Time- and Space- Resolved 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 revisions, as accepted by my examiners.

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

ABSTRACT

Although solid-phase microextraction (SPME) technique has gained wide applications from *in vitro* environmental investigations to *in vivo* pharmacokinetic studies, there are still challenges for utilizing SPME to track fast concentration change over time at a specific location in a heterogeneous system, such as studying the tissue- specific metabolism or bioaccumulation of pharmaceuticals in a living animal. In this case, the technique must be adaptable for *in situ* analysis with highly temporal and spatial resolutions. The goal of the research presented was not only to address this issue but also to develop new analytical methods that were more effective for *in vivo* study using SPME.

In order to improve the temporal resolution, fast SPME sampling technique based on pre-equilibrium extraction must be adopted. However, more efforts need to be placed into calibration so as to guarantee the accuracy of the analysis. In this thesis, firstly, the kinetic calibration was proposed for adsorptive SPME fibres that were widely used for biological samples, which paved the way for performing fast sampling for *in vivo* dynamic monitoring. Secondly, the kinetic calibration was applied for *in vivo* pharmacokinetic study with beagles, with which not only solid experimental evidence was obtained for the calibration theory, but also an example was shown to address the quantitative capability of *in vivo* SPME. The developed method showed comparable sensitivity to traditional blood analysis (linear range 5 – 2000 µg/L and limit of detection: 5µg/L). Furthermore, the traditional kinetic calibration based on isotopically labelled standards was simplified to a single time-point calibration, and a single

standard calibration was developed for multiple analytes. Therefore, the fast *in vivo* sampling could be accomplished in a simple but accurate measure; compared to the established equilibrium SPME technique, statistically no significant difference ($P<0.05$) was observed by using one-way ANOVA and the post-hoc Turkey's test for multiple comparisons.

The second aspect of the thesis addressed the spatial resolution of SPME for *in situ* analysis. Firstly, the sampling of the SPME with high spatial resolution was modeled with multilayered gel system with the mini-sized SPME fibres. The feasibility of the SPME for *in vitro* application was demonstrated by sampling in an onion bulb with heterogeneous structure. Afterwards, the miniaturized fibre was successfully applied to the *in situ* analysis of the concentration distribution of Ochratoxin A in semisolid cheese samples with acceptable sensitivity (Detection limit was 1.5 ng/mL and the linear range was 1.5-500 ng/mL) and comparable accuracy to the standard methods such as liquid extraction and microdialysis. Finally, the *in vivo* application of the space- and time- resolved SPME was implemented to study the tissue-specific bioaccumulations of pharmaceuticals in fish adipose fins and muscle tissues. The results were validated by the standard method liquid extraction, and they were also comparable to the literature results.

The research presented here demonstrated the application potential of the time-and space- resolved SPME for *in situ* dynamic and static analysis in a living system such as a beagle or fish, and in a non-living system such as a cheese piece or an onion bulb.

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DEDICATION

I dedicate this thesis to my son Stephen Pei-en Zhang and my wife Xiaorong Nie.

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ABBREVATIONS

a Time constant

A Surface area of a SPME fiber or microdialysis membrane

α Effective volume fraction in a tissue

C_0 Initial analyte concentration

C_0' Calculated initial analyte concentration

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_{free} Free concentration of drug

Cs 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_{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

Ds Diffusion coefficient in the sample matrix

EP Entrance potential

ESI Electrospray ionization

FAO Food and Agriculture Organization

GC Gas chromatograph

hf Mass transfer coefficient in the fiber coating

HPLC High performance liquid chromatography

hs Mass transfer coefficient in the boundary layer

HS-SPME Headspace Solid-Phase Microextraction

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

MRM Multiple reaction monitoring

MS Mass spectrometer/spectrometry

n Amount of analyte absorbed/adsorbed onto the fiber

n_0 Amount of analyte absorbed/adsorbed onto the fiber at equilibrium

log P Octanol-water partition coefficient

PDMS Poly(dimethlysiloxane)

PPB% Percent 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 loaded on the fiber

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

SPE Solid phase extraction

Sr-SPME Space-resolved solid phase microextraction

t Sampling time

Z Diffusion path length

δ_f The thickness of the fiber coating

δ_s The thickness of the boundary layer

Chapter 1

Introduction

1.1 General Introduction of Solid-Phase Microextraction (SPME)

As a solvent-free sampling and sample preparation technique, Solid Phase Microextraction (SPME) technique was first introduced by Dr. Janusz Pawliszyn's group in 1990.[1] The basic idea is to use a small volume of the extraction phase, usually in the order of 1 microlitre or less, to extract analytes from the sample. Since its early development stage, SPME has been considered an important advancement for the extraction of volatile organic compounds from environmental samples, [1-3] which was significantly boosted by the development of Headspace SPME (Hs-SPME).[4] By integrating sampling, sample pre-concentration, sample preparation and sample introduction into a single step, SPME provides many significant advantages over the traditional sample preparation methods, such as simplicity, portability, time-effectiveness and cost-effectiveness. Therefore, it is not only suitable for laboratory research but also for on-site field sampling. Moreover, with limited extraction amount, sampling can be performed in such a way that only a small portion of the total free compounds are removed from the system. Thus, the disturbance of the normal balance of chemical components is negligible. This is beneficial in the nondisruptive

analysis of very small tissue sites or samples.[5-6] Last but not least, SPME provides a simple means to monitor the free concentration of the analytes in a complicated sample matrix.[5-8] Usually, only the free concentration is the active portion in pharmaceutical and environmental studies. All these strengths offer it versatile applications, from environmental studies to food chemistry and pharmaceutical studies, and from *in vitro* monitoring to *in vivo* analysis. [9-12]

1.1.1 SPME Device

The elementary SPME sampler consists of a support component and a small sized extraction phase. The support component of the early SPME samplers was usually a fused silica fiber; however, due to its fragility, the silica has been replaced by metal wire such as stainless steel wires of different size, i.e., those from Small Parts Inc. (Miami Lakes, FL), which offers more size options and also the robustness and durability of the fiber material.

The most important component of the SPME sampler is the extraction phase. The extraction phase can be either high molecular weight polymeric liquid for absorption, similar to stationary phases in chromatographic column, or a solid sorbent for adsorption similar to the sorbent used in solid phase extraction (SPE). The first used material for the extraction phase is the

rubbery material polydimethylsiloxane (PDMS), which is considered a typical liquid coating since in physicochemical nature its extraction behavior is based on partitioning. On the contrary, there are some solid coating materials, of which the extraction is based on adsorption, where the extraction of the analyte molecules just occurs on the porous surface of the coating, rather than uniformly partitioning into the extraction phase as absorption does.[13] Currently, the commercially available liquid coating materials are PDMS and polyacrylate (PA), while the solid coating materials include PDMS/divinylbenzene (DVB), carbowax (CW)/DVB, carboxen (CAR)/PDMS, DVB/CAR/PDMS and CW/templated resin (TPR). The liquid coating SPME fiber is usually with higher capacity and good sensitivity, but also longer equilibrium time, which can be deduced from the equations for time constant as follows.[14]

$$a = \frac{2Ah_f h_s}{V_f(2h_f K + h_s)} = \frac{Ah_s}{V_f(K + h_s/2h_f)} \qquad (1.01)$$

where a is the time constant, V_f is the fiber volume, A is the surface area of the fiber, h_s is the mass transfer coefficient of the analyte in the sample matrix, h_f is the mass transfer coefficient of the analyte in the fiber coating, and K is the distribution coefficient of the analyte between the coating surface and the sample matrix.

When h_f is much bigger than h_s, $h_s/2\ h_f$ is close to zero, which gives the equation for a solid coating fiber as follows,

$$a = \frac{Ah_s}{KV_f} \qquad (1.02)$$

Since $h_s / 2h_f$ is always bigger than 0, the *a* value from eq.1.2 should be always bigger than the *a* value calculated from eq.1.01. As a result, it could be concluded that the solid coating should have longer equilibrium time (smaller time constant) than the liquid coating if all the conditions, such as fiber materials and agitation, are the same for the two types of fibers. Since the materials that make solid coatings are never the same as those for the liquid coatings, often the solid coating fiber exhibits fast adsorption kinetics and is suitable for fast sampling.[15]Another important advantage for solid coating SPME is that the appropriate fiber can be chosen to extract polar or ionic analytes and compatible for liquid chromatography, which then opens the possibility for using SPME for pharmaceutical analysis and further *in vivo* pharmacokinetic studies.[16-21]

However, there are some limitations for the solid coating fibers. According to the theory of extraction by porous solid SPME coatings, the number of effective surface binding sites where adsorption can take place is limited.[13] When all such sites are occupied, no more analyte molecules can be extracted. This indicates that analyte extraction is a competitive process in which a molecule with higher affinity for the binding sites can replace a molecule with lower affinity. As a result, the linear range of the probe is low and both the linearity and the slope are affected by the concentration of other

competitive compounds, thus resulting in seriously affected calibration. This problem can be significant in complicated biological and environmental matrices such as whole blood or sewage water where many endogenous compounds exist.[15,22-23] Therefore, it is necessary to evaluate the competition effect when using a solid coating in complicated sample matrix.

For volatile analytes, SPME fibers could be directly injected into the gas chromatograph for quantification. The extracted analyte is thermally desorbed into the instrument in the injector and then transported into the column by the gaseous mobile phase. However, for nonvolatile compounds, the extracted analyte must be desorbed into an amount of appropriate liquid solvent, or referred to as desorption solution, usually some organic solvent in which the solubility of the analytes is high. Afterwards, the solvent is submitted to instrumental analysis, for example, the mobile phase solution for the liquid chromatograph could be used for chromatographic separation. In some cases when further concentration of the analyte in desorption solution is needed for the sake of improved sensitivity, the solvent is evaporated and the analyte is re-dissolved in a smaller amount of reconstitute-solution. In addition to increasing the analyte concentrations, the reconstitute-solution is more compatible to the direct instrumentation.

1.1.2 Equilibrium Sampling and Pre-equilibrium Sampling

The classic SPME sampling is based on the partitioning equilibrium of the target analytes between sample matrix and fiber coating. There are two types of sampling in terms of the extraction time: equilibrium sampling and pre-equilibrium sampling.

Equilibrium SPME is the most established method. During the sampling process, the analytes are extracted by the fiber until a partitioning equilibrium is established between the sample matrix and the extraction phase. A linear relationship between the amount of analytes extracted, n_e, and the sample concentration, C_0, is described by the following equation.[24]

$$n_e = \frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s} C_0 \qquad (1.03)$$

where V_f and V_s are the volume of the fiber coating and the sample, respectively. K_{fs} is the fiber coating/sample distribution coefficient of the analyte.When $V_s >> K_{fs}V_f$, the eq 1.03 can be simplified to eq 1.04,

$$n_e = C_0 K_{fs} V_f \qquad (1.04)$$

The strength of this method is its high sensitivity, since the possibly maximal amount of analytes is extracted at a certain concentration; but the weakness is that it has a long extraction time, sometimes from several hours to several days, depending on the sorbent material, matrix, temperature, and agitation. Therefore, it may lead to reduced temporal resolution when it is used for dynamic process monitoring such as a pharmacokinetic study.

In order to achieve fast sampling, the pre-equilibrium extraction method was developed. The milestone is the finding of the quantitative relation between the extracted amount in pre-equilibrium extraction and that in equilibrium extraction by studying the absorption kinetics, shown as equation 1.[25]

$$\frac{n}{n_e} = 1 - \exp(-at) \qquad (1.05)$$

where n is the amount of analyte in the extraction phase after sampling time t, n_e is the amount of analyte in the extraction phase at equilibrium, a is the time constant that is dependent on the volume of the extraction phase and sample, mass transfer coefficients, distribution coefficients, and the surface area of the extraction phase. By combining this equation and the equation for equilibrium extraction, eq 1.03, the linear relationship between sample concentration and the amount of analyte extracted in pre-equilibrium condition can be obtained.

$$n = \frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s} [1 - \exp(-at)] C_0 \qquad (1.06)$$

It deserves noting that the pre-equilibrium extraction shortens the sampling time to achieve fast sampling, but the sensitivity and reproducibility are somewhat compromised, since the sensitivity of the SPME is positively proportional to the amount of analytes extracted, and the reproducibility is often affected by susceptibility of the pre-equilibrium

extraction to agitation of the sample matrix. Therefore, when performing fast sampling, the sensitivity and reproducibility should be evaluated to meet the experimental requirements.

1.1.3 External Calibration *versus* Kinetic Calibration

In principle, SPME is an equilibrium based sampling technique rather than an exhaustive extraction method; therefore the SPME results must be calibrated to obtain the concentration of the analyte in the sample. Generally, calibration helps relate the analytical signal of the analyte to the initial concentration of the analyte in the sample. Specifically for SPME, two types of calibration are needed subsequently to achieve the goal. The first stage is the calibration of instrument response. For example, using mass spectrometry (MS) as a detector, an external calibration curve needs to be constructed to calibrate the response factors that relate the amount of analyte introduced into the instrument with its response. Afterwards, as demonstrated in Fig. 1.1, the linear regression calibration curve of instrument response against the quantities of injected standards is developed, through which the responses from different batches of samples can be calculated.

The second stage is the calibration for the sampling method, which is defined as a process that relates the amount of analyte extracted by the SPME device to the initial concentration of the analyte in the sample.

Currently, there are two important approaches to calibrate the SPME results: calibration curve method and standard-on-fiber method.

Theoretically, the calibration curve method, also referred to as the external calibration method, is applicable to both equilibrium extraction and pre-equilibrium extraction because the amount of analyte extracted is linearly proportional to the initial concentration of analyte in the sample. In spite of the inconvenient and tedious procedures associated with the preparation of the standard solutions and controlling the experimental conditions, usually, this method is simple, with the only requirement that all the experimental conditions for calibration should be the same as for the real sampling. However, it might be difficult or impossible to satisfy the requirement in some cases, such as in on-site field sampling or *in vivo* pharmacokinetic studies, where reproducing the experimental conditions in a laboratory environment is challenging.

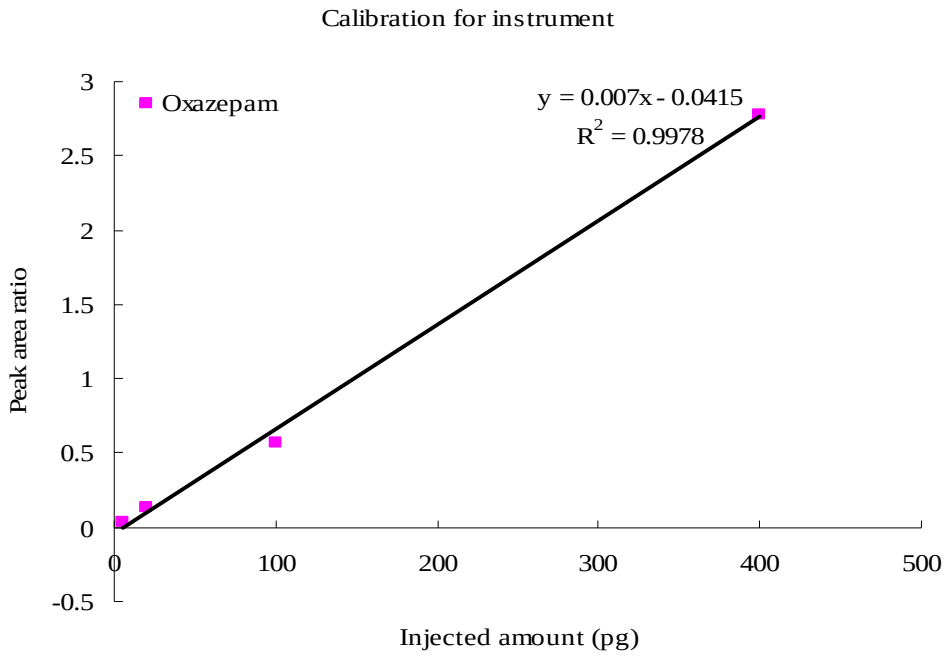

Fig.1.1. Calibration of instrument responses of LC-MS/MS. The injected amounts of standards are 1, 20, 100, 400 pg of oxazepam. The y axis in the plot is the ratio of the peak area of every standard over the peak area of the internal standard lorazepam.

To address the inherent weakness of the calibration curve method, the kinetic calibration method,[14,26] also termed as the on-fiber standardization technique,[27] was developed. This method is based on the symmetric relationship between the absorption process of analytes from a sample matrix to the extraction phase, and the desorption process of pre-loaded standards from the extraction phase to the sample matrix. Thus, extraction of analytes can be calibrated by determining desorption of the pre-loaded standards, usually isotope-labeled compounds. To date, the symmetrical

phenomenon for both solid coating and liquid coating was observed and well explained by the diffusion based mass transfer model.[14,28] Experimentally, this method is simpler than the calibration curve method. More importantly, the method compensates for the effects of agitation and the sample matrix, and it provides accurate quantification of target analytes, especially during on-site or *in vivo* sampling, where standard addition and external standard calibration methods are not practical to use.[27-30]

The relationship of the absorption and desorption processes is expressed by eq. 1.07,[14]

$$\frac{n}{n_e} + \frac{Q}{q_0} = 1 \qquad (1.07)$$

where, Q is the amount of standard remaining in the extraction phase after exposure of the extraction phase to the sample matrix for a sampling time, t, and q_0 is the amount of standard that is pre-loaded in the extraction phase. In eq 1.07, n_e can be easily calculated since n, q_0, and Q can be determined through experiments. Afterwards, the initial concentration of the target analyte, C_0, can be calculated according to eq 1.04 for on-site or *in vivo* sampling, where, $V_s >> K_{fs}V_f$ is satisfied.

1.2 *In vivo* SPME

There are several rationales for conducting *in vivo* analysis. Essentially, studying of chemical processes *in vivo*, such as drug metabolism

in the real physiological environment of a living system, has scientific significance, especially when it is difficult to remove representive samples from the living system for study. For example, when mice blood and tissue are sampled for pharmacokinetic studies, usually a large number of animals are needed to obtain profiles with sufficient data points, since the amount of blood or tissue that can be obtained from an individual animal is limited. If blood and tissue were not removed from mice, small number of animals would be enough and the quality of the data would be improved by reduced individual variation. In addition, if sampling can be performed in such a way that a small proportion of the total free compounds is removed, the disturbance of the normal balance of chemical components is avoided, since compounds of interest are not exhaustively extracted from the investigated system. This is called negligible SPME.[5-6,12] On the contrary, if significant depletion of the free fraction takes place, it results in release of the bound fraction until a new associating equilibrium is established. This may engender the nondisruptive analysis of very small tissue sites or samples.

Currently, ultrafiltration (UF) and microdialysis (MD) are widely used approaches for *in vivo* sampling[31-35]. However, these approaches are inconvenient for field sampling since the sampling systems require pumps, tubing and other appliances. In addition, for ultrafiltration, the sampling process affects the local dissociation equilibrium between the bound and free analytes, thus interfering with the biological system under study; for

microdialysis, the sensitivity could be a concern since sample preconcentration is poor.

The use of SPME in vivo can serve as an ideal alternative because it eliminates the above mentioned problems. For instance, SPME is a nonexhaustive extraction technique where the extracted analytes exist in equilibrium with the extracting phase and sample matrix. According to the SPME theory, above a certain sample size, sample volume does not impact the results [24]. Therefore, it is not necessary to define a specific sample size for the analysis, which is very convenient for *in vivo* sampling. Additionally, SPME directly extracts a small fraction of free analyte. So, a negligible depletion of the free fraction occurs after extraction. Finally, the technology is amenable to miniaturization, and is useful for both small living systems and microanalytical instruments or techniques. These advantages make SPME a promising analytical tool for *in vivo* analysis[21,36].

1.2.1 Requirements of the SPME for *In vivo* Applications

The *in vivo* applications usually set much higher requirements for both SPME fiber materials and sampling strategies than *in vitro* applications.

Firstly, the SPME fiber coating must be biocompatible. Currently, there is no solid research to evaluate the biocompatibility of any SPME fibers. However, the first fiber material used *in vivo* was polypyrrole (PPY) which

is a well known biocompatible material used for fabrication of biosensors.[37-38] Therefore, it is assumed that the PPY fiber is biocompatible without direct evidence supporting the biocompatibility of PPY fibers for the SPME use. Indirect evidence of biocompatibility could be the half-year survival of the animals after the SPME treatment.

Secondly, the linear range of the fiber extraction should be broad enough to cover the expected concentration range of analytes in blood, especially during the early stage or beginning of pharmacokinetic studies where blood concentrations are very high. For example, the C18-bonded silica-polyethylene glycol (PEG) probe has a broad dynamic range (0.5-2000 μg/mL) for diazepam, nordiazepam and oxazepam that ensured detection over the sample concentration range in whole blood during the pharmacokinetic studies of diazepam.[22-23]

Thirdly, the matrix effect should be considered. In fact, the matrix effect is the most complicated and difficult factor, and it can be classified into several categories. Generally, matrix binding, matrix competition, fouling effect, pH effect and salt effect are the commonly encountered matrix effects that affect the quantitative capability of SPME in biological samples and environmental samples.[39-44] Currently, there are dozens of reports discussing the matrix binding and free concentrations, but only a little discussion about the competition effect.[13] Actually, the competition effect should be considered with any SPME method, especially using solid

coating fiber for *in vivo*pharmacokinetic studies, where competition can come from the metabolites and the drug, due to the similarity of their molecular structures. It might also come from the complicated matrix, such as whole blood samples which contain endogenous compounds. Usually, the competition effect is the main problem associated with porous solid coating SPME, since, according to the developed theory, [13,45-47] the number of effective surface sites where adsorption can occur is limited. Thus, the extraction of analytes by solid coating SPME is a competitive process in which several similar molecules compete for one binding-site, and a molecule with a higher affinity for the surface can replace a molecule with a lower affinity. As a result, both the amount of target analyte extracted and the linear range of the extraction decrease, while the decrease is dependent on the concentration of the competitors. An example could be the serious competition effect on PPY fibers that occurred at 50 µg/mL of oxazepam and diazepam during a sampling time of 10 s.[22]

Fouling effect is an important aspect to be addressed for *in vivo* SPME. But there was little experimental research to study the fouling effect, although Dr. Hermens mentioned this in a resourceful review about using negligible depletion SPME to measure the free concentration.[12] The characterization experiment for biofouling effect was conducted by comparing the extraction behaviors of the fibers in direct contact with and without tissue samples. Both the kinetic parameter, time constant and the

thermodynamic parameter, partitioning constant were compared between the tissue-treated fiber and non-treated fiber. For the home-made PDMS fibers used in fish–tissue sampling (Chapter 7), there was no change observed for the two parameters.

Furthermore, the sensitivity of the SPME fiber is a factor that must be considered for quantitative analysis, since it determines the limit of detection and the accuracy of the method when the sample concentration is low, such as during the late-phase of the pharmacokinetic studies. Here, the sensitivity of the fiber is defined as the amount of analyte extracted in a given time, which is determined by the kinetics of the extraction, as shown by eq 1.05. In order to have high sensitivity, the fiber should have a fast equilibrium time, such as the C18-PEG and PPY fibers, for which the equilibrium times were around 5 min during static extraction in whole blood. Meanwhile, the partitioning coefficient (K_{fs}) should also be high enough to have good capacity for the analyte. Eq 1.06 shows that the sensitivity of the SPME, denoted by the fiber volume, sample volume, partitioning constant, time constant and sampling time. When the time is known, the fiber volume and partitioning constant determine the extracted amount of analyte from the sample matrix, while the sample volume affects a little, as evidenced by Eq 1.04.

In addition to the high sensitivity requirements for the SPME fiber coating, the appropriate sampling strategies should also be considered.

Usually, when conducting *in vivo* analysis, such as, a pharmacokinetic study, the analyte's concentration changes rapidly. In order to track the concentration profile, the analytical approach should have a fast response time. Specifically for SPME, the fast sampling is desired to obtain high temporal resolution. In most cases, pre-equilibrium extraction method is preferred over equilibrium extraction unless the equilibrium time of the fiber is quite short, for example, for C18-PEG fibers, the equilibrium time is ~ 5 min in static extraction mode. But, usually, the equilibrium time is quite long, from several hours to days.

Accordingly, to improve the temporal resolution and efficiency of the SPME technique, kinetic calibration was developed where pre-equilibrium sampling is conducted for dynamic monitoring. This involves the extraction of analytes from the sample to the SPME fiber, and then, it is calibrated by determining the desorption of the pre-loaded standards from the fiber to the sample matrix. This method is not only simpler than the calibration curve method, but also compensates for the effect of agitation, temperature and matrix, thus providing more accurate quantitation, especially for on-site and *in vivo* sampling.

The equilibrium SPME coupled to external calibration is not suitable for *in vivo* SPME. The reason is that it is impossible to reproduce the *in vivo* experimental conditions in the laboratory when conducting an *in vitro* experiment to develop an external calibration curve, especially the blood

flow rate during an *in vivo* experiment. In addition, it is often not easy to obtain enough blank sample matrix from the living organism to prepare the standard solutions that are needed for the external calibration. For example, it was found that when conducting the pharmacokinetic studies in beagles, the composition of the commercially available whole blood was significantly different from the whole blood from the experimental animals (Chapter 3). The composition of the sample matrix would affect the matrix binding, fouling and competition for active sites, thus affecting the accurate calibration of the SPME. Moreover, increasing the sampling time and amount of extraction of free analytes may result in non-negligible depletion and significant disturbance to the system under study. Finally, only the SPME fibers that exhibit a fast equilibrium time can be used for equilibrium sampling; otherwise, temporal resolution may be lost for the dynamic monitoring to follow fast metabolism processes, such as pharmacokinetic studies.

But, it must be noted that equilibrium sampling coupled to external calibration could be feasible for *in vivo* SPME, as long as the requirements or parameters for fast equilibration could be satisfied, as mentioned above.

1.2.2 Requirements of Temporal and Spatial Resolution for *In vivo* Applications

The term “temporal resolution” was adopted to describe the requirement of fast sampling for dynamic monitoring of analytes *in vivo*. Here, the temporal resolution of the SPME technique is defined as its capability to accurately determine the sample concentrations at a specific time point on the continuum of time and clearly resolve two different concentrations temporally close to each other, for instance, to monitor the drug concentrations at the time points of “5 min” and “10 min” after the drug administration in a pharmacokinetic study. In this case, the sampling time of the SPME fiber should be less than the duration between the two time-points (i.e., 5 min); otherwise, the SPME fibers cannot tell the difference if the sampling time is long, e.g, 10 min. Generally, the temporal resolution of the SPME is determined by its response time or extraction time. Therefore, to improve the temporal resolution, it is necessary to shorten the sampling time. As mentioned above, a SPME fiber with higher temporal resolution should have high sensitivity; thereby, it is capable of tracking the concentration change. The next task is to develop accurate calibration procedures in order to deliver accurate quantitative results.

Another important aspect of performing *in vivo* studies is to improve the spatial resolution of the SPME fibers. In this study, the spatial resolution of an *in situ* sampling technique is defined as its capability to accurately determine the local concentrations of analytes and clearly resolve two different concentrations spatially close to each other. The rationale for

improving spatial resolution is due to the fact that the uneven distribution of certain substance within a natural system is much more common than uniform distribution because of the heterogeneous nature of the system. Therefore, for tracking the dynamic physiochemical process of a chemical in a heterogeneous system, for example, studying the distribution of pharmaceuticals within a small-sized animal organ or plant tissue, it is not necessary to place the normal-sized SPME fiber throughout the whole organ or tissue to gain a spatially averaged concentration of the organ or tissue. In this case, the fiber should be small-sized, and thereby suitable for the *in situ* sampling on different spots within the organ or tissue.

Generally, the spatial resolution of SPME is determined by the size of its extraction phase; so, it can be improved by reducing the fiber size. However, the sampling time, that determines the temporal resolution of the technique, should also be considered at the same time, since the diffusion during a long sampling time tends to uniform the concentration distribution in the adjacent area, thus making spatial resolution meaningless. Consequently, for SPME experiments, the effect of reduced sampling time should be considered together with shrinking the fiber dimension.

The space-resolved SPME usually results in negligible depletion of the analyte from the sample due to its short sampling time and small-sized extraction phase. Thus, the relationship between the spatial resolution and

temporal resolution of a SPME fiber can be described as equivalent to the relationship between the fiber size and sampling time (eq 1.08).

$$l_m = \frac{L}{SK_{fs}C_s[1-\exp(-at_m)]} \qquad (1.08)$$

where, C_s is the sample concentration, S is the cross section area of the fiber, L (instrumental detection limit) is the minimal amount of analyte that generates a meaningful signal with the instrument, l_m is the minimal length of the fiber, and t_m is the minimal time that generates a significant result. This equation indicates that the sampling time has a negative correlation with the fiber length when all the other conditions, including the cross section area of the fiber, are set. In addition, the spatial resolution and temporal resolution are related to the sample concentration, instrument sensitivity, the cross section area of the fiber, the partitioning coefficient and the time constant of the sampling. This is quite understandable, for example, when the sample concentration or the partitioning coefficient is high, it takes less time to extract the detectable amount of analytes; or even if the fiber is shorter, the extracted amount of analyte is still enough to be detected by the instrument. Furthermore, using an instrument with improved sensitivity, the size of the fiber and sampling time can be reduced.

When the sampling time, *t*, is short as fast sampling, and the amount of analyte extracted is in the linear regime of the extraction time profile, eq 1.08 can be approximated to eq 1.09 by the first order Taylor expansion.

$$l_m = \frac{IDL}{SK_{fs}C_s at_m} \qquad (1.09)$$

eq 1.09 can be rearranged to eq 1.10.

$$l_m t_m = \frac{IDL}{SK_{fs}C_s a} \qquad (1.10)$$

This equation presents the linearly reverse correlation between the temporal and spatial resolution of a SPME sampling. On the other hand, it is important to consider the diffusion of the analyte molecules within the sample matrix as it determines the minimal sampling time that could be used for SPME in heterogeneous system. The diffusion could be described by the integral form of the Fick's first law of diffusion

$$x^2 = 2D * t \qquad (1.11)$$

where, x is the migration distance of the analyte via diffusion, and t is the time duration of the molecule migration via diffusion. While conducting a sampling, the distance of molecule migration in the sample matrix should be shorter than the fiber length so that the *in situ* sampling can be significant. Otherwise, the determined concentration is only a spatially averaged one in a large area since the sphere of molecule diffusion is larger than the probe size. From this perspective, the relation between fiber length and minimal time for an *in situ* sampling (e.g., when x = the spatial resolution or fiber length, l_m) can be described as eq 1.12.

$$l_m^{\ 2} = 2D * t_m \qquad (1.11)$$

For example, it is assumed that the diffusion coefficient of the drug molecules in a semisolid tissue sample matrix, e.g., muscle, is 10^{-9} m^2/s, and the fiber length is 1 mm. Then, the minimal sampling time could be calculated as 500 s or 8 min. Strictly, a sampling time more than 9 min is not an *in situ* study. Similarly, when the sampling time is set as, for example, 10 s, the spatial resolution can be as high as 0.1 mm. There is no practicle significance of using 0.1 mm spatial resolution or fiber length for SPME sampling. However, it indicates that the fiber can be miniaturized that the invasiveness of the *in vivo* sampling can be further decreased. On the other hand, when D is large, e.g., for the volatile molecules in gas, 10^{-4} m^2/s, and the sampling time is 100 s, the diffusion distance is more than 14 cm, thus making the spatial resolution meaningless.

In summary, Eqs 1.08 to 1.10 describe the effect of SPME's sensitivity (including fiber, sample concentration and instrument conditions) on the minimal fiber size and sampling time. But eq 1.11 presents the effect of the diffusivity of the analyte in the sample matrix on the spatial and temporal resolution. For a real sample analysis, both need attention and evaluation.

1.3 Research Objectives

The main objective of this dissertation is to improve the temporal resolution and spatial resolution of SPME and its applications. The research consists of two aspects, as outlined below.

The first aspect addresses the temporal resolution of the SPME technique. The highly time-resolved SPME could be easily achieved by adopting a series of pre-equilibrium sampling procedures coupled to the classic and simplified kinetic calibration methods. Therefore, the first work was the development of kinetic calibration for solid coating SPME, as described in Chapter 2. This part provides the foundation for the time-resolved pharmacokinetic studies. Then, the kinetic calibration with deuterated standards was applied to calibrate the *in vivo* sampling for pharmacokinetic studies, as described in Chapter 3. This work not only provided a solid experimental procedure to conduct quantitative *in vivo* analysis by SPME, but also presented a way to calibrate the pre-equilibrium extraction based on fast SPME. For further simplification of the fast SPME procedures, the non-deuterated standard calibration, the single time-point calibration, and the single standard calibration were proposed and discussed in Chapter 4. Therefore, the first section demonstrated that *in vivo* SPME could be accomplished rapidly, simply and cost-effectively based on the understanding of the kinetic behavior of the *in vivo* SPME.

The second aspect of the research is the development of the space-resolved SPME and its application to both *in vitro* and *in vivo* analysis. Firstly, as described in Chapter 5, the sampling of the SPME with high spatial resolution was modeled with multilayer gel system, and the

feasibility of the SPME for *in vitro* use was demonstrated using a heterogeneous system, an onion bulb. The second application, as described in Chapter 6, is the *in situ* sampling of the spatial distribution of Ochratoxin A, in a piece of semisolid cheese sample. The third investigation was an *in vivo* study of the tissue-specific bioaccumulations of pharmaceuticals in fish adipose fin and muscle tissue. The results are presented in Chapter 7. Finally, an overall summary of the scientific advancement from the work presented in this dissertation and future work are discussed in Chapter 8.

