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ARTICLE

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Sampling-Rate Calibration for Rapid and Nonlethal Monitoring of Organic Contaminants in Fish Muscle by Solid-Phase Microextraction

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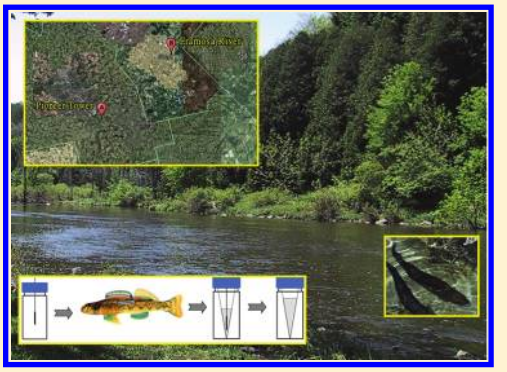
Supporting Information

ABSTRACT: Solid-phase microextraction (SPME) is a promising technique for determining organic contaminants within biotic systems. Existing *in vivo* SPME-kinetic calibration (SPME-KC) approaches are unwieldy due to the necessity of predetermining a distribution coefficient for the analyte of interest in the tissue and the preloading of a calibrating compound to the fiber. In this study, a rapid and convenient SPME alternative calibration method for *in vivo* analysis, termed SPME-sampling rate (SPME-SR) calibration, was developed and validated under both laboratory and field conditions to eliminate such presampling requirements. Briefly, the SPME probe is inserted into tissue, in this study fish dorsal-epaxial muscle, for 20 min allowing the concentrations of target analytes in the fish muscle to be determined by the extracted amount of analyte and the predetermined sampling rates. Atrazine, carbamazepine, and fluoxetine were detected nonlethally in the low ppb levels within fish muscle, with both laboratory and field-derived results obtained by *in vivo* SPME-KC comparable (within a factor of 1.27) to those obtained by lethal sampling followed by tissue liquid extraction. The technique described in this study represents an important advance which broadens the application of SPME *in vivo* sampling technology.



INTRODUCTION

There has been considerable research interest in developing techniques to monitor biologically active compounds in living systems, both in a laboratory context and within natural environments.[1−5] An *in vivo* sampling approach can eliminate errors and reduce the time associated with sample transport and storage, resulting in more accurate, precise, and timely analytical data.[4] A nonlethal *in vivo* approach that permits repeated sampling of individual organisms will greatly enhance temporal studies by reducing the error associated with the use of different individuals at different time-points and provide a better indication of the presence of transient analytes.[5] Further, from an animal ethics perspective, a relatively noninvasive approach that minimizes experimental animal use is increasingly expedient.

Investigating the uptake, bioaccumulation, and depuration of contaminants in fish and other biota is an important research area for environmental scientists. Fish uniquely acquire contaminants both from dietary sources and across gill membranes resulting in tissue burdens up to a million times greater than their ambient water.[6] Such marked uptake by two independent mechanisms poses risks both to aquatic ecosystems and, in the case of commercially exploited species, human health.[7]

Sensitive and relatively noninvasive sampling and analytical approaches are critical to understanding the pharmacokinetics of emerging contaminants such as pharmaceuticals and personal care products (PPCPs), pesticide residues, and a myriad of other compounds which are present in environmental matrices or bioaccumulate at low to very low levels.[8,9] Traditional fish tissue assessments integrate contaminant fluctuations over time, thereby reducing temporal sensitivity needed to detect transient compounds but also invariably requiring lethal sampling. Soxhlet, liquid extraction (LE), accelerated solvent extraction (ASE), and solid phase extraction (SPE) are frequently used sample extraction methods that require homogenized fish (or portions thereof) which may be difficult to obtain for rare or protected species.[10−13] While fish contaminant data within the United States, Canada, China, and Europe is relatively abundant,[6,14−17] measured bioaccumulation data are limited compared to the breadth of existing commercial chemicals. Extensive sample preparation time and resources associated with contemporary approaches limit broader assessments of contaminant distributions in fish.[18] Rapid, nonlethal, and relatively inexpensive sampling

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techniques for monitoring contaminants in fish are an attractive alternative to traditional lethal methods.[19]

