COMPARISON OF MICRODIALYSIS WITH SOLID-PHASE MICROEXTRACTION FOR *IN VIVO* STUDY

by

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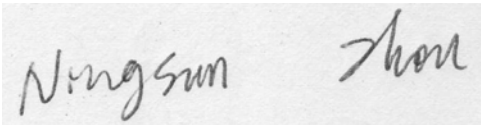
DECLARATION

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

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



ABSTRACT

Although microdialysis (MD) and solid-phase microextraction (SPME) are widely used sampling techniques, a comparison study has not been performed to date. The goal of the research presented was not only to address this issue but also to develop new analytical methods that were more suitable for *in vivo* study using MD and SPME.

A new calibration method called kinetic microdialysis was developed for *in vivo* sampling. Two MD probes with different flow rates were simultaneously inserted into the symmetric parts of sampling system. A simple empirical equation was proposed to calculate the analyte concentrations in the sample matrix using two different dialysate concentrations. Several factors that influenced the correction factor in this equation were discussed. An excellent correlation was observed between the calculated and theoretical value. This method was subsequently applied for *in vivo* sampling, for the measurement of pesticide allocation in the different leaves of a jade plant (*Crassula ovata*). Compared to the other reported MD calibration methods, this novel approach offers several advantages including simplicity, speed, robustness, and increased accuracy.

The on-fiber standardization technique for solid-coated SPME was studied and a theoretical model is proposed for the isotropic behavior of adsorption and desorption, based on Fick's law of diffusion and the Langmuir model. The isotropy of the adsorption and desorption of analytes onto and from the surface of porous solid SPME fiber was validated with the use of a commercially available fiber, a 50 μm carbowax/templated resin (CW/TPR) for carbamate pesticide analysis in various *in vitro* sample matrices. Time constants were comparable for the adsorption and desorption processes. Equilibrium constants and fiber capacities were calculated with the Langmuir Isotherm

Model. A kinetic method was developed to calibrate adsorption using desorption. This calibration corrected for the sample matrix effects and minimized displacement effects as a pre-equilibrium extraction. The technique was successfully applied to the analysis of pesticides in river water and white wine. This developed method could be potentially applied for *in vivo* study.

A new kinetic calibration was developed using dominant pre-equilibrium desorption by SPME. The calibration was based on isotropism between absorption and desorption, which was proved theoretically and experimentally in an aqueous solution and semi-solid matrix. This approach allows for the calibration of absorption using desorption to compensate for matrix effects. Moreover, concentration profiles are initially proposed to verify isotropism between the absorption and desorption, while providing a linear approach to obtain time constants for the purpose of quantitative analysis. This linear approach is more convenient, robust and accurate than the non-linear version with the previously used time profiles. Furthermore, the target analytes are used as the internal standards, thus radioactive or deuterated internal standards are not necessary. In addition, dominant pre-equilibrium desorption utilizes the pre-equilibrium approach and offers a shorter sample preparation time, which is typically suitable for *in vivo* sampling. This kinetic calibration method was successfully applied to prepare samples of polycyclic aromatic hydrocarbons (PAHs) in a flow-through system and *in vivo* pesticide sampling in a jade plant (*Crassula ovata*).

Previous field studies utilizing SPME predominantly focused on volatile compounds in air or water. Earlier *in vivo* sampling studies utilizing SPME were limited to liquid matrices, namely blood. In this study, SPME was developed for *in vivo*

laboratory and field sampling of pharmaceuticals in fish muscle. Pre-equilibrium extraction was used to shorten *in vivo* sampling time. The use of pre-equilibrium desorption rates are proposed as a means to calibrate pre-equilibrium extractions. Excellent linearity was found between the free concentrations determined by SPME from the muscle of living fish and the waterborne concentrations of several pharmaceuticals. It is also firstly proposed a simple SPME method to determine free and total concentrations simultaneously in a living tissue using the known protein binding value. The utility of *in vivo* SPME sampling under field conditions was evaluated in wild fish collected from a number of different river locations under varying degrees of influence from municipal wastewater effluents. Diphenhydramine and diltiazem were detected in the muscle of fish downstream of a local wastewater treatment plant. Based on this study, SPME technique has demonstrated several important advantages for laboratory and field *in vivo* sampling. The development of a rapid, robust, easy to deploy technique which combines sampling, extraction and concentration into one step is a potentially important tool for use *in vivo* field-based sampling.

MD and SPME methods have been developed and compared through *in vitro* and *in vivo* study. For *in vitro* study (juice, milk and orange jelly), both methods offered accurate and precise results (recovery: 88-105% with RSD < 15%) for complex sample matrices by standard addition method. The limits of quantification (LOQs) of the two methods developed were below the tolerance levels in milk set by the United Nations Food and Agriculture Organization (FAO). Compared to MD, the fully automated SPME procedure offered several advantages including high-throughput and more efficient sampling, less labor intensity, and capability for batch analysis. For *in vivo* study, kinetic

calibrations were performed using retrodialysis and in-fiber standardization techniques for MD and SPME, respectively. Quantitative analysis was performed to measure pesticide concentrations in living tissue, i.e., the leaves of a living jade plant (*Crassula ovata*). Although both techniques provided sampling with minimal perturbation to the system under study, SPME was more sensitive, precise and accurate, suitable for field sampling and had a wider application than MD. It demonstrated that SPME has the potential to replace MD for *in vivo* study.

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Finally, I would like to thank my family for their love, care, support, understanding, and encouragement.

DEDICATION

I dedicate this thesis to Luke and Judy.

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1.1 Solid-Phase Microextraction

1.1.1 Introduction

Solid-phase microextraction (SPME) is a sample preparation and sample introduction technique invented by Pawliszyn and colleagues.[1] The first home-made SPME device was carried out by combining the coated fiber into a microsyringe as shown in Figure 1.1.[1] A stainless steel microtubing was used to substitute for the metal rod that served as the piston in a microsyringe. The microtubing had an inside diameter slightly larger than the outside diameter of the fused silica rod. After the first 5 mm of the coating was removed from a 1.5 cm long fiber, it was inserted into the microtubing. Then the fiber was immobilized by high temperature epoxy glue. This design allowed the coated fiber to move into and out of the stainless steel needle. The fiber was protected by the stainless steel needle when the fiber was stored or not in use, and guided into the injector during use. Movement of the fiber out of the needle allowed the coating to be exposed to a sample matrix to perform extraction, or to a gas chromatography (GC) injector to achieve thermal desorption. The behavior of sample injection is like standard syringe injection.

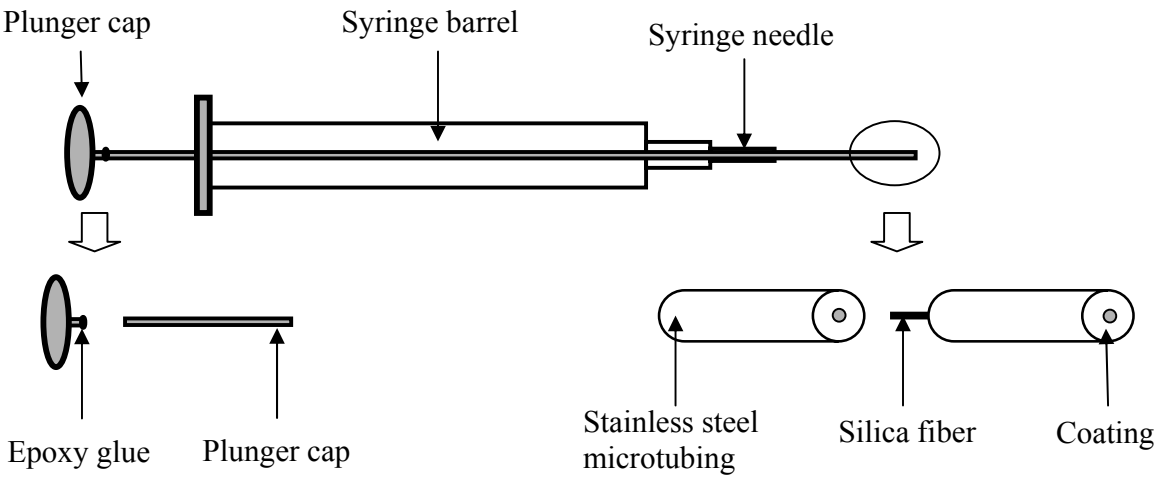

Figure 1.1 The first SPME device based on the Hamilton 7000 series syringe.

Compared to conventional analytical methods, SPME presents several advantages including less organic solvent-consumption, low cost, portability, and combination of sampling, isolation and enrichment into one step. Originally, SPME has been directly coupled to GC or GC/ mass spectrometry (MS) to analyze a wide variety of volatile and semi-volatile chemicals from the environmental, food, and clinical and pharmaceutical samples. More recently, it has also been applied successfully to the extraction of nonvolatile or thermally labile compounds that are suitable for high-performance liquid chromatography (HPLC), LC-MS and LC-MS/MS.[2, 3]

To date, two different groups of SPME fiber coatings are available from a manufacturer. [4] The first group includes polydimethylsiloxane (PDMS) and polyacrylate (PA) that belong to the liquid coatings, i.e., homogeneous pure polymers. The second one is solid coatings including PDMS/divinylbenzene (DVB), carbowax (CW)/DVB, carboxen (CAR)/PDMS, DVB/CAR/PDMS and CW/templated resin (TPR).

The performance of liquid and solid coatings is significantly different. A liquid coating allows the analytes to dissolve in the coating molecules, or partition into the extraction phase. If the coating is thin, within a certain extraction time, the target analytes can penetrate the entire liquid coating through molecular diffusion. Figure 1.2 (A) illustrates the procedure for the liquid coating. Molecular partitioning between the sample matrix and extraction phase occurs. Partition constant can be used to calculate the target analyte concentrations in both the liquid coating and the sample matrix.

Figure 1.2 (B) presents a solid coating that has a glassy or a well-defined crystalline structure. The material density somewhat forbids molecular diffusion within the structure. During the short period of experimental time, the pores of the solid-phase allow adsorption happen. However, after a long extraction time, displacement or competition effect occurs. Chemicals with a high affinity to the extraction phase normally displace molecules that weakly bind or those that are present in the sample at low concentrations. The displacement effect is caused by the limited surface area available for adsorption. When displacement or competition effect occurs, the extracted amounts at equilibrium can change with the concentrations of both the target and other analytes.[5]

Selection of a suitable coating is usually the first step to develop a new SPME method. To date, the commercially available fibers include 7, 30 and 100 μm for PDMS, 85 μm for PA, 75 and 85 μm for CAR/PDMS, 65 μm for PDMS/DVB, 65 and 75 μm for CW/DVB, 50 μm for CW/TPR and 50 μm/30 μm for DVB/CAR/PDMS. For nonpolar analytes, a nonpolar PDMS fiber is the first choice because of the principle of “like dissolves like”. Selection of coating thickness of PDMS fibers depends on the efficiency required, the extraction time and the nature of the analyte. Although a thicker coating

provides the higher capacity, a thinner one can reduce the partition equilibrium time.[6] In the case of more polar compounds, PA or the mixed coating fibers can be evaluated for extraction efficiency.

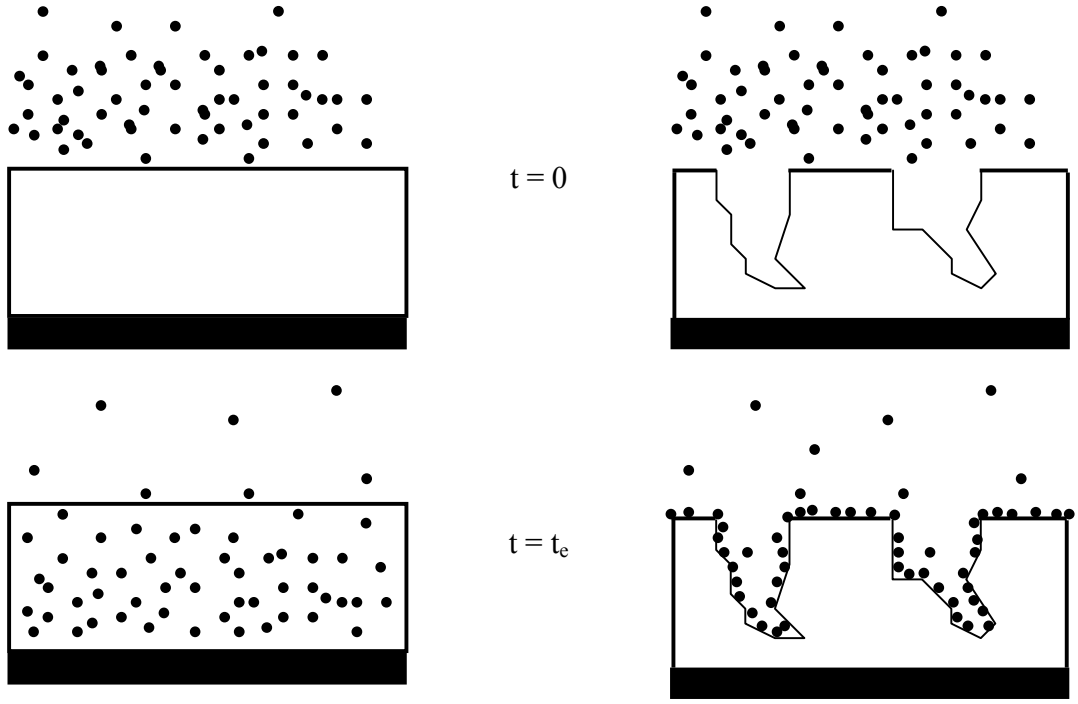

Figure 1.2 Extraction via absorption (A) and adsorption (B) immediately after exposure of the phase to the sample (t = 0) and after completion of the extraction (t = t_e).

Three approaches have been developed for extraction using SPME, direct immersion, headspace, and membrane protection, which are presented in Figure 1.3. The direct immersion approach as shown in Figure 1.3 (A) allows the fiber coating to expose the sample matrix. The approach is straightforward for the analytes to transport from the sample to the extraction phase. Agitation is desirable to achieve a rapid extraction to allow analytes to transport from the bulk of the solution to the surrounding area of the

fiber. In the case of gaseous samples, rapid equilibration is easy to accomplish by the rapid molecular diffusion and natural convection of air. For aqueous sample matrices, molecular diffusion of compounds in a liquid solution is typically slow. Therefore, employing more efficient agitation techniques is desirable, including fast sample flow, rapid fiber or vial movement, stirring or sonication. Agitation reduces the effect caused by the "depletion zone" produced close to the fiber as a result of fluid shielding.[7, 8] Another important parameter, "salting-out" effect, can be used to improve extraction efficiency in most of the aqueous matrices. A certain percent of salt in the solution drives more analytes from the sample matrix to the fiber coating.

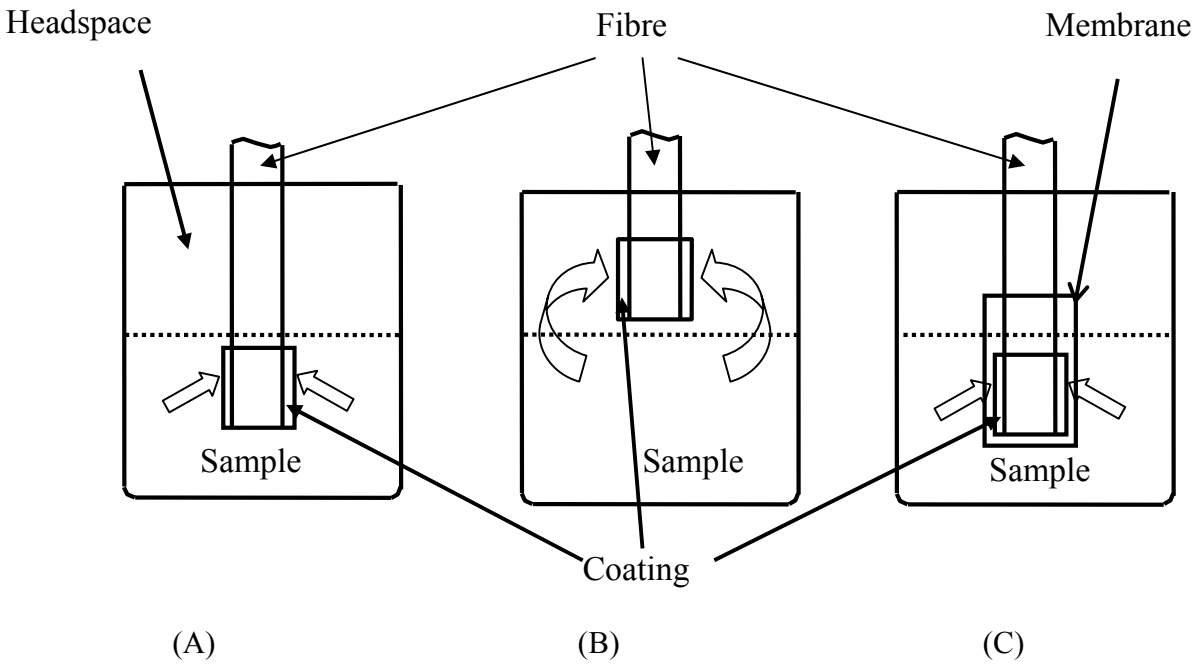

Figure 1.3 Three modes of SPME operation: (A) direct immersion, (B) headspace SPME (C), membrane-protected SPME.

The headspace configuration, Figure 1.3 (B), has to let the analytes pass through the barrier of air before they can contact the coating. Compared to direction immersion, this design offers three advantages. First, without damaging the fiber, the matrix can be adjusted, for example, the modification of the solution pH value. Second, the fiber coating can be free from damage caused by irreversible interaction between the coating and large molecules or other interferences in the sample matrix such as proteins or particular matter. Third, the headspace mode offers a shorter equilibrium time for volatile compounds than direction extraction under similar experimental conditions. Two factors contribute to this outcome. One is the large amount of the analytes in the headspace prior to extraction. Another is that the molecular diffusion in the gaseous phase is typically four orders of magnitude larger than in the liquid matrix.

In the headspace mode, temperature plays an important role in improving the extraction efficiency, which can be explained through extraction kinetics. When a fiber coating is exposed to the headspace of a sample matrix, the analytes in the headspace can reach the fiber coating first. The analytes in the matrix have to escape from the matrix to the headspace before extracted by the fiber. It is illustrated in Figure 1.3 (B). Typically, mass transfers rates from the sample to the headspace limits overall mass transfer to the fiber. To increase the vapor pressure of the analytes, temperature improves extraction kinetics. The second parameter, agitation, can be optimized to shorten equilibrium time, which has a similar mechanism as the above-mentioned direction immersion mode. The third parameter is the ratio between the volume of the sample matrix and the headspace. Although the ratio influences the extraction efficiency, its improvement is not as significant as temperature.

The application of the headspace is limited to volatile and semivolatile compounds. Compared to semivolatile compounds, volatile analytes present at a higher concentration in the headspace and have faster mass transport rates to the headspace. Thus, it is easier for the fiber to extract volatiles. Semi-volatile analytes typically need a longer extraction time. To improve this situation, very efficient agitation and higher temperature can be utilized. [9]

Figure 1.3 (C) illustrates the approach of membrane-protected SPME, which can prevent the fiber from damage when a very dirty or complex sample matrix is used. In this case, it shares the same advantage as the headspace mode. Nevertheless, membrane-protected SPME can be extended to non-volatile compounds, which are not applicable for headspace. Also, after a type of material with specific characteristics is used for the membrane, selective extraction can be improved to a certain degree.

However, the extraction kinetics of membrane-protected SMPE is significantly slower than that of direct immersion. The reason is that the analytes have to pass through the membrane before they can reach the coating. Obviously, the thicker membrane slows down the mass transfer through the membrane. A strategy is to use a thin membrane and increase extraction temperature to shorten the extraction time.[10]

After extraction, analytes must be desorbed to GC or LC for separation and qualitative or quantitative analysis. For the volatile and semivolatile compounds, analytes are easy to desorb from the fiber coating to any GC injector. As soon as the septum-piecing needle penetrates the GC injector septum, the SPME device plunger is depressed to allow the fiber coating to expose the GC injector. The high temperature in the injector lets analytes thermally vaporize from the fiber coating, which is analogous to a

conventional injection using a syringe. To date, there are many commercially available SPME devices directly coupling any GC injector. A well-developed autosampler has eliminated any manual involvement and can automatically perform the whole procedure including incubation, agitation, extraction, and desorption.

In the case of nonvolatile or thermally labile compounds, LC has to be used for separation. Liquid desorption from the fiber is required prior to injection of analytes to LC. However, liquid desorption is a relatively slow process because of the slower diffusion kinetics in the condensed phase compared to a thermal desorption occurring in a GC injector. As a result, there is a process that is analogous to conventional liquid injection to LC. To date, three major liquid desorptions have widely been used for this purpose, direct interface, in-tube SPME, and off-line desorption. Direct interface and in-tube SPME offer good sensitivity because it allows all of the extracts to enter the LC system. However, direct interface must be operated manually and it is difficult to perform automation. For in-tube SPME, *in vivo* or on-site sampling is not easily to perform. Compared to direct interface and in-tube SPME, although off-line desorption can not provide sensitivities as good as the other two designs, it is applicable to any commercially available LC autosampler. The off-line desorption approach has gradually become the most frequently used one for LC application.

1.1.2 Calibration Methods

To date, several calibration approaches that have been developed for SPME are listed as follows:

- Equilibrium Extraction

- Exhaustive Extraction
- Pre-equilibrium Extraction
- Diffusion
- Kinetic Calibration

Figure 1.4 shows the direction immersion approach for SPME. The labeled parameters are necessary to perform calibration.

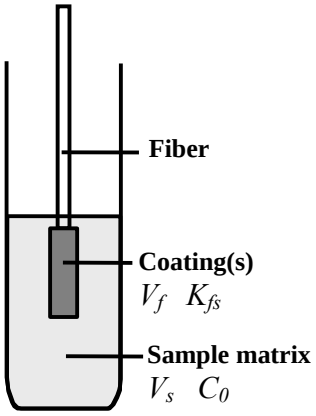

Figure 1.4 Microextraction with SPME. V_f, volume of fiber coating; K_{fs}, fiber/sample distribution coefficient; V_s, volume of sample; C_0, initial concentration of analyte in the sample.

1.1.2.1 Equilibrium Extraction

Equilibrium extraction is the most repeatedly used method. When the extraction time is long enough, concentration equilibrium is established between the extraction phase and the sample matrix. After equilibrium conditions are reached, analytes do not accumulate more although the coating is exposed to the sample matrix for a longer time.

When the analyte concentration reaches equilibrium between the sample matrix and the fiber coating, SPME extraction is typically considered to be complete. The law of mass conservation can be applied to the equilibrium situation. When equilibrium is reached between two phases, the sample matrix and the fiber coating, the following equation can be obtained,[11]

$$n_e = \frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s} C_0 \qquad \text{Equation 1.1}$$

where n_e is the number of moles extracted by the coating, K_{fs} is the fiber coating/sample matrix distribution coefficient, V_f is the fiber coating volume, V_s is the sample volume, C_0 is the initial concentration of a given analyte in the sample.

Equation 1.1 shows that the amount of analyte extracted onto the coating (n_e) is linearly proportional to the analyte concentration in the sample (C_0). The equation is the basis for analytical quantification for SPME.

This equation can only practically apply to liquid polymeric phases such as PDMS, where partitioning equilibrium occurs. For solid sorbent coatings, based on the assumption of constant porosity of the sorbent, the total surface area available for adsorption is considered to be proportional to the coating volume. Therefore, the equation above can be applied to a solid-coated fiber when analyte concentrations are low. For high analyte concentrations, saturation of the surface can cause nonlinear isotherms. Also, displacement or competition effect occurs under this situation.

Equation 1.1 can be modified to apply to more complicated conditions. For example, in the case of a headspace mode, the volumes of the individual phases and the appropriate distribution constants must be considered.

When the sample volume is very large, i.e. $V_s >> K_{fs}V_f$, Equation 1.1 can be simplified to:

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

This equation indicates that the amount of extracted analyte is independent of the volume of the sample. If the fiber is exposed directly to the ambient air, water, production stream, etc., it is not necessary to collect a defined sample prior to analysis. The amount of extracted analyte is independent on the sample volume, and corresponds directly to its concentration in the matrix.

1.1.2.2 Exhaustive Extraction

Exhaustive extraction might occur in SPME under the condition of a very small sample volume. The following equation can be used for exhaustive extraction,

$$n = V_s C_0 \qquad \text{Equation 1.3}$$

The equation indicates that all analytes in the sample matrix are extracted onto the fiber coating. Calibration for exhaustive extraction is relatively simple.

1.1.2.3 Pre-equilibrium Extraction

Pre-equilibrium extraction is proposed to address two issues. The first issue is that an equilibrium extraction time is too long to perform in terms of lab practice. The second issue is that the displacement effect may occur when porous coatings are employed. Before equilibrium is reached, extraction can be interrupted. Calibration is still practical at the constant agitation and the fixed extraction time. The kinetics of absorption of analytes onto a liquid fiber coating is expressed as,[12]

$$n = [1 - \exp(-at)]\frac{K_{fs}V_fV_s}{K_{fs}V_f + V_s}C_0 \qquad \text{Equation 1.4}$$

where t is the extraction time, and a is a time constant, representing how fast an equilibrium can be reached.

1.1.2.4 Diffusion-based Calibration

The diffusion-based calibration method is very useful for field sampling. This method offers two advantages, elimination of the use of conventional calibration curves and feasibility of fast on-site analysis and long-term monitoring. Two approaches have been developed for diffusion-based calibration. One is grab sampling using various empirical mass transfer correlations, another is time-weighted average (TWA) sampling based on Fick's First Law of diffusion. In the case of grab sampling using SPME, the SPME coating is directly exposed to the sample matrix. The equation below can estimate the concentration of analyte in the sample for rapid sampling with solid sorbents having a high affinity to the analyte,[13, 14]

$$C_g = \frac{n\delta}{B_3 D_g A t} \qquad \text{Equation 1.5}$$

where C_g is the analyte concentration in the bulk of the sample, n is the mass of analyte extracted (ng) in a sampling time (t), δ is the thickness of the boundary surrounding the extraction phase, B_3 is a geometric factor, D_g is the gas-phase molecular diffusion coefficient, and A is the outer surface area of the sorbent.

For TWA sampling, the SPME-in-needle device can be used if the sorbent is "zero sink" for the target analytes. The concentrations of analytes in the sample can be calculated with eq 1.6,[15-17]

$$C = \frac{nZ}{ADt} \qquad \text{Equation 1.6}$$

where C is the TWA concentration of the target analyte in air or water during the sampling time t, Z is the diffusion path length, A is the cross-sectional area of the needle, D is the diffusion coefficient of the target analyte in air or water, and n is the amount of analyte that is extracted by the fiber during time t.

1.1.2.5 Kinetic Calibration

Kinetic calibration is based on the isotropic behavior between absorption and desorption in the SPME liquid-coated fiber. This method is also called in-fiber standardization technique. It uses desorption of the standards, which are preloaded in the extraction phase, to calibrate the extraction of the analytes. The initial concentrations of target analytes in the sample matrix, C_0, can be calculated using the following equation,[18, 19]

$$C_0 = \frac{q_0 n}{K_{fs} V_f (q_0 - Q)} \qquad \text{Equation 1.7}$$

where q_0 is the amount of preloaded standard in the fiber, q and Q is the amount of the standard remaining in the fiber after exposure of the fiber to the sample matrix for the sampling time.

This approach is very important for an *in vivo* sampling because it is difficult to use an *in vitro* system to mimic the real living environment. Moreover, a long equilibrium extraction is typically impractical in a living system.

1.1.3 Application for *In Vivo* Sampling

SPME offers several unique characteristics including the small dimension, the portable SPME devices, non- or less-solvent consumption, fast and easy extraction procedure, no biological sample processing, and easy coupling to highly specific instruments such as GC-MS and LC-MS/MS. These characteristics allow SPME to become an ideal *in vivo* sampling technique. For SPME *in vivo* applications reported in the literature, SPME provides sensitivities and precision as good as or better than other sample preparation techniques employed for the same sample matrices.[20, 21] When a SPME fiber is exposed to a living system, only a small amount of the total analyte is removed. Therefore, the SPME extraction procedure does not disrupt the ordinary balance of the chemical components. This also results in relatively broader applications by SPME compared to some other sampling techniques, which may severely damage the live organisms or sacrifice the whole living system.[22]

Biocompatible materials can be directly exposed to the flowing blood of living organisms or other living systems without causing any side-effects or toxic consequences. Therefore, the development of biocompatible extraction phases for SPME has resulted in significant improvements in *in vivo* sampling. To date, researchers have developed and used different biocompatible SPME coatings including restricted access materials (RAM), PDMS, polypyrole (PPY), poly(ethylene glycol) (PEG)/C18, and polyacrylonitrile (PAN).[23-27]

Previous SPME *in vivo* sampling paid attention to odors released by insects, fungi and bacteria. Further investigations included biogenic volatile organic compounds produced by animals and plants. More recently, SPME was extended to the *in vivo*

sampling to determine drug concentrations in a living animal. Two SPME probes, PPY, and PEG/C18, were developed. Its high efficiency and accuracy were demonstrated through pharmacokinetic study. These results were consistent with those produced by the conventional sampling method, liquid-liquid extraction.[25, 26, 28]

1.2 Microdialysis

1.2.1 Introduction

Microdialysis (MD) is a sampling technique containing a short length of hollow-fiber membrane (or probe) and mostly used in a biological system. A MD system consists of a syringe pump, the connective tubings, a probe, and a microvial to collect the dialysate, which is shown in Figure 1.5.[29] MD has been developed for *in vitro* experiments and *in vivo* sampling of endogenous and exogenous compounds from body fluids of selected tissues.

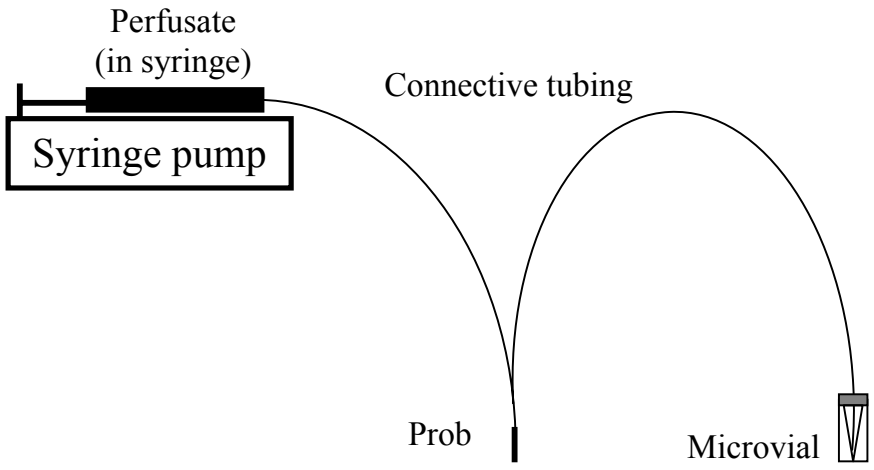

Figure 1.5 A basic microdialysis system consisting of a syringe pump for perfusate delivery, a microdialysis probe and connective tubing.

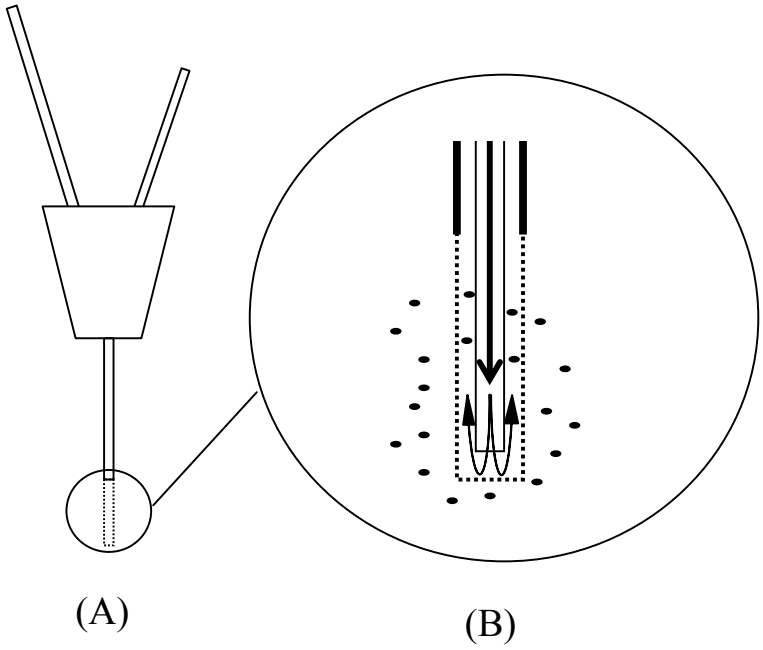

Figure 1.6 Concentric probe (A) and enlarged section of the same probe (B). Small molecules diffuse in and out the semipermeable membrane by concentration gradient. The black arrows indicate the direction of the flow through the probe.

A concentric probe is the most common used design as shown in Figure 1.6. It is composed of a thin dialysis tube with an inner diameter in the range of around 0.15–0.3 mm and a semipermeable membrane at the tip of the probe. The system pump delivers the perfusate to enter the probe through the inlet tubing. Then the perfusate passes the membrane and is transported through the outlet tubing. Finally, the dialysate is collected in a microvial at the end of the outlet tubing. Typically, the flow rate of MD ranges between 0.5 and 5 μl/min, which depends on experimental requirements. For *in vivo* sampling, the perfusate is usually an aqueous solution that has a similar composition of the surrounding medium. It can keep the close osmotic pressure between inside and outside of the probe, so the excessive migration of molecules through the membrane can

be avoided. Molecular diffusion crossing the membrane is driven by the concentration gradient. Therefore, molecules can diffuse into (recovery) or out of (loss) the membrane.

As an analyte-free perfusate solution continues to pass through the probe, non-equilibrium conditions always remain with the exception of method of no-net-flux. The constant concentration gradient makes the concentration in the dialysate (C_d) lower than the concentration in the periprobe fluid or sample matrix (C_0), i.e., $C_0 > C_d$.

The ratio between the concentration in the dialysate and the concentration in the periprobe fluid is defined relative recovery (R_R) expressed as,

$$R_R(\%) = \frac{C_d}{C_0} \times 100\% \qquad \text{Equation 1.8}$$

The relative recovery is dependent on the following parameters,

- velocity of the diffusion process across the membrane which depends on
  - temperature
  - weight cut-off and membrane area
  - concentration gradient
- composition of perfusate
- flow rate
- tortuosity of the sample matrix

1.2.2 Calibration Methods

Before any conclusions can be drawn about concentrations in the periprobe fluid, calibration has to be performed for a MD probe because relative recovery does not reach 100% (not in the case of no-net-flux). Several different methods have been developed for

calibration and thus for determination of relative recovery that produce results of diverse quality. They are listed as follows,

- Method of flow rate variation
- Method of no-net-flux
- Retrodialysis
- Endogenous reference substance

1.2.2.1 Method of Flow Rate Variation

Method of flow rate variation is a technique to obtain the value of relative recovery by varying the perfusate flow rate.[30] The lower the flow rate, the higher relative recovery. At a rate of zero, equilibrium conditions are reached. Thus the dialysate concentration exactly represents the actual sample concentration. When the dialysate concentration against the flow rate is plotted, extrapolating to zero flow can yield the concentration at zero flow rate as well as the relative recovery of the probe. This process can be expressed with the following exponential function,

$$\frac{C_d}{C_0} = 1 - \exp(-rA/F) \qquad \text{Equation 1.9}$$

where r is the mass transfer coefficient, A is the cross area of the membrane, and F specifies the chosen flow rate.

This method has a major disadvantage, time consumption or the long sampling time especially for low flow rates that thus yield poor temporal resolution. Moreover, it is a non-linear approach resulting in more inconvenience and uncertainty. In addition, the actual concentration in the sample matrix is only able to obtain through extrapolating to zero flow with the presumed exponential function. Hence, it is highly recommended to

include measurements at very low flow rates in the experimental setting in order to minimize the degree of uncertainty of the estimated value at zero flow.

1.2.2.2 No-net-flux Method

The no-net-flux method (or zero-net-flux method) is a calibration method using several different perfusates, which are made from the solutions of the interested compound with different known concentrations.[31] A line can be drawn by substance loss or gain measured in the dialysate in relation to the perfusate versus the different perfusate concentrations. The concentration in the periprobe fluid is equal to the value of the intercept at x-axis. Compared to the method of flow rate variation, the no-net-flux method offers an interpolation approach instead of extrapolation. Thus, it is not necessary to presume about the analyte and its behavior pattern in the periprobe fluid. However, with different perfusate concentrations, this method is really time-consuming. Also, it requires steady state conditions

1.2.2.3 Retrodialysis

Retrodialysis is a method using a perfusate spiked with the radio-labelled analyte in a known concentration.[32] Since the radio-labelled analyte (internal standard) shares similar physicochemical properties with the analyte in the sample matrix, it can assume that the relative loss is quantitatively equal to the relative recovery during MD *in vivo* sampling. The concentration of the radio-labelled analyte in the perfusate is known. Also, the concentrations of both of the analyte and the internal standard in the collected

dialysate can be measured. Therefore, the relative recovery of the analyte can be calculated by this equation,

$$R_R(\%) = (1 - \frac{C_d}{C_p}) \times 100\% \qquad \text{Equation 1.10}$$

where C_p is the concentration of the radio-labelled analytes. Afterwards, the concentration of the analyte in the periprobe fluid can be calculated using the measured concentration in the dialysate and the relative recovery. The advantage of this method is that any recovery changes can be observed by the variation of relative loss during *in vivo* sampling.[33]

1.2.2.4 Endogenous Substance as a Reference

An endogenous substance as a reference was used to determine relative recovery. The advantage of this approach is that it is less time-consuming and does not require steady state conditions. Urea was used as an endogenous recovery marker because it has a relatively constant concentration in an animal. Also, it can freely diffuse in and out of the MD membrane due to its small molecular size. It was assumed that that the relative recovery ratios between the interested analyte and urea should be close for both *in vitro* and *in vivo* sampling. Based on this assumption, urea as an endogenous recovery marker was used for *in vivo* studies. The experimental results were in agreement with this assumption. Thus a conclusion was drawn that urea was suitable for a recovery marker.[34, 35] However, an opposite experimental observation was reported.[36] Therefore, more studies have to be conducted to prove if this calibration approach is viable for *in vivo* studies.

1.2.3 Application for *In Vivo* Sampling

Compared to other methods for *in vivo* sampling in biological fluids and tissues, MD offers several advantages. First, it is a less labor intensive technique. The MD membrane with the property of a certain molecular weight cut-off yields a very clean dialysate. Therefore, a cleanup step is not typically required. Second, MD provides a temporal profile because of its ability for continuous sampling, for example, the fate (absorption, distribution, metabolism, etc.) of the pharmaceutical drug at different tissue sites (liver, kidney, brain, muscle etc.) and in different fluids (blood, bile, brain or extracellular fluid). Third, the experimental animal is disturbed less by MD because of the tiny size of the probe. Also, during MD sampling, no blood is withdrawn from the animal, and no fluid is added to or removed from the target tissue. Therefore, the metabolic process can go normally. Forth, the number of experimental animals can be significantly reduced for a given study. One single MD probe can be used to monitor a whole metabolic process before, during, and after the drug administration. The cross-over experiments are easy to perform because each animal serves as its own control. Compared with isolated tissue studies, continuous sampling provides more information from each experimental animal. Fifth, MD avoids problems associated with intra-animal variation. Multiple MD probes can be put multiple sites in a single animal to conduct simultaneous sampling. Conventional pharmacokinetics studies have to use a population of animals for this purpose. Sixth, the risk of enzymatic degradation can be avoided. The dialysate collected in the microvial is isolated from the proteins and other macromolecules or can be frozen immediately. Base on these advantages, MD has been developed for *in vivo* sampling and applied to many disciplines such as neuroscience

research, pharmacodynamic and pharmacokinetic studies. More recently, MD was applied for environmental monitoring studies.

