient of $R^2 > 0.99$ (Figure 3.4)

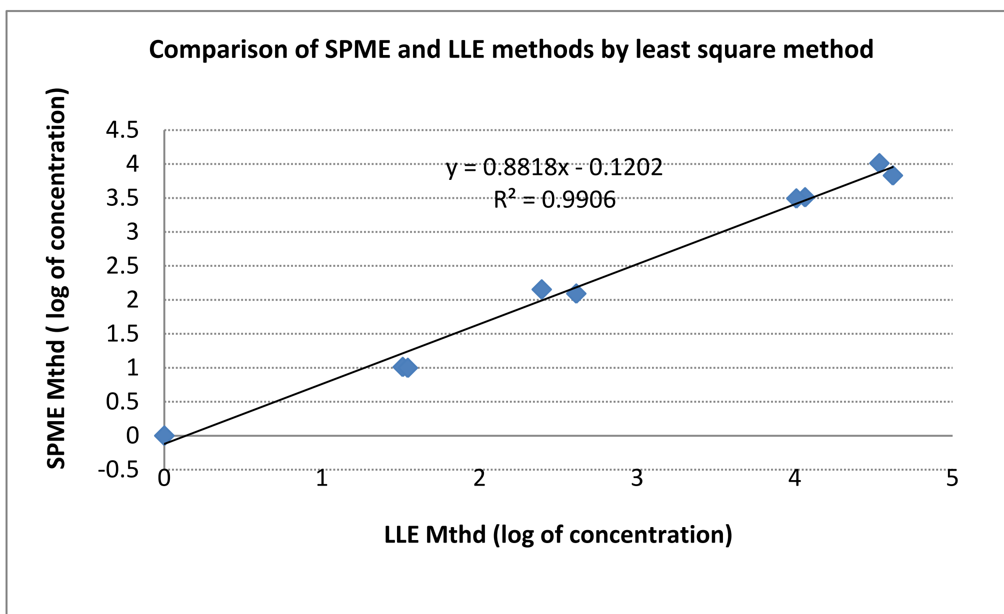


Figure 3:4 Comparison of SPME and liquid-liquid extraction (LLE) method results for fluoxetine.

3.4.2 Single compound laboratory exposure of Rainbow Trout

Fluoxetine (FLX) accumulation in fish bile during the single-compound exposure was correlated with the aqueous exposure concentration (R^2= 0.86 - 0.98) and both fluoxetine and its primary

metabolite norfluoxetine were detected in fish bile, indicating some biotransformation activity. When treated with deconjugating enzymes, the amount of fluoxetine detected in the trout bile was 8 times higher than untreated (conjugated) bile (as shown in Figure 3.5A).

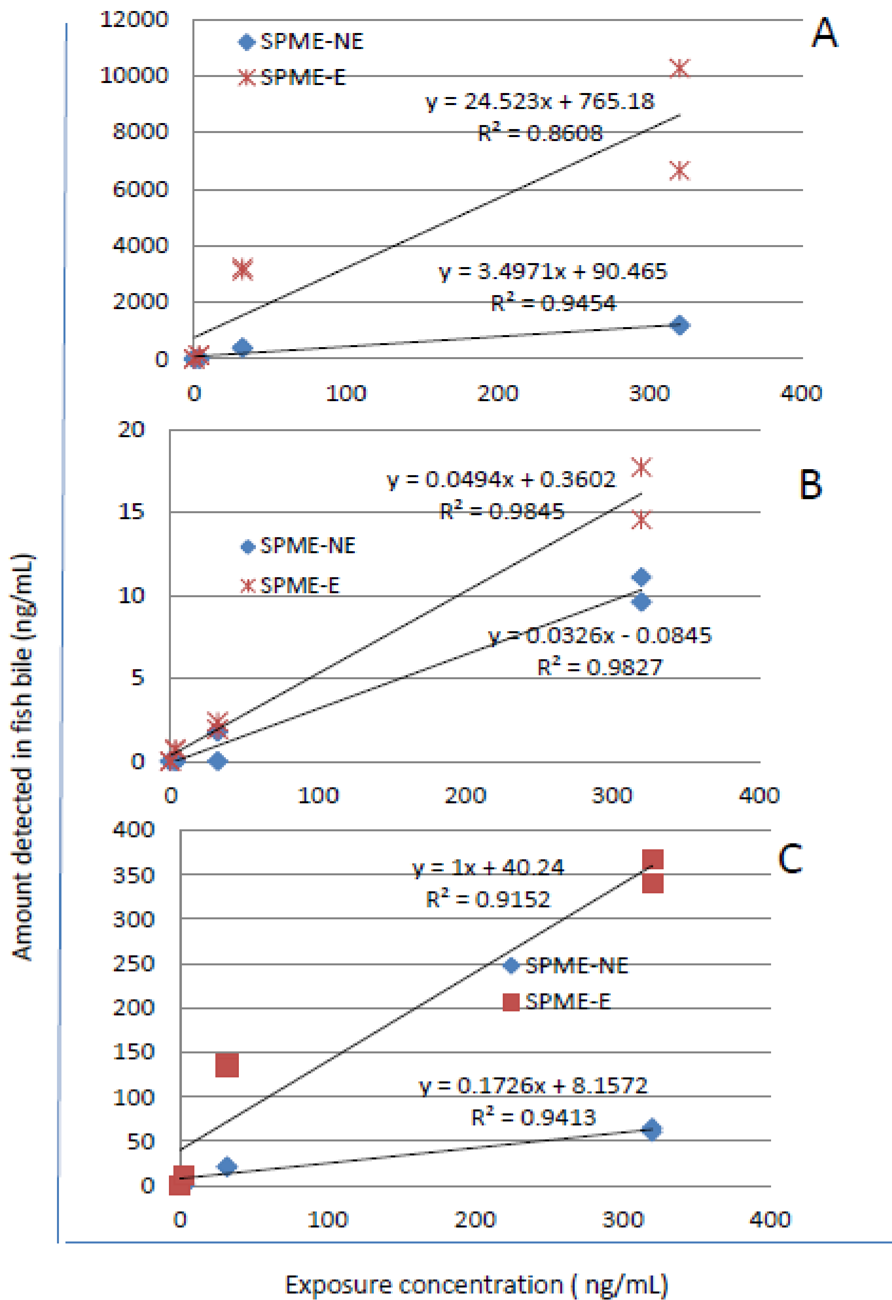


Figure 3:5 Comparison of amount extracted before and after enzyme assisted hydrolysis for A) fluoxetine, B) carbamazepine, C) norfluoxitine (SPME-NE = solid phase microextraction without enzyme hydrolysis, SPME-E = solid phase microextraction with enzyme)

Fluoxetine strongly bioconcentrated in bile (bioconcentration factors, BCFs of 83 – 481) while BCFs of carbamazepine in bile were less than 1 (Table 3.2). The bioconcentration factor was calculated as the ratio of concentration of the compound in fish tissue and measured water concentration. Actual water concentration was used for the calculation for BCF since it was different from nominal exposure concentration. In addition, the bioconcentration of FLX in fish muscle ranged from 3-63, indicating tissue distribution in fish tissue. The observed difference between the measured and nominal water concentration may be due to degradation of the compound or possibly due to adhesion to the aquarium tank. Low concentration of fluoxetine was detected in control fish, possibly from trace contamination of water. Background concentrations were determined and subtracted prior to the calculation of BCF. The low CBZ BCF in fish muscle and bile may be due to a higher biotransformation capacity of carbamazepine in fish, as confirmed in the literature.[126-128]. Also taken into consideration is the fact that carbamazepine is water soluble enough to be excreted by fish.

Table 3:2 Bioconcentration factor of carbamazepine, fluoxetine in Rainbow Trout muscle and bile of single compound laboratory exposure

Exposure conc. (ng/mL)	Measured water conc. (ng/mL) n = 8	Conc. in bile (ng/mL)	RSD (%) n = 3	BCF (bile)	Conc. in muscle (ng/g)	BCF (muscle)
Fluoxetine						
Control	0	0	-	NA	0	NA
3.2	0.3 ± 0.1	132.9	10	416	0.92	3
32	6.7 ± 0.7	3171.3	3	481	428.8	63
320	102.7 ± 7.9	8519.4	29	83	ND*	ND*
Carbamazepine						
Control	0	0	-	NA	0	NA
3.2	2.9 ± 0.1	0.7	4	0.26	0.28	0.10
32	24.7 ± 0.4	2.2	13	0.09	1.68	0.07
320	175.3 ± 10.9	10.3	14	0.06	28.3	0.16

ND* - The muscle concentration was not determined because fish were not healthy
NA - not applicable

3.4.3 Multi-component laboratory exposure of RBT

In a similar manner to single fluoxetine or carbamazepine exposure, the mixture of pharmaceuticals displayed varying BCFs determined for pharmaceuticals in fish bile exposed in the laboratory to 3 μg/L for a range of chemicals. The varying BCF value for these pharmaceuticals may be due to their physicochemical properties which modify uptake (Table 3.3). The concentration of pharmaceuticals in fish muscle, which was determined by Zhang et al.[119], was compared to the corresponding pharmaceuticals in bile extracted from the same set of fish and the result is shown in Table 3.3. In addition, the BCF value for most of the pharmaceuticals were greater in bile compared to fish muscle except for carbamazepine. Therefore, bile samples can also be used for monitoring the exposure of fish to organic

contaminants, particularly where the trace concentration of organic contaminants may be difficult to detect in fish tissues.

Table 3:3 Physico-chemical properties of pharmaceuticals[107, 129-130] and concentration of the compounds measured in exposure water, Rainbow Trout muscle and bile after 8-day laboratory exposure (3 ng/mL)

Analytes	LOD ng/mL	Water solubility (mg/L) at 25 °C	Log K_{ow}	pKa	Water Conc. (ng/mL)	Conc. In bile (ng/mL)	BCF_{bile}	Conc. in muscle[22] (ng/mL)	BCF_{muscle}
Carbamazepine	0.6	17.7	2.45	13.9	2.91 ± 0.15	ND	NA	1.0-1.6	0.3 - 0.6
Fluoxetine	0.4	38.6	4.05	8.7	2.76 ± 0.13	56.2 -289.2	20 -104	106 -189	38 -68
Norfluoxetine	0.3	126.9	2.30	9.1	NA	2.9-35.5	NA	NA	NA
Ibuprofen	0.7	21	3.97	4.9	2.79 ± 0.16	2.3 -3.5	1	3.1-4.4	1.1 - 6.6
Bisphenol A	5.0	300	3.64	9.6	2.65± 0.16	234.6 -697	89-263	ND	NA
Naproxen	3.9	15.9	3.18	4.2	2.88 ± 0.9	ND	NA	ND	NA
Gemfibrozil	0.4	4.96	4.77	4.7	2.65 ± 0.14	372.9 - 2686.7	141-1014	0.5 -1.5	0.2 - 0.6
Diclofenac	1.4	2.4	4.51	4.2	2.86 ± 0.17	< LOQ	NA	ND	NA

NA – Not applicable, ND – Not detected, n = 3.

Muscle data were obtained from Zhang (2010)[47]

In the case of exposures done with multiple pharmaceuticals, the BCF of fluoxetine in bile was 20-104, while the concentration of its metabolite norfluoxetine was found to be 2.9 – 35.5 ng/mL (Table 3.3). The detection of this metabolite (norfluoxetine) showed that the parent compound may have been biotransformed during phase I and II metabolism. In the case of bisphenol A and gemfibrozil, the BCF was found to be 89- 263 and 141-1014 respectively. This shows that a greater concentration of bisphenol A and gemfibrozil were bioconcentrated in the Rainbow Trout bile. Although diclofenac was also detected in the fish bile, its concentration was below the limit of quantitation. Naproxen and carabamazepine were not detected in Rainbow Trout bile. It is very likely the physicochemical properties of the compounds not detected in bile exhibit limited bioconcentration due to their rapid excretion. Also, efficient biotranformation of diclofenac, naproxen and ibuprofen to their respective metabolites may have occurred since these compounds are acidic drugs that can be easily metabolized.[131]

3.4.4 Field exposure of fathead minnow

The antidepressant sertraline was detected in bile of fish at all sites with mean concentrations at the two upstream sites of 1.14 ± 0.3 and 0.72 ± 0.1 ng/mL, while the three downstream sites where sertraline was detected had concentrations of 0.85± 0.2, 0.68 ± 0.1, 0.22 ± 0.04 ng/mL at sites 3, 4 and 5 respectively (Table 3.4a). Conversely, atorvastatin was detected only in the bile of fish caged at one upstream site (site 1) with a concentration of 2.1± 2.5 ng/mL while downstream locations were surprisingly below detection limits, potentially due to up-regulated excretion mechanisms at the more polluted downstream stations. In addition, antidepressant paroxetine was detected in fish bile in the upstream sites 1 and 2 with concentrations of 2.84 ±2.0 ng/mL and 1.06±0.3 ng/mL. Also, the amount of paroxetine detected in downstream site 3

was 1.0 ±0.2 ng/ml. Although target pharmaceuticals were detected in the bile, the concentrations were lower when compared to the concentrations found during static laboratory exposure in which different fish species were used. In the case of field exposure of caged fish in the Grand River, it is likely that the fish were feeding on algae/biofilms; thereby reducing the volume of the bile collected from each fish (volume of bile in each fish was less than 20 μL). As a result, the gallbladder, in which bile is stored, may have been periodically emptied to the intestine to aid digestion due to feeding. Therefore, the concentration of the pharmaceuticals in the bile would be reduced and this factor may explain the low concentration of the pharmaceuticals in the fish bile. Another possible explanation is based on the bioavailability of the drugs in the river. The concentration of the pharmaceuticals in river water is likely to fluctuate, thereby affecting direct uptake by fish. In addition, the lipid content in this fish species may have been lower, thereby affecting the bioconcentration of the drugs in fish tissue. Overall, the BCF of most of the compounds in the bile of the compounds were larger at site 3 and 4 when compared to that determined in the muscle.

Table 3:4a Summary of the concentration (ng/mL, ±standard deviation) and bioconcentration factor of pharmaceuticals detected in fish bile of fathead minnow caged in the field near a municipal effluent outfall (n=6).

	Site 1		Site 2		Site 3		Site 4		Site 5	
Analytes	Bile conc. (µg/L)	BCF_{Bile}	Bile conc. (µg/L)	BCF_{Bile}	Bile conc. (µg/L)	BCF_{Bile}	Bile conc. (µg/L)	BCF_{Bile}	Bile conc. (µg/L)	BCF_{Bile}
Carbamazepine	ND	NA	ND	NA	ND	NA	ND	NA	ND	NA
Fluoxetine	< LOQ	NA	ND	NA	< LOQ	NA	< LOQ	NA	< LOQ	NA
Atorvastatine	2.1 ± 2.5	150	0.50±0.04	70	0.41±0.05	9	ND	NA	< LOQ	NA
Venlafaxin	0.44 ± 0.05	16	0.35 ± 0.01	15	0.33 ± 0.01	1	0.30 ± 0.01	1	0.30 ± 0.02	3
Norfluoxetine	0.46 ± 0.10	153	0.20 ± 0.01	115	0.26 ± 0.03	130	0.27 ± 0.05	135	< LOQ	NA
Sertraline	1.14 ± 0.30	163	0.70 ± 0.10	144	0.85 ± 0.20	65	0.68 ± 0.05	68	0.22 ± 0.04	28
Paroxetine	2.8 ± 2.0	710	1.10 ± 0.30	29	1.07 ± 0.20	214	ND	NA	0.57 ± 0.30	1

Note- The high standard deviation observed in the result is due to bile sample variability from fish to fish.

Table 3.4b: **Summary of the concentration (ng/mL, ±standard deviation) and bioconcentration factor of pharmaceuticals detected in fish muscle of fathead minnow caged in the field near a municipal effluent outfall (n=6).**

	Site 1		Site 2		Site 3		Site 4		Site 5	
Analytes	Muscle conc. (ng/g)	BCF_{muscle}	Muscle conc. (ng/g)	BCF_{muscle}	Muscle conc. (ng/g)	BCF_{muscle}	Muscle conc. (ng/g)	BCF_{muscle}	Muscle conc. (ng/g)	BCF_{muscle}
Carbamazepine	ND	NA	ND	NA	ND	NA	ND	NA	ND	NA
Fluoxetine	4.0 ± 2.0	800	1.7 ± 1.0	425	< LOQ	NA	< LOQ	NA	3.0 ± 1.7	753
Atorvastatine	ND	NA	ND	NA	ND	NA	ND	NA	ND	NA
Venlafaxin	17 ± 13	607	7.27 ± 4.0	303	< LOQ	NA	< LOQ	NA	2.3 ± 1.2	22
Norfluoxetine	ND	NA	ND	NA	ND	NA	ND	NA	ND	NA
Sertraline	1.3 ± 1.0	186	2.1 ± 1.0	418	< LOQ	NA	< LOQ	NA	0.43±0.1	54
Paroxetine	0.3 ± 0.2	82.5	0.4 ± 0.3	10	0.14 ± 0.20	28	< LOQ	NA	0.40±0.30	133

NA – Not applicable, ND – Not detected, <LOQ – less than limit of quantitation

Table 3.4c: Summary of the concentration (ng/mL) of field wastewater samples determined by solid phase extraction (SPE) method near a municipal effluent outfall

Analytes	Site 1	RSD (%) n=3	PAT 2	RSD (%) n=3	PAT 3	RSD (%) n=3	PAT 4	RSD (%) n=3	PAT 5	RSD (%) n=3
Carbamazepine	0.022	9	0.024	10	0.117	3	0.109	5	0.049	5
Fluoxetine	0.005	9	0.004	11	0.005	10	0.004	14	0.004	5
Atorvastatine	0.014	10	0.007	11	0.047	16	0.043	14	0.018	7
Venlafaxin	0.028	6	0.024	5	0.264	3	0.245	5	0.106	2
Norfluoxetine	0.003	12	0.002	14	0.002	11	0.002	3	ND	
Sertraline	0.007	9	0.005	10	0.013	11	0.010	9	0.008	10
Paroxetine	0.004	5	0.037	7	0.005	3	0.004	6	0.003	8
Ibuprofen	0.025	9	0.028	2	0.730	14	0.660	5	0.170	8
Gemfibrozil	0.009	12	0.009	3	0.017	13	0.016	10	0.010	9

ND – not detected

Of those compounds detected at higher concentrations at upstream sites, it is possible the additional nutrients at the wastewater-exposed downstream stations would have resulted in additional feeding opportunities for the caged fathead minnow. If they were feeding regularly, the periodic release of bile to the duodenum would have routinely flushed the bile, while periods of fasting have been known to further concentrate bile analytes[131]. Finally, some of the pharmaceuticals may partition to organic carbon or suspended particulates in the wastewater, making them less bioavailable. Consequently, these various factors may reduce the amount of selected pharmaceuticals bioconcentrated in fish, resulting in low levels being detected (or below detection limits) in the tissue and bile.

3.4.5 Comparison of SPME and traditional methods

The SPME method was validated against traditional solvent extraction (liquid-liquid), with good agreement (R^2= 0.99, slope = 0.88, Figure 3.3). Consequently, SPME is a viable alternative for determining the concentration of pharmaceuticals in fish tissue, with the benefit of simplicity, as it integrates sampling and sample preparation into a single step. In contrast, the traditional liquid extraction method is relatively laborious, involving multiple steps which can affect the precision of the analytical method. While SPME is environmentally friendly in that it uses little or no solvent during the sample preparation, traditional approaches rely on substantial use of solvents, creating potential health and environmental concerns. SPME provides a rapid and inexpensive technique for biopharmaceutical analysis, where analyte loss during sample preparation is reduced due to the integration of multiple steps into a single step while sampling complex matrices such as bile.[132]

3.5 Conclusion

In this study, SPME provides a simple and rapid method for monitoring the bioconcentration and excretion of target pharmaceuticals in fish bile. For many analytes, detection is only feasible in the bile as normal physiological functions naturally bioconcentrate contaminants in the bile prior to excretion. However, as a complex matrix, bile is difficult to analyze. SPME can be a useful sampling technique which limits analytical interference on compounds of interest from a co-extracted matrix. This SPME approach successfully analyzed target pharmaceuticals and target metabolites of fluoxetine and carbamazepine in fish bile following laboratory exposures and exposure to complex mixtures in fish caged near wastewater outfalls. Uptake and bioconcentration of pharmaceuticals in tissues and bile will vary based on their properties, environmental conditions, as well as across species.

Chapter 4

Determination of pharmaceutical residues in wastewater using automated thin film solid phase microextraction with liquid chromatography-tandem mass spectrometry

4.1 Preamble

This chapter has been submitted for publication in the Journal of Chromatography A which is under peer-review. This chapter will be published as a paper: Oluranti P. Togunde, Erasmus Cudjoe, Ken D. Oakes, Fatemah S. Mirnaghi, Mark R. Servos, and Janusz Pawliszyn "Determination of pharmaceutical residues in wastewater using automated thin film solid phase microextraction with liquid chromatography-tandem mass spectrometry". The figures and tables are reprinted from this manuscript with the permission of Elsevier (Copyright Elsevier 2012).

4.2 Introduction

The frequent detection of pharmaceutical residuals of human and veterinary origin in surface and wastewater at trace to ultra-trace concentrations (ng/L to µg/L) [3, 23, 30,133-137] has engendered both public and scientific concern. Such apprehension surrounding the now near ubiquitous reports of pharmaceuticals detected in surface water adjacent to urban areas or areas of intensive agriculture is focused on the anticipated subtle effects such unregulated exposure might produce in aquatic organisms and humans[12,138-139]. One source of these "emerging contaminants" has been attributed to incomplete removal, and hence near continuous environmental infusion, of pharmaceutical residues from municipal wastewater treatment plants [4,140-141]. Agricultural wastewater is another source of these emerging contaminants due to use of veterinary drugs;

however, such introductions to surface water are more seasonal than continuous, often corresponding to the application of manure to fields in the spring and fall. Leachate from solid waste landfills and the application of biosolid as an inexpensive nutrient amendment to agricultural fields have also been identified as sources of pharmaceutical entry to aquatic environments.

