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Analysis of Pharmaceuticals in Fish Using Liquid Chromatography-Tandem Mass Spectrometry

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A liquid chromatography−tandem mass spectrometry (LC−MS/MS) screening method has been developed targeting 23 pharmaceuticals and 2 metabolites with differing physicochemical properties in fish tissue. Reversed-phase separation of target compounds was achieved using a C18 column and a nonlinear gradient consisting of 0.1% (v/v) formic acid and methanol. Eluted analytes were introduced into the mass analyzer using positive or negative electrospray ionization, as appropriate. A variety of extraction solvents, differing in polarity, pH, or both, were investigated in order to assess recovery of target compounds from 1-g tissue homogenates. Among 10 solvents tested, a 1:1 mixture of 0.1 M aqueous acetic acid (pH 4) and methanol was identified as optimal, resulting in extraction recoveries for 24 of 25 compounds exceeding 60%. Tissue extracts were found to influence the LC−MS/MS response for several analytes. Consequently, matrix-matched calibration standards were employed to determine analyte concentrations in environmental samples. Statistically derived method detection limits were <6 ng/g for most analytes. The method was subsequently used to screen for target analytes in fish from an effluent-dominated stream. Diphenhydramine, diltiazem, carbamazepine, and norfluoxetine were detected in 11 of 11 environmental samples at concentrations ranging from 0.11 to 5.14 ng/g.

The occurrence of pharmaceuticals and personal care products (PPCPs) in the environment has received broad interest over the past decade.[1−4] PPCPs have been increasingly detected in water, wastewater, soil, sediments, and biosolids. More recently, reports from our laboratory[5] and others[6−10] have demonstrated that environmental exposures to PPCPs may result in accumulation of parent compounds, their metabolites, or both in tissues of aquatic organisms. These reports have heightened interest in secondary effects of PPCPs and impart a sense of urgency to research focused on understanding fate and partitioning of these compounds in aquatic systems.

Analytical protocols for determination of PPCPs in water, sediment, and biosolids are numerous and have been summarized in recent reviews.[11−14] Due to the complexity of environmental samples, analyses typically employ detailed sample preparation followed by chromatographic separation of analytes and mass spectrometry detection. While methods focused on a single compound or unique compound class (e.g., antibiotics) continue to be reported,[15−20] increasing emphasis on simultaneous analysis of compounds with dissimilar physicochemical properties is evident in recent literature.[11,21−25] This shift in philosophy stems from a desire to gain diverse knowledge with minimum analytical expenditure.

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At present, analytical methodologies for determination of PPCPs in aquatic organisms are limited not only in scope, but also in number. Protocols for measuring select compounds in fish tissues have been reported for diclofenac,[26] two antidepressants and their active metabolites,[5] 4 UV filters and methyl-triclosan,[6,7] 14 musk fragrances,[8,9] 4 tetracycline antibiotics,[27] and 8 veterinary antibiotics representing three structural classes.[28] The general approach employed for analysis of personal care products involved extraction of homogenized tissue with nonpolar solvents, followed by successive size-exclusion and silica gel cleanup procedures prior to gas chromatography−mass spectrometry (GC/MS) analysis.[6−9] In contrast, pharmaceuticals were extracted from tissue using relatively polar solvents (e.g., aqueous buffer or acetonitrile), and extracts were cleaned by solid-phase extraction prior to GC/MS,[5,26] HPLC,[27,28] or LC−MS[28] analysis.

Herein, we report the first multiresidue screening method for pharmaceuticals representing multiple therapeutic classes in fish tissue. This protocol enables simultaneous monitoring of 25 compounds using LC−MS/MS. Key steps in method development involved optimizing extraction of acidic, basic, and neutral analytes from 1-g tissue homogenates and using matrix-matched calibration to compensate for observed matrix interference. As compared to previous methods for analysis of PPCPs in fish tissue, developed methodology offers relatively simple sample preparation in that tissue extracts are centrifuged and directly injected into the LC−MS/MS, following reconstitution in chromatographic mobile phase. The method was subsequently applied to assess the occurrence of target analytes in environmental samples. Four pharmaceuticals were detected in all analyzed specimens, and accumulation of three of these compounds in fish tissues is reported here for the first time.

EXPERIMENTAL SECTION

Pharmaceutical Standards and Reagents. All chemicals were reagent grade or better, obtained from commercial vendors, and used as received. The positive ESI internal standard 7-aminoflunitrazepam-d_7 (100.0 μg/mL in acetonitrile), surrogates (100.0 μg/mL in acetonitrile) acetaminophen-d_4, fluoxetine-d_6, and diphenhydramine-d_3, and reference standards (1000.0 μg/mL in MeOH) fluoxetine, norfluoxetine, sertraline, codeine, diphenhydramine, propranolol, and ibuprofen were purchased as certified analytical standards (Cerilliant Corp., Round Rock, TX). Atenolol was purchased in solid form (99% purity), also from Cerilliant. The negative ESI internal standard meclofenamic acid and reference standards, 1,7-dimethylxanthine, acetaminophem, caffeine, miconazole, carbamazepine, erythromycin, gemfibrozil, trimethoprim, diltiazem, cimetidine, warfarin, thiabendazole, sulfamethoxazole, lincomycin, metoprolol, tylosin, and clofibric acid, were purchased in the highest available purity (Sigma-Aldrich, Milwaukee, WI). Surrogates (100.0 μg/mL in acetonitrile) carbamazepine-d_{10} and ibuprofen-$^{13}C_3$ were purchased from Cambridge Isotopes Lab. Inc. (Andover, MA). Distilled water was purified and deionized to 18 MΩ with a Barnstead Nanopure Diamond UV water purification system.

