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Analysis of selected pharmaceuticals in fish and the fresh water bodies directly affected by reclaimed water using liquid chromatography-tandem mass spectrometry

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Abstract A comprehensive method for the analysis of 11 target pharmaceuticals representing multiple commonly used therapeutic classes was developed for biological tissues (fish), reclaimed water, and the surface water directly affected by irrigation with reclaimed water. One gram of fish tissue homogenate was extracted by accelerated solvent extraction with methylene chloride followed by mixed-mode cation exchange solid phase extraction (SPE) cleanup and analyzed by liquid chromatography-tandem mass spectrometry. Compared to previously reported methods, the protocol produces cleaner extracts resulting in lower method detection limits. Similarly, an SPE method based on Oasis HLB cartridges was used to concentrate and cleanup reclaimed and surface water samples. Among the 11 target compounds analyzed, trimethoprim, caffeine, sulfamethoxazole, diphenhydramine, diltiazem, carbamazepine, erythromycin, and fluoxetine were consistently detected in reclaimed water. Caffeine, diphenhydramine, and carbamazepine were consistently detected in fish and surface water samples. Bioaccumulation factors for caffeine, diphenhydramine, and carbamazepine in mosquito fish (*Gambusia holbrooki*) were calculated at 29±26, 821±422, and 108±144, respectively. This is the first report of potential accumulation of caffeine in fish from a water body directly influenced by reclaimed water.

Keywords Pharmaceuticals · Fish · LC-MS/MS

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Introduction

Because of increasing reports of their occurrence in water, wastewater, soil, sediment, and biosolids [1–5], pharmaceuticals including drugs and their active metabolites have been recognized as emerging environmental contaminants. Municipal wastewater has been identified as one of the main routes bringing these pharmaceuticals into the environment [1]. This is likely because wastewater treatment plants (WWTPs) are not specifically designed to remove most of the pharmaceuticals, thus these compounds are continuously released into reclaimed and surface waters in a wide range of concentrations [2, 3]. Fish are sensitive indicators for substances that enter aquatic ecosystems [6]. Numerous studies have shown that long-term exposure to pharmaceuticals and personal care products (PPCPs) may result in accumulation of parent compounds, their metabolites, or both in tissues of aquatic organisms, suggesting that further studies on secondary effects of PPCPs on aquatic organisms are necessary [4, 5, 7–13].

Due to the complexity of environment matrices and the multiple functionalities of emerging contaminants as target compounds, recently described analytical protocols for the determination of PPCPs in both wastewaters [14–16] and biological organisms [7, 9, 10, 17] have focused on rather specific classes of compounds. Antidepressants [7, 9, 14] and antibiotics [10, 15, 16] are the two classes that have been

studied the most. As the list of compounds found in field collected fish samples expands, the need to develop simultaneous screening methods for multiple classes of drug residues increases as well [18–22]. At present, the general approach employed for the analysis of multi-class pharmaceuticals in fish involves extraction of homogenized tissue with 1:1 mixture of 0.1 M acetic acid and methanol [5], or acetonitrile combined with limited cleanup to back-extract lipid material followed by liquid chromatography–mass spectrometry (LC-MS) analysis [13]. Additionally, the use of solid-phase microextraction (SPME) techniques has also been explored as surrogates to estimate the potential occurrence and uptake of pharmaceuticals in living fish by assuming similar partition behavior between the SPME devices and the fish tissues placed in contaminated environments [12].

Accelerated solvent extraction (ASE) involving high pressure and temperature has been shown to efficiently extract a wide range of compounds from fish tissues [9, 10, 17, 23–29]. Meanwhile, HLB polymeric cartridges are routinely used as SPE sorbent for cleanup of complex environmental samples prior to LC-MS analysis. However, due to considerable variation in lipophilicity and p*K*a among different classes of pharmaceuticals, optimum cleanup efficiency can be compromised by differential retention behavior of target analytes on the sorbent. In contrast, mixed-mode cation exchange (MCX) cartridges can accommodate both neutrals and cations providing better selectivity during elution steps. For instance, Chu et al. [9] developed a method to determine paroxetine, fluoxetine and its metabolite in fish tissue using accelerated solvent extraction followed by MCX cleanup offering better recoveries and minimized matrix interferences. Because of the capabilities mentioned above, mixed-mode MCX cartridges will likely provide the needed retention for the multiple classes of pharmaceuticals proposed herein.