Since pharmaceuticals are usually detected in surface water and wastewater at trace or ultra-trace concentrations, sensitive analytical methods are required for determination of these compounds in environmental samples. The use of high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (LC/MS/MS) has been the technique of choice due to the high selectivity and sensitivity achieved by this method. In general, pharmaceuticals are designed to be polar and relatively non-volatile, thus, they can be readily measured using HPLC without the additional step of derivatization as required by gas chromatography [4,142-143]. Off-line solid phase extraction (SPE) is commonly used as a sample preparation technique to extract and pre-concentrate pharmaceuticals from environmental samples [144-149]. One of the major drawbacks associated with manual (offline) SPE is that it is a laborious and time consuming multi-step sample preparation method. Although the use of automated online SPE method is increasing [150-152], relatively expensive instrumentation is required to perform the extraction. In addition, SPE is subject to clogging during extraction, particularly when handling very dirty environmental water samples without prior filtration. Hence, sample pre-treatment processes such as filtration are therefore required prior to SPE extraction, but loss of matrix-bound analytes during the filtration process is a valid concern.

Recently, solid phase microextraction coupled with liquid chromatography-tandem mass spectrometer (LC/MS/MS) has been gaining popularity for determining pharmaceutical

concentrations in water samples, although the desired sensitivity could be a challenge due to the limited volume and type of extraction phase used [140,153.] To address the drawbacks associated with traditional solid phase microextraction (SPME) methods, a new SPME configuration based on thin film configuration (using C_{18} extraction phase) is proposed, which can improve the method sensitivity and provide faster analysis of pharmaceuticals in water. The new configuration provides large surface area-to-volume-ratio, in which C_{18} particles are immobilized on a flat thin stainless steel. Since the geometry of the extraction phase is different from traditional SPME fibre, the new configuration used for this study is called thin film solid phase microextraction for the purpose of clarity. This new TF-SPME can be used in a 96-well plate format, thereby improving current high throughput analysis of pharmaceuticals in environmental water samples. The aim of this study is to develop a simple, fast, and sensitive analytical method based on automated SPME (SPME/LC/MS/MS) for determining concentrations of selected pharmaceuticals in wastewater in a high throughput 96-well plate format. To this end, the SPME configuration was modified and coupled with a very sensitive mass spectrometer to improve method sensitivity while maintaining the major advantages of SPME as a solvent free, environmentally friendly extraction technique that integrates sampling and sample preparation into a single step. The amount of analyte extracted by SPME at equilibrium is governed by the distribution constant between the extraction phase and sample matrix as shown in equation (1.1) [38]. When equilibrium between the extraction phase and the sample matrix is achieved, the initial concentration of analyte in the sample can be determined, since the amount on the extraction phase is directly proportional to the concentration of the analyte in the sample.

4.3 Experimental

4.3.1 Chemical and material

All chemicals and reagents utilized in this study were obtained at the highest available purity and were used without further refinement. Fluoxetine, diazepam, and diazepam-d_5 were purchased as certified standards from Cerilliant Corp (Round Rock, TX, USA). Carbamazepine was obtained from Sigma-Aldrich (Oakville, ON, Canada), while paroxetine, paroxetine-d_4, sertraline, and sertraline-d_3 were purchased from Toronto Research Chemical (Toronto, ON, Canada). Isotopically-labeled standards carbamazepine-d_{10} and fluoxetine-d_5 were purchased from CDN Isotope Inc (Point-Claire, QC, Canada). Chemical stock solutions were prepared in methanol with working solutions diluted as aliquots of this stock. Dilution water was obtained from a Barnstead Nanopure water purification system deionized to 18 Ω. HPLC-grade methanol (MEOH), HPLC-grade acetonitrile (ACN) and acetic acid were purchased from Fischer Scientific (Ottawa, ON, Canada). Individual 1mg/mL stocks for each pharmaceutical (except for diazepam which was purchased as 1 mg/mL in methanol) were prepared in methanol and stored at -20°C. A 10 mg/L mixture of all selected pharmaceuticals was prepared by diluting individual stocks with methanol. The C_{18} thin film extraction phase used in this study was obtained from Supelco (Bellefonte, PA, USA), and polypropylene 96-well deep plates were purchased from VWR International (Mississauga, ON, Canada).

4.3.2 Automation of sample preparation steps with PAS concept 96 auto sampler

All SPME steps including conditioning of the extraction phase, extraction, and desorption were automated by the Professional Analytical System (PAS) Concept 96 auto sampler (PAS Technology, Magdala, Germany). In this study, TF-SPME performed parallel sample preparations of up to 96 samples using multi-well plate technology. The automation of SPME

provides a high throughput analysis of pharmaceuticals in wastewater without user intervention, with the exception of placement of samples into the multi-well plate. All experiments were performed using the PAS concept auto sampler, as described in detail elsewhere [154-155].

4.3.3 Instrumental analysis (LC/MS/MS)

Analysis was carried out using an Accela™ instrument from Thermo Scientific equipped with a binary pump, vacuum degasser, and an auto sampler coupled with a MS/MS system. System control and data analysis were performed by X-Calibur® software version 2.0.7 provided by Thermo Scientific. Chromatographic separation was performed using a Waters® reversed phase C_{18} column of 100 mm × 2.1 mm i.d, 3 μm particles. The mobile phase consisted of a mixture of eluent A (90% water, 10% ACN and 0.1% acetic acid) and B (90% ACN, 10% water and 0.1% acetic acid) at room temperature with a flow rate of 0.5 mL/min. Optimal pharmaceutical separation was achieved using the following solvent gradient elution: mobile phase B was increased from 0% to 95% over 2 min, held for 1 min, and ramped back to 0% over 2 min. Analysis run time was 5 min including re-equilibration of the analytical column. The sample injection volume was 10 μL and samples were kept in the auto sampler at 5 ^{o}C. Identification and quantification of the analytes were carried out using a sensitive TSQ Vantage™ (triple stage quadrupole) mass spectrometer equipped with ESI sources. Heated nitrogen gas (N_2) was used as both nebulizer and desolvation gas in the ESI sources, while argon was used as a collision gas for collision induced dissociation. Full scan and MS/MS spectra of the individual standard were optimized by direct infusion of 1 mg/L of individual standard solution at a flow rate of 10 μL/mL. All analytes formed protonated molecular species (MH+) and were detected using multiple reaction monitoring (MRM) transitions.

4.4 Thin film solid phase microextraction procedure

4.4.1 Extraction phase: Comparison of C_{18} fibre and thin film

Development of any SPME procedure requires the selection of an appropriate extraction phase with affinity for the target compound(s). In the present study, during the preliminary stage of extraction phase selection, in-house polydimethysiloxane (PDMS) and polymeric C_{18} extraction fibre (Supelco, Bellefonte PA, USA) were compared using pure water spiked with pharmaceuticals to determine the relative affinity of the target compounds. Preparation details for the in-house PDMS extraction phase have been described elsewhere [88]. Relative extraction efficiencies and repeatability for the PDMS and C_{18} extraction phases were evaluated and compared using 2 ng/mL spiked water for equilibrium extraction (n = 12). Based on SPME fundamental principles, the amount of analyte extracted is a function of the physico-chemical properties of the extraction phase. As the coating length or volume increases, so increases the amount of extracted analyte from the sample, thereby resulting in enhanced method sensitivity. In this study, method sensitivity was improved by changing the geometry of the SPME extraction phase to a thin film in order to increase the amount of target analyte extracted from the sample. In other words, the method sensitivity is enhanced due to the increase in the coating surface area and volume. This new configuration based on thin film C_{18} extraction phase was used in a 96-well plate to achieve high throughput analysis of the pharmaceuticals in water samples. The extraction efficiency and reproducibility of the method were also evaluated in spiked water as described previously.

4.4.2 Extraction and desorption time determination

Extraction and desorption time are important parameters affecting SPME performance, with the highest possible sensitivity afforded by extended sampling time allowing for equilibrium extraction. Consequently, equilibrium extraction time was optimized and determined for each of the selected pharmaceuticals by extracting the target analyte from spiked water over time frames of 20-180 min under agitation. Prior to extraction, the extraction phase was conditioned in a 1:1 mixture of methanol and water under agitation (1000 rpm) for 30 min to activate the sorbent. Subsequently, a 1.8 mL aliquot of spiked water (2.0 ng/mL, n = 3) was dispensed into a 2.0 mL 96-well plate prior to the introduction of the extraction phase, with extraction carried out in the well under agitation (1000 rpm) to accelerate extraction by reducing the boundary layer thickness. After extraction, the extraction phase was removed from each well in the 96-well plate and desorbed in the desorption solution for 1 h with agitation at 1500 rpm. Optimization of desorption solvents was carried out using differing ratios of methanol/water, with a 60:40 mixture maximizing the removal of the target analytes from the extraction phase with an observed carryover of less than 1%. Different desorption times were also evaluated, ranging from 30 to 120 min to ensure timely but thorough desorption of analyte. Preconditioning, extraction, and desorption steps were automated with PAS concept software, with the samples extracted at each time point analyzed by a sensitive TSQ Vantage™ (triple stage quadrupole) mass spectrometer. Following optimization, subsequent desorptions utilized 0.6 mL of desorption solvent (60% methanol aqueous) to ensure that the entire extraction phase was immersed in the desorption solution. In order to minimize the variability from the instrument and method procedure, isotopic labeled internal standards were used to correct injection variability and for quantification of the compounds.

4.4.3 Effect of salt and pH on extraction efficiency

The effect of salt on extraction efficiency was evaluated by preparing spiked water samples (2 ng/mL, n = 12) with different concentrations of sodium chloride (0-20 % *w/v*) added. The sample was homogenized for 5 min before extraction and desorption; analysis of analyte at each salt concentration was performed, as previously described. In addition, the effect of pH on extraction efficiency and method sensitivity was evaluated using pH values ranging from 3 to 8. Nanopure water was adjusted with 0.1M solutions of HCl and NaOH using a Thermo Fisher Scientific ORION 3-Star pH meter to achieve the desired pH value, and then the sample was spiked to a final concentration of 3 ng/mL of analytes. After thorough homogenization (2 min), extraction of the spiked water with different pH values was carried out at equilibrium time under 1000 rpm agitation. After extraction, a 1 h desorption of analytes from the extraction phase under agitation at 1500 rpm was immediately conducted under the desorption conditions already described.

4.4.4 Application to real samples

The application of an automated TF-SPME for analysis of selected pharmaceuticals in wastewater was evaluated by analyzing Burlington, ON municipal wastewater treated under a variety of pilot-scale conditions from July to September 2011. Wastewater samples were treated by either conventional activated sludge (CAS), conventional activated sludge with nitrification (CAS-N), or conventional activated sludge with Biological Nitrifying Reactor (CAS-BNR). All samples were analyzed and quantified by the new method with liquid chromatography-tandem mass spectrometry as described previously. The feasibility of the newly developed automated method for monitoring pharmaceuticals in the environment was evaluated using wastewater samples collected from effluent-influenced reaches of the Grand River below the Kitchener and

Waterloo wastewater treatment plants. Samples were collected in glass amber bottles (1000 mL) and transported to the laboratory on ice. Following transportation, the samples were subsequently preserved using 0.2 g/L sodium azide and 0.05 g/L ascorbic acid (to inhibit microbial degradation of analyte) and stored at 4°C until extraction.

4.4.5 Matrix effect study

The effect of matrix on the method was evaluated by comparing the MSMS response of a known amount of deuterated standard of the target compounds spiked in neat solvent and matrix extract from wastewater (post extraction spiked method at one concentration level). The matrix effect (i.e signal suppression or enhancement) was assessed using the method described by Matuszewski et al. [156], which involved relating the peak area of standard obtained from a neat solvent to the peak area of the corresponding analytes in a matrix extract sample. In this case, the wastewater sample and pure water were extracted with the TF-SPME method and the extract was spiked with deteurated standard of the target analytes at one concentration level (5 ng/mL). Next, the extract was injected to the LC/MS/MS system for the quantification of the analytes. This experiment was performed in triplicate (n = 3). In addition, the effect of matrix was assessed at multiple concentration levels based on the post extraction spike method in order to generate the regression line. The assessment of the matrix effect was based on determining the slopes of regression lines in neat solvent (S_1) and wastewater extract (S_2). The pure solvent used in this case was the desorption solution (60% methanol, 40% water) which was spiked at different concentration levels (0.039 – 20 ng/ml). Also, the wastewater sample was extracted by the TF-SPME method, after which the extract was spiked at the same concentration level as the pure solvent. Samples from both matrices were injected into the LC/MS/MS system for quantification.

The slopes of regression line in pure solvent and matrix extract were compared. The matrix effect calculation is shown below:

ME (%) = S_2/S_1*100

S_1 = slope of regression line in solvent

S_2 = slope of regression line in wastewater

4.5 Results and Discussion

4.5.1 Selection of extraction phase

One of the parameters affecting the extraction efficiency and sensitivity of the method is the selection of appropriate type of extraction phase (coating). In this study, the extraction efficiency of the method using different extraction phases and configurations was evaluated in spiked pure water, in-house polydimethysiloxane (PDMS) tubing (165 µm thickness), C_{18} fibres (45µm thickness), and C_{18} thin film (45 µm thickness). The coating length for all extraction phases was 1.5 cm. At an earlier stage of method development, extraction efficiencies of in-house PDMS and commercially-available C_{18} fibres (45 µm thickness) extraction phases were evaluated in spiked water (buffered at pH 7.4) at equilibrium extraction times. Although both extraction phases have affinity for the compounds of interest, non-polar PDMS fibres have less affinity for hydrophilic compounds such as carbamazepine, and C_{18} fibres have shorter extraction times (equilibration times) than PDMS coatings due to coating thickness. Since the coating thickness in PDMS is three times greater than C_{18} fibre, this will result in longer extraction time. Previous evaluations have demonstrated that equilibrium extraction of pharmaceuticals with PDMS can take up to 3 or 4 hours, while C_{18} fibres can equilibrate in as short as 60 min, again due to the thickness of the coating. An additional comparison between C18 cylindrical fibres and C_{18}

configured in its thin film geometry was evaluated in spiked water. The greater extraction phase volume (8 times higher) and surface area (1 order of magnitude greater) of the C_{18} thin film configuration relative to the C_{18} fibre configuration resulted in higher extraction efficiencies for all the analytes examined (Figure 4.1) when compared to the traditional approach using C_{18} fibre.

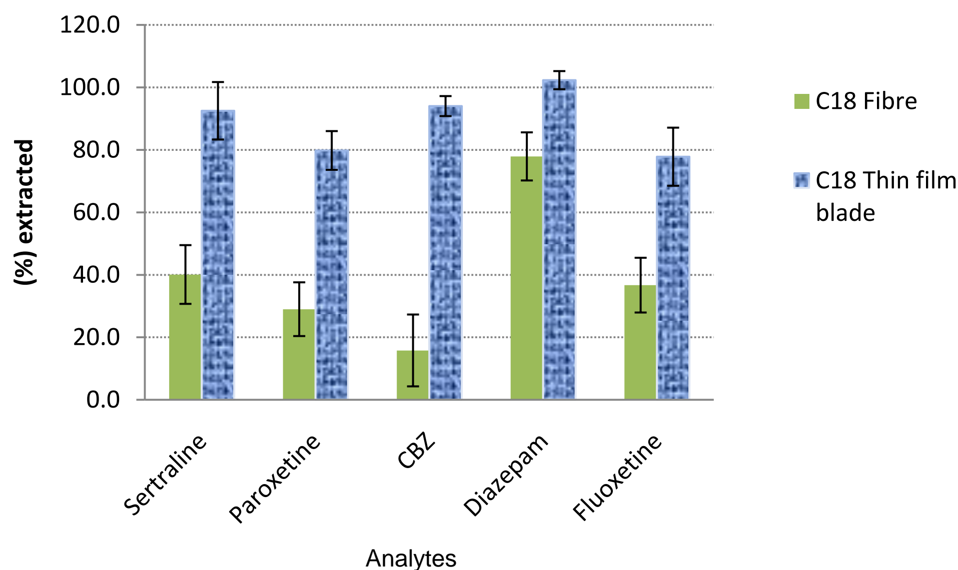


Figure 4:1 Extraction efficiency of C18 fibre and thin film coating for the target pharmaceuticals spiked phosphate buffer solution at pH = 7.4

This result shows a good agreement with the results that were reported elsewhere based on the use of thin film configuration [155]. Consequently, the C_{18} thin film geometry was selected for this study, due both to its enhanced extraction abilities and its ease of automation in a 96-well plate format, improving high throughput analysis of pharmaceuticals in wastewater samples. As a result of the larger volume of the extraction phase in the thin film geometry, the amount of target analytes extracted with C_{18} thin film is improved compared to C_{18} fibre (Figure 4.1). SPME

extraction sensitivity, or the amount of analyte extracted at equilibrium, is improved with C_{18} thin film geometry, and the higher extraction rate is distinctly advantageous in this study.

4.5.2 Effects of salt and pH on extraction efficiency

Extraction efficiency of an analytical method can be greatly influenced by sample pH due to ionization of analyte [157-158]. As the pH changes, the extractable form of the analyte is affected; consequently, the extraction efficiency of the C_{18} thin film geometry was studied over a pH range of 3-8, since receiving environment chemistry can change over time. Thus, if increases in acidity were realized, these would decrease the extraction efficiencies for the anti-depressant drugs of sertraline, fluoxetine, and paroxetine, due to protonation of these basic compounds in acidic medium (Figure 4.2), which was also reported elsewhere in the literature [158]

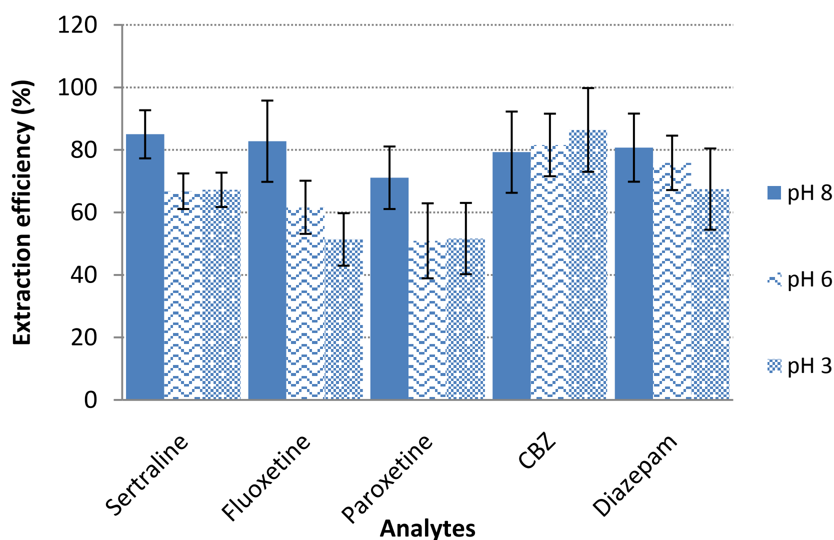


Figure 4:2 Effect of pH on the extraction efficiency of the newly developed SPME thin film method

In this study, however, carbamazepine and diazepam, being neutral compounds, did not vary significantly in their extraction efficiencies as the pH varied between 3 and 8. The effect of ionic strength on SPME extraction efficiency as modified by the addition of sodium chloride (NaCl, 0 – 20% (*w/v*) proportionally increased extraction efficiency up to 10% (*w/v*) salt, which was consistent with previous reports [153, 159]. However, as Figure 4.3 illustrates, increasing salinity from 10 to 20% (*w/v*) produced a gradual decrease in extraction efficiency, perhaps due to interactions between analyte and salt molecules [153, 159, 160]. It can be deduced from the results that the increase in the extraction efficiency was not significant compared to the sample without the addition of salt.

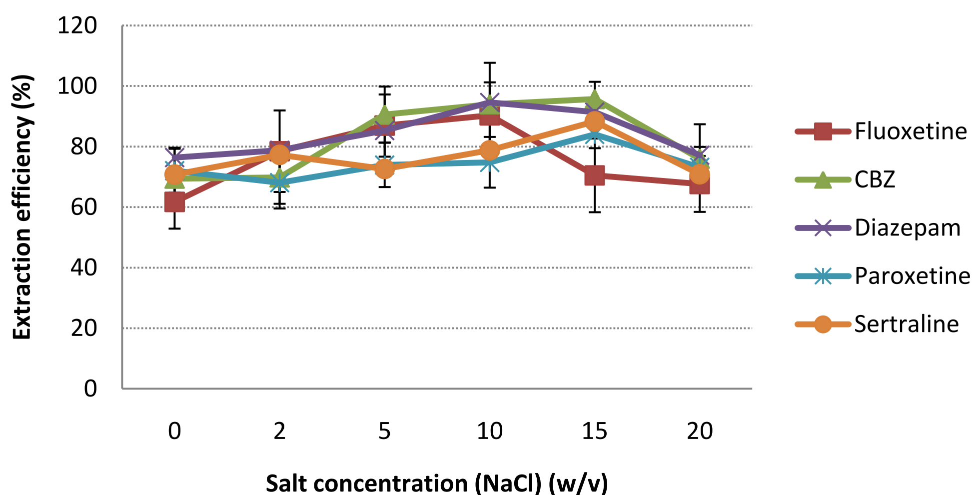


Figure 4:3Effect of ionic strength on the extraction efficiency of the method

4.5.3 Optimization of extraction and desorption conditions

Extraction time and desorption conditions are important factors affecting SPME performance; in the present study, method optimization was performed by sequentially varying one parameter at a time. Optimum extraction times were determined in spiked water (2 ng/mL n = 12) as the

shortest extraction time achieving equilibrium, which required approximately 70 min for the selected pharmaceutical analytes (Figure 4.4).