Chapter 2

Kinetics and Kinetic Calibration for Adsorption-Type SPME

2.1 PREAMBLE and BACKGROUND

2.1.1 PREAMBLE

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2.1.2 Introduction

In the recent years, solid phase microextraction (SPME) has gained extensive application and recognition in many areas. [1-3] It addresses the need for fast sampling and sample preparation for chromatography by integrating sampling, sample preparation and sample introduction into one step. With the development of simple but accurate kinetic calibration method,[4-10] it shows unique potentialities in the field sampling, also called on-site sampling, and *in vivo* sampling as well.[10, 11] In these cases, the effect of sample volume on the results of analysis can be neglected when the sample volume is much larger than the fiber capacity KV_f (K = fiber/sample partition coefficient and V_f = fiber volume). [12, 13]

SPME fiber coatings can be classified into two distinctive types according to the mechanism of the extraction: absorption-type and adsorption-type. For absorption-type coatings, the extraction is based on partitioning of the target analyte between the extraction phase (the SPME fiber) and sample matrix, or in the fiber coating-sample-headspace ternary phase system, and either equilibrium extraction method or pre-equilibrium extraction method is applicable. The most established method is equilibrium extraction, where the fiber, coated with a liquid polymeric film, is placed in a sample matrix until a partitioning equilibrium is reached. The amount of analyte extracted by the fiber at equilibrium, n_e, is

linearly dependent on the initial sample concentration, C_0, as expressed by Eq 2.01,

$$n_e = \frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s} C_0 \qquad (2.01)$$

where, V_f and V_s are the volume of the fiber coating and the sample volume, respectively, and K_{fs} is the fiber coating/sample partition coefficient of the analyte. For on-site or *in vivo* sampling where $V_s >> K_{fs}V_f$, the concentration of target analyte can be calculated by eq 2.02, the simplified form of eq 2.01. [2]

$$n_e = C_0 K_{fs} V_f \qquad (2.02)$$

External standard calibration method can be employed for quantification as long as the experimental conditions for calibration remain same as for the sampling. Usually the equilibrium extraction method provides the highest sensitivity because of the fact that the largest amount of analyte can be extracted out of the sample matrix. However, the extraction time might be too long to reach the equilibrium, thereby resulting in lower temporal resolution for kinetic monitoring. In addition, the equilibrium extraction method is not applicable for adsorption-type coating, also known as solid coating, where the extraction is based on the adsorption of the analyte onto the porous surface of the solid coating since the extracted amount of the analyte under equilibrium condition could be nonlinear with the initial concentration of the analyte in a sample of high concentration.[14] In order to address these problems, Ai developed a

pre-equilibrium extraction method for SPME based on the finding that the amount of analyte extracted by the fiber before equilibrium, *n*, is also linearly dependent on the initial sample concentration, C_0. [15, 16] For quantification, however, the external standard calibration method is not applicable for pre-equilibrium extraction (for field or *in vivo* sampling), since it is difficult to keep the experimental conditions for calibration same as in field- or *in vivo*- sampling. In order to address the inherent incapability of the calibration curve method in this case, the kinetic calibration method,[4-6] also called in-fiber standardization technique,[7] was developed based on the symmetric relationship between the absorption process of analytes from sample matrix to the extraction phase and the desorption process of the preloaded standards from the extraction phase to the sample matrix. Therefore, the extraction of analytes can be calibrated by determining desorption of the preloaded standards (isotope labeled compounds are usually used as standards). This method compensates the effect of agitation, temperature and matrix, thus providing accurate quantification, and especially it is suitable for field- or *in vivo* sampling where standard addition and external standard calibration methods are not practical. [4-10] To date, all the studies for kinetic calibration have been only restricted within absorption-type of SPME fiber coatings[4-6, 10] or pure liquid extraction phase in liquid phase microextraction (LPME), [7-9] without consideration of adsorption-type SPME coating. So, it is not practical to

apply the kinetic calibration method developed on absorption-type fiber to adsorption-type fiber coatings directly. Furthermore, although the kinetics of absorption and desorption process for liquid coating (absorption-type) SPME and LPME have well established theoretical basis for kinetic calibration, there is a lack for a mathematical description of the adsorption and desorption kinetics for adsorption-type SPME coating. Therefore, investigation of kinetics of adsorption and desorption and development of a kinetic calibration method for adsorption-type coating have unique theoretical importance and practice significance.

In this chapter, the kinetics of the adsorption and desorption onto and from the porous solid SPME coating are presented, meanwhile, the symmetric relationship between adsorption and desorption was demonstrated. In order to show its feasibility, the developed kinetic calibration method was successfully applied to correct the matrix and agitation effects in the drug analysis of clinical blood and plasma samples.

2.2 THEORETICAL CONSIDERATIONS

2.2.1 Adsorption Kinetics for adsorption-type SPME fibers

When a SPME porous solid fiber is exposed to an agitated sample matrix, adsorption of the analytes from the sample to the fiber surface occurs (Figure 1, A). Compared to the three-layer model (analytes' diffusion through a boundary layer, partition in the fiber/sample interface,

and then diffusion within the fiber coating) for absorption-type fiber,[4,15] such as the PDMS fiber, the model for adsorption only has two layers, where the analytes diffuse through the boundary layer and then adsorb on the coating surface, without entering the inner part of the fiber. In experimental practice, it was reported that only negligible amount of analyte molecules entered the inner part of the solid coating, while almost all analyte molecules adsorbed on the surface, during a brief extraction,[14] contrary to an extraction with liquid-coating SPME.

The mass transfer of the analytes based on diffusion through the boundary layer is considered as the rate-determining step.[18] Thus, Fick's first law of diffusion (eq 2.03) can be applied to describe this process at the sample matrix/SPME coating interface region:

$$J \equiv \frac{1}{A}\frac{\partial n}{\partial t} = -D_s \frac{\partial C^s}{\partial x} \qquad (2.03)$$

where, J is the mass flux of the analyte from sample matrix to the fiber coating, A is the surface area of the fiber, ∂n is the amount of the analyte adsorbed to the fiber surface during time period of ∂t and D_s is the diffusion coefficient of the analyte in the sample matrix. A steady-state mass transfer can be established when agitation is applied effectively in the sample matrix. Therefore a linear concentration gradient in the boundary layer is assumed:

$$\frac{1}{A}\frac{\partial n}{\partial t} = -D_s \frac{\partial C^s}{\partial x} = -\frac{D_s}{\delta_s}\left(C_s - C_s^{'}\right) \qquad (2.04)$$

where, δ_s is the thickness of the boundary layer, C_s is the concentration of the analyte in the bulk of the sample matrix, and C_s is the concentration of the analyte in the boundary layer at the interface of the fiber coating and the boundary layer. The mass transfer coefficient of the analyte 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. Thus, eq 2.04 can be rewritten as follows.

$$\frac{1}{A}\frac{\partial n}{\partial t} = -h_s\left(C_s - C_s^{'}\right) \qquad (2.05)$$

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

$$K = \frac{C_f}{C_s^{'}} \Rightarrow C_s^{'} = \frac{C_f}{K} \qquad (2.06)$$

where, K is the distribution coefficient of the analyte between the coating surface and the sample matrix, and C_f is the concentration of the analyte on the surface of the fiber coating. If it is assumed that the SPME coating has a uniform pore distribution and surface area throughout its bulk, i.e., the surface area is linearly proportional to the volume of the coating, the concentration of the analyte on the surface of the fiber coating, C_f , can be treated as bulk concentration in the fiber coating. Thus,

$$C_f = \frac{n}{V_f} \qquad (2.07)$$

where, V_f is the fiber volume, and n is the extracted amount of analyte onto the surface of the fiber coating after the exposure time t. Thus C_s can be solved by combining eqs 2.6 and 2.07:

$$C_s^{'} = \frac{n}{KV_f} \qquad (2.08)$$

and in the bulk of sample matrix,

$$C_s = C_0 - \frac{n}{V_s} \qquad (2.09)$$

where, C_0 is the initial concentration of the analyte in the sample matrix, and V_s is the sample volume. Substitution of Eqs,2.08 and 2.09 into Eq 2.05 gives

$$\frac{1}{A}\frac{\partial n}{\partial t} = -h_s\left(\mathrm{C}_0 - \frac{n}{V_s} - \frac{n}{KV_f}\right) \qquad (2.10)$$

Let

$$a = Ah_s\left(\frac{1}{V_s} + \frac{1}{KV_f}\right) \qquad (2.11)$$

When $V_s >> K_{fs}V_f$, Eq 2.13 can be simplified as

$$a = \frac{Ah_s}{KV_f} \qquad (2.12)$$

Let

$$b = Ah_sC_0 \qquad (2.13)$$

Then eq 2.10 can be simplified as

$$n^{'} + an = b \qquad (2.14)$$

eq 2.14 can be solved with the initial condition: t = 0, n = 0

$$n = (b/a)[1 - \exp(-at)] \qquad (2.15)$$

Combination of Eqs 2.12 and 2.13 gives

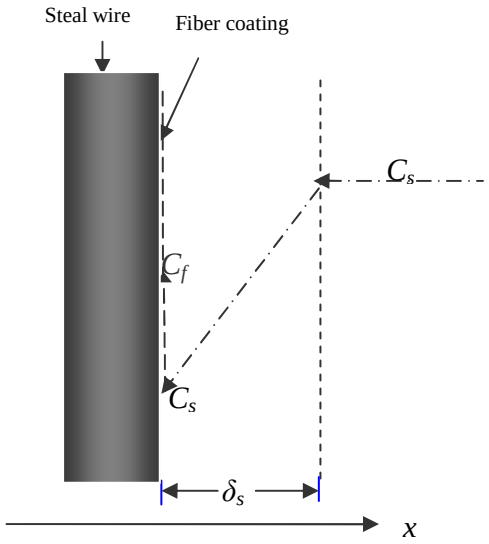
$$b / a = V_f K C_0 \qquad (2.16)$$

Substitution of eqs 2.16 and 2.2 into eq 2.15 gives

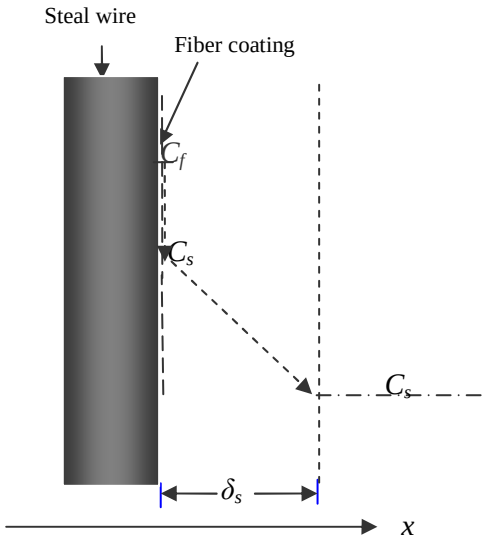
$$n = n_e[1 - \exp(-at)] \qquad (2.17)$$

$$\frac{n}{n_e} = [1 - \exp(-at)] \qquad (2.18)$$

where, t is the exposure time of the fiber into the sample, and a is the time constant that is used to describe how fast the equilibrium can be reached, as defined by eq 2.12. The value of a is dependent on the dimension of the fiber coating, mass transfer coefficients and distribution coefficients in a given sample matrix.



(A)



(B)

Figure 2.1. Interface of porous solid coating SPME fiber in contact with an aqueous solution. A steady-state diffusion is assumed in effectively agitated aqueous sample. The concentration gradient in the boundary layer is assumed to be linear. (A) Adsorption process and (B) Desorption process.

2.2.2 Desorption Kinetics for adsorption-type SPME fibers

When a SPME porous solid fiber, preloaded with an analyte, is exposed to an agitated sample matrix, desorption of the analyte from the coating surface to the sample occurs (Figure 1, B). The desorption process can be treated as the reversed process of the adsorption. Similarly, the equations for describing the desorption process can be derived based on the steady-state diffusion model, in brief,

$$J \equiv \frac{1}{A}\frac{\partial q}{\partial t} = -h_s\left(C_s - C_s^{'}\right) \qquad (2.19)$$

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

$$C_f = \frac{q_0 - q}{V_f} \qquad (2.20)$$

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

$$C_s = \frac{q}{V_s} \qquad (2.21)$$

where, C_s is the concentration of the analyte in the sample matrix after time t.

At the interface of the fiber coating and the boundary layer, quick partition equilibrium of the analyte between the sample matrix and the coating surface is assumed:

$$K = \frac{C_f}{C_s^{'}} \Rightarrow C_s^{'} = \frac{C_f}{K} \qquad (2.22)$$

where, K is the partition coefficient of the analyte between the coating surface and the sample matrix. Combination of Eqs 2.20 and 2.22 gives

$$C_s^{'} = \frac{C_f}{K} = \frac{q_0 - q}{KV_f} \qquad (2.23)$$

Substitution of Eqs 2.21 and 2.23 into eq 2.19 gives a differential equation (eq 2.24).

$$q' = -h_s A\left(\frac{1}{V_s} + \frac{1}{KV_f}\right)q + \frac{Ah_s}{KV_f}q_0 \qquad (2.24)$$

Using the same way as derivation of eq 2.11 to eq 2.16, Eq 2.24 can be solved and arranged as

$$q = q_0[1 - \exp(-at)] \qquad (2.25)$$

in which, parameter a is defined in eq 12. If $Q = q_0 - q$ and Q is the amount of analyte remained on the coating surface after time t. Then,

$$\frac{Q}{q_0} = \exp(-at) \qquad (2.26)$$

2.2.3 The symmetric relationship of adsorption and desorption for adsorption-type SPME fibers

If the desorption and adsorption processes occur under the same experimental conditions, the time constant (*a*) should be the same or similar for the same compounds or similar compounds. Thus, the sum of Q/q_0 (the fraction of the standard remaining on in the extraction phase after sampling time *t*) and n/n_e (the fraction of the analyte adsorbed on the extraction phase after the same sampling time *t*) should be 1 at any desorption/absorption time (eq 2.27).

$$\frac{n}{n_e} + \frac{Q}{q_0} = 1 \qquad (2.27)$$

Therefore, the symmetric relationship between the adsorption process and desorption process has been proved in theory.

2.3 EXPERIMENTAL SECTION

2.3.1 Chemicals and Supplies

Benzodiazepines (diazepam, nordiazepam and oxazepam, 1 mg/mL in methanol) were chosen as the analytes and purchased from Cerilliant (Austin, TX, USA). These were diluted in methanol and phosphate-buffered saline (PBS) pH 7.4, or dog blood/plasma to use in instrument calibration and sample preparation, respectively. Lorazepam was used as the internal standard in sample preparation to calibrate the sample loss in sample preparation as well as sample introduction into the instrument. Beagle whole blood (EDTA as anticoagulant) was obtained from Biological Specialties Corp. (Colmar, PA, USA). Plasma was prepared with the whole blood by

centrifugation and stored frozen at -20 °C until use, and whole blood was stored at 4 °C. HPLC grade acetonitrile and acetic acid (glacial) for HPLC mobile phase, methanol for desorption solution were bought from Fisher Scientific (Unionville, ON, Canada). Water was obtained from a Barnstead Nanopure water system.

Polypyrrole (PPY) fibers that were extensively used for fabricating biosensors due to its conductivity and biocompatibility, were chosen as the model adsorption-type coating for drug analysis.[19-20] The fibers with the same capacity were assumed to have the same surface area. It was verified that the extraction capacity is proportional to the porosity (as determined by scanning electron microscopy).

All fibers used in the desorption experiments were preloaded with deuterated or nondeuterated standards. The loading solution was prepared by spiking deuterated standards (diazepam-d_5, nordiazepam-d_5 and oxazepam-d_5) or non-deuterated standards into 25 mL of sterile deionized-water at 50 µg/L for each. Then the selected PPY fibers were exposed into the loading solution during 40 minutes for standard loading and then kept in tubes for use.

2.3.2 Instrumental Analysis

A CTC-PAL autosampler/Shimadzu 10 AVP LC/MDS Sciex API 3000 tandem MS system was used for the analysis of the drugs and their deuterated standards. The assay conditions were the same as described elsewhere,[20] with the exception that the transition monitored for diazepam-d_5 was m/z 290.2/154.1.

2.4 RESULTS AND DISCUSSION

2.4.1 Verification of the kinetics of Adsorption and Desorption for PPY fibers

In order to verify the kinetics of adsorption and desorption for porous solid fiber coating, absorption and desorption experiments were conducted simultaneously. PPY fibers preloaded with deuterated diazepam were exposed to a flowing solution (linear flow velocity: 7.2 cm/s), diazepam in PBS (pH 7.4, 50 µg/L), for different times to study the time profile for absorption and desorption. Since, eqs 2.18 and 2.26 can be rewritten as Eqs 2.28 and 2.29, respectively,

$$\ln\left(1-\frac{n}{n_e}\right)=-at \qquad (2.28)$$

$$\ln\frac{Q}{q_0}=-at \qquad (2.29)$$

the adsorption and desorption time profiles can be linearized. [7] Figure 2 illustrates the linearized adsorption and desorption time profiles at 25 ^{o}C (i.e., room temperature) where, ln(Q/q_0) or ln(1- n/n_e) is Y axis and – a is the

regression slope (from eqs 2.28 and 2.29). It showed a good linear relationship between ln (Q/q_0) or ln (1- n/n_e) and time ($R^2 > 0.995$). This result demonstrates that Eqs 2.18 and 2.26 accurately describe the kinetics of SPME desorption and absorption based on PPY fibers for drug analysis.

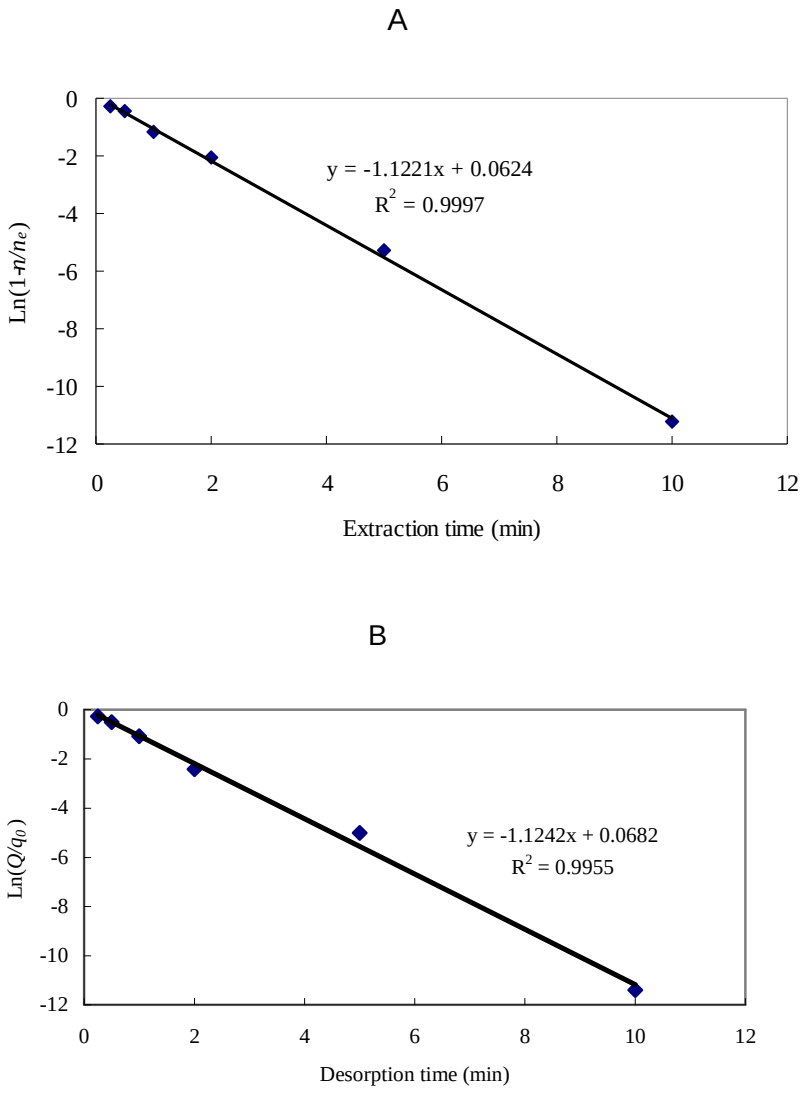


Figure 2.2. The adsorption time profiles (A) of diazepam and desorption time profiles (B) of diazepam-d_5. The adsorption and desorption were performed in

standard solution prepared by PBS (pH 7.4, 50 µg/L) in a flow system at a rate of 7.2 cm/s and 25 ˚C.

Figure 2.3 presents the values of Q/q_0 calculated from the desorption time profile and n/n_e calculated from the absorption time profile. The sum of Q/q_0 and n/n_e is close to 1 at each point (the range is 0.92-1.11), which validates the symmetric relation between the adsorption and desorption.

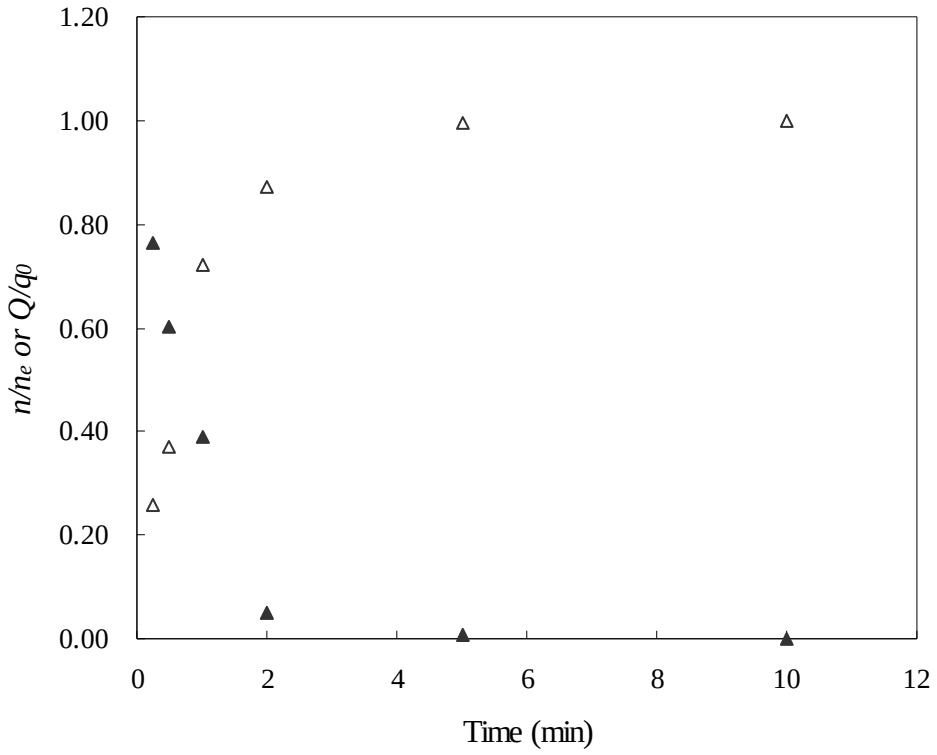


Figure 2.3. The adsorption and desorption time profiles for diazepam and diazepam-d_5.

2.4.2 Pre-equilibrium extraction for Adsorption-type SPME

As mentioned earlier that, equilibrium extraction method is not suitable for adsorption-type SPME due to the narrow linear range, competition effect for the limited binding sites and displacement effect between analytes and their analogues on the coating surface. [14] Thereby, pre-equilibrium extraction method is more desirable for solid coating SPME. When, eqs 2.01 and 2.02 are true for solid coating SPME within the linear range, then combination of eqs 2.18 and 2.01 or 2.02 gives the following equations.

$$n = n_e[1-\exp(-at)] = C_0 \frac{K_{fs}V_fV_s}{K_{fs}V_f + V_s}[1-\exp(-at)] \quad (2.30)$$

$$n = n_e[1-\exp(-at)] = C_0K_{fs}V_f[1-\exp(-at)] \quad (2.31)$$

The above equations illustrate the linearly proportional relationship between *n*, the amount of analyte adsorbed on the coating before equilibrium was reached ($t < t_e$), and C_0, the initial concentration of the analyte in the sample matrix.

2.4.3 The Rate of Adsorption and Desorption

The rate of adsorption and desorption can be judged by the value of the time constant, *a*, which is defined by eqs 2.11 and 2.12. Usually, $V_s >> K_{fs}V_f$ is always true because the number of the binding sites on the surface of the porous coating is much less than those in a liquid coating when the volume

of the two fibers is the same, assuming that the liquid coating fiber also has binding sites within. So the effect of sample volume on *a* value can be neglected. eq 2.12 can be taken as an accurate definition of the time constant *a* for a porous solid coating.

eq 2.12 can be rearranged to

$$a = \frac{A}{V_f} \cdot \frac{D_s}{\delta_s} \cdot \frac{1}{K} \qquad (2.32)$$

where, A/V_f is the surface area in a unit volume of fiber coating which is linearly proportional to the specific surface area for a given coating material, and describes the porosity of a porous solid coating. Temperature can affect D_s and K, thus a value, and the agitation will affect δ_s, the thickness of the boundary layer. In summary, the overall mass-transfer resistance (K/h_s) is contained in the boundary layer for a given fiber. In another word, the agitation condition of the sample matrix can affect the rate for adsorption and desorption directly. So, calibration for the agitation effect during pre-equilibrium extraction is a must for quantitative analysis.

Another point needs to note is that the distribution constant, K, in eq 2.12 is not always the same as that in eqs 2.01, 2.02, 2.30 and 2.31. The K in eq 2.12, defined by eq 2.6, is the real distribution constant, where C_s is the free concentration of the analyte in the boundary layer, i.e., close to the free concentration of the analyte in the sample matrix. Therefore, the K value remains constant in different matrix for a given analyte and fiber coating. However, the distribution constant, K_{fs}, in Eqs. 2.01, 2.02, 2.30 and 2.31,

defined by C_f/C_s, is apparent distribution constant, where C_s can be either the free concentration or the total concentration in the standard solution. The value of K_{fs} changes in different matrices. So, both free concentration and total concentration can be obtained depending on what K_{fs} value is used.

2.4.4 Kinetic calibration: Calibration of Adsorption by Desorption in Pre-equilibrium Extraction

Similar to absorption-type SPME, the kinetic calibration method for solid coating SPME can be developed for calibration in pre-equilibrium extraction based on the symmetric relationship between the adsorption and desorption.

To determine the concentration of analyte in a sample matrix, the fiber was loaded with a known amount of isotopically labeled standard, q_0. Then the preloaded fiber was exposed to the sample matrix for a short time period, t, during which an amount of the analytes were adsorbed onto the fiber surface while a part of labeled standard desorbed from the fiber to the sample matrix. According to eq 2.27, with the known loading amount of standard ($q_{0)}$, the remaining amount of standard (Q) and the extracted amount of analyte (n), the amount of analyte can be extracted from the sample in equilibrium (n_e) can be calculated. Then, the initial sample concentration, C_0, can be calculated using Eq. 2.02. Another approach is the

use of Eq. 2.26 or 2.29 to obtain the value of *a*, and then sample concentration can be calculated using eq 2.31.

No matter which method is chosen, according to eq 2.02 or 2.31, another two parameters, the volume of the fiber, V_f, and the fiber coating/sample distribution coefficients of the analytes, K_{fs}, need to be known to calculate the sample concentrations, C_0. Traditionally, scanning electron microscopy (SEM) is used to determine the thickness of the fiber coating, and then V_f can be calculated. However, this method is not only tedious but also not accurate for porous solid coating. A simple but efficient way to achieve the goal is the use of calibration curve to obtain the product of V_f and K_{fs} directly, as shown in Figure 2.4. A seven-point calibration (n = 3, 5-1500 µg/L) was conducted. The regression slope presents the product of V_f and its K_{fs}. The standard solutions were prepared by spiking standards into the dog blood. Static extraction was performed until equilibrium reached (9 min) at 37 ℃. Proper care was taken to keep the matrix and temperature the same to the real sample, and the extraction time was long enough to reach the equilibrium.

But it needs to be noted that the application range of the aforementioned kinetic calibration is also limited in the linear range (5 - 1000 µg/L) of the calibration curve, where 5µg/L is the limit of detection with the conditions we used, although the symmetry of the adsorption and desorption is true without concentration limit.

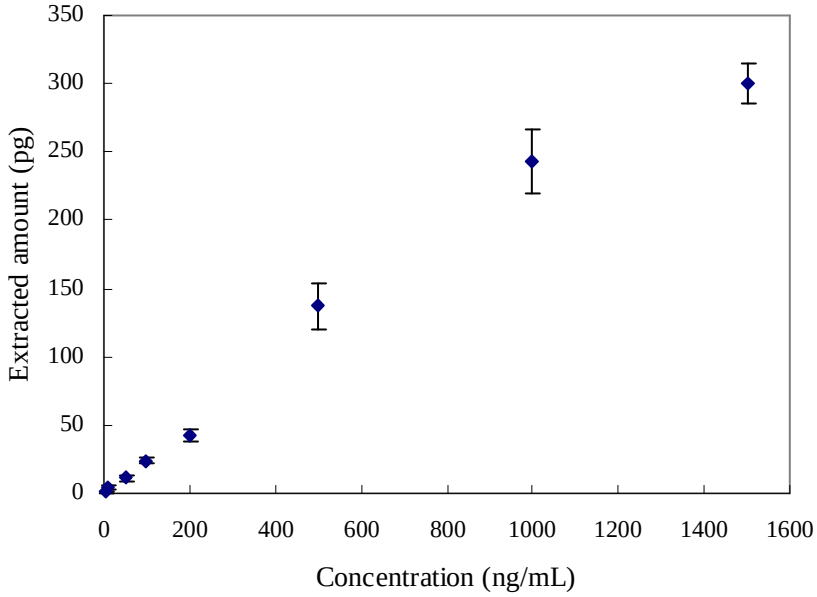


Figure 2.4. SPME calibration with PPY fibers for diazepam (♦) extraction. A seven-point calibration (n = 3), 5 to 1500 µg/L, was conducted.

2.4.5 Application: Drug Analysis in Plasma and Whole Blood Sample

In order to test the feasibility, the kinetic calibration method was used to quantify diazepam in clinical samples, plasma and whole blood with PPY fibers.

The spiked diazepam in the plasma and whole blood matrix is 10 ppb (µg/L), 50 ppb, 200 ppb and 500 ppb. The extraction from plasma was conducted by agitation in a flow system with a linear velocity of 7.2 cm/s, while extraction from blood was conducted at static condition to avoid breaking of red blood cells by agitation.

The product of V_f and K_{fs} for diazepam was determined from the regression slope of the calibration curve as shown in Figure 2.4, where the

standard solution was prepared by whole blood. Thus, the total concentration of diazepam in whole blood can be calculated (based on the product of V_f and K_{fs}) from Figure 2.4. Similarly, the total concentration of diazepam in plasma can be calculated from spiked diazepam in plasma. In addition, the free concentration of diazepam in plasma and whole blood can be determined from the spiked diazepam in physiological saline solution. Here, the product of V_f and K_{fs} the distribution coefficient between fiber and physiological saline sloution, was determined to calculate the concentration.

The relative recoveries and relative standard deviations (RSD) for diazepam in plasma and whole blood are summarized in Table 2.1. The results demonstrate the highly quantitative capability of the kinetic calibration method. This method would be very suitable for fast field and *in vivo* sampling with porous solid SPME fibers, where correctly performing the external calibration method for pre-equilibrium extraction is usually troublesome.

Table 2.1. Calculated relative recoveries of diazepam from plasma and whole blood sample at different concentrations with kinetic calibration.

	Relative recovery (%) (RSD, %; n = 6)	
Concentration (µg/L)	Plasma sample	Whole blood sample
10	91.2 (13.6)	104.1 (12.8)
50	93.3 (9.8)	107.2 (13.2)
200	97.2 (11.9)	111.4 (16.9)
500	103.0 (16.5)	105.1 (18.7)

2.5 CONCLUSION and ADDENDUM

2.5.1 Conclusion

In this chapter, the kinetics of adsorption and desorption for adsorption-type SPME coating based on a steady-state diffusion model is proposed. There are several important conclusions. Firstly, the mathematical expression of the adsorption kinetics provides a directly proportional relationship between the amount of analyte adsorbed on solid coating SPME fiber and its initial concentration in the sample matrix. This relationship suggests the potentiality of porous solid SPME fiber to be used for

quantitative analysis in pre-equilibrium extraction. Secondly, the kinetic calibration method was developed for accurate calibration in pre-equilibrium extraction using porous solid SPME fibers based on the symmetric relationship between adsorption and desorption. However, the quantification is limited within the linear range of the given fiber. In this study with whole blood, the detection limit of the method for diazepam is 5 ng/mL and linear up to 1000 ng/mL. In addition, the rate for adsorption and desorption is controlled by boundary layer, and the time constant was determined by the porosity of the fiber coating, the agitation, the matrix effect and the real distribution constant of the analyte between fiber coating and sample matrix. The fact of boundary layer controlled extraction further points out the importance and necessity of the kinetic calibration to compensate for the agitation and matrix effect during extraction with porous SPME fibers. The theoretical model is verified by the experimental data, and a good agreement between them was observed.

Finally, using PPY fibers, the developed kinetic calibration method was applied for drug analysis in clinical samples (plasma and whole blood), and accurate results were obtained.

2.5.2 Addendum

The text in this chapter is revised compared to the published journal article.

Chapter 3

Kinetic Calibration of Solid-Phase Microextraction for *In Vivo* Pharmacokinetic Studies

3.1 Preamble and Introduction

3.1.1 Preamble. This chapter has been published as a part of the paper: Zhang, X.; Es-haghi, A.; Musteata, F.; Ouyang, G; Pawliszyn, J. Quantitative *in vivo* Microsampling for Pharmacokinetic Studies Based on an Integrated Solid-Phase Microextraction System. *Anal. Chem.* **2007**, *79*, 4507-4513. The contributions of Es-haghi, A, the co-author, was involved in the experimental work with *in vivo* sampling and manuscript revision. The contributions of Musteata, F. and Ouyang, G., the co-authors, involved experimental suggestions and manuscript revision. Tables and Figures are reprinted with permission from Analytical Chemistry (Copyright 2007 American Chemical Society).

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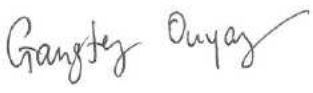
Signature: 

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3.1.2 Introduction

Solid-phase microextraction (SPME) has gained extensive application and recognition in many areas since it was introduced in 1990, [1-3] due to its unique advantages for rapid sampling. It integrates sampling, sample preparation, and sample introduction into one step, thus greatly simplifying the total process of chemical analysis. Moreover, as with limited extraction amount, it does not result in significant disturbance to the system under study. All the strengths indicate that SPME can be used for *in vivo* sampling in a living system. However, in order to achieve accurately quantitative analysis, care must be taken for using appropriate calibration methods except meeting the demanding requirements for the *in vivo* use of the SPME probes.

The first *in vivo* SPME was applied to pharmacokinetic studies, in which dog blood concentrations of benzodiazepines were monitored during 12 hours after injection of a dose of diazepam by using polypyrrole (PPY) fibers.[4] External standard calibration method, also called calibration curve method, was used for quantification. The standard solutions were prepared by spiking benzodiazepine standards into commercial dog blood at known concentrations, and then *in vitro* extraction, solvent desorption, and instrumental analysis were conducted following the same procedure as for *in*

vivo SPME. The detected signals (the amount of analytes extracted from standard solutions) were plotted over the blood concentrations as calibration curves. Since the blood used to prepare standard solutions was not from the dogs in the *in vivo* SPME experiments, and the extraction conditions such as temperature between *in vitro* calibrations were not the same as *in vivo* SPME, the quantified results were more approximated.

In order to address the inherent incapability of the calibration curve method, the kinetic calibration method,[5,6] also called in-fiber standardization technique,[7] was developed based on the symmetric relationship between the absorption process of analytes from sample matrix to the extraction phase and the desorption process of the preloaded standards from the extraction phase to the sample matrix; therefore, the extraction of analytes can be calibrated by determining the desorption of the pre-loaded standards (isotope labeled compounds are usually used as standards). This method is simpler than calibration curve method. And more importantly, it compensates the effect of agitation, temperature, and matrix, thus providing accurate quantification, especially for on-site sampling where standard addition and external standard calibration methods are not practicable.[5-11] Since *in vivo* sampling is a specific case of on-site sampling, kinetic calibration is presumably applicable for *in vivo* SPME if the appropriate SPME fiber is chosen for the given analytes.

In this report, the feasibility and the application conditions of the kinetic calibration for *in vivo* sampling by SPME are demonstrated. And the simplified one-point kinetic calibration and non-isotope labeled standard calibration method were developed based on the finding that the time constant, *a*, is independent from sample concentrations, which were well validated by the *in vitro* and *in vivo* SPME experiments.