Sample Collection and Preservation. Pecan Creek and Clear Creek (two streams located in Denton County, TX) were chosen for field sampling activities. Clear Creek is not impacted by effluent discharges and is routinely used as a local reference stream by the City of Denton, Texas Watershed Protection program. In contrast, annual flows in Pecan Creek are composed almost entirely of effluent discharge from the Pecan Creek Water Reclamation Plant. Effluent-dominated streams are likely worse case scenarios for investigating environmental exposures to PPCPs. Because these streams receive limited upstream dilution, wastewater contaminants may be considered "pseudopersistent", and resident organisms may receive continuous life cycle exposures. Fish (*Lepomis* sp.) were sampled from Pecan Creek (n = 11) and Clear Creek (n = 20) to serve as test and reference specimens, respectively. The approximate size of fish collected from these sites was similar and ranged from 8.8 to 11.5 cm (total length) and 29.4 to 49.0 g. Lateral fillets were dissected from fish collected at both sites and homogenized using a Tissuemiser (Fisher Scientific, Fair Lawn, NJ) set to rotate at 30 000 rpm. Pecan creek homogenates were stored individually, while Clear Creek homogenates were composited into a single sample. All tissues were stored at −20 °C prior to analysis. No target analytes were detected in the Clear Creek composite. Accordingly, this tissue is hereafter referred to as "clean".

Analytical Sample Preparation. Approximately 1.0 g of tissue was combined with 8 mL of extraction solvent (see Figure 2 for tested solvent compositions) in a 20-mL borosilicate glass vial (Wheaton; VWR Scientific, Rockwood, TN), and the mixture was homogenized using a Tissuemiser (Fisher Scientific) set to rotate at 30 000 rpm. Five surrogates were added to each sample: acetaminophen-d_4 (454 ng), fluoxetine-d_6 (636 ng), diphenhydramine-d_3 (8.9 ng), carbamazepine-d_{10} (38.5 ng), and ibuprofen-$^{13}C_3$ (789 ng). Samples were shaken vigorously and mixed on a rotary extractor for 5 min. Following extraction, samples were rinsed into 50-mL polypropylene copolymer round-bottomed centrifuge tubes (Nalge Co.; Nalgene Brand Products, Rochester, New York) using 1 mL of extraction solvent and centrifuged at 16 000 rpm for 40 min at 4 °C. The supernatant was decanted into 18-mL disposable borosilicate glass culture tubes (VWR Scientific), and the solvent was evaporated to dryness under a stream of nitrogen at 45 °C using a Zymark Turbovap LC concentration workstation (Zymark Corp., Hopkinton, MA). Samples were reconstituted in 1 mL of mobile phase, and a constant amount of the internal standards 7-aminoflunitrazapam-d_7 (100 ng) and meclofenamic acid (1000 ng) was added. Prior to analysis, samples were sonicated for 1 min and filtered using Pall Acrodisc hydrophobic Teflon Supor membrane syringe filters (13-mm diameter; 0.2-μm pore size; VWR Scientific, Suwanee, GA). In a typical preparation, a single analyst can manually prepare 12 homogenized tissue samples for LC−MS/MS analysis in less than 5 h.

LC−MS/MS Analysis. A Varian ProStar model 210 binary pump equipped with a model 410 autosampler was used in this study. Analytes were separated on a 15 cm × 2.1 mm (5 μm, 80 Å) Extend-C18 column (Agilent Technologies, Palo Alto, CA) connected with an Extend-C18 guard cartridge 12.5 mm × 2.1 mm (5 μm, 80 Å) (Agilent Technologies). A binary gradient consisting of 0.1% (v/v) formic acid in water and 100% methanol was employed to achieve chromatographic separation and is

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Table 1. Time-Scheduled Gradient Elution Program

time (min)	mobile-phase composition, % 0.1% formic acid	methanol
0	93	7
2	93	7
7	85	15
12	85	15
21	52	48
28	52	48
34	41	59
45	2	98
50	2	98
51	93	7
65	93	7

defined in Table 1. Additional chromatographic parameters were as follows: injection volume, 10 μL; column temperature, 30 °C; flow rate, 350 μL/min. Eluted analytes were monitored by MS/MS using a Varian model 1200L triple-quadrupole mass analyzer equipped with an electrospray interface (ESI).

To determine the best ionization mode (ESI + or −) and optimal MS/MS transitions for target analytes, each compound was infused individually into the mass spectrometer at a concentration of 1 μg/mL in aqueous 0.1% (v/v) formic acid at a flow rate of 10 μL/min. All analytes were initially tested using both positive and negative ionization modes while the first quadrupole was scanned from m/z 50 to [M + 100]. This enabled identification of the optimal source polarity and most intense precursor ion for each compound. Once these parameters were defined, the energy at the collision cell was varied, while the third quadrupole was scanned to identify and optimize the intensity of product ions for each compound. Additional instrumental parameters held constant for all analytes were as follows: nebulizing gas, N_2 at 60 psi; drying gas, N_2 at 19 psi; temperature, 300 °C; needle voltage, 5000 V ESI+, 4500 V ESI−; declustering potential, 40 V; collision gas, argon at 2.0 mTorr.