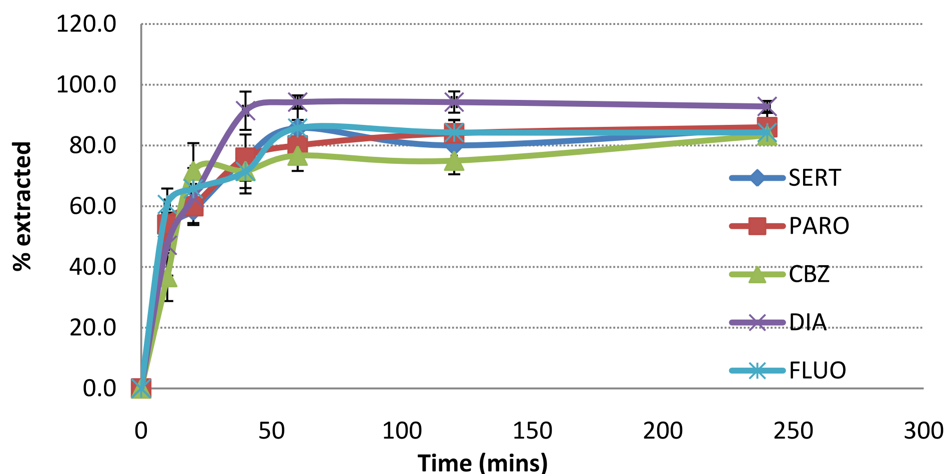


Figure 4:4 Extraction time profiles of the selected pharmaceuticals in spiked water

Since the amount of analyte extracted at equilibrium yielded the highest sensitivity, these extraction times were used for all subsequent experiments. Desorption solvents of varying concentrations of aqueous methanol were also evaluated in preliminary experiments; 1:1 ratios of methanol: water resulted in incomplete desorption of compounds such as diazepam and sertraline from the extraction phase, resulting in higher carryover (3-6%). Conversely, as the percentage of the organic (methanol) phase increased to 70%, analyte peak shapes in the chromatogram were broadened. As a result, a 60:40 ratio of methanol: water was used as the desorption solvent, resulting in efficient desorption of analytes from the extraction phase with negligible carryover (≤ 1%). In order to ensure that there was no cross contamination between each extraction step, the extraction phase was always pre-conditioned in conditioning solution (MeOH: water, 1:1). Table 4.1 summarizes the optimized parameters for the SPME method used in this study.

Table 4:1Optimized extraction and desorption conditions for analysis of the selected pharmaceuticals in spiked water

Parameters optimized on automated SPME robotic system	
Extraction volume	1.8 mL
Extraction time	70 min
Extraction agitation speed	1000 rpm
Desorption solvent composition	MeOH/Water (60:40)
Desorption time	60 min
Desorption volume	600 µL
Desorption agitation speed	1500 rpm

4.6 Method performance

4.6.1 Extraction efficiency (recovery) and sensitivity

Method extraction efficiency was evaluated in spiked wastewater samples (n = 8) at 0.5 ng/mL and 2 ng/mL from surface water from the Grand River upstream of wastewater releases, as downstream samples are strongly influenced by the wastewater discharges. For each spiked concentration, extraction efficiency was determined by comparing the amount of analyte extracted from the spiked samples. Some of the target compounds were detected in the blank (non-spiked) water sample; although it was collected upstream of the Kitchener wastewater discharge, it was influenced by the wastewater released 20 km upstream at Waterloo. Therefore, the amount of the target compound detected in the blank water sample was subtracted from each of the spiked water values. Extraction efficiency (recovery) of the target compounds in water

spiked at the two concentrations ranged from 71-87.5%, with acceptable repeatability (relative standard deviation, RSD, $n = 8$) of 9-15% (Table 4.2).

Table 4:2 Method performance in wastewater effluent spiked with 0.5 and 2 ng/ml of analyte

	0.5 ng/ml			2 ng/mL	
Analytes	Extraction efficiency (%)	RSD(%) $n = 8$	Analytes	Extraction efficiency (%)	RSD (%) $n = 8$
Paroxetine	78.3	11	Paroxetine	74.1	12
Fluoxetine	73.4	13	Fluoxetine	71.2	9
Sertraline	77.9	11	Sertraline	74.3	14
CBZ	83.7	14	CBZ	80.0	15
Diazepam	85.7	11	Diazepam	87.5	15

All test analytes were satisfactorily determined in spiked samples at both 0.5 and 2 ng/mL concentrations with acceptable reproducibility over different days. As method sensitivity is directly proportional to the amount of analyte extracted, the larger surface area of the C_{18} thin film geometry conferred 2 to 3 times more sensitivity than traditional C_{18} fibre SPME methods, without necessarily compromising the extraction time.

4.6.2 Linear range, detection and quantification limits

The method linearity for each compound was evaluated between 0.01 – 10 ng/mL (n = 3) in spiked wastewater samples, with all compounds being linear within the spiked concentration range (correlation coefficient > 0.99). Generally, the linear response of the compounds covered 2-3 orders of magnitude and the calibration curves were generated using linear regression analysis. The quantitative performance of the method was satisfactory since most of the compounds could be quantified within the linear range. The detection limit was determined as the minimum detectable amount of an analyte in the spiked wastewater extract based on S/N ratio of 3. Three replicate wastewater samples were spiked to obtain a final concentration of 500 ng/L and taken through the entire analytical method procedure. Limit of detection (LOD) was determined based on the signal-to-noise ratio (S/N) method as 3× S/N in wastewater. The limit of quantitation (LOQ) for the extraction of the target compounds calculated was based on 10× S/N. All LODs were in the range of 2-13 ng/L while the limit of quantification ranged from 10-50 ng/L, with acceptable reproducibility (Table 4.3). The precision of the chromatographic method was determined by the repeated injection of quality control standard on different days at a concentration of 1.25 ng/mL (n = 5) at the beginning and end of each sample, with an RSD of 5-10%.

Table 4:3Detection, quantification limit, correlation co-efficient (r^2), and weighting

Analytes	LOD (ng/mL)	LOQ (ng/mL)	R^2	Weighting index
Sertraline	0.006	0.02	0.9993	1/x
Fluoxetine	0.009	0.03	0.9960	1/x
Paroxetine	0.013	0.05	0.9952	1/x
Carbamazepine	0.002	0.01	0.9991	1/x
Diazepam	0.005	0.02	0.9998	1/x

4.6.3 Inter-day reproducibility in spiked wastewater

The inter-day reproducibility was determined in wastewater spiked using 2 ng/mL of the analytes of interest over 4 days and 10 repeated analyses (*n* = 8/time-point). These assessments were performed using the high-throughput automated 96-plate format, which yielded good inter-day reproducibility with an RSD of 4-15% (Figure 4.5). Using this approach, the average extraction efficiency of paroxetine was 80%, with an inter-day RSD of 6-12%. Average extraction efficiencies of fluoxetine, sertraline, carbarmazepine, and diazepam were 77-94 % with inter-day reproducibility (RSDs) of 4-12% (Figure 4.5), which was deemed acceptable. Instrument performance (reproducibility and sensitivity) was always checked at the beginning and end of each experiment by injecting quality control standards until satisfactory performance was achieved.

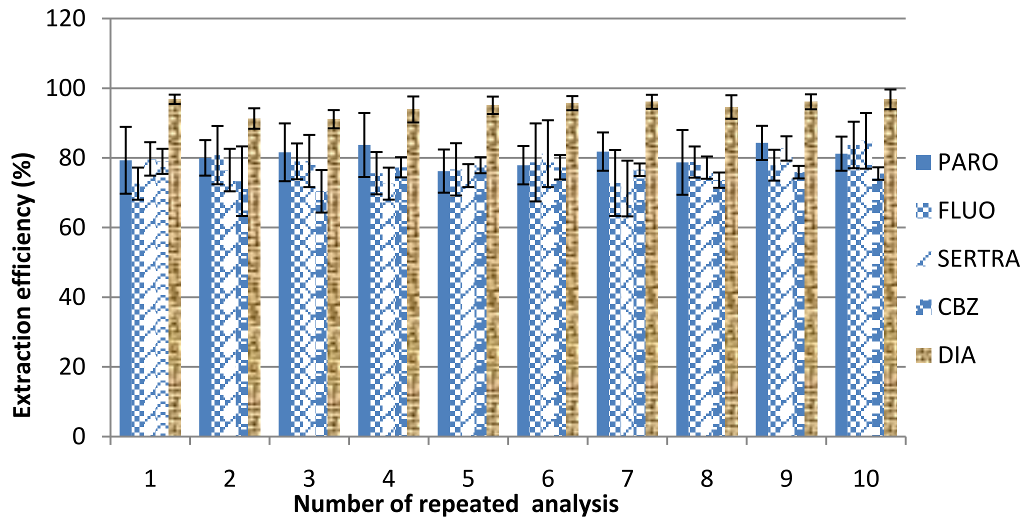
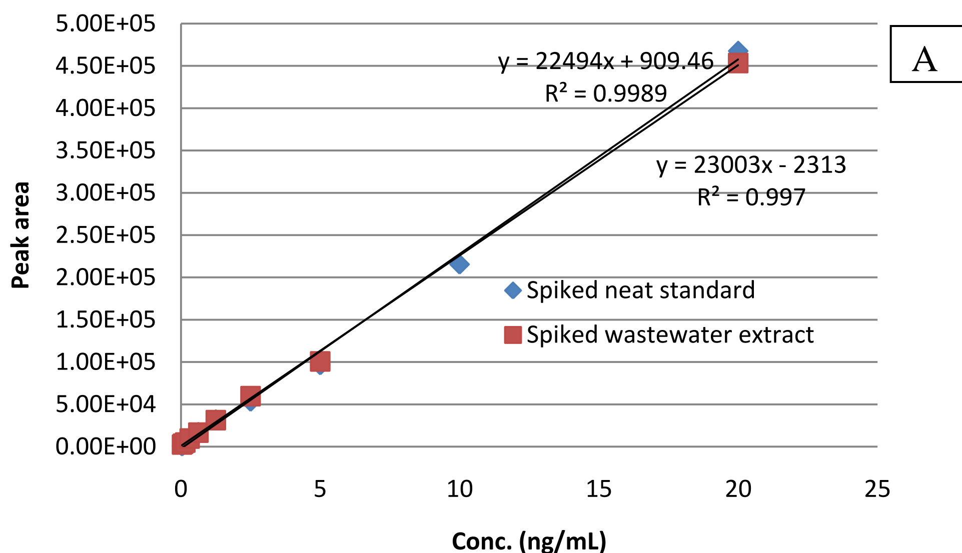


Figure 4:5 Investigation of extraction efficiencies and reproducibility of the method in 10 discrete repeated analyses over 4 days in 2 ng/mL water spiked with target compounds

4.6.4 Matrix effect

The slope of the spiked extract calibration curve was related to the slope of the corresponding spiked neat standards. There was no significant difference between the slopes of the linear regression of the target compounds investigated in both matrices, suggesting that matrix influences on the quantitation of these compounds were negligible (Figure 4.6). In this study, there was no significant matrix effect when using the developed method to quantify the target compounds in wastewater.



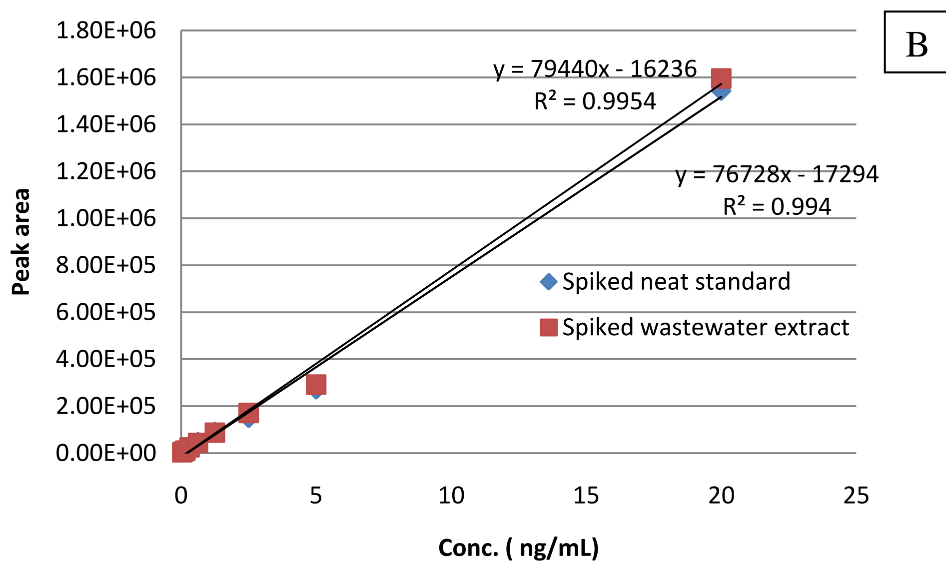


Figure 4:6 Comparisons of slopes of calibration in spiked d5-diazepam (A) and d_{10}-carbamazepine (B) in neat standard and waste sample extract

Also, it is worth mentioning that matrix interference was not observed with the method even when internal standard was not used, when comparing the slope of linear regression in pure neat

standard and wastewater extract (Figure 4.6). As shown in Table 4.4, the results indicate that the effect of matrix co-extraction was not observed for these compounds being investigated.

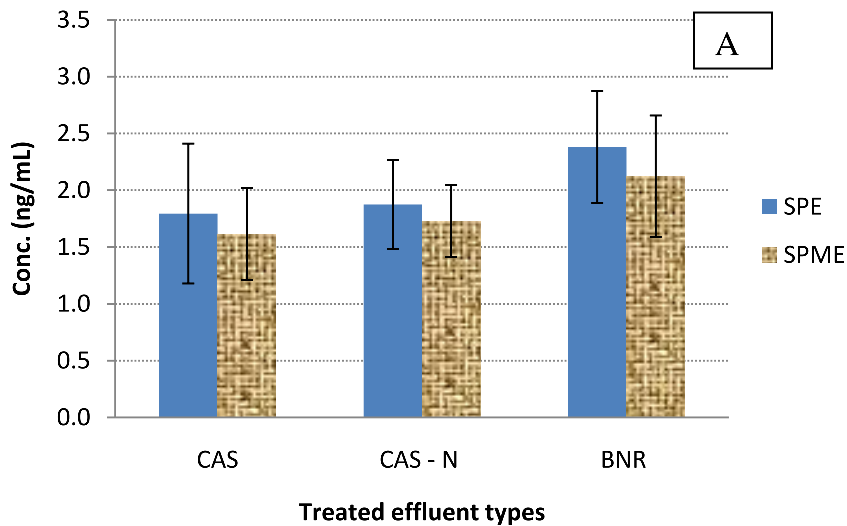
Table 4:4 Comparison of the slope of linear regression of deteurated standard in spiked neat (pure) solvent and wastewater extract over dynamic range (0.039 -20 ng/ml) (n = 3)

Compounds	Slope (S_1)	R^2 correlation	Slope (S_2)	R^2 correlation	ME (%)
d_4-Paroxetine	437	0.9852	460	0.9924	105
d_5-Fluoxetine	2956	0.9985	2805	0.9978	95
d_3-Sertraline	33534	0.9982	34059	0.9929	102
d_{10}-Carbamazepine	79440	0.9954	76728	0.9940	97
d_5-diazepam	22494	0.9989	23003	0.9970	102

This shows that SPME extract was relatively clean, and may not be causing ionization suppression or enhancement. The present results demonstrate that wastewater matrix components that can interfere with the method performance were not co-extracted in the SPME extraction. In addition, the instrument response was linear ($r^2 > 0.99$) in the dynamic range (0.039 – 20 ng/ml) except for d_4-paroxetine, whose correlation co-efficient was greater than 0.98. The use of isotope dilution was also employed to compensate for any matrix effects, particularly when dealing with complex samples such as wastewater [161-168]. The use of SPME as a sample preparation technique is also an effective way to deal with matrix effect, particularly when the internal standard approach is used [140, 153, 167].

4.6.5 Validation of SPME with traditional solid phase extraction (SPE)

The optimized SPME method was validated with the well-established SPE method during the analysis of the Burlington pilot-scale wastewater effluent samples using isotopically-labelled standards in both approaches. With both methods, the concentrations of carbamazepine and fluoxetine were comparable with acceptable precision in all treatment types, with no significant differences in the concentrations determined by the two methods (Figure 4.7).



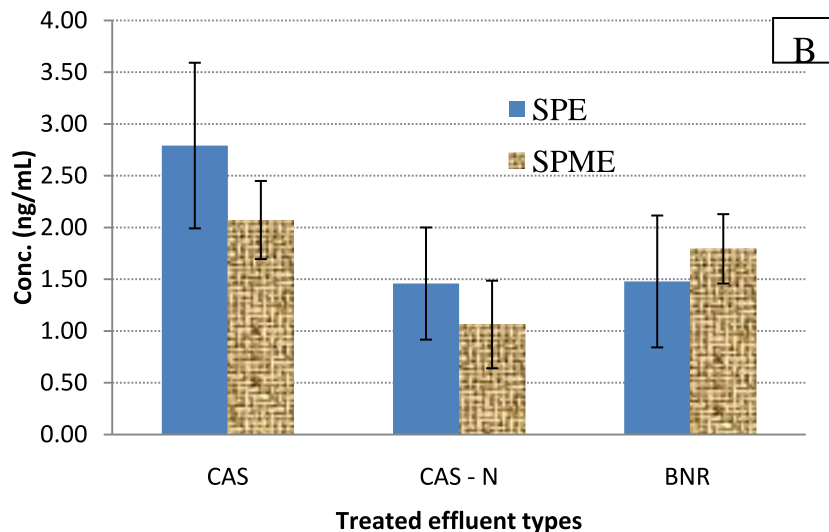


Figure 4:7 Comparisons of concentrations of fluoxetine (A) and carbamazepine (B) between SPME method and traditional method (SPE)

However, the use of automated SPME proved to be more rapid and simpler when compared to the traditional SPE approach. It is notable that the developed method required no sample pre-treatment or vacuum pump and was not subject to matrix clogging of the extraction phase as occurs with SPE, thereby potentially limiting extraction efficiency. For SPE extraction, the samples have to be filtered to avoid clogging. However, while the reproducibility of both methods is comparable, the detection limit of SPE was lower than SPME, reflecting the exhaustive extraction of analytes by SPE leading to higher pre-concentration factors because of the larger volume of sample used. Notwithstanding, the newly developed SPME method can be useful for high-throughput analysis of pharmaceuticals in wastewater samples with a sensitive instrument being used in tandem with the thin film geometry extraction phase. In addition, the TF-SPME system used in this study is relatively less expensive as it does not require a pump to push the sample through the sorbent bed.

4.6.1 Application of the method to wastewater (effluent) from Burlington pilot plant and Grand River water samples

To demonstrate the application of the automated SPME method in urban wastewater samples, the approach was applied to undiluted Burlington, ON wastewater effluent treated by three processes (CAS, CAS-N, and CAS-BNR) at pilot-scale treatment plants. The samples, which visibly varied in clarity and matrix between treatment types (CAS most opaque, CAS-BNR clearest), also varied in detected analyte concentrations over the intervals collected, perhaps reflecting variations in influent concentrations or treatment effectiveness (Table 4.5).

Table 4:5 Concentration (ng/mL) of the target pharmaceutical compounds detected by thin film microextraction method (n = 3)

Compounds	24-Aug			28-Aug			30-Aug		
	BNR	**CAS**	**CAS-N**	**BNR**	**CAS**	**CAS-N**	**BNR**	**CAS**	**CAS-N**
Paroxetine	0.24 ± 0.06	0.35 ± 0.09	0.32 ± 0.06	0.27 ± 0.06	0.30 ± 0.11	0.35 ± 0.07	0.56 ± 0.10	0.71 ± 0.15	0.74 ± 0.23
Fluoxetine	1.05 ± 0.11	1.45 ± 0.15	1.23 ± 0.32	0.83 ± 0.15	0.98 ± 0.12	0.81 ± 0.13	0.81 ± 0.09	1.26 ± 0.16	1.00 ± 0.17
Sertraline	1.41 ± 0.27	1.93 ± 0.18	1.91 ± 0.34	1.38 ± 0.18	2.19 ± 0.32	1.88 ± 0.24	1.38 ± 0.32	1.90 ± 0.33	1.85 ± 0.41
Carbamazepine	0.64 ± 0.12	0.75 ± 0.08	0.69 ± 0.11	0.53 ± 0.13	0.63 ± 0.12	0.51 ± 0.12	0.64 ± 0.07	0.64 ± 0.02	0.64 ± 0.03
Diazepam	ND	ND	ND	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

	16-Sep			18-Sep			20-Sep		
Compounds	**BNR**	**CAS**	**CAS-N**	**BNR**	**CAS**	**CAS-N**	**BNR**	**CAS**	**CAS-N**
Paroxetine	0.32 ± 0.06	0.37 ± 0.14	0.61 ± 0.13	0.55 ± 0.16	0.79 ± 0.18	0.70 ± 0.20	1.04 ± 0.19	0.86 ± 0.27	0.96 ± 0.27
Fluoxetine	2.29 ± 0.41	3.82 ± 0.32	3.01 ± 0.46	1.12 ± 0.08	1.74 ± 0.25	1.44 ± 0.22	ND	ND	ND
Sertraline	1.31 ± 0.24	1.52 ± 0.19	1.47 ± 0.18	1.42 ± 0.30	1.88 ± 0.32	1.81 ± 0.27	ND	ND	ND
Carbamazepine	0.46 ± 0.11	0.53 ± 0.09	0.44 ± 0.10	0.39 ± 0.07	0.45 ± 0.06	0.41 ± 0.06	< LOQ	< LOQ	< LOQ
Diazepam	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

Most target compounds, with the exception of diazepam, were detected and quantified in all treatments, with antidepressants such as fluoxetine, sertraline, and to a lesser extent, paroxetine, being detected at relatively higher concentrations. However, there was an instance when sertraline and fluoxetine were detected but not quantified in two wastewater samples, since the compounds were below the limit of quantitation of the method. In addition, anti-convulsant carbamazepine was detected in the treated effluent at lower concentrations of 390-640 ng/L. Although diazepam was detected in some of the effluents analyzed, the concentrations were below the limit of quantification, either due to relatively low rates of use in the populace or efficient removal by the pilot-scale treatment plants. Another possible reason could relate to microbial activity, which may degrade the organic contaminants including pharmaceuticals, particularly when conventional activated sludge treatment is used for the removal of contaminants. The feasibility of the newly developed method was demonstrated in wastewater samples collected from Grand River at Kitchener and Waterloo downstream sites. Figure 4.8 indicates the chromatogram of some of the ubiquitous compounds detected in the wastewater effluent samples analyzed.