The objectives of this study were to develop a comprehensive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the analysis of pharmaceuticals representing multiple therapeutic classes in fish tissue, reclaimed and surface water, and to achieve better sensitivity and recoveries for the determination of pharmaceuticals in biological tissues by using a combination of accelerated solvent extraction followed by mixed-mode SPE cleanup and LC-MS/MS detection.

Experimental

Reagents, standards and solutions

The LC-MS grade methanol, water, and formic acid used for mass spectrometry analysis and the Optima grade methylene chloride and acetonitrile used for ASE extraction were purchased from Fisher Scientific (Atlanta, GA). Distilled water for SPE cleanup was purified and deionized with a Barnstead Nanopure water purification system. Reference standards lincomycin, trimethoprim, caffeine, sulfamethoxazole, diphenhydramine, diltiazem, carbamazepine, fluoxetine, erythromycin, norfluoxetine, and sertaline were purchased in the highest available purity (Sigma-Aldrich, Milwaukee, WI). Five surrogates, covering the range of functionalities, were used for the method. Caffeine-$^{13}C_3$ was purchased from Cambridge Isotopes Lab. Inc. (Andover, MA). Sulfamethoxazole-d_4, erythromycin-^{13}C, d_3, and paroxetine-d_4 were purchased from Toronto Research Chemicals Inc. (North York, Ontario). Carbamazepine-d_{10} was purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec). Fluoxetine-d_6 was purchased from Cerilliant Corp. (Round Rock, TX). All stock solutions and working solutions were made in methanol and stored at −20 °C in the dark to prevent degradation. The 0.1 % formic acid solution used for mobile phase was prepared daily before analysis. Ancillary solutions used as modifiers, i.e., acetic acid (pH 3.2), 5 % ammonium hydroxide in methanol and methanol–0.1 % formic acid 50:50 (*v*/*v*) were prepared daily.

Sample collection and storage

Water and mosquito fish (*Gambusia holbrooki*) were collected from a freshwater pond and a saltwater marsh located at Florida International University Biscayne Bay Campus (North Miami Beach, FL). The saltwater tidal marsh is not impacted by reclaimed water and is hydrologically connected with Biscayne Bay while the freshwater pond is isolated from the tidal influence of the bay and continuously receives storm water runoff and reclaimed water from a local WWTP through daily sprinkler irrigation. Therefore, the organisms living in this pond are likely to be chronically exposed to the contaminants from reclaimed water. Seven composite mosquito fish samples were collected from each sampling site using unbaited minnow traps deployed at depth between 20 and 30 cm. The sizes of the fish collected in the traps ranged from 2.0 to 4.2 cm (total length) and individuals weighed from 0.3 to 2.1 g. All tissue samples were brought to the laboratory, sorted, classified, and stored at or below −20°C until time of analysis. Fish were pooled to obtain at least 30 g of material and homogenized with a ULTRA TURRAX IKA T18 stainless steel tissuemiser (Wilmington, NC) set to rotate at 10,000 rpm. The homogenates were stored at −20 °C and thawed at 4 °C for approximately 10 h before extraction. Reclaimed water (1 L) was directly drawn from irrigation sprinklers during a period of 2 months, while pond water samples were collected in 1-L pre-cleaned amber glass bottles at the site where fish were collected. All water samples were transported to the laboratory and stored in dark at or below 4 °C until they were filtered and processed. Water extraction was conducted within 7 days of collection to prevent losses due to biodegradation.

