Depth-Profiling of Environmental Pharmaceuticals in Biological Tissue by Solid-Phase Microextraction

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Depth-Profiling of Environmental Pharmaceuticals in Biological Tissue by Solid-Phase Microextraction

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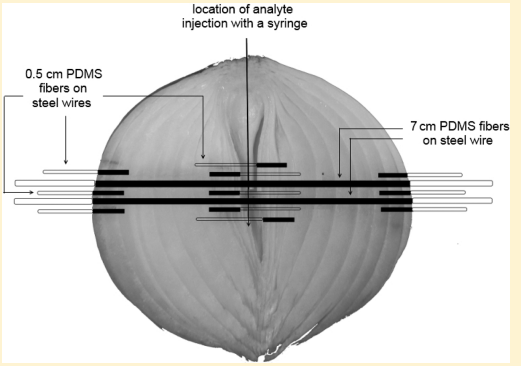
Supporting Information

ABSTRACT: The parallel in vivo measurement of chemicals at various locations in living tissues is an important approach furthering our understanding of biological uptake, transportation, and transformation dynamics. However, from a technical perspective, such measurements are difficult to perform with traditional in vivo sampling techniques, especially in freely moving organisms such as fish. These technical challenges can be well addressed by the proposed depth-profiling solid-phase microextraction (DP-SPME) technique, which utilizes a single soft, flexible fiber with high spatial resolution. The analytical accuracy and depth-profiling capability of DP-SPME was established in vitro within a multilayer gel system and an onion artificially contaminated with pharmaceuticals. In vivo efficacy was demonstrated by monitoring pharmaceutical distribution and accumulation in fish muscle tissue. The DP-SPME method was validated against pre-equilibrium SPME (using multiple small fibers), equilibrium SPME, and liquid extraction methods; results indicated DP-SPME significantly improved precision and data quality due to decreased intersample variation. No significant adverse effects or increases in mortality were observed in comparisons of fish sampled by DP-SPME relative to comparable fish not sampled by this method. Consequently, the simplicity, effectiveness, and improved precision of the technique suggest the potential for widespread application of DP-SPME in the sampling of heterogeneous biotic and abiotic systems.



Monitoring drug distributions in animal tissues is important for pharmaceutical discovery and development, as well as from a toxicological perspective where an understanding of the uptake, pharmacokinetics, and biotransformation of drugs (in animal or human experimental subjects) is often required. For toxicologists, the measurement of waterborne organic contaminants (including pharmaceuticals and personal care products, PPCPs) in fish muscle is critical to understanding the toxicokinetics and potential impact on humans via the food chain.[1−7] Using traditional ex vivo or in vitro sampling procedures, a large number of animals must be sacrificed (due to significant interanimal variation) to obtain sufficient statistical power.[8] Mounting pressure (animal ethics, fiscal constraints) to reduce the number of experimental animals used, while simultaneously improving data quality, dictate that an in vivo sampling technique with good precision, moderate invasiveness, and insignificant lethality is desirable.

For in vivo fish sampling, solid-phase microextraction (SPME) is a promising approach due to the simplicity and analytical sensitivity of the method relative to traditional in vivo sampling techniques such as microdialysis (MD) and ultrafiltration (UF). Sophisticated surgery is often required for MD or UF sampling, and several days are routinely required for wound recovery prior to sample collection. Both MD and UF require syringe pumps and power supplies, rendering these approaches cumbersome for field applications,[9−13] while MD cannot be easily used for depth-profiling analysis due to the unwieldy configuration of the probe. The in vivo SPME technique has been successfully employed to monitor the toxico-kinetics of pharmaceuticals and pesticides in rainbow trout (*Oncorhynchus mykiss*) dorsal-epaxial muscle.[14−16] Recently, the bioconcentration of several widely detectable PPCPs partitioning to muscle tissue was simultaneously monitored relative to that partitioning to the adipose fin using small segmented fiber coatings which improved spatial resolution.[14,16] While the segmented fiber design introduced the potential for sampling heterogeneous sample systems using SPME, this technique precluded the use of single fiber coating probes (such as those available commercially) for spatially resolving analytes within heterogeneous sample systems. During our fish experiments, an important concern arose

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regarding how representative localized SPME-measured fish muscle contaminant burdens were of the larger tissue. However, no techniques were available to simultaneously and nonlethally determine the distribution of contaminants across large tissues, but it is recognized that such an approach would be of significant advantage for pharmacokinetic studies in fish.[16] We recognized the progress in novel mass spectrometry techniques such as laser ablation electrospray ionization (LAESI) which allowed for depth-profiling of small portions of living tissue,[17,18] but the feasibility of this approach for use in aquatic organisms such as fish has not been demonstrated.