Extraction Recoveries. Two groups of control samples prepared from "clean" tissue were employed to determine extraction efficiency for target analytes. Group 1 samples were spiked with internal standards and each analyte, and group 2 samples were spiked with internal standards only. Both groups of samples were carried through the sample preparation procedure described above. Following syringe filtration, group 2 samples were spiked with the same amount of each analyte added to group 1. All samples were analyzed by LC−MS/MS, and individual analyte recoveries were calculated using the following equation:

$$\text{recovery} = \frac{A_{X1}/A_{IS1}}{A_{X2}/A_{IS2}} \times 100\%$$

where A_{X1}, A_{IS1}, A_{X2}, and A_{IS2} represent peak areas for the analyte (X) and internal standard (IS) in groups 1 and 2, respectively.

RESULTS AND DISCUSSION

LC−MS/MS Methodology. Three factors were considered in selecting target analytes (Table 2): (i) number of prescriptions dispensed in the United States during 2005,[29] (ii) variability in structure, physicochemical properties, and therapeutic use, and (iii) relative frequency of occurrence in soils, sediments, and biosolids. Excluding potential ion-exchange phenomena, the physicochemical properties favoring compound partitioning from water to solid environmental matrixes may also promote accumulation of water-borne chemicals in aquatic biota via diffusion across biological membranes. Additionally, compounds residing in sediment may be taken up by aquatic organisms via ingestion. Furlong et al. summarized results from several U.S. Geological Survey occurrence studies targeting PPCPs in environmental matrixes and demonstrated that the frequency of detection for fluoxetine in analyzed sediment, soil, and biosolid samples (64−100%) was much higher than in water (5%).[30] This general trend was observed for 17 additional compounds assessed in their work. Since fluoxetine was previously shown by our group to accumulate in fish tissues,[5] it seemed reasonable to target compounds with a similar occurrence pattern.

Compound-dependent mass spectrometry parameters were investigated by direct infusion of individual analytes into the electrospray source. Optimized MS/MS transitions and collision energies employed for detection and quantitation of each analyte are provided in Table 2, along with the molecular structure and most common therapeutic use for each analyte. With the exception of erythromycin, selected precursors represent the molecular ion $[M + H]^+$ or $[M - H]^-$ for each analyte. The most abundant precursor for erythromycin was found to be the $[M + H - H_2O]^+$ ion at m/z 716, consistent with previous observations.[13,31] Selected product ions represent the most abundant fragment observed for each precursor at the noted collision energy.

Once suitable MS/MS transitions were identified for each analyte, an aqueous mixture of reference standards was employed to optimize chromatographic parameters. A nonlinear gradient consisting of 0.1% (v/v) formic acid and methanol resulted in near-baseline resolution of the majority of analytes in ~50 min (Figure 1). A 15-min isocratic hold (93:7 formic acid−methanol) was added to the end of each run to allow for column equilibration between injections. While the majority of analytes were eluted as single peaks, erythromycin was consistently eluted as two partially resolved peaks. Similar chromatographic behavior for erythromycin has been observed previously and attributed to differing retention characteristics for presumed sterioisomers.[31,32]

Additionally, isotope effects on retention behavior were observed for carbamazepine-d_{10} and fluoxetine-d_6. As evident in Figure 1 (peaks 18 and 19), the observed retention time for carbamazepine-d_{10} (30.08 min) was shorter than that observed for carbamazepine (30.53 min) by almost 30 s. Though not evident in Figure 1 due to coelution of norfluoxetine (35.13 min), a 20-s difference in retention time was also observed for fluoxetine-d_6 (34.58 min) relative to that observed for fluoxetine (34.93 min). These differences are admittedly small but were very reproducible,

(29) RxList The Internet Drug Index, Top 300 Prescriptions for 2005 by Number of U.S. Prescriptions Dispensed. http://www.rxlist.com/top200.htm (accessed November 2006).

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Table 2. Analyte-Dependent Mass Spectrometry Parameters for Target Compounds