1.3 Thesis Objective

Both MD and SPME are the most powerful sampling techniques, which have been previously employed for *in vivo* study. However, there are still numerous fields that have not been explored using either MD or SPME. In addition, a comparison study has not been previously performed.

The overall objective of this thesis is to develop new analytical methods for *in vivo* sampling and sample preparation using SPME and MD. The main focus will be the *in vivo* sampling in soft tissues. Comparison of two sampling techniques will be performed to address advantages and disadvantages for *in vivo* sampling.

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Chapter 2

Kinetic Microdialysis for *In Vivo* Sampling

2.1 Preamble and Introduction

2.1.1 Preamble. So far, there is not a third co-author involving this project. This chapter is not submitted to date.

2.1.2 Introduction. Systemic pesticides are absorbed by plants or animals and move throughout the treated plants to kill certain insects. Some pesticides such as carbofuran only move upwards in plants. When such pesticides are applied to the root zone, they are taken up by roots and travel throughout the plant. This kind of systemic pesticides is also called soil-applied pesticides.[1] Pesticides play an important role in maintaining food supply and environment by protecting plants from disease and pests. However, residues of these pesticides can remain in or on the foods and environment, which causes a significant public concern. The development of simple, fast and accurate pesticide sampling techniques for quantitative analysis is of key importance to prevent and minimize human exposure to pesticides. In addition, such techniques provide information on the concentration and translocation of pesticides within living plants thus enabling the study of pesticide mode of action as well as plant physiology and the evaluation of plants for decontamination of pesticide-contaminated environments.[2, 3]

Microdialysis is one of the powerful tools for *in vivo* sampling, and has been widely used to sample low molecular weight substances from a variety of biological systems.[4] MD is based on the dialysis principle and consists of a membrane that is

permeable to water and small solutes. This membrane is continuously flushed on one side with a solution devoid of the substances of interest, whereas the other side faces the interstitial space of the system under study. A concentration gradient is created and causes diffusion of substances from the interstitial space into the dialysis membrane (and vice versa). The continuous flow through the membrane carries substances from the sampling site for further analysis.[5] Microdialysis has two main advantages. First, it provides continuous sampling of low molecular weight compounds with a minimal perturbation to the system under study. Second, the sample preparation time is typically short when compared to other destructive techniques.

At typical perfusion rate during MD sampling, the continuous flow of perfusion fluid through the probe does not permit the analyte concentration inside the probe to come into equilibrium with the analyte concentration outside the probe. Most commonly, relative recovery is used to calculate the analyte concentration in the sample matrix, which is expressed as,

$$\%R_r = \frac{C_d}{C_0} \times 100\% \qquad \text{Equation 2.1}$$

where $\%R_r$ is the percent relative recovery, C_d is the concentration of the analyte in dialysate and C_0 is the concentration of the analyte in the sample matrix. Under these non-equilibrium conditions, relative recovery will never reach 100% except in the no-net-flux situation. This necessitates the calibration of microdialysis probes to determine relative recovery prior to the determination of analyte concentrations in the periprobe fluid.[6] Moreover, for *in vivo* sampling, a number of factors can influence the value of $\%R_r$ including perfusion flow rate, sample flow rate, temperature of the tissue or dialysis

target, diffusion of the substance within the sample medium, diffusion rate in dialysis membrane, molecular weight cutoff of the dialysis membrane, chemical interaction between the analyte and the membrane, surface area of the dialysis membrane (length and diameter of the probe), blood flow rate, metabolism rate, uptake into cells, extent of tissue vascularization and so on. Among these factors, some can not be controlled such as membrane fouling during sampling, or change of conditions around the membrane. As a result, relative recovery may change, so the use of an appropriate calibration method during MD sampling is crucial in order to obtain reliable quantitative analysis.

So far, there are many methods for MD calibration and thus for determination of relative recovery. The most commonly used calibration techniques for microdialysis are method of flow rate variation, method of no-net-flux, dynamic no-net-flux, and retrodialysis.[7] However, there are limitations for these calibration methods and they yield results of diverse quality. The method of flow rate variation needs a long sampling time with small flow rates, thus resulting in poor temporal resolution. The method of no-net-flux is very time-consuming because of the need to calibrate with different perfusate concentrations and the necessity of steady state conditions. Dynamic no-net-flux is labour intensive due to the fact that a large number of study individuals are required for calibration procedures. When retrodialysis is used, an internal standard, the radiolabeled analyte or calibrator, has to be found. Radiolabled compounds are typically expensive or may be unavailable. Also, a calibrator might not be easy to find because the target analyte and the calibrator have to exhibit a similarity in their physical properties.[6, 7]

To overcome the limitations mentioned above, one novel calibration method called kinetic microdialysis was developed for *in vivo* sampling. In this method, two

microdialysis probes at different flow rates were simultaneously inserted to the symmetric parts of sampling system. The perfusate is just the physiological fluid of the sampling system. An empirical formula was proposed to calculate the analyte concentrations in the sample matrix using the values of two different dialysate concentrations. An excellent correlation was observed between the calculated and theoretical value for all pesticides and drug tested. This approach was applied for *in vivo* sampling.

2.2 Experimental

2.2.1 Chemicals and Supplies

Carbofuran, propoxur, carbaryl, aldicarb and promecarb were supplied from Riedel-de Haën, with greater than 99.5% purity. Acetonitrile and methanol (HPLC grade) were obtained from Fisher Scientific (Nepean, ON, Canada). Stock standard solutions (1000 mg/L) of five pesticides were prepared in acetonitrile and stored at 0–4 °C in the dark. Benzodiazepine standards (1 mg/mL in methanol) were purchased from Cerilliant (Austin, TX). Working solutions were prepared by a series of tenfold successive dilution in methanol or phosphate-buffered saline, pH 7.4 (PBS), to prepare mixtures of various concentrations for use in sample preparation, instrument calibration and quantitative analysis. For small-volume analysis, a polyethylene insert (Supelco, Bellefonte, CA, USA), which has a volume of 200 μL, was positioned in a 2-mL vial. The jade plants (*Crassula ovata*) were purchased from a local greenhouse.

2.2.2 Instrumental Analysis

The HPLC analysis was performed with Agilent 1200 series autosampler, column oven and a binary pump (Mississauga, ON, Canada). For the separation of pesticides, a Symmetry 300^{TM} C4 column (50 × 2.1 mm, 3.5 μm, Waters, Milford, MA, USA) was used. The column oven temperature was set at room temperature, 25˚C. Mobile phases consisted of (A) acetonitrile/water (10:90 v/v) with acetic acid (0.1% v/v) and (B) acetonitrile/water (90:10 v/v) with acetic acid (0.1% v/v). The flow rate was set at 0.5 mL/min. Starting mobile phase conditions were 0% B for the first 0.5 min. This was ramped to 100% B over 2.5 min, held for 1.0 min, and finally returned to 0% B for 1.0 min. This resulted in a total run time of 5.0 min, including reconditioning. In the case of benzodiazepine separation, a Finesse Genesis C18 column (150 × 2.1 mm, 4 μm, Chromatographic Specialties Inc., Brockville, ON, Canada) was used. Mobile phases consisted of (A) acetonitrile/water (10:90 v/v) with acetic acid (0.1% v/v) and (B) acetonitrile/water (90:10 v/v) with acetic acid (0.1% v/v). The flow rate was set at 0.5 mL/min. 35% was applied for the first 2.5 min. This was ramped to 100% B over 0.5 min, held for 1.5 min, and finally returned to 35% B for 2.5 min. This resulted in a total run time of 7.0 min, including reconditioning. A 5 μL injection volume was used for the each experimental sample. To eliminate any potential carry-over from a previous injection, the washing time for the injection needle was 30s in the flush port and the sample flush factor was set to be 20 before any sample injection. No carry-over was found even after injection of a high concentration sample. The eluted analytes were monitored by triple-quadrupole tandem mass spectrometer (MS/MS) using a QTrap 3200 system with a TurboIonSpray source (Applied Biosystems/MDS Sciex, ON, Canada).

Each transition was monitored for 200 ms. Compound specific mass spectrometer settings were determined for each compound separately by infusing a 0.5 μg/mL solution (methanol/water, 1:1 v/v) at 3 μL/min using the integrated syringe pump. Mass spectrometer response sensitivity and linearity were monitored before and after each set of experimental samples with the injection of 5 μL of a series of standards (0.1–100 ng/mL) prepared in methanol and water (1:1 v/v) containing the internal standard, promecarb or lorazepam for pesticides or drugs, respectively. Analyst version 1.4 software (Applied Biosystems) was used to control all components of the system and for data collection and analysis. External calibration curves were performed for microdialysis with good precision (RSD < 5%) and linearity ($R^2 > 0.998$).

2.2.3 *In Vitro* Experiments

Agarose gel (1% w/v) was spiked with various concentrations of pesticides or drugs for *in vitro* experiments to establish the relationship between the analyte concentration in the sample matrix and two dialysate concentrations. The gel was made by mixing 0.5 g Agarose (Agarose 15, BDH Chemicals Ltd., Poole, England) with 50 mL phosphate-buffered saline, pH 7.4 (PBS), in a beaker. After the mixture was heated and became transparent and homogeneous, an aliquot of a 1.9-mL solution was transferred to a 2-mL vial, which contained a 100 μL PBS aqueous solution with a certain amount of pesticides or drugs. The 2-mL vial was then capped and vortexed. Finally, the mixture was cooled to ambient temperature, yielding a semisolid gel. These agarose gels were prepared with pesticide or drug concentrations of 0, 50, 100, 250, and 500 ng/mL. The

agarose gels were used for further experiments after two or three hours of stabilization. All of the experiments using the gel medium were performed under a static mode.

A model 100 digital syringe pump from KD Scientific (VWR, Mississauga, ON, Canada) was employed to deliver the perfusate with the flow rate of 2.0 or 4.0 μL/min at the room temperature. A concentric type probe with 10 mm membrane (BioAnalytical Systems, IN, USA) was directly inserted in gel medium in 2 mL vial. PBS buffer was employed as the perfusate with a sampling time of 20 min. Each dialysate solution was collected in each 200 μL insert positioned in a 2-mL vial. Promecarb and lorazepam methanol solutions were used as LC-MS/MS internal standard solutions for pesticide and drug dialysates, respectively, to calibrate for sample loss during instrumental analysis. The same volume of promecarb or lorazepam methanol solution (100 ng/mL) as the dialysate solution was added to each dialysate solution. After the mixture was vortexed, 5 μL of each solution was automatically injected to LC-MS/MS for quantitative analysis.

2.2.4 *In Vivo* Application

Jade plants were exposed to pesticides via soil-application and then employed for *in vivo* sampling. 0.1 g of each pesticide (Chem Service, West Chester, PA, USA) was weighed and mixed. The mixed fine particles were placed on the surface of the soil. Water was then sprayed to allow the solid pesticides to gradually dissolve and diffuse into the soil. Around 15-mL water was applied to each plant every five days. 122 days after pesticide soil-application, the residues of pesticides in the center of leaves were sampled. The sampling setup is shown in Figure 2.1. Two syringe pumps were employed to deliver the perfusate with the flow rates of 2.0 and 4.0 μL/min at room temperature. As

soon as two holes were drawn using two 18 gauge hypodermic needles in the center of leaves, two concentric type probes were directly inserted into the pairs of leaves. PBS buffer was used as the perfusate with a sampling time of 20 min. Each dialysate solution was collected in 200 μL insert positioned in a 2-mL vial. In the final step, 40 and 80 μL of 100 ng/mL promecarb methanol solutions were added to the dialysate solutions obtained from 2.0 and 4.0 μL/min sampling experiments, respectively. After the mixture was vortexed, 5 μL of each final solution was automatically injected to LC-MS/MS for quantitative analysis.

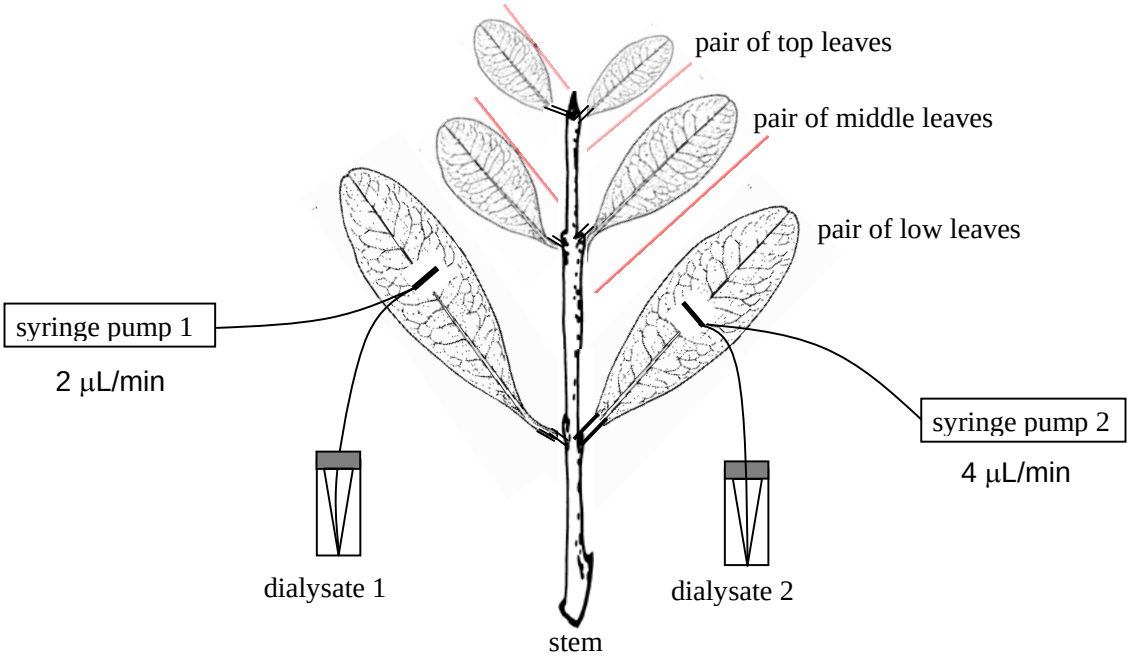


Figure 2.1 Schematic of kinetic microdialysis sampling in the pairs of leaves. Two flow rates, 2.0 and 4.0 μL/min, were employed to deliver the perfusate to pass through the center of leaves.

2. 3 Results and Discussion

2.3.1 Optimization of LC-MS/MS

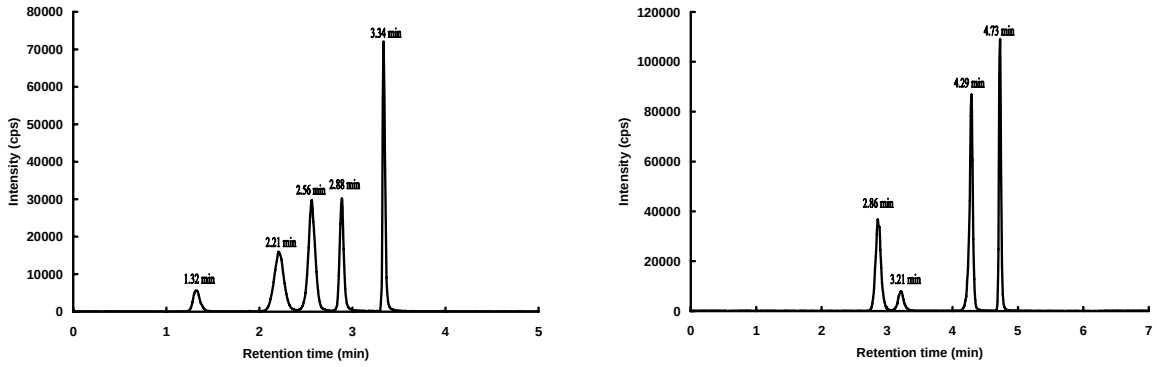
The exact composition of a sample from a living system or *in vivo* sampling is unknown and may be very complex. Multiple reaction monitoring (MRM) was used for *in vivo* study due to its high selectivity, sensitivity and reproducibility as well as good performance on applications and sample types. Compound-dependent mass spectrometry parameters were investigated by direct infusion of individual analyte into the electrospray source. The optimized MS/MS transitions and parameters for detection and quantitation of each analyte are provided in Table 2.1. Once suitable MS/MS transitions were identified for each analyte, a mixture of reference standards was employed to optimize chromatographic parameters. The chromatographic parameters were chosen in such way to reduce total LC run time and to obtain highly efficient separation minimize potential electrospray ionization competition and suppression. The mixture of water and acetonitrile plus acetic acid (0.1% v/v) was used because this mixture offers lower viscosity and back pump pressure as well as better mass transfer between solvent and stationary phase compared to that of water and methanol. Carbamate pesticides and benzodiazepines belong to two different types of chemicals. The chemical properties are quit different as well. Carbamate pesticides are less lipophilic than benzodiazepines. Selecting an appropriate stationary phase can also help to improve the efficiency of method development.[4] Thus, a C4 and C18 LC columns were employed for the separation of carbamate pesticides and benzodiazepines, respectively. A linear gradient consisting of water and acetonitrile plus acetic acid (0.1% v/v) resulted in an excellent separation for pesticides in 5 min (A) and for benzodiazepines in 7 min (B), which are

observed in Figure 2.2. All of the analytes plus the internal standard were eluted as single peaks. Excellent reproducibility was observed for each retention time between each run.

Table 2.1 Optimal parameters for the MRM detection in LC-ESI-MS/MS analysis*

compound	m/z for MRM detection		Ionization and collision parameters (V)			
	Q1	Q3	DP	EP	CE	CXP
Aldicarb	116.2	89.0	47.3	9.0	13.9	2.2
Carbofuran	222.2	123.1	36.1	8.0	29.9	1.9
Propoxur	210.1	111.2	31.4	8.0	19.3	2.0
Carbaryl	202.2	145.0	32.0	9.0	21.9	2.0
Promecarb	208.2	109.2	49.0	3.0	21.4	2.3
Diazepam	285.1	154.1	60.0	5.0	37.0	3.0
Oxazepam	287.2	241.2	50.0	5.0	30.0	3.0
Nordiazepam	271.1	140.0	60.0	5.0	40.0	2.0
Lorazepam	321.1	275.1	69.7	5.9	27.0	4.8

* abbreviations: Q1, quadrupole 1; Q3, quadrupole 3; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential



(A) (B)

Figure 2.2 LC-MS/MS total ion chromatogram resulting from pesticides (A) and drugs (B). Peak identifications are as follows: (A) aldicarb (1.32 min), propoxur (2.21 min), carbofuran (2.56 min), carbaryl (2.88 min), and promecarb (3.34 min), and (B) oxazepam (2.86 min), lorazepam (3.21 min), nordiazepam (4.29 min), and diazepam (4.73 min). The injected amount for each compound was 0.25 ng.

2.3.2 Gel Medium as Mimic System

An agarose gel spiked with pesticides was chosen as a model system for *in vitro* study based on the following reasons. A certain concentration of a gel prevents the liquid convection but does not obstruct free diffusion significantly.[8] Although an agarose gel is different from a living tissue, *in vitro* experiments can be very useful to find mass transfer phenomena in both MD membrane and a semisolid matrix. Such experiments may also yield relevant information for *in vivo* study. Table 2.2 shows excellent linearity obtained when sampling from agarose gel medium as well as the data of relative recoveries using two pairs of flow rates. Figure 2.3 is one of examples showing excellent linearity.

Table 2.2 Slopes and the relative recoveries using different MD flow rates*

Flow rate (μL/min)	2.0	4.0	2.0	4.0
	$Slope_1$ (R^2)	$Slope_2$ (R^2)	$\%R_{r1}$	$\%R_{r2}$
Aldicarb	0.0028 (0.998)	0.0016 (0.998)	21.54	12.31
Carbofuran	0.0099 (0.999)	0.0060 (0.996)	18.44	11.17
Propoxur	0.0093 (0.999)	0.0060 (0.997)	18.13	11.70
Carbaryl	0.0062 (0.996)	0.0034 (0.999)	18.24	10.00
Oxazepam	0.0066 (0.991)	0.0056 (0.991)	22.55	19.15
Diazepam	0.0047 (0.991)	0.0040 (0.991)	23.26	19.52
Nordiazepam	0.0050 (0.993)	0.0042 (0.993)	23.62	19.49

* Four data points were used to obtain each slope.

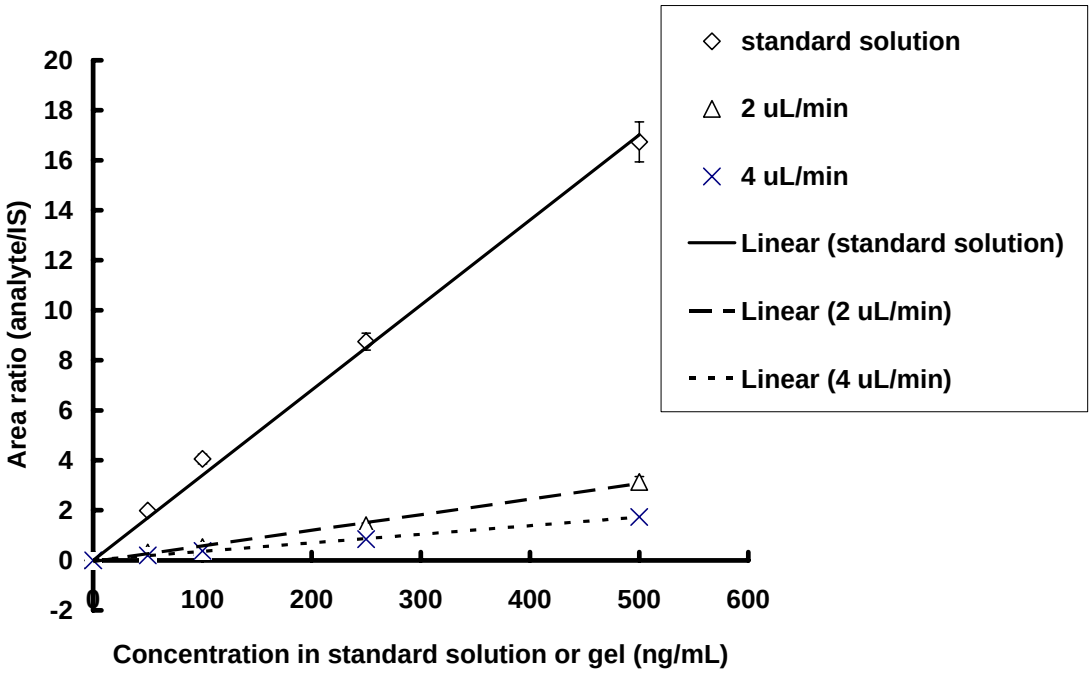


Figure 2.3 Carbaryl calibration curve and extraction curves. ◇, the calibration curve that was obtained from standard solutions. Δ and ×, extraction calibration curves that were obtained from 2.0 and 4.0 μL/min, respectively, in agarose gel (1%, w/v) with a static mode and 25 ˚C.

2.3.3 Development of Kinetic Microdialysis

Kinetic microdialysis employed two identical microdialysis probes that were simultaneously inserted into the symmetric parts of sampling system with different flow rates. Different dialysates were then collected from each probe. Empirically, it should be possible to calculate the initial analyte concentration in the sample matrix from the concentrations of two dialysates. Based on the large experimental data, the empirical equation is proposed to be the following,

$$C_0^{'} = \frac{5kC_1^2}{2C_1 - C_2} \qquad \text{Equation 2.2}$$

where $C_0^{'}$ is the calculated initial analyte concentration, C_1 and C_2 are the concentrations of dialysate 1 and dialysate 2, respectively, and *k* is the correction factor, which is related to the properties of the molecule as well as the pair of the flow rates that are used in the experiments. Eq. 2.2 is applicable under the condition that the flow rate to get dialysate 1 is 2-fold higher than that to obtain dialysate 2.

By re-arranging Eq. 2.2 and changing $C_0^{'}$ to C_0, correction factor, *k*, can be obtained from the equation,

$$k = \frac{C_0}{\frac{5C_1^2}{2C_1 - C_2}} \qquad \text{Equation 2.3}$$

where C_0 is the spiked concentration in sample matrix (real or theoretical concentration).

From Eq. 2.3, it is clear that correction factor, *k*, can be obtained from *in vitro* experiments. The correction factor obtained from *in vitro* experiments should be directly applicable to *in vivo* situation because the sample matrices used for both *in vitro* and *in vivo* experiments are semi-solid. Table 2.3 lists the empirical value of *k* from *in vitro* experiments in gel (1%, w/v) with different pairs of flow rates. Several factors that might influence *k* value were examined including MD flow rates, analyte hydrophobicity and mass transfer coefficients.

Table 2.3 Correction factor (*k*) and octanol-water partition coefficient (log P)

Compound	k [a]	k [b]	log P
Aldicarb	1.37 ± 0.11	0.90 ± 0.08	1.00
Carbofuran	1.52 ± 0.08	1.01 ± 0.06	1.94
Propoxur	1.52 ± 0.06	1.01 ± 0.06	2.15
Carbaryl	1.60 ± 0.05	1.00 ± 0.06	2.41
Oxazepam	0.99 ± 0.07	N/P	2.24
Diazepam	1.53 ± 0.09	N/P	2.80
Nordiazepam	1.40 ± 0.07	N/P	N/A

[a] *k* was obtained from the pair of flow rates, 2.0 and 4.0 μL/min.

[b] *k* was obtained from the pair of flow rates, 1.0 and 2.0 μL/min.

[c] Four data points were used to calculated each correction factor.

N/P: experiments were not performed.

N/A: not found

First factor was the pairs of flow rates that were found to slightly influence *k* value. The values of *k* that obtained from the pair of flow rates, 2 and 4 μL/min is between 1.0 and 1.6 for the selected pesticides and drugs. For the pair of 1 and 2 μL/min, *k* is very close to 1. It suggests that the pair of flow rates slightly influence the *k* value. Also, the pair of higher flow rates (2 and 4 μL/min) exhibited a little larger *k* than that of lower flow rates (1 and 2 μL/min). This indicates that the pair with higher flow rates gives a little higher *k* value. In addition, the pair of flow rates, 2 and 4 μL/min, exhibited a better correlation efficient than that of 1 and 2 μL/min. This may be caused by the

larger flow rate deviation from the used syringe pump when a low flow rate was delivered. Therefore, the pair of flow rates, 2 and 4 μL/min, was used for further investigation.

Second factor influencing *k* values was the molecular properties such as hydrophobicity. Table 2.3 lists the logarithm of octanol-water partition coefficient (log P) as well.[9-11] It is also observed that k varies in terms of different log P. Generally speaking, *k* increases with increasing log P for the same group of chemicals. Although the selected chemicals have various log P values, *k* still remains close for pesticides and clinic drugs. It also indicates that Eq. 2.2 is generally applicable.

Third factor was the mass transfer coefficient, or whether the mass transfer coefficient influences the correlation between the real and calculated concentrations obtained by the empirical equation, Eq. 2.2. The relation between the dialysate concentration and flow rate can be described by the following exponential function,[12, 13]

$$\frac{C}{C_0} = 1 - \exp(-rA/F) \qquad \text{Equation 2.4}$$

where r is the mass transfer coefficient, A is the cross area of the membrane, and F specifies the chosen flow rate. Eq. 2.4 can be rearranged to Eq. 2.5,

$$rA = -F \ln(1 - \frac{C}{C_0}) \qquad \text{Equation 2.5}$$

Here, $\frac{C}{C_0}$ can be replaced by relative recovery. [11] So Eq. 2.5 leads to Eq. 2.6

$$rA = -F \ln(1 - R_r) \qquad \text{Equation 2.6}$$

Eq. 2.6 can be employed to obtain the product of mass transfer coefficient and the cross area of the membrane, which is listed in Table 2.4. The cross area of the membrane,

A, can be assumed to be constant during microdialysis sampling. It is observed that the mass transfer coefficient, *r*, increases with the increasing flow rate, which is in agreement with literature reports.[12, 13] Obviously, the change of the mass transfer coefficient caused by different flow rates can not be negligible. However, correction factor does not change a lot considering different pairs of flow rates according to the data in Table 2.3. It suggests that the mass transfer coefficient should slightly influence *k* or the prediction of the concentration in the sample matrix by using the proposed empirical equation. Likely, Eq. 2.2 could compensate the variation of mass transfer coefficient factor.

Table 2.4 Products of the mass transfer coefficient and the cross area of the membrane [a]

rA	Flow rate (μL/min)		
	1.0	2.0	4.0
Aldicarb	0.32 ± 0.03	0.26 ± 0.01	0.53 ± 0.02
Carbofuran	0.25 ± 0.01	0.41 ± 0.02	0.47 ± 0.02
Propoxur	0.25 ± 0.03	0.40 ± 0.02	0.50 ± 0.02
Carbaryl	0.14 ± 0.01	0.40 ± 0.01	0.42 ± 0.02
Oxazepam	N/P	0.51 ± 0.03	0.85 ± 0.04
Diazepam	N/P	0.53 ± 0.03	0.87 ± 0.03
Nordazepam	N/P	0.54 ± 0.03	0.87 ± 0.02

[a] Four data points were used to obtain each product of mass transfer coefficient and the cross area of the membrane.

N/P: experiments were not performed.

The factors mentioned above influenced the relative recoveries. The relative recoveries are shown in Table 2.2. In spite of different recoveries, Eq. 2.2 offers good correlation between calculated and real (or spiked) concentrations, which suggests that this empirical equation has a general application. Table 2.5 shows the excellent correlation when the pair of flow rates, 2.0 and 4.0 μL/min, were used. Figure 2.4 is one of examples showing good correlation. Linearity is good with the correlation coefficient (R^2 > 0.998). The slope is very close to 1, which suggests that the calculated concentration is close to the real concentration.

Table 2.5 Correlation between calculated and real (or spiked) concentrations in the gel (1%, w/v) *

	Correlation	Correlation coefficient, R^2
Aldicarb	y = 1.129x	0.998
Carbofuran	y = 1.002x	0.998
Propoxur	y = 1.009x	0.998
Carbaryl	y = 1.005x	0.998
Oxazepam	y = 0.972x	0.999
Diazepam	y = 1.058x	0.999
Nordazepam	y = 1.025x	0.999

* Four data points were used to produce each curve and slope, intercept was set to be 0.

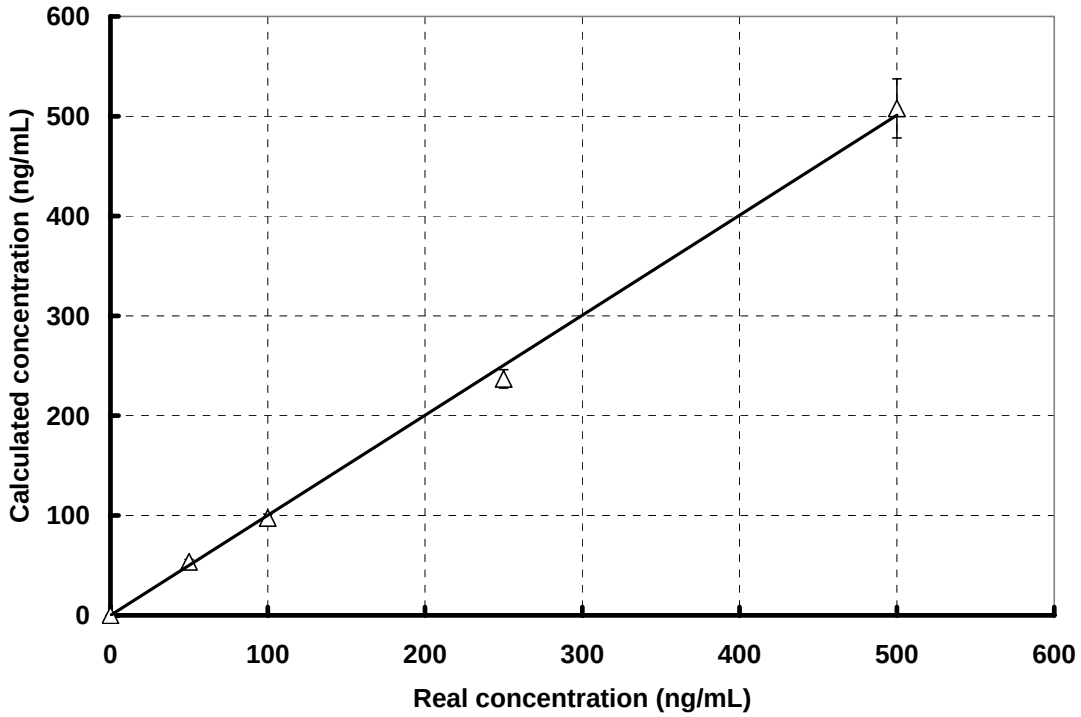


Figure 2.4 Correlation between calculated and real (or spiked) concentrations for carbofuran. Spiked concentrations were known through sample preparation. Calculated concentrations were obtained by Eq. 2.2. Experiments were performed in gel (1%, w/v) with the pair of flow rates, 2 and 4 μL/min. The carbofuran concentration in the dialysates was measured by LC-MS/MS.

2.3.4 Application for *In Vivo* Sampling

As mentioned above, since the change of relative recoveries did not influence the performance of the empirical equation, it indicates that Eq. 2.2 can be applied for *in vivo* sampling. Pesticide allocation in the different leaves of a jade plant was obtained by kinetic microdialysis and calculated by the empirical equation, Eq. 2.2. A close amount of analytes were extracted when the pairs of left and right leaves were sampled at the

same flow rate using MD. Thus, the sampling design shown in Figure 2.1 was used for *in vivo* sampling. Table 2.6 lists the concentrations of pesticides determined in the center of leaves. Compared to lower level leaves, the upper level leaves had lower concentrations of pesticides. This indicates that the concentration in the leaf reflected the procedure of time accumulation. The lower level leaves grew first, so they tended to accumulate more pesticides. Also, the concentration of propoxur was found to be the highest compared to carbofuran and carbaryl. Aldicarb was not detected. This might be caused by selective uptake effects among the four systemic pesticides that were used. Aldicarb is the least lipophilic one of the four tested, so it may not be as easy as others to partition into the hydrophobic cutin. Another reason may be that aldicarb was more likely to leak out from soil when water was applied to soil. Even 122 days (around 17 weeks) following the direct systemic pesticide application to the soil, most of systemic pesticides still provided residual activity, especially for propoxur and carbofuran. 122 days was chosen because a long-term pesticide residual activity is an interesting topic. In other reports, up to 12 weeks (84 days) of residual activity has been reported.[14] The ability to observe residual activity for longer time periods than previously reported may be due to the better sensitivity of the proposed method. Limits of detection (LODs) and limits of quantitation (LOQs) were obtained using microdialysis by performing ten times experiments in the leaf pair of a jade plant that was not exposed to pesticides. Limits of detection (LODs) and limits of quantitation (LOQs) were obtained using microdialysis to perform ten times experiments in the leaf pair of a jade plant without any pesticide application. Table 2.7 presents the data of LODs and LOQs for four pesticides. LOD was calculated as three times the standard deviation of the blank, and LOQ was equal to ten times the standard

deviation of repetitive measurements on a blank. The leaves used in this study were newly grown. The pesticide concentrations in these leaves were different from those older one during the early period of pesticide soil-application. This finding should be useful for study of pesticide action mode and plant physiology as well as evaluation of plants for decontamination of pesticide-contaminated environments.[2, 3]

Table 2.6 Pesticide allocation in different leaves after 122-day pesticide soil-application.

Branch	leaf	Concentration (ng/mL)			
		Aldicarb	Carbofuran	Propoxur	Carbaryl
Branch 1	top	N/A	398.53	3165.25	24.33
	middle	N/A	698.92	4795.87	36.99
	low	N/A	1073.81	7235.73	103.35
Branch 2	top	N/A	53.39	691.21	N/A
	low	N/A	129.13	1510.27	N/A
Branch 3	top	N/A	144.84	1505.76	N/A
	low	N/A	162.88	2069.94	N/A

N/A: detected concentrations were lower than LOD

Table 2.7 Limits of detection (LODs), and limits of quantitation (LOQs) for *in vivo* sampling using kinetic microdialysis.

compound	LOD (ng/mL)	LOQ (ng/mL)
Carbofuran	5	15
Propoxur	20	54
Carbaryl	10	49
Aldicarb	8	26

Compared to the reported calibration methods, kinetic microdialysis offers several advantages. First, this method is simple from both experimental and data processing perspective. Only physiological fluid is used for delivery from the syringe, which simplifies experimental procedures. For the method of no-net-flux or dynamic no-net-flux, different levels of target analytes have to be spiked to the perfusate, which make sample preparation more tedious. When retrodialysis is used, an internal standard, the radiolabeled analyte or calibrator, has to be found. Radiolabled compounds are typically expensive or unavailable. Also, a calibrator might not be easy to find because the target analyte and the calibrator have to exhibit a similarity in their physical properties. Second, this method is fast because a relatively high flow rate can be used for kinetic microdialysis, thus allowing the sampling time to be short. This also results in good temporal resolution. For the method of flow rate variation, a long sampling time is needed due to small flow rates. Third, this approach is robust. This can be observed by correction factor that exhibits little variation even for different analytes with different

properties. Finally, kinetic microdialysis shows a good accuracy, as shown by the excellent correlation between calculated and real concentrations. However, microdialysis exhibits an inherent disadvantage. Pumps and other appliances are required, which makes microdialysis more suitable for laboratory use than for field sampling. Another *in vivo* sampling approach, solid-phase microextraction, is much simpler and more suitable for on-site and *in vivo* sampling.[15-18]

2.4 Conclusion and Addendum

2.4.1 Conclusion. Although microdialysis has been used widely for *in vivo* sampling, the current calibration methods exhibit some limitations. This issue was fully addressed by the novel calibration method, kinetic microdialysis, in this study. This technique employed two different flow rates with two identical microdialysis probes, which were simultaneously inserted into the symmetric parts of sampling system. The empirical equation was proposed to calculate the analyte concentrations in the sample matrix using the values of two different dialysate concentrations. An excellent correlation was observed between the calculated and real values. This method was successfully applied for *in vivo* sampling, measurement of the pesticide allocation in the different leaves of a jade plant (*Crassula ovata*). This approach should also be applicable to other applications such as pharmacokinetic study in animals as long as the symmetric parts can be found and have the same or close concentrations of the target analytes. Compared to the other reported microdialysis calibration methods, this new method offers several advantages including simplicity, speed, robustness, and increased accuracy.

2.4.2 Addendum. N/A

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Chapter 3

Kinetic Calibration for Solid-Coated Solid-Phase Microextraction

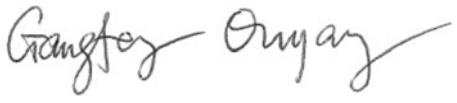
3.1 Preamble and Introduction

3.1.1 Preamble. This chapter has been published as a part of the paper,

Zhou, S. N. S.; Zhang, X.; Ouyang, G.; Es-Haghi, A.; Pawliszyn, J. On-Fiber Standardization Technique for Solid-Coated Solid-Phase Microextraction, *Analytical Chemistry* **2007**, *79*, 1221-1230.