To address these issues, we developed a simple depth-profiling SPME technique (DP-SPME) based on SPME's spatial resolution theory for in vivo measurement of analyte distributions across fish dorsal epaxial muscle using a single SPME fiber with results validated through comparisons with multiple short SPME fibers in individual fish. The resulting data not only demonstrated the efficacy of the depth-profiling approach for sampling semisolid tissues, but also revealed significant improvements in analytical precision when either DP-SPME or multiple fibers were employed in individual fish (relative to multiple fish sampled with a single fiber each).

MATERIALS AND METHODS

Chemicals and Materials. All chemicals purchased were used without further purification. Atrazine was ordered from Chem Service Inc. (West Chester, PA). Deuterated standards were purchased from CDN Isotopes Inc. (Pointe-Claire, Québec, Canada). HPLC grade acetic acid (glacial) and methanol for the HPLC mobile phase were purchased from Fisher Scientific (Unionville, ON, Canada). Agar was ordered from Sigma-Aldrich (St. Louis, MO). Helix medical silicone tubing (ID = 0.31 mm; OD = 0.64 mm, Carpinteria, CA) and stainless steel wire (OD: 0.41 mm; Small Parts Inc., Miami Lakes, FL) were assembled into homemade biocompatible PDMS probes for in vivo SPME application as described previously.[14−16]

DP-SPME in the Model Sample System. To evaluate the depth-profiling capability of the probe, a multilayered agar gel with varying drug concentrations in each layer was utilized to simulate a heterogeneous sample system.[16] Five layers of 1% agar containing differing concentrations of fluoxetine (FLX) and ibuprofen (IBU) in each layer were prepared in five PYREX glass Petri dishes (100 mm diameter) as described in detail elsewhere.[16] The pH of the gel was fixed at 7.4 by adjusting the phosphate buffered saline during gel preparation. The concentrations of both FLX and IBU varied in each 0.5 cm thick gel layer cast into individual Petri dishes with concentrations of 0, 10, 40, 100, and 500 ng/mL. Two 3 cm ×3 cm square gel portions were cut from each Petri dish with a scalpel, with one portion added to each of two different 5-layer gel model systems (Figure 1A). The FLX and IBU concentration gradients in each of the two 5-layer gel model systems differed: in the first gel model system, the gradient was sequential and incremental top to bottom (0, 10, 40, 100, 500 ng/mL), while the second gel model had concentration gradients arranged nonsequentially (500, 10, 40, 0, 100 ng/mL, top to bottom) to capture potential signal crosstalk resulting from interlayer diffusion. Three PDMS fibers (2.5 cm in length) were deployed vertically into each of the gel model systems to simultaneously sample each layer of differing concentration for a 5 min duration. After sampling, the fibers were cut into 0.5 cm segments corresponding to the thickness

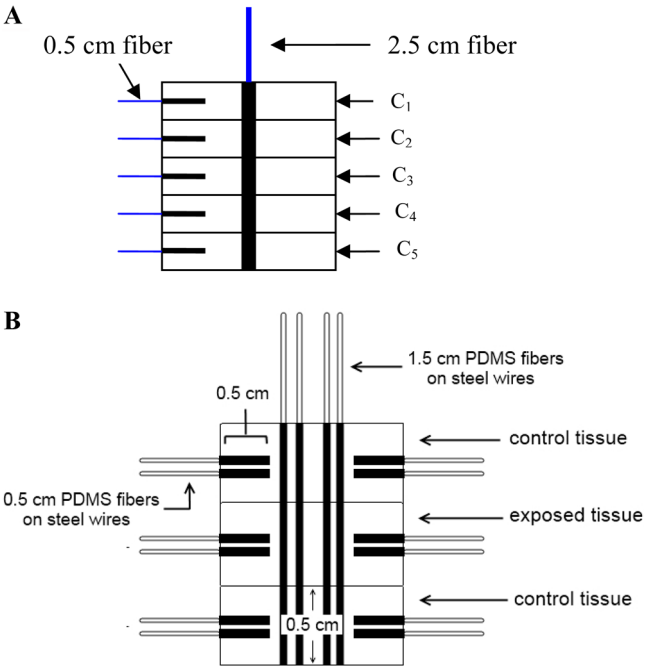

Figure 1. (A) The single long SPME fiber (2.5 cm in length, n = 3) was vertically introduced into the multilayer gel to determine the local concentrations of drugs (IBU and FLX) in each gel layer, with results confirmed by deployment of laterally inserted short fibers (0.5 cm, n = 3) in each layer. (B) The long (depth-profiling) fibers (1.5 cm, n = 2) were vertically deployed to measure the drug concentrations in, and diffusion across, the three layer of fish muscle over time (2 and 48 h) at 4 °C. The laterally inserted short (0.5 cm, n = 2) fibers in each layer were for validation. The middle layer of muscle was excised from drug (CBZ and FLX) contaminated fish, while the top and bottom layers did not initially contain CBZ or FLX as they were excised from fish not exposed to these drugs.