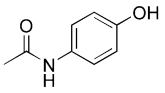
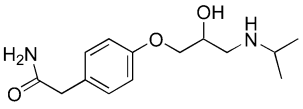
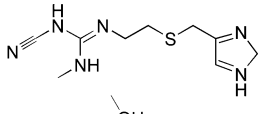
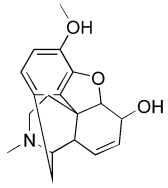
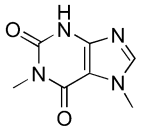
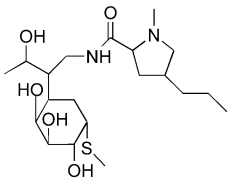
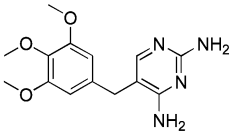
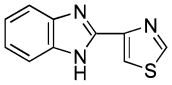
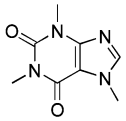
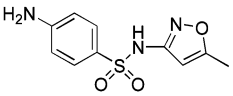
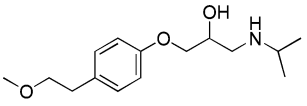
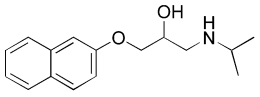
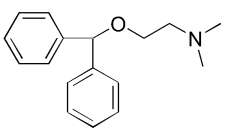
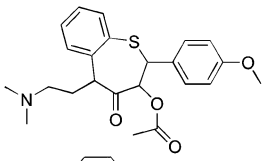
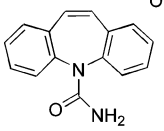
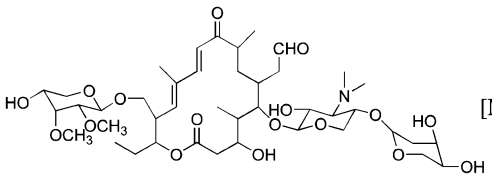
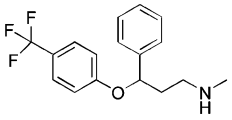
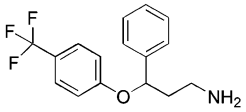
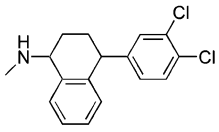
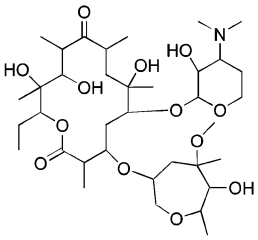
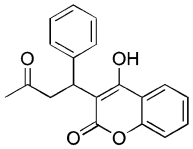
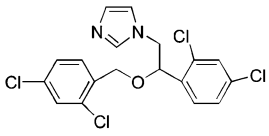
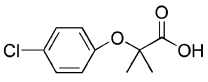
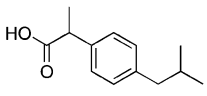
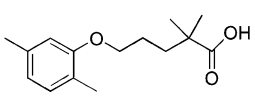
Compound	Use	Structure	Precursor Ion	Collision Energy (eV)	Product Ion	pKa[a]
ESI POSITIVE ANALYTES						
Acetaminophen	analgesic		152 $[M + H]^+$	-11.0	110	9.86
Atenolol	anti-hypertension		267 $[M + H]^+$	-21.5	145	9.16
Cimetidine	anti-acid reflux		253 $[M + H]^+$	-13.5	159	7.07
Codeine	analgesic		300 $[M + H]^+$	-38.0	215	8.25
1,7-dimethylxanthine	caffeine metabolite		181 $[M + H]^+$	-15.5	124	8.50
Lincomycin	antibiotic		407 $[M + H]^+$	-15.5	359	8.78
Trimethoprim	antibiotic		291 $[M + H]^+$	-17.5	261	7.20
Thiabendazole	antibiotic		202 $[M + H]^+$	-23.0	175	
Caffeine	stimulant		195 $[M + H]^+$	-16.0	138	
Sulfamethoxazole	antibiotic		254 $[M + H]^+$	-13.0	156	5.81
Metoprolol	anti-hypertension		268 $[M + H]^+$	-15.5	191	9.17
Propranolol	anti-hypertension		260 $[M + H]^+$	-11.0	116	9.14
Diphenhydramine	antihistamine		256 $[M + H]^+$	-11.5	167	8.76

Table 2 (Continued)

Compound	Use	Structure	Precursor Ion	Collision Energy (eV)	Product Ion	pKa[a]
ESI POSITIVE ANALYTES						
Diltiazem	anti-hypertension		415 $[M + H]^+$	-22.0	178	8.94
Carbamazepine	anti-seizure		237 $[M + H]^+$	-13.5	194	
Tylosin	antibiotic		916 $[M + H]^+$	-31.5	174	7.39
Fluoxetine	antidepressant		310 $[M + H]^+$	-6.0	148	10.1
Norfluoxetine	fluoxetine metabolite		296 $[M + H]^+$	-4.5	134	9.05
Sertraline	antidepressant		306 $[M + H]^+$	-11.0	275	9.47
Erythromycin	antibiotic		716 $[M + H - H_2O]^+$	-18.0	558	8.16
Warfarin	anti-coagulant		309 $[M + H]^+$	-14.0	163	4.50
Miconazole	antibiotic		417 $[M + H]^+$	-27.5	161	6.67
ESI NEGATIVE ANALYTES						
Clofibric Acid	antilipemic		213 $[M - H]^-$	15.4	127	3.18
Ibuprofen	analgesic		205 $[M - H]^-$	7.0	161	4.41
Gemfibrozil	antilipemic		249 $[M - H]^-$	13.0	121	4.75

[a] Calculated values obtained from the SciFinder database (© 2006 American Chemical Society).