Solid phase extraction for water samples

To remove suspended materials, water samples were filtered through 0.5 μm pre-combusted glass fiber filters (GE Water & Process Technologies, Trevose, PA) within 24 h after collection. 50 μL of surrogate standards solution were added to each 250 mL of filtered reclaimed water or pond water sample. Oasis HLB (3 cc/60 mg, Waters Corp., Franklin, MA) cartridges were conditioned with 3 mL of methanol followed by 3 mL of distilled water. Solid phase extraction was performed on an ALLTECH 12-port vacuum manifold (Deerfield, IL). After the samples had passed through the HLB cartridges, they were washed with 4 mL of 5 % methanol in water prepared daily. The analytes were eluted with 3 mL of methanol. Each methanol eluent was mixed with 50 μL of paroxetine-d_4 internal standard solution and evaporated to dryness under a gentle stream of purified nitrogen gas. Dried residues were reconstituted with 200 μL of methanol–0.1 % formic acid 50:50 (*v*/*v*). After brief sonication and vortex, the samples were ready for LC-MS/MS analysis.

ASE extraction for fish tissues

Fish samples were extracted using a Dionex ASE 200 accelerated solvent extraction system (Dionex Corp., Sunnyvale, CA). Twenty-five grams of Na_2SO_4 was placed in the 33-mL stainless steel extraction cell containing a glass fiber filter in the outlet side. Approximately 1 g of fish homogenate was placed on top of the Na_2SO_4. Five surrogates including caffeine-$^{13}C_3$ (412.0 ng), sulfamethoxazole-d_4 (100.0 ng), carbamazepine-d_{10} (29.5 ng), fluoxetine-d_6 (31.0 ng), and erythromycin-^{13}C, d_3 (100.0 ng) were added to each sample. The cell was topped with another glass fiber filter, firmly capped and extracted. Methylene chloride was employed as the extraction solvent. The ASE conditions are as follows: oven temperature 80 °C, pressure 1,500 psi, heat for 5 min, one static cycle, static time 10 min, flush volume 60 %, and purge for 120 s. After each extraction cycle, 30 mL methylene chloride extract was flushed into a 60-mL glass vial and dried under gentle nitrogen stream. The dried extract was then reconstituted with 30 mL of acetic acid (pH 3.2) in water. The glass vial was sonicated for 10 min and the sample was ready for MCX-SPE cleanup.

Cleanup for fish tissues

Oasis MCX cartridges (3 cc/60 mg; Waters Corp., Franklin, MA) were employed for the fish extract cleanup. The cartridges were conditioned with 3 mL of methanol followed by 3 ml of acetic acid in water (pH 3.2). Care was taken not to dry the cartridges during loading process. A cartridge flow rate of less than 1 mL/min was operated under the vacuum of 20 psi. Once the samples had passed through the cartridges, they were washed with 2 mL acetonitrile and dried for 5 min. The cartridges were then eluted with 3 mL of 5 % ammonium hydroxide in methanol. 50 μL of paroxetine-d_4 internal standard solution were added, and samples were dried using a centrifuge concentrator (Labconco Corp., Kansas City, MO). 200 μL of methanol–0.1 % formic acid 50:50 (*v*/*v*) were added to each sample. After 10 min sonication and 1 min of vortex, samples were transferred and analyzed by LC-MS/MS.

LC-MS/MS analysis

The liquid chromatography system consists of a Thermo PAL CTC autosampler and a quaternary Thermo Scientific Accela pump. Analytes were separated on a Hypersil GOLD 50×2.1 mm, 3 μm particle size and 175 Å pore size column, from Thermo Scientific (Bellefonte, PA). A simple binary gradient consisting of A, 0.1 % formic acid in water (*v*/*v*) and B, 100 % methanol was employed for chromatographic separation. The gradient was (methanol %): 0 min 3 %, 4 min 3 %, 10 min 97 %, 13 min 3 %, and 16 min 3 %. The flow rate was maintained at 300 μL/min. The injection volume was 20 μL.