3.2 EXPERIMENTAL SECTION

3.2.1 Preparation of SPME Probes

PEG-C18 SPME probes and devices were prepared using the method described elsewhere.[11] The C18 particles (10 micron) and PEG were donated by Supelco (Bellefonte, PA, USA) as a research sample. All fibers with the same capacity were selected out for *in vivo* experiments. The fibers with the same capacity were assumed to have the same volume, which was verified by finding that the extraction capacity is proportional to the fiber volume by scanning electron microscopy.

3.2.2 *In vitro* Experiments

A systematic investigation was conducted on the extraction behaviors of the probes including extraction time profile, desorption time profile, matrix effect of plasma and whole blood, and dynamic range. By doing these

in vitro experiments, the experimental conditions were optimized for the *in vivo* experiments.

All fibers used in the dog experiments were preloaded with deuterated standards. The loading of standard on fibers was performed as follows: All the deuterated standards for diazepam, nordiazepam, and oxazepam were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The loading solution was prepared by spiking deuterated standards (diazepam-d_5, nordiazepam-d_5, and oxazepam-d_5) into 25 mL of sterile deionized-water at 50 µg/L for each. Then the sterilized probes were exposed into the loading solution during 30 minutes for standard loading and then kept in the sterile Falcon tubes for use.

3.2.3 LC-MS/MS Analysis

A CTC-PAL autosampler/Shimadzu 10 AVP LC/MDS Sciex API 3000 tandem MS system was used for the analysis of the drugs and their deuterated standards. The assay conditions were the same as described before,[4] except that the transitions monitored for the deuterated standards were: m/z 290.2/154.1 for diazepam-d_5, 276.1/140.0 for nordiazepam-d_5, and 292.1/246.1 for oxazepam-d_5 respectively.

3.2.4 Animal Experiments

All animal experiment procedures with beagles were approved by the Animal Care Committees at University of Guelph, and the experiments were performed in the Central Animal Facility of the University of Guelph (Guelph, ON, Canada) similarly as described previously [11] except the sterilization was conducted by immersing the samplers in 8 % formaldehyde and 70 % alcohol for 18 hours to avoid fiber damage during steam autoclaving.

3.2.5 Conventional Blood Analysis

In order to compare the SPME data with conventional blood assay, 1 mL of blood was withdrawn after each SPME extraction and 2 mL of blood was withdrawn before diazepam injection for calibration. [4] Then 500 µL of acetonitrile was added to 100 µL of the whole blood in a 1.5-ml microcentrifuge tube, followed by vortex and centrifugation (5000 rpm, 10 min). And then 400 µL of supernatant was taken out for evaporation under nitrogen gas. Samples were reconstituted in 50 µL of reconstitute solution (methanol/pure water: 1/1, and containing 10 ppb of lorazepam as internal standard to calibrate the sample loss during instrumental analysis. The linear range was 5-2000 µg/L). A six-point calibration (n = 3) from 5-2000 µg/L for diazepam, nordiazepam, and oxazepam based on aforementioned approach was performed at 25 ℃. The standard solutions were prepared by spiking standards into the dog blood collected before drug administration.

3.3 RESULTS AND DISCUSSION

The kinetic process for the absorption of analytes onto a SPME liquid coating fiber can be described with eq 3.1:[12]

$$\frac{n}{n_e} = 1 - \exp(-at) \qquad (3.1)$$

where n is the amount of analyte in the extraction phase after sampling time t, n_e is the amount of analyte in the extraction phase at equilibrium, a is the time constant that is dependent on the volume of the extraction phase and sample, mass transfer coefficients, distribution coefficients, and the surface area of the extraction phase.

The desorption of the preloaded standard from a SPME liquid coating can be expressed by eq 3.2,[5]

$$\frac{Q}{q_0} = \exp(-at) \qquad (3.2)$$

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

If the desorption and absorption processes occur under the same experimental conditions, the time constant a should be the same or similar for the same compounds or similar compounds. Thus the sum of Q/q_0 (the fraction of the standard remaining on in the extraction phase after sampling time t) and n/n_e (the fraction of the analyte absorbed into the extraction

phase after the same sampling time *t*) should be 1 at any desorption/absorption time, which is expressed by eq 3.3, [5]

$$\frac{n}{n_e} + \frac{Q}{q_0} = 1 \qquad (3.3)$$

In eq 3, n_e can be calculated easily since n, q_0, and Q are detectable. Afterwards, the initial concentration of target analyte, C_0, can be calculated according to eq 3.4,[2]

$$n_e = \frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s} C_0 \qquad (3.4)$$

where V_f and V_s are the volume of the fiber coating and the sample, respectively. K_{fs} is the fiber coating/sample distribution coefficient of the analyte. For on-site or *in vivo* sampling, since $V_s >> K_{fs}V_f$, the concentration of target analyte can be calculated by the eq 3.5, which is the simplified form of eq 3.4,[2]

$$n_e = C_0 K_{fs} V_f \qquad (3.5)$$

The equilibrium SPME method based on eq 3.5 has been extensively applied to field sampling such as for air sampling[13] and water sampling, [14] which is also the fundamental for *in vivo* sampling, because in these cases, the sample volume does not affect the results.

The aforementioned derivation process indicates that there are two preconditions for on-site or *in vivo* kinetic calibration. One is the symmetric relationship between standard desorption and analyte absorption described by eq 3.3, and another is the linear relationship between the amount of

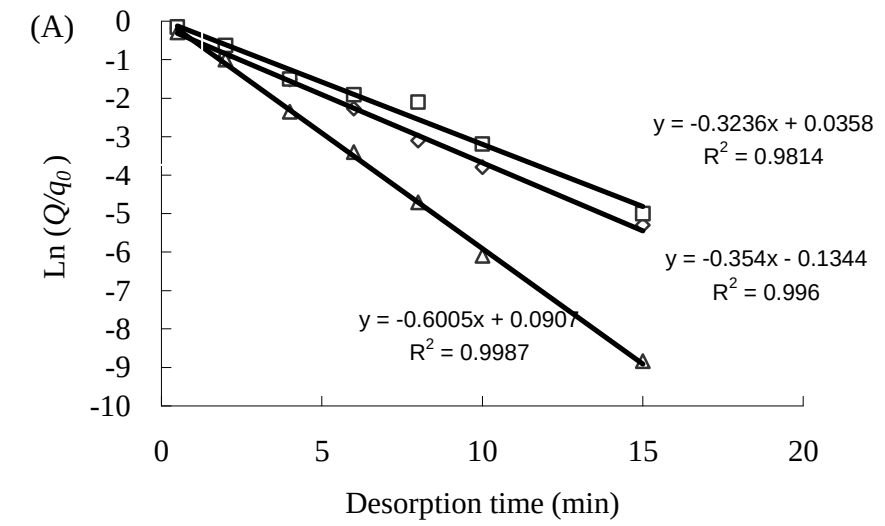
analyte extracted in equilibrium n_e and the initial concentration of target analyte C_0, described by eqs 3.4 and 3.5. Therefore, the application conditions for on-site or *in vivo* kinetic calibration are determined: Firstly, the time constant *a* should be the same or nearly the same for the desorption and absorption, which means the physicochemical properties of the preloaded standard should be the same or very similar to the analyte. Usually the same compound or isotope labeled compound is preferable. Secondly, the experimental conditions such as matrix composition, agitation, temperature, and exposure time for the desorption process should be the same or nearly the same to those in the absorption process; therefore simultaneously conducting desorption and absorption is desirable. Thirdly, the kinetic calibration method only apply to the linear range of the SPME fiber in a given sample matrix. It is not applicable to those cases when the linear relationship between n_e and sample concentration C_0 was broken such as using adsorption-type SPME fibers in high sample concentration.[15] Last but not least, during the time *t*, both the extracted amount *n* and desorbed amount $q_0 - Q$ should be reproducibly detectable, so n/n_e or Q/q_0 within the range of 40-60 % is preferable considering the undesirable variation from fiber making, experimental operation, and instrumental analysis. Thus pre-experimental determination of the appropriate sampling time for a given sampling system is needed before the *in vivo* experiments.

In order to verify the feasibility of the kinetic calibration for blood sampling, *in vitro* experiments were conducted to study the kinetics of the absorption and desorption of the deuterated and non-deuterated drugs in plasma over the linear range (10-2000 μg/L). First, PEG-C18 fibers preloaded with deuterated standard were exposed to a flowing standard solution (flow rate 7.2 cm/s) prepared by spiking a fixed amount of diazepam, nordiazepam, and oxazepam in a given volume of plasma for different times to study the time profile for absorption and desorption. Then the relationship between the time constant *a* and drug concentrations in plasma was studied by varying the concentration of the standard solution. Figure 3.1 illustrates one of the absorption and desorption time profiles at 25 ^{o}C (room temperature) with $\ln(Q/q_0)$ or $\ln(1- n/n_e)$ as *y*-axis, where the regression slope is $-a$ based on eqs 3.1 and 3.2. For each drug analyte, there is a good linear-relation between $\ln (Q/q_0)$ or $\ln (1- n/n_e)$ and time ($R^2 >$ 0.98), which demonstrates that eqs 3.1 and 3.2 accurately describe the kinetics of SPME desorption and absorption based on PEG-C18 fibers for drug analysis. Figure 3.2 presents the values of Q/q_0 calculated from the desorption time profiles and n/n_e calculated from the absorption time profiles. The sum of Q/q_0 and n/n_e is close to 1 at each point (the results range is 0.97-1.15), which demonstrates the symmetry of the absorption and desorption. The averaged value of the sum of Q/q_0 and n/n_e is 1.07,

somewhat higher than 1 due to the little difference of physicochemical properties between a drug analyte and its deuterated analogue.

Moreover, the equilibration time, t_e ($t_{95\%} \approx$ 5-9 min for the three analytes), and the amount of analyte extracted at equilibrium, n_e, can also be obtained from the absorption/desorption time profiles. It is found that both t_e ($t_{95\%}$) and n_e of the extraction based on PEG-C18 fibers is roughly two-folds of PPY fibers in plasma matrix at the concentration of 50 µg/L, which suggested PEG-C18 fibers are more suitable for pre-equilibrium sampling than PPY fibers because both fast extraction time and high sensitivity can be compromised, while it is impossible for PPY fibers. And the sampling time for *in vivo* experiment was set for 2 min so as to keep the n/n_e and Q/q_0 within the range of 40-60 %.

The values of the time constant *a* calculated from each absorption and desorption time profile in plasma samples at different concentrations are listed in table 3.1. The data show that the value of time constant *a* for absorption of benzodiazepines in plasma is close to that for desorption of their deuterated analogues, which suggests that the deuterated standards in this work were suitable for the kinetic calibration. However, it should be noted that the isotope labeled standard can be used for the kinetic calibration only if its time constant *a* for desorption is similar to that of its unlabeled counterparts.



(B)

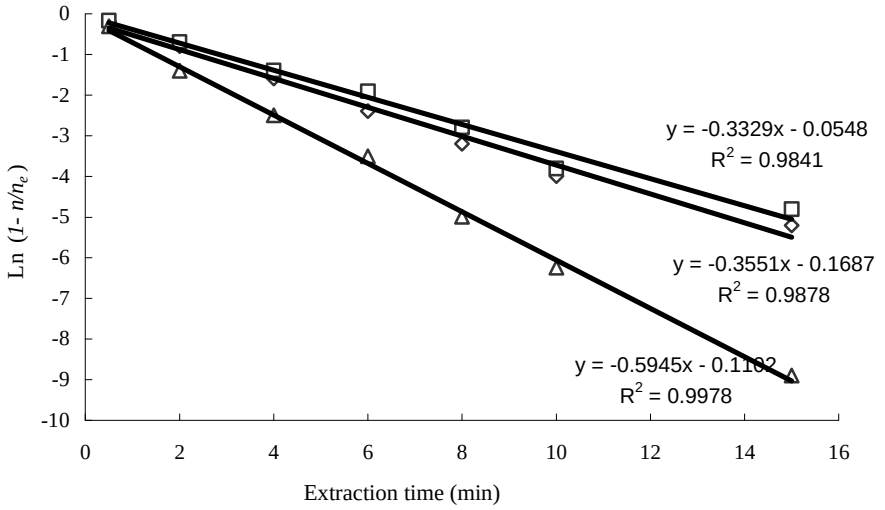


Figure 3.1. The desorption time profiles (A) of diazepam-d_5 (◊), nordiazepam-d_5 (□), and oxazepam-d_5 (Δ) and absorption time profiles (B) of diazepam (◊), nordiazepam (□), and oxazepam (Δ). The absorption and desorption were performed in plasma standard solution in a flow system at a rate of 7.2 cm/s (at 25 ˚C).

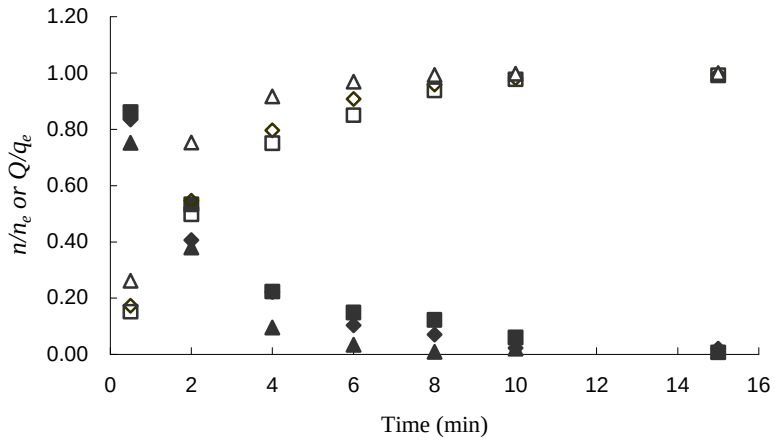


Figure 3.2. The absorption and desorption time profiles for of diazepam (◊), nordiazepam (□), and oxazepam (Δ) and their deuterated analogues: diazepam-d_5 (♦), nordiazepam-d_5 (■), and oxazepam-d_5 (▲).

In order to calculate the sample concentrations, C_0, another two parameters, the volume of the fiber, V_f, and the fiber coating/sample distribution coefficients of the analytes, K_{fs}, need to be determined in eq 3.5. Traditionally, scanning electron microscopy (SEM) is used to determine the thickness of the fiber coating and then V_f can be calculated.[17] However, this method is not just tedious but also not accurate. Here we reported a very simple but efficient way to achieve the goal, i.e., using calibration curves to obtain the product of V_f and K_{fs} directly, as shown in Figure 3.3. The standard solutions were prepared by spiking standards into the dog blood obtained from the same beagles for *in vivo* SPME experiments. Static

equilibrium-extraction was performed because agitation doesn't affect V_f and K_{fs}, but temperature should be the same as *in vivo* SPME because temperature affects K_{fs}. In Figure 4, the regression slope of each trend line is the product of V_f and K_{fs} for the corresponding analyte according to eq 5, where K_{fs} is the fiber coating/blood sample distribution coefficients of the drugs. Actually we do not need to calculate or determine V_f and K_{fs} separately; instead we only need the product of V_f and K_{fs} to calculate the sample concentration, C_0, by using eq 3.5. What deserves mention here is that calibration curves were used for finding out $V_f \times K_{fs}$ rather than calibrating the *in vivo* SPME, where the effect from agitation on the pre-equilibrium extraction cannot be compensated by the calibration curve method.

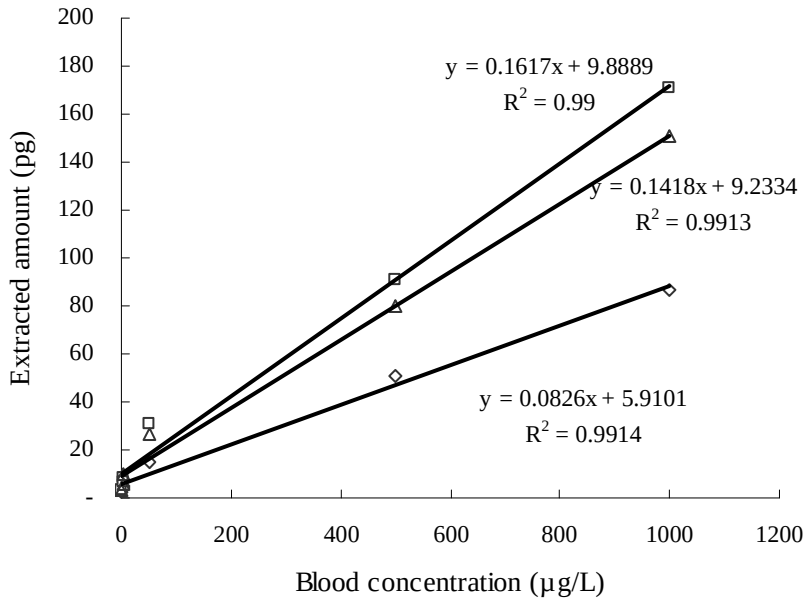


Figure 3.3. SPME calibration with PEG-C18 fibers for diazepam (◊), nordiazepam (□), and oxazepam (Δ). A six-point calibration (n = 3) from 0.5-1000 µg/L was conducted. The regression slope for each analyte presents the product of V_f and its K_{fs}. The standard solutions were prepared by spiking standards into the dog blood obtained from the same beagles for the *in vivo* SPME experiments before drug injection. Static extraction was performed until equilibrium reached (10min) at 37 ℃. The detection limit of the method is shown as 0.5µg/L.

Using the values of $V_f \times K_{fs}$ with Figure 3.3, the sample concentration, C_0, calculated by eq 3.5, is the total concentration of the drug in whole blood, since the K_{fs} used here is distribution coefficient of the drug between the fiber coating and blood matrix. And the free fraction of the drug, which is the active portion of the dose in blood, can be obtained by using the

distribution coefficients of the drugs between fiber and buffer instead. The product of $V_f \times K_{fs}$ for fiber/buffer can be obtained using the calibration curve method in which the standard solutions are prepared with drugs spiked phosphate buffered saline (pH 7.4). Actually, this method for obtaining the product of $V_f \times K_{fs}$ from calibration curve can apply to determining the total and free concentrations in any sample matrix as long as the standard solutions with the same sample matrix. With this method, the binding affinity of the drugs to blood matrix can be calculated by using a similar way described elsewhere. [18]

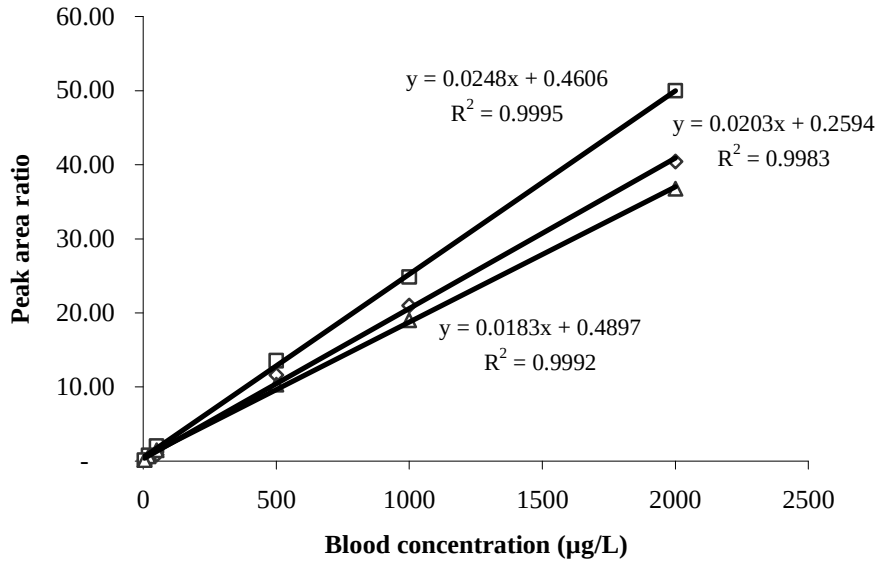


Figure 3.4. Calibration for the conventional blood analysis. A six-point calibration (n = 3) from 5-2000 µg/L for diazepam (◊), nordiazepam (□), and oxazepam (Δ) based on chemical assay was performed at 25 ℃. The standard solutions were

prepared by spiking standards into the dog blood obtained from the same beagles for the *in vivo* SPME experiments before drug injection. The detection limit of the method is 5μg/L.

In previous studies, the conventional blood draw followed by plasma preparation, and chemical analysis was conducted to obtain the total concentration of drugs in plasma. And the resulting plasma concentrations were used to validate the blood concentrations by SPME without considering the absorption of the drugs by blood cells.[4, 11] In this research, the partitioning of the drug analytes between blood cells and plasma was determined. The percentages of the three analytes diazepam, nordiazepam, and oxazepam partitioned in the plasma are about 59 %, 63 %, and 68 %, respectively, where the drug concentrations in whole blood were taken as 100 %. Consequently, the total concentrations obtained from conventional chemical analysis were used to validate the total concentrations from SPME experiments. The procedure of the chemical assay is described in the experimental section, and calibration curves, as shown in Figure 3.4, were employed to calibrate the concentrations in blood. The linear correlation coefficients (R^2) are better than 0.998, which demonstrates the validity of the chemical assay over the linear range (5-2000 μg/L). The detection and limit is 5μg/L.

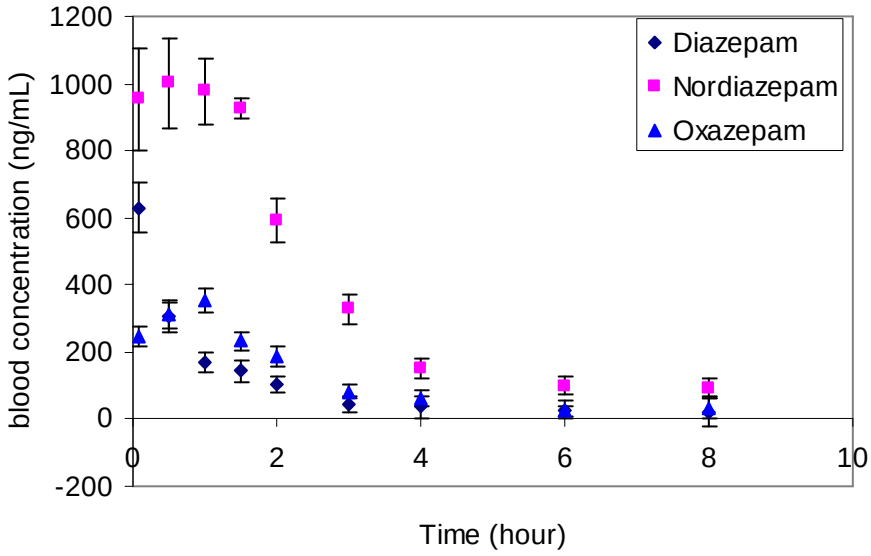


Figure 3.5. Averaged pharmacokinetic profiles of diazepam, nordiazepam, and oxazepam were monitored by *in vivo* SPME over 8 hours on three dogs (n = 6 for the last point, and n = 9 for all the other points). Kinetic calibration based on deuterated standards was performed during the experimental course.

For *in vivo* SPME experiments, diazepam pharmacokinetics in dogs was studied to evaluate the performance of PEG-C18 probes and the kinetic calibration method. The pharmacokinetic profiles of diazepam and the two significant metabolites nordiazepam and oxazepam were monitored by *in vivo* SPME over 8 hours, as shown in Figure 3.5. Accordingly, the results of conventional blood analysis were presented in Figure 3.6. The results from *in vivo* SPME showed good consistence with the results from conventional assay. The correlation coefficients (r) between them are close to 1 (0.97-

0.99). The results showed that the metabolism rate for diazepam in beagles was rapid, as evidenced by the fact that nordiazepam had higher concentrations even in the early stage after drug administration.

Compared to the previous studies,[4, 11] this work provides more detailed and accurate information in the pharmacokinetic profiles ascribed to combining the following advantages together: the accuracy and high sensitivity resulted from the large linear range and high capacity of non-competitive PEG-C18 probes, the fast extraction time decided by pre-equilibrium extraction, as well as the proved merit that the kinetic calibration is inherently more accurate than calibration curve method.

3.4 CONCLUSION and ADDENDUM

3.4.1 Conclusion

In this work, the feasibility of the kinetic calibration of SPME for *in vivo* sampling was demonstrated by theoretical considerations and experimental verification. And the application conditions of this method deserve more attention. Up to now, all the applications of kinetic calibration are restricted in liquid coating SPME, while further investigation for solid coating need to conduct in future. As a result, firstly, the nature of the SPME fiber coating must be determined before using; secondly, the linear range, capacity, equilibration time of the extraction in a given sample matrix need

to be determined to decide the appropriate sampling time so as to keep the n/n_e and Q/q_0 within the range of 40-60 %.

In order to obtain the concentration, a simple method was proposed to calculate the product of V_f and K_{fs}, instead of using the traditional Scanning Electron Microscopy method, which is more tedious and more expensive. Therefore, either the total drug concentration or free concentration can be obtained by using K_{fs} for fiber/blood (or other matrix) or fiber/ buffer respectively. The developed method is sensitive for blood sampling (detection limit: 0.5 ng/mL) and broad linear range (0.5-1000 ng/mL).

Finally, the PEG-C18 fibers were applied to the *in vivo* sampling, more detailed information in the pharmacokinetic profiles were obtained because of the larger linear range and higher capacity of PEG-C18 probes, as well as the faster extraction time. In addition, a simple chemical assay was developed to determine the total concentrations of drugs in whole blood (detection limit: 5 ng/mL; linear range 5-2000 ng/mL), and the results were highly consistent with the SPME results.

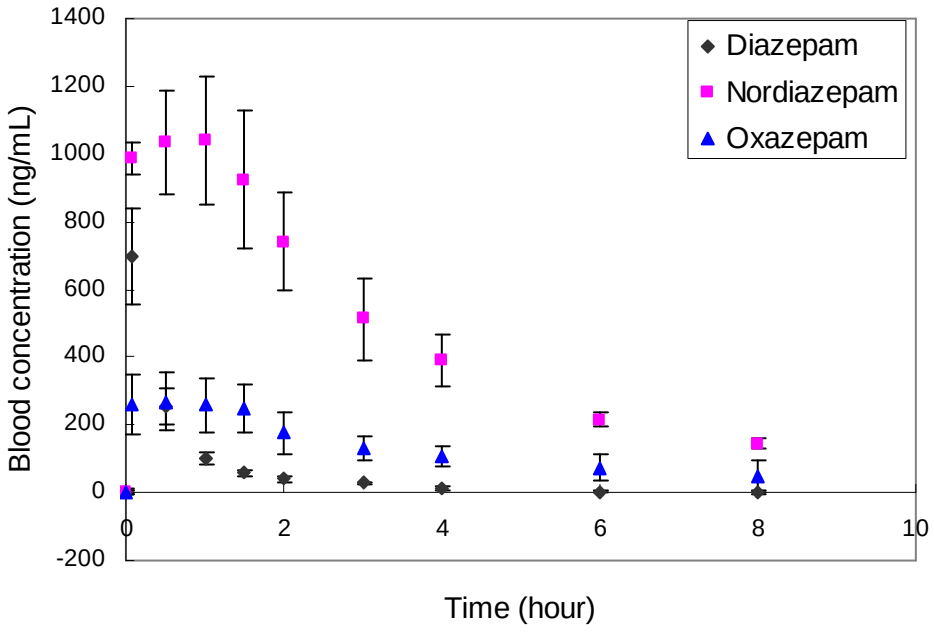


Figure 3.6. Averaged pharmacokinetic profiles of diazepam, nordiazepam, and oxazepam were monitored by conventional blood draws and *in vitro* chemical assay over 8 hours on three dogs (n = 3). Calibration was based on standard curves shown in Figure 3.4.

3.4.2 Addendum

The text of this chapter has been rewritten compared to the published paper.

Chapter 4

Simplified Kinetic Calibration of Solid-Phase Microextraction for *In Vivo* Dynamic Monitoring

4.1 Preamble and Introduction

4.1.1 Preamble

This chapter has not been published. Ali Es-haghi and Jibao Cai contributed to this project. The contributions of Es-haghi, A. involved the *in vivo* experiment. And Cai, J. contributed to the *in vitro* microdialysis experiment and manuscript revision.

I, Ali Es-haghi, authorize Xu Zhang to use the material for his thesis.

Signature:

I, Jibao Cai, authorize Xu Zhang to use the material for his thesis.

Signature:

4.1.2 Introduction

Solid-phase microextraction (SPME) has been demonstrated to be a promising approach to *in vivo* sampling for pharmacokinetic studies, due to its simple instrumentation and implementation, its time-effectiveness and cost-effectiveness, its miniaturized format, and the little disturbance it causes to the system under study. [1-4] However, improving the quantitative capability and automatic potential of SPME for *in vivo* application remains the main task for further development. These improvements can be addressed significantly by bettering the calibration method, because the operational procedure is mainly determined by the calibration approach adopted. On one hand, the simpler the operation, the easier the automation of the procedure; on the other hand, the accuracy of SPME approach, as a distribution equilibrium based sampling technique, depends on whether the appropriate calibration procedure is adopted. Moreover, it would be desirable to have an analytical method with high temporal resolution for dynamic monitoring in a living system, as *in vivo* pharmacokinetic studies. Therefore, fast sampling based on pre-equilibrium SPME would be more suitable than the widely-used sampling strategy based on equilibrium extraction. However, the well-established external calibration method is not suitable to *in vivo* pre-equilibrium sampling due to its inability of correcting

for unknown blood flow, which can seriously affect SPME results in pre-equilibrium conditions.

Accordingly, quantitative analysis was conducted by adopting the kinetic calibration method, or standard-on-fiber calibration, by which analyte extraction is calibrated by determining the desorption of the pre-loaded standards on the fiber. [5-6] Normally, isotope-labeled analogues of the analyte compounds are used as standards and a mass spectrometer serves as the detector. This method is not only simpler than the calibration curve method, but also compensates for the effects of agitation, temperature, and matrix, and thus provides accurate quantification, especially for on-site sampling where the traditional standard addition calibration is not applicable. [7-10] However, the classic kinetic calibration is based on the use of often expensive or even unavailable isotopically labeled standards; this restricts the method from widely application. In addition, at every sampling time-point during the whole experiment fiber with preloaded-standard is needed even in the same sample system, which not only increases the cost, but may also cause experimental error. For example, there must be some loss of standard and/or its activity within the standard-preloaded fibers, since all the fibers are loaded simultaneously but not used at the same time. The loss could be significant when using volatile compounds as standards or radioisotope-labeled standards with short half-lives. Finally, the mass spectrometer (MS) must be required to differentiate the analytes and

deuterated standards, thus not suitable for on-site monitoring for field sampling; moreover, the MS instrument might not be available in every lab and institution, especially in developing countries due to its high price.

There have been some efforts to address this problem. Recently a dominant kinetic calibration method was proposed in which the normal standard instead of the deuterated standard is employed as the calibrant.[11] But the method is inherently limited because the extracted analyte affects quantification of the detected standard signal and results in systematic errors. To reduce error, it is thus necessary that the amount of extracted analyte is negligible compared to the amount of standard remaining on the fiber after desorption. Accordingly, the sampling time must be very short; however, a short sampling time decreases the method's sensitivity and makes it inapplicable for low concentration samples. When sample concentration is high, systematic error from the interference of the signals of analytes with those of standards can be significant. In addition, the experimental operation is time-consuming and labor-intensive because it uses four fibers for standard desorption and an additional fiber for sampling in different sampling sites—which may further decrease the accuracyof the SPME analysis by overlooking variation in concentration among sampling sites. The technique may be useful for studying a static and uniform sample system, but it is not suitable for highly dynamic systems, as in

pharmacokinetic studies of veins where blood flows and drug concentrations change quickly due to a fast metabolism rate.

In this report, based on the finding that the time constant *a* is independent from sample concentrations, a single time-point kinetic calibration was proposed. Afterwards, a normal standard (non-isotope-labeled) kinetic calibration was developed when applying the single time-point calibration before the drug was dosed into the living system. Furthermore, the use of single-standard to accurately calibrate multiple analytes was brought forward, and the quantitative relationship between different analyte concentrations was derived. Finally, a single-point self-calibrated SPME approach was developed for *in vivo* pharmacokinetic studies. Based on these simplified calibrations, the *in vivo* SPME approach for pharmacokinetic studies can be operationally simple, cheap, but more accurate.

To clarify, a brief comparison of two groups of terms is given as follows. First, sampling time-point (also called time-point for sampling or simply time-point) describes the specific instances during the whole pharmacokinetic course when SPME sampling is performed. For example, in the current study, the whole pharmacokinetic course was 8 hours, and there were 9 sampling time-points: 5 min, 0.5 h, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6h, 8h. By contrast, the term “sampling time” describes the length of time the SPME fiber is in direct contact with the target sample. For example, in the

current *in vivo* study, sampling times were set at 2 min and 1 min; the sampling time started after the sampler was introduced into sample and the SPME fiber was exposed to venous blood, and stopped when the fiber was withdrawn from the vein.

Second, for SPME experiments, there are usually two types of calibration. One is calibration for the instrument response. For example, when using HPLC-MS/MS as a detector, an external calibration curve must be constructed in order to calibrate the response factors (the process relating the amount of analyte introduced into the instrument with its response). The second is calibration for the sampling approach or the SPME method, the process relating the amount of analyte extracted by the SPME fiber with the initial analyte concentration in the sample. This study focused on calibration of the SPME for *in vivo* dynamic monitoring, where whether it is necessary to use chemical standards for quantification depends on the calibration method adopted. However, the standards must be used to calibrate the instrument response.

4.2 EXPERIMENTAL SECTION

4.2.1 Preparation of SPME Probes

PEG-C18 SPME probes and devices that were prepared by the method were described elsewhere.[3] The C18 particles (10 micron) and PEG were

from Supelco (Bellefonte, PA). The home-made fibers were with quite large inter-fiber variation (up to 70-80%) in terms of the amount of analyte extracted at equilibrium; therefore, all fibers that had the same capacity were selected for *in vivo* experiments.

4.2.2 *In vitro* Experiments

The extraction behaviors of the probes including extraction time profile, desorption time profile, matrix effect of plasma and whole blood, and dynamic range were characterized in previous work. In this project, to test the feasibility of the simplified calibration methods under a precisely controllable condition, all *in vitro* experiments were conducted within a simple artificial circulation system, which was designed to simulate the blood flow in dog's vein and the schematic was reported in previous report.[4] Phosphate buffered saline (PBS, pH 7.4) and EDTA-anticoagulated beagle whole blood and plasma from Biological Specialties Corp. (Colmar, PA) were used as sample matrix and flowed within the artificial circulation system and the flow rate was set by the peristaltic pumps.

The deuterated standards (diazepam-d_5, nordiazepam-d_5, and oxazepam-d_5) were from Cambridge Isotope Laboratories (Andover, MA) and the non-deuterated standards (diazepam, nordiazepam, and oxazepam) were from Cerilliant (Austin, TX). For loading the fibers with standards for calibration, the loading solution was prepared by spiking deuterated

standards or non-deuterated standards into deionized-water at 50 µg/L for each. For *in vivo* use, the fibers should be sterilized by immersing the samplers in 8 % formaldehyde and 70 % ethanol for 18 hours. Then the sterilized probes were exposed into the loading solution during 30 minutes for loading and then kept in the sterile Falcon tubes for use. For *in vitro* experiments, the fibers and the deionized-water were not sterilized; the other conditions were the same.