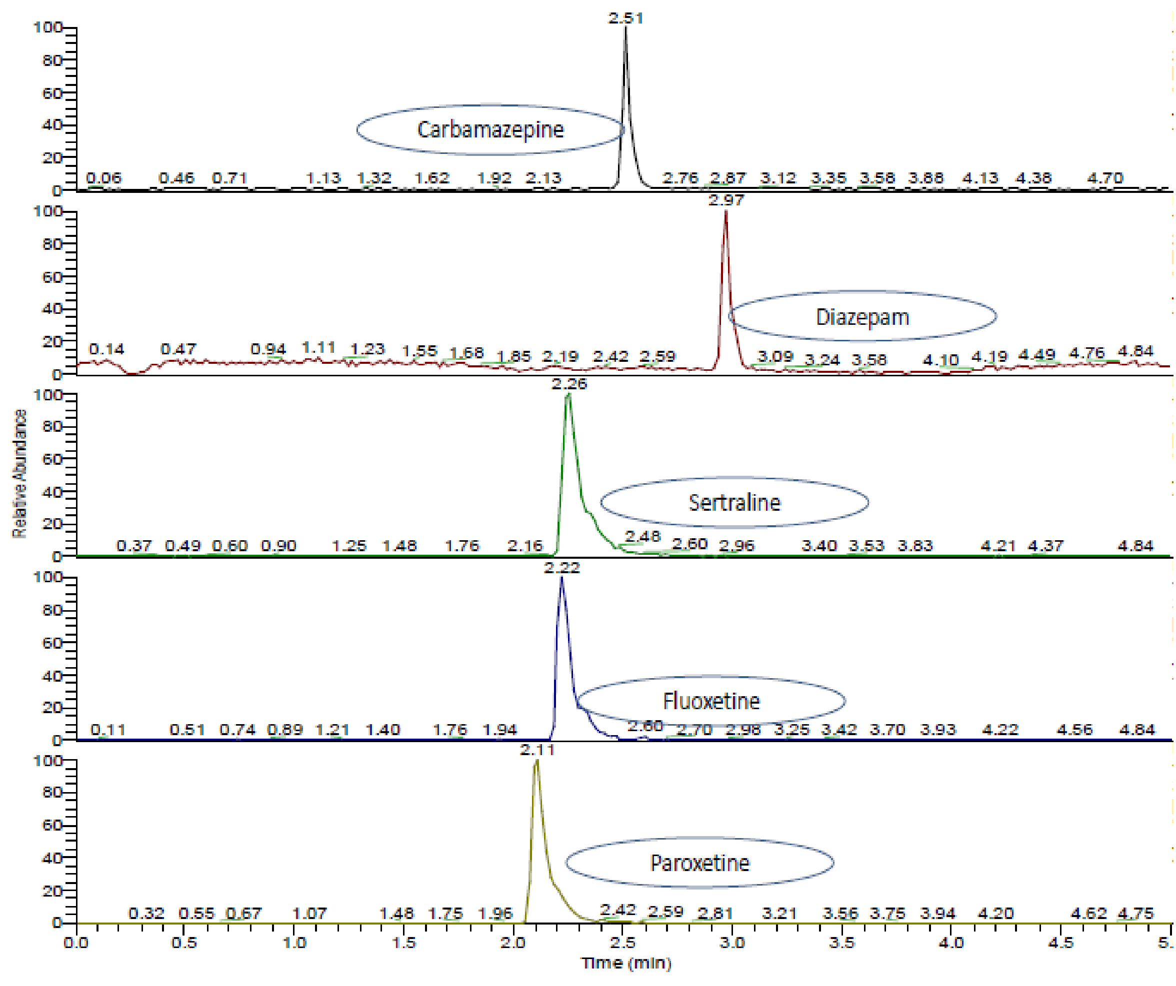


Figure 4:8 Chromatogram of the sample extract from wastewater collected from Grand River at Kitchener downstream site

Figure 4.8 demonstrates that this method is capable of detecting the selected pharmaceuticals in wastewater collected along the Grand River in Waterloo and Kitchener downstream sites. As shown in Figure 4.9, the concentration of these compounds ranged from 200 – 650 ng/L with anti-depressant drugs such as fluoxetine and sertraline having higher concentrations.

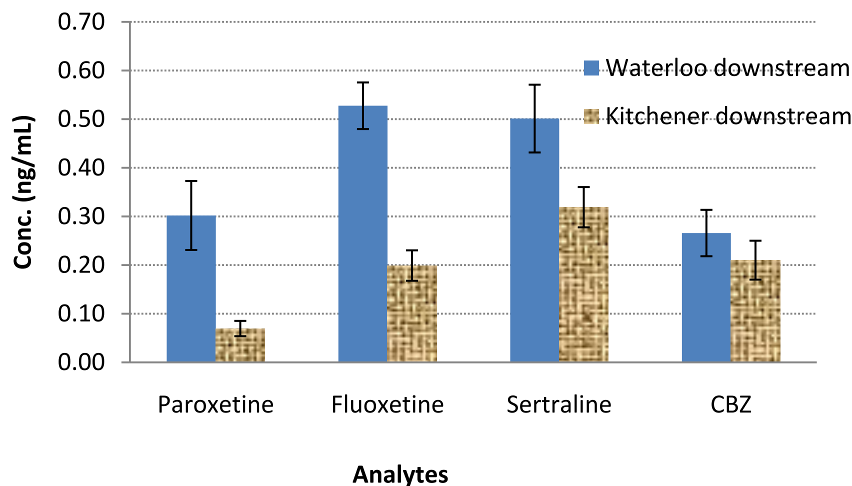


Figure 4:9 Concentration of selected pharmaceuticals detected and quantified in Grand River at Waterloo and Kitchener downstream sites

Again, diazepam was detected but was not quantified because it was lower than the limit of quantitation of the method. The present results demonstrate a simplified methodology for the determination of emerging contaminants such as pharmaceutical residue in the environment. Based on the proposed method, a large number of wastewater samples were processed within a short time frame due to the automation of the extraction and desorption steps, in addition to the use of 96-well plate format for high throughput analysis. In addition, the author's laboratory is currently developing more polar and mixed coatings to advance the future of the TF-SPME technology, which will lead to wider applications for analysis of different classes of pharmaceuticals[168].

4.7 Conclusion

This study demonstrates the efficacy of the automated TF-SPME approach in detecting pharmaceuticals in water samples with varying degrees of matrix and good reproducibility.

Based on the thin film configuration of the extraction phase, the sensitivity of the method is improved when compared to the traditional SPME method. The sensitivity of the method is contingent on the enhanced extraction efficiencies afforded by the surface area of the thin film extraction phase and the sensitivity of the analyzing mass spectrometer. In addition, the automated TF-SPME provides high throughput analysis of pharmaceuticals in water samples. This method is simple, reproducible, and consistent with analyte determinations quantitated by traditional solid phase extraction. The study demonstrates that automated TF-SPME (based on SPME principle) is a promising method of pharmaceutical analysis in wastewater effluents and effluent-influenced surface waters with acceptable detection limits capable of identifying the selected pharmaceuticals in environmental water. In addition, no matrix effect was observed with this technique for the pharmaceuticals being investigated in water samples.

Chapter 5

Optimization of solid phase microextraction for non-lethal *in vivo* determination of selected pharmaceuticals in fish muscle using liquid chromatography – mass spectrometry

5.1 Preamble

This chapter has been modified and published as a paper: O.P. Togunde, K. D. Oakes, M. R Servos, and J. Pawliszyn. 2012. "Optimization of solid phase microextraction for non-lethal *in vivo* determination of selected pharmaceuticals in fish muscle using liquid chromatography – mass spectrometry" *Journal of ChromatographyA*. 2012, 1261, 99-106. The figures and tables are reprinted from this manuscript with the permission of Elsevier (Copyright Elsevier 2012).

5.2 Introduction

Recently, there has been sustained global scientific interest surrounding the detection and potential impact of pharmaceuticals released into the environment on exposed organisms [12,169-171]. The detection of pharmaceutical residues in surface waters influenced by municipal wastewater effluents (MWWEs) and agricultural runoff has raised concerns from regulatory agencies around the globe [23,172-173]. As a consequence of the continuous release of human pharmaceuticals in MWWEs, fish inhabiting these receiving environments can bioconcentrate considerable amounts of these bioactive compounds in their tissues, despite their relatively water soluble properties [40,174]. Various analytical methods have been proposed and utilized to determine the concentrations of environmental pharmaceuticals in fish [14, 31, 39,174-177]. Traditional methods include solid phase extraction (SPE) [40], liquid extraction (LE) [31], and matrix solid phase

dispersion (MSPD) [175]. However, none of these approaches can be applied *in vivo* to determine concentrations of organic contaminants in fish. Traditional analytical methods require lethal sampling of a large number of fish to obtain sufficient tissue and variance estimates to accurately determine bioconcentration rates. From the perspective of animal care, such sampling procedures are undesirable, and in some jurisdictions, unacceptable. Recently, SPME has proven itself a simple alternative analytical method that is particularly relevant for the non-lethal *in vivo* sampling of pharmaceutical residues in fish muscle [43, 61,127,178-179]. However, the small extraction surface area and volume of PDMS fibres limited sensitivity and presented challenges for detecting fairly polar pharmaceuticals (*i.e.* carbamazepine) or those with low bioconcentration factors under pre-equilibrium sampling conditions. To overcome these limitations, a new SPME configuration based on C_{18} thin film geometry (a modification of SPME) was proposed, with potential to improve the sensitivity of the method. Furthermore, the proposed SPME configuration has the potential to improve extraction kinetics under pre-equilibrium sampling conditions relative to traditional PDMS or C_{18} fibre configurations. The objective of this study is to investigate and optimize the new octadecyl (C_{18}) thin film geometry to improve SPME sensitivity and *in vivo* extraction kinetics when quantifying pharmaceuticals in fish.

5.3 Theoretical considerations

The volume and type of the extraction phase is a critical parameter modifying the distribution constant between the extraction phase and sample matrix, as well as the amount of analyte extracted. SPME extraction kinetics and sensitivity (recovery) can be improved by changing the configuration (geometry) of the extraction phase. Theoretically, the initial rate of SPME

extraction is directly proportional to the surface area of the extraction phase [180] as described in equation (1):

$$\frac{dn}{dt} = (D_s A / \delta) C_s \qquad \text{.. 5.1}$$

where dn/dt is the rate of extraction, D_s is the diffusion coefficient of the analyte in the sample matrix, A is the surface area of the extraction phase, δ is the thickness of the boundary layer surrounding the extraction phase and C_s is the concentration of analyte. Based on this equation, extraction kinetics can be improved (particularly during pre-equilibrium extraction) when the traditional cylindrical fibre geometry is changed to a thin film configuration, which will provide a faster extraction rate for the analytes of interest. SPME sensitivity is a function of extraction phase type and volume[89, 181] as the amount of analyte extracted from a sample matrix at equilibrium is proportional to the volume of the extraction phase as shown in equation (1.1). Equation 1.1 can be further simplified to equation 1.2 when sampling large volumes, such that $K_{fs}V_f << V_s$

Based on equation 1.2, the amount of analyte extracted in fish can be related to the volume of the extraction phase and partition co-efficient between the extraction phase and fish sample matrix. Therefore, the use of C_{18} thin film geometry will theoretically improve pre-equilibrium SPME extraction kinetics relative to traditional C_{18} coated fibre geometries for *in vivo* detection of pharmaceuticals in fish.

5.4 Experimental

5.4.1 Chemicals and materials

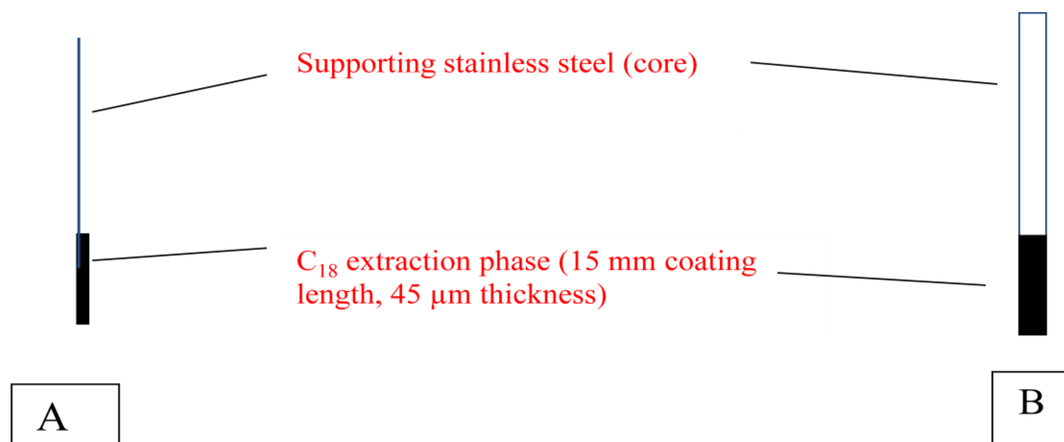
All reagents and pharmaceuticals standards were of the highest purity grade available. Fluoxetine, diazepam, nordiazepam, and diazepam-d_5 were purchased as certified standards from Cerilliant Corp (Round Rock, TX, USA). Gemfibrozil, atorvastatin, ibuprofen, carbamazepine, diclofenac, naproxen, and bisphenol-A (BPA) were obtained from Sigma-Aldrich (Oakville, ON, Canada), while paroxetine and sertraline were purchased from Toronto Research Chemical (ON, Canada). Isotopically-labeled atorvastatin-d_5, atrazine-d_5, BPA-d_{16}, carbamazepine-d_{10}, diazepam-d_5, diclofenac-d_4, fluoxetine-d_5, gemfibrozil-d_6, ibuprofen-d_3, and ^{13}C-naproxen-d_3 were purchased from CND Isotope Inc (Point-Claire, QC, Canada) while sertraline-d_3 and paroxetine-d_4 were purchased from Toronto Research Chemical. Chemical stock solutions were prepared in methanol and stored at -20°C while working solutions were diluted aliquots of these stocks. Dilution water was obtained from a Barnstead Nanopure diamond UV water purification system deionized to 18 Ω. Acetonitrile (HPLC grade), methanol (HPLC grade), and glacial acetic acid were purchased from Fisher Scientific (Ottawa, ON, Canada). Phosphate-buffered saline (PBS) solution, pH 7.4 was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.2 g of potassium phosphate, and 1.44 g of sodium phosphate in 1 L of purified water and by adjusting the pH to 7.4 using 1M sodium hydroxide (NaOH). The C_{18} thin film extraction phase (45 µm, 1.5cm coating length) and traditional C_{18} fibres (45µm, 1.5 cm coating length) were obtained from Supelco (Bellefonte, PA USA). A 10 mg/L mixed solution containing all the pharmaceuticals was obtained by diluting the individual stocks with methanol.

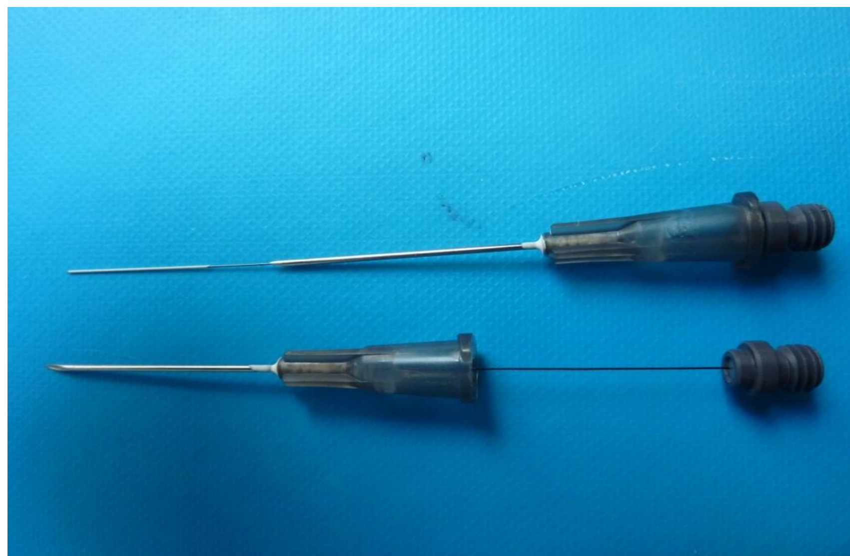
5.4.2 Fish exposure

Immature Rainbow Trout (*Oncorhynchus mykiss*) were purchased from Silvercreek Aquaculture (Erin, ON, Canada) and fathead minnow *(Pimephales promelas*) were purchased from Silhanek Baitfish (Bobcaygeon, ON, Canada). All animal experimental procedures were approved by the local Animal Care Committee at the University of Waterloo (AUP #'s 04-24, 08-08). For the laboratory exposure, Rainbow Trout were acclimatized to laboratory conditions in de-chloraminated municipal water and fed every other day with 2.0 Pt floating commercial trout ration (Martin's Feed Mill, Ontario) until 4 d prior to the onset of the experiment. Subsequently, the fish were exposed to municipal effluent diluted with dechloraminated municipal water. In the field caging experiment, fathead minnow (*Pimephales promelas*), a small-bodied fish, were caged in the Grand River watershed (southern Ontario, Canada) adjacent the Doon Wastewater Treatment Plant (43° 24'03.29"N; 80°25' 12.04"W) at 2 upstream and 3 downstream sites for 14 d in October 2010. Following the exposure, pharmaceuticals extracted from the fish tissues were desorbed in the desorption solution (methanol: water, 3:2) and analyzed by liquid chromatography–tandem mass spectrometry. The quantification of target pharmaceuticals extracted from free-moving Rainbow Trout was performed using a well-established kinetic calibration approach [71,182]. Kinetic calibration is a pre-equilibrium calibration approach utilizing known amounts of preloaded deuterated analyte desorbing from the extraction phase, to calibrate the rate of adsorption of the target analytes from the fish tissue to the extraction phase (based on the symmetric relationship between extractions and desorption kinetics). The concentration of analytes in fish tissue can be determined by equation 1.3

5.4.3 Thin film extraction phase preparation

Traditional C_{18} SPME fibres were obtained from Supelco (Bellefonte PA, USA) and consisted of a flexible stainless steel core with a 1.5 cm coating immobilized on the bottom portion (Figure 5.1a). In contrast, for the thin film extraction phase configuration, a slurry of biocompatible binder polyacrilonitrile (PAN) containing 5 μm C_{18}-coated porous silica particles were immobilized on the metal core (Figure 5.1b) with coating thickness of 45µm and 1.5 cm coating length.





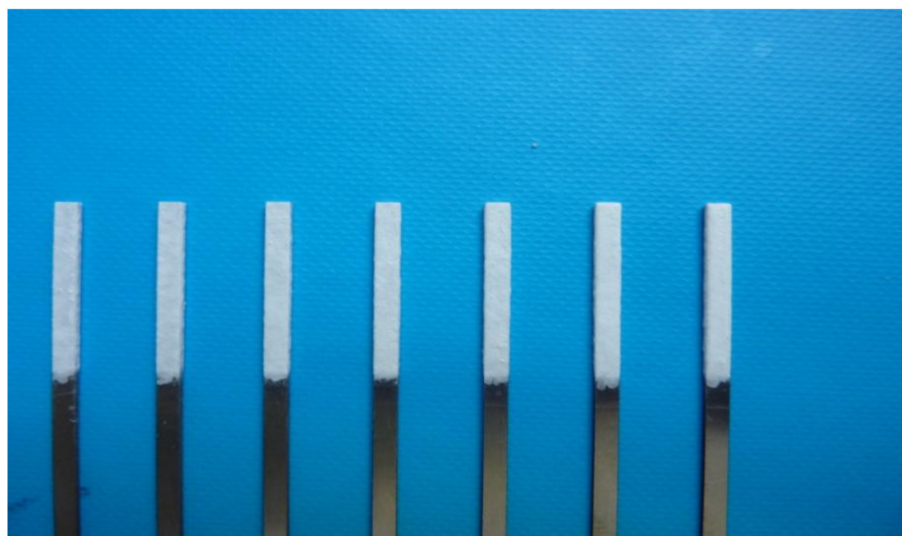


Figure 5:1Schematic diagram of the C_{18} SPME extraction phase geometries used in this study. Both configurations utilize 45 µm coating thicknesses over a 1.5 cm coating length. A is a C_{18} cylindrical fibre (core of 200 µm diameter, length of 40 mm, extraction phase surface area of 8.1 mm^2 (shown in hypodermic needle); B is C_{18} thin film extraction phase (core of 40 mm × 2.1 mm × 0.07 mm and extraction phase surface area of 65.4 mm^2.

The flattened metal support for the thin film configuration was obtained from Professional Analytical System Technology (PAS, Magdala, Germany), the surface upon which the C_{18} particles were immobilized as described in detail elsewhere [183]. Briefly, the blade metal is etched with concentration HCl for 1 hprior to rinsing with water for 30 min. The metal blade is then dried in an oven at 150 ^{o}C for 30 min. The slurry of C_{18} particles (5 µm discovery silica-based-C_{18} particles, Supelco, PA) is made with a mixture of proprietary biocompatible binder PAN (10 *w/w*) and N, N-dimethylformamide (DMF) purchased from Caledon Laboratories (ON, Canada). Coatings (mass of 0.28±0.05 g) were immobilized on the metal fibre core using a flask type sprayer connected to a nitrogen gas line (250 mL Erlenmeyer flask with a sprayer head). After spraying, the coated blade was dried at 180 ^{o}C for 2 min, with the spraying and drying processes repeated at least three more times until a desired coating thickness was achieved. C_{18} fibre is cylindrical with a metal fibre core of 200 µm in diameter, while C_{18} thin film is rectangular with surface area of 0.625 cm^2. Figure 5.1 illustrates the relative physical dimensions of both C_{18} configurations.