All the samples were analyzed with a Thermo TSQ Quantum Access triple quadrupole (QqQ) mass spectrometry equipped with a heated electrospray ion source (HESI). Tandem mass spectrometry detection was performed in selected reaction monitoring (SRM) mode. Collision energy (CE) and tube lens voltage for each compound were optimized through direct infusion into mass spectrometer at concentration of 1 μg/mL and at the flow rate of 15 μL/min. Additional instrumental parameters for all analytes were as follows: Spray voltage at 3,500 V, Capillary temperature and vaporizer temperature at 300 °C, sheath gas (N_2) and Aux gas (N_2) at 30 arbitrary units, and ion sweep gas (N_2) at 10 arbitrary units. Scan time was set to 0.5 s. Precursor ions, product ions, and SRM transition parameters are listed in Table 1.

Matrix effects

Matrix effects could have a severe detrimental effect in LC-MS/MS analysis especially when coupled with ESI sources [30, 31]. Both signal suppression and enhancement are commonly observed due to co-eluting matrix interferences. The exact mechanism still remains unknown but it has been widely discussed in previous work with respect to PPCPs [30–32]. In order to evaluate matrix effects, replicates of clean fish tissues (1 g) were extracted and cleaned up as described above. One of the extracts was spiked with a known amount of analytes and surrogates and brought to a final volume of 200 μL with methanol–0.1 % formic acid (50/50, *v*/*v*). Simultaneously, a methanol–0.1 % formic acid (50/50, *v*/*v*) solution spiked with the same concentration of analytes and surrogates was analyzed as a matrix-free

Table 1 SRM transition parameters

Compounds	SRM 1	Collision energy (V)	SRM 2	Collision energy (V)
Lincomycin	407→126	31	407→359	18
Trimethoprim	291→230	24	291→260	25
Caffeine	195→138	18	195→110	21
Sulfamethoxazole	254→156	17	254→108	29
Diphenhydramine	256→167	15	256→152	40
Diltiazem	415→178	25	415→150	39
Carbamazepine	237→194	19	237→192	21
Erythromycin	717→540	18	717→558	15
Fluoxetine	310→148	8	310→44	12
Norfluoxetine	296→134	6	296→31	46
Sertraline	306→275	12	306→159	28

reference sample. Matrix effects were evaluated using the following equation [30]:

$$\text{Matrix effects}\,(\%) = \left(\frac{R_{m+s} - R_m}{R_0} - 1\right) \times 100$$

where R_{m+s} is the response ratio of the analyte in spiked matrix, R_m is the response ratio of the analyte in unspiked matrix, and R_0 is the response ratio of the analyte in matrix-free reference sample.

Results and discussion

The target compounds were selected based on the results of an EPA pilot study for PPCPs in fish tissue [33] and a previously reported PPCP screening method [5]. According to the EPA pilot study, carbamazepine, diltiazem, diphenhydramine, fluoxetine, norfluoxetine, and sertraline have been detected in fish collected from five effluent-dominated streams in various regions of the United States. Ramirez et al. [5] have also