4.2.3 LC-MS/MS Analysis

A CTC-PAL autosampler/Shimadzu 10 AVP LC/MDS Sciex API 3000 tandem MS system was used for the analysis of the drugs and their deuterated standards. The assay conditions were the same as described before,[1,3] except that the transitions monitored for the deuterated standards were: m/z 290.2/154.1 for diazepam-d_5, 276.1/140.0 for nordiazepam-d_5, and 292.1/246.1 for oxazepam-d_5 respectively.

4.2.4 Animal Experiments

All animal experiment procedures were approved by the Animal Care Committees at University of Guelph, and the experiments were performed in the Central Animal Facility of the University of Guelph (Guelph, ON, Canada) as described previously.[3] The dosage of diazepam was 0.5 mg/kg. About 20 minutes before the intravenous administration of diazepam, three

sequential desorption experiments were conducted by consecutively introducing three probes that were preloaded with non-deuterated diazepam into the cephalic vein through the implanted catheter. The desorption lasted 2 min, and then after a brief rinse with deionized water and drying with Chemwipes, the probes were withdrawn and kept in sterile tubes in dry ice for lab analysis. Blood concentrations of diazepam, nordiazepam and oxazepam were monitored for 8 h. At each sampling time-point, one probe loaded with deuterated standard was introduced for 2 min to perform pre-equilibrium extraction after blood drawing for conventional analysis. At the 2 h and 3 h time points, two blank probes (without standard) were introduced sequentially before the introduction of the probe with deuterated standards. The sampling time is 1 min and 2 min for the two blank probes, respectively. Therefore, at each of these two sampling time-points, three probes were employed, the first two of which were blank probes and the last one was preloaded with deuterated standards.

4.2.5 Conventional Blood Analysis

The conventional blood assay was conducted as previously described.[3] One mL of blood was withdrawn after SPME extraction at each time-point, and 2 mL of blood was withdrawn before the three diazepam-loaded fibers were introduced. Five hundred µL of acetonitrile were mixed with 100 µL of whole blood in a 1.5-ml microcentrifuge tube, followed by vortex and

centrifugation (5000 rpm, 10 min). Then 400 µL of supernatant were collected and evaporated under nitrogen gas. Samples were reconstituted in 50 µL of reconstitute solution (methanol/pure water: 1/1, containing 10µg/L of lorazepam as an internal standard). A six-point calibration (n = 3) from 5-2000 µg/L for diazepam, nordiazepam, and oxazepam was performed at 25 ℃. The standard solutions were prepared by spiking standards into the dog blood collected before drug administration.

4.3 RESULTS AND DISCUSSION

A unique advantage of the SPME technique is its efficacy and simple implementation in on-site sampling, of which *in vivo* sampling is a specific case where sample concentrations change quickly and the matrix effect is complicated. It requires not only sampling probes with fast response times, but also appropriate calibration methods. However, the traditional standard addition method and internal standard method are not applicable for *in vivo* sampling because it is impossible to handle the sample—for example, to mix the sample with spiked standards very well and to prevent metabolic degradation of the standards. The well-established calibration curve method can deliver accurate results for on-site or *in vivo* SPME, but only when equilibrium extraction is performed. Only kinetic calibration using a preloaded standard on the fiber can achieve accurate calibration for pre-equilibrium sampling.[5] When kinetic calibration is applied in *in vivo*

sampling, it is interesting to find that, *Q* (the amount of preloaded standard remaining on the fiber after 2-min sampling) was almost constant at different sampling time-points (Fig. 4.1). This finding suggested that we can further simplify the kinetic calibration without sacrificing its precision and accuracy. After careful investigation of desorption and extraction processes, some very useful applications were developed.

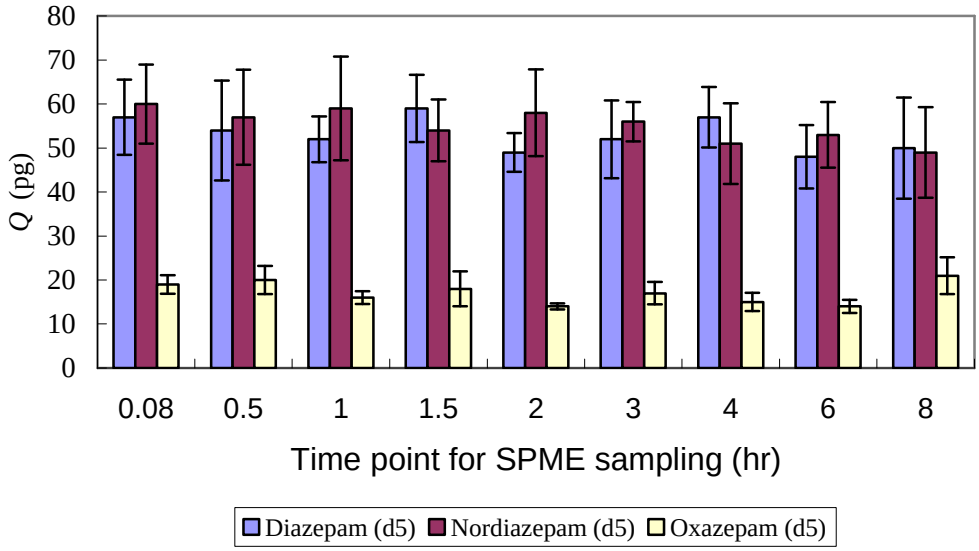


Figure 4.1. The amount of deuterated standard remaining in the fibers (*Q*) after exposure of the preloaded fibers in vein for 2 min at each time point for SPME sampling during the 8-hour *in vivo* experiment.

4.3.1 One-Point Kinetic Calibration for Pharmacokinetic Studies

The classic kinetic calibration is based on the symmetric relationship between absorption (or adsorption) of analytes onto the fiber and the

desorption of the preloaded standard into the sample matrix, as described by eq 4.01:[5]

$$\frac{n}{n_e} + \frac{Q}{q_0} = 1 \qquad (4.01)$$

where n is the amount of analyte in the extraction phase after sampling time t, n_e is the amount of analyte in the extraction phase at equilibrium, Q is the amount of standard remaining in the extraction phase after exposure of the extraction phase to the sample matrix for the sampling time, t, and q_0 is the amount of standard preloaded in the extraction phase.

In the *in vivo* pharmacokinetic studies, we observed that Q was a constant at every time point (from 5 min to 8 h) after the fiber was exposed into the venous blood for 2 min (t, sampling time). If it is true through the duration of 8-hour experiment, 1 - Q/q_0 should also be a constant, since q_0 was a constant, which was set when the fibers were selected out. Consequently, n/n_e is a constant according to eq 4.01. Thus the purpose of the calibration is to find the constant value of n/n_e or 1 - Q/q_0. As Q is a constant at every sampling time-point, one time-point is enough to find the Q value instead of conducting the standard desorption at all the time-points. The only benefit for conducting standard desorption at multiple time-points is decreasing the standard deviation by increased repetition times. However, we could achieve this by repeating desorption several times at one single time-point rather than multiple time-points. Therefore the assumption that Q is a constant during the pharmacokinetic studies provides the basis for the

simplification of the classic kinetic calibration to single time-point calibration.

In order to verify this basic assumption, both theoretical and experimental studies were conducted. In theory, it is easy to find that only sampling time, *t*, and the time constant, *a*, affect the *Q* value according to the desorption kinetics of the preloaded standard, [5]

$$\frac{Q}{q_0} = \exp(-at) \qquad (4.02)$$

In the experiment, *t* is always 2 min for all the fibers. Therefore, *Q* would be a constant as long as *a* value keeps constant. For diffusion in boundary layer based SPME using C18-PEG fibers, *a* value is dependent on fiber parameters, the fiber-sample distribution coefficient, and the sample agitation, as described by eq 4.03.

$$a = \frac{D_s A}{\delta_s K_{fs} V_f} \qquad (4.03)$$

where D_s is the diffusion coefficient of the analyte molecules in the sample matrix, δ_s is the thickness of the boundary layer, which is mainly affected by the agitation,[12] K_{fs} is the distribution coefficient of the analyte between fiber coating and sample matrix, A is the surface area of the fiber coating, V_f is the fiber volume; for an adsorptive coating, the quotient of A/V_f is the specific surface area of the fiber coating, which describes the porosity of the coating material. The eq 4.03 indicates that in the same sampling system, where all the conditions such as the analyte, fibers, sample matrix, temperature and

agitation are fixed, *a* value should keep constant, and thus makes *Q* value constant. It is easy to be understood that, in the beagle experiment, the physiological conditions of the dogs such as the temperature, flow rate and composition of the venous blood did not fluctuate too much to create significant variation of *a* value and *Q*. The data from the *in vivo* experiment support the single-point calibration. For example, as shown in Fig 2, the averaged *Q* value of oxazepam-d_5 (16 ± 1 pg, $n = 3$) at the time-point of 1.5 hour after drug administration was used to calibrate the sampling at all the 9 time-points. The results were very consistent with those from the conventional multiple time-point calibration.

The single-point kinetic calibration would be more timesaving and cost-effective since it uses much less standards and standard loaded fibers. Meanwhile, there would be no error from the loss of standard and/or its activity (e.g., radioactivity) in the fibers since all the fibers are not only loaded but also used simultaneously.

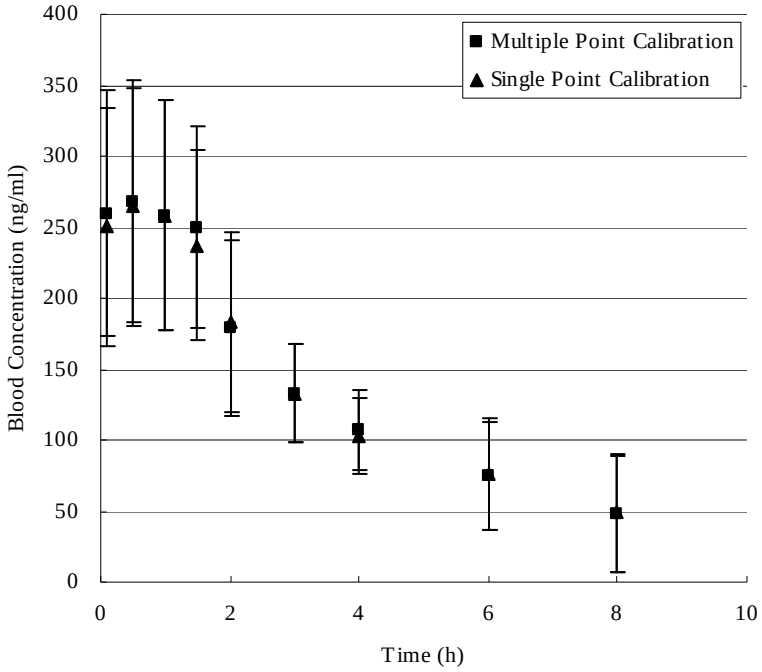


Figure 4.2. The blood concentrations of oxazepam calibrated by single time-point kinetic calibration and by multiple time-point calibration. The single time-point calibration was based on the desorption of oxazepam-d_5 at the time-point of 1.5 hour after drug administration.

In addition, the observation that *Q* value kept constant during the 8-hour indicated that *a* value was not affected by sample concentration, which changes dramatically during the pharmacokinetic profile. Actually, in eq 4.03 there is not a factor determined by the analyte concentration as long as the concentration does not change so much that it affects the sample composition significantly. In order to verify this argument, an *in vitro* experiment was conducted to test the relationship between *a* value and sample concentration. Standard solutions were prepared in dog plasma with

different concentrations from low concentration 10 ng/ml to high concentration 2000 ng/ml. The standard solutions circulated in the flow system at a linear velocity of 7.2 cm/s (at 25 ˚C). The SPME fibers loaded with deuterated standards were exposed into the standard solutions in different time to obtain desorption and extraction time profiles. The values of the *a* constant could be easily obtained from the slopes of the logarithmic form of extraction/desorption time profiles (Fig 4.3); Table 4.1 presented *a* values that were calculate by using $(1/t)\ln[n_e/(n_e-n)]$ with extraction data or $(1/t)\ln(q_0/Q)$ with desorption data. The data confirm that the *a* value is independent of the sample concentrations. This conclusion is also consistent with the observation that the sample concentration has no impact on the equilibrium time of SPME.[13]

Table 4.1. Time constant *a* for absorption and desorption of benzodiazepines in plasma samples at different concentrations

		time constant *a* (*SD*)		
absorption (25 ℃)	C_0 (μg/L)	diazepam	nordiazepam	oxazepam
	10	0.3498 (0.0321)	0.3338 (0.0232)	0.5791 (0.3241)
	50	0.3611 (0.0343)	0.3404 (0.03105)	0.5823 (0.4322)
	500	0.3551 (0.0239)	0.3329 (0.0347)	0.5945 (0.0512)
	2000	0.3429 (0.0312)	0.3358 (0.0344)	0.601 (0.0459)
desorption (25 ℃)		diazepam (d_5)	nordiazepam (d_5)	oxazepam (d_5)
	10	0.3532 (0.0328)	0.3576 (0.3124)	0.5878 (0.0602)
	50	0.3681 (0.0396)	0.3697 (0.0421)	0.5714 (0.0475)
	500	0.354 (0.0402)	0.3236 (0.0332)	0.6005 (0.0575)
	2000	0.3601 (0.0399)	0.3427 (0.0198)	0.6167 (0.0626)

This finding has two important implications. Firstly, if the absorption (for extraction) or desorption (for calibration) is optimized for a given concentration, the equilibrium time and the time constant *a* will be the same for other concentrations. This proves the feasibility of SPME for dynamic monitoring. Secondly, as mentioned before, in dynamic monitoring like a pharmacokinetic study, the information of the desorption obtained from any single time-point can be used to calibrate the data for absorption from all

other sampling time-points through the whole experimental duration, as long as the experimental conditions such as sample matrix, agitation, and temperature do not change significantly over the experiment course. This provides the basis for simplifying the multiple time-point kinetic calibration to a single time-point calibration.

However, for practical use of the single-point kinetic calibration, three experimental issues deserve further discussion. The first is how many times repetitive desorption should be conducted at the single time-point in order to ensure the method's precision. According to statistical principles, the standard error will not be improved rapidly after 3 - 4 replicates.[14] Therefore we chose three-time repetition in this study.

The second issue is the length of the sampling time (also desorption time). As an analytical method with high quantitative capability, the single time-point SPME strategy should be highly accurate and precise. This requires a relatively long sampling time, so that the difference between Q, the remaining amount of standard on the fiber, and q_0, the amount of standard preloaded on the fiber, is significant. Thus the longer the sampling time, the more accurate the results—but also the worse the temporal resolution of the sampling for tracking drug metabolism in a dynamic monitoring situation where sample concentrations change rapidly. To compromise between temporal resolution and accuracy, both the extracted amount (n) and desorbed amount ($q_0 - Q$) should be reproducibly detectable;

therefore, a n/n_e or Q/q_0 within the 30-70% range is preferable, considering the undesirable variation in fiber manufacturing, experimental operation, and instrumental analysis. The *in vitro* experiment showed that 2-min was good for both desorption and extraction.

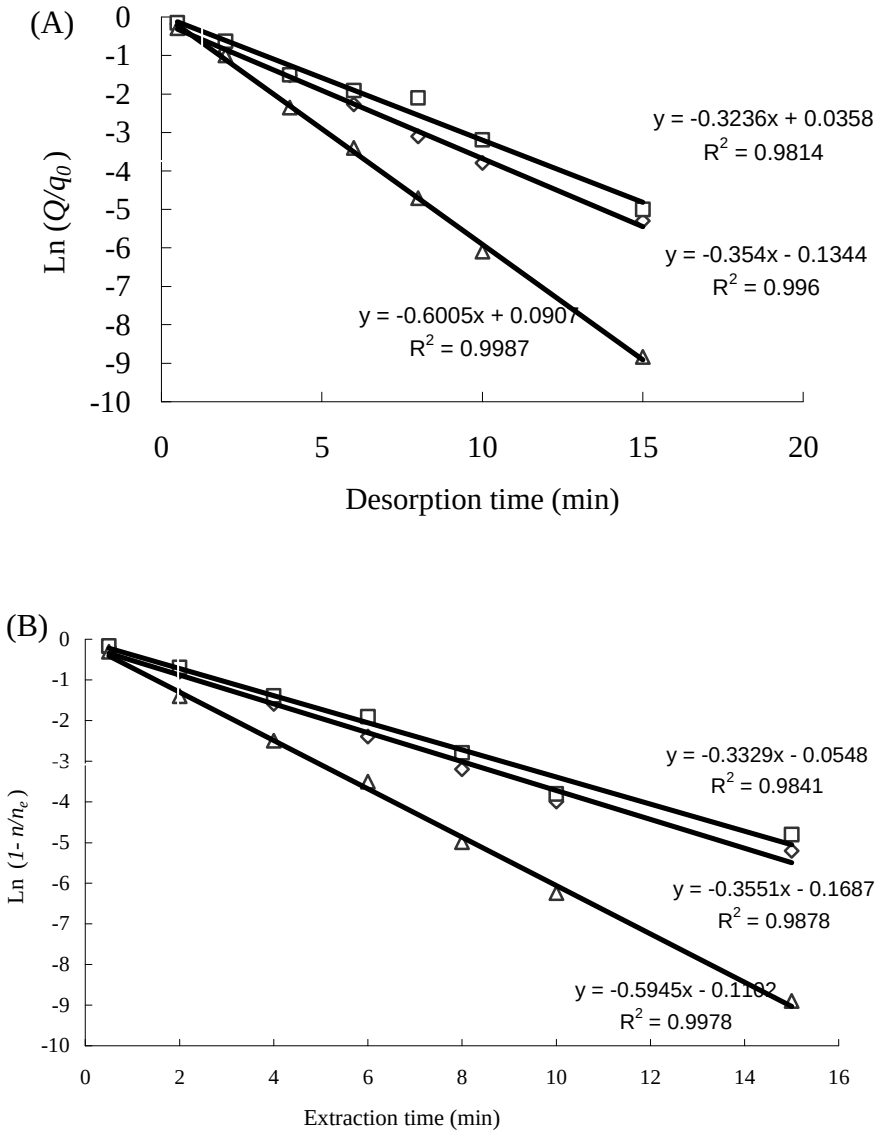


Figure 4.3. The desorption time profiles (A) of diazepam-d_5 (◊), nordiazepam-d_5 (□), and oxazepam-d_5 (Δ) and absorption time profiles (B) of diazepam (◊), nordiazepam

(□), and oxazepam (Δ). The absorption and desorption were performed in plasma standard solution in a flow system at a rate of 7.2 cm/s (at 25 ˚C).

The last issue is determining which time-point during the whole pharmacokinetic duration should be selected for the standard desorption. Theoretically, as long as the blood flow (agitation of the sample) does not change significantly, all time-points can be chosen for the standard desorption. However, in practice, time-points at a sufficient time interval from the next sampling is preferable, in order to ensure enough time for performing several sequential desorption experiments; this is because it is not practical to conduct parallel operations, given the dimension of dog's forearm vein. In this pharmacokinetic study, flowing time-points, such as 2h, 3h, 4 h, 6h and 8 h, are preferable because the 1-2 hour interval is enough for performing the standard desorption in all three dogs.

4.3.2 One-Point Kinetic Calibration by Non-isotope Labeled Standard

Interestingly, performing the standard desorption before drug administration brought a more important simplification of the kinetic calibration. As discussed above, the *Q* value will remain constant as long as blood flow does not change significantly. This indicates the possibility of performing the desorption with the non-isotope labeled standards (i.e., the normal standards for the analytes) in order to obtain *Q* before drug administration, when there is no endogenous source (from metabolism or

secretion) of drug analyte in the blood to interfere the Q value. This new approach might be called “non-isotope labeled standard kinetic calibration”. Theoretically this approach is more accurate than using isotope-labeled compounds as standards, because there is no difference in the physicochemical properties between the analyte and the standard (i.e., they are the same), and because the sum of Q/q_0 and n/n_e is exactly 1. By contrast, the difference between the isotope-labeled standards and their non-labeled counterparts affects the accuracy of the calibration. For example, in previous studies, the sum of Q/q_0 and n/n_e was always somewhat different from 1.[5,8-9] Moreover, when the isotope-labeled compounds are not available, the kinetic calibration based on non-labeled standards is the only option. Furthermore, it is cost-effective since isotopic labeled standards are usually much more expensive than non-labeled ones.

This novel calibration method was verified by the *in vivo* experiment. Before the drugs were administrated, the PEG-C18 fibers, which were preloaded with non-deuterated diazepam/nordiazepam/oxazepam standards, were introduced into the venous blood for 2 min. There is no significant difference between the resulting Q values with non-labeled standards (Diazepam: 54 ± 5 pg; nordiazepam: 57 ± 6 pg; oxazepam: 17 ± 3 pg, n = 6) and those obtained with using deuterated standards.

4.3.3 Single Standard Kinetic Calibration for Multiple Analytes

In pharmacokinetic studies, the concentrations of both the dosed drug and its metabolites need to be monitored simultaneously. More analytes are addressed in some environmental screening studies.[15-17] In all cases for quantitative analysis of multiple analytes, it would be desirable to use a single standard to calibrate all analytes simultaneously. However, there were significant discrepancies between the true concentrations and the calibrated ones when using classic kinetic calibration.[5]

To address the problem, we modified the classic eq 4.01 to eq 4.04.

$$\frac{n}{n_e} + \frac{Q}{q_0} = 1 - e^{-a_1 t} + e^{-a_s t} = \mathrm{b} \qquad (4.04)$$

where a_1 and a_s are the time constant for the analyte and the preloaded standard respectively. When the exposure time, t, is fixed, the sum of Q/q_0 and n/n_e is a constant, b. Since n, q_0, and Q are detectable, n_e can be calculated by eq 4.05,

$$n_e = \frac{n}{b - \frac{Q}{q_0}} \qquad (4.05)$$

Afterwards, the initial sample concentration, C_0, can be calculated using eq 4.06,

$$C_0 = \frac{n}{K_{fs} V_f \left(b - \frac{Q}{q_0} \right)} \qquad (4.06)$$

Furthermore, the concentration ratio of any two analytes can be calculated by eq 4.07,

$$\frac{C_1}{C_2} = \frac{n_1 K_{fs2}\left(b_2 - \frac{Q}{q_0}\right)}{n_2 K_{fs1}\left(b_1 - \frac{Q}{q_0}\right)} \qquad (4.07)$$

where K_{fs1} and K_{fs2} are the fiber coating/sample distribution coefficients of target analyte 1 and 2 respectively; n_1 and n_2 are the extraction amount of target analyte 1 and 2 respectively; and C_1 and C_2 are the sample concentrations of target analyte 1 and 2.

Eq 4.07 is suitable for pre-equilibrium sampling. By substituting eq 4.04 into eq 4.07, when $V_s >> K_{fs}V_f$, the concentration ratio of two analytes can also be derived for equilibrium sampling by eq 4.08,

$$\frac{C_1}{C_2} = \frac{n_{e1} K_{fs2}}{n_{e2} K_{fs1}} \qquad (4.08)$$

where n_{e1} and n_{e2} are the amount of two analyte that can be extracted in equilibrium.

The values of a_1 and a_2 can be obtained by doing desorption experiment once or absorption experiment twice in standard solutions. In the *in vivo* sampling, *a* values for diazepam, nordiazepam, and oxazepam are 0.7, 0.65, and 1.1 min^{-1} respectively. Actually, when two *a* values of two analyes are not very different, such as those for diazepam and nordiazepam, eq 4.08 can be directly used to calculate the concentrations of nordiazepam by using those of diazepam. The ratio of K_{e2}/K_{fs1} can be obtained by comparing the slopes of the two calibration curves in the same standard solution with

equilibrium extraction.[3] In the cases when PDMS fibers are employed for aqueous samples, the ratio can be estimated with their K_{ow} values. [18-20]

With these equations (eq 4.06-4.08), the concentrations of other analytes can be calculated when the concentration of any one analyte is obtained. Furthermore, with eq 4.07, the quantitative relationship of the concentrations between different analytes was established. As a result, the one standard kinetic calibration method was developed.

An *in vitro* experiment was conducted to verify the theory. From the natural logarithm format of the absorption time profiles in PBS in the flow system at 6.5 cm/s (at 25 ˚C), we calculated the *a* values for the three analytes and deuterated standard (*a* = 0.27, 0.25, 0.48, and 0.26 for diazepam, nordiazepam, and oxazepam, and diazepam-d_5 respectively). Afterwards, we used the desorption of diazepam-d_5 to calibrate the absorption of diazepam, nordiazepam, and oxazepam. The sample concentrations are from low to high: 10, 50, 200, 500 ppb of diazepam, nordiazepam, and oxazepam in PBS buffer. C18 fibers preloaded with diazepam-d_5. 2 min of extractions were performed. *b* constant can be calculated by eq 4.04 (*b* = 1.01, 0.99, 1.21 for dizapem, nordiazepam, and oxazepam respectively). As listed in Table 4.2, the concentrations of dizapem, nordiazepam, and oxazepam were calculated by eq 4.06. The data showed that the calibrated results were very consistent with the true concentrations, as illustrated in Fig 4.4.

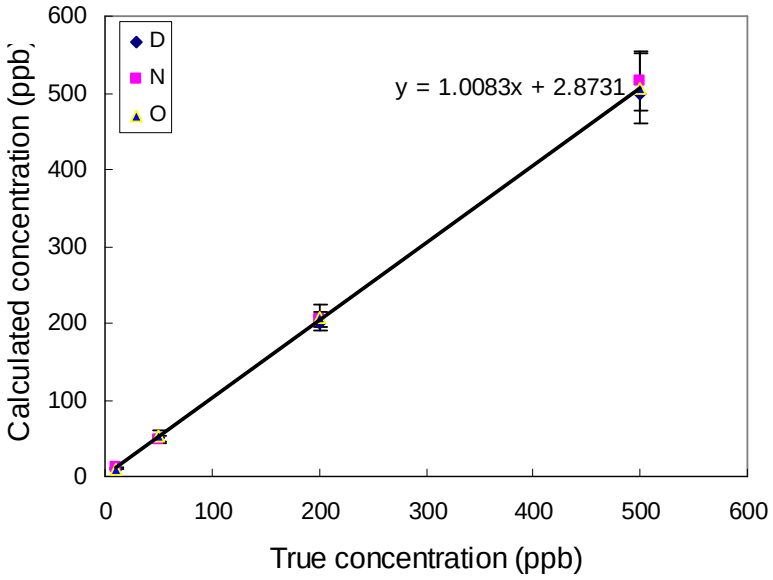


Figure 4.4. The calculated concentration Vs the true concentration of the three analytes: diazepam (◆), nordiazepam (■), and oxazepam (▲).

Table 4.2. The calculated concentration Vs the true concentration of dizapem, nordiazepam, and oxazepam (n = 3) with the single standard (dizapem-d_5) calibration.

Spiked	Calculated concentration (ppb) and relative recovery		
concentration (ppb)	Diazepam	Nordiazepam	Oxazepam
10	10.0±0.4 (96-104%)	11.4±1.1 (103-125%)	10.7±1.9 (88-126%)
50	50.3±1.2 (98-103%)	48.7±4.8 (88-107%)	53.5±6.6 (94-120%)
200	201±3 (99-102%)	204.8±8.9 (98-112%)	207.9±17.3 (95-113%)
500	498±11 (97-102%)	516.2±38.3 (96-111%)	505.7±46.2 (92-110%)

4.3.4 Calibrant-Free Kinetic Calibration for Multiple Analytes

So far all the kinetic calibration methods developed are based on the symmetric relationship between absorption/adsorption and desorption. Therefore, the standard is needed to track the mass transfer kinetics of the analyte during sampling. However, the kinetics of absorption/adsorption can be characterized by the sampling itself, if we perform the sampling twice with different sampling time for each, as shown in the eq 4.09,[21]

$$t_2 \ln(1-\frac{n_1}{n_e}) = t_1 \ln(1-\frac{n_2}{n_e}) \qquad (4.09)$$

where t_1 and t_2 are the sampling time for the first and second sampling, n_1 and n_2 are the amount of analytes extracted during t_1 and t_2 respectively, and n_e is the amount of analyte that could be extracted when equilibrium extraction is performed. In this equation, n_1 and n_2 are detectable and t_1 and t_2 are known, so the n_e value can be calculated. Then we can calculate the sample concentration, C_0, with the established equilibrium-extraction equation: $n_e = C_0 K_{fs} V_f$. The *in vitro* experiment has been conducted in plasma sample spiked with high concentration (500 ng/ml) and low concentration (1 ng/ml) of diazepam, nordiazepam and oxazepam. The sampling time is 1 min and 2 min respectively. The results were without significant difference from those using the traditional kinetic calibration with standard and the equilibrium sampling method (Table 4.3).

Table 4.3. The calculated concentration Vs the true concentration of dizapem, nordiazepam, and oxazepam (ppb) with the different calibration methods. C_0: the initially real concentrations of the plasma sample. C_0^1: the detected concentrations with equilibrium extraction. C_0^2: the detected concentrations with pre-equilibrium extraction and multiple deuterated standards. C_0^3: the detected concentrations with pre-equilibrium extraction and nondeuterated standard. C_0^5: the detected concentrations with pre-equilibrium extraction free of standards.

Analyte	C_0	C_0^1	C_0^2	C_0^3	C_0^4
Diazepam	1	0.9±0.1	1.5±0.9	1.3±0.4	1.3±0.5
Diazepam	500	512.2±24.3	511.4±22.7	541.3±51.8	533.4±54.1
Nordiazepam	1	1.1±0.2	1.1±0.3	0.9±0.4	0.6±0.6
Nordiazepam	500	504.2±17.9	539.5±29.7	479.3±54.3	486.8±69.2
Oxazepam	1	0.9±0.2	1.2±0.2	1.3±0.6	1.6±0.7
Oxazepam	500	470.1±46.0	454.3±30.4	542.9±66.5	562,1±58.7

Actually, the calibrant-free approach can be performed in a single time-point mode to monitor a dynamic process. The basic idea is that the values of *a* constant of each analyte can be calculated by the following equation,

$$a = \frac{\ln(n_e - n_1) - \ln(n_e - n_2)}{t_2 - t_1} \qquad (4.10)$$

Since a value is independent of sample concentrations and keeps constant through all the time-points, we can use it to calculate the values of n_e at every time-point according to eq 4.11,

$$n_e = \frac{n}{1 - \exp(-at)} \qquad (4.11)$$

Then n_e can be used to calculate the sample concentration at that time-point.

The calibrant-free calibration method provides the most cost-effective SPME sampling strategy for multiple analytes with comparable quantitative capability. Furthermore, the multiplexing capability can be unlimited in theory because in nature it is a self-calibration method for every analyte; therefore any analyte can be calibrated as long as its difference in quantity can be reliably detectable between the two samplings. Experimentally, the sampling can be performed in parallel with two fibers, or in series with one fiber for two sequential sampling, but in the latter case, one desorption step should be conducted in between and the fiber must be reusable without changing its performance after the first use in complicated matrix.

4.3.5 Evaluation of the Quantitative Capability of the Simplified Calibration Methods

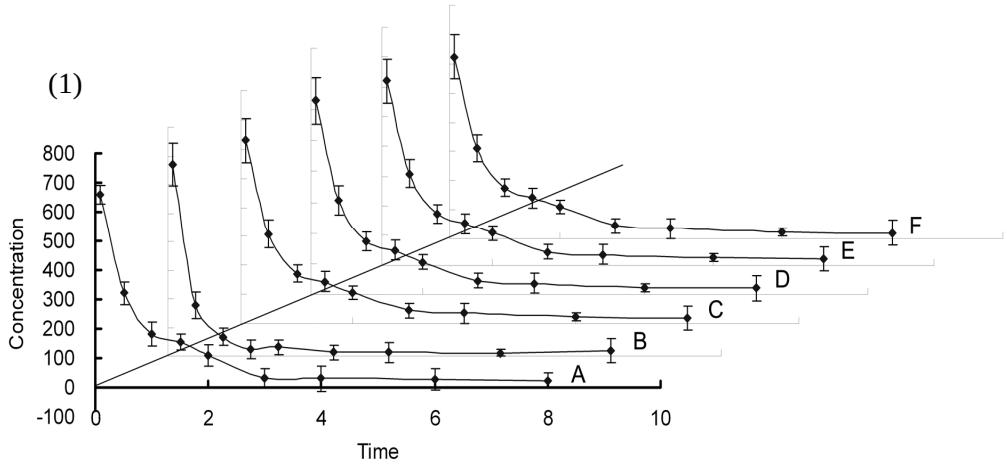
Quantitative capability is one of the most important virtues of SPME technique. We want to simplify the sampling procedures but not sacrificing

the quantitative capability. Therefore, the following five calibration methods were compared in terms of accuracy for *in vitro* monitoring in plasma samples: the equilibrium sampling with calibration curve method and the pre-equilibrium sampling based on kinetic calibration with multiple deuterated calibrants, single deuterated calibrant, single non-deuterated calibrant, and without calibrant. The results were shown in Table 4.3, which showed that there was no significant statistic difference (one-way ANOVA and post-hoc Tukey's test, $P<0.05$) between these kinetic calibration methods, although the standard deviations were a little bit larger than those from the equilibrium extraction, which was ascribed to the less stability of pre-equilibrium SPME than equilibrium SPME, rather than the calibration method itself. And it must be noticed that the relatively higher recovery of the equilibrium SPME in the *in vitro* monitoring does not ensure the better accuracy for the dynamic pre-equilibrium *in vivo* monitoring. Actually as discussed before, the external calibration has inherent limitations in quantifying the pre-equilibrium *in vivo* sampling due to the difficulty of reproducing the matrix and agitation effect of *in vivo* situations in an *in vitro* environment.

Regarding sensitivity issue, the calibration does not affect the detection limit of the methods; however, the sampling strategies adopted affect the sensitivity. For example, in this study, the pre-equilibrium SPME (detection limit: 5-7 ng/mL for diazepam, nordiazepam and oxazepam in blood, 2 min

sampling) has lower sensitivity than equilibrium SPME (detection limits: 1.4-2.8 ng/mL for diazepam, nordiazepam and oxazepam in blood, 15 min sampling). Therefore, as above-mentioned, the sampling time should be set to ensure that there are 30% n_e of analyte extracted.

For the *in vivo* pharmacokinetic studies, we compared the SPME results from the kinetic calibration with multiple deuterated calibrants, with a single deuterated calibrant, with a single non-deuterated calibrant, and without calibrant; afterwards, the SPME results were validated by the conventional chemical analysis, as shown in Fig 4.5. The statistics (one-way ANOVA and post-hoc Tukey's test) showed that there was no significant difference between the each of the four groups of data from pre-equilibrium SPME and the equilibrium SPME at $P<0.05$. The latter is highly correlated with the data from conventional analysis. The correlation coefficients (r) between them are close to 1 (0.97- 0.99).