5.4.4 Instrumental analysis – LC/MS/MS

Analyses were performed on an AB-Sciex 3200 QTrap Mass Spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems Sciex, Foster City, CA, USA). Liquid chromatography (LC) was performed on an HP1100 HPLC system (Agilent Technologies) equipped with a degasser, binary pump, an autosampler, and a column oven. Chromatographic separation was performed on a Zorbax Eclipse XDB C_{18} (150 mm × 21 mm, 3.5µm) column which was preceded by a C_{18} guard column at a flow rate of 0.8 mL/min with mobile phase A (95% water, 5% MeOH, 0.1 % acetic acid) and B (95% MeOH, 5% water, 0.1% acetic acid). The

injection volume for analysis of all the samples was 20 µL. The elution gradient was programmed as follows: mobile phase B was increased from 10% to 50% over 0.5 min and 50% to 100% over 7.5 min, held at 100% for 2 min, and then reduced to 10% over 1 min. Analyst® version 1.4.2 software was used for the data analysis. Quality control samples (10 ng/mL of target compounds) were utilized at the beginning and end of each run to ensure instrumental stability. All analytes were analyzed using selective reaction monitoring (SRM) of the transitions with electrospray ionization (ESI). Optimal MS/MS transitions for the target analytes were determined with either positive or negative ionization mode.

5.4.5 *In vitro* evaluation of performance of thin film and fibre geometries in spiked fish tissue

Extraction efficiency of the two SPME configurations (C_{18} fibre and C_{18} thin film) was evaluated in spiked fish tissue under pre-equilibrium sampling conditions. Evaluation of recovery by the extraction phases was carried out using homogenized (Teflon™ homogenizer at 1100 rpm for 20 min) Rainbow Trout dorsal-epaxial muscle (4.0 ± 0.2 g) spiked with 200 µL of 2 mg/L solutions of target compounds. Subsequently, the 100 ng/g spiked muscle sample was vortexed for another 20 min to ensure complete analyte mixture within the tissue prior to 2 h incubation at 4°C. After incubation, extraction was performed for 24 h in the spiked fish muscle sample using C_{18} fibre and thin film extraction phases. After the 24 h extraction, both C_{18} extraction phase configurations were washed with distilled water for 5 sec, and gently wiped with a Kimwipe® tissue. The C_{18} fibres were immediately desorbed in 100 µL of desorption solvent (3:2 methanol: water) for 90 min at 1000 rpm using a multi-tube vortex (model DVX-2500, VWR International, Mississauga). For the thin film extraction phase, 300 µL of the same desorption solvent removed

analytes from the extraction phase. The extraction and desorption conditions are summarized in Table 5.1

Table 5:1Condition of extraction and desorption of spiked compounds in fish tissue at room temperature (25 ^{o}C)

Extraction conditions		**Desorption conditions**	
Extraction time	24 h	Desorption time	90 min
Extraction sample	spiked fish	Desorption solvent	MeOH/H_2O (3:2)
Spiked concentration	100 ng/g	Desorption volume	100 and 300 µL
Extraction mass	4 g	Agitation speed	1000 rpm
Agitation condition	static		

5.4.6 Extraction kinetics of pharmaceuticals

Extraction kinetics of both extraction phase configurations for drugs utilized in this study were determined in spiked 1% *w/v* agarose gel samples due to limited availability of fish muscle and previous use of 1% *w/v* agarose gel in other studies determining *in vitro* mass transfer phenomenon [181]. Agarose gel was used for *in vitro* study as both the gel and fish muscle are semi-solids of comparable porosity and tortuosity, allowing free diffusion of drugs along their concentration gradient with mass transfer governed by Fick's law in both sample matrices [38,96] The detailed gel preparation procedure has been discussed elsewhere [43]. In brief, agarose was weighed and dissolved in hot phosphate buffer (pH = 7.4) and allowed to solidify at room temperature for 3 h. Just prior to solidification, a known volume of agarose gel was spiked (100 ng/mL n = 3) with a mixture of the selected drugs and vortexed for 10 min in a 4 mL vial to homogenize the drug distribution in the gel. After gel solidification, pre-equilibrium extraction

time profiles were determined (20, 30, 40, 50, and 60 min) using C_{18} fibres and C_{18} thin film extraction phases, which simultaneously performed extractions from different vials for each time point. After each interval, both extraction phases were removed from their respective spiked gels, rinsed gently with distilled water, and patted dry with a Kimwipe®. Immediately thereafter, the fibres and thin film extraction phases were desorbed in desorption solvent (60:40 methanol-water) for 90 min as previously described. Extracts from both C_{18} configurations at each extraction interval were quantified by LC/MS/MS.

5.4.7 Laboratory exposure of Rainbow Trout to wastewater effluent

The juvenile Rainbow Trout used in this study (14.4±0.34 cm; 25.4±1.46 g, mean ±SE, n = 40) were acclimated to laboratory conditions in 1400 L holding tanks continuously receiving de-chlorinated clean water, and fed every other day with floating commercial trout ration (Martin's Feed Mill, Elmira, ON). Acclimation within the 34 L exposure aquaria (containing previously acclimated clean water) began 4 d prior to the onset of the experiment. The experiment began when fish were exposed to wastewater collected from three pilot treatment plants receiving raw effluent generated from the City of Burlington (Burlington, ON). Wastewater samples were treated by Conventional Activated Sludge (CAS), Conventional Activated Sludge with Nitrification (CAS-N), or Conventional Activated Sludge with Biological Nitrifying Reactor (CAS-BNR). A detailed description of the properties and process operation of each type of wastewater treatment has been provided elsewhere [184]. The exposure involved two controls and six different treatments: CAS-20%, CAS-50%, CAS-N-20%, CAS-N-50%, CAS-BNR-20%, and CAS-BNR-50%. Each 34 L aquaria housed 5 fish in each tank and the exposure duration was 4 d with the exposure medium renewed every 48 h, using the de-chlorinated acclimation water as

diluents. Exposure water quality was checked daily and maintained at conditions considered optimal for trout (12.5 ± 0.05^{o}C; pH 8.17 ± 0.06; ammonia 23.5 ± 1.5 μg/L) under a 12 h:12 h light:dark cycle.

The thin film extraction phase was preloaded with deteurated standard (50 ng/mL in spiked phosphate buffer solution) by direct extraction under agitation (500 rpm) for 3 h on a shaker platform. At the conclusion of the 4 d exposure period, *in vivo* sampling by thin film microextraction (TFME) was performed in dorsal-epaxial muscle of anaesthetized (0.1% ethyl 3-amino benzoate methanesulfonate) fish for 30 min (under pre-equilibrium conditions). After 30 min, the extraction phases were removed from fish muscle and rinsed with distilled water prior to immersion in desorption solvent (60:40 methanol-water) for 90 min; an aliquot of desorption solution was injected for LC/MS/MS instrumental analysis. After removal of the extraction phase from their muscle tissue, fish were killed by spinal severance in accordance with protocols approved by our local Animal Care Committee (AUPP 10-17).

5.4.8 Field caging of fathead minnow in municipal wastewater

Fathead Minnow (*Pimephales promelas*), a small bodied fish (5.58±0.03 cm; 1.43±0.02 g, *n* =300) were caged in the Grand River watershed (southern Ontario, Canada) adjacent the Doon Wastewater Treatment Plant (43° 24'03.29"N; 80°25' 12.04"W) at 2 upstream and 3 downstream sites for 14 d in October 2010. The most upstream of the two reference sites is located 1.2 km upstream of the Doon municipal effluent outfall, but 19.45 riverine km downstream of the municipal wastewater discharge from the City of Waterloo. The Waterloo WWTP serves a population of more than 120,055 and the Kitchener MWWTP serves more than 190,000 individuals with a daily discharge of 23,802 m3/day and 77,768 m3/ day respectively. The

second reference was 0.5 km upstream of the Doon outfall. The three downstream stations were 0.5, 1.7, and 5.6 km below the Doon wastewater effluent release, respectively. At each site, two cages (Rubbermaid™ containers) were deployed, each containing two commercial baitfish buckets (FlowTroll®, Frabill Inc, Jackson, WI) holding 15 fish each (30 fish/cage). The Rubbermaid containers were perforated on all surfaces with 2 cm holes and contained a 60 x 60 cm concrete patio stone beneath the bait buckets for weighting. The cages were anchored to the substrate with t-posts fastened to cable running through a homemade pipe frame. This caging design exposed fish to minimal current, thereby reducing exposure stress from constant swimming, yet allowing water to pass freely through the enclosure. After 2 weeks of exposure, fish were anesthetized and sampled as described for the laboratory-based Rainbow Trout exposures.

5.4.9 Results and discussion

5.4.9.1 Extraction efficiency of the method

The influence of C_{18} fibre and thin film extraction phase geometry on method extraction efficiency was determined by the amount of analyte extracted by each configuration under identical conditions. As the extraction phase/sample matrix partition coefficients should be the same for both C_{18} configurations, the influence of the extraction phase volume on extraction efficiency can be directly compared in spiked fish tissue**.** As shown in Table 1, the amount of pharmaceuticals extracted by the thin film extraction phase was significantly higher than that of the fibre geometry, attributable to its greater volume. According to SPME fundamental principles, as the volume of the extraction phase increases, the amount of extracted target analyte will increase proportionally for equilibrium extraction. The C_{18} thin film geometry can extract more analyte than can be extracted by the cylindrical fibre configuration due to the increased

surface area of the thin film, particularly under pre-equilibrium sampling. The use of C_{18} thin film for *in vitro* sampling of selected pharmaceuticals in spiked fish dorsal-epaxial muscle demonstrates that both extraction efficiency and method sensitivity in terms of recovery can be enhanced when compared to traditional C_{18} fibre configurations. In both extraction phases, acceptable reproducibility (4-22% RSD) was achieved for the suite of pharmaceuticals examined (Table 5.2).

Table 5:2 Relative extraction efficiencies of C_{18} thin film and cylindrical fibre configurations using fish tissue spiked with 100 ng/g analytes of interest

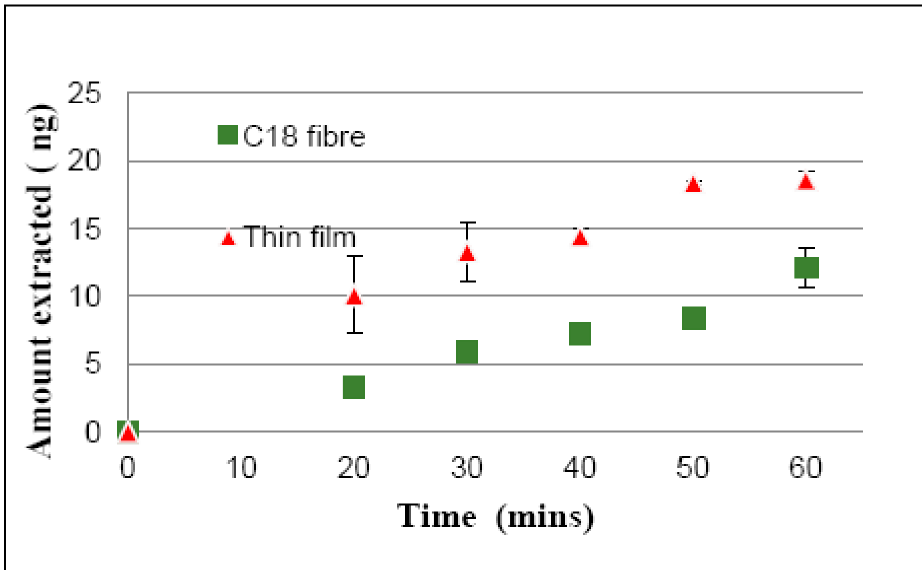
	Fibre		**Thin film**		
Analytes	**Amount extracted (ng)**	**RSD % (*n*= 4)**	**Amount extracted (ng)**	**RSD % (*n*= 4)**	**Ratio of m_{TFME}/m_{SPME}**
Carbamazepine	0.55	4	4.62	13	8
Fluoxetine	0.02	5	0.17	14	9
Diazepam	0.24	16	2.72	17	11
Norfluoxetine	0.03	22	0.19	11	6
Velanfaxin	0.03	11	0.33	16	11
Ibuprofen	0.31	5	2.38	9	8
Gemfibrozil	0.03	4	0.36	18	12

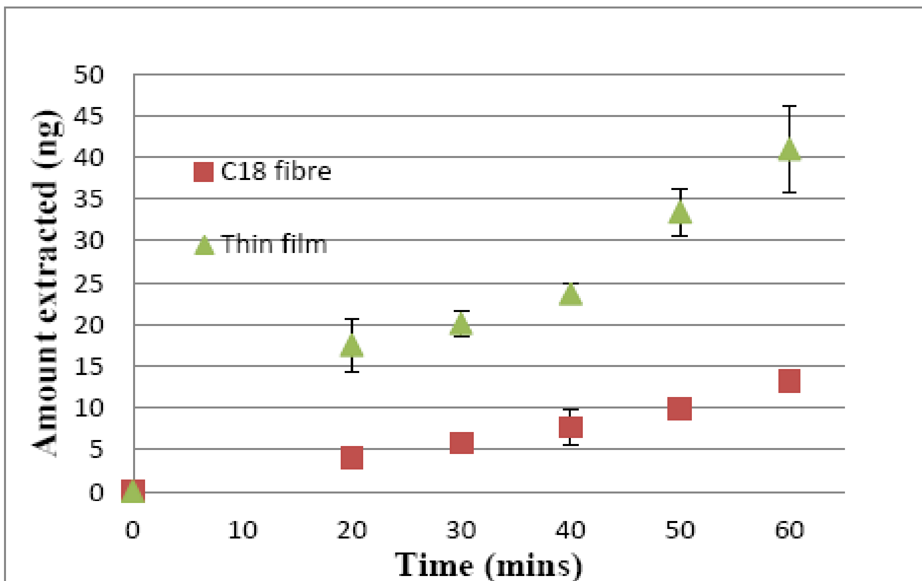
M_{TFME} is the amount of analytes extracted by thin film extraction phase and m_{SPME} is the amount of analytes extracted by solid phase fibre

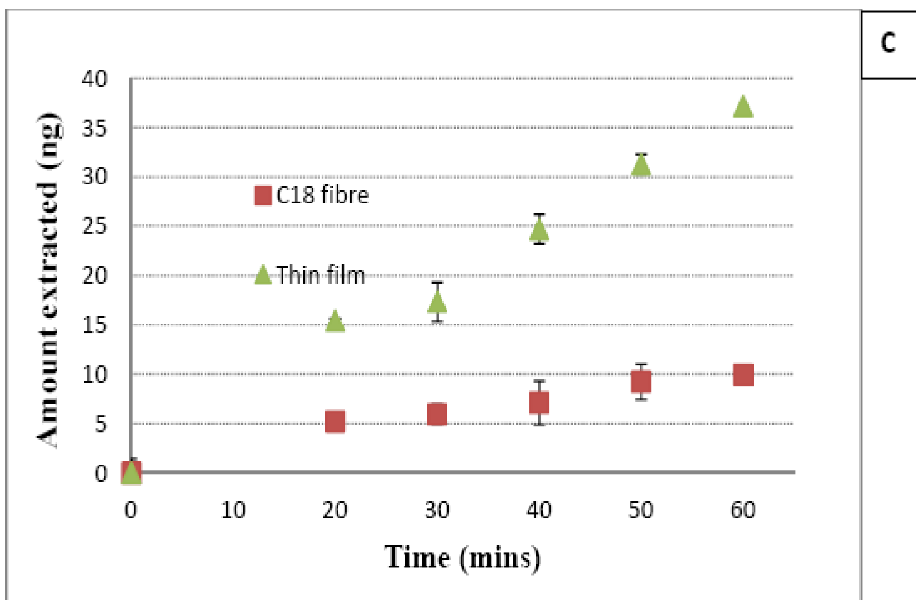
5.4.9.2 Pre-equilibrium extraction kinetics

Based on equation 5.1, the extraction rate is expected to be proportional to the surface area of the extraction phase. The extraction kinetics of the thin film and fibre geometries were compared in

spiked gel with the aim to improve *in vivo* extraction rates under pre-equilibrium conditions such as detecting pharmaceuticals in fish. The surface area of the rectangular thin film C_{18} extraction phase (0.21 cm x 1.5 cm) is approximately 0.625 cm^2 while the surface area of a C_{18} cylindrical fibre is approximately 0.08 cm^2; therefore, the surface area of the thin film extraction phase is approximately eight times greater than that of the fibre. Based upon the pre-equilibrium extraction of selected drugs from the 100 ng/mL spiked gel ($n = 3$) for 30 min under static conditions, it is evident that the extraction rates of carbamazepine, ibuprofen, gemfibrozil and fluoxetine were higher when using the C_{18} thin film configuration relative to the comparable fibre of the same material (Figure 5.2).


A
Amount extracted (ng)
25
20
15
10
5
0
C18 fibre
Thin film
0
10
20
30
40
50
60
Time (mins)


B
Amount extracted (ng)
50
45
40
35
30
25
20
15
10
5
0
C18 fibre
Thin film
0
10
20
30
40
50
60
Time (mins)



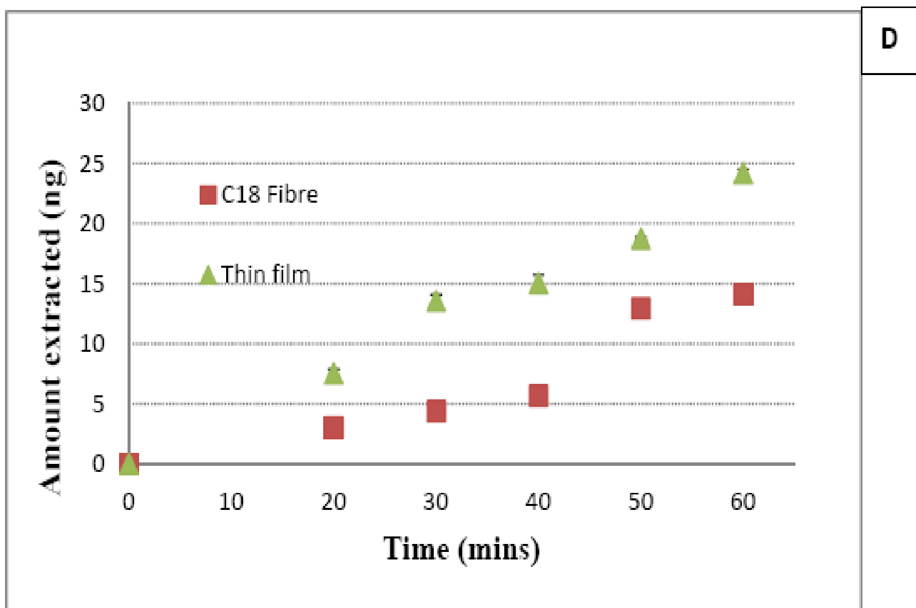


Figure 5:2 Extraction kinetics of fluoxetine (A) and carbamazepine (B) in spiked gel (1% *w/v*) using C_{18} thin film and fibre extraction phase geometries, extraction kinetics of ibuprofen (C) and gemfibrozil (D) in spiked gel (1% *w/v*) using C_{18} thin film and fibre extraction phase geometries

The use of C_{18} thin film extraction geometries would improve extraction kinetics of pharmaceuticals, particularly under pre-equilibrium sampling conditions, due to increased surface area leading to faster extraction rates. In addition, the amount of analyte extracted under pre-equilibrium conditions (30 min) by the thin film extraction phase is higher than the fibre configuration due to the larger surface area in contact with the sampled matrix.

5.4.9.3 *In vivo* determination of pharmaceuticals in fish

In this study, the concentration of pharmaceuticals from fish muscle exposed for 4 d to wastewater effluent was successfully determined using thin film microextraction under pre-equilibrium conditions. The physico-chemical properties of the pharmaceuticals, the inter-fish variability in the uptake, as well as inducible depuration enzymes affect the bioconcentration of the drugs in fish tissue and contribute to the high fish-to-fish variability observed in this study (RSD 15-90 % n = 5). Consequently, the concentration of the pharmaceuticals in fish muscle is expressed as minimum to maximum value, as summarized in Table 5.3

Table 5:3 The measured bioconcentrations factors of target pharmaceuticals in dorsal-epaxial muscle (ng/g *n*=5) of Rainbow Trout (*Oncorhynchus mykiss*) using thin film SPME. The concentration is expressed as min-max. Note that fish exposed to 50% CAS N (*v/v*) and 50% CAS (*v/v*) did not survive the 4 d exposure due to high concentrations of ammonia found in the effluent in these treatments.

	CAS N 20% effluent			CAS 20% effluent			BNR 20% effluent			BNR 50% effluent		
Analytes	**Conc. in fish muscle (ng/g)**	**Water conc. ng/mL, *n*=3 ±S.D**	**BCF**	**Conc. in fish muscle (ng/g)**	**Water conc. ng/mL, *n*=3 ±S.D**	**BCF**	**Conc. in fish muscle (ng/g)**	**Water conc. ng/mL, *n*=3 ±S.D**	**BCF**	**Conc. in fish muscle (ng/g)**	**Water conc. ng/mL, *n*=3 ±S.D**	**BCF**
Fluoxetine	36.6-259	0.50±0.10	73-518	28.8-101.1	0.45±0.01	58-202	29.1-65.2	0.83±0.01	37-79	1.7-119.9	1.00±0.01	2-133
Velanfaxin	<0.09	0.23±0.01	NA	<0.09	0.30±0.01	NA	<0.09	0.30±0.01	NA	<0.09	0.60±0.01	NA
Sertraline	7.5-160.8	0.36±0.01	19-402	1.0-46.1	0.40±0.02	20-334	13.5-39.5	0.37±0.01	34-99	11.2-75.8	0.12±0.01	112-758
Paroxetine	5.3-13.4	<0.06	NA	3.4-16.7	<0.06	NA	2.5-5.73	<0.06	NA	2.3-18.8	<0.06	NA
CBZ	0.6-1.7	0.75±0.03	1.0-2	0.4-0.5	0.80±0.01	1	0.5-4.9	0.86±0.01	5.0-49	0.2-2.4	0.23±0.01	1.0-12
Ibuprofen	<0.84	1.30±0.05	NA	<0.84	0.13±0.02	NA	<0.84	0.10±0.01	NA	<0.84	0.20±0.02	NA
Gemfibrozil	<0.40	0.30±0.03	NA	<0.40	0.23±0.01	NA	<0.40	0.23±0.01	NA	<0.40	0.70±0.01	NA

CBZ: Carbamazepine, BCF_m: Bioconcentration factor in muscle

Velanfaxin was detected in all exposed fish, but below the method limit of quantitation.