of each gel layer prior to their individual desorption. The amount of analyte extracted by each SPME fiber segment should reflect the analyte concentration of the gel layer in which the fiber segment was deployed. Validation of the DP-SPME analysis was performed by comparing the amount of analyte extracted by the vertically inserted 2.5 cm fiber with that extracted by three laterally inserted 0.5 cm fibers (introduced from the side wall of the gel) (Figure 1A). The calibration curve was developed by performing an identical 5 min extraction using 0.5 cm fibers in the remnants of the single gel layers not excised from the Petri dishes in which they were cast. No cross-talk occurred during the calibration experiment as every gel layer with a unique analyte concentration was kept isolated within their original Petri dish.

To further confirm the depth-profiling capability of the DP-SPME probe in rainbow trout muscle, an experiment similar to that of the multilayered agar gel was conducted, using three layers of dorsal-epaxial muscle (Figure 1B). The top and bottom muscle layers were from clean (uncontaminated) fish, while the middle layer was taken from a fish exposed to carbamazepine (CBZ) and FLX. The contaminated fish muscle was obtained by exposing fish for 24 h in water containing a mixture of 300 μg/L CBZ and FLX spiked in 50 μL of pure ethanol. The SPME probes were deployed as shown in Figure 1b, where 0.5 cm probes were introduced laterally in each single layer (and exposed to either clean or contaminated tissue alone) and used to validate the 1.5-cm probe vertically inserted

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across the contamination gradient to assess depth profiling potential across the 3 layers of the drug distribution. The probes were withdrawn and analyzed following deployment in the fish tissue for either 2 or 48 h at 4 °C.

To determine intrafiber diffusion of the compounds, 2 cm SPME fibers (n = 2) had the distal 0.5 cm exposed to a standard solution containing 100 ng/mL of CBZ, IBU, and FLX, for 10 min. After this brief exposure, the probes were briefly wiped to remove any attached solution before storage at 4 °C for 48 h. After 48 h, the probe was cut into 2 mm segments and desorbed in 100 μL of desorption solution for instrumental analysis. The desorption of SPME fibers and instrumental analysis with LC-MS/MS were performed in the same way as described previously and detailed in Supporting Information.

Depth-Profiling of the Pharmaceutical Distribution in a Contaminated Onion. To evaluate the efficacy of DP-SPME within relevant biological matrices, an onion bulb (~7 cm in diameter) was chosen as a representative heterogeneous vegetative tissue with discrete layered structure (Figure 2). The

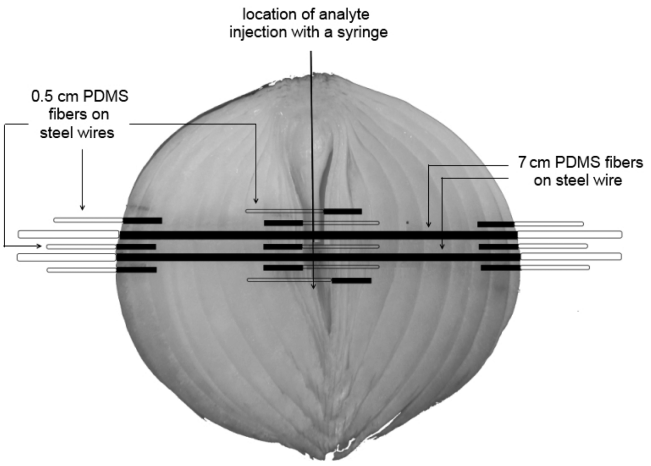

Figure 2. Lateral cross section of the onion bulb injected with 0.5 mL of 2 μg/mL CBZ and FLX (in 10% methanol solution) and the subsequent placement of the 7 cm PDMS fibers for depth-profiling and the 0.5 cm fibres for validating the drug distribution within the discrete layers of the onion.

analyte gradient was introduced by injection of 0.5 mL of 2 μg/mL CBZ and FLX in 10% methanol solution into the center of the onion bulb from the stem side 1 h prior to sampling. Afterward, half of the onion bulb was removed so that the layered tissue was visible for deploying the SPME probes laterally (perpendicular to the stem) into the exact position for analyte extraction. Two probe lengths (7 cm (n = 2) probes for depth-profiling across concentrations; 0.5 cm (n = 12) probes for validation of the local concentrations within individuals layers transected by the 7 cm probes) were deployed in the onion, and removed after 20 min and wiped with Kimwipe tissue to remove any adhering material. The 7 cm depth-profiling fibers were cut into 0.5 cm segments prior to desorption in solvent (50% methanol in pure water) for instrumental analysis (Supporting Information), as were the uncut 0.5 cm validating probes.