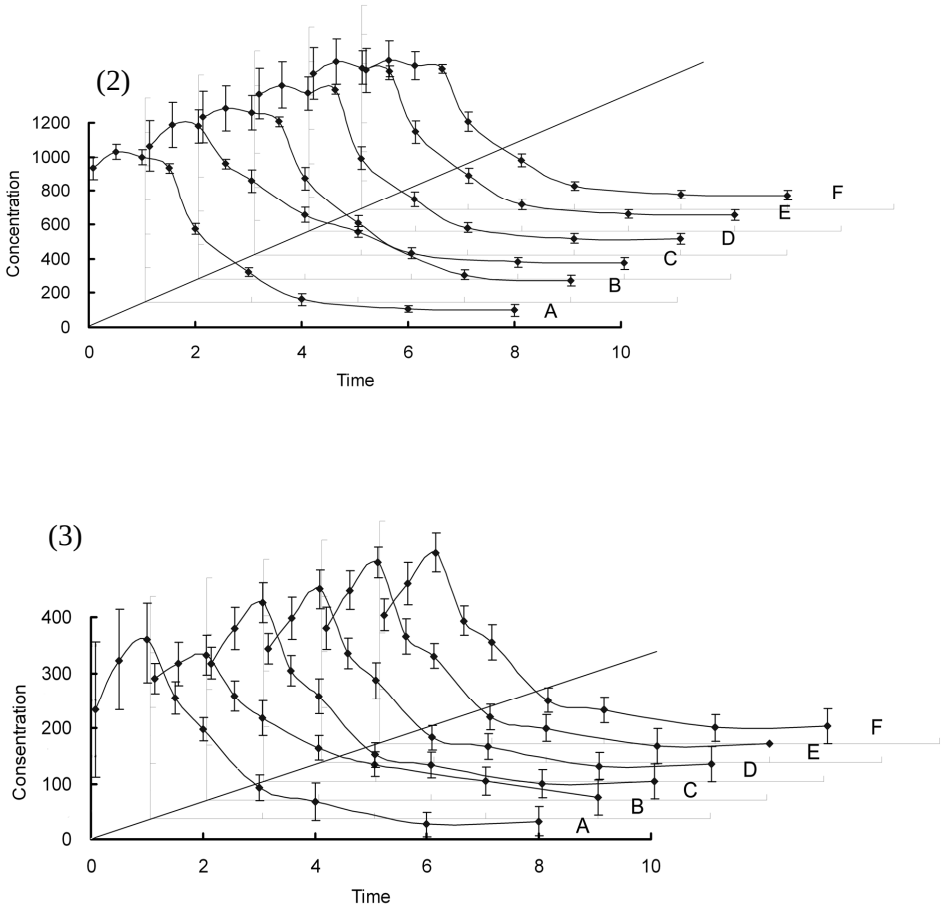


Figure 4.5. Pharmacokinetic profiles of diazepam (1), nordiazepam (2), and oxazepam (3) were monitored by conventional assay and *in vivo* SPME over 8 hours on three dogs (n = 6 for the last point, and n = 9 for all the other points). Error bars were based on standard deviations (S. D.) A: Conventional assay; B: *in vivo* SPME based on equilibrium extraction; C: *in vivo* SPME based on equilibrium extraction; B: *in vivo* SPME based on equilibrium extraction; D: Pre-equilibrium SPME based on kinetic calibration with multiple deuterated standards; E: Pre-equilibrium SPME based on kinetic calibration with single deuterated standard (diazepam-d_5); F: Pre-equilibrium SPME based on kinetic calibration with single non-deuterated standard (diazepam) F: Pre-equilibrium SPME based on standard-free calibration.

4.4 Conclusion

4.4.1 Conclusion

In order to improve the automation potential and quantitative capability of SPME, we proposed three ways to simplify traditional kinetic calibration. The first simplification is temporal, where the single time-point desorption based kinetic calibration was proposed to replace the traditional multiple time-point method. The second approach focused on quantity, where the traditional multiple standards based calibration was simplified into the single-standard calibration and the standard-free calibration method. Thirdly, the kinetic calibration based on non-isotope labeled standards was developed as an alternative to the isotope-labeled standards employed in the traditional kinetic calibration method to obtain reliable calibration. The three simplifications can be combined; for instance, the single time-point kinetic calibration with a single nondeuterated standard or without any calibrant might be used to calibrate multiple analytes at multiple time-points throughout an experimental course. Thus *in vivo* SPME can be preformed not only easily and rapidly, but also accurately, so as to yield high quantitative capability and temporal resolution. Finally, it is worth mentioning that these simplified sampling and calibration approaches are not limited to *in vivo* applications, but are also applicable to *in vitro* dynamic monitoring.

Chapter 5

Development of High Spatial Resolution Solid-Phase Microextraction for *In Situ* Sampling

5.1 Preamble and Introduction

5.1.1 Preamble

This chapter has not been published. Jibao Cai and François Breton contributed to this project. The contributions of François Breton involved the experimental suggestion, and Jibao Cai contributed to the instrument analysis.

I, François Breton, authorize Xu Zhang to use the materials for his dissertation.

Signature:

I, Jibao Cai, authorize Xu Zhang to use the materials for his dissertation.

Signature:

5.1.2 Introduction

As a fast, simple and solvent free sampling and sample preparation method, Solid-phase Microextraction (SPME) has gained wide application from environmental studies to *in vivo* pharmacokinetics;[1-8] however, up to date most applications have been restricted to relatively homogeneous sample systems, such as lake water, air or blood. In some cases, when heterogeneous sample, such as soil, is to be analyzed, headspace SPME provides the operational convenience and avoids the complicated matrix effects.[9] In these situations, spatial resolution does not need to be considered. Consequently, only spatially averaged results along the longitudinal dimension of the extraction phase are obtained. But, there are some situations where *in situ* sampling for heterogeneous systems is conducted, for example, studying the local concentrations in plant or animal tissues where spatial resolution of the SPME fiber plays an important role in accurately quantitative analysis. However, the traditional SPME approaches are not compatible with this aim due to the relatively big size of the fiber coating compared to the sampling locus. For example, to study the concentration and translocation of pesticides within living plants, commercial SPME fibers were applied for herbicides in an onion bulb, but the results were quite qualitative rather than quantitative.[10] There are several

reasons for this outcome. Firstly, the detected tissue concentrations of the herbicides in a specific locus were not in real-time but time-averaged. This is because of the fact that diffusion occurring in the 1 hour of sampling time obscured the concentration difference between the locus under study and its neighborhoods. Secondly, the 1 cm length of the fiber utilized for the onion's heterogeneous structure can only obtain spatially averaged concentrations along the 1 cm length; therefore, it provided a poor spatial resolution. Lastly, the cumbersome desorption process seriously affected the quantitative capability and reproducibility of the analysis. Actually, it is the physical dimension of the probe that determines the spatial resolution of the SPME technique, whereas the fast sampling strategy to obtain real-time concentrations is also necessary to ensure the high spatial resolution technique to be significant. In another word, mainly determined by its probe size, the spatial resolution of a sampling technique is related to its temporal resolution too.

The requirements for high spatial resolution SPME can apply to other *in situ* sampling techniques, such as microdialysis (MD). As a well-established approach, high spatial resolution MD has been commercialized and applied for *in situ* sampling in living systems. For example, it has been widely applied to study the neurochemistry in brains and tissue specific pharmacokinetics.[10-12] However, with faster mass transfer kinetics and unique enrichment capability, SPME might have better

sensitivity as well as higher spatial and temporal resolution for *in situ* sampling.

The objective of this chapter was to develop a novel SPME technique with high spatial and temporal resolution for fast *in situ* analysis in heterogeneous samples. Its feasibility to real sample analysis was tested by investigating the concentration distribution of diazepam within an onion bulb, and validated by the established microdialysis approach.

5.2 EXPERIMENTAL

5.2.1 Overview of Experiments.

In this chapter, a model system was developed for high spatial resolution SPME. This model uses a miniaturized format of SPME fibers and a heterogeneous sample system constructed by multilayered agarose gel with varied drug concentrations in different gel layers. The diffusion-controlled mass transfer within the gel medium was investigated. Fast sampling based on pre-equilibrium SPME with kinetic calibration was conducted to study the local concentration of the diazepam within a 1 mm layer of gel. The partition coefficient, K, and the fiber volume, V_f, were calculated from the calibration curve based on equilibrium extraction in the gel matrix. Finally, the high spatial resolution SPME was applied to study the distribution of diazepam in an onion. To demonstrate the feasibility of

the novel SPME method for *in situ* application, the results were validated by microdialysis (MD). All the SPME and MD experiments were conducted at room temperature, unless otherwise mentioned.

5.2.2 Preparation of the Miniaturized SPME Probes

The C18 particles (10 μm) and polyethylene glycol (PEG) were obtained from Supelco (Bellefonte, PA). C18 coated fibers were prepared and utilized according to the method described previously.[14] An additional step was needed to cut or remove the part of coating using a rajor blade thus leaving two separated 1 mm coating segments remaining on the wire, one segment at the tip of the wire with a 5 mm spacing from the other segment, as shown in Fig. 5.1.

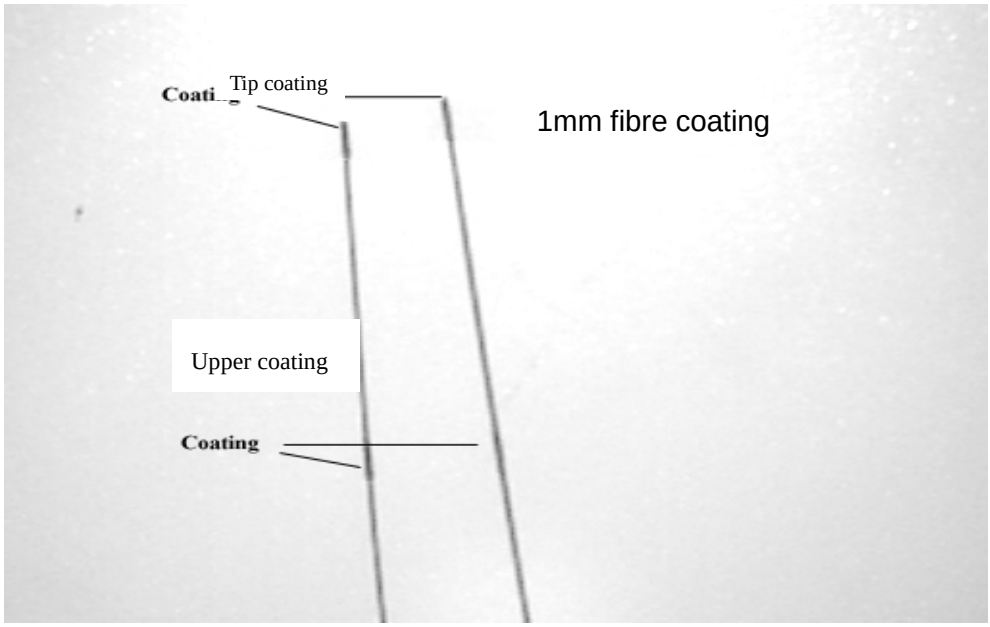


Figure 5.1. The two-segment SPME fibers. The length of each coating segment is on 1 mm.

5.2.3 Loading Deuterated Standards onto SPME Fibers

For performing kinetic calibration, the fibers were preloaded with a deuterated standard (i.e., diazepam-d_5) obtained from Cambridge Isotope Laboratories (Andover, MA). The 50 µg/L loading solution was prepared by spiking diazepam-d_5 into 25 mL of sterile deionized-water. Then, the probes were exposed to the loading solution for 30 min to perform equilibrium extraction (standard loading) and then, stored in clean test tubes for use.

5.2.4 Modeling of Heterogeneous Sample with Multilayered Gel

Agarose gel (1%) prepared in phosphate buffered saline (PBS, pH 7.4) was used to simulate the tissue sample. Every 8 mL gel medium containing a given concentration of diazepam (Cerilliant, Austin, TX) was cast in a 10 ml screw cap vial (Supelco, Bellefonte, PA). The extraction behaviors of the SPME probes, including extraction time profile, desorption time profile, matrix effect of agarose gel, dynamic range of the extraction and symmetry of the extraction and desorption, were conducted in the gel matrices.

To determine the diffusion velocity of diazepam in 1% agarose gel, the gel was cast in the round PYREX crystallization plate (125 X 65 mm) with a thickness of 13 mm, as shown in Fig. 5.2. SPME fibers (8 fibers) were placed in a circle around the plate center with a radius of 30 mm and 45°

angle between each two adjacent fibers. Diazepam (20 µg) in 10 µL methanol was applied in the plate center with a Micropipette. SPME fibers were taken out one by one after every 40 min, briefly rinsed with pure water and then desorbed in 200 µL of HPLC grade methanol (Caledon Laboratories Ltd., Georgetown, ON, Canada) for 10 min. Finally, the methanol was evaporated by nitrogen gas, and the sample was reconstituted in 30 µL of reconstitute solution (50% acetonitrile and 50% nanopure water) with 7.5 ng/mL lorazepam as internal standard.

To model the heterogeneous sample system, gel of multiple layers was cast in a 15 mL plastic Falcon tube. Upward from the bottom, the first 10 cm long supporting gel layer (without diazepam in it) was followed by casting 1 mm thick layer (with 1 ppm of diazepam) and 5 mm long blank gel layer (without diazepam). Afterwards, another 1 mm thick gel layer with 2 ppm of diazepam was cast above the 5 mm blank gel layer. Finally, 10 mm blank gel was cast at the top. It should be mentioned that the following layer was not cast until previous layer had been solidified at 4 °C for 30 min. The positions of the two layers with the drug (i.e., diazepam) were labeled on the tube surface with a marker pen, so that the coating segments of the fibers can be positioned precisely within the layers with diazepam. To introduce the microdialysis probes, two small holes were drilled on the sidewall with a distance of 5 mm at the exact positions of two drug layers. The gel layer system is illustrated in Fig. 5.3.

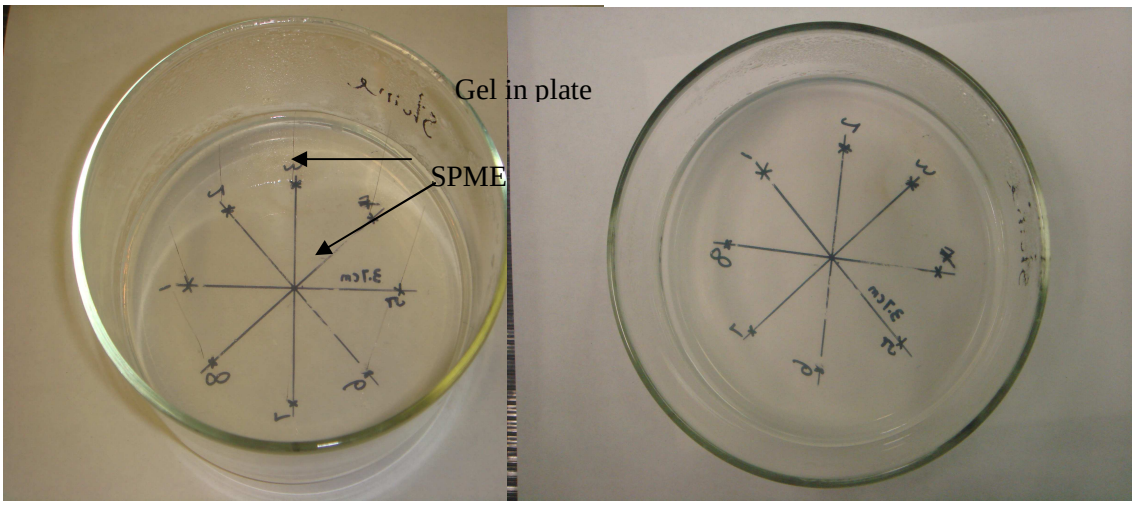


Figure 5.2. Study of diffusion controlled mass transfer. Agarose gel (1%) was cast in the round crystallization plate with a thickness of 13 mm. SPME fibers (8 fibers) were placed in a circle around the plate center with a radius of 30 mm and 45° angle between each two adjacent fibers, as labeled by the numbers from 1 to 8. Diazepam in 10 µL methanol was applied in the plate center at t = 0.

In a triplicate sampling experiment, the segmented fiber preloaded with deuterated diazepam was introduced into the gel and stopped at the exact two layers containing diazepam. Sampling (i.e., extraction) was performed for five minutes. In order to avoid concentration contamination, the fiber was protected with the needle when the fiber was introduced and withdrawn from the gel. A two-step successive desorption was conducted to desorp the extracted analyte and calibrant from the two coating segments into two wells of a 96-well plate, respectively. As shown in Fig. 5.4, first, the fiber was desorped into a well with 50 µL of 100% methanol for 2 min, where the

level of the methanol was only high enough to immerse the first coating segment at the tip of the wire. Then, fiber was put into another well for 2 min containing 200 µL of methanol (100%). The level of methanol was high enough to immerse the second coating segment for desorption. Finally, the methanol in both wells was evaporated, and the samples were reconstituted into 30 µL of reconstitute solution for the analysis.

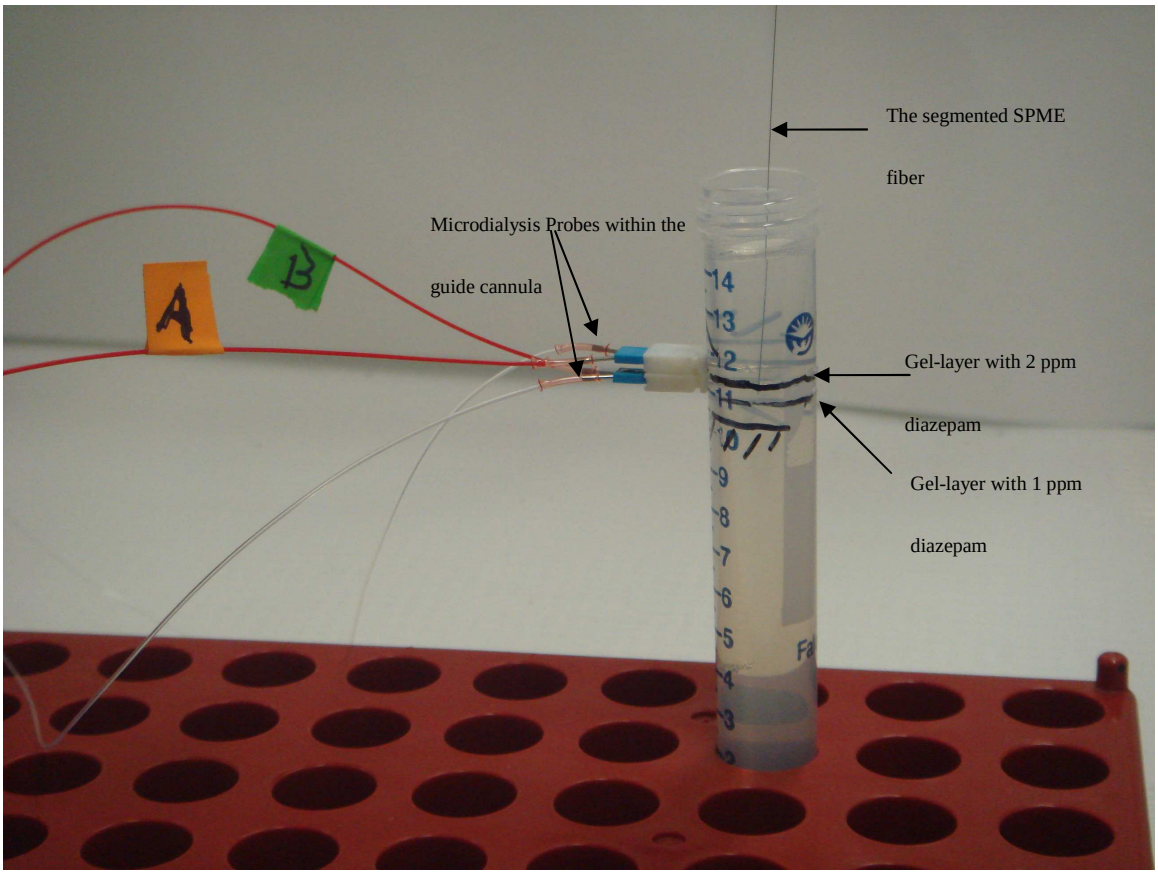


Figure 5.3. Simulation of a heterogeneous sample system with multilayered gel in a plastic Falcon tube. High spatial resolution SPME and microdialysis were conducted to determine the drug distribution in the multilayered gel system.

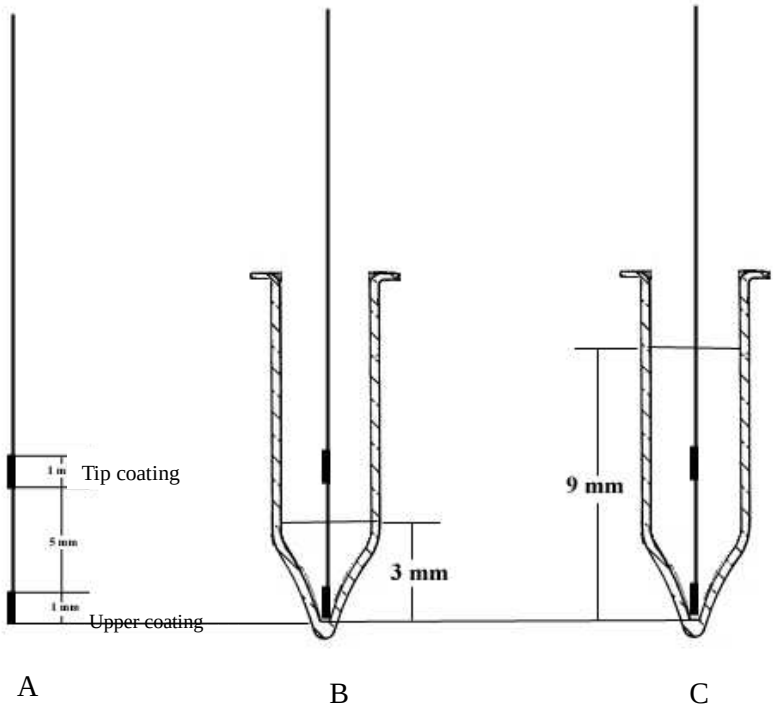


Figure 5.4. The two-step desorption process in a 96-well plate for the extracted analyte and calibrant. A, the segmented fiber; B, the fiber placed into 50 µL of methanol (100%) in a well for 2 min to desorp the analyte from the first coating segment; C, the fiber placed into another well containing 200 µL of methanol (100%) to desorp the analyte from the second coating segment.

For the purpose of validation, two microdialysis probes were placed into the two gel layers, respectively while performing SPME sampling. The microdialysis perfusion fluid was nanopure water, and the flow rate of the perfusion fluid was 2 µL/min. Then, the dialysates were collected in 10 min interval and mixed with 20 µL of acetonitrile for LC-MS/MS analysis.

Quantification of the analyte concentration in microdialysis samples was based on the recovery of the analyte, $C_{dialysate}/C_{perfusate}$.[12]

The limits of detection (LOD) and dynamic range of high spatial resolution SPME and microdialysis were studied separately using standard solutions with various concentrations (0.1 to 500 ppb) in gel medium.

5.2.5 Real Sample Application: Study the Local Concentration of Diazepam in an Onion Bulb

To study the local concentration in a real sample, an onion bulb with a diameter of 6 cm, was chosen because of its layered structure, which made it suitable to serve as a heterogeneous sample model. Methanol/water (10:90, v/v %) of 0.2 mL containing 1 µg/mL of diazepam was injected into the centre of the onion bulb from the stem side 4 hours before the sampling. Towards the axial of the onion, two parallel holes with a length of 1.9 cm were made with a 22 gauge hypodermic needle. The distance between the two parallel holes was 2 mm. The segmented SPME fiber was introduced into one hole while a 4 mm microdialysis probe (CMA/12, CMA/Microdialysis AB, Stockholm, Sweden) was placed into another hole to validate the results from SPME. The schematic for the experimental setup is shown in Fig. 5.5.

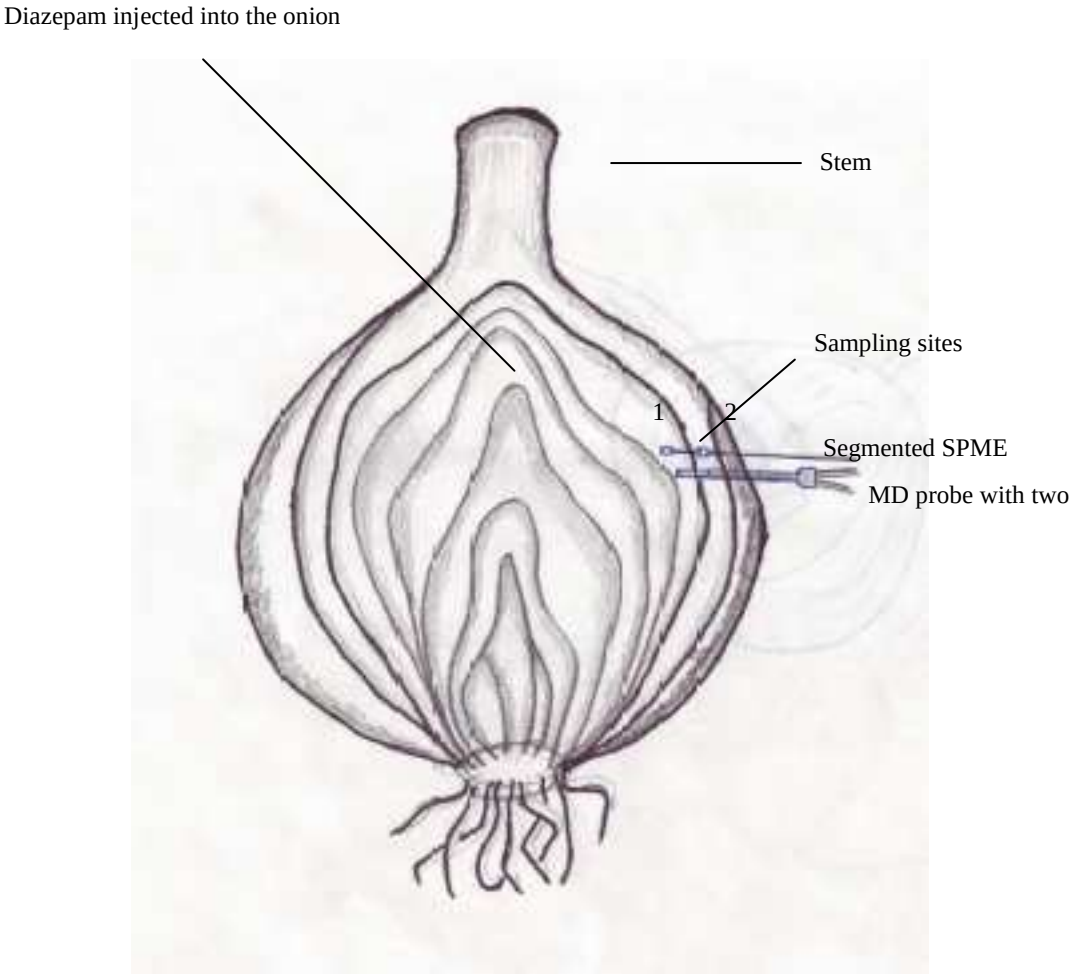


Figure 5.5. The schematic showing the use of the high spatial resolution SPME and microdialysis for *in situ* sampling in an onion bulb.

After 5 min, the fiber was pulled out and briefly rinsed with nanopure water to remove any adhered plant-bulb material, followed by the two-step successive desorption. Afterwards, the fiber was dried in air and stored in 50 mL pure water. The sampling process was conducted for 3 times. The

microdialysis perfusion fluid, flow rate and the calibration procedure were the same as for the gel experiment.

5.2.6 LC-MS/MS Analysis

A CTC-PAL autosampler/Shimadzu 10 AVP LC/MDS Sciex API 3000 tandem Mass Spectrometry (MS) system was used for the analysis of the drugs and their deuterated standards. The assay conditions were the same as described in the literature.[4] Briefly, the column was a Waters Symmetry Shield RP18, 2.1 × 50 mm with 5µm particle size (Millford, MA). Mobile phases were (A) acetonitrile/water (10%: 90%) with 0.1% acetic acid and (B) acetonitrile/water (90%:10%) with 0.1% acetic acid. Mobile phase flow rate was 0.5 mL/min, and the gradient used was 10% B for the first 0.5 min. This was ramped to 90% B over 2.0 min, held for 1.5 min and finally returned to 10% B for 1 min. This provided a total of 5 min run time including reconditioning. For experiments with the onion, LC effluent was directed to waste for the first 1 min of run time to prevent co-extracted compounds from entering the MS instrument. During this divert time, a makeup flow was supplied to the MS via the quaternary pump. The HPLC effluent was analyzed by ESI (positive ion mode) with selected reaction monitoring. Transitions monitored were diazepam: m/z 285.0/154.1, diazepam-d5: m/z 290.2/154.1 and lorazepam: m/z 321.1/275.1.

5.3 RESULTS AND DISCUSSION

5.3.1 Improvement of Spatial Resolution by Reducing the Size of the SPME fiber

Normally, uneven distribution of a substance within a natural system is much more common than uniform distribution due to the heterogeneous nature of the system in most cases. Therefore, for monitoring the dynamic physiochemical process of a substance within a heterogeneous sample system, for example, studying the clinical pharmacokinetics in an animal organ or plant tissue, spatial resolution of the investigating technique is important and deserves more attention. In this study, the spatial resolution of an *in situ* sampling technique is defined as its capability to accurately determine the local concentrations of analytes and clearly resolve two different concentrations spatially close to each other.

The traditional SPME technique has been widely applied to gas or liquid samples, but it has not been used in heterogeneous sample where the spatial resolution is required. As discussed above, the spatial resolution of SPME is determined by the size or dimension of its extraction phase. So, resolution may be improved by reducing the fiber size. However, the sampling time, that determines the temporal resolution of the technique, should also be considered at the same time. This is because of the fact that the diffusion of analytes during a long sampling time tends to uniform the concentration distribution in the adjacent area, thus making the spatial

resolution meaningless. Consequently, for SPME experiments, the effect of reducing sampling time should be considered together with shrinking the fiber dimension. Furthermore, the mass transfer kinetics (diffusion-controlled) in a soft tissue is quite slow compared to that in headspace gas or agitated liquid. Therefore, the SPME coating should possess a fast equilibrium time and high extraction capacity to meet the requirements. In this research, C18-bonded silica particle (5 µm) coated fibers were selected as the extraction phase because of its relatively high extraction capacity and fast equilibrium time. [14] To improve the spatial resolution, the length of the coating was chosen to be 1 mm. In static extraction condition, it was able to extract 30-40 pg of diazepam from 1% gel sample in a fast equilibrium time, i.e., 15 min.

5.3.2 Study of Drug Diffusion and SPME Extraction in the Gel Medium

In a small heterogeneous dynamic living system, such as, an onion bulb, obtaining the real-time *in situ* concentration with traditional techniques is really challenging. Actually, it is impractical to obtain the instant concentration with equilibrium-based SPME, because, during the time to reach equilibrium between sample matrix and extraction phase, the analyte concentration at a specific locus would change due to diffusion within the heterogeneous system. To address this issue, non-equilibrium SPME technique (fast SPME) was adopted to obtain nearly real-time

concentrations. However, the concentration variation resulting from the diffusion effect depends not only on the sampling time, but also on the diffusion velocity of the analyte species under a certain temperature in the sample matrix. Therefore, we determined the linear velocity for the diffusion of diazepam molecules in the gel medium at room temperature. It was found that diazepam could not be extracted by the SPME fiber until 5 hr was passed. This meant that it took 5 hr for the molecules to pass through 30 mm distance in the gel medium and reach the fiber coating. Thus, the diffusion velocity was calculated to be ~ 1.67 μm/sec. This indicated that the concentration changed quite slowly. Therefore, in the gel medium, the concentration change due to the diffusion during 5 – 15 min was negligible. Accordingly, 5 min was selected as the sampling time for the multilayered gel system.

Additionally, the diffusion coefficient of the analyte molecules in the gel medium, *D*, could be determined with the SPME technique according to the integral form of the Fick's first law of diffusion

$$D = \frac{x^2}{2t} \qquad (5.1)$$

where, *x* is the moving distance of the analyte via diffusion, and *t* is the time duration of the molecule migration via diffusion. The calculated diffusion coefficient was in the range of 1×10^{-9} m^2/s.

It should be mentioned that the main cause of concentration variation in the gel model system is the physical diffusion. However, for *in vivo* analysis

in a living system, such as detecting a metabolite in a living animal, the situation could be different, where metabolism plays an important role in varying the drug concentration as physical diffusion does. Thereby, the temporal resolution of the analytical approach deserves more attention. In those cases, investigating the contribution of physical diffusion and chemical metabolism to local tissue concentration of target analytes, such as a drug or neurotransmitter, would be quite suggestive to reveal its functional mechanism.

5.3.3 Kinetic Calibration for the *in situ* Sampling

For non-equilibrium extraction, as discussed previously, kinetic calibration outweighs calibration curve method providing more accurate and precise calibration. [14-17] This approach is especially suitable for achieving real time *in situ* sampling. However, the first prerequisite of kinetic calibration to be met is the symmetric relationship between the extraction and desorption processes of the analyte in sample matrix. Therefore, absorption and desorption experiments were conducted simultaneously to investigate the symmetry. The fibers preloaded with deuterated diazepam were exposed to a gel medium containing 200 µg/L diazepam (pH 7.4) for different times to study the fraction of the standard remaining in the extraction phase after sampling times (t). Figure 5.6 presents the values of Q/q_0 that was calculated from the desorption time profile and n/n_e from the

absorption time profile. The sum of Q/q_0 and n/n_e is close to 1 at each time point, which validates the symmetric relationship between the adsorption and desorption. In addition, the requirements for negligible depletion (nd-SPME) should be met; so that the sampling does not disturb the system under study, and thus, this approach can be used to detect the free concentration of analytes in a complicated sample matrix.[18] The newly developed approach is featured by the negligible depletion during sampling due to its small fiber volume resulting from miniaturized dimension and shortened sampling time. In a1 mm thick gel sample with total volume > 0.2 mL, only 2-4% of the free fraction of diazepam was extracted in 5 min. As a result, the detected sample concentration is not affected by the sample volume; thus making the quantification simpler and more convenient. In addition, sensitivity of the 1 mm SPME fiber should be considered. In the gel experiment, it was shown that the detection limit of 1 mm high spatial resolution SPME was 2.5 ppb, and the dynamic range was linear up to 500 ppb.