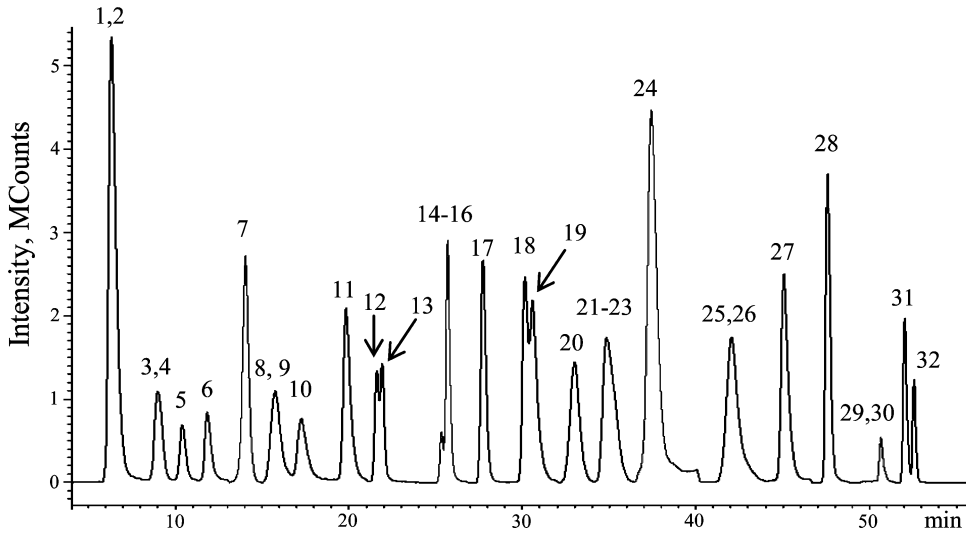


Figure 1. LC−MS/MS total ion chromatogram resulting from analysis of clean tissue spiked with a mixture of pharmaceutical standards. Peak identifications are as follows: (1) acetaminophen-d_4, (2) acetaminophen, (3) atenolol, (4) cimetidine, (5) codeine, (6) 1,7-dimethylxanthine, (7) lincomycin, (8) trimethoprim, (9) thiabendazole, (10) caffeine, (11) sulfamethoxazole, (12) 7-aminoflunitrazepam-d_7 (+IS), (13) metoprolol, (14) propranolol, (15) diphenhydramine-d_3, (16) diphenhydramine, (17) diltiazem, (18) carbamazepine-d_{10}, (19) carbamazepine, (20) tylosin, (21) fluoxetine-d_6, (22) fluoxetine, (23) norfluoxetine, (24) sertraline, (25) erythromycin, (26) clofibric acid, (27) warfarin, (28) miconazole, (29) ibuprofen-$^{13}C_3$, (30) ibuprofen, (31) meclofenamic acid (−IS), and (32) gemfibrozil.

and observed behavior for these analytes is consistent with previous studies demonstrating stronger retention for unlabeled compounds than deuterated analogues in reversed-phase chromatography.[33−35] Presumably, isotope effects were not observed for acetaminophen (peaks 1 and 2) and diphenhydramine (peaks 15 and 16) due to a lower degree of deuterium substitution and decreased resolution at shorter retention times.

Extraction of Target Analytes from Fish Tissue. Due to considerable variation in lipophilicity and pK_a among pharmaceuticals, a systematic study of extraction behavior was conducted to identify an optimal solvent system for extracting target analytes from muscle tissue. Ten solvents, differing in pH or polarity, were tested in a side-by-side comparison. Mean recoveries ($n = 3$) were calculated for individual analytes in each solvent system and are available as Supporting Information (Table S-1). Individual analyte recoveries were averaged for each solvent system and are charted in Figure 2. "Error bars" in this plot represent one standard deviation from the average and provide an assessment of variability among mean recoveries for individual analytes. While these data have no statistical relevance, they clearly provide a convenient metric for comparing overall solvent performance (i.e., the most effective solvents are those displaying maximum recovery and minimum "error").

In general, moderate-polarity solvents were found to be most effective at removing target analytes from tissue. Among tested organic solvents, efficiency increased with increasing polarity (i.e., dichloromethane−hexane < dichloromethane−methanol < acetonitrile−methanol). However, aqueous solvents (the most polar solvents tested) resulted in relatively poor extraction efficiency. Aqueous−organic mixtures proved to be efficient over the entire range of investigated pH conditions (pH 2.4−6), and with exception of tylosin and miconazole, substitution of acetonitrile for methanol had a negligible effect on solvent performance when combined with acetic acid. Since most analytes included in this study are basic (Table 2) and expected to be protonated at pH ≤6, it is not surprising that pH had little effect on recovery as charted in Figure 2. Recovery of acidic analytes into these solvents was expected to decrease with decreasing pH. However, mean recoveries for clofibric acid, ibuprofen, and gemfibrozil were greater for pH 2.4 TFA−methanol and pH 6 phosphate buffer−acetonitrile (~90% in both cases) than for pH 4 acetic acid−methanol (~60%). The origin of this behavior is presently unknown.

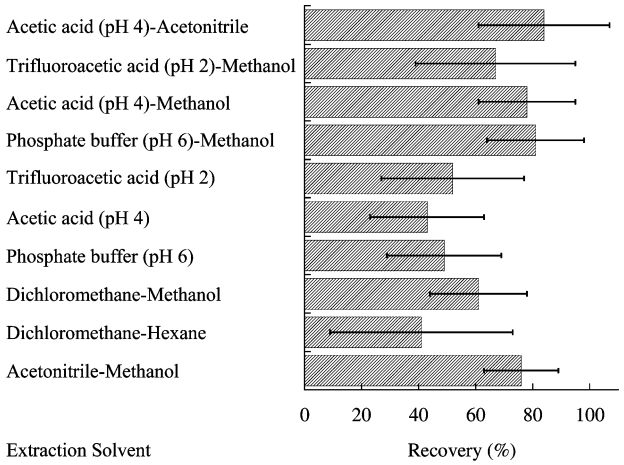


Figure 2. Average recoveries for extraction of target analytes from clean muscle tissue using the noted solvents. See text for details. Solvents were prepared by combining equal volumes of liquid in a binary mixture. Nominal aqueous concentrations of acetic acid and phosphate buffer were 0.1 M. The nominal concentration of trifluoroacetic acid (TFA) was 0.1% (v/v).

Matrix Effects. It is widely recognized that coextracted matrix components can affect analyte ionization in analyses employing

(33) Tanaka, N.; Thornton, E. R. *J. Am. Chem. Soc.* **1976**, *98*, 1617−1619.
(34) Shibasaki, H.; Furuta, T.; Kasuya, Y. *J. Chromatogr., B* **1997**, *692*, 7−14.
(35) Kato, K.; Jingu, S.; Ogawa, N.; Higuchi, S. *J. Pharm. Biomed. Anal.* **2000**, *24*, 237−249.