To further validate the SPME results, organic solvent extraction of the onion tissue (45.8−67.1 mg/piece collected from the layers corresponding to the 0.5 cm probe locations, taken from the onion bulb half that was not treated by SPME probes) with 0.5 mL methanol was performed for 4 h. Each piece of small onion tissue was further minced into very small pieces by a blade prior to adding methanol (containing 10 ng/mL of the deuterated standards CBZ-d_{10} and FLX-d_5) for extraction. The mixture was briefly spun down (6000 rpm for 5 min) and 20 μL of supernatant was injected into the LC-MS/MS for quantification. The matrix effect and variations in injected volume were compensated for by the deuterated standards. The analyte concentrations determined by SPME analysis were calculated using the partitioning coefficient (K_{fs}) obtained in PBS-based standard solutions.[19]

In Vivo Study of the Spatial Distribution of Pharmaceuticals in Fish Muscle by DP-SPME. All experimental procedures involving fish were conducted in the Hagen Aqualab facility at the University of Guelph in accordance with protocols approved by institutional Animal Care Committees (AUP #s 07−16; 08−08, University of Waterloo; 07R123, University of Guelph). Immature rainbow trout (*Oncorhynchus mykiss*, n = 152, 13.4 ± 1.7 cm, 22.9 ± 3.1 g) were exposed to fresh City of Waterloo municipal wastewater effluent (MWWE) for intervals up to 12 d with 2 d static renewals. Twenty-seven glass aquaria (34 L) containing 6 fish/tank were randomly assigned treatments of 20, 50, and 90% MWWE (v/v) diluted with well water, or well water only controls (all treatments in triplicate aquaria). With the exception of un-ionized ammonia (0.41 mg/L 20%, 1.0 mg/L 50%, and 1.6 mg/L in 90% MWWE), water quality was maintained at conditions considered optimal for this species (11.7 ± 0.05 °C, 10.7 ± 0.07 mg/L dissolved oxygen, 8.7 ± 0.02 pH, n = 220). To demonstrate the utility of the developed approach for in vivo measurements in biological systems of significant complexity, a depth-profiling analysis of free concentrations of bioconcentrated pharmaceuticals in rainbow trout was conducted. *In vivo* pre-equilibrium SPME (PE-SPME) measurements were performed on exposure days 8 and 12 for durations of 20 min, 2 h, or 2 d; the kinetic calibration sampling procedure was as described previously.[14−16] Briefly, fish were anaesthetized (0.1% ethyl 3-aminobenzoate methanesulfonate) until loss of vertical equilibrium, whereupon each fish received two 0.7 cm and a single 5 cm PDMS fiber(s) preloaded with deuterated standards (the loading was performed overnight in 500 mL of PBS buffer containing 100 ng/mL of deuterated standard mixture). All three fibers were deployed symmetrically (as shown in Figure 3) into the dorsal-epaxial muscle above the lateral line, with the two 0.7 cm fibers corresponding to the beginning and end of the 5 cm fiber. After fiber insertion, the stainless steel internal fiber supports were withdrawn using forceps, leaving the soft fiber coating in the muscle for 2 h and 2 d sampling intervals. After the fish were euthanized, the fiber coatings were excised from the muscle and wiped with a wet Kimwipe tissue. The 5 cm fibers were cut into seven 0.7 cm segments and desorbed individually in 100 μL of desorption solution concurrent with the individual desorption of the two 0.7 cm fibers from the symmetric side of the fish body. Since no statistical differences in measured concentrations were observed across the fiber segments, 50 μL of desorption solution from each 0.7 cm segment from the same fish were transferred into a single 1.5 mL amber GC vial. This pooled solution was evaporated under nitrogen gas prior to reconstitution into 50 μL of methanol solution (80% MeOH in pure water) to improve method sensitivity. Total analyte (free and bound) concentrations in the fish tissue were measured by methanolic extraction as described in detail previously.[15,20,21]

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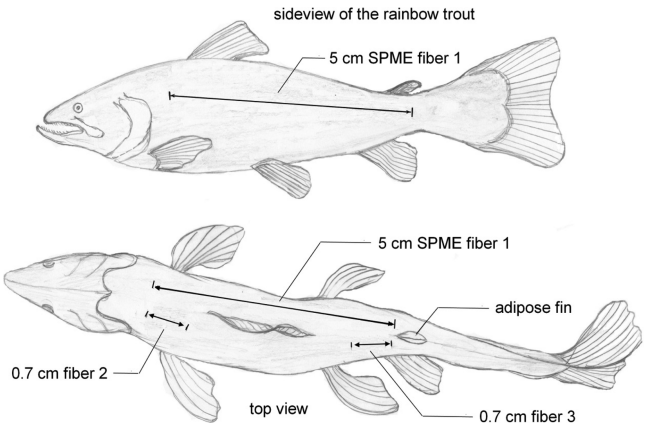

Figure 3. Lateral and dorsal views of the placement of the two 0.7 cm and single 5 cm PDMS fibers for in vivo sampling of rainbow trout (*Oncorhynchus mykiss*) dorsal-epaxial tissue.