The contributions of Gangfeng Ouyang, the co-author, involved experimental suggestions and manuscript revision. The drug analysis part (in the paper) generated by X. Zhang and A. Es-Haghi have not been written into this chapter, so it is considered that their permissions are not required. Tables and Figures are reprinted with permission from Analytical Chemistry (Copyright 2007 American Chemical Society).

I, Gangfeng Ouyang, authorize Simon Ningsun Zhou to use the material for his thesis.

Signature: 

3.1.2 Introduction. Solid-phase microextraction (SPME) is a valuable sampling technology that is fast, simple, solvent-free, and combines sampling, sample preparation and pre-concentration to the extraction phase into one single step.[1] SPME has been broadly applied in many fields including environmental monitoring, industrial hygiene, process monitoring, clinic, forensic, food, flavor, fragrance and drug analyses, in laboratory and on-site analyses.[2] Currently, two types of SPME coatings, liquid- and solid-phase, have been employed. Polydimethylsiloxane (PDMS) and polyacrylate (PA) belong to liquid coatings. PDMS has been widely employed for the determination of volatile organic compounds (VOCs) such as benzene, toluene, ethylbenzene and xylene (BTEX), polycyclic aromatic hydrocarbons (PAHs), odor and flavor. Both PDMS and PA absorb during the procedure of extraction.[4] A liquid coating has the advantage to analyze higher concentration ranges without a displacement effect and is normally integrated with gas chromatography. In other case, when a solid-coating such as divinylbenzene (DVB), Carboxen (CAR; a carbon molecular sieve), and Carbowax (CW; polyethylene glycol) or in different coating combinations, blends or copolymers, is utilized, an adsorption mechanism is frequently observed.[3]

Based on a diffusion-controlled mass transfer process, a dynamic SPME model was proposed.[4, 5] According to this model, isotropic behavior between absorption and desorption in the SPME liquid coating fiber was demonstrated and a new calibration method, kinetic calibration, was developed.[6, 7] This kinetic calibration method is also referred as the in-fiber standardization technique.[8] This approach utilizes the desorption of the internal standards, which are pre-loaded in the extraction phase, to calibrate the extraction of the analytes in the sample matrix. This kinetic calibration method was

successfully investigated for both in-vial SPME[8] and LPME.[9, 10] In addition, based on this kinetic calibration technique for field time-weighted average (TWA) water sampling, two passive samplers, the SPME PDMS-rod and the SPME PDMS-membrane, were estimated.[11, 12] More recently, this technique was further applied for pharmacokinetic study to directly determine the concentrations of pharmaceutical drugs and metabolites in blood.[13]

Although kinetic calibration has been investigated using liquid-coated SPME fibers, a systematic evaluation of this technique with a solid-coated fiber has not been performed to date. Moreover, the sensitivity of a solid-coated SPME fiber was found to be much higher than a PDMS coating when polar compounds such as pesticides[14] and VOCs were analyzed.[2, 3]

In this chapter, we examined the possibility of kinetic calibration for a solid-coated fiber. The kinetic processes between adsorption and desorption were investigated using one solid-coated fiber. The isotropic behavior was demonstrated in different sample matrices for two carbamate pesticides, carbofuran and carbaryl. For the purpose of the fast and accurate analysis of various interested compounds, the developed approach was applied to river water and wine samples, which corrects the matrix effects and limits the displacement effects as a pre-equilibrium approach.

3.2 Theoretical Considerations

3.2.1 Langmuir Model. The process of adsorption and desorption with a solid porous coating SPME fiber can be well described using Langmuir model. An assumption can be

made that a monolayer of the adsorbate can be formed on the surface. The following equation can be used to calculate the concentration of analyte adsorbed by the fiber:[3]

$$C_{fA}^{\infty} = \frac{C_{f\max} K C_{sA}^{\infty}}{1 + K C_{sA}^{\infty}} \qquad \text{Equation 3.1}$$

where C_{fA}^{∞} is the equilibrium concentration of analyte on the fiber surface in mol cm^{-2}, C_{fmax} is the maximum active site concentration on the surface in mol cm^{-2}, K is the equilibrium constant of adsorption in cm^2 mol^{-1}, and C_{sA}^{∞} is the analyte equilibrium concentration in the solution in mol cm^{-3}. By rearrangement of eq 3.1, eq 3.2 can be obtained,[15]

$$\frac{1}{n_{fA}^{\infty}} = \frac{1}{n_{f\max}} + \frac{1}{n_{f\max} K C_{sA}^{\infty}} \qquad \text{Equation 3.2}$$

where n_{fA}^{∞} is the equilibrium amount of analyte adsorbed on the fiber, and $n_{f\max}$ is the maximum amount of the analyte adsorbing on the active sites of the fiber, which reflects the maximum amount of active sites by assuming a 1:1 ratio of active sties to adsorbed analyte. From eq 3.2, a plot of $\frac{1}{n_{fA}^{\infty}}$ versus $\frac{1}{C_{sA}^{\infty}}$ produces a straight line with a slope of $\frac{1}{n_{f\max} K}$ and a y-intercept of $\frac{1}{n_{f\max}}$. Therefore, the values of $n_{f\max}$ and K can be obtained from the linear regression equation. This approach is named as a "Reciprocal Langmuir Analysis".[15] C_{sA}^{∞} is equal to the initial concentration of analyte (C_0) minus $\frac{n_{fA}^{\infty}}{V_s}$. Thus, eq 3.2 can be further rewritten to eq 3.3,

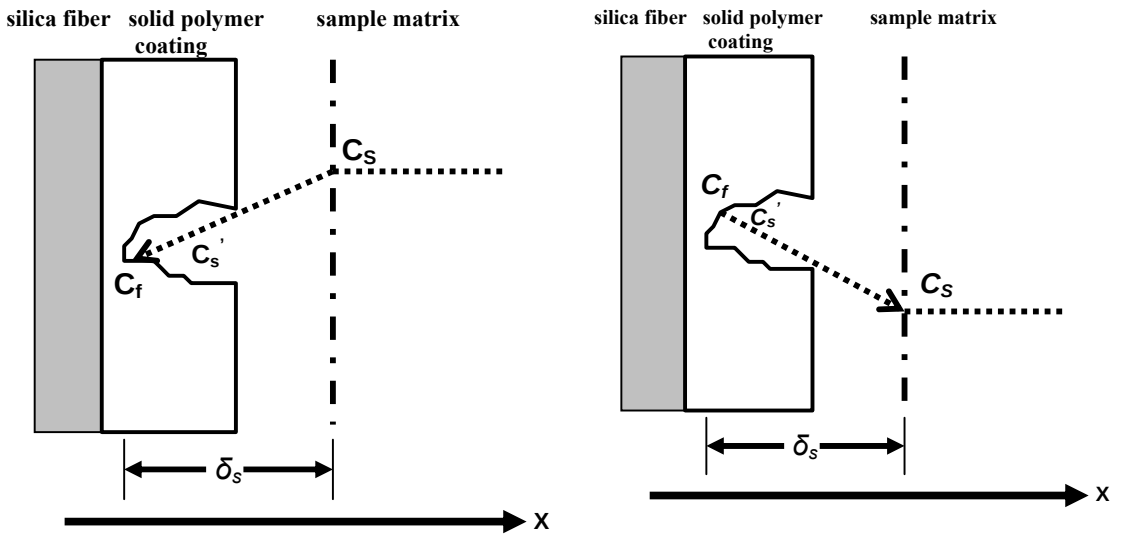
$$C_0 = \frac{n_{fA}^{\infty}}{K(n_{f\max} - n_{fA}^{\infty})} + \frac{n_{fA}^{\infty}}{V_s} \qquad \text{Equation 3.3}$$

where V_s is the volume of the sample matrix. When $C_0 >> \frac{n_{fA}^{\infty}}{V_s}$, which is true for on-site or *in vivo* sampling, eq 3.3 can be made simpler,

$$C_0 = \frac{n_{fA}^{\infty}}{K(n_{f\max} - n_{fA}^{\infty})} \qquad \text{Equation 3.4}$$

The initial analyte concentration in a solution can be calculated from n_{fA}^{∞}, the extracted equilibrium amounts of the target analyte., n_e is utilized to replace n_{fA}^{∞} in the following context to simplify the symbol.

3.2.2 Kinetic Adsorption. When a SPME porous solid-coated fiber is directly immersed to an agitated aqueous sample matrix, the adsorption of the analytes from the sample to the fiber porous surface occurs due to high affinity of the fiber coating material to the analytes, as shown in Figure 3.1 A.



(A) (B)

Figure 3.1 Schematic of the adsorption and desorption processes between the porous surface of SPME fiber and the aqueous solution. A linear concentration gradient is assumed in both the pore and the boundary layer when the aqueous solution is agitated constantly. (A) is the adsorption process and (B) is the desorption process. C_s **is concentration of the analyte in the sample matrix,** C_s' **is the concentration of the analyte in the boundary layer at the interface of the fiber coating and the boundary layer,** C_f **is the concentration of the analyte on the surface of the fiber coating, and** δ_s **is the thickness of the boundary layer**

The rate-determining step is considered to be the mass transfer of the analytes based on diffusion through the boundary layer.[16] Therefore, Fick's first law of diffusion can be used to express this process at the region of the sample matrix/SPME coating interface,[5]

$$J \equiv \frac{1}{A}\frac{\partial n}{\partial t} = -D_s \frac{\partial C_s}{\partial x} \qquad \text{Equation 3.5}$$

where J is the analyte mass flux from the sample matrix to the fiber coating, A is the fiber surface area in cm^{-2}, ∂n is the analyte amount adsorbed to the fiber surface during the period of time ∂t, D_s is the analyte diffusion coefficient in the sample matrix, and C_s is analyte concentration in the sample matrix. When agitation is applied well in the sample matrix, a steady-state mass transfer can be established. Thus, it is assumed that there is a linear concentration gradient in the boundary layer ,

$$\frac{1}{A}\frac{\partial n}{\partial t} = -D_s \frac{\partial C_s}{\partial x} = \frac{D_s}{\delta_s}\left(C_s - C_s^{'}\right) \qquad \text{Equation 3.6}$$

where δ_s is the boundary layer thickness, and C_s' is the analyte concentration in the boundary layer at the interface of the fiber coating and the boundary layer. The analyte mass transfer coefficient in the fiber coating, h_s, can be defined as D_s/δ_s, a constant for a steady-state diffusion process in an effectively agitated sample matrix. Therefore, eq 3.6 can be expressed as:

$$\frac{1}{A}\frac{\partial n}{\partial t} = h_s\left(C_s - C_s^{'}\right) \qquad \text{Equation 3.7}$$

Langmuir model can be described for this process. There is a quick equilibrium for the analyte between the sample matrix and the coating surface at the interface of the fiber coating and the boundary layer,[1]

$$K = \frac{C_f}{SC_s^{'}} \Rightarrow C_s^{'} = \frac{C_f}{KS} \qquad \text{Equation 3.8}$$

where K is the analyte equilibrium constant between the coating surface and the sample matrix, C_f is the analyte concentration on the surface of the fiber coating in mol cm^{-2}, and S is the unoccupied site concentration on the surface of the sorbent in mol cm^{-2}. When the limited sites are occupied, S is typically a constant. It is assumed that the SPME coating

has a uniform pore distribution and surface area throughout its bulk. Thus, the analyte concentration on the surface of the fiber coating, C_f, can be measured by eq 3.9,

$$C_f = \frac{n}{A} \qquad \text{Equation 3.9}$$

where n is the extracted analyte amount on the surface of the fiber coating in mol after the period of the exposure time t. Therefore, by combining eq 3.8 and eq 3.9, C_s' can be obtained:

$$C_s^{'} = \frac{n}{KSA} \qquad \text{Equation 3.10}$$

And in the sample matrix,

$$C_s = C_0 - \frac{n}{V_s} \qquad \text{Equation 3.11}$$

The insignificant amounts of analytes are typically extracted from the bulk sample solution to the fiber surface, i.e., $C_0 >> \frac{n}{V_s}$, especially for on-site and *in vivo* sample preparing. Therefore, it results in eq 3.12,

$$C_s = C_0 \qquad \text{Equation 3.12}$$

Substitution of eqs 3.10 and 3.12 into eq 3.7 leads to eq 3.13

$$\frac{1}{A}\frac{\partial n}{\partial t} = h_s\left(\mathrm{C}_0 - \frac{n}{KSA}\right) \qquad \text{Equation 3.13}$$

Let

$$a = \frac{h_s}{KS} \qquad \text{Equation 3.14}$$

Let

$$b = Ah_sC_0 \qquad \text{Equation 3.15}$$

Then eq 3.13 can be expressed as

$$n' + an = b \qquad \text{Equation 3.16}$$

At the initial condition: $t = 0$, $n = 0$, eq 3.16 can be solved,

$$n = (b/a)[1 - \exp(-at)] \qquad \text{Equation 3.17}$$

Eq 3.18 can be obtained by combining eqs 3.14 and 3.15,

$$b/a = AKSC_0 \qquad \text{Equation 3.18}$$

Since the analytes are usually insignificantly extracted from the bulk sample solution to fiber surface, the initial concentration, C_0, is typically close to the analyte concentration C_{sA}^{∞}, at equilibrium. Based on the Langmuir model, the concentration of analyte on the fiber at equilibrium is

$$C_{fA}^{\infty} = \frac{n_e}{A} = KSC_{sA}^{\infty} = KSC_0 \qquad \text{Equation 3.19}$$

It is noticed that n_e is equal to b / a from eqs 3.18 and 3.19. Eq 3.17 becomes eqs 3.20 and 3.21,

$$n = n_e[1 - \exp(-at)] \qquad \text{Equation 3.20}$$

$$\frac{n}{n_e} = 1 - \exp(-at) \qquad \text{Equation 3.21}$$

where t is the time of the fiber exposed to the sample, and a is the time constant. The time constant, defined by eq 3.14, is employed to illustrate how quickly the equilibrium can be reached.

3.2.3 Kinetic Desorption. Figure 3.1 B shows desorption of the analytes from the coating surface to the sample after a SPME porous fiber, pre-loaded with an analyte, is exposed to an agitated sample matrix. The desorption process can be considered as the reverse process of the adsorption. In a similar manner, the equations to describe the

desorption process can be derived based on the steady-state diffusion model. It can be briefly described by the following,

$$J \equiv \frac{1}{A}\frac{\partial q}{\partial t} = -h_s\left(C_s - C_s^{'}\right) \qquad \text{Equation 3.22}$$

where ∂q is the analyte amount desorbed from the fiber surface during the period of time ∂t. When the initial analyte amount preloaded on the coating surface is q_0, the remaining analyte concentration on the fiber surface after the exposure time t can be described as:

$$C_f = \frac{q_0 - q}{A} \qquad \text{Equation 3.23}$$

where q is the analyte amount desorbed from the coating surface into the sample matrix after the period of the exposure time, t. In the bulk of the sample matrix,

$$C_s = \frac{q}{V_s} \qquad \text{Equation 3.24}$$

where C_s is the analyte concentration in the sample solution after the period of time, t. Typically, the sample volume is very large, C_s is equal to zero, especially for on-site and in-vivo sampling.

It is assumed that there is a quick equilibrium for the analyte between the sample matrix and the coating surface At the interface of the fiber coating and the boundary layer,

$$K = \frac{C_f}{SC_s^{'}} \Rightarrow C_s^{'} = \frac{C_f}{KS} \qquad \text{Equation 3.25}$$

Combining eqs 3.23 and 3.25 results in,

$$C_s^{'} = \frac{C_f}{KS} = \frac{q_0 - q}{KSA} \qquad \text{Equation 3.26}$$

Substitution of eqs 3.24 and 3.26 into eq 3.22 gives the differential equations as,

$$q' + \frac{h_s}{KS} q = \frac{h_s}{KS} q_0 \qquad \text{Equation 3.27}$$

In a similar manner employed to derive eq 3.12 to eq 3.17, eq 3.27 can be arranged as eq 3.28,

$$q = q_0 [1 - \exp(-at)] \qquad \text{Equation 3.28}$$

where a is the time constant, which is previously defined in eq 3.14. Let $Q = q_0 - q$, and Q is the analyte amount remaining on the coating surface after the period of time, t. This leads to eq 3.29,

$$\frac{Q}{q_0} = \exp(-at) \qquad \text{Equation 3.29}$$

3.3 Experimental

3.3.1 Chemicals and Supplies. Carbofuran, deuterated carbofuran(carbofuran-d_3), carbaryl and promecarb were obtained from Riedel-de Haën, with greater than 99.5% purity. Carbaryl-$^{13}C_6$ was purchased from Cambridge Isotope Labs (Andover, MA, USA). Stock standard solutions (1000 μg/mL) of carbofuran, carbaryl and promecarb were made in acetonitrile. 0–4 °C in the dark was used as the storage condition for stock solutions. By diluting the primary standard solution in acetonitrile, a secondary standard solution (20 μg/mL) was made. Working solutions were made by spiking with different volumes of the secondary standard solution for quantitative calibration and sample preparation.

Prior to use, the fiber (Supelco, Oakville, ON, Canada), 50μm CW/TPR, was conditioned at room temperature in acetonitrile for 5 min in static mode and then in nanopure water for 30 min with 500 rpm agitation. A polyethylene insert with a volume

of 200 μL was mounted in a 2-mL vial for the purpose of small-volume analysis. The river water was got from the Laurel Creek (Waterloo, ON, Canada). A white wine (Jackson-triggs Vininers) was supplied from a local liquor store. The fine particles in the river water and white wine were removed using 0.2 μm Acrodisc® Syringe Filters (Ann Arbor, MI, USA).

3.3.2 Instrumental Analysis. The instrumental system included a Shimadzu gradient LC system with a model SCL 10 AVP system controller, two model LC 10 AVP dual piston pumps and a model DGU 14A on-line mobile phase degasser supplied from Mandel Scientific (Guelph, ON), a CTC analytics model HTS PAL autosampler from Leap Scientific (Carrboro, NC) with a 20 μL injection loop and a Sciex model API 3000 turbo ionspray tandem mass spectrometer (Toronto, ON). A Waters Symmetry Shield RP18, 2.1mm×50 mm column with 5 μm particle size was supplied from Waters Corporation (Milford, MA). Mobile phases included (A) acetonitrile/water (10:90) with 0.1% acetic acid and (B) acetonitrile/water (90:10) with 0.1% acetic acid. The total flow rate was 0.5 mL/min. A gradient with 10% B was applied for the first 0.5 min. This was ramped to 90% B over 4.0 min, held for 2.0 min, and finally returned to 10% B for 1.5 min. As a result, a total analytical run-time including reconditioning was only 7.0 minutes. A injection volume was 20 μL for all of the experimental samples. Each transition monitored was 200 ms. The monitored transitions were: carbofuran, mass to charge ratio (m/z) 222.2/123.1; carbofuran-d_3, m/z 225.2/123.1; carbaryl, m/z 202.2/145.0; carbaryl-$^{13}C_6$, m/z 208.2/151.0; and promecarb, m/z 208.2/109.2. By infusing a 100 μg/mL methanol solution at 15 μL/min using a model 100 digital syringe pump from kd

Scientific (VWR, Mississagua, Canada), compound specific mass spectrometer settings were monitored for each individual compound. Before and after each set of experimental samples, mass spectrometer response sensitivity and linearity were checked on a daily basis with the injection of 20 μL of a series of standards (1–200 ng/mL) prepared in acetonitrile:water (1:1) containing IS, promecarb. All components of the system and for data collection and analysis were controlled by Analyst version 1.4 software (Applied Biosystems). The time of chromatographic hold-up was around 20 s. After 2.5 min, the first analytical peak appeared. Figure 3.2 illustrates the chromatograms collected for each pesticide.

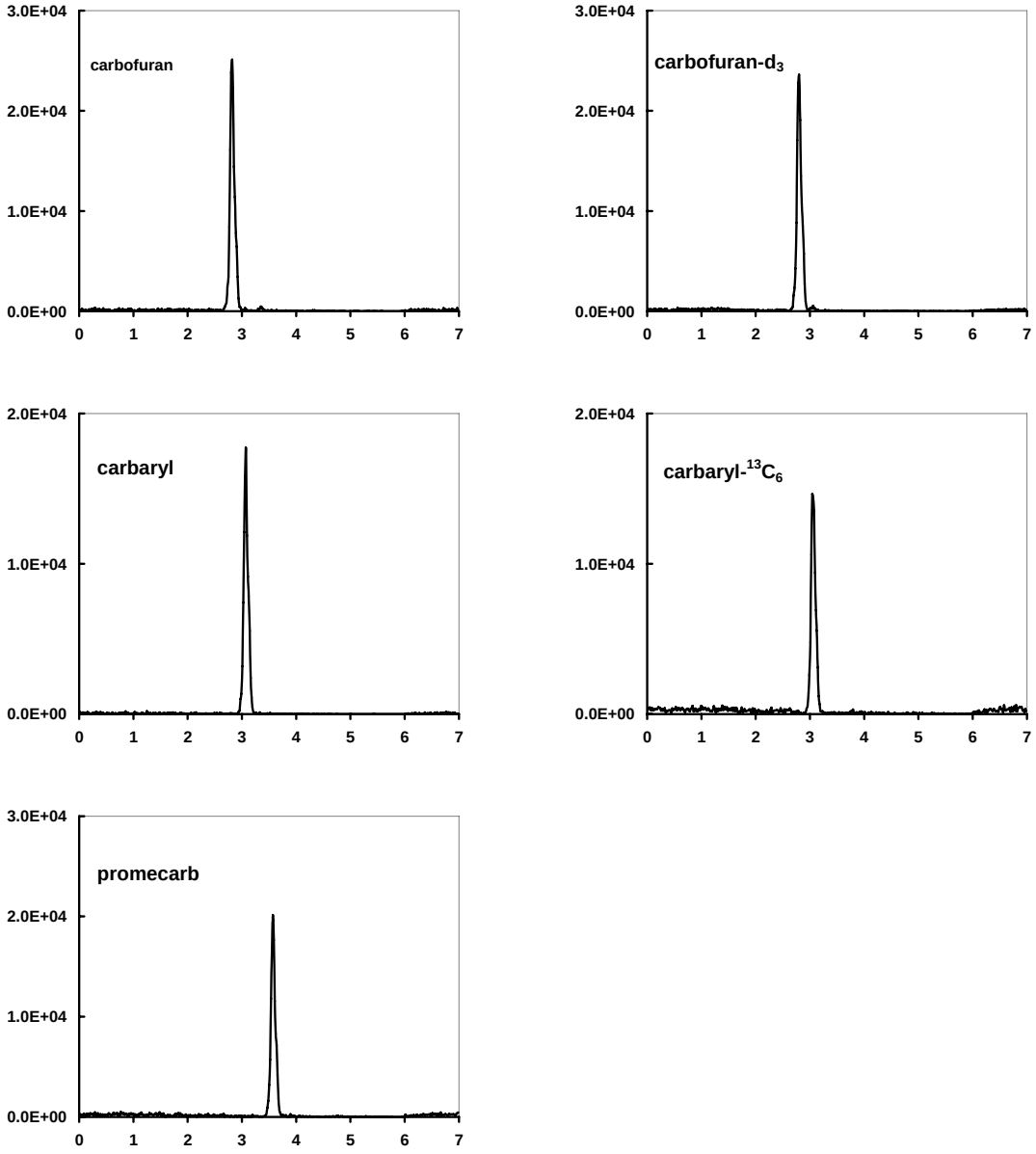


Figure 3.2 Chromatograms for selected reaction monitoring analysis of analytes (10 ng/mL each). X-Axis is retention time (minute), and Y-Axis is signal intensity (counts/s). Transitions monitored are as reported in the text.

3.3.3 Adsorption and Desorption. For the experiments with a 2 min pre-agitation period (at 500 rpm) and 500 rpm agitation for adsorption and desorption at 25 ºC, a CTC CombiPal autosampler (Zwingen, Switzerland) utilizing the associated Cycle Composer software (Version 1.4.0) was used.

The direct immersion mode for adsorption and desorption was used to all of the SPME experiments for pesticide analysis. Two kinetic methods were investigated. The first one is that the adsorption and desorption were achieved separately, and the second is that the processes were conducted simultaneously. In the case of the first method, the extraction experiment was conducted by adding a 75 μL acetonitrile solution containing 20 μg/mL, carbofuran and carbaryl to a 10-mL vial. Then, acetonitrile was evaporated in the fume hood. An aliquot of a 7.5 mL sample solution was transferred to the vial. The solution was vortexed and then left to homogenize for 30 min. Afterward, the vial was left in the CTC Combipal for SPME direct extraction for a specific period of time (1, 2, 3, 5, 10, 15 or 25 min). After each extraction, the analytes adsorbed on the CW/TPR fiber were desorbed in 75 μL acetonitrile in a 200 μL insert for 5 min under a static mode. Prior to next extraction, to remove any organic solvent residue on the fiber surface, the same fiber was conditioned at room temperature in nano-pure water for 10 min with an agitation of 500 rpm. 75 μL of 50 ng/mL promecarb aqueous solution, the internal standard for LC-MS/MS to compensate for the variation of injection volume, was transferred to the desorption solution in the 200 μL-insert. After the solution was mixed, a 20 μL aliquot of the final solution was injected into LC-MS/MS using the auto-sampler for quantitative analysis.

The pre-loading solution was made in 1% NaCl aqueous solution for the desorption experiment. The loading was conducted for 10 min at room temperature under agitation at 500 rpm. The fiber was directly immersed in a new solution with 1% NaCl for a specific period of time mentioned above, to perform desorption. The organic solvent desorption, internal standard addition, and LC-MS/MS injections were the same as mentioned above.

The second kinetic method was conducted by simultaneous adsorption and desorption. After carbofuran-d_3 and carbaryl-$^{13}C_6$ in an aqueous solution (containing 1% NaCl) were pre-loaded on the CW/TPR fiber for 10 min, the fiber was directly immersed into a new sample solution containing 200 μg/L of carbofuran and carbaryl under agitation at 500 rpm at room temperature for simultaneous adsorption and desorption.

3.4 Results and Discussion

3.4.1 Considerations of SPME Fibers. Carbofuran and carbaryl are the thermally labile compounds. It was observed that thermal decomposition occurred under normal GC/MS conditions, which agree well with in the literature reports.[17] A conclusion can be drawn that that LC was the best of choice for quantitative analysis. A 50 μm CW/TPR fiber manufactured exclusively for the purpose of HPLC was used for the determination of the target pesticides.

3.4.2 Internal Standards Loading. A 1% NaCl aqueous solution containing either carbofuran, carbaryl, carbofuran-d_3, or carbaryl-$^{13}C_6$ was employed for the standard pre-loading on the fiber. This way can wet the surface of the fiber with a layer of water prior

to desorption. Also, this wetting approach kept air bubble away from trapping in the pores of the fiber, which affects the fiber performance. This guaranteed the same conditions on the porous surface of the fibers prior to each extraction step. It also ensured the isotropic behavior for adsorption and desorption. Compared to a pure aqueous solution or higher percentage salt solutions, it was noticed that 1% NaCl aqueous solutions offered improved reproducibility between loadings. Moreover, those samples containing 1% NaCl exhibited good extraction and desorption profile. The same result was obtained in PBS buffer (pH 7.4) that contains around 1% salt. In addition, 10 min loading time for those analytes were sufficient.

Good reproducibility was also accomplished with the fully automated CTC Combipal autosampler. The whole experimental procedure included the internal standard pre-loading, adsorption and desorption processes. Previously, this strategy has been employed for automated headspace liquid-phase microextraction.[10]

3.4.3 Proof of the Isotropism. Two methods are utilized to prove the isotropic processs between adsorption and desorption. The first one is to find out the sum of n/n_e (the fraction of the analyte adsorbed into the fiber coating after a period of sampling time t) and Q/q_0 (the fraction of the standard remaining on in the fiber coating after a period of sampling time t). When the same experimental conditions were applied to the desorption and adsorption processes, the values of time constant a should be close or similar for same/similar compounds. According to eqs 3.21 and 3.29, the sum of n/n_e and Q/q_0 should be close to 1 at any desorption/absorption time. It can be described as,

$$\frac{n}{n_e} + \frac{Q}{q_0} = 1 \qquad \text{Equation 3.30}$$

It was observed that the sum of n/n_e and Q/q_0 was close to 1 for various sample matrices including nano-pure water, an aqueous solution containing 5% acetonitrile, PBS buffer (pH = 7.4), river water and white wine (CW/TPR). Good agreement was achieved between the experimental data and the theoretical prediction. One example of isotropic profiles is shown in Figure 3.3.

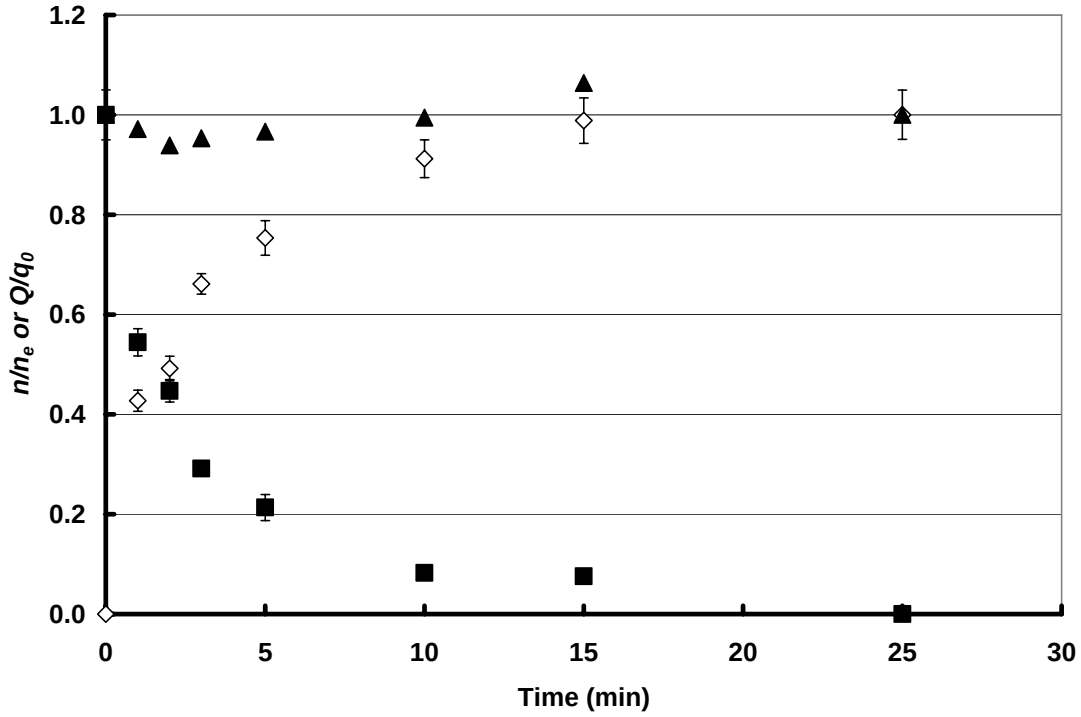


Figure 3.3 Carbaryl extraction (◆) and desorption (■) isotropic profile. The adsorption and desorption were performed in a filtered white wine containing 200 ng/mL of carbofuran and carbaryl at the agitation speed of 500 rpm and 25 ˚C. ▲, the sum of n/n_e and Q/q_0

The second method is to compare the value of time constant from adsorption with that from desorption in order to evaluate the isotropism. Eqs 3.21 and 3.29 can be re-arranged as eqs 3.31 and 3.32, respectively,

$$\ln\left(1-\frac{n}{n_e}\right)=-at \qquad \text{Equation 3.31}$$

$$\ln\frac{Q}{q_0}=-at \qquad \text{Equation 3.32}$$

Both time profiles of adsorption and desorption can be linearized.[7] It is expected to have the equal slopes that represents the values of time constants.

Table 3.1 presents some time constants of adsorption and desorption in different sample matrices as well as the achieved linearity. Although different matrices were applied, time constants between adsorption and desorption kept close within 5% relative standard deviation (RSD) observed in most cases, which further suggested the isotropic behavior between adsorption and desorption.

The rate of adsorption and desorption can be evaluated by the value of the time constant, a, as defined in eq 3.14. This equation represents an accurate definition for the time constant a for a porous solid coating.

$$a=\frac{h_s}{KS}=\frac{D_s}{\delta_s}\frac{1}{K}\frac{1}{S} \qquad \text{Equation 3.33}$$

According to eq 3.33, the value of time constant, a, is influenced by several factors, the analyte structure and properties, the sample matrix components, the properties and dimension of the fiber coating, agitation speed, and temperature. The agitation speed affects the thickness of the boundary layer, δ_s. Thus, it influences time constant. Moreover, there is not relationship between the time constant and the analyte

concentration. In addition, it was noticed that time constants kept close for either separated or simultaneous modes when the same sample matrix and fiber were utilized.

Table 3.1 Time constants *a* for adsorption and desorption of carbofuran and carbaryl using CW/TPR (seven data points were used to calculate each time constant)

Sample matrix	Time constant a (min^{-1}) (R^2)			
	Carbofuran		Carbaryl	
	Adsorption	Desorption	Adsorption	Desorption
Nano-pure water	0.350 ± 0.020 (0.966)	0.375 ± 0.013 (0.980)	0.180 ± 0.004 (0.974)	0.195 ± 0.002 (0.989)
Aqueous solution containing 5% acetonitrile	0.428 ± 0.021 (0.978)	0.413 ± 0.028 (0.964)	0.299 ± 0.009 (0.964)	0.293 ± 0.015 (0.966)
PBS buffer (pH = 7.4)	0.478 ± 0.019 (0.965)	0.480 ± 0.018 (0.962)	0.143 ± 0.002 (0.979)	0.146 ± 0.008 (0.988)
River water	0.590 ± 0.025 (0.985)	0.572 ± 0.043 (0.870)	0.303 ± 0.013 (0.993)	0.318 ± 0.016 (0.999)
White wine	0.660 ± 0.023 (0.968)	0.620 ± 0.012 (0.978)	0.228 ± 0.009 (0.972)	0.232 ± 0.019 (0.952)

When a solid-coated fiber is used, competition or displacement effect should be considered. Equilibrium extraction experiments demonstrated that the linear range was up to around 100 ng/mL of the pesticide concentration tested. A conclusion could be drawn that competition adsorptions occurred at higher concentration. Moreover, $n_{f\max}$ is the maximum amount of the analyte, which can be adsorbed on the active sites of the fiber or the active sites of the fiber for target analyte. $n_{f\max}$ varied in different sample

matrices, especially in white wine. It demonstrated competition effect. However, an isotropic behavior was observed for a 400 ng/mL of solution. The results indicated that displacement effects did not affect the calibration, at least under the experimental conditions tested in this study.

3.4.4 Real Sample Matrices. The data obtained above indicated the isotropic behaviors between adsorption and desorption and also suggested the feasibility of the real application using kinetic calibration for the purpose of quantitative analysis.

3.4.4.1 Equilibrium Constants and Capacities. Based on eq 3.3, to calculate the initial concentration of the analyte in a solution, the equilibrium constant and fiber capacity should be obtained. Langmuir analysis can be performed through equilibrium extraction experiments as mentioned previously. For equilibrium extraction, the experimental conditions were the same as those utilized for extraction procedure as described above. For the CW/TPR fiber, the equilibrium extraction time was 15 min. The equilibrium constants (K) and fiber capacities (n_{fmax}) were generated from the data utilizing equilibrium extractions.

A solid-coated fiber is usually good for the extraction of low concentrations of analytes in a sample matrix. When CW/TPR was utilized for extraction at equilibrium, it was observed that less than 0.04% carbofuran and 0.1% carbaryl were taken from the river water sample. Compared to the initial concentration, the extracted amounts were negligible. One example for Reciprocal Langmuir Analysis is presented in Figure 3.4. Good agreement was observed between the Langmuir model and the experimental data

for restricted concentration ranges. In Table 3.2, the data of the equilibrium constants (K) and capacities (n_{fmax}) calculated from the Reciprocal Langmuir Analysis lists. It was observed that different matrices did not significantly change the values of K when the same fiber was employed. Nevertheless, the components of the sample matrix more affect the values of n_{fmax}. It was also noticed that a clean matrix such as nano-pure water presented higher linearity compared to complicated matrices, such as river water, for equilibrium extraction. Good agreement was found between the present results and previous studies that have used solid-coated fibers for headspace extraction.[4] However, this calculation keeps widely empirical. When the parameters of a rationally isothermic adsorption are obtained, empirical data is valuable for reasonably reliable results, which can be got for the extent of surface coverage under different conditions.[18] A conclusion can be draw that solid-coated SPME fibers offer a valuable tool for the study of isotherm adsorption chemistry.

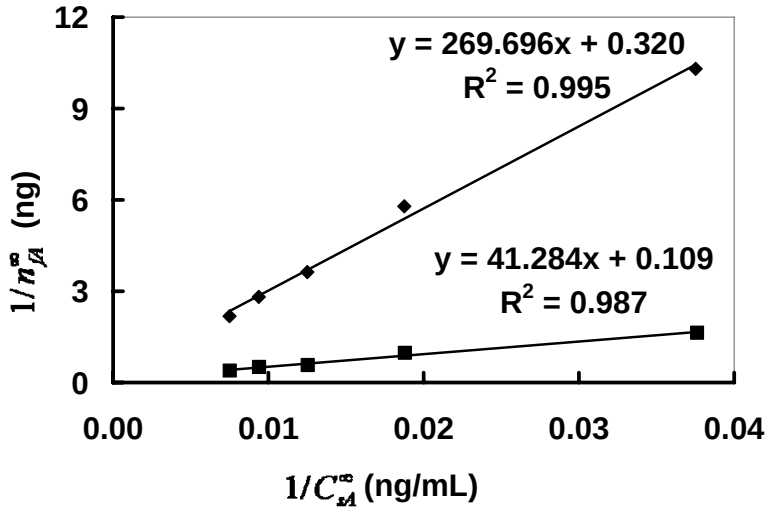
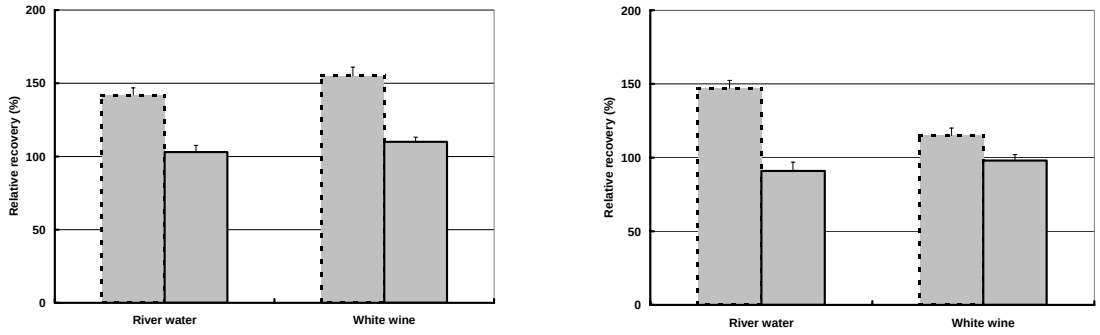


Figure 3.4 Reciprocal Langmuir Analysis for carbofuran (◆) and carbaryl (■). The experiments were performed in filtered river water containing 200 ng/mL of carbofuran and carbaryl at the agitation speed of 500 rpm and 25 ˚C.