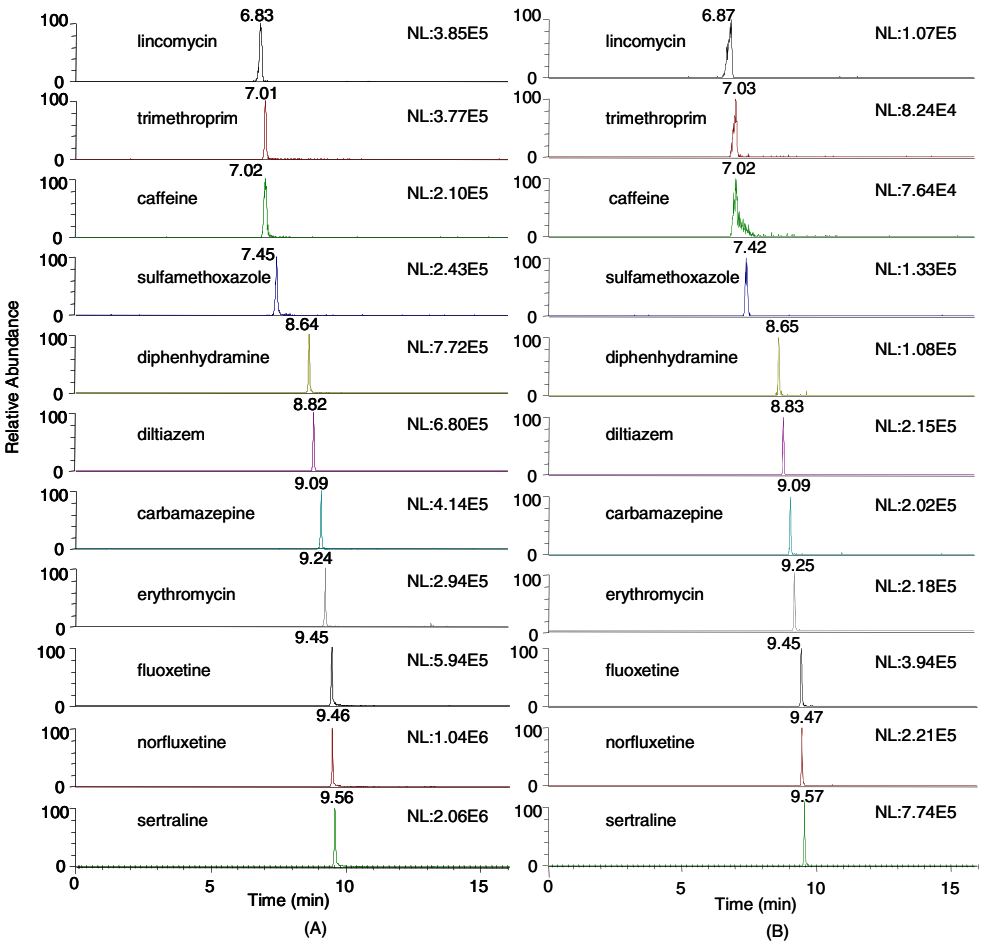

Fig. 1 Representative chromatograms of all selected pharmaceuticals standards on HESI (**A**) and ESI (**B**) sources at the same spiking level (Table 4)

reported the presence of diphenhydramine, diltiazem, carbamazepine, and norfluoxetine in fish collected from an effluent-dominated stream in Texas. In addition to the target compounds mentioned above, other pharmaceuticals such as trimethorprim, caffeine, sulfamethoxazole, and erythromycin that have been routinely detected in the reclaimed water used for ground irrigation at Florida International University Biscayne Bay Campus (North Miami Beach, FL) were also included in this study to assess potential accumulation from the reclaimed water [34].

Mass spectrometry

In order to achieve similar ionization behavior as those expected during real sample analysis, optimization of analyte responses was performed while the mobile phase was infused along with the standard solution through a tee connector at a speed of 50 μL/min. The most abundant molecular ion was selected as the precursor ion for that particular analyte. With the exception of erythromycin, protonated $[M+H]^+$ was found to be the most abundant precursor ion for all analytes. $[M+H-H_2O]^+$ was found to be the most abundant precursor ion for erythromycin which was in agreement with Hirsh et al. [35] who showed that erythromycin has already lost a water molecule when present in the aquatic environment. Once the precursor ion has been identified, Q3 was scanned to define product ions and to optimize the collision energy for each compound. Two SRM transitions were selected to avoid false positives and to give 4 identification points in accordance with EU Commission Decision 2002/657/EC [36]. All optimized parameters can be found in Table 1.