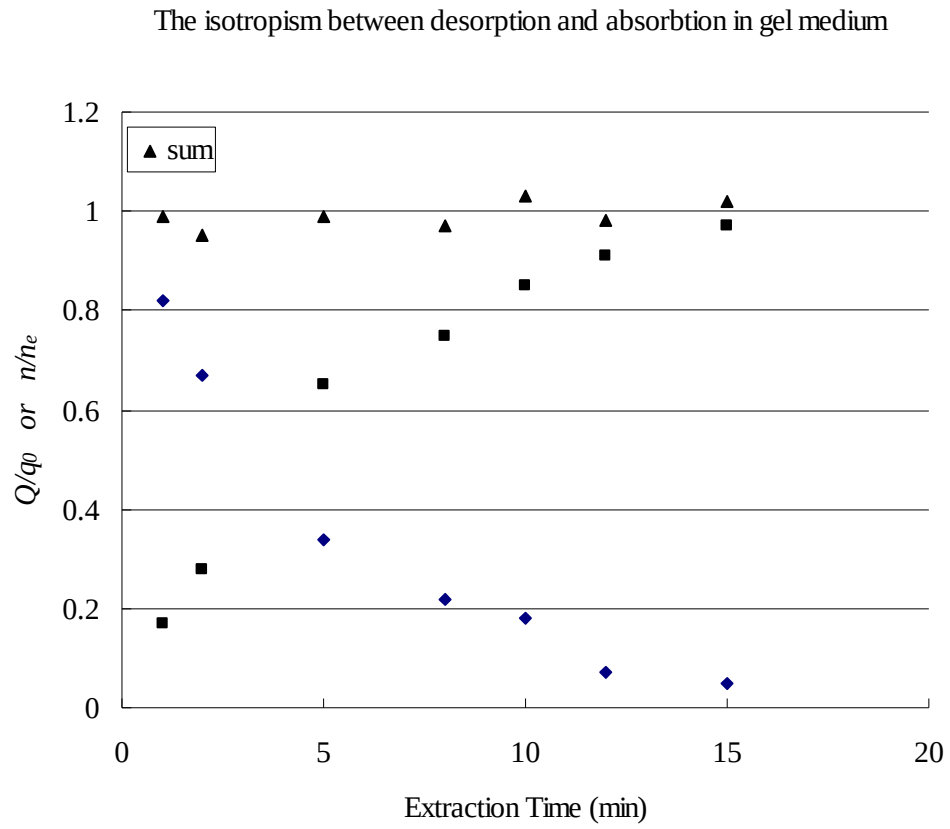


Figure 5.6. The absorption and desorption time profiles for diazepam (◊) and its deuterated analogues, diazepam-d_5 (♦) in 1% agarose gel (at 25 ˚C).

In order to obtain the sample concentration, external calibration method was used to determine the product of V_f and K_{fs}, the slope of the calibration curve for equilibrium extraction in gel-prepared standards at room temperature. Consequently, the free concentration and fraction of the analyte can be obtained easily by comparing the calibration in gel medium and that in PBS buffer. It was found that the free fraction of diazepam in gel medium is ~ 73%. It suggests that the bound fraction of the analyte to the agarose gel is around ~ 27%.

5.3.4 Real Sample Analysis by SPME and Validation by Microdialysis

To test the feasibility of the high spatial resolution SPME, the local concentrations at two sampling sites (labeled as 1 and 2 in Fig. 5.5), that were located in different depth along the needle-made-hole in the onion bulb, were monitored. The calculated concentrations (ppb or ng/mL) at the two sampling sites based on the results from the triplicate extractions are presented in Table 5.1. Data from microdialysis experiments are also presented in Table 5.1 to compare with the data from SPME experiments. It was found that there was no significant difference between the three extractions at each location; however, the concentrations that are reflected by the extracted amount were significantly different between the two locations with the two coating segments. The extracted amount at the tip coating was about 4 - 5 times higher than that from the upper part coating. This result indicated the very heterogeneous nature of the onion bulbs; meanwhile, the difference demonstrated the spatially resolved capability of the segmented SPME. However, the determined concentration from the microdialysis probe in the 1.9 cm long hole is just 3 times lower than that from the SPME fiber segment at sampling site 1, but 2 times of that from the SPME fiber segment at the sampling site 2. Apparently, the calculated concentrations from SPME results did not match those from microdialysis. In fact, with 4mm-probe (lower spatial resolution than 1mm SPME fiber),

the microdialysis probed the averaged concentrations in a much wider range along the lengthwise holes, i.e., 4 mm in length from the tip, rather than the 1 mm range concentration sensed by the SPME fiber. Therefore, the concentration from the microdialysis probe in the 1.9 cm long hole could be regarded as the spatially weighted average of the concentration as determined by the two SPME segments. This rationale was supported by introducing 4 mm C18-coated SPME fibers into the 1.9 cm hole along with the MD probe for a 5 minutes extraction. The obtained concentrations agreed with those from MD probe. Therefore, the results demonstrate that the quantitative capability of the SPME is comparable to the microdialysis, making SPME device suitable for the quantitative *in situ* analysis.

Table 5.1. Application of the segmented fibers for *in situ* analysis of real time local concentrations in two loci with 5 mm distance in an onion bulb. The SPME data were validated by microdialysis, and concentration unit was ppb (ng/mL).

Sampling sites	Site 1			Site 2	
Trial #	SPME [1]	SPME[2]	MD	SPME[1*]	MD
1st	633	275	298	131	N/A
2nd	625	267	289	127	N/A
3rd	626	261	277	119	N/A

Footnote:

1. SPME [1]: The 1 mm fiber segment at the tip of the wire located at site 1 in the

onion bulb.

2. SPME ^{1*}: The 1 mm upper fiber-segment located at site 2 with 5 mm in distance from the site 1.
3. SPME ^{2}: The 4 mm fiber coating at the tip of the wire located at site 1 in the onion bulb together with the MD probe.
4. No MD probe at site 2 (N/A: not applicable).

Compared to the high spatial resolution SPME that can detect the concentrations in the two adjacent locations simultaneously with a segmented fiber, each microdialysis probe can only be applied to one single determination. Furthermore, the multiplexing capability of SPME can be further improved by preparing multiple segmented fiber coating on a single wire. On the contrary, there is a lack of multiplexing capability for microdialysis technique. Moreover, the SPME device is much simpler and more compact than the microdialysis sampling as the latter includes a needle syringe, tubing and microinjection (syringe) pump. For calibration, the kinetic calibration is not only accurate, but also time-saving so that it ensures the temporal resolution of the SPME sampling. However, for microdialysis, determining the % recovery is more time consuming and labor intensive. More importantly, from the gel experiment, the limit of detection for a 4 mm microdialysis probe (i.e., 5 ppb) is 2 time higher than that of 1 mm high spatial resolution SPME (i.e., 2.5 ppb). Therefore, the SPME method could have higher sensitivity than the microdialysis if the

MD probe could be made in the same size. However, the microdialysis sampling has some advantages. As a continuous online-sampling technique, it is easy to be completely automated for the whole analysis process including sampling, sample preparation and sample introduction into an instrument for quantification, for example, coupling with a capillary electrophoresis device.[19-22] In addition, MD probes can be employed as delivery tools for drugs or chemicals, while that is not suitable for SPME.[23] On the contrary, SPME is a discontinuous sampling technique, so more efforts are needed to achieve full automation for the bioanalysis, especially when studying nonvolatile molecules using LC-MS. Nevertheless, for *in situ* sampling, high spatial resolution SPME provides irreplaceable effectiveness and efficiency.

5.3.5 Spatial-profile Sampling with the Segmented SPME Probes

During *in situ* sampling in a heterogeneous sample system, the purpose was to investigate the spatial distribution of target analytes at a given time. In order to achieve this goal, many probes were to place in the representative sampling sites. Moreover, all the individual samplings should be conducted simultaneously. This is too difficult or even impractical with the traditional sampling techniques, such as MD or traditional SPME.[10] But the segmented design of the miniaturized SPME fibers and the stepwise successive desorption procedure provided the possibility to perform two parallel

samplings simultaneously with only a single probe. The multiplexing capability can be improved by increasing the coating segments to achieve spatial-profile sampling; however, the current 2-segment design serves as an excellent prototype for the one-dimensional spatial-profile probes.

5.4 Conclusion

5.4.1 Conclusion

In conclusion, a high spatial resolution SPME approach was developed and evaluated in this work. The novel SPME method also showed high temporal resolution. A multilayered gel system was constructed to simulate a heterogeneous sample. Finally, real application was conducted for drug analysis in a very heterogeneous system, such as an onion bulb. Compared to the microdialysis approach, the results demonstrated the feasibility, accuracy (relative recovery: 93%), sensitivity (LOD: 2.5 ng/mL) and efficiency (5 min) of the newly developed SPME sampling device. Moreover, the segmented design of the SPME fibers and stepwise successive desorption procedure offer not only the spatial resolution, but also the multiplexing capability for parallel spatial-profile samplings with a single probe.

Chapter 6

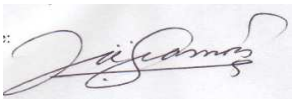
In Situ Monitoring of Ochratoxin A in Cheese Sample with Miniaturized Solid-Phase Microextraction

6.1 Preamble and Introduction

6.1.1 Preamble. This chapter has not been published. Erasmus Cudjoe and Dajana Vuckovic contributed to this project. The contribution of Erasmus Cudjoe was involved in the cheese sampling. The contribution of Dajana Vuckovic involved in experimental suggestions and manuscript revision.

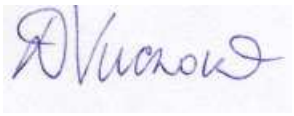
I, Erasmus Cudjoe, authorize Xu Zhang to use the material for his thesis.

Signature:



I, Dajana Vuckovic, authorize Xu Zhang to use the material for his thesis.

Signature:



6.12 Introduction

Ochratoxin A (OTA), a secondary metabolite produced by several common toxigenic fungi (moulds), is one of the most widely-occurring nephrotoxic, carcinogenic and immunosuppressive toxins and is considered to be involved in severe pathological response from humans and animals.[1-3] Currently, it has become a major health concern and there is an increasing

need for accurate monitoring of this mycotoxin in food products.[4-8] However, traditional sample preparation approaches such as liquid extraction (LE) and solid-phase extraction (SPE), have proven to be time-consuming and labor-intensive. Antibody-based immunoaffinity chromatography (IAC) and active protein based affinity separation has been introduced for this purpose.[9-10] However, the cost and the fragility of the non-reusable column prevent it from being usable with the complicated cheese sample matrix. Therefore, the need to develop fast and low-cost sample preparation approaches for OTA analysis in semi-solid food samples cannot be overemphasized.

As an effective sampling and sample preparation method, solid-phase microextraction (SPME) has gained extensive application and recognition in many areas since it was introduced. [11-14] One of the key reason is its unique capability in integrating sampling, sample preparation, and sample introduction into one single step, and thus the total process of chemical analysis is greatly simplified. Although, SPME method was previously applied to the analysis of mycotoxins in cheese samples with good detection limits,[15-16] the SPME fiber was only applied to the reconstituted sample extract after a liquid extraction step, thus making the procedure tedious and time-consuming.

One of the objectives of this work was to introduce the SPME fiber directly into the cheese sample so as to demonstrate the effectiveness of the

technique in performing *in situ* sampling and sample preparation. The chosen approach was effective and simple compared to previous liquid extraction methods. The extraction phase was acidified prior to sampling in order to improve extraction efficiency of OTA. Moreover, the miniaturized size of the extraction phase coupled with fast *in situ* sampling would provide an effective space- and time-resolved approach for the detection of OTA levels at different sites in one small sized cheese sample and also the concentration variations with time. Generally the temporal resolution of the SPME is determined by its response time, or sampling time, while the spatial resolution is determined by the size of the extraction phase. Because the proposed method could provide good spatial (1 mm) and temporal resolution, it was further investigated whether the data could be used to determine if the OTA contamination was originating from the fungus such as *Penicillium verrucosum* or from the raw materials that the cheese was made of.

6.2 EXPERIMENTAL SECTION

6.2.1 Chemicals and Materials

OTA, Ochratoxin B (OTB) and other chemicals were of analytical grade and ordered from Sigma-Aldrich. (Oakville, Ontario, Canada). Stock solutions of OTA and OTB were prepared in methanol and stored at -20° C. Carbon-tape was from TAAB Laboratories Equipment Ltd (Calleva Park,

Reading Berkshire, United Kingdom). 0.01 inch stainless steel wires were purchased from Small Parts Inc. (Miami Lakes, FL, USA) and cut into pieces of 5-cm length.

6.2.2 Preparation and Characterization of SPME fibers

Carbon-tape fiber was used as the extraction phase for the sampling.[17] The carbon tape was cut into 1 mm X 1mm pieces and each piece was immobilized onto the end of one 5-cm stainless steel wire. The resulted carbon-tape based SPME fibers were 1 mm in length. All fibers were acidified with hydrochloric acid and preloaded with internal standard simultaneously. The loading solution was prepared by spiking OTB standard into 50 mL of diluted HCL aqueous solution (pH 2.0) at 100 µg/L. Then the fibers were exposed into the loading solution during 8 hours for standard loading.

A systematic investigation was conducted to characterize the extraction behaviors of the fibers including extraction time profile, desorption time profile, pH effect on the extraction, and dynamic range. All the characterization experiments were performed in 1% agarose gel medium to optimize the experimental conditions for cheese samples.

6.2.3 Sampling and Sample preparation with SPME

Three semisolid cheese samples were monitored in this study. The aged Cheddar cheese was donated by Dr. Pawliszyn, while the “no name”® skim milk cheese were obtained in a local supermarket. The cheese was separated into three different sets. Set A comprised aged cheese allowed to develop mould stains while set B had no mould stains. Set C (No name®) had no mould stain however it was less matured compared to sets A and B. For set A cheese with the mould stain, two SPME fibers were introduced into two sampling sites at different distances from the mould stain. In the case of set B and C, three fibers were introduced in a triangular manner and a fourth fiber was placed at the center of this triangle. Sampling was done for 20 min after which the fibers were cleaned with Kimwipes® to remove any cheese residues attached to the surface of the coating. The fibers were later put into wells inside a 96-well plate containing 250 µL Polypropylene inserts and then 150 µL of pure methanol was used for desorption of the analytes. Desorption was completed in 15 min with agitation on a mechanical KV-300 shaker set at the speed of 100 rpm. Finally, 20 µL of desorption solution was injected in LC-MS/MS system for quantification.

6.2.4 LC-MS/MS Analysis

A CTC-PAL autosampler/Shimadzu 10 AVP LC/MDS Sciex API 3000 tandem MS system was used for the analysis of the OTA and OTB. Briefly, the column was a Waters Symmetry Shield RP18, 2.1 × 50 mm, 5 µm

particle size (Millford, MA). Gradient elution was performed with a flow rate of 0.5 mL min^{-1} for separation with mobile phase (A) acetonitrile/water (10: 90) with 0.1% acetic acid, and (B) acetonitrile and acetic acid 100:0.1. The gradient started with 10% B for the first 0.1 min, followed by a linear increase to 40% B in 6 min, and then it was ramped to 100% B in 0.1 min, held for 3 min before decreased to 10% B in 0.01 min. This provided a total 8 min run time including reconditioning the column. For experiments using cheese samples, LC effluent was directed to waste for the first 2 min of run time, to eliminate co-extracts from entering the MS. The mass spectrometer worked in the negative ion mode. The m/z transitions were m/z 402.1/357.9 and 402.1/314.0 for OTA, and 368.0/133.1 for OTB.

6.2.5 Conventional Cheese Analysis

In order to validate the SPME data, the traditional liquid extraction with methanol as the extraction phase was performed. About 0.05 g of cheese sample (n = 3) was weighed by difference in a preweighed 2 ml amber vial with a PTFE sealed screw cap. 1 ml pure methanol was added and the resulted mixture was sonicated for 30-min, followed by a 20-min centrifugation (15000 g). 900 µl supernatant was divided into 3 identical 300-µL aliquots in 3 new 2-mL amber vials. 15 µL of standard solutions (0, 100, and 200 ng/mL in pure methanol) were added into the three vials, respectively. Afterwards the solvent in the three samples was evaporated

under nitrogen gas. The residue was reconstituted with 100 µl mobile-phase A and subjected to instrumental analysis.

In addition, standard addition was adopted to calibrate the conventional analysis in that it is suited to deduce how much endogenous analyte was in the original cheese sample with complicated matrix effect.[18] Care should be taken to ensure that the calibration requires a linear response to the quantity of the analyte plus standard.

6.3 RESULTS AND DISCUSSION

Previous studies showed that the carbon-tape SPME fiber had significant affinity for OTA.[17] It exhibited high extraction capacity and efficiency compared to the commercial fibers. However, the carbon-tape SPME fibers were applied to only homogenous and acidified liquid samples, which is totally different from performing *in situ* sampling in a semisolid sample such as a cheese. In the latter case, for *in situ* studies, to avoid damage the whole sample, it is impossible to treat the sample with chemicals; therefore, it requires a fiber with good sensitivity for the non-treated samples. OTA is a weak acid with the carboxylic group on the phenylalanine moiety (pK_a = 4.4) and shows a strong dependence of extraction yield on sample pH. All the previous SPME experiments for the analysis of OTA were conducted around pH 3 by the adjustment of matrix pH.[19-20] Considering the adjustment of matrix pH was not an option in the current study, a new strategy was

proposed which involved the acidification of extraction phase prior to sampling. Secondly, *in situ* analysis requires the SPME fiber to have high spatial resolution; i.e., the fiber size must be minimized to probe local concentrations in a small-sized sample. To address this requirement, the dimensions of SPME extraction phase were reduced to 1 mm x 1 mm.

Finally, on-fiber standardization approach was used for quantitative analysis in order to keep sampling times as short as possible.

6.3.1 Development of Acidified Mini-Carbon-Tape-Fibers

Selection of the carbon-tape SPME fibers in this work was due primarily to its high extraction capacity.[17] The extraction effeciency for OTA was strongly affected by the pH environment of the sampling. To address that, the fibers were acidified prior to sampling and tested in gel matrix. A pH series (pH = 1, 2, 3, 4, 5) of aqueous HCl solutions were prepared and the carbon tape-fibers (n = 3) were immersed in 10 ml of each of the acidic solutions in a 40-ml vial for 8 hours at room temperature. Afterwards, the fibers were used for extraction in agarose gel containing 10 ppb of OTA. As presented in Fig. 6.1, the results showed the strong pH dependence of the extracted amount in both gel matrix and in the cheese. In addition, this data showed that the carryover for all extractions was similar, so the extraction difference was due to the different affinity of the fibers to

the analyte rather than the carryover. For subsequent experiments, aqueous solution of HCl at pH 2 was used to acidify fibers prior to sampling.

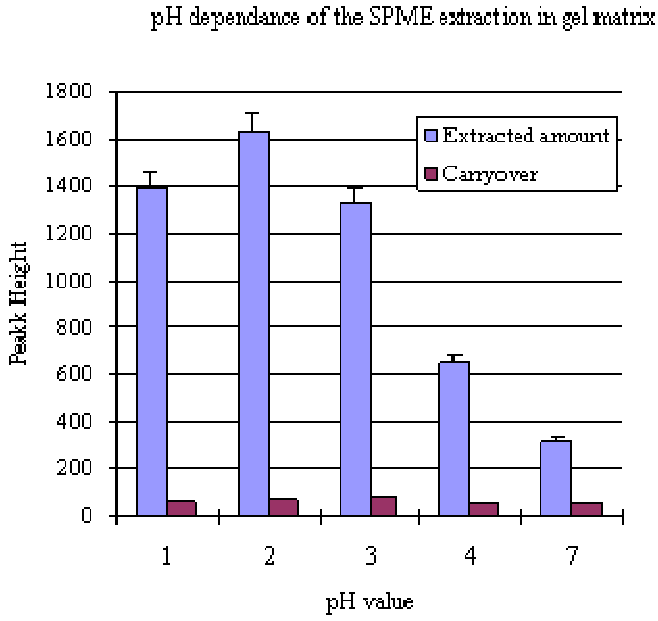


Figure 6.1. The pH dependence of OTA extraction using the mini-carbon-tape SPME fibers in gel matrix (n = 3).

Another aspect for characterizing the acidified fiber was to study its extraction kinetics. We compared the extraction time profile of acidified fibers in a non-acidified sample matrix (1% agarose gel) with that of the acidified fibers in acidified gel matrix (1% agarose gel, pH = 3). As shown in Figure 6.2, the two profiles were consistent, though the precision for the acidified fibers in a non-acidified sample matrix (RSD ~10%) was somewhat larger than sampling in the acidified gel (RSD ~5%). The agreement of results indicated that acidifying fibers was equivalent to

acidifying sample in terms of kinetic behavior (indicated by no change in time required to reach equilibrium) and thermodynamics (indicated by no change in the amount of analyte extracted at equilibrium).

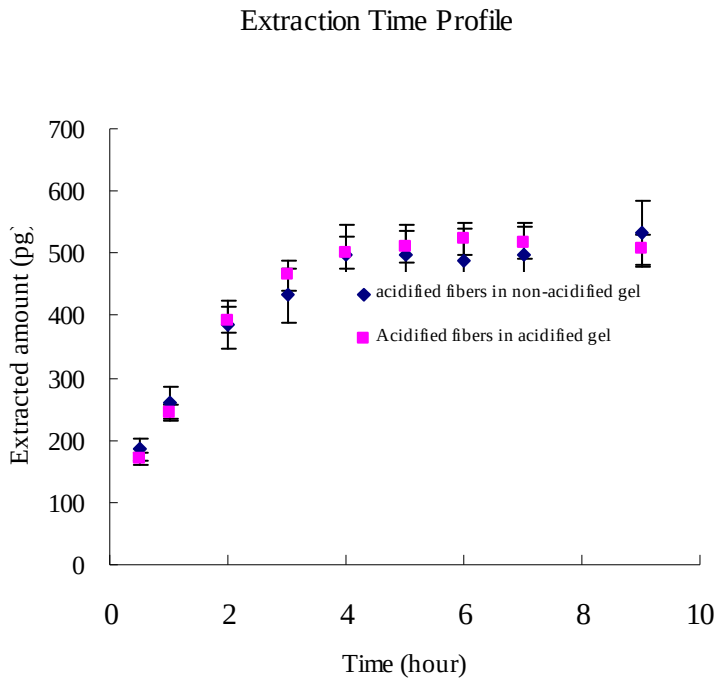


Figure 6.2. Comparison of OTA extraction time profiles by using acidified fibers in non-acidified gel prepared sample with using acidified fibers in acidified gel sample.

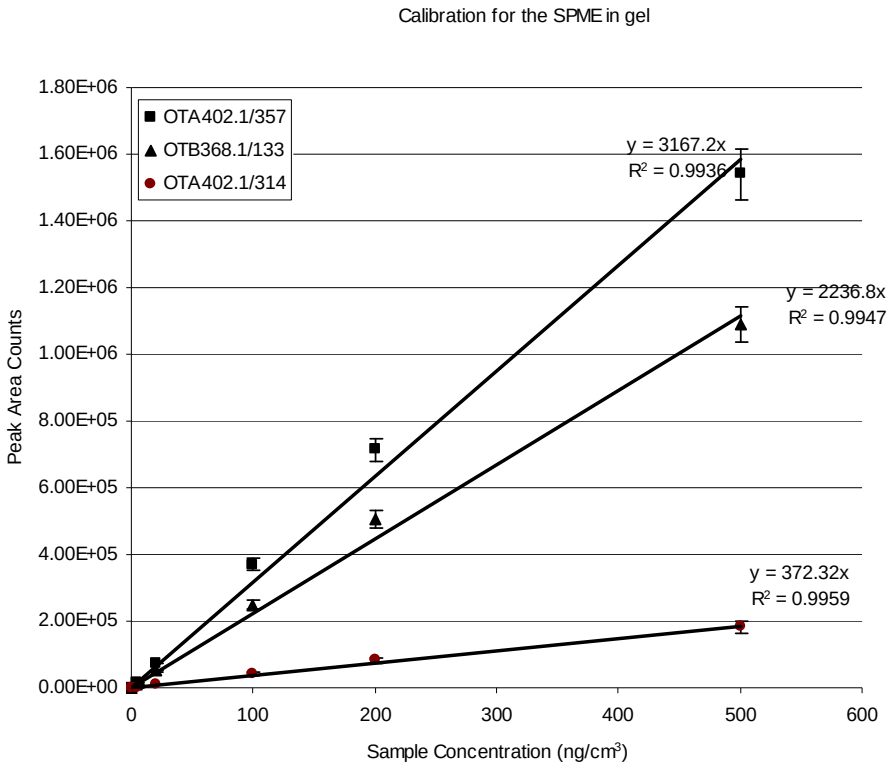


Figure 6.3. The SPME calibration curves of OTA and OTB developed with the acidified fibers in standards prepared in gel. 30-min samplings were performed under room temperature.

Finally, we studied the linear relationship between the sample concentrations and the instrumental response, which was the pre-requisite for a quantitative analysis. The experiment was performed in 1% agarose gel with different OTA concentrations. Figure 6.3 presents the linearly proportional relation, which indicated the feasibility of using the acidified fibers for quantitative analysis. The linear range of the carbon-type SPME was 1.5-500 ng/mL.

Furthermore, for *in situ* analysis, the spatial resolution of the SPME fiber must be improved by a reduction in the size of the fibers. However, the reduction of the coating volume also decreases the sensitivity, so it was important to ensure that the amount extracted by the miniaturized fiber was still sufficient for this analysis. The results showed that the limits of detection and quantification for the 1 mm fiber in gel matrix was 1.5 and 3.5 ng/mL respectively, which was with fairly good sensitivity for the analysis of Ochratoxin A in cheese sample according to the following SPME studies.

6.3.2 On-fiber Standardization for Calibration

For *in situ* analysis of a cheese sample with SPME, another challenge is the correct calibration, since the three traditional calibration methods including external calibration curve, standard addition method and internal standard method, are not applicable for the *in situ* study. However, the on-fiber standardization method, or called kinetic calibration, provides the solution for accurate on-site sampling for it compensates for the agitation effect and matrix effect.[21-25] As a specific case of on-site sampling, *in situ* sampling is theoretically appropriate for on-fiber standardization. The calculation is based on the following equation.

$$C_0 = \frac{nq_0}{q_0 - Q} \bullet \frac{1}{k_{fs} V_f} \qquad (6.1)$$

where C_0 is the initial concentration of analyte, Q is the amount of standard remaining in the extraction phase after exposure of the extraction phase to

the sample matrix for the sampling time, V_f is the volume of the fiber, K_{fs} is the fiber coating/sample distribution coefficients of the analytes, and q_0 is the amount of standard that is pre-loaded in the extraction phase.

However, the typical kinetic calibration is based on the use of isotopically labeled compounds as standards, which was not applicable for the OTA analysis because its isotopically labeled analogue is not commercially available. Herein we use OTB as the standard to calibrate OTA extraction. However, two requirements must be satisfied so that OTB can be directly used for the on-fiber standardization of OTA: (1) there should be no endogenous OTB in the sample; (2) the time constant of the extraction of OTA should not be significantly different from that of OTB in the same matrix. In addition, it is worth noting when calculating the OTA concentration based on eq. 6.1, K_{fs} must be the distribution coefficient for the analyte OTA, rather than that for the analogue OTB, which was used as the standard. In order to satisfy these requirements, firstly, it was confirmed that no OTB was present in the cheese sample using traditional liquid extraction. Secondly, the extraction time profiles for OTA and OTB in gel matrix were compared. As shown in Fig. 6.4, there was no significant difference between OTA's and OTB's equilibration time, and equibration time is an indicator of the magnitude of time constant. Actually, the time constant, *a*, could be calculated by fitting the extraction time profile into the following mathematical model:

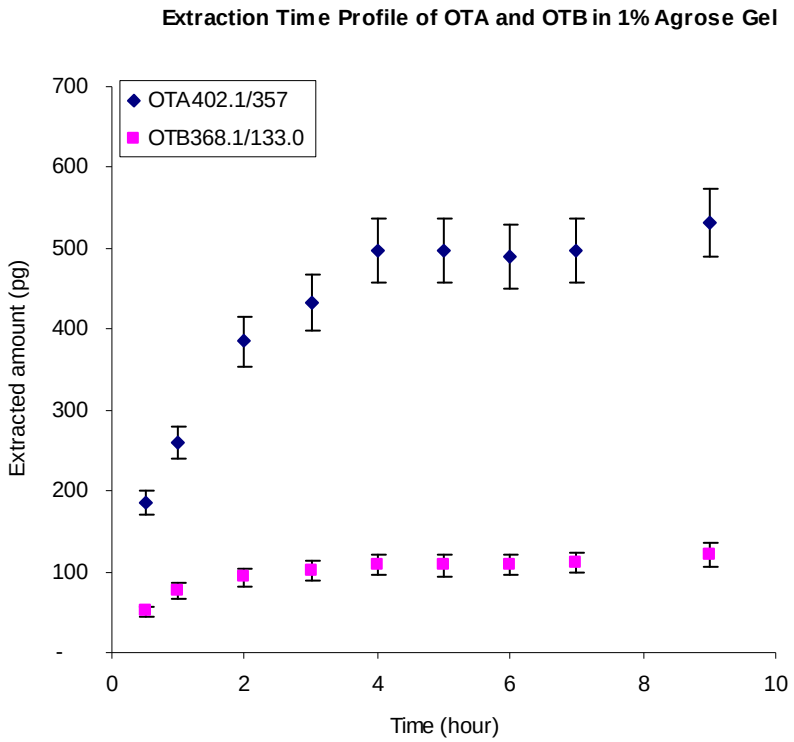


Figure 6.4. The extraction time profiles of OTA and OTB in gel sample under room temperature.

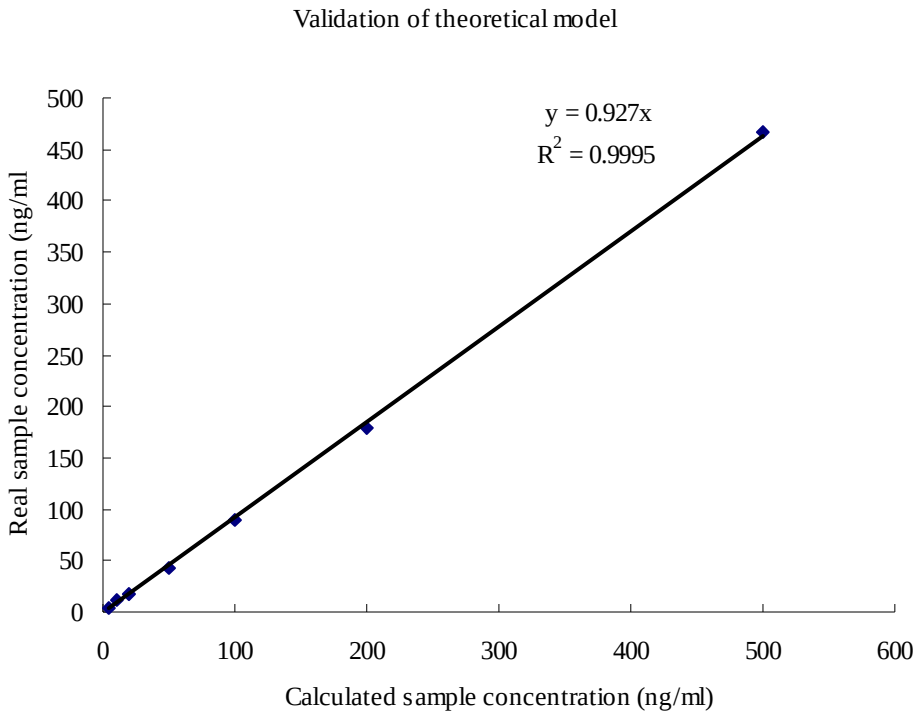


Figure 6.5. Validation of the on-fiber standardization using OTB as the standard for OTA. The calculated relative recoveries were around 93% (5 - 500 ng/cm^3).

$$\frac{n}{n_e} = 1 - \exp(-at) \qquad (6.2)$$

where n is the amount of analyte in the extraction phase during each sampling time t, n_e is the amount of analyte in the extraction phase at equilibrium, a is the time constant. Here, the a values were calculated as 0.85 and 0.77 hr^{-1} for OTA and OTB respectively. The small difference in the magnitude of a value would not result in significant deviation from the isotropism relation between desorption and adsorption, which was explained in detail elsewhere.[21-22] Therefore, OTB could serve as the standard for OTA monitoring.

Moreover, a proof of principle experiment was conducted in gel matrix to test the validity of using OTB to calibrate OTA. As shown in Fig. 6.5, the calculated relative recoveries were around 93% (5 - 500 ng/mL), shown as the slope of the linear regression, which demonstrated the accuracy of the method.

Last but not least, in order to obtain sample concentrations with on-fiber standardization, the product of V_f and K_{fs} of the analyte must be determined.[26] In this report, a simple method was proposed to determine the $K_{fs} \cdot V_f$ values of the carbon tape fiber in semisolid cheese sample. The definition of the partitioning coefficient, K_{fs}, gives

$$K_{fs} = \frac{C_f}{C_s} = \frac{n_e / v_f}{c_s} \qquad (6.3)$$

The eq. 6.2 can be rewritten as

$$K_{fs} V_f = n_e / C_s \qquad (6.4)$$

where n_e is the amount of OTA extracted in equilibrium SPME and C_s is the sample concentration of OTA in cheese samples. Herein, we used the equilibrium SPME method to obtain n_e and then detected the sample concentration with traditional organic solvent (methanol) extraction. First of all, the SPME fibers were put into the cheese sample for 10 hours to ensure the equilibrium extraction.[17] Then the traditional liquid extraction was calibrated by standard addition method to compensate for the matrix effect. Finally, the calculated $K_{fs} \cdot V_f$ value ranged from 0.15-0.22 μl for three different semi-solid cheese samples.

Experimentally, to streamline the operation procedure we combined the fiber treatment with acids and the standard loading into one single step. The experimental results showed that there was no significant difference between performing the two steps separately and simultaneously (data not shown). Therefore the carbon-tape fibers were loaded from acidic OTB aqueous solution (pH = 2) in all subsequent experiments.

6.3.3 *In Situ* Cheese Analysis

The application of the proposed *in situ* SPME approach to real sample analysis was demonstrated by analyzing 3 different semi-solid cheese samples as described in the experimental section, in which both the spatial concentration-distribution and the concentration change over time were studied. The results are presented in Table 6.1. It was found that for Set A with a mould stain, the OTA concentration had an inverse relation with distance between the sampling site and mould stain. However, the amount of OTA detected was directly proportional to storage time for mouldy cheese (set A), as shown in Fig. 6.6. This data indicated presence of live fungi in the cheese. If the fungi were not intentionally inoculated into the cheese during manufacturing, it could be safely speculated that the fungi were contaminated during transportation, storage or manufacturing, for example, from flaws of the packages. For the Set B without mould stains, the uniform distribution of the OTA concentration was observed; moreover, the OTA

concentration did not change during the 2 weeks. This result indicated that there were no active fungi that produce OTA in the cheese and the raw materials were the likely origin of contamination. In the case of Set C cheese sample, there was no detectable OTA. Based on these results, it is safe to conclude that the *in situ* SPME approach could be used to track the possible OTA contamination sources in cheese products.