Table 3.2 Log values of equilibrium constants (*K*) and capacities (n_{fmax}) for Reciprocal Langmuir Analysis

		Carbofuran	Carbaryl
Nano-pure water	Log *K* (M^{-1})	8.32	8.65
	n_{fmax} (ng)	4.79	16.42
5% ACN aqueous solution	Log *K* (M^{-1})	8.32	8.53
	n_{fmax} (ng)	3.78	11.55
PBS buffer (pH = 7.4)	Log *K* (M^{-1})	8.63	8.68
	n_{fmax} (ng)	2.70	14.64
River water	Log *K* (M^{-1})	8.42	8.72
	n_{fmax} (ng)	3.12	9.19
White wine	Log *K* (M^{-1})	7.60	8.66
	n_{fmax} (ng)	0.124	0.0792

3.4.4.2 Kinetic calibration for Pesticide Analysis. Good precision (RSD < 5%) and linearity (R^2 > 0.999) were obtained from 1 ng/mL to 200 ng/mL for carbofuran and carbaryl when external calibration analysis was done using the standard solutions in acetonitrile:water (1:1, v/v), containing carbofuran and carbaryl with promecarb as the internal standard. In the case of real samples, particulate matter should be removed before SPME experiments to prolong the fiber life.[19] Based on eq 3.21, the amount of analyte in the extraction phase at equilibrium, n_e, can be calculated using time constant, a, obtained from the desorption. The carbafuran and carbaryl concentrations were obtained using eq 3.3. Figure 3.5 illustrated the relative recoveries of the 200 ng/mL spiked samples, which are obtained using 1 min with a CW/TPR fiber. The results indicate that the on-fiber standardization technique generated a more accurate result of the tested pesticides in various matrices. Moreover, this approach shortened the sampling time as a pre-equilibrium sampling technique, reducing displacement effects and competition adsorptions for a solid-coated SPME.[3, 20, 21]



(A) (B)

Figure 3.5 Calculated recoveries of carbofuran (A) and carbaryl (B) in river water and white wine with external calibration (▭) and kinetic calibration (■) using 50 μm CW/TPR fiber. The same experimental conditions were set as Figure 3.3 and 3.4.

3.5 Conclusion and Addendum

3.5.1 Conclusion. Kinetic calibration was studied using solid-coated SPME fibers. It was theoretically predicted that there is an isotropic behavior between the adsorption of the analytes from the sample matrix to the SPME solid-coated fiber and desorption from the fiber to the sample matrix based on Fick's law and the Langmuir model. Using a commercially available fiber, 50 μm CW/TPR, this research was performed to analyze carbamate pesticides in different sample matrices. Good agreement was obtained between the experimental results and the theoretical prediction with close time constants between the processes of adsorption and desorption. The isotropic processes were used for kinetic calibration for adsorption using desorption to correct for the sample matrix effect. This pre-equilibrium technique also shortened the sampling time. In addition, the solid-coated fiber offered for a shorter period of time for the desorption of the concentrated analytes on the fiber into an organic solvent for liquid chromatography analysis, which is particularly useful for the analysis of thermally labile or non-volatile compounds. The kinetic calibration approach was used to determine pesticides in river water and white wine. For the future work, research could be conducted to apply this technique for different *in vivo* applications, such as systemic pesticide translocation study in plants, various toxicokinetic processes in animals, and TWA on-site air or water sampling.

3.5.2 Addendum. Compared to the part of the published paper, the text of this chapter has been fully revised.

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Chapter 4

In-fibre Standardization Technique Using Dominant Pre-equilibrium Desorption for Solid-Phase Microextraction

4.1 Preamble and Introduction

4.1.1 Preamble. This chapter has been published as the paper,

Zhou, S. N.; Zhao, W.; Pawliszyn, J. Kinetic Calibration Using Dominant Pre-equilibrium Desorption for On-Site and in Vivo Sampling by Solid-Phase Microextraction, *Anal. Chem.* **2008**, *80*, 481-490.

The contributions of Wennan Zhao, the co-author, involved the performance of PAHs experiments together with Simon Ningsun Zhou. Tables and Figures are reprinted with permission from Analytical Chemistry (Copyright 2008 American Chemical Society).

I, Wennan Zhao, authorize Simon Ningsun Zhou to use the material for his thesis.

Signature: 

4.1.2 Introduction. Compared to conventional sampling methods, solid-phase microextraction (SPME) offers several advantages, including less organic solvent-consumption, low cost, portability, and combination of sampling, isolation and enrichment into one step. This technique has found many applications in numerous disciplines.[1] So far, the equilibrium extraction method is the most well-established and widely used quantification method for SPME. In this method, a partitioning equilibrium between the sample matrix and the SPME extraction phase is reached. The amount of analyte extracted by fiber, n_e, and the initial concentration of the analyte in the sample matrix, C_0, follow the relation,[2]

$$n_e = \frac{K_{fs} V_f V_s}{V_s + K_{fs} V_f} C_0 \qquad \text{Equation 4.1}$$

where K_{fs} is the distribution coefficient, V_f is the volume of the fiber coating, and V_s is the volume of sample matrix. Typically, the volume of the sample matrix is large. Compared to V_s, when the product of K_{fs} and V_f is fundamentally insignificant, Equation 4.2 can be derived from Equation 4.1,

$$n_e = K_{fs} V_f C_0 \qquad \text{Equation 4.2}$$

Pre-equilibrium extraction with a short sampling time can also be employed for this purpose. When the sampling conditions such as convection and agitation are constant, there is a relation between the amount of analyte extracted and the extraction time. Based on the timed accumulation of analytes in the polymer coating, quantification can be performed. Ai first developed this pre-equilibrium approach, and proposed a dynamic SPME model according to a diffusion-controlled mass-transfer process. [3, 4] A further investigation had been performed by Chen et al., who demonstrated an isotropic

behavior between absorption and desorption in the SPME fiber. It led to a new calibration method, in-fibre standardization technique.[5, 6] The in-fibre standardization technique utilizes the desorption of the internal standards that are pre-loaded in the extraction phase and then used to calibrate the extraction of the analytes. This method was also applied to solid-coated SPME, which is based on the isotropy of adsorption and desorption.[7] So far, the in-fibre standardization technique has been successfully used in environmental monitoring, food, and clinic drug analysis, in laboratory, on-site analyses, as well as *in vivo* studies.[7-13] In addition, a number of academic reviews have discussed this method.[14-16]

However, the previous in-fibre standardization techniques have to employ radioactive or deuterated compounds as the pre-loaded internal standards. The radioactive or deuterated substances are either relatively expensive or inadequately available. Moreover, their suitability for *in vivo* studies is a concern. Furthermore, for the *in vivo* sampling of tissues or plants, in-fibre standardization technique has not been utilized.[17] In addition, with extraction or desorption time profiles, a non-linear approach has been used previously to obtain the time constant by for in-fibre standardization technique. The non-linear is not suitable for an extraction with a longer equilibrium time such as tissue sampling.

To address the issues mentioned above, one new calibration method utilizing dominant pre-equilibrium desorption was developed for on-site sampling or soft tissue sample preparation and quantitative analysis. In this approach, the interested analytes function as the internal standards. Thus, radioactivity or deutarated standards are not required. Concentration profiles were originally utilized to validate isotropy between

absorption and desorption. It is found that concentration profiles are more accurate and convenient than previously used time profiles. Dominant pre-equilibrium desorption have two advantages, a shorter sampling preparation time and a linear approach to obtain time constants. To our best knowledge, this report is the first time to use SPME for *in vivo* tissue sampling and quantitative analysis.

4.2 Theoretical Considerations

If a SPME fiber is inserted to a simple liquid sample matrix, the rate-determining step is considered to be mass diffusion of an analyte from the sample to the SPME polymer coating and from the surface of the fiber coating to its inner layers. This step slows down the period of an equilibrium time.[18] Fick's first law of diffusion can be used to describe this process:[4, 19]

$$J \equiv \frac{1}{A}\frac{\partial n}{\partial t} = -D_f \frac{\partial C_f}{\partial x} = -D_s \frac{\partial C_s}{\partial x} \qquad \text{Equation 4.3}$$

where J is the mass flux of the analyte from the fiber to the sample, A is the surface area of the fiber coating, ∂n is the amount of analyte that has absorbed to the fiber surface during time period ∂t, D_f and D_s are the diffusion coefficients of the analyte in the SPME fiber coating and the liquid sample matrix, respectively, and C_f and C_s are the concentrations of the analyte in the fiber and the liquid sample matrix, respectively. In a simple liquid matrix, the dynamic processes and isotropic behavior have been previously studied.[4, 6] Figure 4.1 shows another situation, a tissue with a total volume V and interstitial space volume V_0. The flux J to the fiber is:[19]

$$J \equiv \frac{1}{A}\frac{\partial n}{\partial t} = -D_f \frac{\partial C_f}{\partial x} = -\alpha \overline{D}_s \frac{\partial C_s}{\partial x} \qquad \text{Equation 4.4}$$

where α is the effective volume fraction (V_0/V), and $\overline{D}_s$ is the diffusion coefficient in the interested soft tissue. Flux of diffusion is proportional to the α value, which is typically lower than 1. Obviously, the diffusion path length *in vivo* is clearly greater than that existing in a free solution due to the tortuosity (λ) of the route, which can be observed in Fig. 4.1. Thus, the diffusion coefficients in a free liquid matrix (D_s) are typically higher than those found in a porous matrix such as a soft tissue.

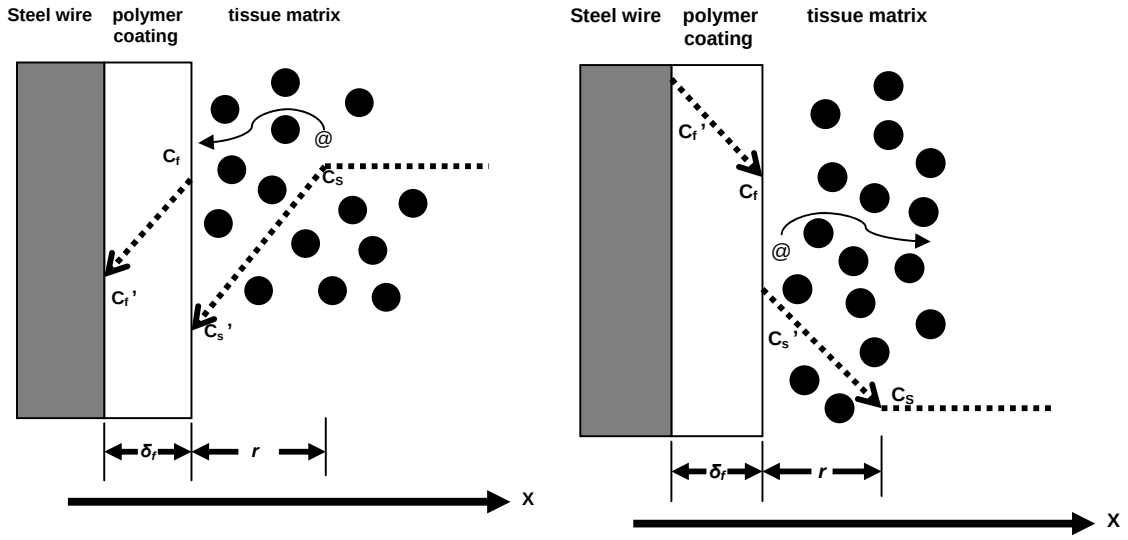
By the same mathematical treatment of the absorption process, the following equation can be derived from Equation 4.4,[4]

$$\frac{n}{n_e} = 1 - \exp(-at) \qquad \text{Equation 4.5}$$

where n is the amount of analyte extracted on the surface of the fiber coating after the exposure time t, n_e is the amount of the target analyte extracted at equilibrium, and a is the time constant describing how quickly the equilibrium can be reached, which is defined by Equation 4.6.

$$a = \frac{2AD_f\alpha\overline{D}_s(KV_f + V_s)}{2KV_sV_fD_fr + V_sV_f\alpha\overline{D}_s\delta_f} \qquad \text{Equation 4.6}$$

where r is the distance measured perpendicular to the considered area, and δ_f is the thickness of the SPME polymer coating. Equation 6 demonstrates that the time constants are different between a soft tissue and a simple liquid matrix.[4]



(A) (B)

Figure 4.1 Schematic of the absorption and desorption processes between the liquid coating of SPME fiber and the tissue matrix. A linear concentration gradient is assumed in both the fiber coating and the tissue medium when the experimental conditions are constant. (A) is the absorption process and (B) is the desorption process. C_s **is concentration of the analyte in the tissue matrix,** C_s' **is the concentration of the analyte in the sample at the interface of the fiber coating and the tissue,** C_f **is the concentration of the analyte in the coating at the interface of the fiber coating and the tissue,** C_f' **is the concentration of the analyte in the coating at the interface of the fiber coating and the stainless steel wire, and** δ_f **is the thickness of the fiber coating, r is the distance measured perpendicular to the area considered for an analyte, @ represents an analyte molecule with the arrow line indicating the diffusion route, and ● is the solid tissue, which the analyte does not pass through.**

In Figure 4.1, B, when a SPME liquid coating fiber preloaded with an analyte is exposed to a tissue matrix, desorption of the analyte from the fiber occurs. By the same mathematical treatment for the process of desorption, the following equation can be derived from Equation 4.4,[6, 8]

$$\frac{Q - q_e}{q_0 - q_e} = \exp\left(-at\right) \qquad \text{Equation 4.7}$$

where Q is the remaining amount of the analyte in the fiber at time t, q_0 is the preloaded amount of the analyte, q_e is the remaining amount of analyte in the fiber at equilibrium, and a is the time constant. When K_{fs} is large, q_e is not negligible. Based on eqs 4.5 and 4.7, at any absorption/desorption time, the sum of n/n_e and $(Q-q_e)/(q_0-q_e)$ should be 1. This can be expressed by,

$$\frac{n}{n_e} + \frac{Q - q_e}{q_0 - q_e} = 1 \qquad \text{Equation 4.8}$$

As previously mentioned, eq 4.8 shows the feasibility of in-fibre standardization technique using SPME. Nevertheless, when a long equilibrium time exists for a living tissue sampling, q_e may be difficult to obtain. Moreover, if a fiber is placed for an extensive period in the living tissue, wound effects could occur. To address this issue, eq 4.7 can be re-arranged as

$$Q = [\exp(-at)]q_0 + [q_e - q_e \exp(-at)] \qquad \text{Equation 4.9}$$

From eq 4.9, at a fixed pre-equilibrium desorption time and constant experimental conditions, a plot of Q versus q_0 produces a straight line with a slope, $\exp(-at)$, and a y-intercept, $[q_e - q_e \exp(-at)]$. Therefore, the time constant value can be obtained from the slope of the linear regression equation, and q_e is calculated from the intercept value.

Moreover, eq 4.9 can be used for both a simple liquid matrix and semi-solid medium. By eqs 4.5 and 4.2, the value of the time constant can be employed to obtain the initial concentration of the analyte in the sample matrix. After a fiber preloaded with a large amount of the analyte is exposed to the sample matrix, dominant desorption occurs and extraction can be negligible. By this dominant desorption, calibrate can be performed. It is named as in-fibre standardization technique using dominant pre-equilibrium desorption, which is suitable for on-site sampling preparation and *in vivo* sampling.

4.3 Experimental

4.3.1 Material. With greater than 99.5% purity, carbofuran, Propoxur, carbaryl, aldicarb and promecarb were obtained from Riedel-de Haën. Acenaphthene, fluorene, anthracene, and pyrene were supplied from Supelco (Bellefonte, PA, USA). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Nepean, ON, Canada). Helium with ultra high purity was obtained by Praxair (Kitchener, ON, Canada). Stock standard solutions (1000 mg/L) of five pesticides were made in acetonitrile and stored at 0–4 °C in the dark. For the purpose of quantitative calibration and sample preparation, working solutions were prepared by a series of tenfold successive dilution.

For the analysis of polycyclic aromatic hydrocarbons (PAHs), the SPME fiber holder and 100 μm polydimethylsiloxane (PDMS) fiber were purchased from Supelco (Oakville, ON, Canada). Prior to use, the 100 μm PDMS fiber was conditioned at 250 °C for 1 h. Custom-made 165 μm PDMS fibers were employed for both *in vitro* and *in vivo* pesticide sampling. PDMS hollow fiber membrane tubing (Helixmark, Carpinteria, CA, USA) was cut into 1.0 cm portions. Stainless steel wires made from the plungers of 10

μL microliter syringes (Hamilton, Nevada, USA) were sonicated in deionized water and acetone to ensure their cleanliness. Then each wire was used to immobilize one PDMS portion. Prior to use, the 165 μm PDMS fibers were conditioned in methanol (one hour) and then in nano-pure water (one hour). A polyethylene insert with a volume of 200 μL was positioned in a 2-mL vial for small-volume analysis,.

The standard PAH aqueous solution generation system has been reported previously.[20] With a temperature controller (Omegalux, US), the permeation chamber temperature was kept at 30 ± 1 °C to decrease the effect of the temperature on the flow-through system. The jade plants (*Crassula ovata*) were obtained from a local greenhouse.

4.3.2 Instrumental Analysis. The analysis of the PAHs was performed in a Saturn 3800 GC/2000 ITMS system with a SPB-5 column (30 m, 0.25 mm i.d., 0.25 μm film thickness) (Supelco, Mississauga, ON, Canada). The electron ionization (EI) mode tuned with perfluorotributylamine (PFTBA) was used for the MS system. Helium was used as the carrier gas with a flow rate of 1 mL/min. The 1079 injector at 270 ºC was used as the SPME injections. In the case of the liquid injections, it was set at 40ºC and then increased to 250 ºC at a rate of 100 ºC/min. The column temperature was at 40 ºC for the first 2 min and then increased to 250 ºC by 30 ºC/min. After the temperature was held for 5 min, it was increased by 30 ºC/min increments to 280ºC, and held for 15 min. The GC run time was 30 min. A mass scan was set from 40 to 300. The base peak of each interested chemical was selected and integrated. A liquid midpoint calibration standard was used to monitor the instrument daily. If any deviation in the area counts greater than 15 %, re-injection of that standard was performed. In the case of the deviation with constantly

greater than 15 %, the instrument was re-calibrated with a five-point calibration plot. To ensure that the chromatography was within the required specifications, peak shape quality, resolution, and retention times were also carefully checked.

As described in Chapter 3, an LC-MS/MS system consisting of a Shimadzu 10AVP liquid chromatograph with a system controller and dual binary pumps interfaced to a CTC-PAL autosampler and an MDS Sciex API 3000 tandem mass spectrometer was used to analyze pesticides. Beyond carbofuran and carbaryl, the mass transitions of propoxur and aldicarb were monitored from m/z 210.1/111.2, and m/z 116.2/89.0, respectively.

4.3.3 Absorption and Desorption. In the case of PAH analysis, a CTC CombiPal autosampler (Zwingen, Switzerland) with the associated Cycle Composer software (Version 1.4.0) was used for the experiments with a 2 min pre-agitation period (at 500 rpm) and 500 rpm agitation (25 ºC) for absorption, analyte loading and desorption. Verification of isotropism between absorption and desorption in the aqueous solutions was designed using four experiments. The first experiment was designed to produce absorption time profiles (n~t) by absorption with a constant concentration and variable time. The 100 μm fiber was exposed to 10-mL aqueous solutions with 0.100 μg/mL acenaphthene, fluorene, anthracene, and pyrene, for a specified period of time (5, 10, 15, 30, 45, 60, 90, 120, 180, or 240 min). The second experiment was designed to generate desorption time profiles (Q~t) by desorption with a constant concentration and variable time. The fiber was exposed to an aqueous solution containing 0.100 μg/mL PAHs in a vial for one hour to pre-load the analytes. Subsequently, each pre-loaded fiber was directly immersed in 10-mL PAH-free water in each vial to perform desorption for a

specified period of time (5, 10, 15, 30, 45, 60, 90, 120, 180, or 240 min). The third experiment was designed to yield absorption concentration profiles (n~n_e) by absorption with variable concentrations and a constant pre-equilibrium extraction time. The conditioned fibers were directly immersed in the vials containing 0.080, 0.040, 0.020, or 0.010 μg/mL PAHs for 20 min (to obtain n) or 240 min (to obtain n_e), to achieve absorption. The final experiment was designed to make desorption concentration profiles (Q~q_0) for desorption with variable concentration and a constant pre-equilibrium desorption time. The fiber was directly immersed in a 10-mL aqueous solution containing 0.080, 0.040, 0.020, or 0.010 μg/mL PAHs for 30 min, to perform analyte pre-loading. Each fiber was then directly immersed in 10mL PAH-free water in each vial for 20 min to carry out desorption (to get Q). To obtain q_0, desorption was not done in water. After absorption or desorption, the analytes in the fiber were thermally desorbed in the GC/MS injector for quantitative analysis.

In the case of pesticide analysis, to verify isotropism and kinetic calibration, agarose gel (1% w/v) contained a specified concentration of pesticides for *in vitro* absorption and desorption experiments. The gel was generated by mixing 0.5 g of agarose (Agarose 15, BDH Chemicals Ltd., Poole, England) with 50 mL phosphate-buffered saline, pH 7.4 (PBS), in a beaker. After heat was applied, the mixture became transparent and homogeneous. 1.9-mL mixture was transferred to a 2-mL vial containing a 100 μL aqueous PBS pesticide solution. Then each 2-mL vial was capped and vortexed. After the mixture was cooled to ambient temperature, a semi-solid gel was yielded. The agarose gels were ready for further experiments after two or three hours of stabilization. All of the experiments using the gel were done under a static mode. As mentioned above to verify

isotropism between absorption and desorption, four experiments were designed. The first experiment was to generate absorption time profiles. The conditioned fiber was exposed to the gel medium in the vial containing the gel with 2.0 μg/mL carbofuran, propoxur, carbaryl, and aldicarb, for a specified period of time (2, 5, 10, 20, 30, 60, 90, 120, 150, 200, 250, or 300 min). The second experiment was to produce desorption time profiles. Each fiber was exposed to a PBS solution containing 2.0 μg/mL of pesticides in a vial for one hour to perform the analyte pre-loading. Then the pre-loaded fiber was inserted to a vial containing the pesticide-free agarose gel to perform desorption, for a particular period of time (2, 5, 10, 20, 30, 60, 90, 120, 150, 200, 250, or 300 min). The third experiment was to generate absorption concentration profiles. The conditioned fiber was directly inserted to the agarose gel containing the pesticides (0.0625, 0.125, 0.25, or 0.50 μg/mL) for 20 min (to obtain n) or 300 min (to obtain n_e), for absorption. The final experiment was to produce desorption concentration profiles. The fiber was inserted in a vial containing the PBS (pH 7.4) aqueous solution containing 0.25, 0.50, 1.00, or 2.00 μg/mL pesticides for one hour to perform analyte pre-loading. The fiber was then inserted to a vial with pesticide-free agarose gel for 20 min (to obtain Q) to perform desorption. Desorption was not performed in the agarose gel to obtain q_0. After absorption or desorption mentioned above, the analytes in the PDMS fiber were desorbed to 75 μL of acetonitrile for one hour under a static mode. Then, 75 μL of a 50 ng/mL promecarb aqueous solution, the LC-MS/MS internal standard, to compensate for the variation of injection volume, was added to the desorption solution. After the solution was mixed, a 20 μL aliquot of the final mixture was automatically injected into the LC-MS/MS for quantitative analysis.

4.3.4 Dominant Pre-equilibrium Desorption. For PAHs, the pre-loading was done for 30 min at 500 rpm in an aqueous PAH solution with a concentration of 10.0, 20.0, 40.0, or 80.0 ng/mL. Three aqueous solutions were examined for desorption. The first was the PAH-free solution. The second was the solution containing PAHs with 4-fold lower concentrations (2.5, 5.0, 10.0, or 20.0 ng/mL, respectively) compared to the pre-loading solution. The third was the solution containing 8-fold lower concentrations (1.25, 2.5, 5.0, or 10.0ng/mL, respectively). The desorption was achieved at 500 rpm for 20 min. In the case of pesticide analysis, the pre-loading was achieved in the agarose gel containing 0.25, 0.50, 1.00, or 2.00 μg/mL pesticide, for 20 min. Then the fibers with the preloaded analytes were inserted to the pesticide-free gel for 20 min, as well as the gel containing 0.0625, 0.125, 0.25, or 0.50 μg/mL pesticides, respectively, which were 4-fold lower compared to the concentration in the preloaded gel medium.

4.3.5 Distribution Constants. In the case of pesticide analysis, two methods were employed to obtain the distribution constants (K_{fs}) between the fiber coating and the sample matrix. The first method used jade leaf juice as the sample matrix. The pesticide-free leaves from a jade plant were cut and ground with a mortar and pestle. 0.2-μm Acrodisc Syringe Filters (Ann Arbor, MI) were used to filter the juice to remove the fine particles. The amount of pesticides was added to the leaf juice to allow the concentration to be 0.25 μg/mL. The PDMS fiber was directly immersed in the juice matrix for extraction for 180 min at 500 rpm and 25 ˚C. The second approach to get K_{fs} was to utilize the agarose gel (1%, w/v) prepared from PBS (pH 7.4) with a pesticide

concentration of 0.25 μg/mL. The extraction was achieved under static mode and 25 ˚C with a 300 min equilibrium time.

4.3.6 On-site and *In Vivo* Study. SPME direct extraction was used to determine the PAH concentrations in the flow-through system. The extraction was achieved for 20 min in the sampling cylinder at ca. 0.25 cm/min flow rate. Triplicate was performed using the same fiber. Then, dominant pre-equilibrium desorption was performed for in-fibre standardization technique. The pre-loading was achieved in the aqueous solutions for 30 min at 500 rpm. The concentrations in the pre-loading solutions for fluorene, anthracene, and pyrene were 10.0, 20.0, 40.0, or 80.0 ng/mL. The concentration of acenaphthene was 4-fold higher in the pre-loading solutions. The fiber was exposed to the sampling cylinder to achieve dominant pre-equilibrium desorption for 1 h. Using the same fiber with the different pre-loading amounts, this process was repeated.

For *in vivo* sampling, the jade plants with the pesticide soil-application were utilized. 0.01 g for each pesticide (Chem Service, West Chester, PA, USA) was mixed. The mixed fine particles were spread on the soil. Then water was sprayed to make the solid pesticides gradually dissolve and diffuse to the soil. Every other day, around 15-mL water was applied to each plant. For technical convenience, *in vivo* experiments were performed at room temperature. Before the *in vivo* extraction, the absorption fibers were conditioned without any analyte pre-loading. The desorption fibers with the pre-loaded analytes were exposed to the PBS (pH 7.4) aqueous solutions with pesticides at concentrations of 1.0, 2.0, 4.0, and 8.0 μg/mL for 20 min. Immediately after the holes were drawn using a 21 gauge hypodermic needle, the absorption fibers and the desorption

fibers were simultaneously inserted into holes in two jade plant leaves for 20 min to achieve absorption and desorption. Figure 4.2 shows the locations for SPME absorption and desorption as well as two sampling points (1 and 2). Four fibers for desorption were located in the same leaf within 1 cm. The analyte desorption from the fibers to the organic solvent, and solution making for LC-MS/MS analysis were the same as described above. (Note: To perform the experiments simultaneously may be difficult. At intervals of 1 min, each fiber was inserted in the sample matrix.)

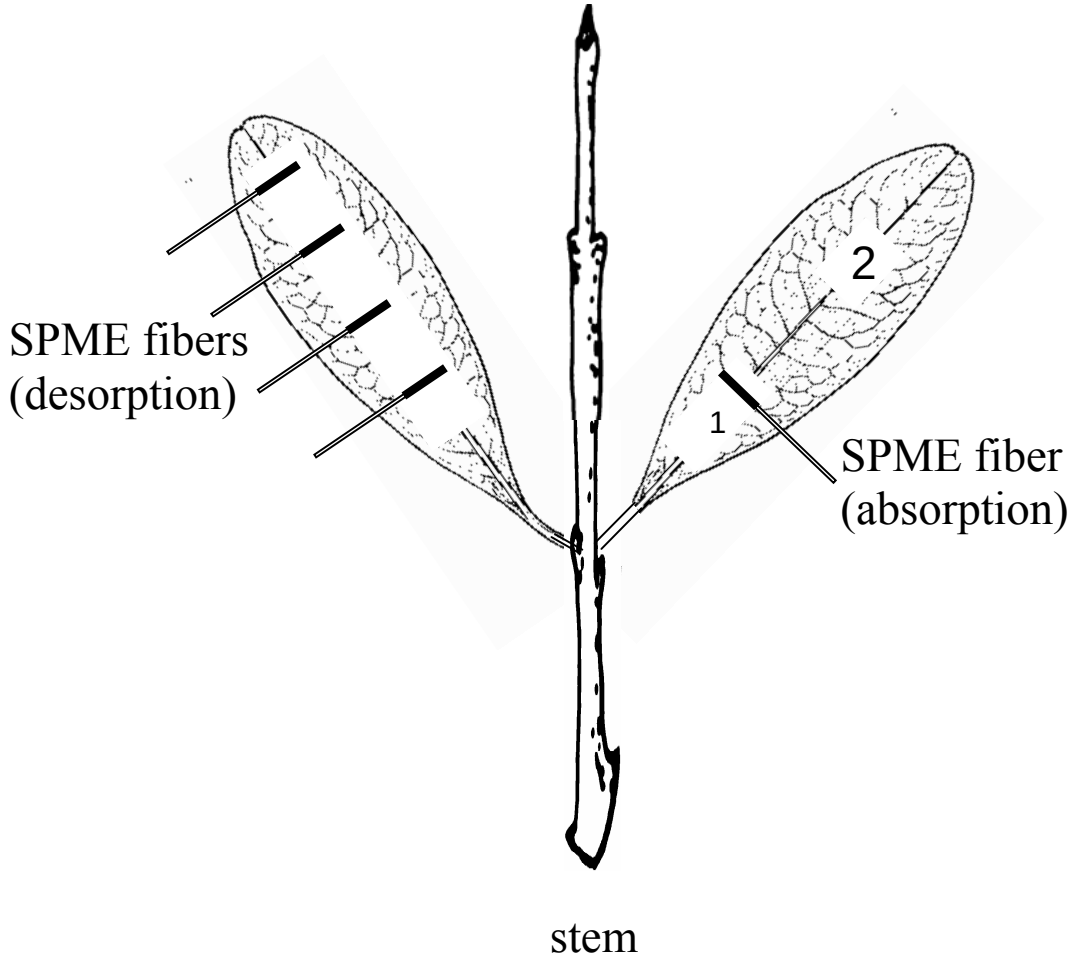


Figure 4.2 Locations for SPME absorption and desorption as well as two sampling points (1 and 2)

4.4 Results and Discussion

4.4.1 Primary Concerns. To choose a suitable fiber is the first step to develop a SPME method. Due to its high affinity to PAHs, the PDMS fiber was selected for the flow-through system and on-site application. Moreover, the PDMS coating was chosen for pesticide sampling because of several following reasons. First, the PDMS coating is biocompatible and high-purity medical grade. Therefore, the method could be further extended to animal or human studies. Second, as a liquid coating, the disadvantage of competition or displacement effects is not a concern. Third, the fibers were made in house because of the consideration of size and cost of using the fibers only one. Usually, fibers from a commercial source are expensive, and are not practical for single-use and *in vivo* experiments.

To gain precise results, internal standard pre-loading to an SPME fiber is important.[21] In this study, the internal standard pre-loading was achieved in aqueous, PBS solutions or gel, instead of the sample headspace. This way allowed the surface of the fiber to be wet with a layer of water prior to any absorption or desorption. This wetting approach prevented the surface of the fiber from drying, by which the fiber performance could be potentially influenced. It is also observed that a big difference did not exist using aqueous, a PBS solution, or an agarose gel for pesticide pre-loading. The intention of pre-loading is to allow a certain amount of analytes to go to the fiber coating. It is acceptable as long as the same pre-loading matrix is employed for the same batch experiment. The headspace pre-loading method leads to the fiber coating surface drying (or air bubble on the fiber surface), which makes for poor reproducibility. Moreover, using a headspace approach under a higher temperature (higher than room temperature),

pesticides can be pre-loaded to the fiber. However, it caused a complicated experimental procedure.

As described in Chapter 2, an agarose gel containing pesticides was utilized as a mimic system for an *in vitro* study. The reasons can be briefly summarized as follows. Agarose gel prevents convection but does not obstruct free diffusion significantly at certain concentration.[22] An agarose gel is different from a living tissue. However, *in vitro* experiments can be valuable to find mass transfer phenomena in both SPME coatings and semi-solid matrices, which may yield relevant information for *in vivo* study.

4.4.2 Isotropic Behavior. To confirm that the desorption of a standard from the extraction phase into the sampling matrix was isotropic to the absorption of the analyte into the extraction phase from the sample matrix, absorption and desorption were done in both the aqueous sample matrix and the agarose gel medium.

Time profiles were preliminary utilized for this validation. In spite of different absorption times, the concentrations of analytes (PAHs or pesticides) in the sample matrix (aqueous solution or gel) kept same. In addition, the pre-loaded amounts of analytes retained equal as the desorption time varying. This method has not been reported previously for a semi-solid medium although it has been achieved in the aqueous solutions.[6, 7, 11, 12] For all of the PAHs and pesticides used in this study, the addition of $\frac{n}{n_e}$ and $\frac{Q-q_e}{q_0-q_e}$ was close to 1, which agrees well with eq 4.8. Figure 4.3 shows one example. To further examine the isotropic behaviour, the time constants from absorption were compared with those from desorption. Rearrangement of eqs 4.5 and 4.7 results in eqs 4.10 and 4.11, respectively,

$$\ln(1-\frac{n}{n_e}) = -at \qquad \text{Equation 4.10}$$

$$\ln(\frac{Q-q_e}{q_0-q_e}) = -at \qquad \text{Equation 4.11}$$

The absorption and desorption time constant profiles can be obtained with $\ln(1-\frac{n}{n_e})$ or $\ln(\frac{Q-q_e}{q_0-q_e})$ as the y-axis, and t as the x-axis. The regression slope is $-a$. In Table 4.1, the time constants of absorption and desorption are listed for the different analytes. Time constants for the same chemical kept close between absorption and desorption with less than 5% relative standard deviation (RSD) observed in most cases. This suggests the isotropic behavior of absorption and desorption. Figure 4.4 is one example that demonstrates that eqs 4.10 and 4.11 accurately describe the kinetic procedures of SPME desorption and absorption in both an aqueous and gel medium. Moreover, the isotropic behavior was observed in the agarose gel medium when the 100 μm PDMS fiber was utilized. The data are not listed in this chapter.

Table 4.1 Time constants *a* for absorption and desorption of PAHs and pesticides from time constant profiles [a]

	Time constant a (min^{-1}) (R^2)	
	Absorption	Desorption
Acenaphthylene	0.0253 ± 0.0022(0.990)	0.0252 ± 0.0024 (0.993)
Fluorine	0.0228 ± 0.0024 (0.996)	0.0217 ± 0.0030 (0.990)
Anthracene	0.0147 ± 0.0018 (0.992)	0.0153 ± 0.0026 (0.984)
Pyrene	0.0092 ± 0.0014 (0.990)	0.0099 ± 0.0012 (0.990)
Carbofuran	0.0130 ± 0.0010(0.990)	0.0126 ± 0.0021 (0.987)
Propoxur	0.0142 ± 0.0016 (0.994)	0.0136 ± 0.0020 (0.991)
Carbaryl	0.0126 ± 0.0010 (0.994)	0.0127 ± 0.0008 (0.991)
Aldicarb	0.0131 ± 0.0012 (0.991)	0.0129 ± 0.0016 (0.991)

[a] Ten and eleven data points were used to calculate each time constant for PAHs and pesticides, respectively.

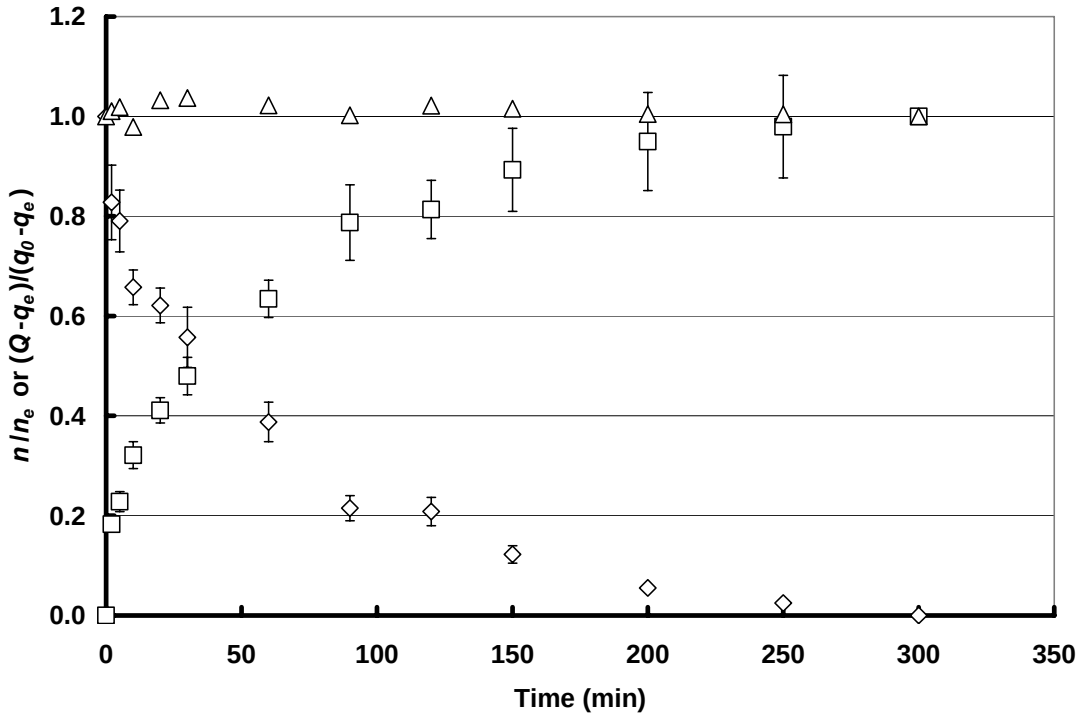


Figure 4.3 Propoxur absorption (□) and desorption (◇) time profiles. The absorption was performed in agarose gel (1%, w/v) containing 2 μg/mL carbofuran, propoxur, carbaryl, and aldicarb in static mode and 25 ˚C. For desorption profile, the analytes were preloaded in a PBS (pH 7.4) aqueous solution containing 2.0 μg/mL pesticides for one hour, and then the fiber was desorpiton in a pesticide-free gel. 165 μm PDMS fibers were used to produce the profiles. Δ, the sum of $\frac{n}{n_e}$ and $\frac{Q-q_e}{q_0-q_e}$

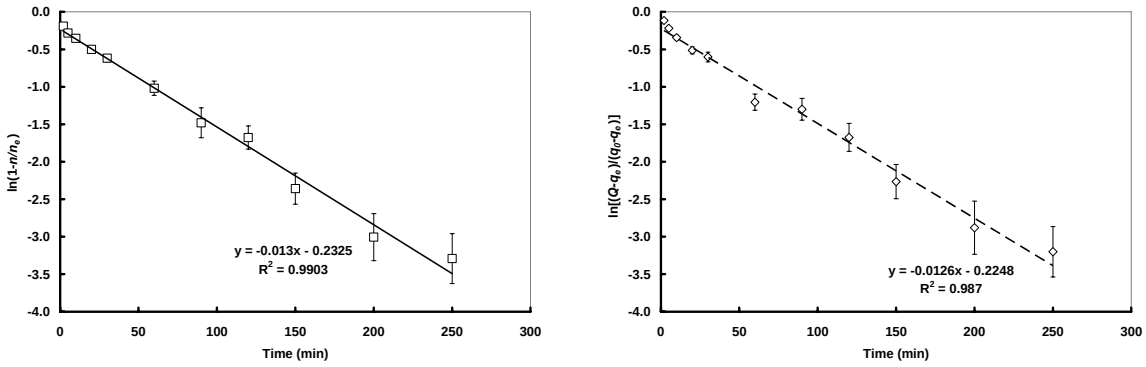


Figure 4.4 Carbofuran absorption (□) and desorption (◇) time constant profiles. The same experimental conditions were set as in Figure 4.3.