Both electrospray ionization (ESI) and heated electrospray ionization (HESI) probes were tested to obtain optimal ionization efficiency. HESI is designed to use ESI in combination with heated auxiliary gas that transforms ions in solution into ions in the gas phase more efficiently. As shown in Fig. 1, HESI probe showed significant signal improvement over ESI for all the compounds, in particular for early eluting compounds such as lincomycin, caffeine, and trimethoprim mainly because of better peak shapes and narrower peak width. Therefore, HESI was selected as the ion source in this study.

Accelerated solvent extraction for fish tissues

The most commonly used methods for extracting multiclass pharmaceuticals from fish tissues involve sonicating or vigorously shaking tissue homogenates with organic solvents such

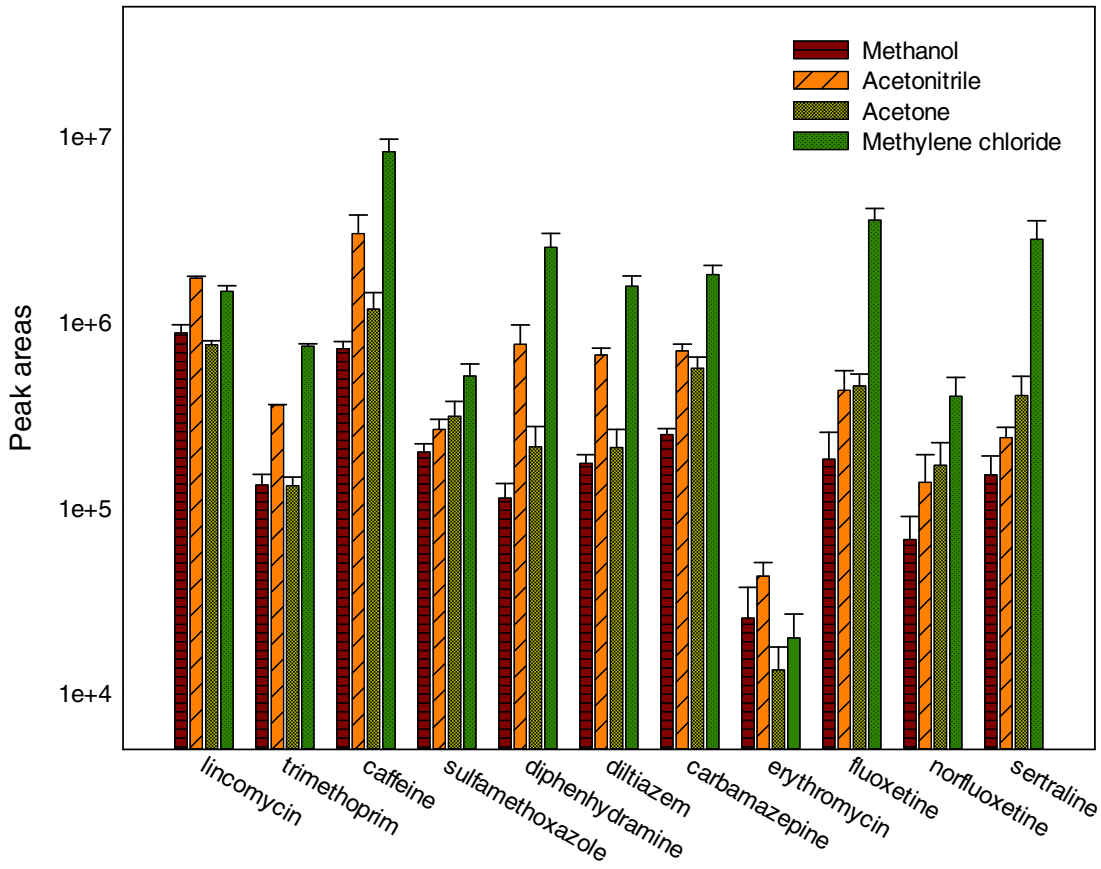

Fig. 2 ASE solvent selection based on absolute recoveries

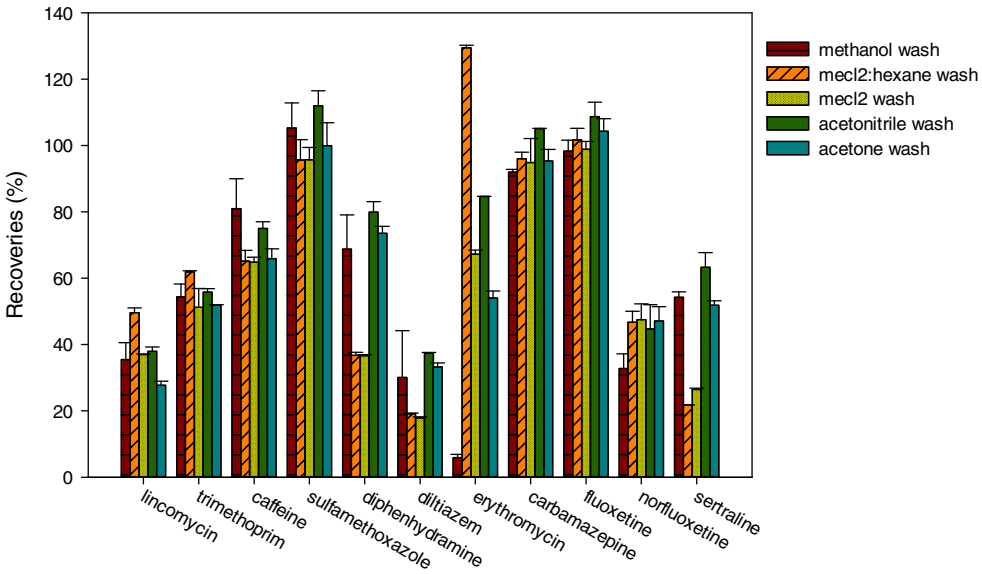

Fig. 3 Averaged (n=3) individual analyte recoveries in each wash solvent system

as acetonitrile and methanol followed by direct LC-MS analysis [5, 8, 13, 33]. However, in order to improve the extraction efficiency and method throughput, ASE was used because of its advantages over traditional methods, including automated extraction, higher recoveries, and smaller volume of extraction solvents [9, 10, 17, 23–29]. The first challenge when developing ASE method is to choose the appropriate extraction solvent. Various solvents including methanol, acetone, acetonitrile, and methylene chloride were tested for fish homogenate samples (1 g) fortified with all the analytes. The