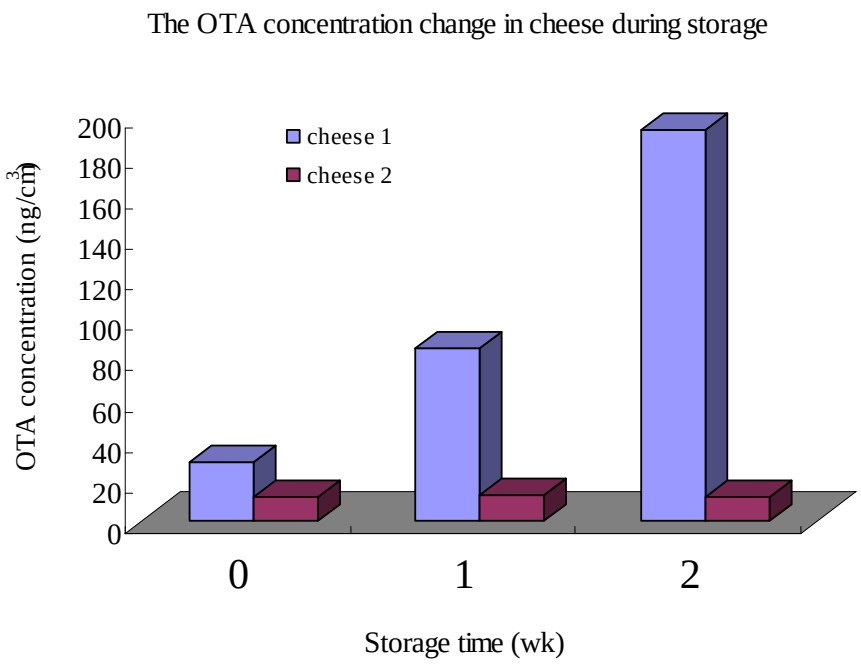


Figure 6.6. The OTA concentration change in the Cheddar cheese during the two week storage in 4 ˚C refrigerator. Cheese 1: the aged Cheddar cheese with a mould stain. Cheese 2: the aged Cheddar cheese without mould stains.

The SPME results were consistent with those obtained by traditional liquid extraction (LE) that was calibrated by standard addition, as shown in Table 6.1, which proved the validity of the *in situ* SPME analysis. Meanwhile, the SPME sampling with on-fiber standardization method was simpler and faster than using the LE coupled to standard addition. It must be admitted that the latter is necessary to obtain K_{fs} value for SPME analysis, but much less analyses were needed since the K_{fs} value should be the same for the whole sample; on the contrary, the traditional LE needs to be performed for every sampling site in each sample.

Table 6.1. Summary of the *in situ* SPME results for OTA occurrence in the three cheese samples. Cheese 1: the aged Cheddar cheese with a mould stain. Cheese 2: the aged Cheddar cheese without mould stains. Cheese 3: the "no name"® skim milk cheese

Cheese #	Sampling site	*S-con(ng/cm^3)	SD	**L-con.(ng/cm^3)	SD
1	$1(close)	42	5.2	39	3.7
	&2 (far)	20	3.5	18	2.1
2	1	11	1.4	13	1.3
	2	12	1.6	12	1.5
	3	11	0.8	10	1.1
	4	12	1.5	11	1.2
3	1	§nd		nd	

2	nd	nd
3	nd	nd
4	nd	nd

Notes:

* Sample concentration obtained by SPME technique;

** Sample concentration obtained by Liquid Extraction technique;

$ The #1 sampling site that is close to the mould stain in the cheese;

& The #2 sampling site that is further than #1 site to the mould stain in the cheese;

§ Non-detectable.

In addition, to confirm the identity of OTA in real cheese samples, two transitions m/z 402.1/357.9 and 402.1/314.0 were used for OTA, in addition to LC retention time. Fig. 6.7 shows the chromatogram of OTA and OTB by LC-MS/MS.

Compared to the previous studies,[19-20] this work provides a simple but effective means to conduct *in situ* analysis of pH dependent ionic analytes in semi-solid food matrix.

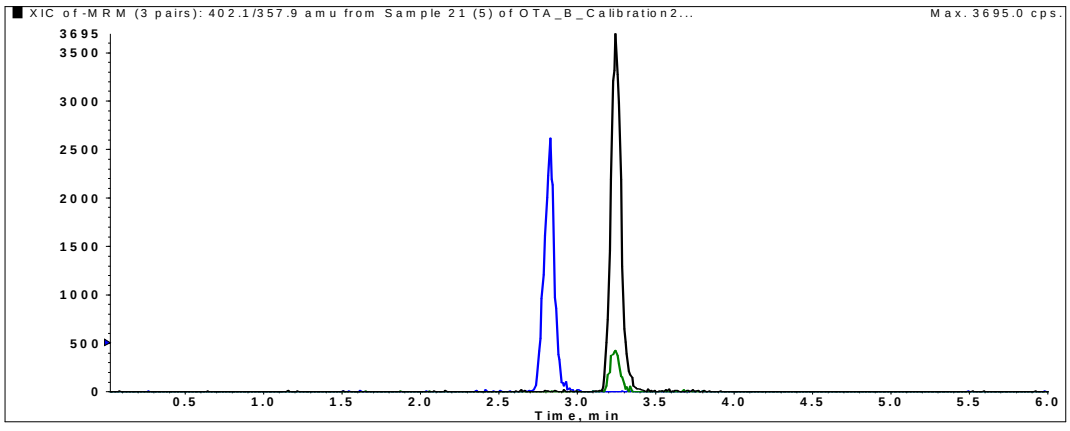


Figure 6.7. The chromatogram of the OTA and OTB by the LC/MS-MS. The Peak with a retention time of 2.8 min is for OTB, the standard. The Peaks with retention at 3.4 min are for OTA, among which the taller one is from the transition of m/z 402.1/357.9, and the shorter peak is for 402.1/314.0.

Compared to the previous studies,[19-20] this work provides a simple but effective means to conduct *in situ* analysis of pH dependent ionic analytes in semisolid food ascribed to the effectiveness of treating the miniaturized carbon-tape fibers with acids and the proved merit that the kinetic calibration is inherently more accurate than calibration curve method in complicated sample matrix.

6.4 CONCLUSION

6.4.1 Conclusion

In situ SPME method was achieved by the acidification of the extraction phase with aqueous HCl solution at pH 2 which improved the extraction sensitivity of the fiber primarily because the analyte was a weak acid and in addition to decreasing analyses time. A new on-fiber kinetic calibration was developed by using OTB as an internal standard. The suitability of the OTB as an effective internal standard was demonstrated by comparing the kinetic behavior of OTA and OTB and showing the

consistence of their time constants (*a* values). In addition, the utilization of the miniaturized SPME with enhanced spatial resolution made it possible for the *in situ* analysis in a small-sized sample. Interestingly, the spatial distribution of the OTA concentrations in cheese sample and the concentration change over time can be used to find if there is active OTA producing fungi in the cheese thus resulting the OTA contamination.

Chapter 7

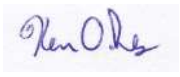
In Situ Monitoring of Tissue-specific Bioaccumulation of Pharmaceuticals in Live Fish with Space-resolved Solid-Phase Microextraction

7.1 Preamble and Introduction

7.1.1 Preamble

This chapter has not been published. Ken D. Oakes, Leslie Bragg and Mark Servos contributed to this project. The contributions of Dr. Oakes included setting up the animal experiment in the wet laboratory, monitoring the water temperature and water chemistry, and advising on composing and revising the manuscript. Dr. Mark Servos provided all laboratory facilities including LC/MS-MS, and a general guidance on the project. Leslie Bragg analyzed the water samples with SPE and helped for instrumental analysis.

I, Dr. Ken D. Oakes, authorize Xu Zhang to use the material for his thesis.

Signature: 

I, Professor Mark Servos, authorize Xu Zhang to use the material for his thesis.

Signature: 

I, Leslie Bragg, authorize Xu Zhang to use the material for his thesis.

Signature: 

7.1.2 Introduction

The detection of human pharmaceuticals and personal care products (PPCPs) in aquatic environments, and their accumulation in non-target aquatic organisms has been an area of increasing research interest in recent years.[1-11] Elucidating the uptake and bioaccumulation profiles of environmental mixtures of pharmaceuticals is essential to furthering our understanding of the environmental fate and ecological risks of these ubiquitously detected compounds. [1-4] The potential for PPCPs to exert adverse effects on exposed aquatic organisms such as fish has been well demonstrated [12-13]. Traditional sampling and sample preparation techniques have been utilized to study the toxicology and distribution of pharmaceutical residues in various fish tissues, organs, and cell lines.[1-3,14-15] However, these techniques are often unsuitable for tracking the dynamic processes of bioaccumulation and metabolism, which are integrated whole organism responses modulated *in vivo* by a myriad of pathways including inducible hepatic detoxifying enzymes and excretion mechanisms.

Recently, the simplicity and robustness of the solid-phase microextraction (SPME) technique has been applied to the *in vivo* determination of pharmaceuticals in fish.[19] A significant advantage of SPME fibers is their ability to extract a variety of trace contaminants from fish tissues without lethal sampling. To date, only relatively large SPME fibers (10 mm in length) have been used, constraining their application to larger

tissues such as muscle. Further, the physical fiber size precluded the spatial resolution required for parallel assessments of contaminant distributions in adjacent tissues. Previous SPME studies in fish were also limited to single pharmaceutical exposures (carbamazepine and fluoxetine in single compound exposures) in what were essentially proof-of-principle studies.[19] Consequently, the performance of *in vivo* SPME techniques under multi-analyte scenarios (such as mixtures of PPCPs within municipal wastewater effluents) has not been evaluated.

In this chapter, both muscle and adipose fin were simultaneously sampled using space-resolved SPME to determine the tissue specific bioaccumulation of compounds commonly detected in urban areas influenced by municipal wastewater effluents, industry and agricultural runoff. Adipose fin was chosen as a tissue of interest since it contains significantly more lipid than does muscle, and should differentially bioaccumulate lipophilic PPCPs relative to muscle tissue.[20-21] To reflect the complex mixtures represented in environmental matrices, fish were exposed to a mixture of nine analytes including seven pharmaceuticals belonging to three therapeutic classes: lipid regulators from the fibrate group (gemfibrozil) and statin group (atorvastatin), non-steroidal anti-inflammatory drugs (ibuprofen, diclofenac, and naproxen), and anti-depressives (fluoxetine and carbamazepine), plus the pesticide atrazine and the hormone-disrupter bisphenol A (BPA) from the polymer industry. Consequently, this study

broadly evaluates the application of Sr-SPME techniques for assessing the bioaccumulation of contaminants in fish under controlled exposure scenarios.

7.2 EXPERIMENTAL SECTION

7.2.1 Chemicals and Materials

All aqueous solutions were prepared using de-ionized water obtained from a Barnstead Nanopure water system. All chemicals purchased were of the highest possible purity and used without further purification. Gemfibrozil, atorvastatin, ibuprofen, carbamazepine, diclofenac, naproxen, and bisphenol A (BPA) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Fluoxetine, lorazepam, and atrazine were obtained from Cerilliant Corp (Round Rock, TX). The isotope labeled standards were purchased from CDN isotopes Inc. (Pointe-Claire, Quebec, Canada). Stock solutions were prepared in methanol and stored at -20 °C. HPLC grade acetonitrile for the HPLC mobile phase, methanol for standards preparation and desorption solution, formaldehyde, ethanol and acetic acid (glacial) were purchased from Fisher Scientific (Unionville, ON, Canada).

7.2.2 Preparation and Characterization of Sr-SPME Fibers

Home-made PDMS fibers were employed as the extraction phase for the *in vivo* sampling. Helix medical silicone tubings with a 0.31 mm ID and

0.64 mm OD (Carpinteria, CA) were used as the SPME coating, and 3.5 cm long stainless steel wires, 0.483 mm in diameter were purchased from Small Parts Inc. (Miami Lakes, FL) to serve as the internal support for the SPME fibers. The silicone tubing was cut into 1 mm long segments and the steel wire was carefully introduced into two tubing segments forming discrete sheaths. The distance between the two segments was adjusted to 4 mm after wetting with methanol. The resultant SPME fiber contained 2 separate 1 mm coating segments with a 4 mm space between them (Fig. 7.1A). All fibers were pre-conditioned in 100% methanol for 24 h, and then in sterile pure water for 2 h to remove the methanol. Before *in vivo* application, each fiber was put into a polypropylene microcentrifuge tube and stored at -20 °C.

A systematic characterization was completed to determine the extraction time profile, desorption time profile, any biofouling effect during extraction, and dynamic range. The characterization experiments were performed in standard spiked phosphate buffered saline (PBS, pH 6.8), 1% agarose gel medium, and dorsal-epaxial fish muscle tissue (~ 1 g) to optimize the experimental conditions for *in vivo* sampling.

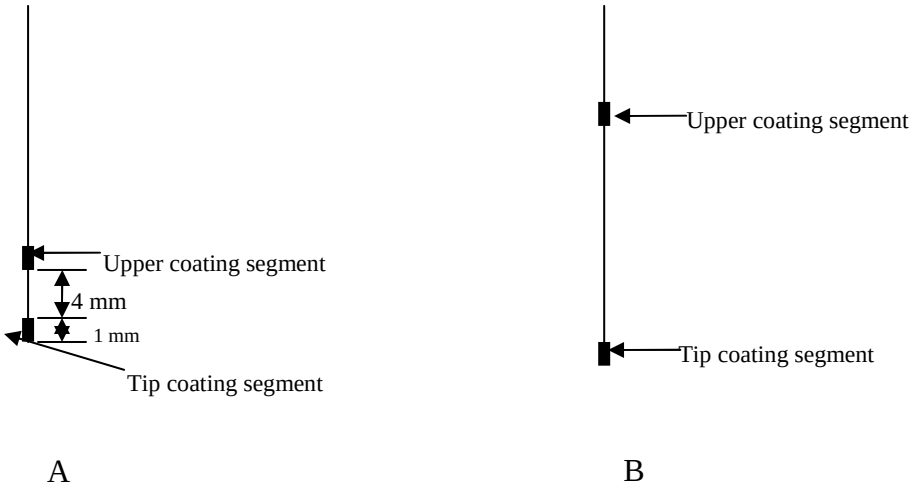


Fig. 7.1. Configuration of the segmented SPME fiber. A: the fiber for in vivo use. B: the fiber ready for desorption in organic solvent.

It was found that the inter-fiber variation was around 8-15%, largely attributable to the small size of the coating segments. When using home-made fibers, the smaller the fiber size, the bigger the inter-fiber variation. This variation was corrected by monitoring the amount of lorazepam extracted from a 50 µg/L aqueous standard solution by each coating segment within 30 min. As the amount of analyte extracted by a fiber is determined by its volume under fixed conditions, the difference in extracted analyte is a surrogate for variations in fiber volume. In this experiment, the upper coating segment must be moved to the opposite end of the wire (leaving a 5 mm space at the tip) in order to differentiate the two

segments by virtue of their positions (Fig. 7.1B). The 30 min extraction and desorption experiment was performed in two successive rounds, each specific for one of the two fibre coating segments. The second extraction was performed after finishing the first round and reversing the direction of the wire to expose the second coating segment to the standard solution. Both the extraction and desorption experiments were conducted in 96-well plates. Desorption of the extracted analytes from the fiber coating was performed in the same way as extraction but each well contained 100 µL of methanol, with each segment positioned at the two ends of the wire sequentially desorbed into methanol within individual wells of the 96 well plate.

In order to evaluate intra-fiber consistency, the same batch of fibers was used in 4 consecutive extraction-desorption cycles, yielding an intra-fiber variation of around 5%, which was deemed acceptable. Further, the results indicated that 30 min was a sufficient desorption period for the fibers when immersed in 100 µL methanol with agitation at 150 rpm on an orbital shaker.

Another important step was to characterize any biofouling potential associated with sampling in fish tissues. The fouling effect was investigated by comparing the extraction behaviors of the fibers in direct contact with and in the absence of contact with fish tissue samples. Specifically, we conducted a series of static extractions in standard solution containing 50 ng/mL of both carbamazepine and fluoxetine in PBS buffer over different

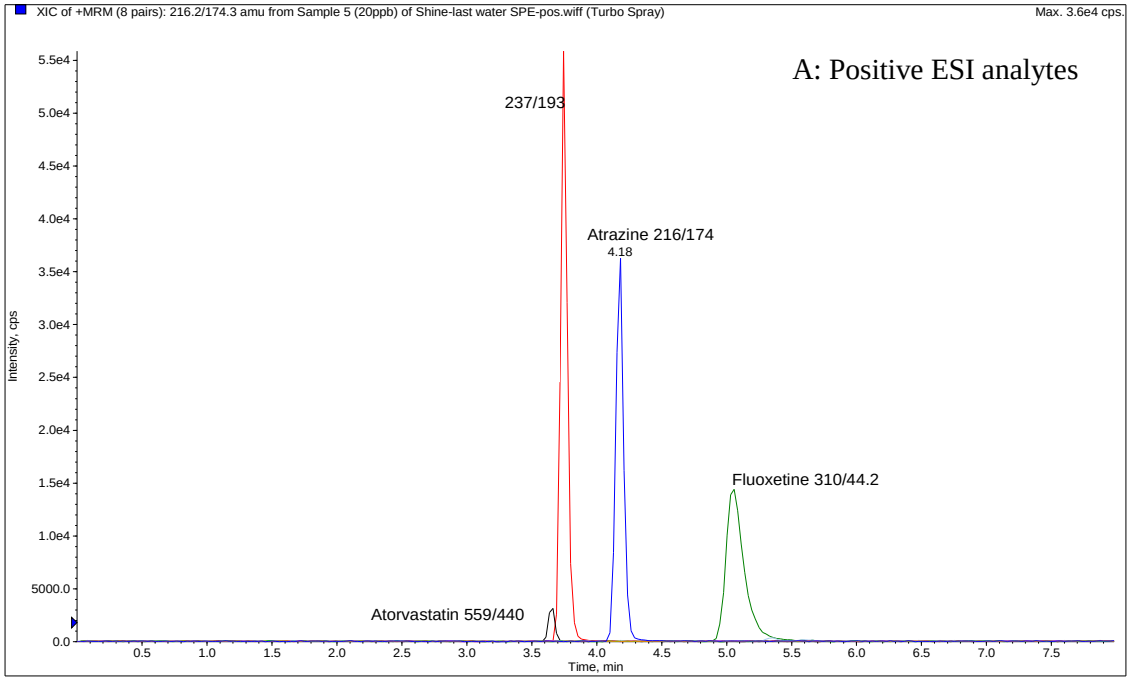
time intervals (5, 10, 20, 30, 60, 90, and 120 min) to develop two time profiles with two groups of fibers. One group was the normally pre-conditioned home-made PDMS fibers; the second being identical fibers exposed to tissue. The latter treatment first introduced the preconditioned fibers into muscle tissue specimens for 30 min. Then the fibers were twice rinsed with de-ionized water and wiped with Kimwipe® tissues prior to implementing a 30 min static extraction in standard solutions. With the time profiles, both the extracted amount in equilibrium (a thermodynamic parameter determined by the partitioning coefficient) and the time constant (a kinetic parameter) could be compared.

7.2.3 Quantification with LC/MS-MS

An Agilent 1200 HPLC/MDS Sciex Q-trap 3200 tandem MS system was used for the analysis of the pharmaceuticals and the isotopic standards. For negative ESI MS analysis, the separation is carried out by using a 4.6 × 150 mm Supelcosil LC-18 column with a 5 µm particle size (Supelco Corp., Bellefonte, PA). Gradient elution was performed with a flow rate of 0.8 mL min^{-1} for separation with mobile phase (A) HPLC grade water (100%) with 5 mM ammonium acetate, and (B) HPLC grade methanol. The gradient started with 10% B for the first 0.5 min, ramping to 60% B in 0.01 min, and then linearly increasing to 100% B over 7.5 min. The 100% B mobile phase was then held for 3 min before decreased to 10% B over 0.01 min. This

provided a total 11 min run time including reconditioning of the column. For the positive ESI MS analysis, the gradient started with 60% B for the first 0.5 min, ramping to 80% B in 0.01 min. The mobile phase was then linearly increased to 100% B over 5.5 min and held for 0.5 min before decreasing to 60% B in 0.5 min. The total run time including column reconditioning was 8 min. The resultant chromatograms are shown in Fig. 7.2.

For quantification, the main mass spectrometer analyte parameters were optimized and are summarized in Table 7.1. The MS parameters for the isotope labeled standards were similar to their non-labeled analogues and are consequently not provided in the table. The instrumental detection limit (IDL) for each analyte is shown, and generally, the positive ESI was more sensitive than the negative ionization. Instrument performance was compromised for atorvastatin and biosphenol A, as later discussed.



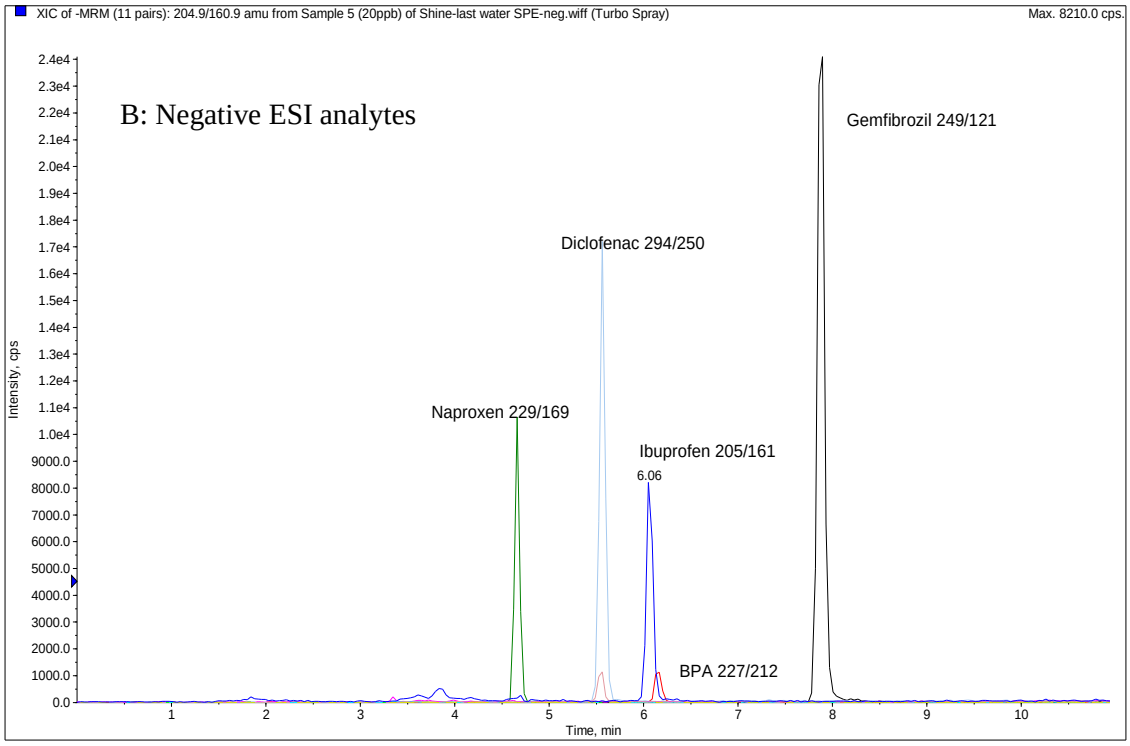


Fig. 7.2. The separation of the analytes in positive ESI and negative ESI modes.

Table 7.1. MS Instrumental detection limits (IDL), Declustering potentials (DP), Entrance potentials (EP): Collision energies (CE), Collision cell exit potentials (CXP) for study analytes: DP, EP, CE, and CXP are provided as voltages.

Compound	Use	Transition	DP	EP	CE	CXP	IDL*
ESI POSITIVE ANALYTES							
Atrazine	pesticide	216/174	67	3.8	27	2.4	0.1
Fluoxetine	antidepressant	310/44	48	2.9	44	7	0.2
Atorvastatin	cholesterol lower	559/440	83	5.9	32	22	0.7
Carbamazepine	anti-seizure	237/195	55	4.9	51	2.7	0.05
ESI NEGATIVE ANALYTES							
Gemfibrozil	antilipemic	249/121	-55	-2	-17	-3	0.15
Naproxen	anti-inflammatory	229/169	-29	-1.9	-25	-4	0.3
Diclofenac	anti-inflammatory	294/250	-46	-2.5	-15	-2	0.4
Ibuprofen	analgesic	205/161	-41	-2.6	-11	-1	0.3
Bisphenol A	hormone-disrupter	227/212	-53	-10	-28	-5	1.0

7.2.4 *In vivo* Sampling with Sr-SPME

Municipal de-chlorinated water (with chlorine and chloramine residuals removed and/or stabilized by aeration and Big Al's Aquarium Water Conditioner (10 mL:40 L, Woodbridge, ON), respectively) was used for all fish experiments. All experimental procedures involving animals were

conducted in the Biology Wet-lab Facility at the University of Waterloo in accordance with protocols approved by the institutional Animal Care Committee (AUP # 07-16). The juvenile rainbow trout (*Oncorhynchus mykiss*) used in this study were 19.8 to 24.5 cm in length and 80.0 to 134.2 g in weight (n = 20). Of these 20 fish, nine were divided into three groups (3 fish /34 L aquaria) and exposed for 8 d to water spiked with the analyte mixture (atorvastatin, atrazine, BPA, carbamazepine, diclofenac, fluoxetine gemfibrozil, ibuprofen, and naproxen; 3 ng/mL of each compound dissolved in 100 uL of methanol). Daily renewals of test solutions on subsequent exposure days replaced half the exposure water volume (17 L) with analytes replenished in test solutions via their addition within 50 uL of methanol. The three 34 L exposure aquaria containing the analyte mixtures (3 fish each, 9 total) were exposed alongside a solvent control (3 fish) with 8 additional fish held as clean water controls. Water quality was monitored daily and maintained at conditions considered suitable for trout (temperature 12.4 ± 0.05 °C; dissolved oxygen 9.64 ± 0.15 mg/L; pH 7.79 ± 0.04; ionized ammonia 54.9 ± 3.6 µg/L).

The *in vivo* sampling was conducted every two days for a period of 8 days using pre-equilibrium SPME with kinetic calibration for quantitation. The sampling procedure with kinetic calibration was similar to that described previously, [17] with minor modifications. Briefly, after the fish was anaesthetized (0.1% ethyl 3-aminobenzoate methanesulfonate) until loss of

vertical equilibrium, a 20 gauge needle was applied to pierced the fish at an angle approximately 30-45˚ (from the vertical) through the adipose fin and into the dorsal-epaxial muscle penetrating the latter tissue approximately 0.8 cm in depth. Subsequent to removing the needle, the Sr-SPME fiber was introduced into the hole until the two coating segments were positioned in muscle and adipose fin tissue respectively, as shown in Fig. 7.3. The fiber coating was embedded in the tissue and the close contact between the tissue and fiber prevented water entry. After fiber placement the fish was put into the fresh reference water for 8 min. Then the fish was anaesthetized again prior to removing the fiber coating, for a total contact time between the fiber and tissue of 10 min. After a brief rinsing with de-ionized water and drying with a Kimwipe® tissue, the fiber was put back into the polypropylene microcentrifuge tube labeled with the fish number and date.

For parallel desorption of the analytes from the fiber, the two coating segments were positioned onto opposite ends of the steel wire by again moving the upper segment along the wire. Then the wire was cut into two sections, each containing one coating segment with each segment desorbed into 100 µL of methanol within a 200- µL polypropylene insert positioned in a 2 ml amber vial (National Scientific, Rockwood, TN). The vials were capped and agitated at 140 rpm for 2 h, after which the wires with coating segments were removed with a magnet, and 60 µL of water containing 20 µg/L lorazepam as the internal standard was added to the methanol followed

by a brief votex. Lorazepam was used to calibrate the sample loss during LC/MS-MS quantification. It deserves noting that the parallel desorption was adopted for the *in vivo* sampling experiment, in which the wire with coatings was cut to two sections to be fitted into the insert in the 2-ml vial for desorption. However, the in series procedure of extraction and desorption was used for monitoring the inter-fiber variation, as described before in the "Preparation and Characterization of Sr-SPME Fibers" part, where only the position of coating segments was changed but the fiber was intact so that it could be reused for the following *in vivo* sampling. On the contrary, the fibers used for the *in vivo* sampling were not reusable after the parallel desorption procedure.

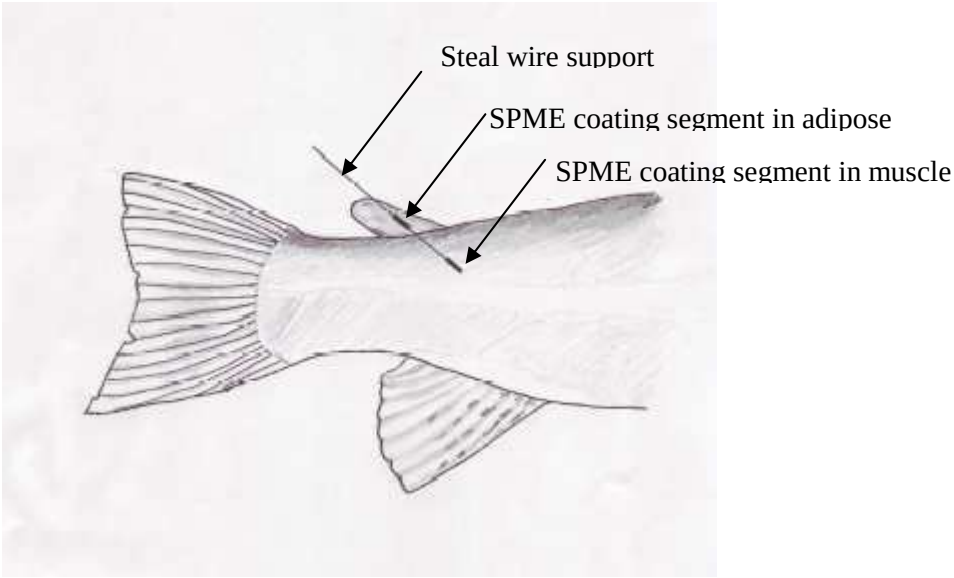


Fig. 7.3. Placement of the segmented SPME fiber into adipose fin and muscle tissue.

7.2.5 Determination of Free Concentrations by Equilibrium SPME and Total Concentrations by Organic Solvent Extraction

The free concentrations of the analytes of interest in muscle and adipose tissue were analyzed using the equilibrium SPME technique, while the accumulated tissue concentrations after 8 days of exposure were determined (following lethal sampling) by organic solvent extraction with methanol and calibration by isotope dilution. These procedures will both validate the sensitivity of the SPME technique, and calculate the distribution coefficient (K_{fs}) between the fiber coating and the tissue sample.

The *in vitro* tissue SPME analysis utilized a clean fiber with a single coating segment (2 mm in length) for insertion into an excised piece of muscle or adipose tissue. The tissue and fiber were held at 4 ˚C for 15 h under static conditions; conditions deemed sufficient to achieve equilibrium based on extraction time profiles from earlier *in vitro* experiments. After equilibration, the fiber was removed from the tissue and rinsed briefly with de-ionized water prior to desorption in methanol. Since the fiber length is only 2 mm, the amount of analyte extracted from the sample tissue is considered to be negligible.

The total analyte concentrations accumulated in fish tissues were determined using traditional liquid extraction (LE) with methanol. Whole tissues were prepared for LE analysis by cutting the sample into approximately 2 mm^2 pieces with a scalpel on a chopping board covered

with alumina foil with mass determined in an analytical balance following transfer to a pre-weighed microcentrifuge tube. Then 500 µL of methanol containing 20 ng/mL isotopically-labeled standard mixture was added into each tissue sample and homogenates generated (4 x 20 seconds/round with a Teflon homogenizer). Following a brief vortex, low-temperature centrifugation (4 ˚C, 15000 rpm, 30 min) separated the tissue pellet from the supernatant, the latter of which (100 µL) was transferred into a 200 µL polypropylene insert within a 2-mL amber vial. Finally, 60 µL of water containing 20 µg/L lorazepam (the internal standard) was briefly votexed into the mixture to produce the final sample for instrumental analysis. The relative recovery of the isotope standards following liquid extraction of muscle and adipose tissue of control fish was used to assess extraction efficiency.

7.2.6 Water analysis with SPME and SPE

To determine the bioaccumulation factor of each compound in the target tissue, SPME techniques were employed to monitor the concentrations of the pharmaceuticals in exposure aquaria water. To facilitate calibration by standard addition, every 40 ml sample was divided into two 20 ml aliquots. One aliquot was spiked with 60 ng of each of the 9 standards dissolved in methanol, while the other received the same amount of pure methanol to compensate for any solvent effects. Concentrations of each unknown were

quantified by comparing the signal intensity of spiked and un-spiked samples. Three fibers with only a single coating segment were used to monitor every water sample. The extraction was performed at 140 rpm on an orbital shaker for 30 min, and the desorption procedure and reagents were the same as previously described. To evaluate the accuracy of the standard addition method, a blank sample was divided into four 10 ml aliquots and spiked with 0, 30, 45, and 60 ng of standards respectively. The linearity (R^2) of the regression line and the averaged standard deviation (RSD) from all three points were used to assess relative recovery.

To evaluate the efficacy of SPME for water analysis, solid phase extraction (SPE) was performed alongside the SPME assessments. Water samples were extracted with the Oasis HLB cartridges (Waters Corp., Millford, MA) on an automated SPE system (12-port Visiprep vacuum manifold coupled to a vacuum pump from Supelco (Bellefonte, PA)). The SPE cartridges were pre-conditioned (in the order) with 5 mL of methyl tertiary butyl ether (MTBE) MTBE, 5 mL of methanol, and 5 mL of HPLC grade water. For calibration by the isotope dilution method, 50 µL of 100 ug/L isotopically-labeled standard mixture was added into each of the 500 mL water samples prior to SPE extraction. The sample was introduced into the cartridge at a flowrate of 15 mL min^{-1}; after sample passage, the cartridges were rinsed with 5 mL HPLC grade water and dried under vacuum for approximately 15 min. When the SPE cartridges were dry,

analytes were eluted with 5 mL of pure methanol and 5 mL of 10:90 (*v/v*) methanol : MTBE, sequentially. The eluted extract was then evaporated completely and re-constituted with 500 µL of methanol reconstitute solution containing 75 ug/L lorazepam as internal standard. Recovery was determined using 500 mL water samples from the control tanks spiked with the same known amount of deuterated standard mixture (5 ng of each standard) to determine analyte recovery with the SPE method.

7.3 RESULTS AND DISCUSSION

Previous studies demonstrated that the SPME technique can be easily applied to *in vivo* analysis of plants and animals tissues, including those of fish. [19,22-25] This technique exhibits high efficiency, simplicity, and convenience compared to conventional sampling and sample preparation methods. However, SPME techniques have previously been applied only to relatively large, uniform tissue samples such as venous blood,[23-25] fish muscle,[19] and porpoise blubber,[26] while the current study demonstrates the possibility of *in vivo* SPME for high-throughput and space-resolved applications. Further, the present study demonstrates the utility of SPME for the *in vitro* sampling of water and tissue samples, and provides a comparative study between SPME and more established techniques such as SPE and LE. Thus this report provides a comparative overview of the

relative merits of several important sampling methodologies common in environmental toxicology.

7.3.1 Development of Segmented Sr-SPME Fibers

The choice of fiber coating material in any SPME study is based on the requirements of the *in vivo* experiment and made with consideration of required attributes such as biocompatibility, sensitivity, and robustness. In the present study, these factors were important, but the focus was on miniaturizing the fibres for tissue specificity while ensuring reproducibility was not compromised.