Fish exposed to 100% effluent experienced high mortality within a few hours of exposure due to high ammonia concentrations. Again, in response to ammonia toxicity, fish exposed to 50% CAS N (*v/v*) and 50% CAS (*v/v*) also did not survive the 4 d exposure. Bioconcentration factors (BCFs) were calculated in surviving fish using the ratio of the analyte concentration in fish muscle to that of the exposure water.

Following exposure to each treated wastewater effluent, the anti-depressant drugs fluoxetine, sertraline and paroxetine were detected in fish dorsal-epaxial muscle, demonstrating both the efficacy of the proposed method and the recalcitrant nature of these compounds. The concentration of fluoxetine detected in fish muscle ranged from 101 ng/g in 20% CAS (*v/v*), to 111.9 ng/g in 50% BNR (*v/v*) effluent; however, up to 259 ng/g were found in fish exposed to 20% CAS N (*v/v*), thereby suggesting all three treatment paradigms allowed passage of significant quantities of this drug for uptake by biota. Fish can readily bioconcentrate fluoxetine, as has been previously reported [185] and to a lesser extent sertraline and paroxetine, despite high rates of prescription and parent compound excretion into wastewater systems of the latter two drugs [186]. Notwithstanding the relatively high concentrations of sertraline and paroxetine entering Canadian wastewater treatment plants, final effluent concentrations are lower than that of the less prescribed fluoxetine, suggesting sertraline and paroxetine are rapidly removed during wastewater treatment [186]. Fluoxetine, despite lower prescription rates and relatively higher rates of metabolism in humans, is frequently found in aquatic environments, and is taken up by biota, presenting a potential environmental risk [185-186].

All targeted pharmaceuticals were detected in the treated municipal wastewater effluents used in this study, as summarized in Table 2. The pharmaceuticals quantified in wastewater were also detected in exposed fish muscle, with the exceptions of gemfibrozil and ibuprofen. Sertraline

was detected in fish dorsal-epaxial muscle tissue up to 160.8 ng/g in 20% CAS (*v/v*) effluent, along with paroxetine up to 18.8 ng/g in 20% CAS effleunt. The detection of sertraline in tissue is consistent with earlier reports in caged fish studies where fathead minnow (*Pimphales promelas*) accumulated 3.83±1.8 μg/kg, although paroxetine was not bioconcentrated in that study [31]. This is somewhat surprising due to modeled rates of removal during wastewater treatment of 85% and 28% for sertraline and paroxetine, respectively (Table S5). Carbamazepine bioconcentration was low (<1.7 ng/g) which is a reflection of its relatively high water solubility (log K_{ow} 2.25) [145] and persistence (2.9% removal during wastewater treatment). Similarly, the highly prescribed antidepressant venlafaxine is predicted to be only sparingly removed during wastewater treatment (8.9% removal) [145] and is dominantly excreted as the parent compound from urine. However, as venlafaxine is fairly water soluble (log K_{ow} of 3.28 [145] excreted unmodified), its rapid excretion in fish also precludes significant bioconcentration, although both venlafaxine and sertraline were detected in fish tissues in the 1-3 ng/g range in other studies[186]. Gemfibrozil and ibuprofen were not detected in muscle of fish exposed to treated effluent; as both pharmaceuticals are acidic drugs, they are easily ionized in the pH range (8-9) of the effluent, limiting their bioconcentration relative to that of their neutral form [145,186]. No target pharmaceuticals were detected in the control fish, and for all compounds studied, analyte concentrations in tissues after 4 d of exposure may have been subjected to metabolism by inducible enzymes systems or eliminated through conjugation with more hydrophilic moieties [187-189].

5.4.9.4 Bioconcentration of pharmaceuticals in field-exposed fathead minnow

In the case of field exposure of fathead minnow in the Grand River, the antidepressant sertraline was also detected in fish muscle at all sites except the intermediate downstream station

(sites 3 and 4) with mean bioconcentration factors at the two upstream sites of 186 and 418, respectively, while the last downstream sites where sertraline was detected had a bioconcentration factor of 54 (Table 5.4)

Table 5:4 Summary of the concentration (ng/mL, ±standard deviation) and bioconcentration factor of pharmaceuticals detected in fish muscle of fathead minnow caged in the field near a municipal effluent outfall (*n*=6). Wastewater samples were extracted by solid phase extraction (SPE). Sites 1 and 2 are 1.2 and 0.5 km upstream of the Doon outfall (respectively), while sites 3, 4, and 5 are 0.5, 1.7, and 5.6 km downstream, respectively.

	Site 1			Site 2			Site 3			Site 4			Site 5		
Analytes	**Muscle conc. (ng/g)**	**Water conc. ng/mL, n=3 RSD (%)**	**BCF**	**Muscle conc. (ng/g)**	**Water conc. ng/mL, n=3 RSD (%)**	**BCF**	**Muscle conc. (ng/g)**	**Water conc. ng/mL, n=3 RSD (%)**	**BCF**	**Muscle conc. (ng/g)**	**Water conc. ng/mL, n=3 RSD (%)**	**BCF**	**Muscle conc. (ng/g)**	**Water conc. ng/mL, n=3 RSD (%)**	**BCF**
CBZ	<0.40	0.022 (9)	NA	<0.40	0.024 (10)	NA	<0.40	0.117 (3)	NA	<0.40	0.109 (5)	NA	<0.40	0.045 (5)	NA
Fluoxetine	4.0±2.6	0.005 (9)	800	1.7 ± 0.95	0.004 (11)	425	< 0.64	0.005 (10)	NA	< 0.64	0.004 (14)	NA	3.01±1.70	0.004 (5)	753
Atorvastatin	<0.25	0.014 (10)	NA	<0.25	0.007 (11)	NA	<0.25	0.047 (16)	NA	<0.25	0.043 (14)	NA	<0.25	0.018 (7)	<0.25
Venlafaxin	17±13	0.028 (6)	607	7.27 ± 4.0	0.024 (5)	303	< 0.09	0.264 (3)	NA	< 0.09	0.245 (5)	NA	2.30±1.20	0.106 (2)	22
Norfluoxetine	<0.64	0.003(12)	NA	<0.64	0.002 (14)	NA	<0.64	0.002 (11)	NA	<0.64	0.0021 (3)	NA	<0.64	ND	NA
Sertraline	1.3±1.0	0.007 (9)	186	2.09 ± 1.0	0.005 (10)	418	< 0.24	0.013 (11)	NA	< 0.24	0.01 (9)	NA	0.43±1.0	0.008 (10)	54
Paroxetine	0.3±0.2	0.004 (5)	82.5	0.37 ± 0.25	0.037 (7)	10	0.14 ± 0.20	0.005 (3)	28	< 0.25	0.004 (6)	NA	0.40±0.30	0.003 (8)	133

NA: Not Applicable

Similarly, fluoxetine was taken up by fish from the wastewater and bioconcentrated in their muscle at sites 1, 2, and 5 with bioconcentration factors of 800, 425, and 753, respectively. Conversely, carbamazepine and atorvastatin were not detected in the muscle of caged fish at any of the sites, suggesting these drugs have lower bioconcentration potential in fathead minnow. Carbamazepine in particular was very frequently detected in wastewater effluents (Table 5.4), but with limited bioconcentration in fish tissues due to its relatively high solubility (log Kow = 2.26) in water. The antidepressant paroxetine was detected in fish muscle in the upstream sites 1 and 2 (reflecting the discharges of the Waterloo municipal wastewater plant) with BCFs of 83 and 10, respectively, and was also bioconcentrated at downstream sites 3 and 5 with BCFs of 28 and 133, respectively. Although target pharmaceuticals were detected in the muscle of field-exposed Fathead Minnow, concentrations were generally lower than those observed during the static laboratory exposure in which Rainbow Trout were used. This may be due to inherent species differences, or fluctuations in the bioavailability of the drugs in the river, which is very likely to change over time with precipitation events, variation in hydraulic and solids retention times in the wastewater treatment plants, differing rates of photolysis in receiving environments, and a myriad of other environmental factors that are controlled within a laboratory context.

5.5 Conclusion

For the first time, non-lethal *in vivo* thin film SPME was used to quantitate target pharmaceuticals in fish muscle under pre-equilibrium conditions. The C_{18} thin film geometry (as a modification of SPME) improves *in vivo* extraction kinetics and sensitivity, allowing for quantification of trace pharmaceuticals in living fish dorsal-epaxial muscle following short term wastewater effluent exposure. The present study demonstrates the uptake of pharmaceuticals in

fish from wastewater in both laboratory and field based exposures without the requirement of sacrificing the fish. *In vivo* SPME based on thin film geometry is a promising non-lethal technique which is suitable for determination of emerging contaminants in fish muscle.

Chapter 6

Development and evaluation of new *in vivo* SPME device (sampler) for rapid sampling of pharmaceuticals in fish using LC/MS/MS

6.1 Preamble

This chapter is to be submitted for publication in the Journal of Seperation Science. The manuscript has been written as: Oluranti P. Togunde, Heather Lord, Ken D. Oakes, Mark R. Servos, and Janusz Pawliszyn "Development and evaluation of new *in vivo* SPME device (sampler) for rapid sampling of pharmaceuticals in fish using LC/MS/MS"

6.2 Introduction

Increasing concerns about the presence of pharmaceutically active compounds in the environment, and in particular aquatic systems, have been documented in the literature due to their potential impact on aquatic organisms such as fish [12, 23,190-192]. As a result, a number of different analytical methodologies have been developed in order to investigate and determine trace concentrations of pharmaceuticals in fish tissue. At present, conventional analytical methods involve sample preparation, such as solvent extraction (SE), solid phase extraction (SPE), accelerated solvent extraction (ASE) and solid phase matrix dispersion (SMD) [39, 40,177,193]. Additionally, passive sampling devices such as the semi-permeable membrane device (SPMD) and polar organic chemical integrative sampler (POCIS) stationary sampling device have been used to mimic fish in order to determine the time-integrated concentration of bioavailable waterborne organic contaminants in fish tissue [194]. The general approach that is used for the

determination of pharmaceuticals in fish tissue involves tissue homogenization, followed by extraction with an organic or polar solvent. Then, the sample extract is cleaned-up with a method such as solid phase extraction before instrumental analysis [195,196]. One of the major limitations of the traditional approach is that the fish sampled have to be sacrificed before tissue samples can be collected and analyzed. Invariably, several fish are usually sacrificed before the concentration of pharmaceuticals in the fish muscle can be determined. In order to overcome this drawback, non-lethal sampling methods (such as tissue biopsy technique, passive sampling device)[197] are increasingly being used to investigate uptake and accumulation of pharmaceuticals in fish tissue. This approach can potentially reduce the need to sacrifice large numbers of fish to determine the bioaccumulation of pharmaceuticals in fish. One of the techniques recently reported involves non-lethal sampling of organic contaminants based on the removal of the adipose fin to determine organic contaminants in fish. However, possible adverse effects associated with the technique, in particular to the fish having its adipose fin removed, have not presently been well-studied yet[197]. Recently, solid phase microextraction was used for the determination of trace pharmaceuticals in fish muscle[43, 61,178]. The aim of this study is to evaluate the use of the newly developed SPME device as a non-lethal sampling technique of organic contaminants in fish based on its simplicity for laboratory and field applications. Previous SPME application involved a two-step sampling process, as well as anesthetization of the fish before the *in vivo* sampling can be carried; however, the new *in vivo* sampler has a unique advantage: target compounds can be sampled in fish without the anaesthetization process. In addition, the new sampler simplifies the process of sampling into a single step. In this study, *in vitro* and *in vivo* fish sampling of pharmaceuticals were evaluated using this new *in vivo* device, where the sampler is used to investigate uptake and bioconcentration potential of waterborne contaminants, such as

pharmaceutical residues in fish muscle. The calibration method for *in vivo* sampling under pre-equilibrium sampling has been described elsewhere [71, 72].

6.3 Experimental

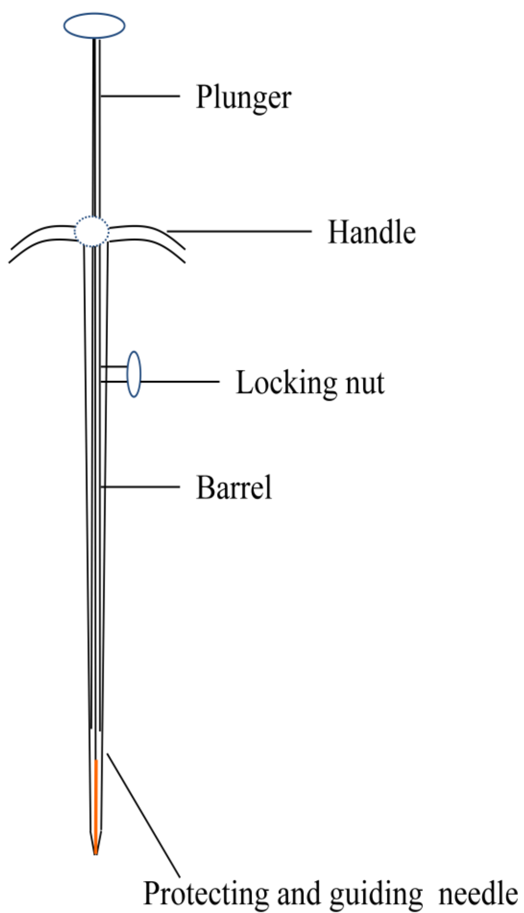
6.3.1 Chemicals and material

All chemicals used for this study were purchased from Sigma-Aldrich (Oakville, ON, Canada) except fluoxetine, diazepam, nordiazepam, lorazepam, and diazepam-d_5, which were purchased from Cerilliant Corp. (Round Rock TX, USA). Paroxetine and sertraline were purchased from Toronto Research Chemical (Toronto, ON, Canada). Agarose (Agarose 15) was purchased from BDH Chemicals Ltd., Poole, England. Rainbow Trout (*Oncorhynchus mykiss*) were purchased from Silver Creek Aquaculture (Erin, ON, Canada). Phosphate-buffered saline (PBS) solution, pH 7.4 was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.2 g of potassium phosphate, and 1.44 g of sodium phosphate in 1 L of purified water. The experimental procedures for the *in vivo* study were approved by local Animal Care Committee at the University of Waterloo (AUP #'s 04-24, 10-17).

6.3.2 New *in vivo* SPME device (sampler)

The new device assembly contains four main parts: (a) the plunger with a nut, (b) the probe containing the extraction phase, (c) hypodermic needle with a sharp end, and (d) overall body of the device (barrel). The plunger is used to move the probe in and out of the device (Figure 6.1). The probe is a long stainless steel wire (20 cm long with a 1.5 cm extraction coating).

A- Fiber is enclosed in the guiding needle



B- Fiber is exposed for sampling

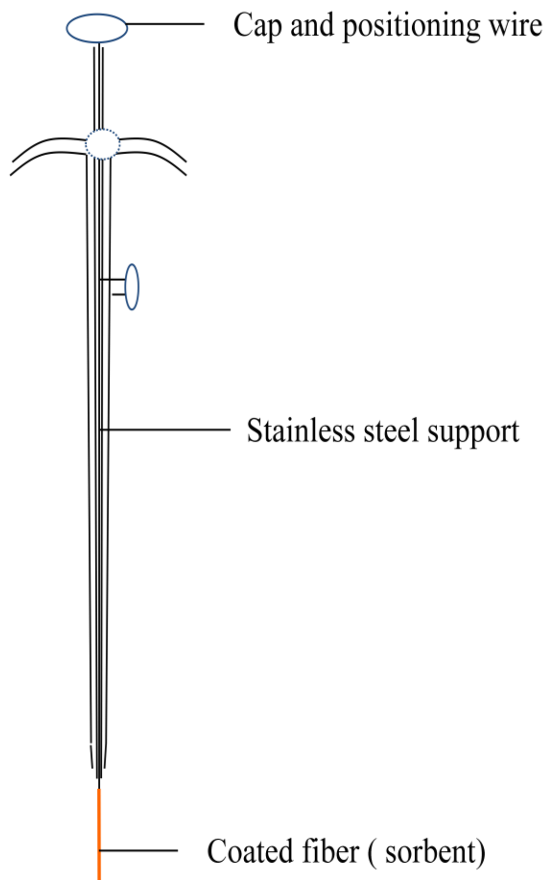


Figure 6:1 Schematic diagram of *in vivo* tissue sampling device (above). *In vivo* SPME device with PDMS biocompatible coating (Top) Depressing the plunger exposes 1.5 cm SPME coating during the extraction. (Bottom) The device is shown retracted for storage. (University of Waterloo machine shop made this device for us)

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When the probe is inserted in the device through the hypodermic needle, the nut on the plunger is used to hold the probe in position. The hypodermic needle is used to protect the extraction phase at the tip of the probe. In addition, the needle is used to pierce the fish muscle so that the extraction phase can easily be inserted in the fish muscle. The new device uses PDMS as the extraction phase, which is biocompatible with fish tissue for *in vivo* sampling. The detailed preparation and assembly of the custom made poly dimethylsiloxane probe (165 μm coating thickness) has been described elsewhere[43]. The new sampler device is designed to make pharmaceutical sampling easier and simpler thereby minimizing tissue damage. During sampling

in fish muscle, the plunger is moved down in order to expose the extraction phase to fish muscle for a specific time (Figure 6.2).

Figure 6:2 ***In vivo* SPME device being used for sampling pharmaceuticals in live fish**

The extraction phase can also remain in the fish tissue as long as 1 hdepending on the sensitivity of the method. After the sampling process, the extraction phase (fibre) is withdrawn from the fish muscle and then gently rinsed with nanopure water, followed by drying the extraction with Kimwipe®. Subsequently, the probe (fibre) is desorbed in the optimized desorption solvent for injection into LC/MS/MS system.

6.3.3 Evaluation of new *in vivo* device in spiked gel and fish muscle

Evaluation of the new device was carried out in spiked gel matrix and fish muscle in order to evaluate its simplicity in the laboratory for sampling. Agarose gel was used for the *in vitro* studies because it allows free diffusion of analytes[81]. All of the experiments were performed

under static mode in order to simulate *in vivo* sampling conditions. The conditioned PDMS fibre was inserted in the device and then directly immersed in the vial containing the gel with 0.1 μg/mL diazepam, nordiazepam and carbamazepine, for a specified period of time (1 h). After the extraction, the fibre was desorbed in desorption solvent (50% acetonitrile: water) for 90 min under agitation speed of 1500 rpm. Furthermore, another *in vitro* experiment was performed using homogenized fish muscle (Rainbow Trout) with the same concentration as used in gel. Following the extraction, the fibre was desorbed in the solvent as previously described. After desorption, 20 μL of the sample extract was injected to the LC/MS/MS for instrumental quantification.

6.3.4 *In vivo* sampling in Rainbow Trout exposed to municipal effluent in laboratory and field monitoring of wild fish

The juvenile Rainbow Trout used in this study (14.4±0.34 cm; 25.4±1.46 g, mean ±SE, n = 18) were acclimated to laboratory conditions in 1400 L holding tanks continuously receiving de-chlorinated water. Municipal wastewater samples were collected from Burlington, ON, which were treated under a variety of pilot-scale conditions from July to September 2011. The wastewater samples were treated by either conventional activated sludge (CAS), conventional activated sludge with nitrification (CAS-N), or conventional activated sludge with Biological Nitrifying Reactor (CAS-BNR). Acclimation of Rainbow Trout began 4 d prior to the onset of the experiment. The extraction phase was preloaded with deuterated standard (50 ng/mL in spiked phosphate buffer solution) by direct extraction under agitation (500 rpm) for 3 h on a shaker platform. At the conclusion of the 8 d exposure period, *in vivo* sampling with the new SPME device was performed in the dorsal-epaxial muscle of anaesthetized fish for 30 min (under

pre-equilibrium conditions), The fish (Rainbow Trout) was anaesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate (M222) until loss of vertical equilibrium, then the *in vivo* sampler with a 20 gauge needle was used to pierce the fish dorsal-epaxial muscle so that the extraction phase can be implanted in the fish muscle. After the PDMS fibre is placed in the fish muscle, the anaesthetized fish was placed into fresh reference water.After sampling the tissue for 30 mins, the fish was then reanaesthetized prior to removing the fiber coating, then the extraction phases were removed from fish muscle and rinsed with distilled water prior to immersion in desorption solvent (60% methanol-water) for 90 min with agitation speed of 1000 rpm; an aliquot of desorption solution was injected for LC/MS/MS instrumental analysis. The applicability of the new device for sampling of pharmaceuticals was further evaluated in live wild fish collected from different rivers located in Paisley, ON. The target species of fish was muskellunge (*Esox masquinongy*), which is only used for catch-and-release sport fishing due to its low population numbers. As such, the use of a non-lethal sampling technique is required for sampling this protected fish species. For larger wild fish, anesthetizing was not required in order to sample the target compounds in fish tissue. The fish were sampled using the device for 30 min and then released into the river after sampling. Analysis of the studies in gel, spiked fish and field studies samples were carried out using an Accela™ LC-MS/MS instrument from Thermo Scientific.