To correlate analyte concentrations in fish with those of their exposure milieu, three water samples (500 mL each) from each exposure aquaria were collected before renewing the exposure effluent and extracted by SPE as detailed previously.[14,16] The method quantification limits (MQLs) were defined as the minimum sample concentrations which could be quantified using the analytical method (including instrument analysis) with a signal/noise ratio over ten. Potential ionization suppression or enhancement of SPME extracts was evaluated by comparing the instrumental response to the same set of calibration standards (ibuprofen-d_3 and fluoxetine-d_5) with and without SPME extracts.[22]

RESULTS AND DISCUSSION

Accuracy and Precision of DP-SPME in Model Sample Systems. The depth-profiling capability of a SPME fiber is based on its spatial resolution, which in turn is determined by fiber dimension, analytical sensitivity, and the mass transfer of target analyte in the sample matrix and within the fiber coating.[16] In this work, the accuracy of DP-SPME was evaluated in two respects: the relative recovery of the measurement, and analyte diffusion within the SPME fiber and within the fish muscle.

DP-SPME accuracy in the multilayer gel was excellent, as relative recoveries (the ratio of the measured concentration over the spiked concentration) for all FLX and IBU concentrations calculated against calibration curves from the original single gel layers were 93−106%. The experimental results indicated no significant interlayer diffusion occurred during sampling (Table 1), which is consistent with the results obtained with the multilayer model using fish muscle, while the precision of the triplicate measurements was demonstrated by the low relative standard deviations (<5%).

To assess the influence of intrafiber diffusion on the depth profiling capability of the DP-SPME fiber, we first monitored diffusion of CBZ, IBU, and FLX along the silicone tubing over a 2 d interval. While our results indicated no detectable intrafiber diffusion over 2 d, over the same interval, we did observe interlayer diffusion in fish muscle where a small fraction of the drugs (about 6% CBZ and 5% FLX) diffused from the contaminated layer to each clean layer (Table S1). Consequently, we conclude that a single fiber can spatially resolve concentration differences within adjacent semisolid samples (such as fish muscle) within a reasonable time frame (i.e., $\sim \leq$ 2 d sampling yielding $\geq$90% relative recovery with high spatial resolution).

In Vitro Application: Depth-profiling of Drugs in a Layered Plant Tissue. To evaluate the efficacy of DP-SPME in a layered plant tissue containing analyte gradients, we monitored the local concentrations of CBZ and FLX with 7 cm depth-profiling fibers validated within each tissue layer in the onion bulb by 0.5 cm probes (Figure 2). The detected concentrations within the onion were highest at the innermost layer adjacent the analyte injection site (219 ± 49 ng/g for CBZ and 533 ± 87 ng/g for FLX), and lower within the distal layers (6 ± 5 ng/g and 18 ± 13 ng/mL), as anticipated. The measured drug concentration distribution in the onion was consistent between extractions using long (depth-profiling) and short (validating) SPME fibers and with methanolic extraction (Figure 4 for CBZ; Figure S1 for FLX). The significantly different analyte concentrations among different locations along the fiber suggest both the appropriateness of onion bulbs as a heterogeneous model, and the capability of DP-SPME to profile different concentrations in adjacent vegetative tissues.

In Vivo Application: Spatial Distribution of Pharmaceuticals in Fish Muscle. To demonstrate the feasibility of the DP-SPME approach under a variety of applications, an in vivo assessment of pharmaceutical distributions in fish muscle was conducted. The ability to routinely perform such experiments would be of interest to toxicologists and chemists, since environmental pharmaceuticals are well publicized emerging contaminants whose near ubiquitous detection in surface waters adjacent urban areas pose a potential threat to human and ecosystem health.[1−3,23−25]