In order to obtain an accurate evaluation of the fate of pharmaceuticals in a small-sized fish tissue, miniaturization of the SPME fiber dimensions is a must. In the present study, the fiber length was reduced to 1 mm, which could effectively be utilized to monitor the analyte bioaccumulation in a small area of approximately 1 mm^3. The miniaturized fiber combined with the segmented design made it possible to probe two separate sampling sites in a small fish using one fiber with two mini-coating segments. Additionally, the sensitivity of the fiber to non-polar pharmaceuticals could be guaranteed with the strong affinity of the PDMS coating for non-polar molecules. The extraction behavior of the mini-coating in standard solution showed that detection limits during a 10 min static extraction were identical for gemfibrozil, carbamazepine, and ibuprofen at 0.2 ng/mL , and 0.05, 1, 4, 5, 7,

and 12 ug/mL for atrazine, fluoxetine, diclofenac, naproxen, atorvastatin and BPA, respectively.

The potential for biofouling was investigated through extraction time profiles using tissue-treated and non-treated fibers, which indicated no significant impairment of the fiber extraction ability resulting from exposure to fish tissue (Fig.7.4). In addition, an effective method to correct the inter-fiber variation was proposed which reduced the RSD from 14% to 6.5% based on 100 coating segments. The reduction in RSD of 7.5% was found to be due to inter-fiber variations, with the remaining 6.5% attributable to experimental error including variations in sampling procedure, instrumental analysis, etc.

7.3.2 Calculating Distribution Constant (K_{fs})

As discussed in previous papers, it is necessary to determine the product of the analyte V_f and K_{fs} to obtain total sample concentrations using on-fiber standardization.[27] In this report, the $K_{fs} \bullet V_f$ product was obtained by combining the data from equilibrium SPME and conventional LE techniques for the *in vitro* tissue sample based on the following equation:

$$K_{fs}V_f = n_e / C_s \qquad (7.1)$$

where n_e is the amount of analyte extracted using the equilibrium SPME approach and C_s is the analyte concentration in the sample tissue. Herein, we used the equilibrium SPME method (15 h equilibration time *in vitro*) to

obtain n_e, and then measured the sample concentration, C_s, with traditional organic solvent (methanol) extraction. This traditional LE approach was calibrated using the isotope dilution method as an internal calibration standard to increase quantitation accuracy by compensating for tissue matrix effects. Using this approach, the relative recoveries calculated from control tissue samples spiked with isotope standards were 94.4-103.1%, which demonstrated the accuracy and reliability of the method.

Listed in Table 7.4, The K_{fs} values, specially refered to as K_{fm} and K_{fa} for distribution coefficient of the analyte between the fiber coating and muscle or adipose fin tissue respectively in this study, were calculated with the products $K_{fs} \bullet V_f$ devided by the fiber volume of the coating segments. When compared with literature K_{ow} values, the roughly proportional relationship is readily obvious; an observation consistent with other environmental samples (water and air) from previous studies.[28-30]

7.3.3 Water sampling with SPE and SPME

Fish exposure water samples analyzed by both SPE and SPME did not differ significantly in their respective estimates of waterborne concentrations of any analyte examined (Table 7.2). The standard addition method used for calibrating the SPME data was highly accurate as evidenced by the linearity of the calibration curve regression line ($R^2 = 0.992$), and average RSD (less than 6.3%).

As with the LE tissue samples, the isotope dilution technique was employed to calibrate the SPE exposure water analysis. Usually, the easiest way to calculate an analytes concentration is using the response ratio of the analyte relative to its deuterated standard to multiply by the spiked concentration of the standard in the sample matrix. The underlying assumption for this method is that the response factor ratio of the analyte would be the same as its' deuterated analogue, with an ideal value of 1. However, as demonstrated in Table 3, this relationship is not always the case, as in the present study the response ratio for the analyte relative to its' deuterated standard differ significantly (range 0.68-1.46). To compensate for the apparent response difference between analytes and their deuterated analogues, the response ratio should not be assumed as 1, but be corrected by the measured response factor ratio on a compound-specific basis.

The SPE procedure was much more time (15 h) and labor intensive than the SPME method (2-3 h) when measuring analyte concentrations in fish exposure water. However, SPE has higher sensitivity than SPME in that the SPE method could detect trace levels of analytes in the control water (shown in Fig. 7.5), which were non-detectable using the SPME technique. The heightened sensitivity of the SPE technique is attributable to the exhaustive extraction method inherent to this approach, while equilibrium-based SPME sampling typically yields lower sensitivities. Despite the higher detection limits associated with the SPME technique, this

method can be used to monitor free (biologically available) analyte concentrations within complex matrices, an application not suited for SPE analysis. [23,27-28]

7.3.4 Study the Tissue Specific Bioaccumulation with Sr-SPME

With the space-resolved SPME technique, the accumulation of pharmaceuticals and bioactive analytes in muscle and adipose tissue were determined simultaneously, demonstrating the utility of the *in vivo* technique for tracking tissue burdens over time (Fig. 7.6). To generate these datasets, total tissue analyte concentrations were measured by LE at the experiments conclusion (8 d), which in turn were used to calculate distribution coefficients for muscle and adipose tissue, as shown in Table 7.4. Thereafter the total concentrations of the pharmaceuticals for each sampling interval could be calculated for tracking the dynamic accumulation process.

Table 7.2. Total analyte concentrations in water samples as determined using SPME and SPE techniques (n = 3; mean ± SD; unit: ng/mL)

	Ibuprofen		Naproxen		Diclofenac	
Day number	SPME	SPE	SPME	SPE	SPME	SPE
2	2.88±0.14	2.82±0.50	2.89±0.12	2.98±0.11	3.02±0.11	2.92±0.25
4	2.75±0.11	2.73±0.12	2.81±0.07	2.81±0.15	2.89±0.10	2.83±0.19
6	2.68±0.07	2.83±0.14	2.73±0.17	2.79±0.06	2.83±0.12	2.80±0.17
8	2.87±0.15	2.79±0.11	2.69±0.11	2.92±0.12	2.97±0.17	2.88±0.13

	Atrazine		Fluoxetine		Atorvastatin	
Day number	SPME	SPE	SPME	SPE	SPME	SPE
2	2.79±0.13	2.88±0.17	2.78±0.27	2.87±0.12	2.85±0.13	2.90±0.16
4	2.64±0.23	2.68±0.16	2.59±0.19	2.64±0.15	2.41±0.24	2.58±0.13
6	2.77±0.12	2.63±0.18	2.67±0.13	2.76±0.11	2.36±0.18	2.63±0.17
8	2.66±0.20	2.67±0.15	2.56±0.22	2.77±0.14	2.53±0.20	2.62±0.21

	Bisphenol-A		Gemfibrozil		Carbamazepine	
Day number	SPME	SPE	SPME	SPE	SPME	SPE
2	2.92±0.11	2.90±0.10	2.86±0.17	2.81±0.24	2.86±0.12	3.03±0.18
4	2.74±0.21	2.71±0.25	2.65±0.13	2.73±0.21	2.81±0.07	2.87±0.17
6	2.87±0.11	2.76±0.19	2.61±0.12	2.67±0.08	2.73±0.17	2.81±0.05
8	2.08±0.23	2.22±0.21	2.65±0.23	2.37±0.16	2.69±0.11	2.91±0.16

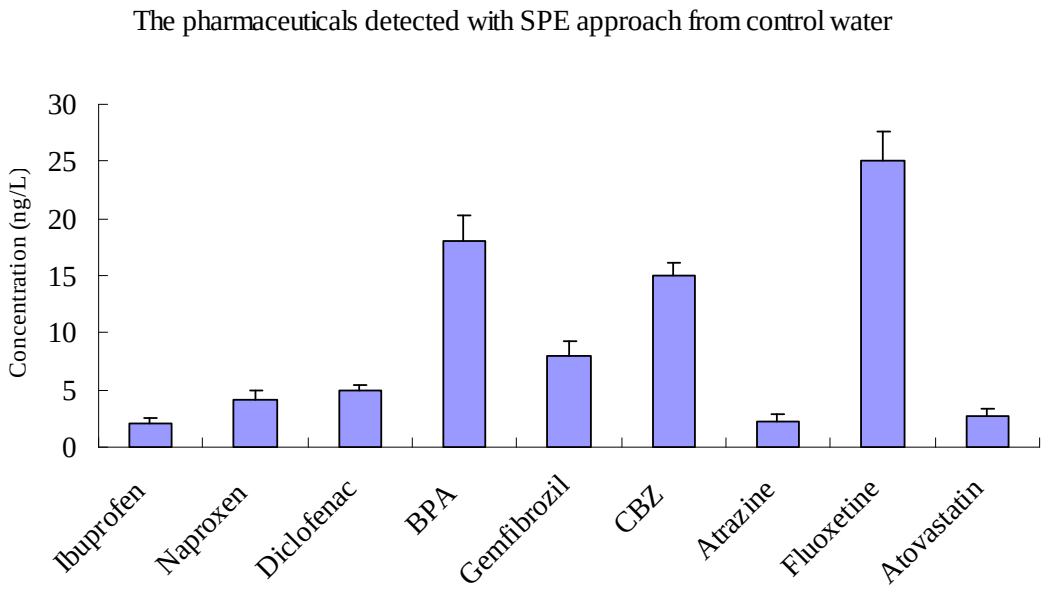


Fig. 7.4. The sample concentrations of the reference water by using SPE method. The detection limits of SPE for ibuprofen, BPA, naproxen, gemfibrozil, diclofenac, atrazine, carbamazepine, fluoxetine and atorvastatin were 1, 10, 0.2, 0.15, 0.15, 0.025, 0.05, 0.2 and 1 ng/L.

Table 7.3. The response ratio for analyte over the same amount of its deuterated standard.

Calculating the response factor ratio (RFR)

Analyte/Deuterated Std	RFR
Ibuprofen/Ibuprofen-d3	0.68
Naproxen/C13-Naproxen-d3	1.46
Diclofenac/Diclofenac-d4	1.15
BPA/BPA-d16	1.31
Gemfibrozil/Gemfibrozil-d6	1.13

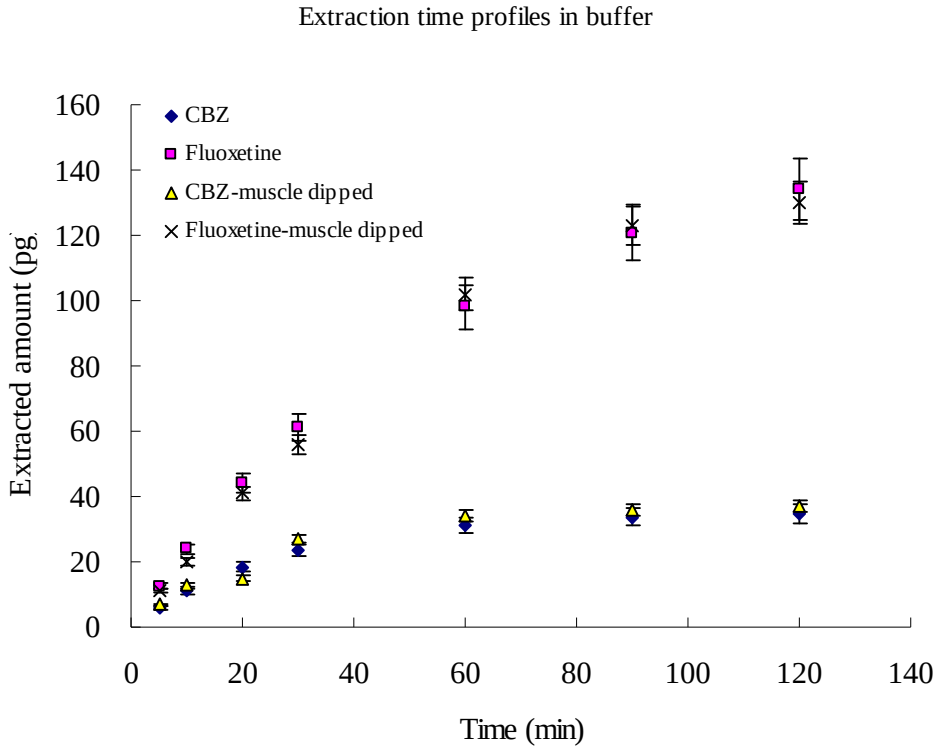


Fig. 7.5. The extraction time profiles with the original fiber and muscle-dipped fiber coating.

Of the nine analytes comprising the exposure test mixtures, only five compounds (atrazine, gemfibrozil, CBZ, ibuprofen, and fluoxetine) were detected in fish tissues by SPME and LE, while the remaining four analytes (atorvastatin, naproxen, BPA, and diclofenac) were not detected. There are several potential explanations for our inability to detect these analytes. One explanation is the detection limit of the instrumentation is simply insufficient to measure select analytes in fish tissues. However, as all analytes were persistent and detected in the exposure water, it would appear all compounds were equally available for uptake across the gills, but four of

the compounds simply did not bioaccumulate within fish tissues. Some of the lower log K_{ow} compounds, or those which readily ionize may have been rapidly excreted, and consequently only present in low concentrations in fish tissues. Alternatively, those analytes which could be quickly metabolized by fish would be present as the parent compound only at low concentrations.[33] Overall, while the sensitivity limits of the Sr-SPME technique may have precluded detection of some analytes, sensitivity should not have been a factor with the LE determination, suggesting the four compounds not being detected by LE are likely absent due to their excretion or metabolism rather than a lack of method sensitivity. Regardless, the five detected analytes represent widely used environmental compounds including two anti-depressant drugs, one anti-inflammatory, one analgesic and one pesticide constituting a diverse group of compounds requisite to assessing the efficacy of Sr-SPME as a robust detection technique in fish.

Overall, there was a roughly proportional relationship between the K_{fm} (fiber-muscle tissue distribution) value and the K_{ow} value for a specific compound; however, there were also exceptions. For example, the log K_{ow} values for carbamazepine and atrazine were approximately equivalent (2.4 and 2.34, respectively) but their K_{fm} values were markedly different (11 and 790, respectively), which could be ascribed to several aspects. First, the distribution occurred in the organism should be different from that in water or air, since more biochemistry processes such as metabolism are involved

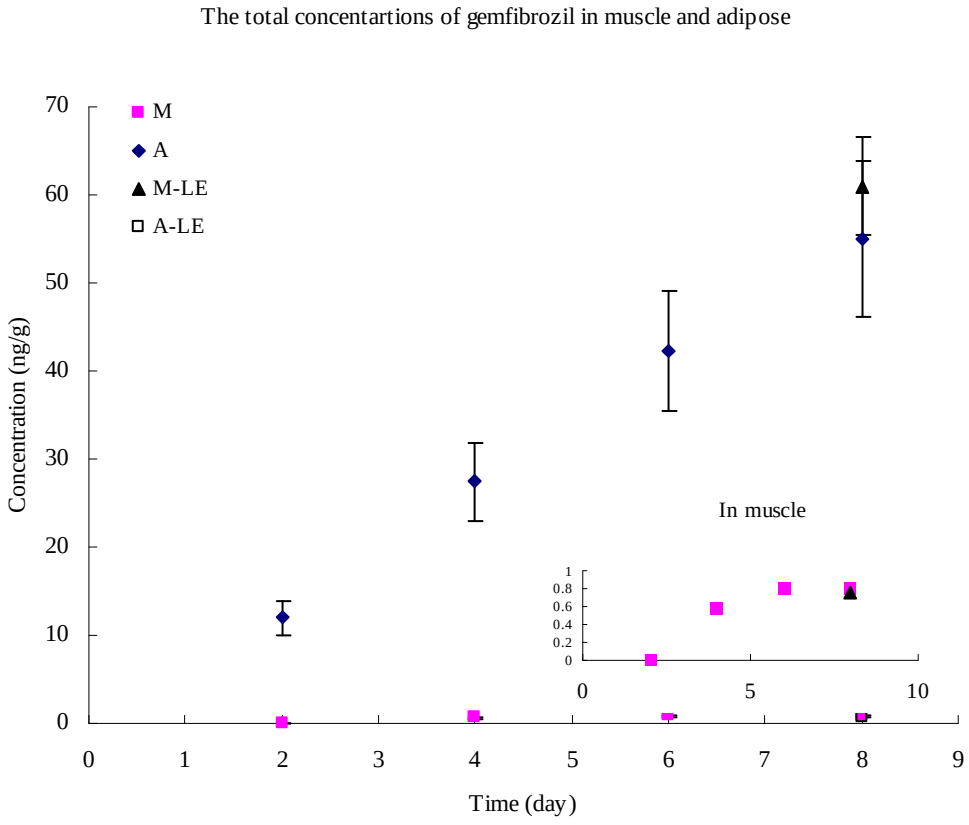
in the organism and make it complicated. Second, the composition of the tissue might vary along with the growth and development of the juvenile fish. Nevertheless, the current SPME technique may provide a simple means to study the *in vivo* dynamic distribution.

Bioaccumulation factors (BAF) were calculated based on the total concentrations of the compounds in the tissue and exposure aquaria. Generally, the results were consistent with previous literature values[4, 19] demonstrating a positive relationship between BAF_s, including BAF_m and BAF_a, and log K_{ow} values. The correlation could be further improved by using lipid normalized analyte concentrations if the amount of lipid in the tissue can be accurately determined.[21] The positive correlation observed in the present study between BAF_m and BAF_a using Sr-SPME and LE, supports previous findings in flathead catfish (*Pylodictis olivaris*).[21] However, this basic relationship is modified by a variety of factors including compound ionization, metabolism, the physiological status of the fish, and also the composition of the pharmaceutical mixtures. Previous studies have demonstrated that ionization of analytes at physiological pH values can strongly affect bioaccumulation potential, and would vary with the pKa of the parent compound and any metabolites or transformation products present.[4] For example, the pK_a value of carbamazepine is 13.4 and ibuprofen is 5.2, which could affect both their polarity/ionization capacity and bioaccumulation potential.[36] Further, interactions between different drugs

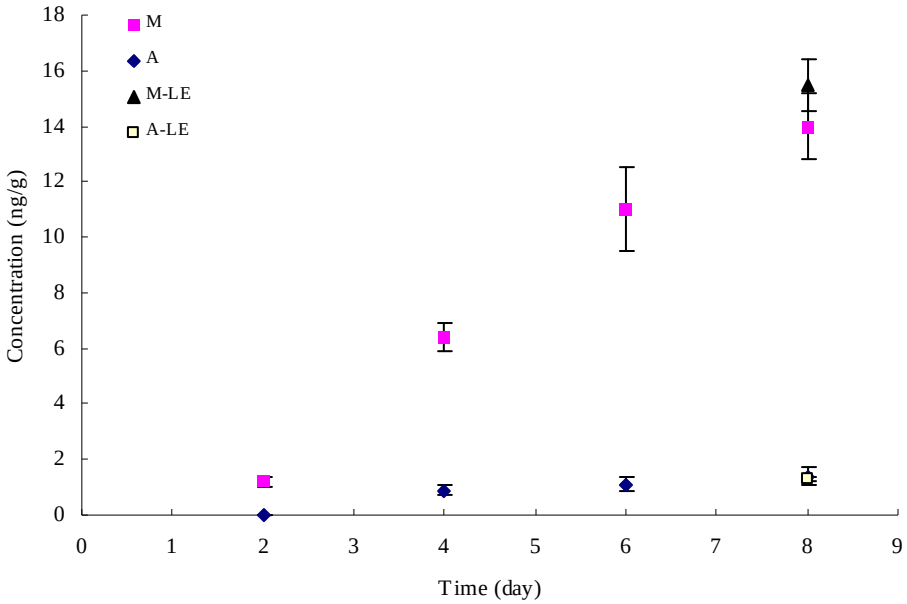
could modify metabolism of co-occurring analytes. For example, the hypolipidemic drugs atorvastatin and gemfibrozil modify lipid metabolism and may influence the bioaccumulation of high K_{ow} analytes such as fluoxetine.

Table 7.4. The distribution and bioaccumulation factors of the test compounds after eight exposure days.

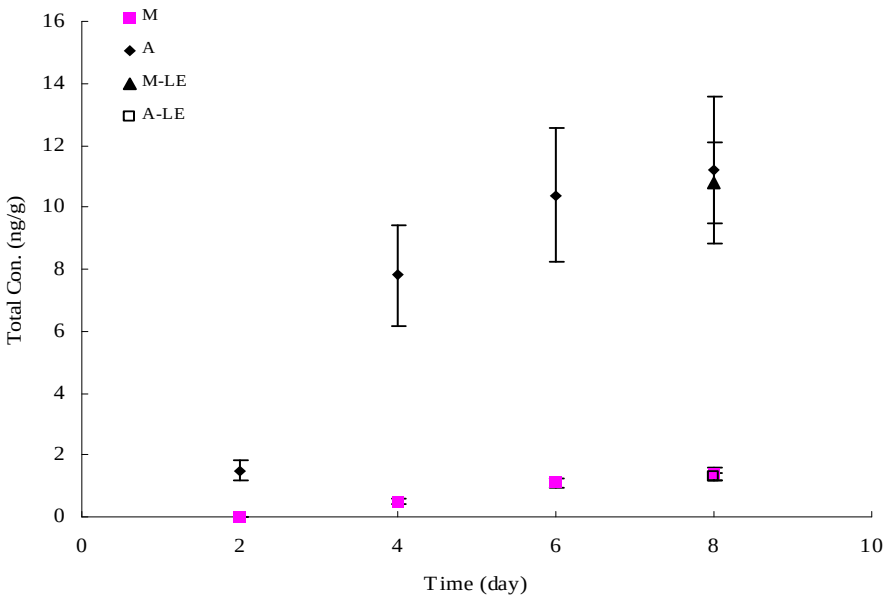
	Atrazine	Gemfibrozil	CBZ	Ibuprofen	Fluoxetine
$K_{f\text{-}m}$	703.2	15.4	11.2	10.3	25.2
$K_{f\text{-}a}$	404.3	1.1	5.0	0.9	0.8
$\log(K_{ow})^*$	2.34	4.77	2.40	3.97	4.64
BAF_m	0.4	0.3	0.5	1.5	58.1
$BAFa$	5.2	20.1	4.3	13.8	112.1



Atrazine in muscle and adipose



Carbamazepine in muscle and adipose



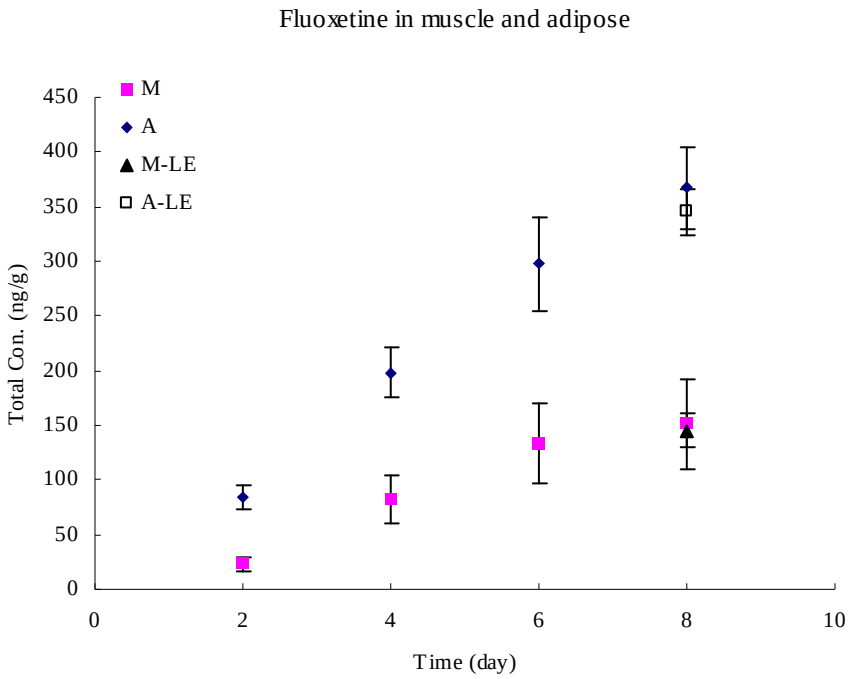


Fig. 7.6. The bioaccumulation of gemfibrozil, ibuprofen, atrazine, carbamazepine, and fluoxetine in fish muscle and adipose fin. M: muscle tissue concentrations by SPME. A: adipose fin concentrations by SPME. M-LE: muscle tissue concentrations by LE. A-LE: adipose fin concentrations by LE.

7.4 CONCLUSION

7.4.1 Conclusion

The Sr-SPME technique was developed to facilitate the *in vivo* analysis of tissue-specific bioaccumulation of pharmaceuticals and pesticides in fish tissues. The segmented miniaturized configuration of the probe offered the requisite spatial resolution for *in situ* application in small fish. The medical grade PDMS fiber coating proved to be biocompatible and highly sensitive.

A standard on-fiber calibration was adopted for accurate quantification within short exposure durations. With this approach, the bioaccumulation of pharmaceuticals and other bioactive analytes in adipose and muscle tissue was simultaneously determined. The results demonstrated differential (but correlated) bioaccumulation of four pharmaceuticals (gemfibrozil, CBZ, ibuprofen, and fluoxetine) and one pesticide (atrazine) within muscle and adipose tissue. Distribution coefficients were determined using equilibrium SPME combined with LE for *in vivo* monitoring of the analyte concentrations in semi-solid fish tissues. SPME and SPE determinations of aqueous analytes were in good agreement. However, the SPME method is much simpler and faster than SPE, although the SPE technique demonstrated superior sensitivity. Overall, the Sr-SPME technique is a novel SPME application that is simple to deploy, with good spatial and temporal resolution, sensitivity, and capacity to simultaneously monitor multiple tissues *in vivo*.

Chapter 8

Summary and Perspective

8.1 Summary

Although solid phase microextraction (SPME) has gained wide applications from *in vitro* environmental investigations to *in vivo* pharmacokinetic studies, it is still difficult to monitor fast concentration change over time at a specific spot or sampling site in a heterogeneous system, where the sampling technique should have high temporal resolution and spatial resolution. Here, the temporal or spatial resolution of the SPME technique is referred to as its capability to clearly resolve two different concentrations that are close to each other temporally or spatially. Generally the temporal resolution of the SPME is determined by its response time or sampling time. Therefore, to improve the temporal resolution, it is necessary to reduce the sampling time. On the other hand, the spatial resolution of SPME is determined by the size of its extraction phase; so it could be improved by reducing the fiber size. However, the sampling time of SPME that determines its temporal resolution, should also be considered simultaneously, because the diffusion during a long sampling time tends to uniform the concentration distribution in the adjacent areas, thus making spatial resolution meaningless. Consequently, for SPME experiments, the

effect of reducing sampling time should be considered together with shrinking the fiber dimension.

It must be noted that reducing the sampling time and fiber dimension always result in compromised sensitivity, as shown in Eq. 1.08, and Eq. 1.10 of Chapter 1. Therefore, in case of time- and space-resolved SPME, the sensitivity of the method must be carefully investigated in advance because the sensitivity of SPME sets the limits for sampling time and fiber dimension. In addition, the spatial resolution is dramatically affected by the diffusion of the analyte molecules in the sample matrix, as described by Eq. 1.12 of Chapter 1. Hence, minimal sampling time must be evaluated to ensure the validity of an *in situ* analysis in a heterogeneous system.

The goal of the research presented in this dissertation was not only to address the issues mentioned above but also to develop new analytical methods that were more efficient and effective for *in vivo* studies using SPME. In order to achieve the goal, the research has been conducted in four steps. Briefly, the first step is the development of kinetic calibration for solid coating SPME coatings. One rationale is that the solid coating SPME can be easily devised to have polar surface chemistry thus more suitable for sampling of polar pharmaceuticals, while liquid coating SPME normally has better affinity and sensitivity for nonpolar analytes. Furthermore, the kinetic calibration overcomes the inherit limitations of the traditional external calibration curve method and delivers accurate calibration for the *in vivo*

pre-equilibrium sampling. Later, the kinetic calibration was successfully applied to the *in vivo* pharmacokinetic studies; moreover, a series of simplified kinetic calibration was developed to improve not only the cost-effective but also the time-effective, or temporally resolved. The third step is the development of SPME technique with miniaturized fiber coating and improved spatial resolution. Finally, the feasibility of the time-resolved and space-resolved SPME for *in vitro* study was demonstrated by studying the drug distribution in the onion bulb with highly heterogeneous structure and the toxin's (i.e., Ochratoxin A) concentration distribution in the cheese and its change over time. Its efficacy for *in vivo* dynamic study was illustrated by investigating the tissue specific accumulation of pharmaceuticals in live fish. The detailed summary of the research is presented in the following paragraphs.

The first work is to study the kinetics of adsorption and desorption of analytes onto and from the surface of porous solid SPME fibers. Theoretical model, based on the assumptions of steady-state diffusion in the boundary layer and quick distribution equilibrium on the interface between the fiber surface and sample matrix, was proposed. It was found that the adsorption kinetics provided a directly proportional relationship between the amount of analyte adsorbed by solid SPME fiber before the equilibrium and its initial concentration in the sample matrix. This observation indicates that quantitative analysis with porous solid SPME fiber can be performed by

pre-equilibrium extraction. Consequently, the kinetic calibration method was developed for accurate calibration in pre-equilibrium extraction using porous solid SPME fibers based on the symmetric relationship between adsorption and desorption. However, the quantification is limited within the linear range of the given fiber. For example, for diazepam in beagle whole blood, the linear range was 5 - 1000 µg/L, where 5 µg/L is the limit of detection. In addition, the rate for adsorption and desorption was found to be boundary layer-controlled. This suggested the importance and necessity of the kinetic calibration to compensate the agitation and matrix effect while using porous SPME fibers in pre-equilibrium extraction. Finally, the resulting kinetic calibration was used for drug analysis in clinical plasma and whole blood using Polypyrrole (PPY) fibers, and accurate results (relative recovery: 91-101%) were obtained.

The second work involved applying the kinetic calibration of SPME for *in vivo* sampling. The kinetics of desorption and adsorption of analytes from and onto the SPME fibers showed the feasibility of the kinetic calibration method for *in vivo* application. Furthermore, for determining the sample concentration, a simple method was proposed to calculate the main parameter, the product of V_f and K_f. To evaluate its validity, diazepam pharmacokinetics was studied with the PEG-C18 probes. Accurate metabolism information was obtained using PEG-C18 probes of large linear range (5-2000 µg/L) and high capacity (~1 ng), along with the fast extraction

time (2 min) defined by the kinetic calibration method. The results were comparable to the results of traditional blood drawing and chemical assay (LOD: 5 µg/L and linear range 5-2000 µg/L). Actually, with the improved temporal resolution, i.e., 2 min sampling time, there could be more sampling points for the pharmacokinetic studies, thus providing much more detailed information for the drug metabolism. The detection limit (0.5 µg/L) of the equilibrium sampling (10 min) was ten times lower than that (5 µg/L) of the pre-equilibrium sampling (2 min).

Afterwards, a series of simplified calibration methods and sampling strategies were developed to improve its accuracy and automation potentiality; meanwhile, the application conditions of these methods were investigated. Through studying the desorption kinetics of the preloaded standards from the SPME fibers, it was found that the time constant, *a*, is independent of the sample concentrations, but it is affected by the sample composition, sample agitation, fiber surface area and composition, and the distribution constant as described by eqs 2.11, 2.12, and 2.32. Consequently, the multiple time-points and isotope-labeled internal standard based traditional kinetic calibration approach was simplified to single time-point and non-isotope labeled standard calibration, further to single-standard calibration for multiple analytes and finally to a single-point self-calibrated SPME without using standards. The simplification was based on the assumption that the blood composition and agitation do not change

significantly during the 8-10 hour *in vivo* experiment. All the methods were verified by *in vitro* and *in vivo* experiments and validated by traditional blood drawing and chemical assay (no significant difference according to one-way ANOVA and the post-hoc Turkey's test for multiple comparisons). The simplified calibration methods guaranteed the temporal resolution (2 min vs. the 10 min sampling with equilibrium sampling), enhanced the quantitative applications of SPME for *in vivo* dynamic monitoring, and improved the multiplexing capability and automation potentiality for high throughput analysis.

The spatial resolution of SPME was addressed in several aspects. Firstly, the sampling of the SPME with high spatial resolution was modeled in multilayered gel system with the mini-sized SPME fibers (1 mm in length), and the feasibility of the SPME for real application was demonstrated in an onion bulb, a heterogeneous system. The results agreed with that from the established microdialysis method (Table 5.1); but methodologically SPME was found to have higher sensitivity and resolution (SPME: LOD: 2.5 ng/mL; patial resolution: 1 mm. MD: LOD: 5 ng/mL; patial resolution: 4 mm), simpler implementation and more cost-effectiveness. Another *in vitro* study was performed using the miniaturized carbon-type fiber for in situ analysis of concentration distribution of Ochratoxin A (OTA) in a piece of semisolid cheese. The limits of detection and quantification for the 1 mm fiber in gel matrix were

1.5 and 3.5 ng/mL and the linear range was 1.5-500 ng/mL. It is interesting that the spatial distribution of the contaminant concentration and the concentration change with the time can be used to monitor the active OTA fungi in the cheese, i.e., an indicator of contamination source. The results were validated by liquid extraction (Table 6.1). Finally, the *in vivo* application of the space- and time- resolved SPME was demonstrated by studying the tissue-specific bioaccumulations of pharmaceuticals in fish adipose fin and muscle. With the segmented design of the SPME fibers, the pharmaceutical residues in fish muscle and adipose fin can be determined simultaneously with only one SPME fiber; thus increasing the throughput of the analysis. The *in vivo* assay showed good sensitivity, for example, the detection limits during a 10 min static extraction were identical for gemfibrozil, carbamazepine, and ibuprofen at 0.2 ng/mL, and 0.05, 1, 4, 5, 7, and 12 ug/mL for atrazine, fluoxetine, diclofenac, naproxen, atorvastatin and BPA, respectively. The results were validated by liquid extraction and solid phase extraction, as shown in Table 7.2 and Fig.7.6. And they were also comparable to the literature results.

The research presented here demonstrated the application potential of the time-and space- resolved SPME for *in situ* dynamic and static analysis in living systems, such as an *in vivo* study and in a non-living system, such as a cheese piece or an onion bulb.

8.2 Perspective

The developed novel SPME technique has many important advantages, such as simple operation, cost-effectiveness and improved quantitative capability. But there are still some fields that deserve in depth exploration.

The first issue could be the matrix competition effect for solid coating SPME. Traditionally, researcher believed that that the competition effect could be eliminated or reduced by shortening the sampling time. But, the observed reduced competition is due to less competition or because of the fact that the analytical signal is not strong enough to reveal the competition. Other matrix effects on the sampling, for example, the effect of biofouling, on the kinetic property of the SPME extraction, need to be addressed clearly.

Secondly, the extraction mechanism, absorption/adsorption or mixed mechanism needs to be studied. This is important because the answer could be used for accurate quantification. For example, it would be important to determine the surface area of a solid coating SPME fiber.

In addition, when many efforts have been put into the field to seek specific extraction sorbents, such as molecularly imprinted polymers, antibody or aptamer immobilized materials, there is less attention onto the universal extraction materials. The non-selectivity might have useful application, especially when the fiber is used for metabolomic studies.

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