Table 3. Observed Matrix Effects for Extracted Tissue Samples[a]

analyte	retention time, min	conc in HCOOH ng/mL	PO_4−MeOH pH 6, ng/g	HAc−MeOH pH 4, ng/g	HAc−MeCN pH 4, ng/g	TFA−MeOH pH 2.4, ng/g	MeOH−MeCN, ng/g
acetaminophen	6.4	220	186(−15%)	194(−12%)	197(−11%)	154(−30%)	195(−11%)
atenolol	9.1	110	141(+29%)	117(+7%)	118(+8%)	98(−11%)	102(−7%)
cimetidine	8.9	60	51(−16%)	57(−5%)	63(+5%)	53(−11%)	53(−11%)
codeine	10.4	330	330(∼0%)	335(+2%)	331(∼0%)	299(−9%)	326(−1%)
1,7-dimethylxanthine	11.8	40	41(+3%)	42(+4%)	43(+7%)	38(−4%)	39(−2%)
lincomycin	14.0	220	239(+9%)	228(+4%)	231(+5%)	201(−8%)	224(+2%)
trimethoprim	15.6	90	97(−8%)	87(−3%)	87(−3%)	81(−10%)	80(−11%)
thiabendazole	15.8	90	83(−8%)	84(−6%)	84(−7%)	81(−10%)	86(−4%)
caffeine	17.2	210	222(+6%)	214(+2%)	228(+8%)	223(−6%)	228(+8%)
sulfamethoxazole	19.8	85	66(−23%)	81(−5%)	81(−4%)	75(−12%)	76(−11%)
metoprolol	21.9	85	83(−2%)	78(−9%)	81(−5%)	80(−6%)	72(−15%)
propranolol	25.3	40	32(−19%)	35(−11%)	30(−24%)	31(−23%)	19(−52%)
diphenhydramine	25.8	4	3.3(−17%)	3.2(−20%)	3.1(−21)	3.1(−23%)	2.2(−45%)
diltiazem	27.7	6	6.3(+4%)	5.8(−4%)	5.6(−6%)	5.7(−5%)	4.0(−34%)
carbamazepine	30.6	40	31(−22%)	36(−11%)	35(−12%)	35(−12%)	35(−12%)
tylosin	32.9	210	344(+64%)	237(+13%)	248(+18%)	261(+24%)	262(+25%)
fluoxetine	34.9	220	92(−58%)	141(−36%)	99(−55%)	72(−67%)	91(−58%)
norfluoxetine	35.2	200	69(−65%)	116(−42%)	87(−56%)	53(−74%)	81(−60%)
sertraline	37.2	220	47(−78%)	117(−47%)	63(−71%)	31(−86%)	93(−58%)
erythromycin	42.0	200	510(+155%)	1000(+400%)	2422(+1111%)	1476(+638%)	1934(+867%)
clofibric acid	42.4	70	165(+136%)	38(−46%)	53(−25%)	238(+240%)	42(−39%)
warfarin	45.0	40	20(−49%)	31(−22%)	29(−27%)	18(−55%)	25(−38%)
miconazole	47.5	400	43(−89%)	87(−78%)	73(−82%)	19(−95%)	196(−51%)
ibuprofen	50.6	1600	4045(+152%)	1271(−20%)	1289(−19%)	5491(+243%)	859(−46%)
gemfibrozil	52.5	200	307(+54%)	129(−35%)	119(−41%)	275(+38%)	110(−45%)

[a] Reported concentrations represent calculated values for each analyte in spiked matrix. See text for details. Values in parentheses represent percent difference in concentration relative to that observed for HCOOH. Negative sign indicates suppression of the analytical response. Solvent notations are as follows: PO_4, phosphate buffer; MeOH, methanol; HAc, 0.1 M acetic acid buffer; MeCN, acetonitrile; TFA, 0.1 M trifluoroacetic acid buffer.

electrospray interfaces.[13,21,31] Accordingly, an approach reported by Vanderford et al.[31] was employed to evaluate matrix effects for extraction solvents promoting recovery >60% in Figure 2. Clean muscle tissue (1 g) was extracted, centrifuged, and reconstituted in 0.1% formic acid. Extracts were spiked with a known amount of each analyte prior to analysis. Aqueous formic acid (0.1% v/v) was also spiked with the same concentration of target compounds and analyzed as a matrix-free reference sample. Concentrations of analytes derived from an internal standard calibration curve prepared using standards constituted in 0.1% formic acid are tabulated in Table 3.

As expected, the degree of matrix interference was found to depend on both analyte and extraction solvent. Coextracted matrix components were found to have minimal effect on the analytical response of early-eluting analytes (retention time <25 min). In contrast, matrix suppression was observed for most other ESI+ analytes; especially for fluoxetine, norfluoxetine, sertraline, and miconazole. Exceptions include tylosin and erythromycin, for which significant signal enhancements were observed in a number of cases. Data for clofibric acid, ibuprofen, and gemfibrozil in Table 3 are misleading for extracts resulting from extraction with phosphate buffer−methanol and trifluoroacetic acid−methanol. In each of these cases, data in Table 3 suggest an apparent signal enhancement. In fact, the analytical response for these analytes was suppressed in all cases, but more pronounced suppression of the ESI− internal standard (meclofenamic acid) in these extracts resulted in calculated concentrations exceeding the reference condition.

Matrix effects identified in Table 3 collectively demonstrate that accurate quantitation of analytes in tissue extracts is not feasible using calibration standards prepared in aqueous formic acid. Common approaches for dealing with matrix interference include spiking each sample with a known amount of labeled analyte(s) prior to analysis (i.e., isotope dilution), employing the method of standard additions, or using matrix-matched calibration standards. While isotope dilution is perhaps the best approach for compensation of matrix interference in analyses employing mass spectrometry,[36] cost and limited availability of labeled standards are problematic for broad screening methods. A primary limitation for standard addition methods is related to sample mass or volume, which is often limited in tissue analyses. Furthermore, quality assessment of standard addition data is difficult to monitor using standard QA/QC performance metrics. In contrast, matrix-matched calibration is relatively simple to implement, provided that clean reference tissue is available.