Table 1. Analyte Concentrations for Ibuprofen (IBU) and Fluoxetine (FLX) as Determined by Depth-Profiling SPME in Multi-Layer Model Gel Systems (n = 3)[a]

	multilayer gel 1		multilayer gel 2[b]	
spiked conc. (ng/mL)	IBU	FLX	IBU	FLX
0	0 (0)	0 (0)	0	0
10	9.8 ± 0.3 (9.5 ± 0.3)	9.6 ± 0.1 (9.7 ± 0.4)	9.9 ± 0.5	10.2 ± 0.4
40	39.1 ± 1.5 (38.7 ± 2.3)	41.0 ± 1.2 (40.2 ± 0.7)	38.7 ± 0.8	38.3 ± 0.8
100	99.2 ± 3.7 (105.1 ± 4.1)	97.8 ± 3.4 (99.5 ± 2.1)	98.1 ± 4.3	102.1 ± 2.9
500	487.9 ± 13.3 (495.0 ± 22.6)	511.5 ± 13.7 (497.8 ± 14.5)	496.0 ± 25.5	508.3 ± 14.5

[a]The validation data obtained by laterally-inserting short fibers in each gel layer are provided in parentheses for comparison. The gradient for both IBU and FLX in Multi-layer Gel 1 was 0, 10, 40, 100, and 500 ng/mL, while that of Multi-layer Gel 2 was 500, 10, 40, 0, and 100 ng/mL, top to bottom (Figure 1). [b]Note: Multilayer Gel 2 was not ascending in concentration (top to bottom) as was Multilayer Gel 1, but rather presented in tabular format in this order to facilitate comparisons.

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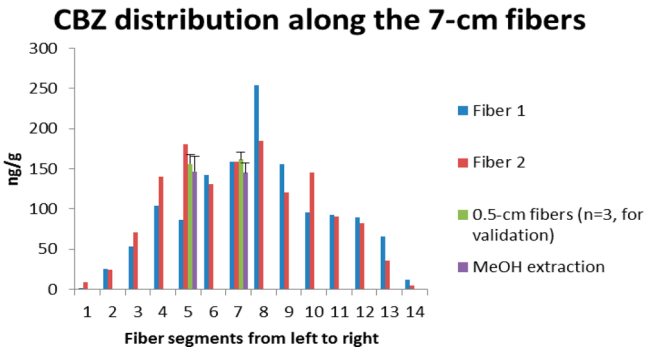

Figure 4. Depth-profiling of the FLX distribution as assessed by depth profiling along the 7 cm SPME fiber spanning the onion bulb. The 7 cm depth-profiling PDMS fibers were cut into seven 0.5 cm segments after the extraction was completed, and sequentially numbered from 1 to 14 (from left to right) corresponding to the position where the fiber was deployed in the onion. The 0.5 cm fibers were deployed laterally in locations corresponding to the depth-profiling (7 cm fiber) segments 1, 5, 7, and 14.

In this study, the sampler configuration and sampling strategy, while novel, are simple and easily replicated. First, a 5 cm probe (fiber) was introduced longitudinally into the fish dorsal-epaxial muscle (from adipose fin to just posterior of the operculum) to achieve in vivo depth-profiling of the pharmaceutical distribution in fish muscle over a relatively long distance with a single fiber. Second, we introduced multiple 0.7 cm SPME fibers on the opposite (symmetric) side of each fish, thus improving the precision of the measurement. Finally, three sampling intervals (20 min, 2 h, and 2 d) were employed, reinforcing the time-effectiveness of PE-SPME over the equilibrium SPME (E-SPME) approach.

Of the 9 environmentally relevant pharmaceuticals we attempted to identify in fish and their exposure water, only ibuprofen and fluoxetine were detected in both matrices (Table 2; S2), indicating some capacity for bioconcentration of these drugs. The bioconcentration factors for IBU and FLX were about 16 and 18, respectively, over the 12 d exposure. It must be noted that the pharmaceutical concentrations in the wastewater effluent, and effluent pH values fluctuated in the fresh effluent (collected every second day) over the course of the exposure, which resulted in lower bioconcentration factors than were derived during previous research performed under fixed experimental conditions.[14,15,26] SPME analysis demonstrated generally consistent tissue concentrations of these pharmaceuticals across the three sampling durations (spanning 20 min to 2 days), as presented in Tables 2 and S2. The free pharmaceutical concentrations in fish approximately reflected exposure milieu concentrations, and were uniformly distributed in the muscle tissue (Table 3). For PE-SPME, sensitivity is proportional to both sampling duration and fiber surface area as illustrated by the method quantification limits (MQLs, Table S3) and our in vivo experiment (Table S2).[27−30] Relative to each single 0.7 cm fiber segment, heightened sensitivity was achieved by pooling the analyte extracted from the 0.7 cm segments (Tables 2 and S2).

The use of multiple SPME fibers per fish improved the precision of free concentration estimates relative to the application of a single fiber. The range of free concentration differences among fish of the same treatment derived using a single fiber was 11−31% and 11−43% for ibuprofen and fluoxetine (n = 42), respectively. When two 0.7 cm fibers were deployed in each fish, the variability in free concentration estimates narrowed considerably to 7−10% for ibuprofen, and 7−12% for fluoxetine (n = 12), presumably by eliminating interfish variation in uptake and depuration of analyte. Therefore, in vivo sampling employing multiple fibers per fish and/or DP-SPME improves the free concentration estimates via improved precision, which in turn decreases the number of experimental animals required.