Concentration profiles were employed for verification of the isotropic behavior between absorption and desorption, too. This approach is investigated for the first time. In spite of the variable analyte concentrations in the aqueous solution or gel for absorption, or the different pre-loaded amounts in the fiber for desorption, the absorption or desorption times kept same. By re-arrangement of Equation 4.5, eq 4.12 can be obtained,

$$n = [1 - \exp(-at)]n_e \qquad \text{Equation 4.12}$$

From the equation above, a plot of n versus n_e produces a straight line with a slope of $[1 - \exp(-at)]$ when the absorption conditions and sampling time are constant. It can also be used to eq 4.9 with a slope of $\exp(-at)$. For a fixed pre-equilibrium time, the sum of the slopes from the absorption and desorption concentration profiles should be close to 1. Figure 4.5 is one example of absorption and desorption concentration profiles. Moreover, Table 4.2 lists the slopes of aborption and desorption lines, as well as the sum of the slopes from two lines, which is very close to 1. It indicates that an isotropic

behavior exists between absorption and desorption. Table 4.2 presents the values of time constants calculated from the slopes, too. It is observed that the values of the time constants from the absorption and desorption are very close.

Table 4.2 Slopes for aborption and desorption from concentration profiles, the sum, and time constants calculated from slopes

	Slope [a]			Time constant, a, (min^{-1}) [b]	
	Absorption	desorption	Sum of slopes	Absorption	desorption
	1-exp(-at) (R^2)	exp(-at) (R^2)			
Acenaphthylene	0.4019 ± 0.0173 (0.995)	0.6166 ± 0.0154 (0.999)	1.0185	0.0257 ± 0.0014	0.0242 ± 0.0013
Fluorine	0.3622 ± 0.0177 (0.980)	0.6526 ± 0.0183 (0.997)	1.0148	0.0225 ± 0.0014	0.0213 ± 0.0014
Anthracene	0.2532 ± 0.0144 (0.993)	0.7252 ± 0.0377 (0.997)	0.9784	0.0146 ± 0.0010	0.0161 ± 0.0026
Pyrene	0.1577± 0.0054 (0.991)	0.8420 ± 0.0539 (0.998)	0.9997	0.0086 ± 0.0003	0.0086 ± 0.0032
Carbofuran	0.2188 ± 0.0090 (0.994)	0.7675 ± 0.0215 (0.996)	0.9863	0.0123 ± 0.0006	0.0132 ± 0.0014
Propoxur	0.2350 ± 0.0089 (0.987)	0.7508 ± 0.0233 (0.997)	0.9858	0.0134 ± 0.0006	0.0143 ± 0.0016
Carbaryl	0.2225 ± 0.0042 (0.988)	0.8037 ± 0.0193 (0.996)	1.0262	0.0126 ± 0.0003	0.0109 ± 0.0012
Aldicarb	0.2255 ± 0.0120 (0.995)	0.7605 ± 0.0274 (0.993)	0.9860	0.0128 ± 0.0008	0.0137 ± 0.0018

[a] four data points were used to obtain each slope.

[b] time constant uncertainty were calculated using propagation of uncertainty from those of slopes.

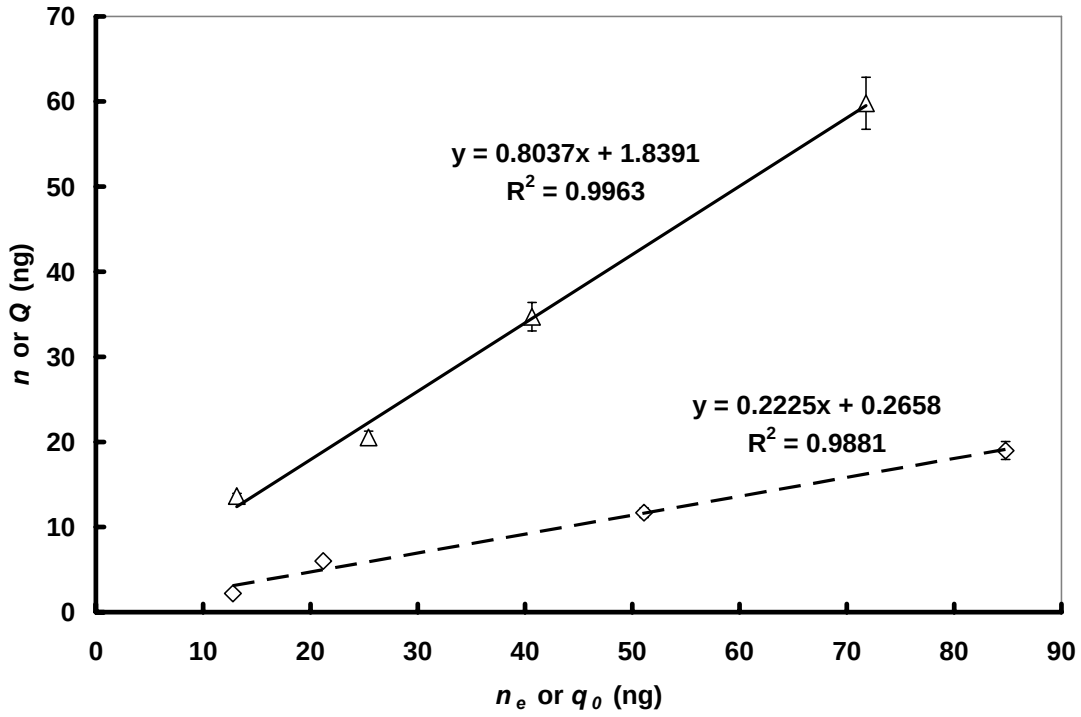


Figure 4.5 Carbaryl absorption (◇) and desorption (Δ) concentration profiles. The absorption was performed by the exposure of the fiber to the agarose gel (1%, w/v) containing 0.0625, 0.125, 0.25, or 0.50 μg/mL pesticides for 20 min (to obtain *n*), or 300 min (to obtain n_e). For the desorption profile, the fiber was exposed to the PBS (pH 7.4) aqueous solution containing 0.25, 0.50, 1.00, or 2.00 μg/mL pesticides for one hour under static mode for analyte pre-loading, and then the analytes was desorbed from the fiber in the pesticide-free agarose gel for 20 min under static mode (to obtain *Q*). To obtain q_0, desorption was not performed in the agarose gel.

Eq 4.6 defines time constant, a, which represents how quick an absorption or desorption can be reached in a semi-solid medium. The value is affected by several factors including the structure and properties of the analyte, the properties and dimension of the fiber coating, and components of the aqueous or tissue matrix. The effective volume fraction, α, and the diffusion coefficient, $\overline{D}_s$, in a living tissue, make different between the value of the time constant in a soft tissue and that of a simple liquid matrix or other tissues. Due to the presence of impermeable materials, the tortuosity factor ($\lambda^2 = \overline{D}_s/D_s$) increases the *in vivo* diffusion pathway.[23] Based on the results presented in Tables 4.1 and 4.2, a significant difference of the time constants is not observed for the four pesticides studied. This is possibly resulted from the large pores in agarose gel.[24]

The values of time constants for the same compound are close for both the absorption and desorption by comparison of Table 4.1 with Table 4.2. This suggests that in-fiber standardization technique could be performed using either time profiles or concentration profiles. However, three advantages associated with concentration profiles exist. Using concentration profiles is more robust and easier to obtain time constants. When an experiment is performed to obtain a time profile, a little variation in the value, n/n_e or $(Q-q_e)/(q_0-q_e)$, close to the equilibrium point could lead to a major change of the time constant because the relationship of the natural logarithm. Moreover, the concentration profiles offers a shorter sampling time. It is naturally suitable for on-site sampling, and especially for *in vivo* sample preparation. A long equilibrium extraction may not be feasible due to the changing of the analyte concentrations in the living system within a short period of time. Another concern is the potential wound effect. Finally, by comparison of the use of time profiles, concentration profiles are more accurate with

lower RSD and smaller error bars, which are observed in Tables 4.1 and 4.2, as well as Figure 4.3, 4.4, 4.5, and 4.6. Based on these findings, concentration profiles were further investigated in this study.

Several steps should be performed to determine the pre-equilibrium absorption and desorption time in order to get concentration profiles. The first step is to get the equilibrium time by extraction experiments or absorption time profiles. Then, 1/10 of the equilibrium time can be used as the pre-equilibrium time for concentration profiles. This time point is the crossing point of absorption and desorption time profiles, which the mathematical treatment of Eq 4.5 and 4.7 can clarify. As shown in Figure 4.3, at this time point, around half of the extracted amount at equilibrium from the sample matrix as well as a certain amount of desorption from the pre-loading fiber can be noticed. In addition, an ideal pre-equilibrium desorption time or the crossing time point can be gotten by choosing the different fiber thickness and the special coating.

4.4.3 Dominant Pre-equilibrium Desorption. When the pre-loaded amount of an analyte in the fiber is larger than the potential extracted amount from the sample matrix, dominant pre-equilibrium desorption was examined to find whether desorption is dominant and extraction is insignificant. The experiment was designed to determine if the desorption rate could be affected by small amounts of interested analytes in the sample matrix. As shown in Figure 4.6, the desorption rate remained unchanged when the pre-loaded amounts of the analyte in the desorption fiber were 4-fold higher than the potential extracted amounts from the sample matrix. Two separate desorption lines lead to close slopes, exp(-*at*), for aldicarb. When an experiment is designed, a certain distance should

be remained between the fibers for desorption and extraction to avoid diffusion of the desorbed analytes to the fibers for extraction because the pre-loaded internal standards are the extracted analytes.

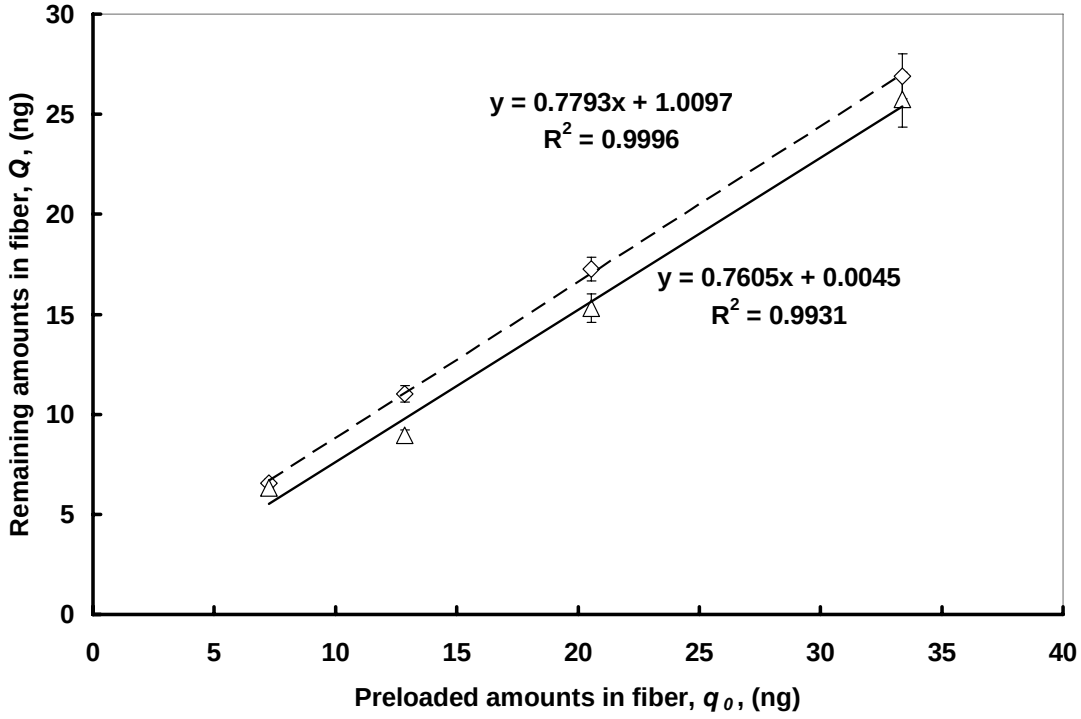


Figure 4.6 Comparison of two desorptions for aldicarb in the agarose gels (1%, w/v). The analyte pre-loading to the fiber was performed in the gels containing four pesticides of 0.25, 0.50, 1.00, or 2.00 μg/mL for 20 min. The analyte desorption from the fiber was performed for 20 min in the pesticide-free gel (Δ), or the gel with 4-fold lower pesticide concentrations, i.e. 0.0625, 0.125, 0.25, or 0.50 μg/mL (◇), compared to the pre-loading concentrations.

4.4.4 Real Sample Matrix. The in-fiber standardization technique was employed to quantify PAHs in the flow-through system with GC-MS and the pesticides in the leaves of jade plants with LC-MS/MS, respectively, to evaluate this approach for on-site and *in vivo* tissue sampling.

4.4.4.1 Distribution Constants. The distribution constants have to be obtained in order to calculate the initial concentrations of analytes in a sample matrix because the in-fibre standardization technique belongs to an equilibrium standardization technique. Previously, distribution constants of PAHs have been determined.[25] For pesticides, agarose gel (1%, w/v) made from PBS (pH 7.4) as the sample matrix was used to test the previous assumption. According to the assumption, extraction is in essence from extracellular fluid that should have close properties to the PBS buffer.[17] The distribution constant using the direct extraction mode can be obtained from the following dependence got from eq 4.13,[2]

$$K_{fs} = \frac{n_e V_s}{V_f (C_0 V_s - n_e)} \qquad \text{Equation 4.13}$$

Table 4.3 shows distribution constants (from both approaches) and LC retention times (from liquid chromatography). It was observed that the K_{fs} values from both approaches were close, which proved the correction of the previous assumption. Moreover, the values of the distribution constants were roughly proportional to those of LC retention times. This can be explained by the molecular properties. Both the PDMS SPME coating and C18 particles in the LC column have the high non-polar properties. The higher hydrophobic property suggests that the compound have the higher value of distribution constant and the longer retention time.

Table 4.3 Distribution constants (K_{fs}) of pesticides as well as LC retention times (t_r)

	K_{fs}[a]	K_{fs}[b]	t_r (min)
Carbofuran	73.2 ± 4.4	81.7 ± 5.4	2.80
Propoxur	57.3 ± 3.1	65.5 ± 4.8	2.76
Carbaryl	83.9 ± 7.0	80.6 ± 6.9	3.05
Aldicarb	40.0 ± 1.7	42.0 ± 2.4	2.34

n = 3

[a] the sample matrix was the agarose gel (1%, w/v) made from PBS (pH 7.4)

[b] the sample matrix was the filtered juice from the jade leaves

Table 4.4 Slopes of desorption concentration profiles obtained from the flow-through system, time constants, *a*, calculated PAH concentrations, limits of detection (LODs), and limits of quantitation (LOQs).

compound	Slope of desorption [a] / exp(-*at*) (R^2)	*a* (min^{-1})	Concentration [b] (μg/L)	LOD (μg/L)	LOQ (μg/L)
Acenaphthylene	0.9393 (0.999)	0.00104	28.54 ± 3.08	6.64	22.13
Fluorine	0.9067 (0.996)	0.00163	41.45 ± 1.87	6.91	23.02
Anthracene	0.8253 (0.998)	0.00320	1.45 ± 0.11	0.31	1.03
Pyrene	0.8493 (0.999)	0.00272	1.92 ± 0.16	0.22	0.73

[a] four data points were used to obtain each slope.

[b] n = 3

4.4.4.2 In-fiber Standardization Technique for PAHs and Pesticides Sampling. In the case of the on-site PAH sampling, the flow-through system was employed. Table 4.4 lists the slopes of the desorption concentration curves, the values of the time constants and the measured PAH concentrations in the flow-through system. When dominant pre-equilibrium desorption with analytes as the internal standards was utilized, excellent linearity was found.

In the case of pesticide sampling, the isotropism was validated in a gel medium. However, the geometry and properties of a constructed gel medium might not be the same as those of a real semi-soft tissue. Passage of the substance between *in vivo* and *in vitro* were expected to be different.[26] It is obvious that *in vitro* calibration has a limited application in SPME *in vivo* application.

A number of factors had to be considered before *in vivo* calibration was performed. First, the soft tissue is considered as homogeneity. Since the diameter of the SPME fibers in this study is by far larger than that of the cells in the living tissue, an assumption can be made that the structure of the sampled tissue is homogenous. Second, the number of desorption fibers that are used to construct a desorption concentration profile should be designed. To improve sensitivity and lead to a non-negligible q_e, a high affinity fiber to the target analyte is desirable. It is found that the intercept is not equal to zero based on eq 4.9. Thus, one point calibration can not be applied for this case. Four desorption points were applied for *in vivo* tissue sampling in this study in order to achieve more accurate results. Third, the sampling positions should not allow the analytes desorbed from the desorption fibers to go to the absorption fiber. An optimized design is illustrated in Fig. 4.2. A neighboring leaf was designed for the purpose of desorption to

correct the matrix effects from sampling the original jade leaf. It is assumed that the neighboring leaf had the same structure and properties. A similar design could be suitable for an animal experiment. It can be designed to perform extraction and desorption in the left leg and the right one of a rat, respectively. The similar scenario may be applied for brain sampling. Fourth, the distance and orientation of the desorption fibers should not allow analytes desorbed from one desorption fiber to disturb desorption from other fibers. As shown in Fig. 4.2, the fiber with the highest amount was close to the tip of the leaf, and the fiber with lowest pre-loaded amount was near the petiole or the stem. One direction from the sink to the shoot system is the characteristics of the transfer of systemic pesticides. Certain distance of the desorption fibers were remained, so disturbance between desorption should not happen, which is confirmed with the excellent linearity obtained from the desorption concentration curves as shown in Table 4.5.

The time constant values obtained from *in vivo* desorption concentration profiles are also presented in Table 4.5. in Figure 4.7, one example is shown. For all pesticides tested in the living leaves of the jade plant, excellent linearity ($R^2 \geq 0.990$) was noticed. Compared to *in vitro* data in Table 4.1 and Table 4.2, the values of the time constants show a difference among the four compounds tested, which indicates that the matrix effects. Table 4.5 presents limits of detection (LODs) and limits of quantitation (LOQs), which were obtained using ten fibers to perform extraction in the leaves of a jade plant without any pesticide application. LOD was calculated as three times the standard deviation of the blank, and LOQ was equal to ten times the standard deviation of repetitive measurements on a blank.[27] LODs and LOQs of PAHs are presented in Table 4.4.

Table 4.5 Slopes obtained from *in vivo* desorption concentration profiles [a], time constants, *a*, limits of detection (LODs), and limits of quantitation (LOQs) for *in vivo* analysis.

compound	Slope of desorption / exp(-*at*) (R^2)	*a* (min^{-1})	LOD (μg/L)	LOQ (μg/L)
Carbofuran	0.8360 (0.990)	0.00896	3	11
Propoxur	0.9353 (0.994)	0.00334	5	16
Carbaryl	0.9903 (0.999)	0.00049	10	44
Aldicarb	0.6633 (0.999)	0.02053	3	11

[a] four data points were used to obtain each slope.

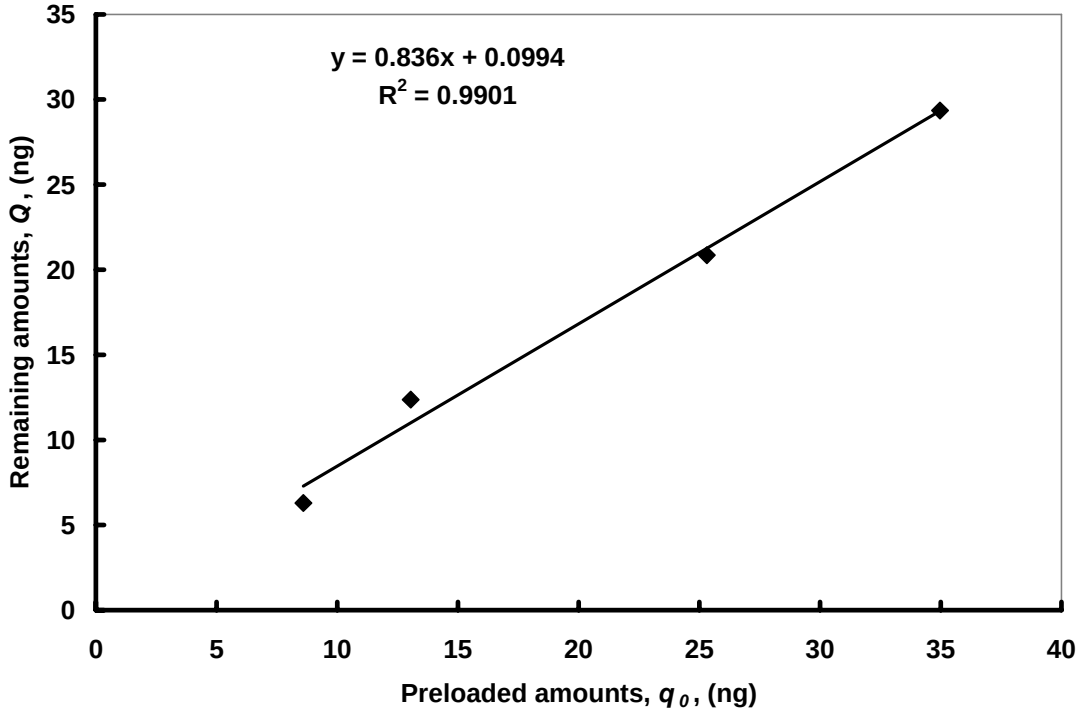


Figure 4.7 Carbofuran *in vivo* concentration desorption profile. The fiber was pre-loaded with carbofuran, propoxur, carbaryl and aldicarb by exposing to the PBS (pH 7.4) aqueous solutions containing 1.0, 2.0, 4.0, or 8.0 μg/mL pesticides for 20 min under static mode. Then four fibers were simultaneously inserted to the same leaf of the jade plant within 1 cm to perform desorption for 20 min at 25 ˚C. It is illustrated in Fig. 4.2.

Inhomogeneous distribution of pesticide in the leaf was was observed. The concentration of the pesticide at the sampling point 1 was higher than that of point 2, as shown in Fig. 4.2. These results suggest that these pesticides be xylem mobile. When a fiber was exposed to a leaf, the whole leaf could be considered as the same environment except pesticides. However, the small amount of pesticides presented in the leaf did not affect the dominant desorption of analytes from the fiber compared to the large amount of pre-loaded pesticides in the fibers. This is the purpose to employ the approach of dominant pre-equilibrium desorption. Sampling point 1 was utilized to get concentration-changing profiles as shown in Fig. 4.8, which was obtained from a jade plant leaf after the systemic insecticides were applied directly to the growing medium (the soil). After systemic pesticides applied to the growing medium and taken up by plant roots for more than 50 days, residual activity was still observed. This agrees well with the literature report with up to 12 weeks.[28] Compared to propoxur and carbaryl, it was observed that a lower concentration of carbofuran was found in the leaf. It suggested that propoxur and carbaryl could be absorbed more by the jade plant than carbofuran, or carbofuran might bind strongly to various constituents of the soil. Several factors affected the absorption amounts and rate of pesticides from the soil to the leaves. the diffusion of pesticides from the soil to the roots can by influenced the soil constituents, moisture and temperature. The entrance of pesticides into the root system can also be affected by pesticide properties such as hydrophobicity. In addition, the pesticide transport in a plant is affected by atmosphere humidity, light intensity, and so on. Fig. 4.8 did not illustrated because the experimental results for aldicarb were not similar to the others. Each pesticide including aldicarb with high amounts (0.2 g) was applied to the soil of one jade plant. A small but

detectable aldicarb peak was found after 40 days. Like propoxur, carbaryl and carbofuran, aldicarb is a soil-applied systemic pesticide, too. However, it is the least favored uptake by the jade plant. This could be caused by selective uptake effects among the four systemic pesticides used. In addition, among the four tested, aldicarb is the least lipophilic one. Thus, it may be the most difficult to partition into the hydrophobic cutin. The error bars were not put in Figures 4.7 and 4.8 due to the following reasons. First, it was impossible to achieve multiple experiments in a single leaf within one day due to the small or limited size of a leaf, so a single experiment was performed to obtain Figures 4.7 and 4.8 at intervals of 5 days. Second, the concentration of a pesticide in different level was different although different levels of leaves (older and newly-grown) were used for sampling. The upper level leaves (newly-grown) contained pesticides with lower concentrations compared to lower level leaves (older). This indicates that the procedure of time accumulation resulted in the different concentration in the different levels of leaves. As the lower level leaves grew first, they were more likely to accumulate more pesticides. A fully mathematical description of the pesticide uptake by a jade plant can be considered as the future work.

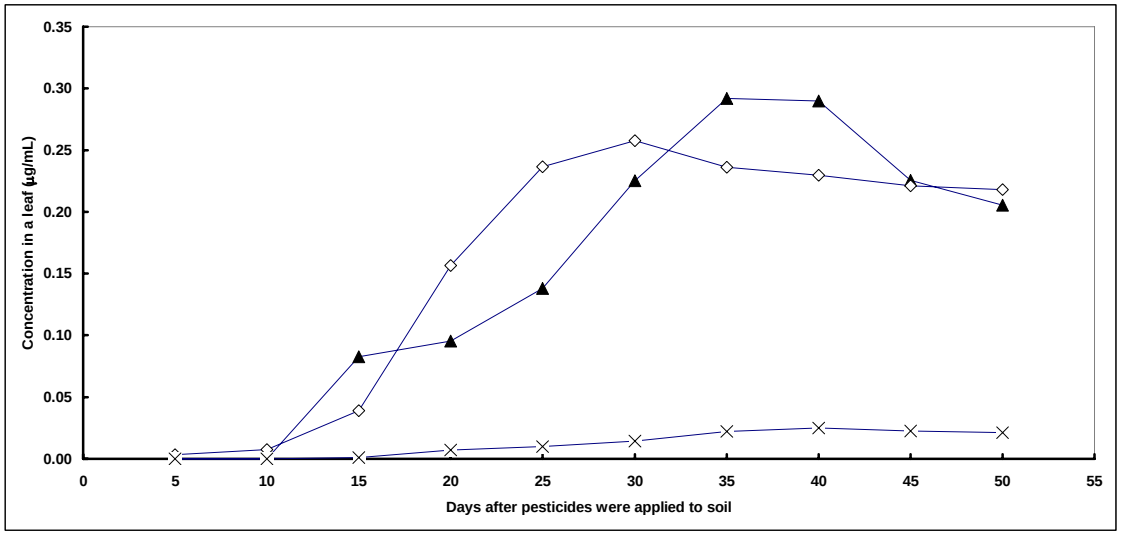


Figure 4.8 Concentration-changing profiles of carbofuran (×), propoxur (◇) and carbaryl (▲). It was obtained from a jade plant leaf in sampling point 1 (Fig. 4.2). The extractions were performed at 25 ˚C for 20 min. 165 μm PDMS fibers were used for extraction.

In vivo SPME sample preparation provides several advantages. It offers continuous sampling with minimal disturbance to the system under study. Fewer plants are employed and the approach is less invasive than other *in vitro* sampling methods or techniques. SPME is simpler and more convenient for on-site sampling or field-based monitoring compared to microdialysis, a widely used *in vivo* technique, which needs a cumbersome syringe or osmotic pump during sample preparation. In spite of the small size of an osmotic pump and non-requirement of a power supply, an osmotic pump delivers a less stable flow rate compared to a syringe pump.[29]

4.5 Conclusion and Addendum

4.5.1 Conclusion. In this study, an accurate and reliable in-fibre standardization technique has been developed using dominant pre-equilibrium desorption for on-site and *in vivo* application by solid-phase microextraction. A newly reported approach using the concentration profile with the pre-equilibrium approach not only confirmed isotropism between absorption and desorption but also offered a linear approach to get a time constant, which was more accurate and convenient than that obtained from a time profile. Early in-fibre standardization technique has been utilized with radioactive or deuterated internal standards that are either expensive or not available. In the present study, solid-phase microextraction utilized the target analytes as the internal standards by dominant desorption, which allowed desorption to calibrate absorption. Dominant pre-equilibrium desorption not only provides a shorter sampling time but also offered time constants for the reason of quantitative analysis. This in-fibre standardization technique was successfully applied to on-site PAH sampling in a flow-through system and *in vivo* pesticide sample preparation in a jade plant (*Crassula ovata*). This developed SPME method is very suitable for *in vivo* sampling in a field-based monitoring compared to microdialysis needing a bulky syringe or osmetic pump. This method with modification might be used for *in vivo* sampling in other biological organ systems. The developed technique could also be applied for field-based contaminant monitoring, such as in a river, as long as the desorbed analytes from the desorption fibers do not reach the absorption fibers.

4.5.2 Addendum. Compared to the published paper, the text of this chapter has been fully revised.

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Chapter 5

Application of Solid-Phase Microextraction for *in Vivo* Laboratory and Field Sampling of Pharmaceuticals in Fish

5.1 Preamble and Introduction

5.1.1 Preamble. This chapter was submitted to the journal, Environmental Science & Technology. Ken Oakes and Mark Servos are the co-authors of the submitted manuscript. The contributions of Ken Oakes involved the proposal writing for animal utilization, the activities in the wet laboratory (the establishment the fish facilities, fish raising, analyte spiking, and helping during SPME sampling), the activities in the field sampling (the selection of the sampling locations, fish capturing, and helping during SPME sampling), and revision for the chapter as well as suggestions for the whole project. Mark Servos provided all of the laboratory facilities including instrumentation, and a general guide for this research. Tables and Figures are reprinted with permission from American Chemical Society.

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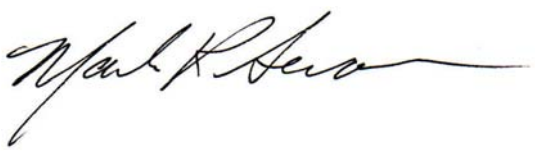
Signature: 

5.1.2 Introduction. There is growing scientific interest[1, 2] and public concern[3, 4] regarding the near ubiquitous detection of Pharmaceuticals and Personal Care Products (PPCPs) in surface waters adjacent urban areas. PPCPs are often released to receiving environments via municipal wastewater effluents (MWWE) as complex and dynamic mixtures containing thousands of chemical substances including prescription and over-the-counter therapeutic drugs, fragrances, and cosmetics.[5] Emerging evidence suggesting that some PPCPs may alter endocrine function in exposed non-target organisms heightened public interest, particularly within the EU. [2, 6] While impacts of estrogenic compounds on fish have been both dramatic and well-studied, the bioaccumulation potential and associated risks of long-term exposure of fish to other bioactive PPCPs have not been well characterized.[2, 7]

Researchers have developed a variety of analytical methods to measure PPCPs in fish tissues. Traditional sample preparation approaches for the analysis of PPCPs required the homogenization of tissue, followed by liquid-liquid extraction (LLE) using organic solvents, or solid-phase extraction (SPE). Pressurized liquid extraction[8] followed by mixed mode SPE cleanup was also developed to extract paroxetine, fluoxetine and norfluoxetine from fish tissues.[9] Following extraction and cleanup, the prepared samples are injected into gas chromatography (GC)[10-15], high performance liquid chromatography (HPLC),[16] liquid chromatography mass spectrometry (LC-MS),[17, 18] or liquid chromatography tandem mass spectrometry (LC-MS/MS)[19, 20] for quantitative analysis.

However, LLE, SPE and PLE, have several disadvantages including device expense, the need for comparatively large quantities of organic solvents, and the relative time and labor required to obtain final samples. Also, these methods only offer the total

concentrations of drugs in a tissue, not free or unbound concentrations. Although solid-phase microextraction (SPME) overcomes the limitations of the sample preparation techniques mentioned above, to date, it has not been evaluated for its utility in the determination of PPCPs in semi-solid tissues.

SPME is a novel sampling and sample preparation technique that offers several advantages over conventional sampling methods by combining sample extraction, concentration, and introduction into a single step. It has found numerous applications in many disciplines.[21] Based on the total time of contact between sample and extraction phase, two methods are currently in use. One is equilibrium extraction, and another is pre-equilibrium extraction. Equilibrium extraction is applied to the case that a partitioning equilibrium between the sample matrix and extraction phase is reached. The amount of analyte extracted by a fiber, n_e, is given by,[22]

$$n_e = \frac{K_{fs} V_f V_s}{V_s + K_{fs} V_f} C_0 \qquad \text{Equation 5.1}$$

where K_{fs} is the distribution coefficient, V_f is the volume of the fiber coating, V_s is the volume of sample matrix, and C_0 is the initial concentration of the analyte in the sample matrix. The volume of a sample matrix is typically large. When the product of K_{fs} and V_f is essentially negligible compared to V_s, eq 5.1 can be simplified to

$$n_e = K_{fs} V_f C_0 \qquad \text{Equation 5.2}$$

Pre-equilibrium extraction can be utilized to shorten sampling time. If convection and agitation are constant in the sample, then the amount of analyte extracted is related to the time of extraction. Quantification can then be performed based on the timed accumulation of analytes in the coating. This pre-equilibrium extraction was developed

and proposed a dynamic SPME model based on a diffusion-controlled mass-transfer process.[23, 24] On the basis of this process, an isotropic behavior between absorption and desorption on the SPME fiber was confirmed. It led to a new calibration method, kinetic calibration.[25, 26] The kinetic calibration method is based on the desorption of internal standards, which are pre-loaded in the extraction phase and then used to calibrate the extraction of the analytes. This method was also extended to solid-coated SPME, based on the isotropy of adsorption and desorption.[27]

In vivo field sampling addresses the need to study chemical processes in association with the normal biochemical milieu of a living system in a complex environment. *In vivo* sampling may be the only alternative when lethal sampling is not practical or feasible, and may yield more representative results with unstable or readily metabolized compounds. A major advantage of *in vivo* SPME is the rapid sampling time, which may be efficacious in the monitoring of both environmental spills and bioaccumulation potential of emerging compounds of concern. A particularly important potential *in vivo* SPME application is in studying contaminant dynamics in threatened aquatic species, such as the black (*Moxostoma duquesnei*) and river redhorse (*M. carinatum*) where lethal sampling is not possible. To date, SPME has been applied in a variety of field, or *in vivo* studies. However, previous SPME field sampling has been limited to volatile compounds in air or water quantified by gas chromatography/mass spectrometry (GC/MS) [28-33]. At present, SPME *in vivo* sampling has been restricted to liquid matrices (blood) in studies conducted within a controlled laboratory environment. [34-37] Previously published SPME kinetic calibration approaches required the use of radio-

labelled or deuterated standards that, if available for the compound of interest, are often prohibitively expensive.

The purpose of this study was to evaluate the efficacy of SPME techniques as an analytical tool under a variety of applied *in vivo* laboratory and field conditions. The *in vivo* analysis of pharmaceuticals in wild fish muscle tissue, the application of SPME to assess human pharmaceutical bioaccumulation in laboratory fish exposures, and the development of techniques to facilitate rapid and simple field assessments are all novel aspects of this study not previously evaluated. As part of the validation for SPME in muscle, an objective of this study was to use pre-equilibrium desorption rates as a means to calibrate pre-equilibrium extractions. This technique, which compensates for matrix effects using concentration profiles, provides a linear approach for kinetic calibration, while eliminating the necessity for radio-labeled or deuterated internal calibration standards. In addition, to the best of our knowledge, this report is the first to utilize the *in vivo* SPME approach combining with liquid extraction (LE) for the determination of muscle protein binding of drugs. When the protein binding is known, *in vivo* SPME can be used to measure free and total concentration simultaneously in a living tissue.

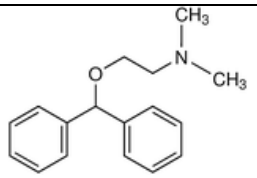
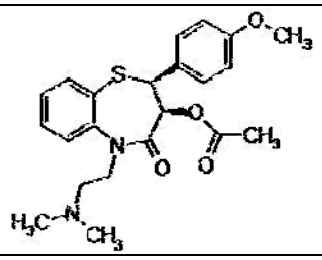
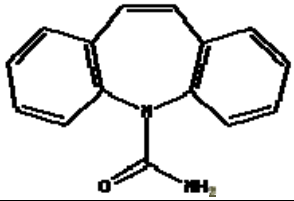
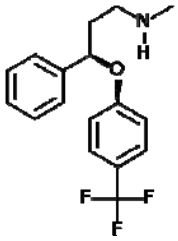
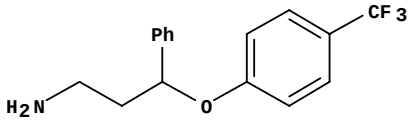
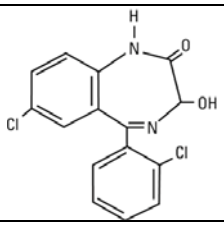
5.2 Experimental

5.2.1 Chemicals and Supplies. Reference standards for fluoxetine, norfluoxetine, and diphenhydramine (1000 μg/mL in MeOH) were purchased as certified analytical standards (Cerilliant Corp., Round Rock, TX). Reference standards for carbamazepine, and diltiazem were purchased in the highest available purity (Sigma-Aldrich, Oakville, ON, Canada). The internal standard lorazepam was also obtained from Cerilliant Corp. Methanol (HPLC grade) was obtained from Fisher Scientific (Nepean, ON, Canada). Table 1 illustrates the structures of the compounds used in this study. Stock standard solutions (1000 mg/L) of individual drugs were prepared in methanol and stored at 0–4 °C in the dark. Working solutions were prepared by a series of successive tenfold dilutions for quantitative calibration and sample preparation. Distilled water was purified and deionized to 18 MΩ with a Barnstead Nanopure Diamond® UV water purification system. Rainbow trout (*Oncorhynchus mykiss*) were purchased from Silvercreek Aquaculture (Erin, ON, Canada).

Self-made 165 μm thick poly(dimethylsiloxane) (PDMS) fibers were used for both *in vitro* and *in vivo* drug sampling. PDMS hollow fiber membrane tubing (Helixmark, Carpinteria, CA, USA) was cut into 1.0 cm portions. Each PDMS portion was placed over a 4 cm fine stainless steel wire (483 μm in diameter; medical grade; Small Parts Inc., Miami Lakes, FL). Before PDMS coatings were mounted, wires were sonicated in de-ionized water and acetone to ensure their cleanliness. The 165 μm PDMS fibers were conditioned in methanol for 1 h in static mode and then in nano-pure water for 1 h prior to use. The conditioning steps also served to sterilize the SPME fiber prior to insertion into the fish.

An integrated desorption device was designed for field sampling, as shown in Figure 5.1. This device consisted of 200-μL polyethylene inserts (Supelco, Bellefonte, PA), 2-mL vials (National Scientific, Rockwood, TN) and a 54-vial plate (Agilent, Mississauga, ON, Canada). Each insert was positioned in one vial. Immediately after sampling, the fiber coating was briefly rinsed in double distilled water and lightly dried with a Kimwipe® tissue. When dry, the fiber coating was immediately immersed into 50 μL of methanol within the insert. The vial was covered by a cap to prevent any evaporation of methanol during desorption. Each vial was put into an individual hole of the 54-vial plate and the whole device was covered with Parafilm® to immobilize the vials for transportation to the laboratory.