final selection of the extraction solvent was based on the "absolute recovery" calculated by subtracting the peak area of unspiked sample from that of the spiked sample. Absolute recoveries in different solvent systems are shown in Fig. 2, where methylene chloride showed the highest absolute recoveries for all the analytes except for lincomycin and erythromycin. Other ASE conditions such as oven temperature, pressure, static time and cycles, heat-up time, and flush volume were selected with slight modifications according to previously reported methods [9].

SPE cleanup

Although trace analysis at ng/g level can be achieved when using mass spectrometry as the detection method, matrix effects are still problematic due to co-eluting matrix components during the extraction procedures without additional cleanup steps [5, 33]. Gel permeation chromatography (GPC) is generally applied to remove lipid contents from biological tissues. However, this method requires large volume of organic solvent and an extra cleanup step is still needed to make the sample suitable for LC-MS analysis [9]. In contrast, mixed mode cation-exchange (MCX) SPE has been shown to be a successful alternative to cleanup complicated matrices such as fish tissues while still maintaining optimum recoveries of analytes with considerable ranges of p*K*a and lipophilicity [9]. MCX cartridges can provide much cleaner extract than regular HLB cartridges because organic solvents such as methanol or acetonitrile can be used to wash cartridges and eliminate interferences without losing the selectively retained analytes for further elution. The only requirement is that the