6.4 Result and discussion

6.4.1 Evaluation of extraction phase of *in vivo* SPME device

The performance of the extraction phases (PDMS and C_{18} fibre) that are frequently used for sampling in living animals has been described elsewhere[181]. PDMS was chosen as the extraction phase for the *in vivo* sampler because it is an inexpensive method as well as biocompatible in a

complex matrix. Evaluation of the PDMS fibre in spiked phosphate buffered saline shows that the selected pharmaceuticals, and in particular diazepam and nordiazepam, have an affinity for this extraction phase. As shown in Table 6.1, diazepam has the highest percentage extraction recovery, while carbamazepine has the least due to its solubility in water. This observation can be related to the different octanol-water partition coefficients of the analytes.

Table 6:1 Extraction recovery of the target compounds in spiked gel sample at equilibrium

Analytes	**Amount extracted (ng)**	**% extracted**	**RSD (%) (n= 5)**
Carbamazepine	17.5	12	8
Diazepam	106.5	71	7
Nordiazepam	52	35	6

Although the recovery of carbamazepine is the slightest when compared to the two other compounds, overall reproducibility of the extraction phase is very good (RSD < 10%). The limits of detection and quantification of the method in fish and gel matrix is summarized in Table 6.2.

Table 6:2 Limit of detection in homogenized fish tissue and gel

	Gel		Fish tissue	
Compounds	LOD (ng/mL)	LOQ (ng/mL)	LOD (ng/g)	LOQ (ng/g)
Carbamazepine	0.01	0.10	0.07	0.48
Diazepam	0.02	0.19	0.30	0.97
Nordiazepam	0.07	0.20	0.34	0.40

6.4.2 *In vivo* evaluation of new sampler in fish exposed to effluent in the laboratory and wild fish

Results of sampling of target compounds show that the new device can be used to sample pharmaceuticals in fish muscle. Table 6.3 summaries the concentration of the target compounds determined in fish muscle.

Table 6:3 The measured concentration and bioconcentrations factors of target pharmaceuticals in dorsal-epaxial muscle (ng/g , ±standard deviation)) of rainbow trout (*Oncorhynchus mykiss*) in laboratory 8-day exposure using new SPME device. Water concentration was determined by solid phase extraction.

		CAS N 20% effluent			BNR 20 % effluent		
Analytes	**LOQ (ng/g)**	**Conc. in fish muscle (ng/g) n= 3**	**Water Conc. (ng/ml) n= 3±S.D**	**BCF**	**Conc. in fish muscle (ng/g) n= 3**	**Water Conc. (ng/ml) n= 3±S.D**	**BCF**
Carbamazepine (CBZ)	1.5	ND	8.9±1.2	NA	ND	8.5±0.2	NA
Fluoxetine (FLX)	0.1	0.10-0.14	0.7±0.1	0.2	0.73-0.76	0.6±0.1	1.3
Sertraline (SER)	0.2	6.4-7.7	0.9±0.1	8.5	7.2-8.0	1.0±0.1	8
Paroxetine (PAR)	0.2	5.4-6.8	1.4±0.1	5	6.1-7.3	1.4	18
Atorvastatin (ATOR)	0.3	ND	3.6±0.5	NA	ND	5.6±0.3	NA
Diclofenac (DLF)	1.4	<LOQ	1.2±0.2	NA	<LOQ	1.3±0.2	NA
Vanlafaxine (VAL)	0.1	<LOQ	1.9±0.2	NA	<LOQ	1.8±0.2	NA

ND- not detected, NA- not applicable

For compounds that are moderately water soluble like carbamazepine, there is low potential for this compound to bioconcentrate in muscle, and as a result, carbamazepine was not detected in fish muscle at this time. In the case of anti-depressant some of the drugs were dcetected in fish muscle. For example, the concentration of sertraline determined in the fish muscle was 6.4 to 7.7

ng/g and 7.2 to 8.0 ng/g in the two different effluent exposures. Similarly, the concentration of paroxetine in fish muscle ranged from 5.4 to 6.8 ng/g based on CAS-treated effluent exposure and 7.2 to 8.0 ng/g based on BNR-treated effluent. As well, the concentration of fluoxetine determined in the muscle of fish exposed to CAS treated effluent (0.1 to 0.14 ng/g) was found to be lower when compared to the concentration found in the fish (0.73 to 0.76 ng/g) exposed to BNR treated effluent. Surprisingly, atorvastatin was not detected in fish muscle, even though its Log K_{ow} is greater than 3; this may be due to biotransformation of the compounds in fish to more polar metabolites. It is to be noted that while diclofenac and vanlafaxine were detected in the fish muscle, their concentrations were below the limit of quantitation. Likewise, in the case of wild fish collection, carbamazepine was similarly detected in Muskellunge fish muscle, but its concentration was below the limit of quantitation. In sum, the utility of the new SPME device as a non-lethal sampling device is a promising alternative in the evaluation of organic contaminants in fish muscle. This approach would be particularly useful to those threatened or endangered fish species in which lethal sampling may not be feasible.

6.5 Conclusion

This study evaluates and demonstrates for the first time that the newly developed SPME device is a promising device for sampling of organic contaminants in fish. *In vivo* SPME method is demonstrated as a non-lethal sampling technique which can prevent the sacrificing of fish for sampling. In the study, a significant step is demonstrated towards the use of a new *in vivo* SPME device for rapid non-lethal monitoring of pharmaceuticals in fish, both in laboratory conditions as well as field-based exposure. Some of the selected pharmaceuticals were successfully determined in the fish muscle with the new sampler. Presently, there is on-going development on

the new device to provide wider applications in terms of coating development, particularly for high water-soluble compounds and their metabolites.

Chapter 7

Laboratory and field study of bioconcentration of pharmaceutical residues in fish muscle and bile using solid phase microextraction coupled with liquid chromatography-tandem mass spectrometry

7.1 Preamble

This chapter is to be submitted for publication in the Journal of Chromatography A. The manuscript has been written as: Oluranti P. Togunde, Ken D. Oakes, Mark R. Servos, and Janusz Pawliszyn "Laboratory and field study of bioconcentration of pharmaceuticals residues in fish tissue and bile using solid phase microextraction coupled with liquid chromatography-tandem mass spectrometry".

7.2 Introduction

The presence of biologically active xenobiotic compounds in aquatic environments is an emerging concern in many developed and developing countries due to the potential impact on both aquatic organisms and human health [191,192,198-200]. Environmental pharmaceuticals are considered emerging contaminants of concern due to their designed biological potency at low therapeutic dosages, coupled with the near ubiquitous detection of such in adjacent urban areas due to release from human sewage systems [201-203]. As present conventional municipal sewage treatment processes do not completely remove these pharmaceutical contaminants, they eventually end up in aquatic receiving environments within municipal wastewater effluents (MWWE) where they are available for uptake by resident organisms. Although the concentrations of pharmaceuticals detected in aquatic environments are invariably low (usually

in the ng/L to μg/L range), organisms are continuously exposed, resulting in relatively water soluble, readily metabolized drugs behaving as "pseudopersistent" compounds, due to their chemical half-lives being met or exceeded by uptake rates from their MWWE-influenced environments [204]. Generally, the concentration of pharmaceuticals in MWWEs decreases with downstream distance from the effluent outfall in riverine environments due to dilution effects, bio- and photodegradation processes, and sorption to sediments. However, recalcitrant pharmaceuticals may be still detected over longer distances and intervals, and their continuous release via MWWEs sustains their availability over long reaches in aquatic receiving environments [205].

In response to the potential of bioconcentration of environmental pharmaceuticals in tissues of fish and other aquatic organisms, different analytical methodologies have been developed to quantitatively determine the concentrations of these compounds in specific tissues such as brain, muscle, liver, plasma, and bile [14,44,46]. Most of these analytical methods are based on solvent extraction, or solid phase extraction (SPE) as means of sample preparation as well as for isolation and pre-concentration of analytes. Recently, solid phase microextraction (SPME) has been gaining ground as a valuable analytical technique for non-lethal *in vivo* determination of pharmaceutical residues in fish muscle [43, 92]. The present study aimed to develop a simplified and reliable analytical method suitable for field applications that is based upon SPME, in order to investigate uptake and bioconcentration of pharmaceuticals residues in fish bile and muscle.

Improving our understanding of pharmaceutical bioconcentration within fish tissues is important both for our insight into the toxicokinetics of these compounds in exposed biota themselves, but also for assessing the risk to humans consuming them. To the best of the authors' knowledge, this work is the first demonstrating the utility of the SPME approach in determining the

bioconcentration of pharmaceuticals in fish bile and muscle. A significant advantage of SPME is the simplicity of deployment when determining *in vivo* concentrations of target analytes in fish tissues under pre-equilibrium sampling conditions using the kinetic calibration method, as represented by equation 1.3. The concentration of target analytes in fish bile samples can be determined using the equilibrium SPME approach, as represented by equation 1.1

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7.3 Experimental

7.3.1 Chemicals and materials

All reagents and pharmaceuticals standards were of highest purity grade available. Gemfibrozil, atorvastatin, ibuprofen, atrazine, carbamazepine, diclofenac, naproxen, and bisphenol-A (BPA) were obtained from Sigma-Aldrich (Oakville, ON, Canada), while paroxetine and sertraline were purchased from Toronto Research Chemical (ON, Canada). Fluoxetine, diazepam, nordiazepam, and diazepam-d_5 were purchased as certified standards from Cerilliant Corp (Round Rock, TX, USA). Isotopically-labeled atorvastatin-d_5, atrazine-d_5, BPA-d_{16}, carbamazepine-d_{10}, diazepam-d_5, diclofenac-d_4, fluoxetine-d_5, gemfibrozil-d_6, ibuprofen-d_3 and ^{13}C-naproxen-d_3 were purchased from CDN Isotope Inc. (Point-Claire, QC, Canada) while sertraline-d_3 and paroxetine-d_4 were purchased from Toronto Research Chemical. Chemical stock solutions were prepared in methanol and stored at -20°C while working solutions were diluted aliquots of these stocks. Dilution water was obtained from a Barnstead Nanopure diamond UV water purification system deionized to 18 Ω. Acetonitrile (HPLC grade), methanol (HPLC grade), and glacial acetic acid were purchased from Fisher Scientific (Ottawa, ON, Canada). Phosphate-buffered saline (PBS) solution, pH 7.4 was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium

chloride, 0.2 g of potassium phosphate and 1.44 g of sodium phosphate in 1 L of purified water and adjusting the pH to 7.4. The C_{18} thin film extraction phase (45 µm, 1.5cm coating length) and traditional C_{18} fibres (45µm, 1.5 cm coating length) were obtained from Supelco (Bellefonte, PA USA). A 10 mg/L mixed solution containing all the pharmaceuticals studied was obtained by diluting the individual stocks with methanol. Rainbow Trout (*Oncorhynchus mykiss*) were purchased from Silvercreek Aquaculture (Erin, ON, Canada). All animal experimental procedures were approved by the local Animal Care Committee at the University of Waterloo (AUP #'s 04-24, 08-08).

7.3.2 **Laboratory exposure of Rainbow Trout to municipal effluent**

The Rainbow Trout used in this study (19.7±1.50 cm; 59.5±12.4 g, (mean ±SE), n = 120) were acclimated to laboratory conditions in 1400 L holding tanks continuously receiving de-chloraminated municipal water, and fed every other day with 2.0 Pt floating commercial trout ration (Martin's Feed Mill, Elmira, ON). Acclimation within the 34 L exposure aquaria (containing clean water previously acclimated to in the 1400 L tanks) began 4 d prior to the onset of the experiment. The experiment began by exposing fish to wastewater collected from three pilot treatment plants receiving raw effluent generated from the City of Burlington (Burlington, ON). Wastewater samples were treated by Conventional Activated Sludge (CAS), Conventional Activated Sludge with Nitrification (CAS-N), or Conventional Activated Sludge with Biological Nitrifying Reactor (CAS-BNR). Exposure of fish involved two controls and six different treatment exposure concentrations: CAS-20% *v/v*, CAS-50% *v/v*, CAS-N-20% *v/v*, CAS-N-50% *v/v*, and CAS-BNR-20% *v/v*, CAS-BNR-50% *v/v*. Each 34 L aquaria housed 6 fish, for exposure durations of 4 and 8d, with exposure medium renewed every 48h using corresponding freshly

obtained wastewater samples. Exposure water quality was checked daily and maintained at conditions considered optimal for trout (14.1 ± 0.5^{o}C; pH 8.13 ± 0.06; ammonia 23.5 ± 1.5 μg/L).

7.3.3 ***In vivo* and *in vitro* SPME sampling of pharmaceutical residues in fish bile and muscle**

In vivo SPME sampling was conducted under pre-equilibrium conditions (30 min) with quantitation of pharmaceuticals in fish muscle by kinetic calibration. The C_{18} blade (45 μm thick, 1.5cm coating length) used as the extraction phase was preloaded with deteurated standards of the target analytes (50 ng/mL in spiked phosphate buffer solution) by direct extraction under agitation (500 rpm) for 3h on a shaker platform. Detailed information on the procedure and preparation of C_{18} blade including its dimensions has been described elsewhere [206]. At the conclusion of the 4 and 8d exposure period, *in vivo* sampling of target compounds was performed in the dorsal-epaxial muscle of anaesthetized (0.1% ethyl 3-amino benzoate methanesulfonate) fish for 30 min (pre-equilibrium conditions). After 30 min, the extraction phases were removed from fish muscle and rinsed with distilled water prior to immersion in desorption solvent (60:40 methanol-water) for 90 min. An aliquot of desorption solution was then injected for LC/MS/MS instrumental analysis. Following SPME sampling, fish were re-anesthetized prior to euthanasia by cervical vertebrae severance. Bile samples were collected from the excised gallbladders and immediately frozen in liquid nitrogen and stored at -80°C. Prior to analysis, the bile was thawed and a portion was de-conjugated with glucuronidase, sulfatase and α-glucosidase. Specifically, 100 μL of these enzymes [β-glucuronidase (1000 units/mL), Sulfatase (2 units/mL), β-glucosidase] were added to a mixture of 100 μL bile: 200 μL 0.1 M phosphate buffer at pH 6.0. These biliary mixtures were incubated for 16 h at 37°C

prior to the introduction of C_{18} SPME fibres (1.5 cm coating length, 45 μm thickness supplied by Supelco Bellefonte, PA, USA) into the vial for extraction under equilibrium conditions at 1200 rpm continual vortex agitation (model DVX-2500, VWR International, Mississauga ON, Canada). Following sample extraction, analytes were desorbed from the SPME fibres for 1.5h at 1200 rpm into 200 μL methanol: water (1:1) containing 20 ng/ml deuterated analogues of the target compounds as internal standards. Subsequently, 20 μL of the sample extract was injected to the LC/MS/MS for quantification analysis. Concentrations of waterborne pharmaceuticals in the MWWEs fish were exposed to be determined using traditional solid phase extraction (SPE) [207,208]. Water samples, collected in 500 mL amber glass bottles, were preserved with sodium azide (0.2 g/L) and ascorbic acid (0.05 g/L) to prevent analyte degradation by microbial activities. Prior to SPE extraction, these samples were spiked with isotopically-labeled surrogates before being filtered through Whatman® glass/fibre filter paper (Fisher Scientific, Mississauga, ON, Canada). Waters Oasis® HLB cartridges (500 mg) (Milford, MA, United States) were used for extraction after pre-conditioning with 5 mL of HLPC grade Methyl Tertiary Butyl Ether (MTBE), 5 mL of HLPC grade methanol, 5 mL of HLPC grade water. After water sample extraction, SPE cartridges were rinsed with 5 mL of HPLC grade water. Elution of analytes from the cartridge was completed with 5 mL of methanol and 5 mL of 10:90 (*v/v*) methanol/MTBE. Eluted solutions were collected and blow dried under a gentle stream of nitrogen. Finally, each sample was reconstituted in 500 μL of methanol and stored at -20°C until analysis by LC–MS/MS.

7.3.4 **Wild White Sucker sampling adjacent wastewater outfalls**

White Sucker *(Catostomus commersonii)* fish were collected from the Grand River in southern Ontario from reaches downstream of the Kitchener and Waterloo municipal wastewater treatment plant (MWWTP) outfalls in October 2011. White Sucker were selected due to their prevalence in the watershed and their inherent ability to withstand adverse conditions below these discharges that preclude the presence of more sensitive species. Fish were collected using a Smith-Root 12A-POW backpack electroshocking unit (Smith-Root Canada, Merritt, BC) which temporarily stuns, but does not harm, captured fish. Although sizes of fish utilized invariably differed from site to site, efforts were made to use only similar-sized fish at the sites sampled. After collection, fish were transferred to a commercial bait-bucket that received aeration by a battery-powered bubbler. *In vivo* sampling of target analytes in the fish dorsal-epaxial muscle was performed using the identical SPME approach previously described for the laboratory-based Rainbow Trout exposure. Similarly, pharmaceutical concentrations in bile samples were determined using the *in vitro* SPME approach described in section 7.3.3.

7.3.5 Instrumental analysis – LC/MS/MS

Analyses were performed on an AB-Sciex 3200 QTrap Mass Spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems Sciex, Foster City, CA, USA). Liquid chromatography (LC) was performed on a HP1100 HPLC system (Agilent Technologies) equipped with a degasser, binary pump, an autosampler and a column oven. Chromatographic separation was performed on a Zorbax Eclipse XDB C_{18} (150 mm × 21 mm, 3.5µm) column which was preceded by a C_{18} guard column at a flow rate of 0.8 mL/min with mobile phase A (95% water, 5% MeOH, 0.1 % acetic acid) and B (95% MeOH, 5% water and 0.1% acetic acid)

utilizing a 20 μL injection volume. The gradient elution was programmed as follows: mobile phase B was increased from 10% to 50% over 0.5 min and 50% to 100% over 7.5 min, held at 100% for 2 min and then reduced to 10% over 1 min. Analyst® version 1.4.2 software was used for the data analysis. Quality control samples (10 ng/mL of target compounds) were utilized at the beginning and end of each run to ensure instrumental stability.

7.4 Results and discussion

7.4.1 Laboratory verification of pharmaceutical uptake and bioconcentration

The uptake of pharmaceuticals and their bioconcentration in Rainbow Trout muscle using an *in vivo* SPME approach has been previously reported [43, 92]. In the present study, a C_{18} coated blade (45 μm thick, 1.5cm coating length) was used to investigate the uptake of bioactive analytes in muscle tissue of fish by utilizing a new SPME geometry, which improves both sensitivity and analyte sampling rate in fish muscle. Pharmaceutical residues bioconcentrated in muscle of fish exposed to variously-treated MWWEs (Table 7.1) were determined non-lethally, demonstrating the potential of the technique to quantify analyte tissue burdens *in vivo* without sacrificing the animal. Anti-depressant drugs such fluoxetine, sertraline, and paroxetine were preferentially taken up or retained within fish muscle in this laboratory exposure, relative to other more hydrophilic analytes, corresponding to their respective log Kow values (Table 7.1).

Table 7:1 The measured bioconcentrations factors of target pharmaceuticals in dorsal-epaxial muscle (ng/g , ±standard deviation)) of Rainbow Trout (*Oncorhynchus mykiss*) in laboratory exposure using thin film SPME.

	CAS 20% effluent			**CAS N 20% effluent**			**CAS N 50% effluent**		
Analytes	**Muscle Conc. (ng/g) *n*=9**	**Water conc. (ng/mL) *n*= 3**	**BCF**	**Muscle Conc (ng/g) *n*=9**	**Water Conc. (ng/mL) *n*= 3**	**BCF**	**Muscle Conc. (ng/g) *n*=9**	**Water Conc. (ng/mL) *n*= 3**	**BCF**
Carbamazepine	0.36 - 0.54	9.3±1.0	0	0.61 -1.73	8.63±0.2	0	0.17 - 0.82	11±2	0
Fluoxetine	3.47 - 9.10	0.6±0.1	15	3.97 - 6.5	0.6±0.1	11	1.9 - 5.28	1.4±0.2	4
Norfluoxetine	0.16 - 0.6	NA	NA	0.82 - 1.98	NA	NA	< LOQ	NA	NA
Sertraline	0.63 - 7.95	0.9±0.1	9	2.79 - 4.77	0.6±0.1	8	0.44 - 1.26	1.6±0.3	1
Paroxetine	0.19 - 4.2	<LOQ	NA	0.8 - 1.14	< LOQ	NA	0.45 - 0.47	< LOQ	NA
Ibuprofen	0.6 - 1.12	1.5±0.1	1	0.30 - 2.39	1.3±0.2	2	0.4 - 1.20	3.2±0.5	0
Gemfibrozil	0.8 - 1.5	2.3±0.8	1	0.33 - 2.82	0.3±0.1	9	0.52 - 1.71	0.6±0.1	3
Triclosan	1.3-1.77	<LOQ	NA	0.66-0.90	< LOQ	NA	1.0-2.85	< LOQ	NA

	BNR 20% effluent			BNR 50% effluent			CAS 50% effluent		
Analytes	Muscle Conc. (ng/g) *n*=1	Water Conc. (ng/mL) *n*= 3	BCF	Muscle Conc. (ng/g) *n*=9	Water Conc. (ng/mL) *n*= 3	BCF	Muscle Conc. (ng/g) *n*=5	Water Conc. (ng/mL) *n*= 3	BCF
Carbamazepine	4.88	8.7±0.3	0	0.17 - 2.40	12±2	0	< LOQ	23±5	NA
Fluoxetine	7.85	0.6±0.1	13	4.46 - 6.78	1.5±0.2	5	2.72 - 3.65	1.5±0.4	2
Norfluoxetine	0.76	NA	NA	0.01 - 0.36	NA	NA	< LOQ	NA	NA
Sertraline	4.72	0.8±0.2	24	0.69 - 4.09	2±0.5	2	1.72 -2.28	2.3±1.0	1
Paroxetine	1.25	< LOQ	NA	0.11 - 1.56	< LOQ	NA	0.53- 0.76	< LOQ	NA
Ibuprofen	2.1	0.6±0.2	4	0.87 - 1.54	1.5±0.4	1	< LOQ	3.8±0.5	NA
Gemfibrozil	1.89	2.5±0.4	1	0.53 - 1.57	6.2±1.5	0	0.26 - 0.37	5.8±2.0	0
	0.81	< LOQ	NA	0.69-1.18	< LOQ	NA	0.84-0.94	< LOQ	NA

Note- For BNR 20% effluent exposure, only one fish was sampled by SPME method because the remaining two fish were not healthy and died before sampling time.