Solid-phase microextraction (SPME) represents an important development in the field of analytical chemistry.[20] It is a solvent-free sample preparation technique which combines sampling, isolation, and enrichment into one step.[21] Using this approach, micro quantities of solid sorbent or liquid polymer (in appropriate format) are exposed to the sample, with quantification based on the amount of analyte extracted under appropriate conditions. Since its inception, SPME has been widely used in different fields including biology, toxicology, pharmaceuticals, and forensics.[22−24] With the development of novel calibration procedures,[25] SPME has recently emerged as a rapid analytical tool for monitoring contaminants in the environment[26] as well as in pharmacological studies.[27] SPME *in vivo* sampling is possible[28−31] through the inclusion of a standard in the extraction phase,[32] in a process called kinetic calibration (KC).[33] Specifically, this method utilizes the desorption of a preloaded standard from the extraction phase to the tissue to calibrate the extraction of the target analyte from the tissue. As a result, the extracted amount of analyte at equilibrium can be predicted by rapid pre-equilibrium sampling, with the analyte concentration in the sample matrix, C_s, calculated using the following equation[34]

$$C_s = \frac{n}{KV_f(1 - Q/q_0)} \quad (1)$$

where n is the amount of the extracted analyte, q_0 is the preloaded standard, Q is the standard remaining in the SPME fiber coating after sampling, V_f is the volume of the fiber coating, and K is the distribution coefficient of the analyte between the fiber coating and the sample matrix. This method is cumbersome as it requires both the calibrant and target analyte to possess similar physicochemical properties (i.e., typically achieved through the use of isotopically labeled analogues), with the distribution coefficient of the analyte between the extraction phase and the sample matrix determined in advance (which is often inconvenient and time-consuming).[35] For multianalyte applications, existing methodologies require preloading of an isotopically labeled analogue for each analyte, which if available, are often prohibitively expensive.

To address these limitations, many efforts have been made to reduce or eliminate the use of deuterated calibrants, including dominant desorption kinetic calibration (DDKC),[28] standard-free kinetic calibration (SFKC),[36] one-calibrant kinetic calibration (OCKC),[37] and the diffusion-based interface (DBI) model.[38] In DDKC, the desorption of very high concentrations of target analyte serves to calibrate the extraction of the analyte, thereby eliminating the use of isotopically labeled standards. The method has been improved by the use of a segmented fiber, where one coating segment serves as extractor and another as the calibrator, eliminating the need to predetermine the distribution coefficient but still requiring preloading of standard(s).[30] In contrast, all extracted analytes can be calibrated with two independent samplings using the SFKC approach, which eliminates the need to preload any standard to determine the desorption rate constant, but requires prior knowledge of the distribution coefficient. OCKC uses the desorption of a single standard to calibrate all extracted analytes, thereby negating the preloading of multiple isotopically labeled compounds or high concentration standards and simplifying the standard loading. Nevertheless, the OCKC technique requires prior knowledge of the diffusion coefficients of both target analytes and the calibrant in the sample matrix. Consequently, the OCKC approach, while feasible for air or water sampling, is not practical for *in vivo* analysis. Finally, the DBI model does not need a calibrant but requires the diffusion coefficients of the compounds in sample matrix and the thickness of the boundary layer to be known,[38] which limits the application of the method.

To simplify SPME *in vivo* analysis, a sampling-rate (SR) based SPME technique coupled with a LC-MS/MS system for quantitative *in vivo* analysis of organic compounds in fish muscle was investigated. In this study, both SPME-KC and LE were utilized in the same tissues to evaluate the efficacy of the proposed nonlethal SPME-SR sampling method.

EXPERIMENTAL SECTION

Animals. All animal experimental procedures were approved by the local Animal Care Committee at the University of Waterloo (AUP #'s 04−24, 07−16, and 08−08). Rainbow trout (*Oncorhynchus mykiss*) were purchased from Silvercreek Aquaculture (Erin, ON, Canada), while wild greenside darter (*Etheostoma blennioides*) were collected using a Smith-Root 12A-POW backpack electroshocking unit (Smith-Root Canada, Merritt, BC) from locations of interest within the Grand River watershed (adjacent Kitchener and Guelph, ON, Canada).

The rainbow trout (n = 12, length 25.1 ± 1.5 cm; weight 159 ± 29 g) and greenside darter (n = 36, length 6.5 ± 0.6 cm; weight 2.7 ± 0.5 g) utilized in the lab exposures were evenly divided into several aerated 34 L aquaria. One group of each species was maintained as a control in the same water used as diluent for the test solution (3 μg/L of atrazine, carbamazepine, and fluoxetine), which was partially renewed daily (50% volume replacement). Water quality (pH, dissolved oxygen, ammonia, and temperature) was monitored daily and maintained at conditions considered optimal for each species. All fish were exposed to their respective treatments for 7 d prior to SPME *in vivo* sampling.