The DP-SPME sampling was validated using two independent approaches based on previously established methods for validating in vivo sampling.[31−33] First, the free concentrations, measured on the 12th exposure day by SPME were well correlated with total concentrations measured by methanolic extraction (Figure 5). Second, the 20 min and 2 h PE-SPME sampling calibrated by kinetic calibration were validated during the 2 day SPME sampling by the traditional standard curve calibration method.[32] For the 2 d measurements, the SPME extraction would have achieved equilibrium based on equilibrium times determined during earlier in vitro gel experiments (ibuprofen, 3.5 h; fluoxetine, 16 h at 15 °C). The agreement of the data calculated by PE-SPME with that derived from E-SPME analysis confirmed the assay validity. In addition, the consistence of the measured concentrations

Table 2. Ibuprofen Concentrations in Rainbow Trout (ng/g) and Exposure Water (ng/L) Following 8 and 12 d Exposure to Multiple Concentrations (%) of Waterloo Municipal Wastewater Effluent (E)[a]

ibuprofen				muscle-SPME (C_{free})					
days	E (%)	water SPE	muscle LE (C_t)	20 min-S	20 min-P	2 h-S	2 h-P	2d-S	2d-P
8	0	25.2 ± 3.3	ND	ND	ND	ND	ND	ND	ND
	20	89.0 ± 34.4	1.1 ± 0.4	ND	ND	0.2 ± 0.06	0.1 ± 0.04	0.2 ± 0.06	0.2 ± 0.03
	50	251.3 ± 51.8	4.6 ± 0.7	ND	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.1	0.5 ± 0.2
	90	387.1 ± 66.3	7.1 ± 1.9	0.9 ± 0.2	0.9 ± 0.3	0.8 ± 0.2	0.9 ± 0.2	1.1 ± 0.2	0.9 ± 0.2
12	0	35.2 ± 12.5	ND	ND	ND	ND	ND	ND	ND
	20	194.6 ± 45.2	1.9 ± 0.6	ND	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.2
	50	423.4 ± 59.1	11.6 ± 1.7	1.3 ± 0.2	1.2 ± 0.4	1.3 ± 0.1	1.3 ± 0.3	1.4 ± 0.2	1.4 ± 0.3
	90	NA	no fish	no fish	no fish	no fish	no fish	no fish	no fish

[a]Waterborne concentrations were measured by solid-phase extraction (SPE), total concentrations (C_t) in muscle were measured by methanolic liquid extraction (LE), and free concentrations (C_{free}) in muscle were measured by solid-phase microextraction (SPME). 20 min-S, 2h-S and 2d-S and 20 min-P, 2h-P and 2d-P are averaged free concentrations as measured by single (S), and pooled (P) 0.7 cm segmented fibers over 20 min, 2 h, and 2 d, respectively (n = 18). NA = not available; ND = not detectable due to the lower concentrations than the method quantification limits. No fish survived in 90% E over the 12 d interval.

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Table 3. Spatial Distribution of Ibuprofen and Fluoxetine in Dorsal−Epaxial Muscle of Rainbow Trout (*Oncorhynchus mykiss*)[a]

		5 cm SPME fiber							0.7 cm fiber	
	sampling time	1	2	3	4	5	6	7	T1	H1
ibuprofen (ng/g)	20 min	1.1 ± 0.3	1.3 ± 0.4	1.2 ± 0.3	1.4 ± 0.4	1.4 ± 0.4	1.3 ± 0.4	1.3 ± 0.3	1.2 ± 0.3	1.1 ± 0.4
	2 h	1.3 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	1.4 ± 0.2	1.4 ± 0.3	1.2 ± 0.3	1.1 ± 0.3	1.2 ± 0.1	1.3 ± 0.3
	2 d	1.4 ± 0.2	1.5 ± 0.3	1.3 ± 0.3	1.6 ± 0.2	1.3 ± 0.2	1.5 ± 0.2	1.4 ± 0.2	1.5 ± 0.4	1.4 ± 0.3
fluoxetine (ng/g)	20 min	1.0 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	1.0 ± 0.4	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.4	1.1 ± 0.3	1.1 ± 0.3
	2 h	0.9 ± 0.3	1.0 ± 0.2	0.8 ± 0.3	1.0 ± 0.1	1.2 ± 0.3	1.0 ± 0.2	0.9 ± 0.2	1.1 ± 0.3	1.0 ± 0.2
	2 din	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.2	1.2 ± 0.4	1.1 ± 0.3	0.9 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.3

[a]The 5 cm PDMS fibers were cut into seven 0.7 cm segments, sequentially numbered as 1, 2, 3, 4, 5, 6, and 7 (from the tail to head) corresponding to the position where the fiber was deployed in the fish. Two additional 0.7 cm fibers placed in the symmetric side of the fish were numbered as T1 and H1, corresponding laterally to the #1 and #7 segments in the 5 cm fiber. Mean pharmaceutical concentrations (± absolute difference) are from fish exposed 12 d to 50% effluent (n = 2).