Table 5.1 Compounds used in the study and structures.

Diphenhydramine	
Diltiazem	
Carbamazepine	
Fluoxetine	
Norfluoxetine	
Lorazepam	

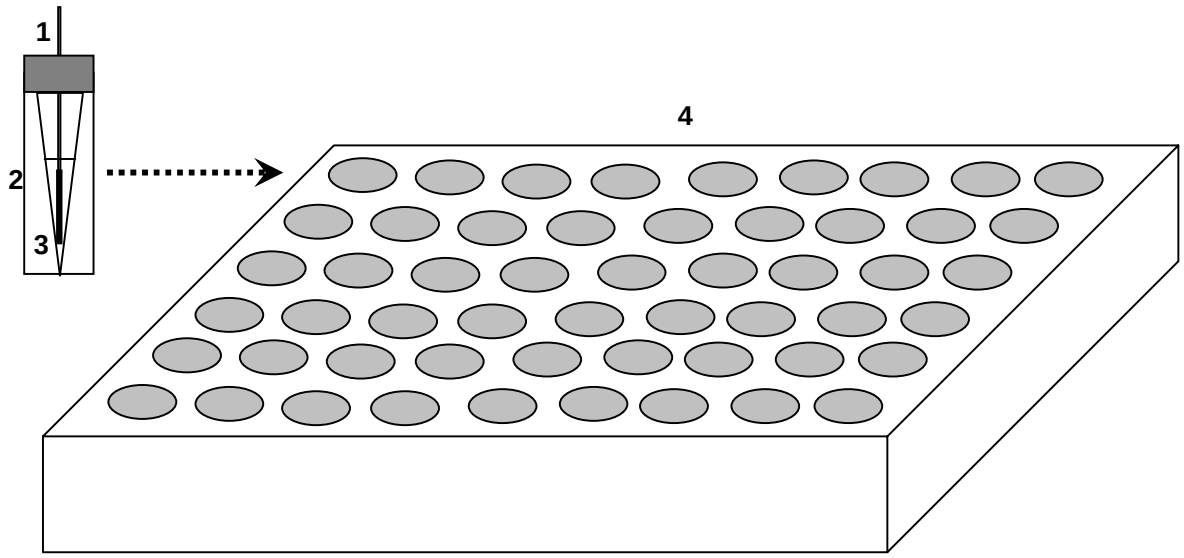


Figure 5.1 Desorption device for *in vivo* and field sampling: (1) SPME fiber, (2) 2-mL vial, (3) 200-μL insert, and (4) 54-vial plate.

5.2.2 Instrumental Analysis. The HPLC analysis was performed using an autosampler, column oven and a binary pump (1200 series, Agilent, Mississauga, ON, Canada). A Finesse Genesis C18 column (150 × 2.1 mm, 4 μm, Chromatographic Specialties Inc., Brockville, ON, Canada) was used for separation. Mobile phases consisted of (A) methanol/water (5:95 *v/v*) with formic acid (0.1% *v/v*) and (B) methanol/water (95:5 *v/v*) with formic acid (0.1% *v/v*). The flow rate was set at 0.4 mL/min and a 0% B gradient was applied for the first 0.5 min. This was ramped to 100% B over 1.0 min, held for 1.5 min, and finally returned to 0% B for 3.0 min. This resulted in a total run time of 6.0 min, including reconditioning. A 10 μL injection volume was used for each experimental sample. Eluted analytes were monitored by triple-quadrupole tandem mass spectrometer (MS/MS) using a QTrap 3200 system with a TurboIonSpray source (Applied Biosystems/MDS Sciex, ON, Canada). Each transition was monitored for 200 ms.

Compound specific mass spectrometer settings were determined for each compound separately by infusing a 0.5 μg/mL solution (methanol/water, 1:1 *v/v*) at 3 μL/min using the integrated syringe pump. Transitions monitored were as follows: diltiazem, *m/z* 415.2/178.2; diphenhydramine, *m/z* 256.1/167.2; carbamazepine, *m/z* 237.1/194.2; fluoxetine, *m/z* 310.1/44.0; norfluoxetinethe, *m/z* 296.3/134.1; and lorazepam, *m/z* 321.1/275.1. Mass spectrometer response sensitivity and linearity were monitored before and after each set of experimental samples through the injection of 10 μL of a series of standards (0.1–100 μg/L) prepared in methanol and water (1:1 *v/v*) containing the internal standard, lorazepam. Analyst® version 1.4.2 software (Applied Biosystems) was used to control all components of the system and for data collection and analysis. External calibration curves were performed with good precision (RSD < 5%) and linearity (R^2 > 0.999).

5.2.3 *In Vitro* Absorption and Desorption. Agarose gel (1% *w/v*) was spiked with various concentrations of pharmaceutical drugs for *in vitro* absorption and desorption experiments to verify isotropism and kinetic calibration. Krebs-Ringer bicarbonate solution (NaCl: 122 mM; KCl: 3 mM; $MgSO_4$: 1.2 mM; $KHPO_4$: 0.4 mM; $NaHCO_3$: 25 mM; and $CaCl_2$: 1.2 mM) was used to make a gel matrix for *in vitro* study as it approximates an internal physiological milieu in the sense that it is isosmotic with plasma [38]. The gel was made by mixing 0.5 g of agarose (Agarose 15, BDH Chemicals Ltd., Poole, England) with 50 mL Krebs-Ringer bicarbonate solution. After the mixture was heated and became transparent and homogeneous, 1.9-mL was transferred to a 2-mL vial, which contained 100 μL of aqueous Krebs-Ringer bicarbonate solution. The 2-mL vial

was then capped and vortexed. Finally, the mixture was cooled to ambient temperature, yielding a semi-solid gel, which was used for *in vitro* experiments after two or three hours of stabilization. All of the experiments using the gel medium were performed under static mode, and were designed to verify isotropism between absorption and desorption. The first experiment was performed to produce absorption time profiles to determine equilibrium time. The conditioned fiber was directly immersed in the vial containing the gel with 2.0 μg/mL diltiazem, diphenhydramine, carbamazepine, fluoxetine, and norfluoxetine, for a specified period of time (5, 10, 20, 30, 60, 90, 120, 150, or 180 min).

The second experiment was performed to develop absorption concentration profiles. The conditioned fiber was directly exposed to the agarose gel containing the same drugs as the first experiment, but with concentrations of 0.10, 0.25, 0.50, or 1.00 μg/mL for 20 min (to obtain n) or 180 min (to obtain n_e), for absorption. The third and last experiment was performed to develop desorption concentration profiles. The fiber was dosed in a vial of Krebs-Ringer bicarbonate solution containing the drugs utilized in the previous two experiments at concentrations of 0.10, 0.25, 0.50, or 1.00 μg/mL for one hour to pre-load analytes. Each fiber was then exposed to a vial that contained drug-free agarose gel for 20 min (to obtain Q) for desorption. To obtain q_0, desorption was not performed in the agarose gel.

Following the experimental absorption or desorption described above, analytes in the PDMS fiber were desorbed (with constant agitation at 400 rpm on an oscillating shaker) in a 200 μL insert containing 50 μL of methanol for 1 h. Then, 50 μL of a 100 ng/mL lorazepam aqueous solution (the internal standard for LC-MS/MS which was used to compensate for the variations in injection volume) was added to the desorption

solution within the 200 μL insert. After the solution was vortexed, a 10 μL aliquot of the final solution was automatically injected into the LC-MS/MS for quantitative analysis.

5.2.4 Distribution Constants (K_{fs}). Distribution constants (K_{fs}) between the fiber coating and the sample matrix were determined in aqueous Krebs-Ringer bicarbonate solution containing 0.10 μg/mL of diltiazem, diphenhydramine, carbamazepine, fluoxetine, and norfluoxetine. The extraction was performed at 400 rpm and 25 ˚C for 180 min. Following extraction, the desorption of analytes from each fiber, the addition of lorazepam, and sample injection into the LC-MS/MS were performed as described above.

5.2.5 Laboratory *In Vivo* Desorption and Extraction. All animal experimental procedures were approved by the Animal Care Committee at the University of Waterloo (AUP #04-24 and 07-16). *In vivo* desorption and extraction experiments were performed in the wet-lab facility of the Biology Department, University of Waterloo utilizing immature rainbow trout (length 15.6 ± 0.3 cm; weight 28.7 ± 1.4g).

Rainbow trout held in clean reference water were used for *in vivo* desorption experiments, which utilized the same procedures for analyte pre-loading to the fibers as those used to produce the *in vitro* desorption profiles described above. The *in vivo* desorption procedure began by anaesthetizing fish in 0.1% ethyl 3-aminobenzoate methanesulfonate until vertical equilibrium was lost (approximately 1 min). Once anaesthetized, a 20 gauge needle was used to penetrate the dorsal-epaxial muscle to a depth of approximately 12-13 mm. This depth assured that the entire SPME coating was embedded in the muscle. The guide needle was removed and the SPME probe containing pre-loaded analytes was immediately inserted into the muscle (Figure 5.2). Following

fiber placement, the fish was placed in a recovery tank containing fresh, aerated reference water for approximately 19 min. During this interval, each fish was continuously observed as it regained equilibrium and consciousness and moved freely around the tank. Following the 19 minute period when both the fish was conscious and the SPME fibre was equilibrating within the muscle tissue, the fish was anaesthetized as before. While anaesthetized, the fiber was quickly taken out from the fish. The total time for the fiber to equilibrate in fish muscle was 20 min. Double distilled water was used to quickly rinse any blood on the surface of the coating and stainless steel wire. Residual water on the fiber was dried with a Kimwipe® tissue. Finally, the fiber was placed in 50 μL methanol for 1 h to allow for desorption of the analytes from the coating.

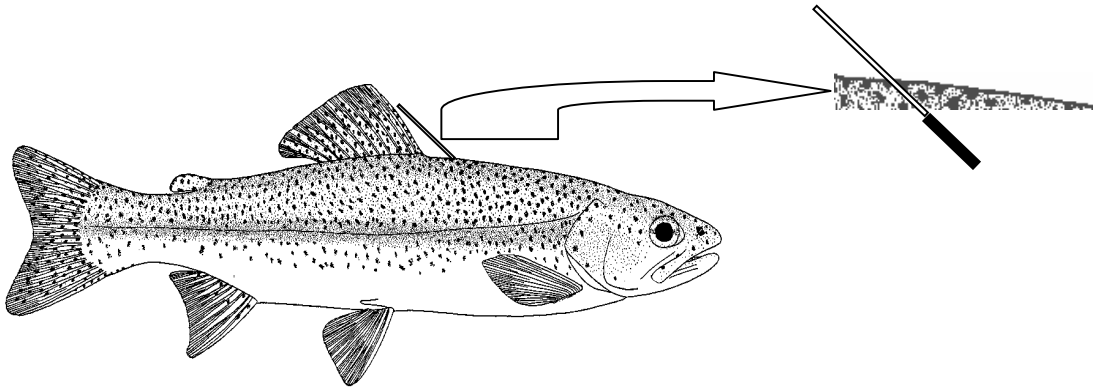


Figure 5.2 Location of SPME fiber used for *in vivo* sampling in the dorsal-epaxial muscle of a rainbow trout, and location of muscle sample excision.

Clean fibers (without any pre-loading of analyte) were employed for the *in vivo* extraction experiments to determine the concentration of a free or unbound drug or its metabolite. Rainbow trout were divided into four groups, which were exposed for 7 or 14

days (d) to 0 (clean reference water, or control), 3.2, 32, and 320 ng/mL fluoxetine with 50% daily renewals of test solutions. The fluoxetine stock solution, 108.80 mg/mL, was made fresh every third day in ethanol. Each exposure aquaria was 34 L, and three fish were placed in each aquaria, with four aquaria replicates (12 fish) per treatment, at a mean loading density of 2.43 g/L. Water quality was assessed daily and was maintained at standards considered optimal for this species (temperature 12.5 ± 0.02 ˚C; dissolved oxygen 10.09 ± 0.03 mg/L; pH 8.17 ± 0.06; unionized ammonia 23.5 ± 1.5 μg/L). Initial dosing of the aquaria utilized a 100 μL aliquot of fluoxetine in ethanol (EtOH, or EtOH control) added to each 34 L aquaria to achieve nominal fluoxetine exposure concentrations of 3.2, 32, and 320 ng/mL (and EtOH control). On subsequent days, to achieve 50% daily renewal, 50 μL of dosing compound was added to each aquaria. After 7 and 14 d of exposure, fish were sampled for the *in vivo* extraction experiments using the fiber insertion procedures described above.

A second *in vivo* extraction experiment, utilizing the anti-epileptic drug carbamazepine, was performed subsequent to the fluoxetine exposure. While both fluoxetine and carbamazepine are neuroactive pharmaceuticals, they have different modes of action as well as physico-chemical properties, necessitating the use of dimethyl sulfoxide, instead of ethanol, as the carrier solvent. All other doses and procedures were identical for carbamazepine as that described for fluoxetine. After SPME procedures were completed and the fish was anaesthetized, approximately 0.75 g of dorsal-epaxial muscle was cut from each fish and stored in a 2-mL vial (Wheaton Science, Millville, NJ, USA) at −80 ˚C prior to liquid extraction.

To calculate fish muscle density (g/mL), a piece of dorsal-epaxial muscle adjacent the SPME sampling area was excised and weighed on an analytical balance, with the volume displaced by the muscle mass determined using a graduated cylinder and verified by calibrated pipette. Using the measured density of 1.07 g/mL, analyte concentration (ng/mL) can be convented to ng/g.

5.2.6 Determination of Total Concentrations. Liquid extraction (LE) was utilized to determine the total concentration of a drug or its metabolite in muscle. Standard addition method was performed using drug-free muscle from control fish to obtain the recovery data. Each piece of fish dorsal-epaxial muscle was thawed to room temperature, then homogenized manually in a 2-mL vial. Immediately following homogenization, 1 mL of extraction solution (methanol:water:formic acide, 95:5:0.1 *v/v/v*) containing spiked drugs (10, 20, 50, or 100 ng/mL) was added to the homogenized drug-free muscle. The same volume of extraction solution without any spiked drugs was added to homogenized muscle from fish which had been exposed to drugs in order to measure the total concentrations in muscle. The mixtures in the vials were shaken vigorously at 2400 rpm using a VWR digital vortexer for 10 hours. After agitation, 450 μL from each vial was transferred to a microcentrifuge tube with a 30 KDa exclusion membrane (Ultrafree-MC, Millipore Corp., USA) for centrifugation 14 000 rpm for 30 min. Of this filtrate, 50 μL was transferred to a 200-μL insert. The addition of lorazepam, and sample injection into the LC-MS/MS were performed as described above.

5.2.7 Field *in Vivo* Application. To assess the efficacy of SPME technique in evaluating *in vivo* pharmaceutical concentrations in wild fish, three field sampling locations were used, two of which were heavily influenced by municipal wastewater effluents (Figure 5.3). Wild 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 the fish species utilized varied somewhat from site to site, efforts were made to overlap species composition. As much as possible, smaller-bodied fish were targeted as these species typically have well-defined home ranges and may reflect the chemistry of their capture environment better than more mobile, larger-bodied species. For small-bodied fish, the SPME fiber was inserted (under anaesthetic) roughly 20° from the lengthwise-axis of the fish, in contrast to the near 45° insertion used on the larger-bodied rainbow trout in the laboratory *in vivo* assessments. An Oakton Con 11 conductivity meter (Oakton Instruments, Vernon Hills, IL) was used to measure the mean conductivity of both 100% MWWE and that of the river at several upstream (reference) locations. The net increase in conductivity attributable to constituent ions of the MWWE was then used to calculate the percentage composition of effluent at downstream sampling locations as a percentage of 100% MWWE.

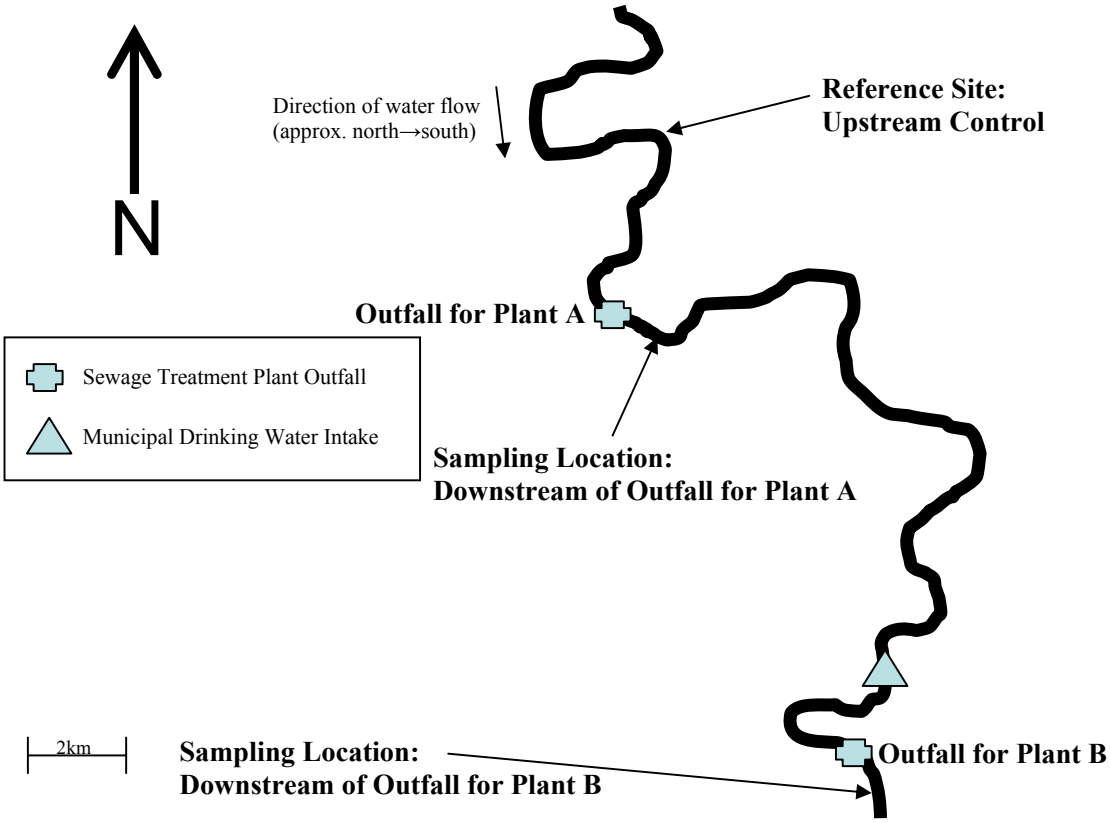


Figure 5.3 Locations of two municipal sewage treatment plant outfalls in a southern Ontario watershed (Grand River). Sites where wild fish were collected for *in vivo* SPME sampling in August 2007 are indicated.

5.3 Results and Discussion

5.3.1 Pre-study Considerations The determination of the optimal fiber coating is considered the first step in the development of a SPME method. PDMS fiber coating was selected for field and *in vivo* sampling as, being comprised of high-purity medical grade materials, this coating is considered biocompatible. Consequently, protein adsorption does not occur when PDMS biomaterial contacts living tissue,[39-41] which provides the added benefit that cleanup is not necessary after SPME extraction. In the present study, no bio-fouling was observed on any fibers after deployment in a variety of fish tissues. This represents a distinct advantage relative other sample preparation techniques such as pressured liquid extraction where further cleanup must be employed prior to LC-MS/MS analysis.[9] Also, as a liquid coating, PDMS does not have the disadvantage of competition or displacement effects observed with solid coatings.

In the present study, the SPME fibers used were constructed "in house", in consideration of both the size of fibers needed, and the relative cost involved in using each fiber only once. Generally speaking, commercially available fibers are expensive, and are not suitable for single-use and *in vivo* experiments. The relative standard deviation (RSD) for "in house" fiber-to-fiber reproducibility was determined to be below 5.5% for each of the 5 drugs evaluated in the equilibrium extraction experiments. This excellent reproducibility was complimented by the good linearity observed throughout both *in vitro* and *in vivo* studies reported in this paper, suggesting the PDMS-coated SPME fibers are appropriate for the study conditions employed.

The integrated desorption device was specifically designed for field sampling. This device was economical and flexible, and could be configured for any number of

vials with each vial independent of the others. The 54-vial plate can be re-used. When Parafilm® is used to cover and immobilize the vials in the plate, the device is portable and compact; attributes important for field studies at inaccessible sites. Preliminary trials found 1 h agitation (at 400 rpm) was adequate to desorb 99% of the analytes from the fiber. After desorption, an internal standard solution was added, and the entire device was placed into the LC-MS/MS instrument for automated analysis. It was not necessary to dry the desorption solution using nitrogen, or to re-condition the samples. The integrated desorption device demonstrated the advantages of high-efficiency, simplicity, and high-throughput.

Internal standard loading to an SPME fiber is an important step in order to obtain precise results.[42] The pre-loading process in this study was performed in aqueous solutions instead of the sample headspace. This approach was used to wet the surface of the fiber with a layer of water prior to any desorption. This wetting procedure kept the surface of the fiber from drying, which could potentially influence the fiber performance. Before the fibers were used for any experiments, one hour of immersion in methanol allowed any contaminants present to desorb off the fiber and into the methanol. Subsequent to the methanol immersion, immersion of the fiber for 1h in water not only removed methanol from the SPME coating, but also allowed the surface of the coating to be covered by a layer of water consist with the aqueous sample matrix.

An agarose gel spiked with drugs was chosen as a mimic system for an *in vitro* study based on the reasons described in Chapter 2. Briefly, agarose gels can prevent convection without significantly obstructing free diffusion.[43] Although an agarose gel is an imperfect model for living tissue, *in vitro* experiments can be very useful to find mass

transfer phenomena between SPME coatings and semi-solid matrices, and may yield relevant information for *in vivo* studies. Five target analytes were selected for the present study, based on previously published work demonstrating diltiazem, diphenhydramine, carbamazepine, and norfluoxetine (the primary metabolite of fluoxetine) were detected in fish tissue in screening experiments.[20] In the current study, the focus was not on screening compounds, but on developing a new analytical method demonstrating the feasibility of SPME techniques for *in vivo* laboratory and field sampling.

5.3.2 Kinetic Calibration Using Pre-equilibrium desorption. When a SPME fiber is inserted into tissue *in vivo*, the tortuosity (λ) of the molecular diffusion route may result in extensive periods of time required for equilibrium to be achieved. Such intervals could produce "wound effects" resulting from a fiber placed for an extensive periods of time in living tissues. Preferable, from an animal husbandry as well as a sampling efficiency perspective, is to use pre-equilibrium extraction to shorten sampling time.

When a SPME fiber is exposed to a semi-solid medium (*i.e*. most biological tissues), mass diffusion of an analyte from the sample matrix to the SPME polymer coating and from the surface of the fiber coating to its inner layers is considered to be the rate-determining step that slows down the process of reaching equilibrium [44]. This absorption process can be described as [24]

$$\frac{n}{n_e} = 1 - \exp(-at) \qquad \text{Equation 5.3}$$

where n is the extracted amount of analyte in the fiber coating after the exposure time t, n_e is the extracted amount of the target analyte at equilibrium, and a is the time constant.

Eq 5.3 is rearranged to eq 5.4,

$$n = [1 - \exp(-at)]n_e \qquad \text{Equation 5.4}$$

From eq 5.4, a plot of n versus n_e yields a straight line with a slope of $[1 - \exp(-at)]$ when the absorption conditions and pre-equilibrium extraction time are fixed. This process serves to generate a pre-equilibrium extraction concentration profile.

After a SPME liquid coated fiber is preloaded with an analyte, it is exposed to a tissue matrix and desorption of the analyte from the fiber occurs. The process of desorption can be expressed to eq 5.5 [26, 45],

$$\frac{Q - q_e}{q_0 - q_e} = \exp(-at) \qquad \text{Equation 5.5}$$

where Q is the remaining amount of analyte in the fiber at time t, q_0 is the preloaded amount of the analyte, and q_e is the remaining amount of analyte in the fiber at equilibrium. The term q_e is not considered negligible when K_{fs} is large and may be difficult to obtain when a long equilibrium time exists for *in vivo* tissue sampling. To solve this problem, eq 5.5 can be expressed as:

$$Q = [\exp(-at)]q_0 + [q_e - q_e \exp(-at)] \qquad \text{Equation 5.6}$$

From eq 5.6, a plot of Q versus q_0 yields a straight line with a slope of $\exp(-at)$ and a y-intercept of $[q_e - q_e \exp(-at)]$ when the pre-equilibrium desorption time and experimental conditions are constant. It produces a pre-equilibrium desorption concentration profile. Thus, the value of a can be calculated from the slope of the linear regression equation, and q_e is obtained from the value of the intercept.

The sum of the two slopes from eqs 5.4 and 5.6 should be 1 at any pre-equilibrium absorption/desorption time. This relationship can be used to verify isotropism

between absorption and desorption, while also indicating the possibility of SPME kinetic calibration. The value of the time constant or [1-exp(-*at*)] can be used to obtain the initial concentration of the analyte in a sample matrix by eqs 5.3 and 5.2. If a tissue density is known or can be determined, it can be used to convert ng/mL to ng/g as described previously in the Experimental Section. This strategy, called kinetic calibration using pre-equilibrium desorption, has been found suitable for field and *in vivo* sampling.

Concentration profiles were used to verify the isotropic behavior between absorption and desorption. The absorption or desorption times remained the same in spite of the diverse *in vitro* analyte concentrations in the gel when determining absorption, or the diverse pre-loaded amounts of analyte in the fiber when determining desorption. It was determined that *in vitro* equilibrium in extraction experiments in gel medium was around 200 min for the drugs selected for the present study. Thus, 20 min was chosen as the pre-equilibrium time to develop the new method, kinetic calibration using pre-equilibrium desorption. Table 5.2 summarizes the slopes of aborption and desorption from the concentration profiles at a fixed pre-equilibrium time (20 min) as well as the sum of the slopes from two lines, which is very close to 1. The sums of these slopes confirm that a nearly perfect isotropic relationship exists between absorption and desorption. Table 5.2 further provides the time constants calculated from the slopes, demonstrating rates of absorption and desorption are similar. In addition, this pre-equilibrium approach was also validated by accuracy, recoveries, which are listed in Table 5.2. The value of n_e can be obtained using eq 5.3, and then C_0 can be calculated from eq 5.2. The recovery can be determined using the calculated C_0 divided by the

spiked or real concentration in gel. Good accuracy suggests that the pre-equilibrium approach could be further applied for real sample matrices.

Table 5.2 Aborption and desorption slopes from concentration profiles, sum of slopes, time constants derived from slopes, and recovery.

Compound	Slope*		Sum of slopes	Time constant, a, (min^{-1}) **		Recovery (%)
	Absorption	Desorption		Absorption	Desorption	
	1-exp(-at) (R^2)	exp(-at) (R^2)				
Diltiazem	0.120 ± 0.006 (0.997)	0.844 ± 0.031 (0.995)	0.964	0.0064 ± 0.0003	0.0085 ± 0.0019	92 ± 5
Diphenhydramine	0.182 ± 0.011 (0.998)	0.763 ± 0.050 (0.999)	0.945	0.0100 ± 0.0007	0.0135 ± 0.0033	90 ± 7
Carbamazepine	0.361 ± 0.026 (0.999)	0.693 ± 0.040 (0.999)	1.054	0.0224 ± 0.0021	0.0183 ± 0.0029	93 ± 4
Fluoxetine	0.253 ± 0.010 (0.990)	0.744 ± 0.040 (0.995)	0.997	0.0146 ± 0.0007	0.0148 ± 0.0027	95 ± 8
Norfluoxetine	0.298 ± 0.014 (0.998)	0.735 ± 0.032 (0.996)	1.033	0.0177 ± 0.0010	0.0154 ± 0.0022	96 ± 6

*four data points were used to obtain each slope.

**time constant uncertainties were calculated using propagation of uncertainty from slopes.

5.3.3 Application to Real Sample Matrices. To evaluate the use of the kinetic calibration method for field and *in vivo* tissue sampling, this technique was applied to quantify the selected pharmaceuticals in laboratory-reared and wild-captured fish using LC-MS/MS.

5.3.3.1 Distribution Constants (K_{fs}). Since kinetic calibration is a derivation of the equilibrium standardization technique, the distribution constants should be known in order to estimate initial concentrations of analyte in the sample matrix. The distribution constant for the direct extraction mode can be calculated from the following dependence obtained from eq 5.7[21],

$$K_{fs} = \frac{n_e V_s}{V_f (C_0 V_s - n_e)} \qquad \text{Equation 5.7}$$

Table 5.3 lists the distribution constants for the pharmaceuticals used in the present study, as determined in Krebs-Ringer solution. These distribution constants can only be used to calculate the free or unbound drug concentrations within a tissue; all concentrations obtained from SPME in the current study are free concentrations in the dorsal-epaxial muscle of fish. The total concentrations (free plus bound drugs) can be obtained from liquid extraction. Table 5.3 also provides the CAS number, *log* K_{ow} values and percentage for theoretical removal during wastewater treatment for the pharmaceuticals used in the present study. The higher the *log* K_{ow} value, the less polar (or water soluble) the compound. PDMS, the fiber coating used in this study, is a non-polar material, which has a high affinity for less polar compounds. Interestingly, with the PDMS fiber coating, the measured K_{fs} values are roughly proportional to *log* K_{ow} values and theoretical removal efficiency in wastewater treatment facilities.

Table 5.3 Pharmaceuticals, CAS numbers, *log* K_{ow}, percentage for theoretical removal during wastewater treatment for the pharmaceuticals, and distribution constants (K_{fs}) between the SPME fiber coating and Krebs-Ringer solution (n = 3).

Compound	CAS #	*log* K_{ow}*	Removal in Wastewater Treatment (%)*	K_{fs}
Diltiazem	033286-22-5	2.79	3.81	439 ± 9
Diphenhydramine	000058-73-1	3.11	8.76	564 ± 12
Carbamazepine	000298-46-4	2.25	2.96	30.0 ± 0.1
Fluoxetine	000002-84-9	4.65	32.4	737 ± 38
norfluoxetine	56161-73-0	N/A	N/A	631 ± 15

*KOWWIN, v1.67 estimate, Episuite, USEPA

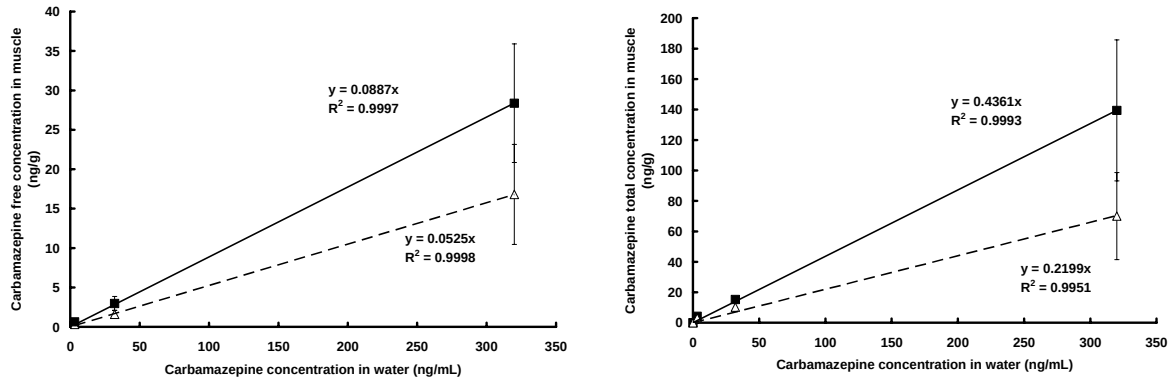
5.3.3.2 *In Vivo* Desorption and Extraction. *In vivo* SPME desorption and extraction experiments were employed to determine the free concentration of a drug in living muscle since SPME techniques remove insignificant quantities of target analyte from sample matrices.[21] Table 5.4 presents slopes for desorption concentration curves and values of the time constants obtained *in vivo* from free-moving laboratory rainbow trout. Excellent linearity was observed using pre-equilibrium desorption of pre-loaded internal analyte standards. Figure 5.4 (A) demonstrates the correlations between free or unbound carbamazepine concentrations in fish muscle and aqueous concentrations in the exposure medium. Excellent linearity ($R^2 \geq 0.99$) was observed for all of the drugs (fluoxetine, norfluoxetine and carbamazepine) tested in the muscle of free-moving fish, confirming the reliability of SPME for *in vivo* tissue sampling.

Table 5.4 Slopes from *in vivo* desorption concentration profiles, time constants (*a*), limits of detection (LODs), and limits of quantitation (LOQs) for *in vivo* SPME analysis in rainbow trout (*Oncorhynchus mykiss*) muscle.

Compound	Slope of desorption* / exp(-*at*) (R^2)	*a* (min^{-1}) **	LOD (pg/g)	LOQ (pg/g)
Diltiazem	0.610 ± 0.059 (0.999)	0.025 ± 0.005	0.2	0.67
Diphenhydramine	0.713 ± 0.096 (0.998)	0.017 ± 0.007	3	11
Carbamazepine	0.554 ± 0.070 (0.998)	0.029 ± 0.006	50	170
Fluoxetine	0.725 ± 0.085 (0.993)	0.016 ± 0.006	8	28
Norfluoxetine	0.689 ± 0.062 (0.999)	0.019 ± 0.004	2	5.3

*four data points were used to obtain each slope (n = 3).

**time constant uncertainties were calculated using propagation of uncertainty from slopes.



(A) (B)

Figure 5.4 Relationships between carbamazepine free or total concentrations in fish muscle and in exposure water. Fish were exposed to carbamazepine for 7 d (■) or 14 d (Δ) at nominal concentrations of 0 (control), 3.2, 32.0, or 320.0 ng/mL of carbamazepine. *In vivo* SPME sampling (A) and liquid extraction (B) of bioaccummulated analytes were performed for carbamazepine (n = 3).

The rate of absorption or desorption can be judged by the value of the time constant, *a*, which is affected by several factors including the structure and properties of the analyte, components of the sample matrix, the properties and dimension of the fiber coating, agitation speed, and temperature. The agitation speed changes the thickness of the boundary layer, which directly affects the time constant. The values of time constant in Table 5.2 are different from those in Table 5.4. It indicates the rate of desorption or absorption is influenced by the different sample matrix as well as sampling conditions. Since the *in vivo* sampling situation is difficult to mimic using the *in vitro* one, it is necessary to utilize desorption to calibrate absorption.

Table 5.4 also presents limits of detection (LODs) and limits of quantitation (LOQs) obtained using ten fibers inserted in the muscle of free-moving fish that were not

exposed to any pharmaceutical drugs. LOD was calculated as three times the standard deviation in the background signal observed from ten SPME samplings in the tissue, and LOQ was equal to ten times the standard deviation of repetitive measurements. SPME can lower LOD and LOQ through two mechanisms. First, SPME functions to pre-concentrate the analyte from the sample matrix to the fiber coating, especially in the case of a large K_{fs}. Secondly, as the PDMS coating used in this study is biocompatible, no fouling was observed on the surface of the coating after *in vivo* sampling, which minimized the matrix effect for the LC-MS/MS instrumental analysis resulting in a larger signal to noise ratio.

5.3.3.3 Determination of the Protein Binding of Drugs in Muscle. Prior to obtaining drug protein binding values in muscle, the total concentration of a drug has to be known. Liquid extraction was used to measure the total drug concentrations in muscle. Based on a modification of a previously reported method, methanol:water:formic acid (95:5:0.1, *v/v/v*) was chosen to extract drugs from muscle tissue [20]. This solution not only provided relatively high recoveries for the determined drugs, but also was compatible with the components of the HPLC mobile phases, which can eliminate the additional step of reconstitution after extraction. Recovery data are listed in Table 5.5. Figure 5.4 (B) demonstrates the correlations between total carbamazepine concentrations bioaccumulated in fish muscle and aqueous concentrations in the exposure medium. Excellent linearity ($R^2 \geq 0.99$) was observed for all of the drugs (fluoxetine, norfluoxetine and carbamazepine) tested in the muscle, which indicates the robustness of this liquid extraction approach for the determination of total concentrations. Compared to LE,

SPME offered a better linearity, which indicates that SPME was more robust with less matrix effects.

Table 5.5 Recovery of analytes following liquid extraction, muscle protein binding (following 7 and 14 d *in vivo* aqueous exposure), and plasma protein binding (literature values) for selected pharmaceutical compounds evaluated in this study.

Compound	Liquid extraction recovery (%)*	Muscle protein binding (%) (7 days)	Muscle protein binding (%) (14 days)	Plasma protein binding (%) (literature)
Diltiazem	53 ± 8	N/A	N/A	81
Diphenhydramine	50 ± 7	N/A	N/A	98
Carbamazepine	90 ± 9	79 ± 3	76 ± 7	76
Fluoxetine	52 ± 5	99.5 ± 0.5	99.2 ± 0.6	95
Norfluoxetine	47 ± 5	99.7 ± 0.4	N/A	N/A

n = 4

Bioaccumulation factor (BAF) can be calculated as the ratio of the concentration of a substance in an organism's tissue to its concentration in the organism water [46]. The value of BAF is the slope of the curve between the measured total concentrations in the tissue and the concentrations in water. One example for BAF is provided in Figure 4 (B). The BAF for carbamazepine after 7 and 14 d exposure are 0.44 and 0.22, respectively. It can be concluded that carbamazepine does not significantly bioaccumulate in fish dorsal epaxial muscle. This finding is attributable to the hydrophobicity of carbamazepine, which has a relatively low *log* K_{ow} and K_{fs} as shown in Table 5.3. Interestingly, both free

and total concentrations of carbamazepine in muscle after 14 d of exposure were lower than those after 7 d, suggesting some capacity for carbamazepine metabolism was up-regulated over time. In contrast, significant bioaccumulation was observed for fluoxetine. It was found that the free fluoxetine concentrations in the living fish muscle following 7 and 14 d exposure were only 0.30 and 0.65 times that of the aqueous concentrations in the exposure medium, respectively. The BAFs for fluoxetine in muscle following 7 d and 14 d of exposure were around 62 and 84, respectively. The bioaccumulation over time in fish muscle can be explained by the higher *log* K_{ow} value of fluoxetine. The higher *log* K_{ow} value a molecule has, the higher BAF. The correlation between *log* K_{ow} and BAF supports previous studies suggesting use of fish for fluoxetine biomonitoring studies [10]. This bioaccumulation of fluoxetine may produce acute toxicity, as seen in the present study where immature rainbow trout did not survive in water with concentrations > 3.2 ng/mL beyond 7 d. Ongoing studies in our laboratory are investigating bioaccumulation and tissue distribution of these drugs in other organs, some of which (liver, brain) are known biochemical targets of these compounds, and bioaccumulate higher concentrations than muscle tissue.[10]

The percentage of drug binding to muscle proteins (*MPB*%) is calculated from the free and total concentration of drug:

$$MPB\% = \frac{C_{total} - C_{free}}{C_{total}} \times 100 = (1 - \frac{C_{free}}{C_{total}}) \times 100 \qquad \text{Equation 5.8}$$

where C_{total} is the total concentration of drug in muscle and C_{free} is the free concentration of drug in muscle.