Chemicals and Supplies. Methanol (HPLC grade) was purchased from BDH (Toronto, ON, Canada). Atrazine, carbamazepine, fluoxetine, d_5-atrazine, d_{10}-carbamazepine, d_5-fluoxetine, and lorazepam were purchased from Sigma-Aldrich (Oakville, ON, Canada). Pure water from a Barnstead Nanopure water system (Dubuque, IA) was used for all chemistry experiments, and fish were held and exposed in the laboratory using dechloraminated municipal tap water.

Instrumental Analysis. The Agilent 1200 series HPLC system (Mississauga, ON, Canada) and a Finesse Genesis C18 column (150 × 2.1 mm, 4 μm, Chromatographic Specialties Inc., Brockville, ON, Canada) were used for separation. Mobile phases consisted of (A) 5 mM ammonium acetate in water and (B) methanol. The flow rate was set at 0.8 mL/min, and a 60% B gradient was applied for the first 0.5 min. This was ramped to 80% B in 0.01 min, held for 4.5 min, then increased to 100% B, held for 1.5 min, and finally returned to 60% B in 0.01 min, and held for 1.5 min. This resulted in a total run time of 8.0 min, including reconditioning. A 10 μL injection volume was used for each experimental sample. Eluted analytes were monitored by a triple-quadrupole tandem mass spectrometer (MS/MS) using a QTrap 3200 system with a TurboIonSpray source (Applied Biosystems/MDS Sciex, ON, Canada) in positive ion mode. Each transition was monitored for 200 ms. Compound-specific mass spectrometer settings were determined for each compound separately by infusing a 0.5 μg/mL methanol solution at 3 μL/min

using the integrated syringe pump. Transitions monitored were as follows: atrazine, m/z 216.2/174.3; carbamazepine, m/z 237.1/193.3; fluoxetine, m/z 310.3/44.3; d_5-atrazine, m/z 221.1/179.3; d_{10}-carbamazepine, m/z 247.2/204.4; d_5-fluoxetine, m/z 315.2/44.2; and lorazepam, m/z 321.1/275.1. Mass spectrometer response sensitivity and linearity were monitored before and after each set of experimental samples through the injection of 10 μL of a series of standards (0.5−500 ng/mL) prepared in methanol containing the internal standard, lorazepam (5 μg/mL). Analyst version 1.4.2 software (Applied Biosystems) was used to control all components of the system and for data collection and analysis. External calibration curves yielded good precision (RSD < 5%) and linearity (R^2 > 0.995).

Fiber Preparation. Custom made 165 μm thick polydimethylsiloxane (PDMS) fibers were used for both laboratory and field sampling (Figure 1). PDMS hollow fiber membrane tubing (i.d. 0.31 mm, o.d. 0.64 mm, Helixmark, Carpinteria, CA) was cut into 1.0 cm segments and placed over a 4 cm long stainless steel wire (483 μm diameter, medical grade, Small Parts Inc., Miami Lakes, FL). Before PDMS coatings were mounted, the wires were sonicated in deionized water and acetone to ensure their cleanliness. The prepared 165 μm PDMS fibers were sonicated in methanol for 15 min and then in Nanopure water for an additional 15 min prior to use.

SPME *in Vivo* Fish Sampling. The procedures associated with *in vivo* SPME fish sampling are comprised of five steps: (A) standard loading, (B) fiber storage, (C) *in vivo* sampling, (D) fiber desorption, and (E) instrumental analysis (Figure 2).

In step A, the conditioned custom-made fibers (see Fiber Preparation section, Materials and Methods) were preloaded with deuterated analogues of each target analyte to serve as a calibrant. Each fiber was immersed in a 2 mL amber vial containing 100 ng/mL aqueous solution and supported by the vial cap septum during vortexing at 1200 rpm for 60 min. The prepared fibers were then removed (with the initial cap and septum) and subsequently suspended in a second empty amber vial until use (step B). In most cases, the fibers were prepared one day before sampling and stored at 4 °C until use.