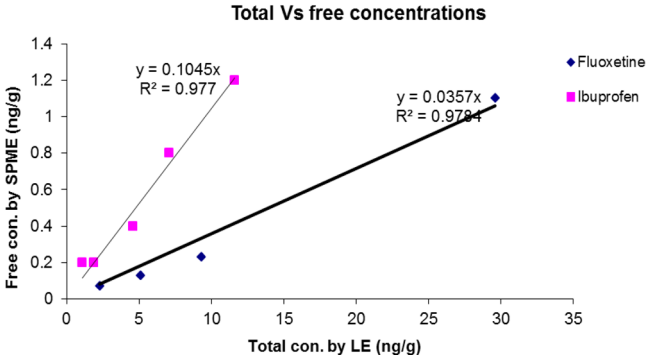

Figure 5. Correlation between the mean free analyte concentrations in fish muscle (ng/g) determined by SPME and that determined by methanolic liquid extraction (LE).

through the whole muscle tissue verified that localized SPME sampling of muscle contaminant burdens were reflective of the larger tissue. During instrumental analysis by HPLC-MS/MS, we evaluated the potential matrix effect on ionization enhancement or suppression by comparing the instrument response to the same amount of deuterated standards with and without matrix.[22] The data showed ∼11% ionization enhancement of IBU-d_3 and 9% suppression of FLX in fish muscle, which was deemed acceptable for this analysis.

During the exposure, 13 of the 72 fish implanted with SPME fibers died, including a single fish from the control treatment (due to a poorly inserted SPME fiber damaging viscera), another in the 50% effluent treatment, and 11 fish in the 90% effluent treatment. We are attributing the mortalities associated with the 50 and 90% MWWE treatments to the generally compromised health of these fish resulting from chronic exposure to very high (1.0 and 1.6 mg/L, respectively) levels of un-ionized ammonia, according to Emerson's study.[34] The mortality rate (13/72) of the SPME sampled group did not differ from that experienced by fish similarly exposed to MWWE, but not implanted with SPME fibers (12/72, p = 0.999, Fisher's Exact Probability Test). Additionally, during the dissection, only a single fish was observed with an infection adjacent the insertion site of the SPME fiber, despite inhabiting a milieu rich in microorganisms associated with a wastewater effluent. In light of these mortality rates, it could reasonably be concluded that DP-SPME, utilizing up to three fibers (including one of relatively large physical dimension (5 cm) for sampling intervals up to 2 d), were not a significant additional stressor for fish exposed to wastewater effluents. In addition, the 2 day in vivo sampling duration, which produced negligible mortality, demonstrates the safety and feasibility of implantation sampling using the soft PDMS fibers. Such implantation approaches may lead to the possibility of future online in vivo monitoring systems utilizing biocompatible materials such as PDMS, although undoubtedly that goal is predicated on technologies as yet undeveloped.

While the present study demonstrates the simplicity and efficacy of this technique to rapidly monitor free concentrations in complex matrices, care must be exercised when performing in vivo experiments. For example, the incident angle during probe insertion into the fish muscle must be shallow; too steep an angle could direct the fiber into the abdominal cavity and kill the fish, and too shallow an angle may unnecessarily open a second puncture wound in the skin. For 2 h and 2 d sampling, the fibers should be completely inserted into the muscle tissue to avoid fiber loss as sinusoidal movements during fish locomotion serve to work out improperly placed fibers over time. We believe the delicate insertion of SPME probes and the flexibility of the 5 cm PDMS depth-profiling fibers were important factors mitigating undue stress responses in the fish under study.

CONCLUSION

The DP-SPME technique was developed to measure pharmaceutical distributions across a fish tissue and an onion bulb with a single PDMS fiber, thus opening the possibility of using commercially available SPME probes for depth profiling applications. The technique showed better analytical precision, was minimally invasive, and reduced experimental animal use relative to traditional approaches. DP-SPME, when coupled to modern chromatography hyphenated tandem mass spectrometry, has the potential to efficiently screen multiple analytes within heterogeneous biotic and abiotic sample systems, such as contaminated soil, river sediment, and plant tissue.

ASSOCIATED CONTENT

Supporting Information

Additional material as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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