For reasons discussed above, matrix-matched calibration was employed to minimize matrix interference in the analysis of environmental samples. Calibration standards were prepared by adding a known amount of each target analyte and five labeled surrogates to 1 g of clean muscle tissue. Tissues were homogenized and carried through the entire sample preparation procedure prior to analysis. Acetic acid (pH 4)−methanol was selected as the extraction solvent, since observed matrix effects were minimized in this extract (Table 3). Linear calibration curves (r^2 > 0.99 for concentration ranges specified in Table 4) were constructed by plotting the response factor for each analyte versus tissue spiking levels (ng/g of tissue) and used to determine analyte concentrations in all subsequent analyses.

(36) Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. *Anal. Chem.* **2003**, *75*, 3019−3030.

Table 4. Investigated Linear Range, LOD, LOQ, and MDL for Target Analytes in Fish Muscle Tissue[a]

analyte	linear range, ng/g	LOD,[b] ng/g	LOQ,[c] ng/g	MDL,[d] ng/g
acetaminophen	3.12−400	0.30	0.99	4.40
atenolol	1.25−160	0.48	1.62	1.48
cimetidine	0.625−80	0.24	0.81	1.04
codeine	4.69−600	1.07	3.55	6.11
1,7-dimethylxanthine	0.625−80	0.17	0.58	1.02
lincomycin	3.12−400	0.63	2.09	5.53
trimethoprim	1.25−160	0.79	2.63	2.15
thiabendazole	1.25−160	0.14	0.47	2.63
caffeine	3.12−400	0.34	1.15	3.93
sulfamethoxazole	1.25−160	0.23	0.76	2.29
metoprolol	1.25−160	0.25	0.85	2.50
propranolol	0.625−80	0.01	0.03	1.07
diphenhydramine	0.0625−8	0.01	0.03	0.05
diltiazem	0.094−12	0.04	0.13	0.12
carbamezepine	0.625−80	0.03	0.12	0.54
tylosin	3.12−400	1.18	3.93	5.02
fluoxetine	4.69−600	0.76	2.54	6.73
norfluoxetine	3.12−400	0.32	1.08	2.90
sertraline	3.12−400	0.21	0.71	3.57
erythromycin	3.12−400	0.85	2.84	6.42
clofibric acid	1.25−160	0.10	0.32	2.69
warfarin	0.625−80	0.09	0.29	0.86
miconazole	3.12−400	0.39	1.32	10.8
ibuprofen	25−3200	3.14	10.4	45.9
gemfibrozil	3.12−400	0.25	0.85	6.68

[a] Clean tissues employed in the determination of these parameters were extracted using a 1:1 mixture of 0.1 M acetic acid (pH 4) and methanol. See text for details. [b] LOD, calculated as 3 times the standard deviation in the background signal observed for replicate analysis of a tissue blank. [c] LOQ, calculated as 10 times the standard deviation in the background signal observed for replicate analysis of a tissue blank. [d] MDL, determined by multiplying the one-sided Student's *t*-statistic at the 99% confidence limit times the standard deviation observed for eight replicate analyses of a matrix spike (spiking level ≤10 × MDL).

Analytical Performance Metrics. Analyte-specific limits of detection (LODs), limits of quantitation (LOQs) and method detection limits (MDLs) are defined and reported in Table 4. Although LOD and LOQ are recognized performance metrics within academic circles, MDL is more appropriate for establishing detection thresholds in environmental analyses with potential regulatory implications. LOD[37] and LOQ[38] are derived from analyses of a "blank" sample, and thus, do not account for potential matrix effects. In contrast, MDL is derived from replicate analyses of a matrix spike and represents the lowest concentration of analyte that can be detected in a given matrix with 99% confidence that the concentration is nonzero.[39] As shown in Table 4, MDLs for fish tissue were typically higher than either LOD or LOQ, irrespective of the level of matrix interference identified in Table 3. These results clearly suggest that use of LOD and LOQ as detection and quantitation thresholds in practical applications of this method could lead to acceptance of questionable data. For this reason, MDLs were employed as a single detection/quantitation threshold in screening analyses (see below). Additionally, it is important to note that relative standard deviations derived from replicate analyses of the matrix spike (data not shown) were ≤6% for all analytes, demonstrating excellent reproducibility.

(37) Kaiser, H. *Anal. Chem.* **1987**, *42*, 53A.
(38) Skoog, D. A.; Holler, F. J.; Nieman, T. A. *Principles of Instrumental Analysis*, 5th ed.; Harcourt Brace & Co.: Philadelphia, PA, 1998; pp 11−15.
(39) Definition and Procedure for the Determination of the Method Detection Limit. *Code of Federal Regulations*, Title 40, Section 136, Appendix B, 1986.

Table 5. Concentrations of Analytes (ng/g of Wet Weight) Detected in Muscle Tissues from Fish Collected in Pecan Creek, Denton County, TX

analyte	range ($n = 11$)	mean ($n = 11$)
diphenhydramine	0.66−1.32	0.96
diltiazem	0.11−0.27	0.21
carbamazepine	0.83−1.44	1.16
norfluoxetine	3.49−5.14	4.37

Analysis of Environmental Samples. In order to confirm the utility of LC−MS/MS methodology for analysis of environmental samples, fish were sampled ~650 m downstream from the effluent discharge into Pecan Creek and screened for target analytes. Four compounds were detected in all analyzed specimens at concentrations exceeding statistically derived MDLs (Table 5). Identification of analytes in environmental samples was confirmed by monitoring two fragment ions and comparing their retention time and relative intensity with a spiked sample. Representative