The *in vivo* SPME sampling (step C) began by anaesthetizing fish in 0.1% ethyl 3-aminobenzoate methanesulfonate (MS-222) until vertical equilibrium was lost. Once anaesthetized, a 20 gauge needle was used to penetrate the skin and dorsal-epaxial muscle to a depth of approximately 12 mm. This depth ensured that the entire SPME coating was embedded in the muscle. When the guide needle was removed, the SPME probe containing preloaded calibrant was immediately inserted into the muscle. At the end of the sampling interval (20 min was used based on ref 35), the fish was anaesthetized as before, and the fiber was quickly removed from the fish. Double distilled water was used to quickly rinse any blood from the surface of the fiber coating and stainless steel wire. Residual water on the fiber was gently dried with a Kimwipe tissue. Finally, the fiber was placed in a 200 μL polypropylene insert (within a 2 mL vial) containing 50 μL of methanol for desorption of the extracted analytes and residual calibrants from the coating (step D). For each sampling occasion, three blank fibers (preloaded with deuterated target analyte) were directly desorbed into 50 μL of methanol to determine the amount of preloaded calibrant.

Following the procedure described above, the vials were put on a shaker and agitated at 1200 rpm for 60 min. Then, the fiber was taken out, and 50 μL of lorazepam (internal standard in methanol; 100 ng/mL) was added to the desorption solution within the insert. After vortexing, a 10 μL aliquot of the solution was automatically injected into the LC-MS/MS for analysis (step E).

Three organic contaminants routinely detected in surface waters adjacent urban and agricultural areas, atrazine, carbamazepine, and fluoxetine, were selected as the target analytes. Two fish species, large-bodied rainbow trout and small-bodied greenside darter, were utilized for testing. Approximate locations of SPME fiber insertion into fish dorsal-epaxial muscle are illustrated for both species (Figure S1).

Although SPME sampling is nonlethal, to compare with traditional LE methods, fish were euthanized at the conclusion of the exposure by an overdose with MS-222, followed by severance of the spinal cord in accordance with approved procedures. Samples of dorsal-epaxial muscle (approximately 0.5 g) were removed from each fish and stored in a 2 mL cryovial (Wheaton Science, Millville, NJ) at −80 °C prior to liquid extraction.

Determination of Distribution Coefficients. SPME kinetic calibration requires prior knowledge of the distribution coefficients between the fiber coating (extraction phase) and the sample matrix (i.e., muscle) for each analyte. Equilibrium extraction is frequently used to determine the distribution coefficient K, which can be calculated from C_f/C_s, where C_f and C_s are concentrations of the analyte in the fiber coating and sample matrix, respectively. SPME equilibrium extraction requires an *a priori* knowledge of the equilibrium time for a given analyte in a given matrix. For semisolid sample matrices such as fish muscle, the equilibrium time is very long and would need to be independently verified for each target analyte. Consequently, using equilibrium

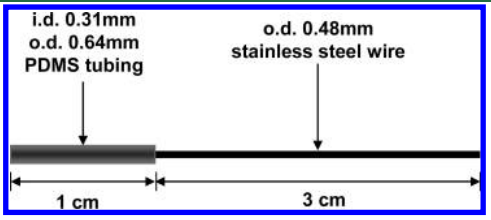

Figure 1. Schematic of the custom-made PDMS fiber.

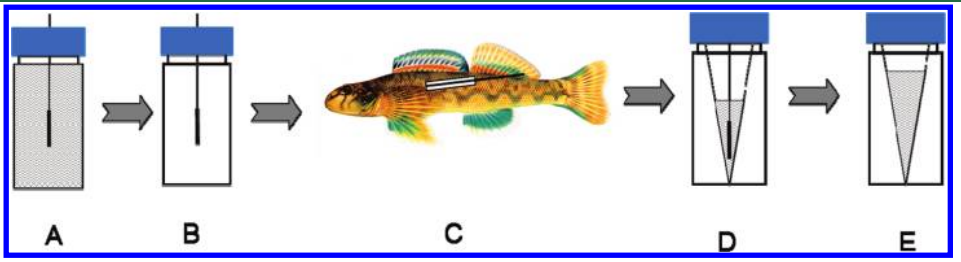

Figure 2. Outline of SPME *in vivo* fish sampling and analysis procedures. A, standard loading; B, fiber storage; C, *in vivo* sampling; D, fiber desorption; E, instrumental analysis. (Steps A-E are required for the SPME-KC approach, whereas only C-E are needed for the proposed SPME-SR method.)