Calculated bioconcentration factors (BCFs; the ratio of the chemical concentration in the fish muscle to that in the MWWEs) for fluoxetine (BCFs 2-15) demonstrate that this anti-depressant partitioned to, and was retained by fish muscle to a greater degree than more water soluble analytes. For example, carbamazepine was not retained in tissues (BCF < 1) despite being present at relatively high concentrations in the MWWEs (Table 1), demonstrating that risk to exposed organisms is a function of environmental concentrations and the physicochemical properties of the compounds. This concept is reinforced by the small but relevant BCFs for fluoxetine in muscle of fish exposed to lower, environmentally-relevant 20% wastewater concentrations (for CAS-N, CAS-BNR, and CAS) which produced BCFs of 11, 13, and 15, respectively). The main metabolite of fluoxetine, norfluoxetine, was also quantified in fish muscle, indicating that the parent compound can be quickly metabolized by fish, and retention alone is not the chief factor mediating fluoxetine tissue concentrations [41]. Rather, the lipophilic nature of fluoxetine drives into tissues from the exposure water, while retaining the somewhat less lipophilic, but equally bioactive metabolite norfluoxetine in the tissues [49]. Both sertraline (BCFs 1-24) and paroxetine were detected in fish, although a bioconcentration factor for paroxetine could not be calculated as its waterborne concentration (determined by solid phase extraction) was below the limit of quantitation. Bioconcentration of acidic pharmaceuticals (such as gemfibrozil and ibuprofen) were lower, possibly due to ionization within the ambient pH environment of the MWWEs evaluated. When in their ionized forms, the relative affinity of these drugs for the wastewater milieu relative to fish tissues is increased, resulting in low tissue burdens [209].

7.4.2 Bioconcentration of pharmaceuticals in fish bile

Following the 4 d laboratory exposure of Rainbow Trout to the various MWWEs, most target pharmaceutical residues could be detected in the bile of exposed fish with the exception of ibuprofen and paroxetine (Table 7.2).

Table 7:2 Summary of the concentration (ng/mL, ±standard deviation) and bioconcentration factor of pharmaceuticals detected in fish bile of Rainbow Trout (*Oncorhynchus mykiss*) in a 4-day laboratory exposure using SPME method.

	CAS 20% effluent			**CAS N 20% effluent**			**BNR 20% effluent**		
Analytes	**Bile Conc (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*= 3**	**BCF**	**Bile Conc (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*= 3**	**BCF**	**Bile Conc (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*= 3**	**BCF**
Carbamazepine	8.7 ± 2.0	9.3±1.0	1	1.66	8.6±0.2	0	0.4±0.1	8.7±0.3	0
Fluoxetine	1.7±0.3	0.6±0.1	3	1.4±0.4	0.6±0.1	2	1.8±0.7	0.6±0.1	3
Sertraline	1.3	0.9±0.1	1	ND	0.6±0.1	NA	1.4	0.8±0.2	2
Paroxetine	ND	<LOQ	NA	ND	< LOQ	NA	ND	< LOQ	NA
Ibuprofen	ND	1.5±0.1	NA	ND	1.3±0.2	NA	ND	0.6±0.2	NA
Gemfibrozil	0.8±0.2	2.3±0.8	0	ND	0.3±0.1	NA	0.7±0.1	2.5±0.4	0
Triclosan	145.1±14	<LOQ	NA	50.1±4	< LOQ	NA	56.9±9	< LOQ	NA

	BNR 50% effluent			**CAS N 50% effluent**			**CAS 50% effluent**		
Analytes	**Bile Conc (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*= 3**	**BCF**	**Bile Conc (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*= 3**	**BCF**	**Bile Conc (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*= 3**	**BCF**
Carbamazepine	0.8 ± 0.7	12±2	0	24±0.17	11±2	2	22.2±1.5	23±5	1
Fluoxetine	2.1±0.2	1.5±0.2	1	2.1±0.2	1.4±0.2	2	2.5±1.5	1.5±0.4	2
Sertraline	1.4	2±0.5	1	ND	1.6±0.3	NA	1.3	2.3±1.0	1
Paroxetine	0.4±0.2	< LOQ	NA	ND	< LOQ	NA	ND	< LOQ	NA
Ibuprofen	ND	1.5±0.4	NA	ND	3.2±0.5	NA	ND	3.8±0.5	NA
Gemfibrizil	1.3±0.3	6.2±1.5	0	ND	0.6±0.1	NA	2.0±0.7	5.8±2.0	0
Triclosan	97.9±16	< LOQ	NA	85.2±19	< LOQ	NA	216±15	< LOQ	NA

Two prominent anti-depressives, fluoxetine and sertraline, were detected in the bile of all effluent-exposed fish (except the CAS exposure in the 50% *v/v* treatment, for which sertraline was not detected). Mean fluoxetine concentrations in the bile of fish exposed to CAS 20%, CAS N 20% ,and BNR 20% were 1.7±0.3, 1.4±0.4, 1.8±0.7 ng/mL (BCFs 2-3), while those of fish exposed to BNR 50%, CAS-N 50%, and CAS 50% were 2.1 ± 0.2, 2.1±0.2 and 2.5 ± 1.5 ng/mL (BCFs 1-2) respectively. Similarly, carbamazepine was also detected in all the bile samples of effluent-exposed fish, although in low amounts (BCF <1). The anti-depressant sertraline was detected in the bile of all fish, with the exception of those exposed to the CAS N 50 % effluent treatment. The low bioconcentration of the parent compounds in the fish bile may be attributed to the fact that the parent compounds have undergone phase I or phase II metabolic processes, thereby reducing the accumulation of the parent compound in the bile.[210-212]

Similar concentrations of pharmaceuticals were detected in bile of fish exposed for 8 d (4 d longer than previous) to variously treated municipal effluents (Table 7.3). Fluoxetine and carbamazepine were again detected in most bile samples, although with lower bioconcentration factors, suggesting excretion/detoxification pathways were up-regulated over time. However, mean concentrations of sertraline (16.1±2.6, 24.9±22.7 mg/mL, BCF 4-19) and paroxetine (9.4±6.2, 10.4±3.7 mg/mL, BCF 6-7) were significantly higher after 8 d in bile of fish exposed to 20 and 50% BNR effluent, suggesting they were not subject to the same removal mechanisms as fluoxetine. Surprisingly, BCFs determined for several target analytes differed with the percentage effluent (*v/v*) in the exposure milieu. Similar responses have been reported previously, with MWWE constituents enhancing or impairing the metabolism of co-occuring effluent constituents

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Table 7:3 Summary of the concentration (ng/mL, ±standard deviation) and bioconcentration factor of pharmaceuticals detected in fish bile of Rainbow Trout (*Oncorhynchus mykiss*) in a 8-day laboratory exposure using SPME method.

	CAS 20% effluent			**CAS N 20% effluent**			**BNR 20% effluent**		
Analytes	**Bile Conc. (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*=3±S.D**	**BCF**	**Bile Conc. (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*=3±S.D**	**BCF**	**Bile Conc. (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*= 3±S.D**	**BCF**
Carbamazepine	10.9±0.9	8.3±0.3	1.2	1.8±0.4	8.9±0.2	0.2	0.9	8.5±0.2	0.1
Fluoxetine	1.3±0.1	0.6±0.1	2	1.2±0.2	0.7±0.1	2	2.1±0.5	0.6±0.1	3.5
Sertraline	1.3	0.9±0.1	1.4	ND	0.9±0.1	NA	16.1±2.6	1.0±0.2	16
Paroxetine	2.8	1.4±0.1	NA	0.9	1.4±0.2	0.6	9.4±6.2	1.4	7
Ibuprofen	ND	1.7±0.5	NA	ND	0.5±0.2	NA	ND	0.89±0.2	NA
Gemfibrozil	0.6±0.4	1.9±0.7	0.3	ND	0.4±0.1	NA	1.3±0.2	1.6±0.4	0.8
Triclosan	91±58	<LOQ	NA	46±15	< LOQ	NA	16±13	< LOQ	NA

	BNR 50% effluent			**CAS N 50% effluent**		
Analytes	**Bile Conc. (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*= 3±S.D**	**BCF**	**Bile Conc. (ng/mL) *n*=9**	**Water Conc. (ng/mL) *n*= 3±S.D**	**BCF**
Carbamazepine	1.0±0.7	10.6±2	0.1	3.6±0.5	11.1±0.4	0.3
Fluoxetine	3.4±0.4	0.8±0.2	4	2.3±0.8	0.9±0.2	3
Sertraline	24.9±22.7	1.3±0.5	19	ND	1.1±0.3	NA
Paroxetine	10.4±3.7	1.8	6	0.5	1.8±0.2	0.3
Ibuprofen	ND	1.1±0.4	NA	ND	0.6±0.1	NA
Gemfibrozil	1.4±0.3	2.0±0.5	0.7	ND	0.5±0.1	NA
Triclosan	52.1±19	< LOQ	NA	82±55	< LOQ	NA

7.4.3 **Bioaccumulation of pharmaceuticals in wild White Sucker**

The uptake and bioconcentration of pharmaceutical residues in wild white sucker collected from an effluent-dominated river (downstream of the MWWTPs servicing the cities of Kitchener and Waterloo) was investigated and determined using the SPME method previously developed and validated within the lab environment. In White Sucker muscle sampled from fish resident below the Waterloo effluent outfall, BCFs for the anti-depressants fluoxetine, venlafaxin, and sertraline were 41-3470, 8-164, and 194-3860 respectively (Table 7.4). As per the laboratory exposures, water-soluble carbamazepine, while present in the environment, did not bioconcentrate in fish muscle. In a similar response, as observed below the Waterloo municipal wastewater discharge, White Sucker resident downstream of the Kitchener outfall had fluoxetine, venlafaxin and sertraline concentrated in their muscle tissues, although with lower BCFs than observed at Waterloo (Table 7.4). Of the analytes examined, the anti-depressants bioconcentrate to the greatest extent in fish muscle, likely due to their relatively high octanol-water partition coefficients (log Kow >3), and subsequent tendency to partition from water to the relatively hydrophobic environment afforded by fish tissues [31,181, 187, 209]. The higher BCFs observed in the White Sucker utilized in the field study relative to that observed with Rainbow Trout in the controlled laboratory exposure may be due to analyte uptake in the field from both water and dietary sources. The uptake of pharmaceuticals by White Sucker across both the gill (water) and gut epithelium (food) would thereby increase the realized body burden in this benthivorous species to levels which would be unattainable via water only, as is the case of the lab-exposed Rainbow Trout. Although there was no definite evaluation made of pharmaceutical burden obtained through the diet of the White Sucker fish, it may be more appropriate to refer to bioaccumulation,

rather than bioconcentration factors to reflect the potential routes of contaminant entrance in this species. Conversely, acidic drugs such as ibuprofen and gemfibrozil were not detected in White Sucker muscle, likely due to ionization, which facilitates rapid excretion/minimal uptake under the ambient pH environment of the MWWEs. In contrast, basic anti-depressives have a neutral charge at these environmental pH values, facilitating their partitioning to fish tissues [92, 213].Therefore, the presence and bioconcentration of ionic forms of compounds is a function of environmental pH and dissociation constants, whereas bioconcentration of neutral, nonpolar forms is attributable to their hydrophobic partitioning tendencies. In all instances in this study, in both experimental laboratory treated MWWEs and under real-world field conditions, ionized acidic drugs or those of low log Kow did not bioconcentrate to the same extent as basic, neutral drugs, which were unchanged under local environmental conditions.

Table 7:4 Bioaccumulation factors of pharmaceutical residues in wild White Sucker muscle collected near a municipal effluent outfall in downstream Waterloo and Kitchener

Compounds	**LOQ (ng/g)**	**Muscle Conc. Waterloo (ng/g) *n* = 11**	**Water Conc. (ng/ml) Waterloo**	**BCF/BAF Waterloo**
Carbamazepine	1.0	< LOQ	0.14	NA
Fluoxetine	0.4	0.41 - 34.7	0.01	41-3470
Atorvastatin	0.3	ND	0.05	NA
Venlafaxin	0.1	0.81 - 16.4	0.10	8-164
Paroxetine	0.5	0.11 - 7.9	ND	NA
Sertraline	0.2	1.94 - 38.6	0.01	194-3860
Gemfibrozil	0.1	ND	0.01	NA
Ibuprofen	0.3	ND	0.05	NA

Compounds	LOQ (ng/g)	Muscle Conc. Kitchener (ng/g) n = 11	Water conc. (ng/mL) Kitchener	BCF Kitchener
Carbamazepine	1.0	ND	0.02	NA
Fluoxetine	0.4	0.45 - 3.3	0.01	45-330
Atorvastatin	0.3	ND	0.44	NA
Venlafaxin	0.1	1.8-3.3	0.04	45-83
Paroxetine	0.5	0.49 - 4.62	ND	NA
Sertraline	0.2	0.7 - 7.1	0.01	70-710
Gemfibrozil	0.1	ND	0.01	NA
Ibuprofen	0.3	ND	0.44	NA

7.4.4 Bioconcentration of pharmaceuticals in wild White Sucker bile

Fluoxetine, venlafaxin, paroxetine and sertraline bioconcentrated in the bile of wild White Sucker collected downstream of local MWWTPs discharging final treated effluent from the cities of Kitchener and Waterloo, ON (Table 7.5)

Table 7:5 Measured bioaccumulation factor of pharmaceuticals in wild White Sucker bile near a municipal effluent outfall in downstream Waterloo and Kitchener

Compounds	LOQ (ng/mL)	Bile Conc. Waterloo (ng/mL) *n* = 11	Water Conc. Waterloo (ng/mL) *n* = 3	BCF/BAF Waterloo
Carbamazepine	0.6	ND	0.14	NA
Fluoxetine	0.4	1.23 ± 0.78	0.01	123
Atorvastatin	0.3	ND	0.05	NA
Venlafaxin	0.1	0.59±0.41	0.10	6
Paroxetine	0.4	1.52±0.77	<LOQ	NA
Sertraline	0.2	8.20±5.05	0.01	820
Gemfibrozil	0.4	ND	0.01	NA
Ibuprofen	0.7	ND	0.05	NA

Compounds	LOQ (ng/mL)	Bile Conc. Kitchener (ng/mL) *n* = 11	Water Conc. Kitchener (ng/mL) *n* = 3	BCF Kitchener
Carbamazepine	0.6	ND	0.02	NA
Fluoxetine	0.4	4.07±1.24	0.09	45
Atorvastatin	0.3	ND	0.44	NA
Venlafaxin	0.1	0.63±0.78	0.04	15
Paroxetine	0.4	12.23±2.00	< LOQ	NA
Sertraline	0.2	6.69±4.48	0.04	167
Gemfibrozil	0.4	ND	0.01	NA
Ibuprofen	0.7	ND	0.44	NA

Water-soluble analytes such as carbamazepine, atorvastatin, ibuprofen and gemfibrozil were not detected in bile and, unlike the anti-depressives studied, have no need to undergo Phase I and II metabolism to facilitate excretion, and thus, are not transferred to bile [49, 181, 213]. Biological biotransformation involves changing the xenobiotic properties, usually by hydroxylation, reduction or oxidation to increase water solubility and facilitate excretion. As a result, Phase I metabolites may be excreted directly, or further solubilized when phase II complexation conjugates these metabolites (or sometimes parent compounds) with a glucuronide, sulfate or glutathione molecule [214]. Relative to other tissue and depending on the lipophilicity of the analyte, a greater portion of metabolites relative to its parent compounds may be found in bile as the hepatic processes delivering these analytes to the bile may have also subjected them to Phase I biotransformation. Similarly, parent compounds or metabolites conjugated to water soluble molecules facilitating excretion of the complex as Phase II conjugates were also detected in high concentrations for higher log Kow compounds.

7.5 **Conclusion**

This study demonstrates the utility of SPME in investigations into the bioconcentration potential of pharmaceuticals in fish, particularly for biological tissues such as bile and dorsal-epaxial muscle. The present study further reinforces the well-recognized thesis that conventional wastewater treatments, both those experimental and in contemporary use by municipalities, are inefficient in removal of anti-depressants, which can bioconcentrate/bioaccumulate significantly in fish tissues. Based on the simplicity of the

method, solid phase microextraction is a promising analytical sampling technique for the *in vivo* and *in vitro* analysis of biological samples for laboratory and field applications.

Chapter 8

Summary and future directions of SPME technique

8.1 Summary of applications of SPME technique

8.1.1 Bioconcentration of pharmaceuticals in fish tissues: Analytical perspective

The presence and bioaccumulation of active pharmaceutical ingredients in aquatic organisms such as fish still remains a subject of concern due its possible impact on both human health and aquatic biosystems. The primary source of pharmaceuticals in the aquatic environment is through treated wastewater (sewage) from municipal wastewater treatment plants, which are not presently designed to completely remove them. One of the main analytical challenges present involved with studying pharmaceuticals in the environment is the sensitivity of the analytical method used. The effect of complex matrices poses yet another challenge, since biological and environmental samples have complex matrices which can affect the performance of analytical methods. As a result, a vast number of analytical methodologies are currently being developed with the aim to identify and quantify contaminant residues in aquatic tissue.

With recent developments, sensitivity of analytical methods has improved, in particular through the use of mass spectrometry with higher capability of selectivity and sensitivity[40,207,215]. Additionally, as a response to recent studies, the municipal and federal authorities have commenced upgrading treatment plants in order to increase the efficiency of contaminant removal prior to releasing wastewater back to the aquatic environment [216-218]. As

was observed throughout this thesis in different studies mentioned prior, the fate and distribution of these environmental contaminants depend a variety of factors which must be taken into consideration such as: physico-chemical properties of the contaminants (water solubility, lipophilicity), environmental conditions, as well as diet and aquatic organism physiology (age, sex, species, and weight). However, we are able to use the concentration of environmental contaminants in fish tissue as an excellent indicator of the quality of water entering the aquatic environment. Therefore, it is important to establish the linkage between exposure of contaminants and the adverse effect in aquatic organisms such as fish [212]. In order to achieve this, a reliable and robust analytical methodology is required.

8.1.2 *In vivo* sampling and monitoring of pharmaceuticals in living organism

Traditional analytical methodologies (Solid phase extraction, Solvent extraction) used for determination of pharmaceuticals in fish tissue are laborious, time consuming, and involve multiple steps in their workflow. Development and improvements achieved with SPME technique have enabled us to address some of the drawbacks associated with the traditional analytical methods, in particular their lack of simplicity, as well as the need for reduction of multiple steps associated with the analysis. Furthermore, the technique developed and discussed in this dissertation (C_{18} thin film SPME) can be used to investigate the concentration of environmental contaminants in living organisms (fish) without major consequences. Hence, the use of SPME as a non-lethal sampling method provides a simplified approach for *in vivo* determination of pharmaceuticals (organic contaminants) in

fish tissue without the need to sacrifice the animal being sampled [61,179]. In view of the simplicity of the method, SPME is a promising technique that can be used for monitoring of environmental contaminants in fish tissue. Based on the *in vivo* sampling approach, the real-time tissue concentration in fish can be determined, since contaminants can undergo continuous metabolic transformation in fish. Also, the technique has the potential to study the uptake and depuration of organic contaminants in fish, which may provide useful information on the bioavailability of contaminants.

8.1.3 High throughput analysis of pharmaceuticals in wastewater

The development of thin film automated multi-sampler has provided the possibility of monitoring environmental contaminants such as pharmaceuticals. Based on thin film geometry (blade), enhanced recovery can be achieved due to increased surface area of the extraction phase. As a result, method sensitivity and precision is enhanced. The automation of SPME procedure (particularly the extraction and desorption steps) coupled with liquid chromatography-mass spectrometer is a great advancement for the technique, which can provide high throughput analysis of organic contaminants in wastewater. Although manual solid phase extraction (SPE) is commonly used for analysis of organic contaminants in wastewater, its multi-step approach and the time consuming sample preparation are of concern. The development of SPME technique for this purpose acts as more precise and sensitive method option, simplifying the sample preparation for extraction of organic contaminants in wastewater without the need for sample pre-treatment steps such as

filtration. In addition, the choice of extraction phase and optimization of fibre coating (extraction phase) is crucial to the success of the method in terms of recovery, sensitivity and reproducibility of the method.

8.2 Future direction of the SPME technique

8.2.1 *In vivo* sampling by SPME for environmental analysis

In vivo SPME technique has many potential applications, such as the investigation and monitoring of organic contaminants in protected or endangered living systems, where the organisms being sampled cannot be sacrificed. In addition, as a non-lethal sampling method, this technique also minimizes the number of animals needed in research or monitoring of pollutants in aquatic organisms such as fish, minimizing the environmental impact on the system. This technique opens new possibilities in the areas of metabolomics study, toxicology and tissue analysis in fish. SPME demonstrated several important advantages such as simplicity, sensitivity, and robustness for *in vivo* application in a dynamic system. Also, the automated blade system (based on SPME principle) is a promising method of pharmaceutical analysis in wastewater effluents and effluent-influenced surface waters with acceptable detection limits, capable of identifying the selected pharmaceuticals for quantitative high throughput analysis in environmental water.

8.2.2 Rapid sampling of organic contaminants in fish with new *in vivo* sampler

The development of a new *in vivo* sampler device for sampling pharmaceuticals in fish is a great advancement toward implanting extraction phase in living organisms with minimal disturbance. Also, the new device can be used to rapidly sample pharmaceuticals in fish muscle without the need for anesthetization. Therefore, advancement in SPME device technology and methodology has opened up new possibilities for SPME applications, especially in combination with powerful mass spectrometers for *in vivo* monitoring of pollutants in living organisms such as fish. However, the development of new coating is necessary in order to widen the application of this device for environmental analysis of other classes of contaminants. Also, there is on-going development on new biocompatible coatings that may widen the applications of SPME for *in vivo* sampling of polar compounds in animals including fish[168]. In summary, SPME is a promising sample preparation technique which may provide useful application and information on a living system due to its simplicity.

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