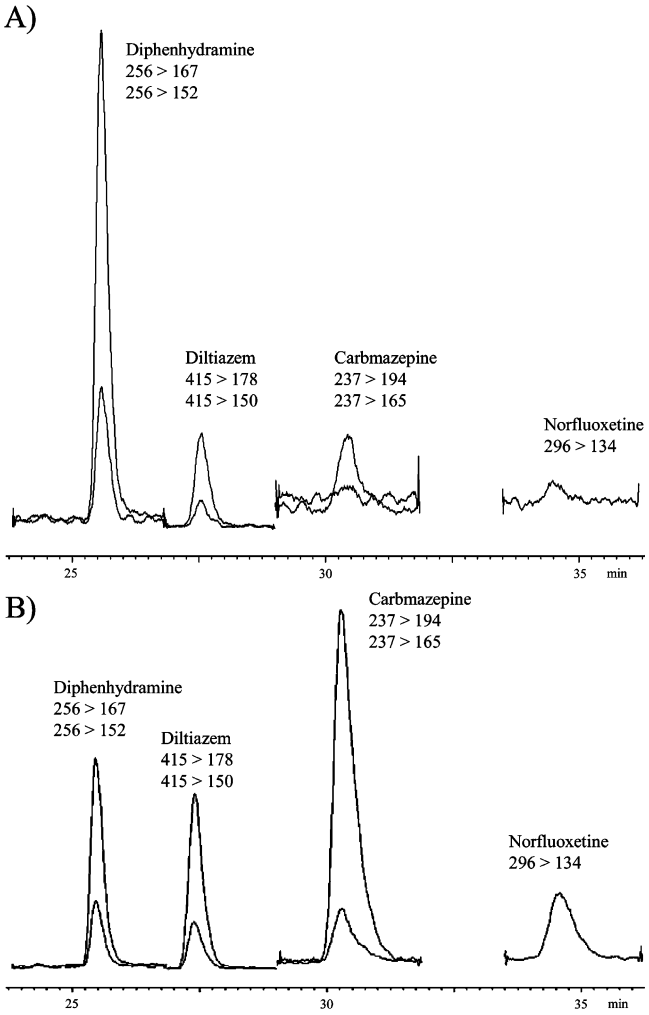


Figure 3. LC−MS/MS reconstituted ion chromatograms displaying analyte-specific quantitation and qualifier ions monitored for (A) a tissue extract from a fish (*Lepomis* sp.) collected in Pecan Creek and (B) an extract from clean' tissue spiked with known amounts of diphenhydramine (1.6 ng/g), diltiazem (2.4 ng/g), carbamazepine (16 ng/g), and norfluoxetine (80 ng/g). The higher *m*/*z* fragment is more intense in all cases.

reconstituted ion chromatograms are shown in Figure 3. Note that collision-induced dissociation of norfluoxetine produced only one fragment ion of sufficient intensity to be observed under these conditions. Fluoxetine and sertraline have been detected previously in fish from Pecan Creek.[5] However, it is not surprising that these compounds were not detected in this study, as reported concentrations in fish muscle (1.1 and 0.34 ng/g, respectively) fall below MDLs defined in Table 4. While norfluoxetine has also been observed previously in fish tissues,[5] accumulation of diphenhydramine, diltiazem, and cabamazepine is reported here for the first time.

Duplicate analysis of a matrix spike prepared from a Pecan Creek tissue specimen was conducted to assess method accuracy. Analyte spiking levels in this sample corresponded to the upper third of the calibration range for each analyte (~15 × MDL). Excepting fluoxetine, norfluoxetine, and sertraline, mean spike recoveries ranged from 88 to 120% (data not shown), demonstrating that target compounds can be quantified with acceptable accuracy in environmental samples. Relative percent difference for duplicate analyses was ≤16% for all compounds. It is important to point out that positive bias was observed for clofibric acid and ibuprofen (recovery, 186 and 145%, respectively) when matrix-matched internal standard calibration was employed. However, improved accuracy for these analytes was achieved (97% recovery in both cases) by using a matrix-matched external standard calibration curve (i.e,, a plot of analyte peak area versus tissue spiking level). Improvements in surrogate recovery for ibuprofen-$^{13}C_3$ in unspiked samples were also observed using the external standard approach. These results suggest that meclofenamic acid is not a suitable internal standard for clofibric acid and ibuprofen in environmental samples.

(40)) Barber, L. G.; Keefe, S. H.; Antweiler, R. C.; Taylor, H. E.; Wass, R. D. *Environ. Sci. Technol.* **2006**, *40*, 603−611.

Mean recoveries for fluoxetine, norfluoxetine, and sertraline were less than quantitative in the matrix spike (44, 64, and 46%, respectively), and surrogate recoveries for fluoxetine-d_6 in unspiked samples ranged from 60 to 97%. Matrix-matched external standard calibration did not significantly improve accuracy for these compounds. However, quantitation of fluoxetine based on isotope dilution with fluoxetine-d_6 resulted in 110% recovery for the matrix spike. It is reasonable to expect that additional compounds, not present in clean tissue extracts, may be present in Pecan Creek samples since this stream is significantly impacted by wastewater effluent and previous studies have demonstrated that wastewater contaminants not classified as PPCPs are accumulated in fish residing in effluent-dominated ecosystems.[7,40] Therefore, it is possible that compounds not monitored in this study coelute with target antidepressants and result in unforeseen matrix effects. To the extent that this proves to be a general occurrence in future applications of reported methodology, it may become necessary to employ isotope dilution for accurate quantitation of these compounds.

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SUPPORTING INFORMATION AVAILABLE

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

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