Table 1. Total Analyte Concentrations (C_s) Determined in Rainbow Trout (*Oncorhynchus mykiss*) and Greenside Darter (*Etheostoma blennioides*) Dorsal Epaxial Muscle by LE and the Calculated Distribution Coefficients, *K*, Determined by SPME

		rainbow trout (n = 3)		greenside darter (n = 9)	
analyte/fiber immersion time		C_s (ng/g)	*K*	C_s (ng/g)	*K*
atrazine	1 day	1.3 ± 0.1	602	1.6 ± 0.2	255
	2 days	1.4 ± 0.2	669	1.8 ± 0.2	263
carbamazepine	1 day	2.3 ± 0.2	9	5.2 ± 0.6	16
	2 days	2.0 ± 0.2	11	5.0 ± 0.8	14
fluoxetine	1 day	141 ± 16	31	54 ± 4	14
	2 days	164 ± 16	35	59 ± 5	15

extraction to determine *K* values is time-consuming and often impractical. In the present study, a pre-equilibrium method was used for the determination of the distribution coefficient *K*. Equation 1 can be rewritten as

$$K = \frac{n}{C_s V_f (1 - Q/q_0)} \quad (2)$$

indicating the *K* value can be determined under pre-equilibrium conditions by simultaneous extraction of the target analyte and desorption of a calibrant.

Liquid Extraction. Liquid extraction (LE) was utilized to determine the total concentrations of target analytes in fish muscle. The standard addition method was applied to drug-free muscle from control fish to obtain analyte recoveries. Pieces of dorsal-epaxial muscle (approximately 0.2 g) were thawed to room temperature and transferred into a microcentrifuge tube (2 mL) prior to homogenization with a dimensionally matched pestle for 20 s. Immediately following homogenization, 1 mL of methanol containing spiked analytes (100 ng/mL) was added to the homogenized drug-free muscle. The same volume of methanol was added to homogenized muscle from fish exposed to target analytes in order to measure total concentrations in muscle. The mixtures in the vials were shaken vigorously at 2400 rpm using a digital vortex for 1 h. After agitation, 450 μL from each vial was transferred to a microcentrifuge tube with a 30 KDa exclusion membrane (Ultrafree-MC, Millipore Corp., U.S.) for centrifugation at 14 000 rpm for 1 h. Of this filtrate, 50 μL was transferred into a 200 μL insert. The addition of lorazepam, and sample injection into the LC-MS/MS were performed as described above.

RESULTS AND DISCUSSION

Distribution Coefficients. A recently published paper indicated homogenization of tissues can decrease their fugacity capacity, suggesting that *in vivo* sampling would require different partition coefficient estimates than sampling homogenized tissues.[39] Accordingly, in this study *in vivo* sampling was used to determine distribution coefficients, with analyte extraction and desorption of the isotopic standards performed simultaneously in the same fish. To obtain reliable *K* values, the SPME fibers were kept in the fish muscle for either one or two days, during which all the fish were healthy and moved normally about the aquaria. Total analyte concentrations in fish muscle (C_s) were determined using the traditional LE method.

K values of the three compounds determined after one day of desorption/extraction were quite similar to those obtained after SPME probes were exposed in the fish muscle for two days (Table 1). Notably, *K* values of the compounds differed between the two species of fish, with some compounds such as fluoxetine markedly higher in trout relative to darters, perhaps reflecting differing lipid content of the two fish species. Regardless, the reproducibility of both C_s and the derived *K* values demonstrates both the potential application and reliability of the pre-equilibrium method for determining target analyte concentrations.

Laboratory Evaluation of SR-SPME *in Vivo* Analysis Method. For passive sampling, the extraction process can be defined according to three paradigms: linear, kinetic, and near equilibrium.[40] Within a linear model, it is assumed that the rate of mass transfer (or sampling rate) remains constant throughout the duration of sampling, and the relationship between the concentration of target analyte in the sample matrix and the extracted amount of analyte can be expressed by

$$C_s = \frac{n}{R_s t} \quad (3)$$

where C_s is the concentration of the analyte in the sample matrix, n is the amount of the extracted analyte, R_s is the sampling rate for the target analyte, and t is the sampling time. In the experiments determining *K* values, increasing amounts of analyte were extracted with time, as evidenced by the 2 d sampling yielding almost twice the extracted amounts of atrazine relative to that extracted after 1 d. During short duration *in vivo* fish sampling (e.g., 20 min), such linear response patterns allow sampling rates of different analytes to be predetermined and used for quantitation purposes when sampling other individual fish of the same species.