Considering that the total drug concentration is directly proportional to the slope of the extraction curve using LE (S_{LE}) and the free concentration is directly proportional to the slope of extraction curve using SPME (S_{SPME}), eq 5.8 becomes:

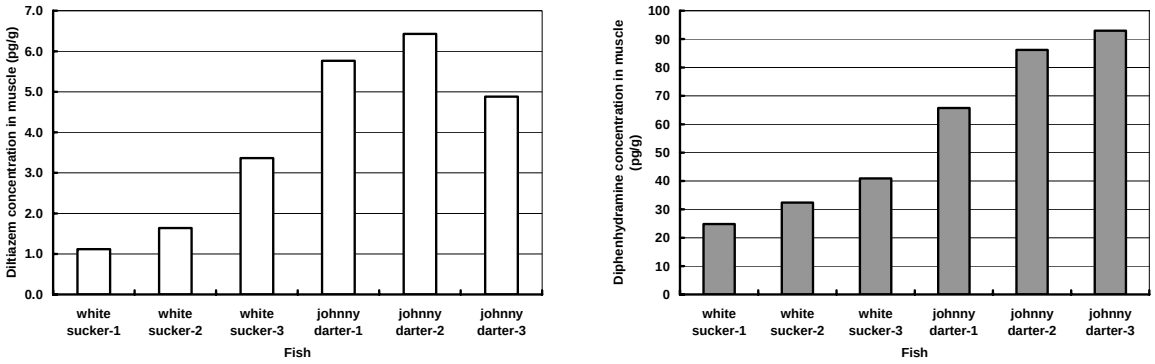
$$MPB\% = (1 - \frac{S_{SPME}}{S_{LE}}) \times 100 \qquad \text{Equation 5.9}$$

Figure 5.4 demonstrates an example of generating the slopes to calculate *MPB*%. Eq 5.9 was applied for the determination of protein binding in fish muscle for the two drugs plus one metabolite, with the results presented in Table 5.5. We are unaware of previous reports determining protein binding of pharmaceuticals in muscle tissue. Thus, the *MPB*% is compared to the percentage of plasma protein binding (*PPB*%) from literature [47-50]. The values of *MPB*% from the present study are close to *PPB*%, especially for carbamazepine. Comparisons can be made on the basis of two variables, the protein content of different tissues and the molecular hydrophobic property of the drug. Although the muscle tissue contains more protein than does plasma, differing protein percentages between these tissues do not influence the *MPB*% of carbamazepine due to its less hydrophobic nature. However, the different protein contents allows greater *MPB*% of fluoxetine than *PPB*% due to the more hydrophobic nature of this compound. Interestingly, the values of *MPB*% from fish at 7 d exposure time were higher than those following 14 d of exposure, further suggesting some capacity for drug metabolism was up-regulated over time. Both free and total concentrations are considered to be important from a toxicological perspective. Free drugs are bioactive, but those bound to proteins can serve as a reservoir, an important consideration which may be directly related to the half-life of a drug. A unique aspect of fluoxetine, which may be a function of protein binding, is the long time needed to reach steady-state blood plasma concentrations, and

the likewise long period for total clearance.[51] Based on eq 5.8 or 5.9, the total concentration of a drug in muscle can be calculated using a determined free concentration from SPME fiber and the known value of protein binding. Consequently, the *in vivo* SPME approach developed herein can have potentially wide-reaching applications in determining free and total concentrations simultaneously in a living tissue using the known protein binding value.

5.3.3.4 Application for Field and *In Vivo* Sampling. Wild fish were collected from below sewage treatment plant effluent diffusers at locations in the river containing the highest available percentage of effluent as determined by conductivity readings (Figure 5.3). At Sewage Treatment Plant A, fish were collected in an area of 55 % effluent (*v/v*), while at Sewage Treatment Plant B, samples were collected from areas comprised of a range of concentrations from 15-42% effluent (*v/v*). Of three field sampling locations, the site downstream of Sewage Treatment Plant B contained the most bioaccumulated pharmaceutical compounds, which is consistent with previous, unpublished field studies demonstrating high concentrations of these compounds in surface water samples (D. Bennie, National Water Research Institute, Burlington, ON). In contrast, all 5 pharmaceuticals selected for analysis in this study were below the LOD at Sewage Treatment Plant A, which serves a smaller population and has recent upgrades which may have enhanced removal of these bioactive compounds. Carbamazepine, fluoxetine, and norfluoxetine were also under the LOD within the muscle tissues for fish collected below Sewage Treatment Plant B. Only the calcium channel blocker diltiazem and the antihistamine diphenhydramine were detected above the LOQ in fish inhabiting the

receiving environment below Sewage Treatment Plant B (Figure 5.5). It was observed that pharmaceutical concentrations in johnny darter (*Etheostoma nigrum*) were higher than those in white sucker (*Catostomus commersoni*). As the small-bodied johnny darter were sexually mature adults defending home ranges, their residency is more permanent than the sexually immature white sucker, which may be more transient in their habitat fidelity. The total concentrations of diphenhydramine were found to be higher than those of diltiazem. This observation agrees well with the reported literature [20]. Further details of plant locations, the fish species, and operating characteristics collected in waters influenced by each plant are provided in Table 5.6.



(A) (B)

Figure 5.5 Free diltiazem (A) and diphenhydramine (B) concentrations in the muscle of wild fish collected below Sewage Treatment Plant B using SPME for field *in vivo* sampling.

Table 5.6 Description of *in vivo* field sampling sites and fish species collected adjacent two municipal sewage treatment plant outfalls in August, 2007.

Sampling location	Fish species*	Fish length (cm)
Reference Site (upstream) (43°30'19"N; 80°28'08"W)	carp	11.0
	white sucker	10.0
	smallmouth bass	8.0
Downstream of Sewage Treatment Plant A *Conventional Activated Sludge With Continuous Phosphorus Removal; Serving Population of 164,000* (43°28'39"N; 80°29'05"W)	carp-1	13.0
	carp-2	10.0
	carp-3	11.0
	carp-4	13.0
	white sucker-1	15.0
	white sucker-2	16.0
	smallmouth bass	11.0
Downstream of Sewage Treatment Plant B *Conventional Activated Sludge With Continuous Phosphorus Removal; Serving Population of 105,100* (43°23'36'N; 80°24'45"W)	white sucker-1	7.0
	white sucker-2	8.2
	white sucker-3	6.9
	johnny darter-1	5.0
	johnny darter-2	5.2
	johnny darter-3	5.4

*carp (*Cyprinus carpio*), white sucker (*Catostomus commersoni*), smallmouth bass *(Micropterus dolomieu*), and johnny darter (*Etheostoma nigrum*)

SPME is suitable for i*n vivo* sampling because it allows for the possibility of non-lethal sampling of even small-bodied animals, with minimal perturbation to the system under study as fewer animals are used relative to other techniques. Compared to microdialysis, a commonly used *in vivo* technique, SPME offers several advantages. First, SPME is simpler, easier and more convenient. Microdialysis requires a cumbersome syringe or osmotic pump during sampling.[52] Secondly, SPME is more sensitive than microdialysis. SPME polymers can pre-concentrate the target analytes from the sample matrix to the coating, especially when K_{fs} is large. With microdialysis, a concentration gradient allows the analyte to passively diffuse through the membrane from the sample matrix to the inside of microdialysis probe. A typical relative recovery of microdialysis for *in vivo* sampling is approximately 20%. Third, although microdialysis has been applied *in vivo* for fish sampling, the experiment had to be performed on a fish surgical table [53, 54], indicating field applications would be difficult. In addition, SPME is ideally suited for field sampling as the PDMS coating is fully supported by the stainless steel wire, which can be left *in situ* during *in vivo* sampling. In contrast, a microdialysis probe is less robust since when sampling in a semi-solid tissue, caution has to be taken to minimize the potential for probe damage. This research represents an important step toward developing powerful biomedical, pharmaceutical, forensic and toxicological applications, as the advantages of SPME would be directly useful for analysis of field and *in vivo* biological samples.

5.4 Conclusion and Addendum

5.4.1 Conclusion. Previous field studies utilizing solid-phase microextraction (SPME) predominantly focused on volatile compounds in air or water. Earlier *in vivo* sampling studies utilizing SPME were limited to liquid matrices, namely blood. The present study has expanded SPME technique to semi-solid tissues under lab and field conditions through the investigation of both theoretical and applied experimental approaches. Pre-equilibrium extraction was used to shorten *in vivo* sampling time. The use of pre-equilibrium desorption rates are proposed as a means to calibrate pre-equilibrium extractions. Excellent linearity was found between the extracted amounts by SPME in the muscle of living fish and the waterborne concentrations of several pharmaceuticals. A simple SPME method for the simultaneous determination of free and total analyte concentrations in a living tissue is also described. The determined protein binding values are close to those from literature, which validates this developed method. The utility of *in vivo* SPME sampling under field conditions was evaluated in wild fish collected from a number of different river locations under varying degrees of influence from municipal wastewater effluents. Diphenhydramine and diltiazem were detected in the muscle of fish downstream of a local wastewater treatment plant. Based on this study, SPME techniques have demonstrated several important advantages for laboratory and field *in vivo* sampling. The development of a rapid, robust, easy to deploy technique which combines sampling, extraction and concentration into one step is a potentially important tool for use *in vivo* field-based sampling.

5.4.2 Addendum. The submitted manuscript had been significantly revised and shortened to three-fourths of the present length of this chapter. Only Table 5.3 with

modification, Table 5.4, and Figure 5.4 remained in the submitted manuscript. Also, the whole section of Conclusion does not appear in the submitted manuscript.

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Chapter 6

Comparison of Microdialysis with Solid-Phase Microextraction for *In Vitro* and *In Vivo* Studies

6.1 Preamble and Introduction

6.1.1 Preamble. This chapter has been accepted as the paper,

Simon Ningsun Zhou, Gangfeng Ouyang, Janusz Pawliszyn, Comparison of microdialysis with solid-phase microextraction for in vitro and in vivo studies, *Journal of Chromatography A*, In Press, 2008.

The contributions of Gangfeng Ouyang, the co-author, involved suggestions for both the written manuscript and response-to-comments. This chapter is reprinted with permission from Journal of Chromatography A (Copyright 2008 Elsevier).

I, Gangfeng Ouyang, authorize Simon Ningsun Zhou to use the material for his thesis.

Signature: 

6.1.2 Introduction. Carbofuran, propoxur, carbaryl and aldicarb belong to carbamate pesticides that are widely used in agriculture. The pesticides have systemic activity when they are applied to the soil. They can enter the roots of a plant and travel to the leaves, where they can then poison insects that feed on the leaves. Their property of chemical stability allows them to be highly persistent in raw milk, water, soil, plants and the atmosphere.[1] They are hazardous by dermal, oral or subcutaneous exposure routes. The severe toxicity caused by these pesticides has raised public awareness of the consequences to ecosystem and human health of their presence in environment, even at trace levels. In view of these facts, the development of a fast and accurate pesticide sampling technique for quantitative analysis is of key importance.

A number of techniques have been employed for measuring pesticides for *in vitro* study, including solvent extraction,[2] single-drop microextraction,[3] hot water extraction,[4] and solid-phase microextraction (SPME).[5-7] SPME is a valuable sampling technology, which is fast, simple, solvent-free or less, and combines sampling, sample preparation and pre-concentration to the extraction phase into one single step.[8,9] It has been widely applied in environmental monitoring, industrial hygiene, process monitoring, clinic, forensic, food, flavor, fragrance and drug analyses, in laboratory and on-site analyses for *in vitro* and *in vivo* study.[10, 11] So far, the SPME technique has been applied for carbamate analysis in a variety of samples such as water,[12] food,[13] serum,[14] human whole blood and urine.[15] Like SPME, microdialysis (MD) also has the advantage of low organic solvent consumption, which is environmentally friendly. MD was applied for the determination of glucose, ascorbic acid, or sulfonamide residues in juice or milk for *in vitro* experiments.[16, 17] Both MD and SPME have the characteristics including as less

destruction and minimal perturbation to the system under *in vivo* study compared to other methods.

To our knowledge, MD has not been reported for pesticide analysis in juice and milk samples for *in vitro* study. For the application of direct immersion SPME to pesticide analysis in the juice, the developed methods needed a tedious centrifugation or filtration of each sample.[18] The reported methods using SPME for the determination of pesticides in milk needed a labor-intensive procedure of protein precipitation.[6] Although a hollow fiber membrane-protected SPME was applied to the analysis of pesticides in milk, it was labor-intensive because automation was not designed for it.[5] Gas chromatographic determination of pesticide residues in royal jelly was developed using liquid phase extraction,[19] which made the sample preparation too complicated. Moreover, there are no reports using MD for *in vivo* pesticide sampling in a plant. The reported SPME method was applied only for semi-quantitative analysis *in vivo* study in plants.[20] In addition, a comparative study for MD and SPME was not investigated.

The aim of this work was to develop and compare two microextraction methods, MD and SPME, to determine pesticide levels for the purpose of *in vitro* and *in vivo* study. For *in vitro* study, when MD was applied to the complex sample matrices (juice, milk and jelly), sample preparation was totally unnecessary. However, automation was not achieved for MD. By comparison, combination of a newly-designed membrane filtration device with SPME led to a little sample pre-treatment, less labor intensity and automation for juice and milk samples. To our knowledge, the design for membrane filtration prior to direct immersion SPME is reported for the first time. This design allowed batch analysis and high throughput sampling without intensive manipulation such as filtration by

centrifugation or protein precipitation. Both developed methods offered accurate and precise results and met the requirement of the Food and Agriculture Organization (FAO). This work is also the first to employ MD and fully automated SPME for pesticide analysis in jelly. In the case of *in vivo* study, addition of internal standards to the extraction phase was applied for *in vivo* tissue sampling. By using retrodialysis for MD or in-fiber standardization technique for SPME, quantitative analysis was achieved to measure the pesticide concentrations in the leaves of the living jade plant (*Crassula ovata*). By comparison, for *in vivo* study, SPME was more sensitive, had wider application, was easier to perform, and was more convenient for field sampling than MD.

6.2 Experimental

6.2.1 Chemicals and Supplies

Carbofuran, deuterated carbofuran (carbofuran-d_3), propoxur, carbaryl, aldicarb and promecarb were supplied from Riedel-de Haën, with greater than 99.5% purity. Carbaryl-$^{13}C_6$ was from Cambridge Isotope Labs (Andover, MA, USA). Acetonitrile and methanol (HPLC grade) were obtained from EM science (Gibbstown, NJ, USA). Stock standard solutions (1000 mg/L) of five pesticides were prepared in acetonitrile and stored at 0–4 °C in the dark. Working solutions were prepared by a series of tenfold successive dilution for quantitative calibration and sample preparation. For small-volume analysis, a polyethylene insert, which has a volume of 200 μL, was positioned in a 2-mL vial. The fiber (Supelco, Oakville, ON, Canada), 60 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) for *in vitro* study, was conditioned at room temperature in acetonitrile for 5 min in a static mode and then in nanopure water for 30 min with 500 rpm agitation

prior to use for *in vitro* experiments. Self-made Polydimethylsiloxane (PDMS) fibers were employed for single-use *in vivo* experiments. The PDMS fiber making procedure was as follows: PDMS hollow fiber membrane tubing (Helixmark, Carpinteria, CA, USA) was cut into 1.0 cm lengths. Each PDMS segment was placed over one stainless steel wire, the plunger of a 10 μL microliter syringe (Hamilton, Nevada, USA). The volume of the coating was around 2.5 μL. Before the PDMS coatings were mounted, wires were sonicated in deionized water and acetone to ensure their cleanliness. The fibers were conditioned in methanol for one hour in a static mode and then in nanopure water for another hour prior to use. The orange juice, milk, jelly powder and jade plants (*Crassula ovata*) were purchased from local stores.

6.2.2 Preparation of Samples for *In Vitro* Experiments

The orange juice and milk were directly spiked with pesticides at different concentration levels before extraction. Jelly was made from the jelly powder according to the instructions on the product. Briefly, about 80 g jelly powder was added to 250 mL boiling water. The mixture was heated and stirred until the powder was completely dissolved. After 250 mL orange juice was added, the mixture was stirred again. Then 1.9 mL aliquots of the hot mixture were transferred to 2 mL vials containing 0.1 mL aqueous solutions with pesticides at different concentration levels. The final solutions were vortexed to allow homogenization, and then the vials containing the solutions were placed in a freezer for 15 min to allow coagulation. After they were taken out and returned to room temperature for two hours, the jelly samples were ready for MD or SPME sampling.

6.2.3 Microdialysis for *In Vitro* Sampling

Juice or milk was directly transferred into a 10-mL vial without any pretreatment for MD sampling, as shown in Figure 6.1. A syringe pump described above with 1-mL syringe (Hamilton, NV, USA) was employed to deliver the perfusate, nanopure water, methonal or acetonitrile. The perfusate flow rate was set at 2.0 μL/min. The linear MD probe with 5 mm membrane (BioAnalytical Systems, IN, USA) was immobilized by passing it through the septum of the 10-mL vial. The membrane was immersed in a 9 mL aliquot of the solution, juice or milk with or without spiked pesticides. The dialysate was collected in the insert of the container. The sampling time was 15 min with an agitation speed of 500 rpm. Then, 30 μL of 10 μg/L promecarb aqueous solution, the internal standard for LC-MS/MS, which is used to compensate for the variation of injection volume, was added to the desorption solution in the 200 μL insert. After the solution was vortexed, a 20 μL aliquot of final solution was automatically injected into LC-MS/MS for quantitative analysis.

In the case of pesticide sampling in jelly, the setup was similar to the above with modification. A concentric type probe with 10 mm membrane (BioAnalytical Systems, IN, USA) was directly inserted into the jelly medium in 2 mL vial. Water as the perfusate with a sampling time of 25 min and no agitation were applied for this extraction procedure. An aliquot of 50 μL 10 μg/L promecarb solution in acetonitrile, the internal standard for LC-MS/MS, was added to the dialysate. After the solutions were vortexed, they were ready for LC-MS/MS analysis.

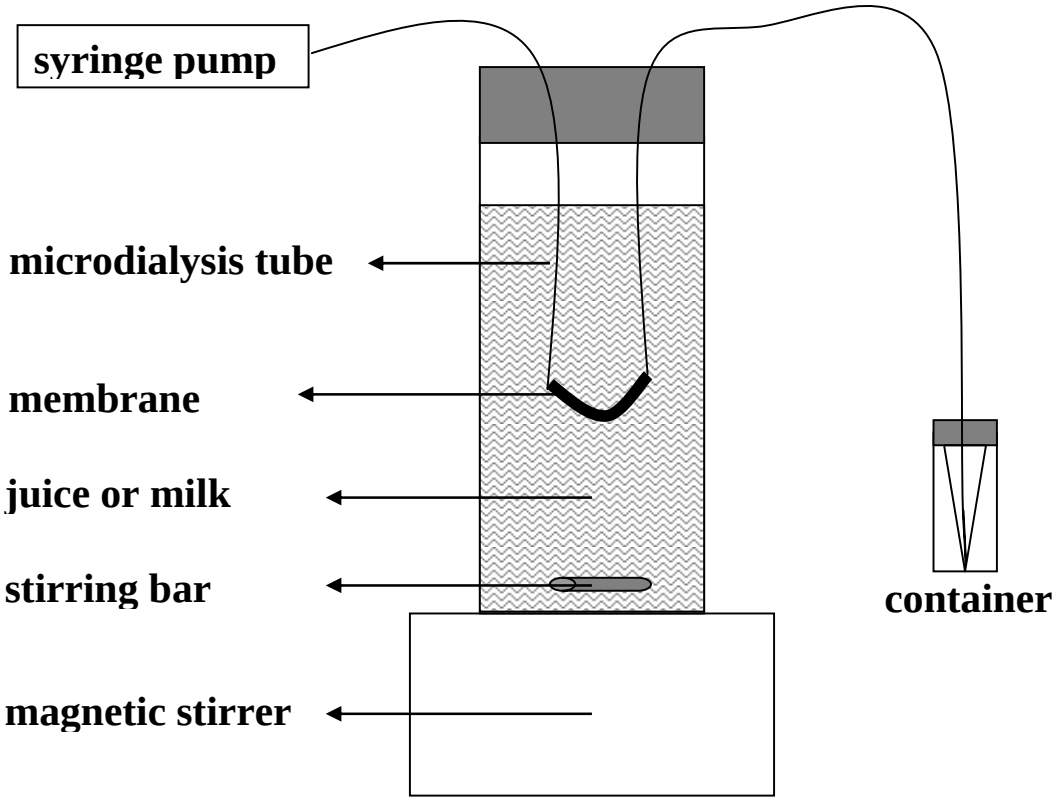


Figure 6.1 Schematic diagram of the microdialysis *in vitro* sampling device.

6.2.4 Solid-Phase Microextraction for *In Vitro* Sampling

Prior to SPME for juice and milk matrices, a membrane filtration device (Figure 6.2) was employed for the sample pre-treatment. 0.45 μm × 47 mm nylon-66 membrane (Supelco, Oakville, ON, Canada) was attached to the tube as soon as the bottom of 1.5 mL microcentrifuge tube (Fisher Scientific, Ottawa, ON, Canada) was melted out on a 400 ºC hot plate. Aliquots of 50μL acetonitrile solutions containing different concentration levels of carbofuran, propoxur, carbaryl and aldicarb were added to a series of 10-mL vials; acetonitrile was then evaporated in the fume hood. Aliquots of 7.5 mL

sample solutions, juice or milk, were added to the series of 10-mL vials. The mixtures were vortexed, and then allowed to stabilize for 30 min. Each 1.5 mL tube with the membrane on the bottom was put inside each 10 mL vial, and then the vials were capped. After the spiked samples in the vials were shaken in the SK-300 shaker (Medline Scientific Limited, Oxfordshire, UK) at 100 rpm overnight, 0.1 mL solution in each tube was transferred to a 200 μL insert in the 2 mL glass vial. The 2 mL glass vials with the inserts inside were put in a CTC CombiPal autosampler (Zwingen, Switzerland) using the associated Cycle Composer software (Version 1.4.0) for SPME with 20 min extraction time in a static mode at room temperature (25 ºC). After each extraction, the analytes on the fiber were desorbed in a 200 μL insert containing 50 μL acetonitrile for 5 min in a static mode. Prior to the next extraction, the fiber was conditioned at room temperature in nanopure water for 10 min while being agitated at 500 rpm, in order to remove any organic solvents on the fiber surface. Then, 50 μL of 10 μg/L promecarb aqueous solution was added to the desorption solution. LC-MS/MS injection was followed as described above.

For pesticide sampling in jelly, the setup was similar to the above with modification. No sample preparation was involved before SPME extraction. The SPME fiber was directly exposed to the jelly matrix in a 2 mL vial. The sampling time was 25 min and no agitation was applied for this extraction procedure.

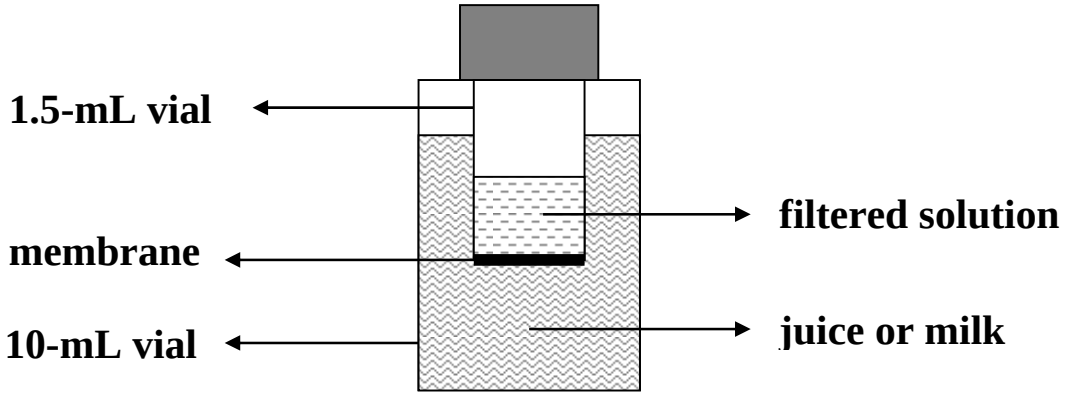


Figure 6.2 Schematic diagram of the membrane filtration device prior to SPME sampling.

6.2.5 *In Vivo* Simulation for Microdialysis Sampling Using a Gel Medium

Agarose gel was used to simulate living tissue sampling for both microdialysis and solid-phase microextraction before *in vivo* experiments were designed. Agarose gels (1% w/v) with a series of spiked non-isotope-labelled pesticide concentrations were used for *in vitro* absorption and desorption experiments to verify isotropism and kinetic calibration. The gel making process was as follows: 0.5 g agarose (Agarose 15, BDH Chemicals Ltd., Poole, England) was mixed with 50 mL phosphate-buffered saline, pH 7.4 (PBS), in a beaker. After the heterogeneous mixture was heated and became transparent and homogeneous, an aliquot of a 1.9-mL hot solution was transported into a 2-mL vial, which contained 100 μL PBS aqueous solution spiked with certain amounts of pesticides. The 2-mL vial with the mixture inside was capped and vortexed to allow homogenization. Finally, the mixture was cooled to ambient temperature, yielding a semisolid gel. The agarose gels were used for further experiments after two hours.

Carbofuran-d_3 and carbaryl-$^{13}C_6$ were used as the internal standards for the experiments of *in vivo* simulation using the agarose gel and *in vivo* sampling using a jade

plant. A series of perfusates were made from PBS (pH 7.4) aqueous solutions containing carbofuran-d_3 and carbaryl-$^{13}C_6$ at the concentrations of 0.05, 0.10, 0.25, or 0.50 μg/mL. A series of the agarose gel matrices also contained the same concentrations of non-isotope-labelled carbofuran and carbaryl. A concentric type probe with 10 mm membrane was directly inserted into the gel in the 2 mL vial. A model 100 digital syringe pump was employed to deliver the perfusate with the flow rate of 2.0 μL/min at room temperature. The sampling time was 20 min for each sample. Then, 40 μL of 10 μg/L promecarb acetonitrile solution was added to each dialysate solution. After vortexed, the mixtures were ready for LC-MS/MS separation and quantitative analysis.

6.2.6 *In Vivo* Simulation for Solid-phase Microextraction Sampling Using a Gel Medium

The process of making the agarose gel was the same as mentioned above. The conditioned PDMS fibers were exposed in the vials containing the PBS (pH 7.4) aqueous solution with carbofuran-d_3 and carbaryl-$^{13}C_6$ concentrations of 0.25, 0.50, 1.00, or 2.00 μg/mL for one hour, to perform internal standard preloading. Then the individual fibers were exposed to the vials containing the agarose gel with carbofuran and carbaryl pesticide concentrations of 0.25, 0.50, 1.00, and 2.00 μg/mL for 20 min, to perform absorption and desorption simultaneously.

After absorption and desorption for 20 min, the chemicals absorbed and remaining in the individual PDMS fiber were desorbed to 75 μL acetonitrile in a 200 μL insert for one hour under a static mode. Then, 75 μL of 10 μg/L promecarb aqueous solution, the internal standard for LC-MS/MS to compensate for the variation of injection volume, was

added to the desorption solution in the 200 μL-insert. After the solution was vortexed, a 20 μL aliquot of final solution was automatically injected into LC-MS/MS for quantitative analysis.

6.2.7 *In Vivo* Sampling Using Microdialysis

The jade plants with pesticide soil-application were employed for *in vivo* sampling. 0.1 g of two pesticides (Chem Service, West chester, PA, USA), carbofuran and carbaryl, were weighed and mixed. The mixed fine particles were spilled onto the surface of the soil. Then water was sprayed to allow the solid pesticides to gradually dissolve and diffuse into the soil. Around 20-mL water was applied to each plant every five days.

Figure 6.3 shows the setup of *in vivo* sampling using MD, which was employed to sample the left leaf of the jade plant. After the holes were punched using a 23 gauge hypodermic needle in the sampling locations, the concentric MD probe was inserted. A series of perfusates were made from PBS (pH 7.4) aqueous solutions containing carbofuran-d_3 and carbaryl-$^{13}C_6$ with the concentrations at 0.05, 0.10, 0.25, and 0.50 μg/mL, to sample the positions 1, 2, 3 and 4, respectively. The sampling time was set at 20 min. The sample preparation after the *in vivo* sampling was the same as that of MD sampling in the gel for *in vivo* simulation mentioned above.

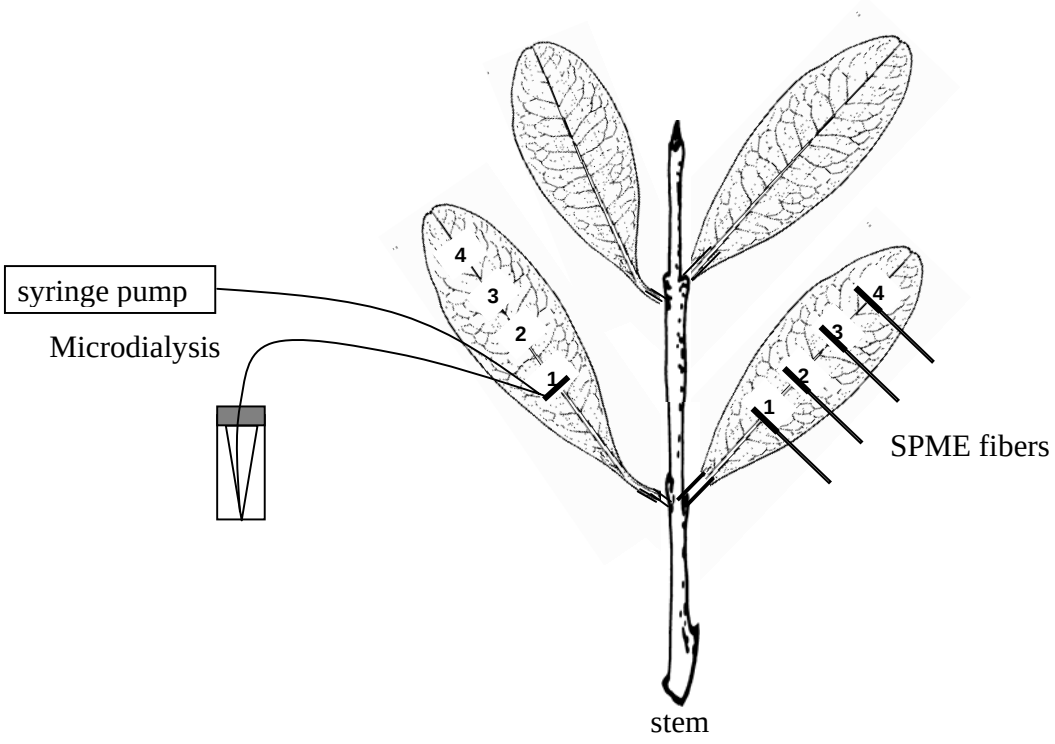


Figure 6.3 Setup for comparison of MD with SPME for *in vivo* sampling

6.2.8 *In vivo* Sampling Using Solid-phase Microextraction

Figure 6.3 also shows the setup of *in vivo* sampling using solid-phase microextraction, which was employed to sample the right leaf of the jade plant. The internal standard pre-loading was performed by exposure of the fibers to the PBS (pH 7.4) aqueous solutions spiked with carbofuran-d_3 and carbaryl-$^{13}C_6$ with the concentrations at 0.25, 0.50, 1.00, and 2.00 μg/mL for 1 hour under a static mode. As soon as the holes were punched using a 21 gauge hypodermic needle in the sampling locations, the fibers with the pre-loaded internal standards were inserted into the leaf of at positions 1, 2, 3, and 4. The sampling time was set at 20 min. The following sample preparation and LC-MS/MS analysis were the same as that of SPME sampling in the gel for the *in vivo* simulation mentioned above.

6.2.9 Instrumental Analysis

Pesticide analyses were performed on an LC-MS/MS system consisting of a Shimadzu 10AVP liquid chromatograph with a system controller and dual binary pumps interfaced to a CTC-PAL autosampler and an MDS Sciex API 3000 tandem mass spectrometer. The assays were carried out as described in Chapter 2 and 4. In addition, the carbofuran-d_3 and aldicarb transitions were monitored from m/z 225.2/123.1 and carbaryl-$^{13}C_6$, m/z 208.2/151.0, respectively.

External calibration curves were performed for both MD and SPME with good precision (RSD < 5%) and linearity ($R^2 > 0.999$).

6.3 Results and Discussion

6.3.1 Method Development for Juice and Milk Using Microdialysis

Standard addition method can be used to perform quantitative analysis for *in vitro* MD sampling. The MD technique employs the dialysis principle.[21] At typical perfusion rate, the continuous flow of perfusion fluid through the probe does not permit the analyte concentration inside the probe to come into equilibrium with the analyte concentration outside the probe. Under these non-equilibrium conditions, the concentration of the analyte in the dialysate is less than the actual concentration in the sample matrix surrounding the probe. The ratio between the fraction recovered to the actual concentration is usually expressed as a percentage, and referred to as "relative recovery" (R_R), which is calculated by eq. 6.1,[22]

$$\%R_R = \frac{C_d}{C_s} \times 100\% \qquad \text{Equation 6.1}$$

where C_d is the concentration of the target analyte in the dialysate, and C_s is the concentration of the analyte in the sample matrix.

To obtain a more precise result, a series of concentrations from standard solutions or dialysate solutions can yield a straight line under fixed conditions. The slope ratio can be used to calculate the R_R, which is described by eq. 6.2,

$$\%R_R = \frac{S_d}{S_s} \times 100\% \qquad \text{Equation 6.2}$$

where S_d is the slope of the curve from the dialysate solutions, and S_s is the slope of the calibration curve obtained from the standard solutions. Eq. 6.2 was used to calculate all of the R_R in this study. When the conditions of MD sampling remain unchanged, R_R remains constant and the initial analyte concentration in the sample matrix can be calculated by eq. 6.3,

$$C_s = \frac{C_d}{R_R} \qquad \text{Equation 6.3}$$

The concentration gradient of an analyte across the MD membrane is driven by the flow rate of the perfusion fluid through the membrane, so the flow rate can influence the relative recovery. In order to obtain an acceptable diffusion relative recovery in an acceptable time, the flow rate and the sampling time were set at 2.0 μL/min and 15 min, respectively. The collected 30 μL dialysate plus 30 μL promecarb solution resulted in 60 μL solution, which was good enough for duplicate injections to the LC-MS/MS system.

The effect of the agitation speed on R_R was investigated next. Three agitation speeds, 0, 500 and 1000 rpm, were examined. It was found that the agitation speed of 500 rpm offered a similar R_R to that of 1000 rpm, which is shown in Figure 6.4. It indicates that the molecular diffusion cross the MD membrane was the rate-controlling

step when the agitation speed was equal to or larger than 500 rpm. Thus an agitation speed of 500 rpm was chosen for further investigation.

The effect of “salting-out” was also examined. It was found that the relative recoveries depended on the sample matrices. Figure 6.5 presents the effect of adding NaCl on the relative recoveries. For carbofuran, propoxur and carbaryl in the juice sample, $\%R_R$ increased at first, and then decreased as more NaCl was added to the sample solution. However, $\%R_R$ of aldicarb always decreased with salt addition. The reasonable explanation is that aldicarb has the highest polarity compared to the other three pesticides. In the case of milk, it was observed that the $\%R_R$ was decreased with the increased amounts of NaCl in the sample. The reasonable explanation was that there was a large amount of organic material in the sample matrices, especially milk, so the “salting-out” effect allowed the pesticides to interact highly with both the organic solvent in the MD probe and the organic material in the matrices. As a result, the extraction efficiencies did not benefit from the effect of “salting-out” in solutions such as milk. To make the experimental procedures simple, the addition of NaCl was not included in the optimized conditions.

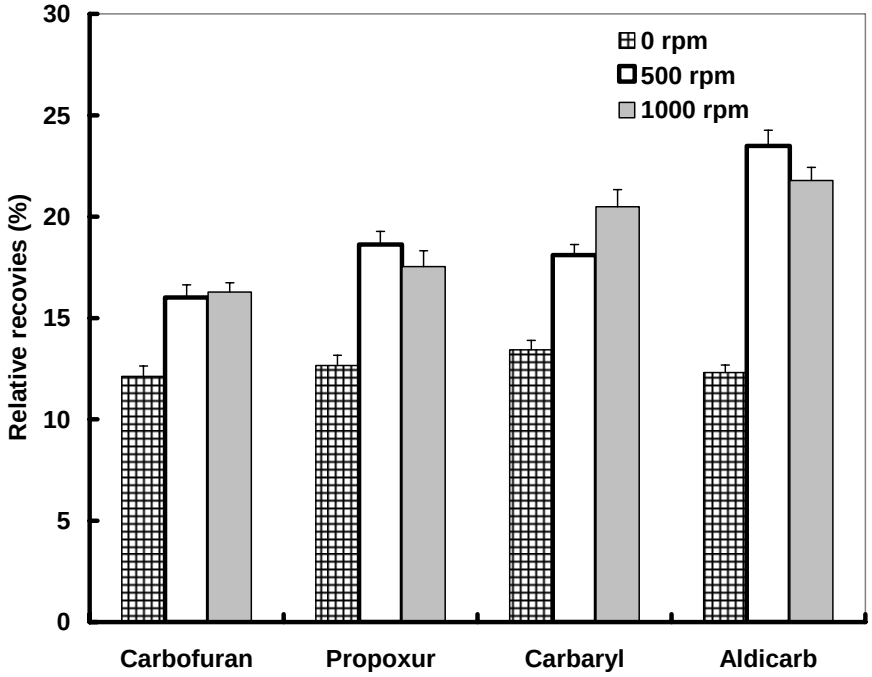
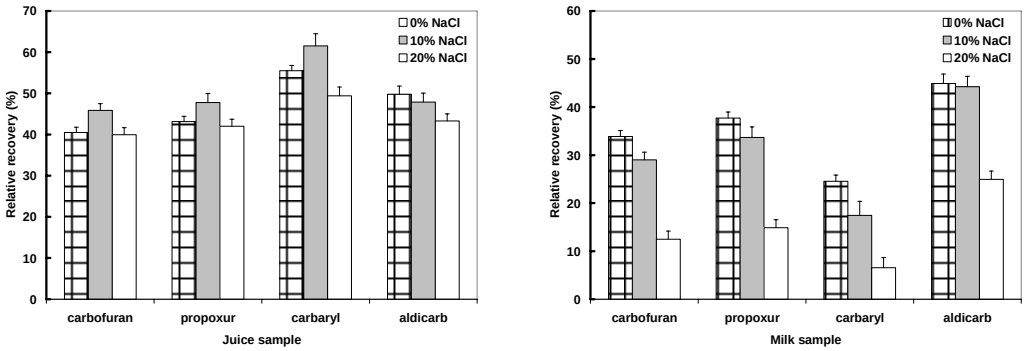


Figure 6.4 Dependence of relative recovery on the agitation speeds. The first, second and third bars represent 0, 500 and 1000 rpm, respectively. The experiments were performed using water as perfusate, at a flow rate of 2.0 μL/min and the pesticides dissolved in nanopure water (n = 3).