Sampling rates of the three analytes in both fish species were determined (using eq 3) after inserting prepared fibers into the dorsal-epaxial muscle of analyte-exposed fish for 20 min. The amount of extracted analyte, n, was quantitated through the injection of standard solutions, with analyte concentrations in fish muscle determined by LE. The determined sampling rates for atrazine, carbamazepine, and fluoxetine were (respectively) 1.37 ± 0.26 mg/min, 0.11 ± 0.01 mg/min, and 0.28 ± 0.04 mg/min in greenside darter and 3.30 ± 0.23 mg/min, 0.17 ± 0.04 mg/min, 0.15 ± 0.03 mg/min in rainbow trout. Based on the kinetic equation proposed by Ai Jiu,[41] both the diffusion coefficient of a compound and its distribution coefficient between the fiber coating and sample matrix will affect the sampling rate. As muscle tissue varies in composition between species, the sampling rates determined for the three compounds differed between the two fish species but were consistent between individuals within a species and could thus be used to calibrate the *in vivo* sampling of other exposed fish. The calculated analyte concentrations in fish muscle derived with SPME-SR predetermined sampling rates are

Table 2. Concentrations (Means ± SE) of the Three Target Analytes in Dorsal-Epaxial Muscle of Rainbow Trout (*Oncorhynchus mykiss*) and Greenside Darter (*Etheostoma blennioides*)[a]

	concentration (ng/g)					
	rainbow trout (n = 3)			greenside darter (n = 9)		
analyte	LE	SPME-KC	SPME-SR	LE	SPME-KC	SPME-SR
atrazine	1.6 ± 0.3	1.1 ± 0.1	1.4 ± 0.3	1.0 ± 0.1	0.7 ± 0.1	1.1 ± 0.1
carbamazepine	2.8 ± 0.5	2.8 ± 0.4	2.5 ± 0.4	4.3 ± 0.8	3.7 ± 0.7	4.1 ± 0.9
fluoxetine	159 ± 13	123 ± 13	171 ± 28	57 ± 13	72 ± 8	69 ± 16

[a] Fish were exposed for 7 d under laboratory conditions with analyte concentrations determined by SPME rapid *in vivo* sampling (quantitated by both kinetic calibration (KC) and sampling rate (SR) approaches) and *in vitro* LE techniques.

very close to those obtained by LE and SPME-KC methods, demonstrating the feasibility of the proposed SPME *in vivo* sampling and analysis method (Table 2).

Field Applications. The SPME method established and validated within the laboratory was subsequently evaluated for its application under field conditions. Two sampling sites in the Grand River Watershed (southern Ontario, Canada), Pioneer Tower (43°23′59.02″N; 80°25′00.41″W) and Eramosa River (43°32′49.02″N; 80°11′49.35″W), were selected for contaminant monitoring in wild greenside darter (Figure S2). This small-bodied fish has a small, well-defined home range and reflects the chemistry of the environment of capture better than larger, more mobile fish. The Pioneer Tower location is immediately downstream of the Doon (Kitchener) municipal wastewater treatment plant, and collected fish were strongly influenced by its plume (12−25% effluent *v/v* based on conductivity measurements performed on site) while the Eramosa River site is influenced by moderate agricultural land use. For each site, 12 fish were collected by backpack electroshocking and sampled immediately near the site of capture using the SPME *in vivo* sampling rate procedure described above. The field collected fish were held in water from the location of capture, being briefly removed only to insert and remove the SPME fiber at the onset and conclusion of the *in vivo* sampling. After fiber removal from the fish, individual fibers were replaced in the 2 mL vials and positioned on wet ice in a 54 vial plate for transportation to the laboratory, where final desorption and analysis were performed. Although the *in vivo* SPME technique does not require lethal sampling, for this experiment, the fish were euthanized, and selected tissues were harvested for comparative studies with LE.

Figure 3A illustrates the sampling rates determined for the three analytes under laboratory conditions and at the two field sampling sites. Marked similarities between sampling rates determined in greenside darter under laboratory and field conditions suggest a linear R_s that, while analyte and fish tissue-specific, is largely independent of sampling conditions (such as temperature, fish activity, and water quality), yet similar between individuals within a species. Further, calculated analyte concentrations for wild fish quantitated using sampling rates determined in laboratory fish do not differ from those obtained by LE, demonstrating the efficacy of the approach (Figure 3B). Consequently, field SPME sampling of fish (of the same fish species in which the sampling rates were derived) can be dramatically simplified since there is no requirement to preload calibrant onto the fibers. As matrix differences between individuals of the same fish species is slight, this approach may be a simple, direct technique for rapidly and nonlethally assessing large numbers of fish under field conditions.

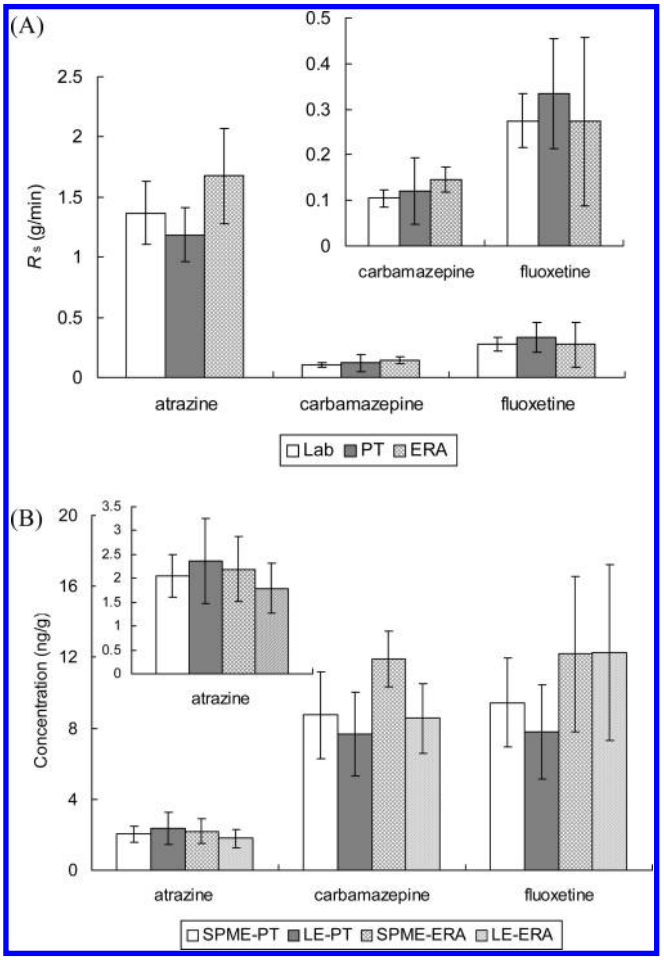

Figure 3. The sampling rates of three analytes determined within the laboratory and at two field sampling sites, PT (urban-influenced) and ERA (agriculturally influenced), (A); absence of statistically significant differences in comparisons of analyte concentrations calculated by laboratory predetermined sampling rates and those determined by LE (one-way analysis of variance, $p < 0.05$) (B).

In vivo SPME offers a less-invasive sampling method over traditional approaches that require invasive biopsy or lethal sampling followed by solvent extraction. This study demonstrated that the SPME-SR method can be used for quantitative *in vivo* analysis of organic contaminants in semisolid animal tissue, thereby significantly simplifying *in vivo* SPME analysis procedures by eliminating both the requirement of predetermining K values and the preloading of standards onto each fiber. A further advantage

of SPME-SR is the avoidance of tissue contamination with dissociated calibrant from preloaded fibers, which allows individuals to be repeatedly sampled. The increased simplification and convenience offered by the SPME-SR can broaden the utility of SPME for use in studies of biologically active compounds and organic contaminants in living systems, with potential to replace conventional methods requiring sacrifice of the animals under study.

The experimental results illustrated sampling rates for selected analytes are similar within a fish species, independent of the sampling conditions. Consequently, the sampling rate of the SPME fiber can be predetermined under laboratory conditions and directly used to quantitate field samples. Further experimentation is needed to determine if sampling rates determined in one area can be applied to other genetically distinct fish stocks in geographically disparate regions or countries, and if SPME-SR can be similarly applied to other tissues in warm and cold-blooded species of various sizes and life-stages. Further, the analytes used in this study were all organic, and only the total concentrations of these compounds were investigated. As some reports indicate a portion of the absorbed or bound analyte fraction can release during *in vivo* SPME sampling,[42−44] further investigations into the feasibility of the sampling rate method for determining free concentrations of a greater variety of compounds in biological samples is required.

■ ASSOCIATED CONTENT

Supporting Information. Figures S1−S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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