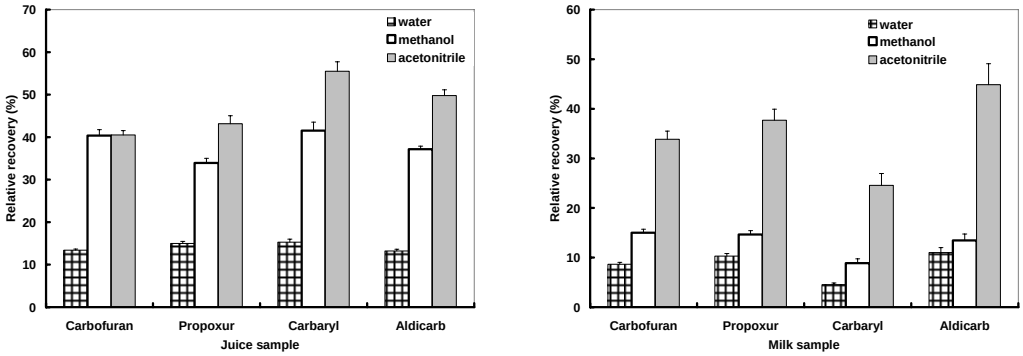


(A) (B)

Figure 6.5 Effect of "salting-out" on the relative recovery. (A) and (B) represent juice and milk sample, respectively. The first, second and third bars stand for 0 %, 10 % and 20 % NaCl in the sample matrix. The experiments were performed using acetonitrile as perfusate, a flow rate of 2.0 μL/min and a sample agitation speed of 500 rpm (n = 3).

The influence of perfusate on the relative recovery was further evaluated. As accommodated LC mobile phase, nanopure water, methanol and acetonitrile were chosen to study R_R. Acetonitrile as the perfusate offered the highest extraction efficiencies for both juice and milk, as shown in Figure 6.6. The results matched the liquid-liquid extraction (LLE) principle. Extraction using MD is superior to LLE because any water miscible solvents can be used for MD. In addition, there is not an emulsion concern when using MD. Interestingly, the relative recovery in juice samples was higher than that from milk. The pesticides could have higher interactions with the milk matrix than the components in the juice sample. Figure 6.6 also presents $\%R_R$ of MD under the optimized conditions with acetonitrile as the perfusate solutions at a flow rate of 2.0 μL/min, and an

agitation speed of 500 rpm. The range of R_R was between 40—55%, and 25%—45%, for juice and milk, respectively.



(A) (B)

Figure 6.6 Dependence of relative recovery on perfusate. (A) and (B) represent juice and milk sample, respectively. The first, second and third bars stand for water, methanol and acetonitrile as the perfusate, respectively. The experiments were performed with a flow rate of 2.0 μL/min and the sample agitation speed of 500 rpm (n = 3).

6.3.2 Method Development for Juice and Milk Using Solid-phase Microextraction

As mentioned above, since it is easy to spike any target analytes in the sample matrix, standard addition method is the easiest and most efficient way to perform quantitative analysis for *in vitro* SPME sampling. The amount of analyte extracted by the coating is linearly proportional to the initial concentration in the sample.[9]

Generally speaking, the first step in developing a SPME method is to select a suitable fiber for the target analytes. Other authors have noted that the PDMS/DVB fiber

has the highest affinity to carbofuran.[12] This fiber was also chosen for the development of the SPME method in this study.

Both juice and milk are the complex or "dirty" samples, so in order to prolong the life of the SPME fiber, it can not be immersed directly into samples without any pre-treatment. The reported methods required either protein precipitation [6] or changing the protected membrane for each single sample.[5] To address the labor- and time-intensiveness issues, a novel membrane filtration device was designed for sample pre-treatment. This device was described in the experimental section. Since the device was easy to make and the materials were inexpensive, one membrane filtration device was used for each sample. It allowed preparing batches of samples simultaneously by using batches of these devices. Thus it is suitable for high throughput sampling.

Two different membranes, nylon-66 and polypropylene, were tested for this purpose. It was found that nylon-66 membrane offered good sealing when both kinds of sample matrices were used. However, polypropylene was not suitable for milk samples because leakage was found from some tubes. This could be explained by the robustness of nylon-66. When they were used for the juice sample, both nylon-66 and polypropylene membrane offered similar results. These results supported the assumption that the membrane itself did not affect the process of the filtration and quantitative analysis. Thus the following data present the results using nylon-66 as the filtration membrane. In addition, it was observed that the liquid level in the plastic tube was lower than that of the sample solution in the glass vial due to the osmotic pressure between two solutions. Following the step of membrane filtration, procedures for SPME were fully automated including extraction, analyte desorption using acetonitrile and fiber conditioning.

Competition effects have been demonstrated in many instances of using a solid-coated fiber.[23] This can cause biases in the quantitative determination of compounds.[24-26] The problem was solved using a short sampling time, i.e. pre-equilibrium extraction. "Zero-sink" occurs when a fiber with a high affinity to the target analyst is employed. The amount of extracted analyte is proportional to analyte concentration in the sample solution.[27-29]

Based on the developed theory, 20 min in a static mode was employed for the pre-equilibrium extraction time. Compared to 7 hrs equilibrium extraction time in a static mode, the sensitivity using 20 min pre-equilibrium extraction was only four times lower. However, the total extraction time was shortened by 95%. After extraction, the concentrated analytes on the fiber surface were desorbed in acetonitrile for 5 min, which was sufficient to remove more than 99% of the analytes. The cleaning-up function of SPME was obvious. After membrane filtration, the solutions from the juice and milk looked orange and turbid, respectively. However, all of the desorption solutions were clean after SPME.

Experimental results demonstrated that different sample matrices, juice and milk, offered close extraction efficiencies, for carbofuran and propoxur. However, in the case of carbaryl, the extraction efficiency in juice is higher than it is in milk. Compared to carbaryl, an opposite phenomenon was observed for aldicarb. Nevertheless, the factors did not affect quantitative analysis due to the good linear response observed.

6.3.3 Comparison of Microdialysis with Solid-phase Microextracion for Juice and Milk Samples

Both techniques belong to microextraction and have the advantage of low solvent consumption, which is environmentally friendly. The standard addition method was applied to both MD and SPME for quantitative analysis because this study is an *in vitro* study rather than *in vivo*. Robustness was demonstrated for both MD and SPME with membrane filtration when they were applied to the complex sample matrices, juice and milk. A MD probe and one single SPME fiber were successfully used through all of the experiments for juice and milk samples. Table 6.1 compares the linear dynamic ranges and regressions for both methods in juice and milk samples. Good linearity ($R^2 > 0.99$) was obtained for both methods.

Table 6.2 presents recovery (%R), which is defined as the percentage ratio between the concentration of an analyte found and the concentration of an analyte added. Good precision (R.S.D < 15%, n = 3) and accuracy (the range of recoveries from 88% to 105%) were observed for both developed methods. It indicates that the experimental data from both methods agree well with the spiked or real levels. In addition, it is noted that precision and accuracy from SPME are better than those from MD. It suggests that SPME be robust and reliable compared with MD.

Table 6.1 Comparison of the linear dynamic ranges and regressions for microdialysis and solid-phase microextraction in juice and milk samples

		MD		SPME	
		linear dynamic range (μg/L)	regressions (R^2)	linear dynamic range (μg/L)	regressions (R^2)
Carbofuran	Juice	0.14-100	0.999	3.4-1000	0.994
	Milk	0.16-100	0.998	3.2-1000	0.996
Propoxur	Juice	0.12-100	0.999	3.3-1000	0.996
	Milk	0.13-100	0.999	3.2-1000	0.996
Carbaryl	Juice	0.12-100	0.999	4.4-1000	0.994
	Milk	0.26-100	0.998	8.6-1000	0.993
Aldicarb	Juice	0.10-100	0.999	7.7-1000	0.998
	Milk	0.11-100	0.998	5.6-1000	0.998

Table 6.2 Recovery (%R), limits of detection (LODs), and limits of quantitation (LOQs) using MD and SPME. Comparison with maximum residue limits set by the Food and Agriculture Organization (FAO) for selected carbamates in milk

		MD			SPME			FAO
		%R (%RSD)	LOD (μg/L)	LOQ (μg/L)	%R (%RSD)	LOD (μg/L)	LOQ (μg/L)	(μg/L)
Carbofuran	Juice	104.2 (3.1)	0.04	0.14	100.9 (2.0)	1	3.3	N/A
	Milk	97.2 (9.3)	0.05	0.16	98.5 (9.1)	1	3.2	50
Propoxur	Juice	93.1 (4.6)	0.04	0.12	105.0 (3.3)	1	3.3	N/A
	Milk	91.2 (14.9)	0.04	0.13	103.5 (8.8)	1	3.2	50
Carbaryl	Juice	104.9 (3.3)	0.04	0.12	96.4 (2.9)	1	4.4	N/A
	Milk	90.0 (8.6)	0.08	0.26	95.0 (5.1)	3	8.6	50
Aldicarb	Juice	91.6 (6.6)	0.03	0.10	102.2 (4.9)	2	7.7	N/A
	Milk	88.3 (12.1)	0.03	0.11	89.8 (9.8)	2	5.6	10

* Juice and milk samples spiked at 50 μg/L level for carbofuran, propoxur and carbaryl and at 10 μg/L for aldicarb (n = 3).

** Maximum residue limits were not found in juice.

Table 6.2 also lists limits of detection (LODs) and limits of quantitation (LOQs) for the two methods as well as comparison of the two methods with maximum residue limits (MRL) set by the Food and Agriculture Organization (FAO).[30] LOD was calculated as three times the standard deviation of the blank, and LOQ was equal to ten times the standard deviation of repetitive measurements on a blank.[31] LOQs of both methods are below tolerance levels set by the FAO for residues of the carbamate insecticides considered in the milk sample. It was noted that MD offered higher sensitivity than the SPME method under the conditions employed in this study.

Batch analysis and high throughput sampling were achieved using the novel membrane filtration device followed by a fully automatic SPME step. This new SPME method avoids intensive manipulation. However, sample extraction had to be done one by one manually using MD. Automatic performance for MD could be investigated in the future. Both techniques could be applied to nutrient analysis in juice, milk and other liquid food samples.

6.3.4 Method Development and Comparison for Jelly Sample

After MD and SPME were developed in liquid matrices, juice and milk, two techniques were investigated further for the semi-solid sample matrix, jelly. For MD, the concentric type probe membrane was used because it was easier to handle than the linear type MD. Although acetonitrile was initially used for pesticide sampling, it was found that the components from jelly were aggregated on the surface of the MD probe membrane, which gradually influenced the diffusion of pesticides from the jelly matrix to

the surface of membrane. After water was employed as the perfusate, no aggregation was observed, and good linearity ($R^2 > 0.99$) was obtained for the target analytes.

When SPME was applied to the jelly matrix, the extraction procedure became simpler than that in the liquid matrices mentioned above. The organic particulates were immobilized in the jelly so that the fiber contamination from the particulates was not a concern. Also, aggregation was not observed on the surface of the fiber. Good linearity was achieved with regression larger than 0.994 for the insecticides.

Table 6.3 shows % recovery, % relative standard deviation, LODs, LOQs, and limits of linearity (LOLs) for both developed methods. Good accuracy (recovery range: 88% — 104%) indicates that the experimental data from both methods agree well with the spiked or real levels. It was noticed that the repeatability using SPME was better than that of MD in the jelly medium. This further suggests that SPME could be reliable and robust compared to MD. Moreover, compared to MD, the automated SPME procedure was less labor intensive.

Table 6.3 Recovery (%R), limits of detection (LODs), limits of quantitation (LOQs), and limits of linearity (LOLs) using MD and SPME in jelly

	MD				SPME			
	%R (%RSD)	LOD (μg/L)	LOQ (μg/L)	LOL (μg/L)	%R (%RSD)	LOD (μg/L)	LOQ (μg/L)	LOL (μg/L)
Carbofuran	103.7 (7.3)	0.09	0.31	200	99.3 (1.8)	0.1	0.47	200
Propoxur	93.0 (12.0)	0.4	1.5	200	103.9 (3.5)	0.2	0.65	200
Carbaryl	94.8 (14.2)	0.2	0.75	200	95.0 (3.1)	0.2	0.74	200
Aldicarb	88.1 (10.7)	2	6.5	200	92.8 (3.5)	0.5	1.6	200

* Samples spiked at 10 μg/L level (n = 3) for the measurement of recovery (%R).

6.3.5 Method Development for *In Vivo* Study Using Microdialysis

The value of R_R for *in vivo* tissue sampling may be different from that from *in vitro* experiments. Also, it is not practical to spike any target analytes in the living sample matrix. An *in vivo* calibration technique should be used for sampling in a living system. Internal standards such as isotope-labelled analytes can be added to the extraction phase (the perfusate of MD) to perform kinetic calibration for quantitative analysis, which is referred to as retrodialysis.[32] It operates by using a perfusate spiked with the analytes in a known concentration. Since the diffusion process is assumed to be quantitatively equal in both directions, the substance loss through the membrane is the same as its *in vivo* recovery. % relative loss (% R_L) can be calculated by the following equation:

$$\%R_L = (1 - \frac{C_d^{'}}{C_p^{'}}) \times 100\% \qquad \text{Equation 6.4}$$

where $C_d^{'}$ is the concentration of the internal standard in the dialysate, and $C_p^{'}$ is the concentration of the internal standard in the perfusate. When R_R is equal to R_L, the initial concentration of the analyte in the living tissue can be calculated by Eq. 6.3.

Before *in vivo* experiments, an agarose gel was chosen as a mimic system for *in vitro* study based on the reasons described in Chapter 2. Briefly, a certain concentration of a gel prevents convection but does not obstruct free diffusion significantly.[33] Although an agarose gel is different from a living tissue, *in vitro* experiments can be very useful to find mass transfer phenomena in both MD or SPME coating and a solid matrix. It may also yield relevant information for *in vivo* study. Only carbofuran-d_3 and carbaryl-$^{13}C_6$ were used as the internal standards for *in vivo* simulation in the agarose gel and *in vivo* sampling in a jade plant because the isotope-labelled propoxur and aldicarb are either expensive or commercially unavailable.

As mentioned above, relative loss should be equal to relative recovery in order to apply retrodialysis for quantitative analysis. Table 6.4 presents the results from an agarose gel and from a jade plant leaf through the MD experiments. It was found that $\%R_R$ was very close to $\%R_L$ for carbofuran in a gel matrix. This suggests that the diffusion process of carbofuran was quantitatively equal in both directions; the carbofuran-d_3 loss through the membrane was the same as carbofuran recovery. It also implies that it can be applied to *in vivo* study for quantitative analysis by substituting $\%R_L$ for $\%R_R$. However, this situation could not be applied to carbaryl. The results in Table 6.4 show that $\%R_L$ had a significant difference from $\%R_R$ for carbaryl although

good linearity was achieved. It suggests that the diffusion of carbaryl was not quantitatively equal in both directions, so only semi-quantitative analysis could be performed by substituting $\%R_L$ for $\%R_R$. Table 6.4 lists % recovery for each target compound as well. Good accuracy (recovery range: 89% — 110%) indicates that the experimental data from both methods agree well with the spiked or real levels. It suggests that this approach could be applied for *in vivo* study.

Table 6.4 %relative recovery (%R_R), % relative loss (%R_L), and % recovery (%R) measured from an agarose gel, and %R_L from a leaf in a jade plant through the microdialysis experiments

	Agarose gel			A leaf of a living jade plant
	%R_R (R^2)	%R_L (R^2)	%R (%RSD)	%R_L (R^2)
Carbofuran	23.78 (0.997)	23.61 (0.984)	90.8 (5.2)	46.60 (0.997)
Carbaryl	13.65 (0.994)	53.42 (0.999)	89.3 (6.2)	11.92 (0.998)
Propoxur	24.86 (0.996)	24.15 (0.996)	90.0 (6.3)	--
Aldicarb	25.53 (0.998)	15.24 (0.999)	109.6 (5.2)	--

* Four data points were used to obtain each slope.

** Recovery (%R) in gel calculated by the measured concentrations divided by the spiked or real values from relative recovery experiments.

*** Relative recovery and relative loss were performed simultaneously for carbofuran and carbaryl, and relative recovery and relative loss were performed separately for propoxur and aldicarb.

6.3.6 Method Development for *In Vivo* Study Using SPME

The same principle can be applied for *in vivo* tissue sampling using SPME: this is referred to as in-fiber standardization technique.[34, 35] When the fiber with the pre-loaded isotope-labelled internal standards is exposed to the sample matrix, an isotropic procedure can be observed for desorption of the internal standards from the fiber to the sample matrix and absorption of the target analytes from the sample matrix to the fiber. The dynamic procedure of extraction can be described by eq. 6.5,[36]

$$\frac{n}{n_e} = 1 - \exp(-at) \qquad \text{Equation 6.5}$$

where n is the extracted amount of analyte in the fiber coating in mol after the exposure time t, n_e is the extracted amount of the target analyte at equilibrium, and a is the time constant that is used to describe how quickly equilibrium can be reached. The desorption procedure can be defined by the following equations.[34, 37]

$$\frac{Q - q_e}{q_0 - q_e} = \exp\left(-at\right) \qquad \text{Equation 6.6}$$

where Q is the amounts remaining of the internal standard in the fiber at time t, q_0 is the preloaded amount of the internal standard, and q_e is the amount remaining in the fiber at equilibrium. The time constant, a, is assumed to be the same for a chemical and its isotope-labelled one, so eq. 6.7 can be obtained by adding eqs. 6.5 and 6.6 together.

$$\frac{n}{n_e} + \frac{Q - q_e}{q_0 - q_e} = 1 \qquad \text{Equation 6.7}$$

Eqs. 6.8 and 6.9 can be obtained by rearranging eqs. 6. 6 and 6.5,

$$Q = [\exp(-at)]q_0 + [q_e - q_e \exp(-at)] \qquad \text{Equation 6.8}$$

$$n = [1 - \exp(-at)]n_e \qquad \text{Equation 6.9}$$

From eq. 6.8, a plot of Q versus q_0 yields a straight line with a slope of $\exp(-at)$ and a y-intercept of $[q_e - q_e \exp(-at)]$ when the desorption time and experimental conditions are constant. It can be called pre-equilibrium desorption. Similarly for eq. 6.9, a plot of n versus n_e yields a straight line with a slope of $[1-\exp(-at)]$ when the extraction time and experimental conditions are constant. Two applications can be obtained from eqs 6.8 and 6.9. First, the sum of two slopes from two straight lines should be equal to one, which can be used to validate the isotropic behavior between extraction and desorption. Second, the value of the slope from desorption, $\exp(-at)$, can be employed to calculate the extracted amounts in equilibrium, n_e, by using eq. 6.9. When the volume of sample matrix (V_s) is larger than that of the fiber coating (V_f), so the initial concentration of the analyte in the living tissue, C_0, can be calculated by eq. 6.10,[9]

$$C_0 = \frac{n_e}{K_{fs} V_f} \qquad \text{Equation 6.10}$$

where K_{fs} is the distribution coefficient between the fiber coating and the sample matrix.

Choosing a suitable fiber was the first step in developing a SPME method. A PDMS coating was selected for the study based on the following considerations. The PDMS coating used is high-purity medical grade, i.e. biocompatible, so the developed method should be further applicable to animal or human study. Also, among all of the SPME fibers, the PDMS coating is the most widely used one, which is good for this primary work. Furthermore, as a liquid coating, PDMS does not have the disadvantage of competition or displacement effects as compared to a solid coating. Moreover, self-made fibers were employed due to the cost considerations. Commercially available fibers are expensive, and are not suitable for single-use and *in vivo* experiments.

Internal standard loading to fibers is an important step in order to obtain a precise result.[38] The preloading process in this study was performed in gel or PBS solution instead of headspace. This approach was to wet the surface of the fiber with a layer of water prior to any absorption or desorption. This wetting procedure also kept the surface of the fiber from drying, which may influence fiber performance.

The kinetics of absorption and desorption were performed in the agarose gel medium to validate that the desorption of a standard from the extraction phase into the sampling matrix is isotropic to the absorption of the analyte from the sample matrix into the extraction phase, under the same conditions. 20 min was used as the absorption and desorption time because it allowed a certain amount of internal standards to desorb from the coating to the sample matrix. Since 20 min is not long, it is also suitable for field sampling. In addition, it matches the sampling time of MD for the purpose of comparison. Table 6.5 shows the values of slopes obtained from both gel matrix and the jade plant using SPME. The sum of the slopes from absorption and desorption was close to that obtained for pesticides through experiment using gels. It suggests isotropism between absorption and desorption. Good accuracy (recovery range: 92% — 99%) is also observed in Table 6.5. It indicates that the experimental data from both methods agree well with the spiked or real levels.

Based on eq. 6.10, the values of K_{fs} should be known in order to calculate the initial concentration of pesticides in the tissue. The leaf juice was obtained by squeezing the leaves and further removing any fine particles from the leaf juice, which was done using a 0.2 μm filter disc. Then equilibrium extraction was employed by using the leaf

juice with the spiked pesticides and the PDMS fiber. The values of K_{fs} were found to be 81.7 ± 5.4 and 80.6 ± 6.9 for carbofuran and carbaryl, respectively.

Table 6.5 Slopes of absorption desorption and recovery (%R) obtained from the agarose gel matrix, and slope of desorption in the leaf of the jade plant using SPME

	agarose gel				jade plant
	Slope				Slope
	Absorption	desorption	Sum of slopes	%R (%RSD)	desorption
	1-exp(-*at*) (R^2)	exp(-*at*) (R^2)			exp(-*at*) (R^2)
Carbofuran	0.1685 ± 0.0063 (0.993)	0.8235 ± 0.0266 (0.987)	0.992	93.2 (4.5)	0.7719 (0.989)
Carbaryl	0.2374 ± 0.0053 (0.999)	0.7943 ± 0.0201 (0.985)	1.032	98.3 (3.5)	0.8993 (0.988)
Propoxur	0.2350 ± 0.0089 (0.987)	0.7508 ± 0.0233 (0.997)	0.986	92.1 (5.2)	--
Aldicarb	0.2255 ± 0.0120 (0.995)	0.7605 ± 0.0274 (0.993)	0.986	96.6 (4.9)	--

* Four data points were used to obtain each slope.

** Recovery (%R) in gel calculated by the measured concentrations divided by the spiked or real values from pre-equilibrium extraction (absorption) experiments.

*** Absorption and desorption were performed simultaneously for carbofuran and carbaryl, and absorption and desorption were performed separately for propoxur and aldicarb.

6.3.7 Comparison of Microdialysis with Solid-phase Microextraction for *In Vivo* Study

Both solid-phase microextraction and microdialysis can be applied for *in vivo* tissue sampling. Figure 6.7 shows the concentrations of carbofuran obtained from both techniques after 20 days of pesticide application to soil. It is observed that the detected concentrations from SPME and MD were close to each other. However, a concentration slight difference was found between the two sampling approaches. The concentration values from MD were lower than those from SPME. Experiments demonstrated that the carry-over existed after each MD sampling. The carry-over was due to pesticide adsorption on the MD membrane as well as the small amount of dialysate remaining in the outlet tube after a MD sampling. In the case of SPME, more than 99% of extracted analytes were desorbed out from the fiber after any SPME sampling. The carry-over could be therefore avoided. It indicates that SPME offered more accurate results than MD.

By comparison, SPME is the more powerful tool for *in vivo* tissue sampling with several advantages over MD, based on this study. First, SPME was more sensitive than MD, due to the higher affinity biocompatible PDMS coating as compared to the PBS aqueous buffer used as the MD perfusate. Table 6.6 shows comparison of LOD and LOQ between MD and SPME for *in vivo* sampling using a jade plant without any pesticide soil application. Second, SPME provided more precise and accurate results than MD based on the data of %RSD and %recovery listed in Tables 6.2, 6.3, 6.4 and 6.5. It also indicates that SPME is more reliable and robust. Third, SPME is more suitable and convenient for field sampling due to its simplicity. For the purpose of perfusate delivery, MD has to be

coupled to a syringe pump, which is bulky and requires a power supply. It is not good for on-site *in vivo* sampling. In addition, SPME in-fiber standardization technique has a wider application for quantitative analysis than retrodialysis. As mentioned above for retrodialysis, some chemicals such as carbaryl did not have equal diffusion in both directions, which led to semi-quantitative analysis. From experiments to perform relative recovery and relative loss separately, it was found that propoxur showed equal diffusion in both directions of the MD probe, but aldicarb did not as shown in Table 6.4. However, with SPME, an isotropic procedure occurred for all of four chemicals, as shown in Table 6.5.

Table 6.6 Limits of detection (LODs), and limits of quantitation (LOQs) obtained from *in vivo* sampling in a leaf of a jade plant using MD and SPME

	SPME (μg/L)		MD (μg/L)	
	LOD	LOQ	LOD	LOQ
Carbofuran	3	11	4	15
Carbaryl	10	44	10	48

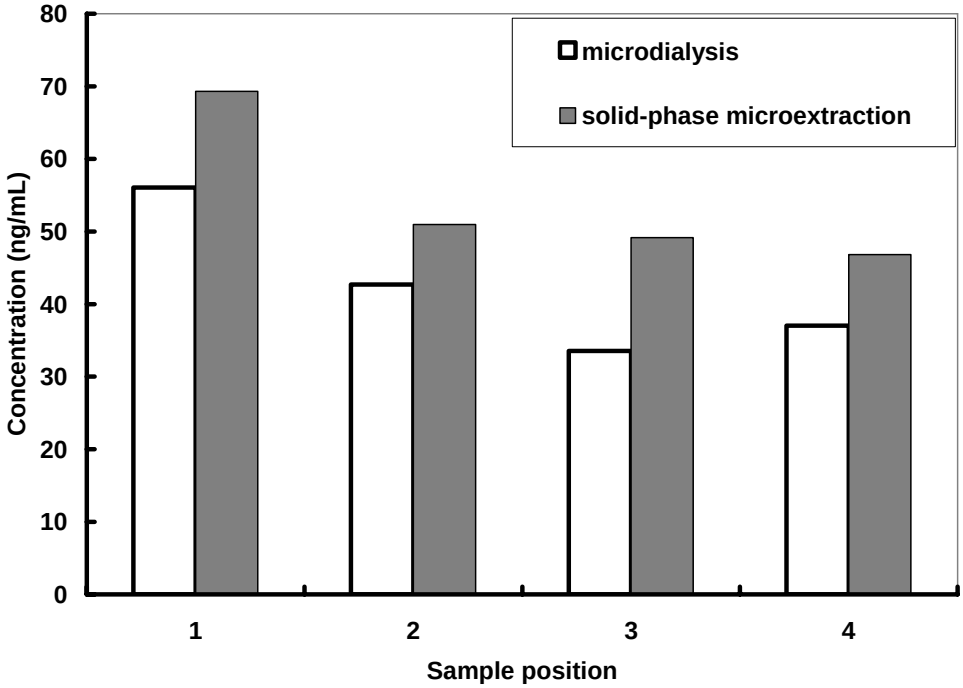


Figure 6.7 Concentrations of carbofuran in the leaves of a jade plant. The sampling time was 20 min after 20 days of the pesticide application to soil. Microdialysis and solid-phase microextraction were used to sample the left and right leaves, respectively, which is shown in Figure 6.3. PBS (pH 7.4) and 165 μm PDMS were the perfusate for microdialysis and the SPME coating, respectively. Sample positions can also be found in Figure 6.3.

6.4 Conclusion and Addendum

6.4.1 Conclusion. Although microdialysis and solid-phase microextraction are the most widely used sampling techniques, a comparison study was not performed previously. This issue was fully addressed for both *in vitro* and vivo study in this paper. For *in vitro* study, two developed techniques were able to be applied to complex sample matrices with accurate and precise results. The two methods proposed allowed the detection of pesticides at the levels established by the FAO. Compared to MD, SPME offered several advantages including full automation, less labor intensity, capability for batch analysis, high-throughput, and highly efficient sampling. In the case of *in vivo* study, although both MD and SPME were invasive, SPME provided several advantages over MD, including higher sensitivity, better precision and accuracy, simplicity for field sampling and wider application. It demonstrated that SPME has the potential to replace MD for *in vivo* sampling. In the future, automation of MD could be done. Both developed techniques could be applied for the determination of other analytes in food samples such as fertilizers, antibiotics, hormones, colorants, and preservatives. Moreover, the two methods could be used for other analytes in other sample matrices such as drug analysis in whole blood. In addition, both techniques with modification might be employed for *in vivo* toxicokinetic studies in animals.

6.4.2 Addendum. Based on the statement from Elsevier,

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dissertation…", most parts of this chapter keeps intact. The mainly modified part in this chapter is the last paragraph of the section of Introduction compared to the accepted manuscript.

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Chapter 7

Summary and Perspective

7.1 Summary

Significant attention has been drawn to the development of techniques to analyze levels of biologically active compounds in living systems in natural environments. These works represent a considerable departure from conventional sampling techniques, where a portion of the system under investigation is removed from its natural environment and the compounds of interest extracted and analyzed in a laboratory environment. Both MD and SPME are powerful sample preparation techniques, which have been developed for *in vivo* study previously. However, there are still numerous fields that have not been explored using either MD or SPME. In addition, a comparison study between MD and SPME has not been performed to date. This thesis not only compared advantages and disadvantages of both techniques, but also focused on the development of new MD and SPME methods that are more suitable for *in vivo* sampling.

Although microdialysis has been used widely for *in vivo* sampling, the current calibration methods exhibit some limitations. This issue was fully addressed by the novel calibration method, kinetic microdialysis. This technique employed two different flow rates with two identical microdialysis probes, which were simultaneously inserted into the symmetric parts of sampling system. The empirical equation was proposed to calculate the analyte concentrations in the sample matrix using the values of two different dialysate concentrations. An excellent correlation was observed between the calculated

and real values. This method was successfully applied for *in vivo* sampling, measurement of the pesticide allocation in the different leaves of a jade plant (*Crassula ovata*). Compared to the other reported microdialysis calibration methods, this new method offers several advantages including simplicity, speed, robustness, and increased accuracy.

An on-fiber standardization technique was studied using solid-coated SPME fibers. Based on Fick's law and the Langmuir model, it was theoretically predicted that there is an isotropic processes between the adsorption of the analytes from the sample matrix to the SPME solid-coated fiber and desorption from the fiber to the sample matrix. This research was conducted using a commercially available fiber, 50 μm CW/TPR, for carbamate pesticide analysis in various sample matrices. The experimental results agreed well with the theoretical prediction with similar time constants between the processes of adsorption and desorption. The isotropic processes were employed for kinetic calibration for adsorption using desorption, which corrected for the sample matrix effect. This pre-equilibrium technique also reduced the sampling time. In addition, the solid-coated fiber provided for a shorter period of time for the desorption of the concentrated analytes on the fiber into an organic solvent for liquid chromatography analysis. This is particularly useful for the analysis of thermally labile or non-volatile compounds. The technique was applied for pesticide analysis in river water and white wine.

An accurate and reliable kinetic calibration method using dominant pre-equilibrium desorption was developed for on-site and *in vivo* application by solid-phase microextraction. A newly proposed approach using the concentration profile with the pre-equilibrium approach not only validates isotropic behaviour between absorption and desorption but also provides a linear approach to obtain a time constant, which is more

accurate and convenient than that obtained from a time profile. Previously, kinetic calibration has been used with radioactive or deuterated internal standards that are expensive or sometimes not available. In the present work, solid-phase microextraction employs the target analytes as the internal standards by dominant desorption, which allowed desorption to calibrate absorption. Dominant pre-equilibrium desorption not only offers a shorter sample preparation time but also provides with time constants for the purpose of quantitative analysis. This kinetic calibration method was successfully applied to on-site PAH sampling in a flow-through system and *in vivo* pesticide sampling in a jade plant (*Crassula ovata*). This developed SPME method is typically suitable for *in vivo* sampling in a field monitoring compared to microdialysis that requires a bulky syringe or osmetic pump.

Previous field studies utilizing solid-phase microextraction (SPME) predominantly focused on volatile compounds in air or water. Earlier *in vivo* sampling studies utilizing SPME were limited to liquid matrices, namely blood. The present study has expanded SPME technique to semi-solid tissues under lab and field conditions through the investigation of both theoretical and applied experimental approaches. Pre-equilibrium extraction was used to shorten *in vivo* sampling time. The use of pre-equilibrium desorption rates are proposed as a means to calibrate pre-equilibrium extractions. Excellent linearity was found between the extracted amounts by SPME in the muscle of living fish and the waterborne concentrations of several pharmaceuticals. A simple SPME method for the simultaneous determination of free and total analyte concentrations in a living tissue is also described. The determined protein binding values are close to those from literature, which validates this developed method. The utility of *in*

vivo SPME sampling under field conditions was evaluated in wild fish collected from a number of different river locations under varying degrees of influence from municipal wastewater effluents. Diphenhydramine and diltiazem were detected in the muscle of fish downstream of a local wastewater treatment plant. Based on this study, SPME techniques have demonstrated several important advantages for laboratory and field *in vivo* sampling. The development of a rapid, robust, easy to deploy technique which combines sampling, extraction and concentration into one step is a potentially important tool for use *in vivo* field-based sampling.

Although MD and SPME are the widely used sampling techniques, a comparison study had not been performed to date. In this study, MD and SPME methods have been developed and compared through *in vitro* and *in vivo* study. For *in vitro* study (juice, milk and orange jelly), both methods offered accurate and precise results (recovery: 88-105% with RSD < 15%) for the complex sample matrices by standard addition method. The limits of quantification (LOQs) of the two developed methods were below tolerance levels in milk set by the United Nations Food and Agriculture Organization (FAO). Compared to MD, the fully automated SPME procedure offered several advantages including high-throughput and more efficient sampling, less labor intensity, and capability for batch analysis. For *in vivo* study, kinetic calibrations were performed using retrodialysis and in-fiber standardization techniques for MD and SPME, respectively. Quantitative analysis was performed to measure pesticide concentrations in the living tissue, i.e., the leaves of a living jade plant (*Crassula ovata*). Although both techniques provided sampling with minimal perturbation to the system under study, SPME was more

sensitive, precise and accurate, suitable for field sampling and had a wider application than MD. It demonstrated that SPME has the potential to replace MD for *in vivo* study.

7.2 Perspective

The newly developed MD and SPME methods mentioned previously have achieved breakthroughs for *in vivo* study. The future research could consist of several new investigations.

First, automation of MD could be developed for *in vitro* sampling as long as the MD membrane is not fouled or clogged by the sample components during the period of sampling process. Increased to automation could tremendously reduce labor intensity. Another advantage of MD is to directly apply to the complex or "dirty" sample matrices. One potential application is to determine a trace amount of target analytes in food samples such as fertilizers, antibiotics, hormones, colorants and preservatives.

Second, kinetic MD should be further applicable for *in vivo* sampling in other living systems as long as the symmetric parts can be found and have the same or similar concentrations of the target analytes. One of examples applicable for pharmacokinetic study would be in a rat or dog. The veins in the left and right legs can be employed as the symmetric parts. The correction factor in the proposed empirical equation might need to be changed slightly if and when the sample matrix and target analytes are different.

Third, the approach of kinetic calibration using dominant desorption by SPME should be applied for *in vivo* sampling in other biological organ systems. One of the potential applications could be *in vivo* toxicokinetic studies in animals. The proposed technique might also be useful for pollutant monitoring in field sampling. Sampling in a

river could be one example as long as the desorbed analytes from the desorption fibers do not diffuse to the absorption or other desorption fibers.

Fourth, the SPME coating should be further miniaturized in order to apply for small size soft tissues such as a brain or liver. The procedure to manufacture miniaturized fibers should be automated to decrease intra- and inter-fiber variation to improve reproducibility. Combining with new materials and new knowledge such as nanotechnology, SPME could become a very useful tool to investigate the mechanism of biochemistry and biology.

Finally, diffusion-based calibration could be applied for *in vivo* tissue sampling. When the SPME sorbents have a high affinity to the target analytes, the molecular diffusion and sampling conditions should remain little variation during the short period of *in vivo* sampling time. Two SPME designs can be utilized for diffusion-based calibration, one is direct-exposure, and another SPME-in-needle. When the approach of direct-exposure is used, the initial analyte concentration in the living system is proportional to the extracted amounts with a short sampling time. Other parameters including the thickness of the boundary layer, the geometric factor, the molecular diffusion coefficient, and the outer surface area of the sorbent can be calculated, found from literature, or obtained by experiments. In the case of SPME-in-needle, the molecular diffusion in the diffusion path length is the rate-controlling step. Thus *in vitro* parameters can be directly applied for *in vivo* study.

Abbreviations

a	Time constant
A	Surface area of a SPME fiber or microdialysis membrane
α	Effective volume fraction in a tissue
B_3	Geometric factor
C_0	Initial analyte concentration
$C_0^{'}$	Calculated initial analyte concentration
C_1	Concentrations of dialysate 1
C_2	Concentrations of dialysate 2
C_d	Concentration of the analyte in dialysate
CE	collision energy
C_f	Analyte concentration in the fiber coating at the interface of the fiber coating and the boundary layer
$C_f^{'}$	Analyte concentration in the fiber coating at the interface of the fiber coating and the fused silica
C_{fA}^{∞}	Analyte concentration on the fiber surface at equilibrium
C_{fmax}	Maximum concentration of active sites on the surface
C_{free}	Free concentration of drug in muscle
C_g	Analyte concentration in the bulk of the gas sample
C_s	Analyte concentration in the bulk of the sample matrix
$C_s^{'}$	Analyte concentration in the boundary layer at the interface of the fiber coating and the boundary layer
C_{sA}^{∞}	Analyte concentration in the solution at equilibrium

C_{total}	Total concentration of drug in muscle
CXP	Collision cell exit potential
CW/TPR	carbowax/templated resin
d	Days
d	Diameter of the fiber
DP	Declustering potential
D_f	Diffusion coefficient in the fiber coating
D_g	Gas-phase molecular diffusion coefficient
D_s	Diffusion coefficient in the sample matrix
$\overline{D}_s$	Diffusion coefficient in the tissue
EP	Entrance potential
ESI	Electrospray ionization
F	Microdialysis flow rate
FAO	Food and Agriculture Organization
GC	Gas chromatograph
h_f	Mass transfer coefficient in the fiber coating
HPLC	high performance liquid chromatography
h_s	Mass transfer coefficient in the boundary layer
J	Mass flux
k	correction factor
K_{fs}	Distribution coefficient
LC	liquid chromatography
LLE	liquid-liquid extraction

LODs	Limits of detection
LOQs	Limits of quantitation
MD	microdialysis
MPB%	drug binding to muscle proteins
MRM	Multiple reaction monitoring
MS	Mass spectrometer
MWWE	municipal wastewater effluents
n	Amount of analyte absorbed onto the fiber
n_0	Amount of analyte absorbed onto the fiber at equilibrium
n_{fA}^{∞}	Amount of analyte adsorbed on the fiber at equilibrium
$n_{f\max}$	Maximum amount of the analyte that can be adsorbed on the active sites on the fiber
log P	octanol-water partition coefficient
PAH	Polycyclic aromatic hydrocarbon
PDMS	Poly(dimethlysiloxane)
PPB%	percentage of plasma protein binding
PPCPs	Pharmaceuticals and Personal Care Products
ppm	Parts per million
r	Mass transfer coefficient of molecule crossing the microdialysis membrane
$\%R_r$	Percent relative recovery
q	The amount of standard desorbed from the fiber
q_0	The initial amount of standard extracted onto the fiber
∂q	Amount of the analyte desorbed from the fiber surface during time period

	∂t
Q	Amount of standard remaining on the fiber
Q1	Quadrupole 1
Q3	Quadrupole 3
SPME	Solid phase microextraction
V_f	Volume of the fiber coating
V_s	Volume of the sample matrix
VOC	Volatile organic compound
S	Concentration of unoccupied sites on the surface of the sorbent
SPE	Solid-phase extraction
S_{LE}	Slope of the extraction curve using LE
S_{SPME}	Free concentration is directly proportional to the slope of extraction curve using SPME
t	Sampling time
TWA	Time-weighted average
Z	Diffusion path length
δ_f	The thickness of the fiber coating
δ_s	The thickness of the boundary layer