Table 2 Observed MDLs matrix effects and recoveries in fish tissue

	MDL (ng/g)	Ramirez's MDL(ng/g) [5]	Matrix Effects (%)	Recovery (%)
Lincomycin	1.05	5.53	−38±0.2	63±8
Trimethoprim	1.03	2.15	−65±1	83±4
Caffeine	0.81	3.93	33±3	64±13
Sulfamethoxazole	0.84	2.29	−54±1	44±4
Diphenhydramine	0.08	0.05	−40±1	85±4
Diltiazem	0.11	0.12	−95±1	60±16
Carbamazepine	0.10	0.54	−26±0.4	61±0.5
Erythromycin	0.51	6.42	20±0.5	28±0.4
Fluoxetine	1.19	6.73	−5±5	66±12
Norfluoxetine	0.41	2.90	−42±5	19±10
Sertraline	0.26	3.57	−26±2	80±1

Table 3 Observed MDLs matrix effects and recoveries in pond water and reclaimed water

	Pond water			Reclaimed water		
	MDL (ng/L)	Matrix effects (%)	Recovery (%)	MDL (ng/L)	Matrix effects (%)	Recovery (%)
Lincomycin	3.3	263±14	136±14	10.9	268±59	152±12
Trimethoprim	2.6	64±2	101±6	266	74±6	69±4
Caffeine	7.6	−7±6	101±8	348	33±6	75±13
Sulfamethoxazole	4.6	−14±3	104±5	67.5	9±4	94±14
Diphenhydramine	0.4	−41±0.8	82±1	53.1	−18±0.3	79±1
Diltiazem	0.2	−36±3	68±0.7	26.9	−49±4	83±0.2
Carbamazepine	1.5	−18±1	90±5	17.8	26±7	117±23
Erythromycin	15	−2±4	112±2	88.0	35±3	115±8
Fluoxetine	3.4	−3±4	95±6	5.0	7±0.9	86±0.1
Norfluoxetine	1.0	−44±2	32±2	1.8	−25±2	21±0.1
Sertraline	1.2	−9±3	56±0.6	4.9	−2±2	40±0.6

samples need to be acidified in order for analytes to be retained on the sorbent by cation exchange reactions. In addition, care must be taken during the pH adjustment because analytes could be lost during the loading and washing steps if the pH is too low [9]. Due to the fact that sulfamethoxazole has the lowest p*K*a of 5.8 among all the analytes [37], ASE extract was adjusted with acetic acid to pH 3.3 which is approximately 2 units below the p*K*a of sulfamethoxazole. Five solvents including methanol, acetonitrile, acetone, methylene chloride, and hexane–methylene chloride (50/50, *v*/*v*) mixture were tested as the wash solvents. Individual analyte recoveries in each solvent system are plotted in Fig. 3, where error bars represent standard deviations from average recoveries (n=3). Because acetonitrile is a stronger eluent than methanol [38], higher recoveries were expected from the methanol wash. However, acetontitrile was found to be the most effective solvent at removing interferences from the cartridge while offering maximum recovery and good overall precision.

Matrix effects

All compounds were analyzed for potential matrix effects in HESI positive mode and results are shown in Table 2 along with the statistically derived method detection limits (MDLs; n=7). Positive values indicate signal enhancement and negative values indicate signal suppression due to the matrix effects. As shown in Table 2, caffeine and erythromycin showed moderate signal enhancements. Similar results were also observed by Ramirez et al. [5]. Other compounds showed various degrees of signal suppression.

For water samples, matrix effects were evaluated using the same procedure as for fish tissues (Table 3). Organic

Table 4 Fortification levels for matrix effect assessment, recovery calculation, and MDL determination in fish tissues and water samples

	Fortification levels in fish tissues (ng/g)			Fortification levels in water (ng/L)		
	Matrix effect assessment	Recovery calculation	MDL determination	Matrix effect assessment	Recovery assessment	MDL determination
Lincomycin	9.9	9.9	1.0	40.0	40.0	4.0
Trimethoprim	9.9	9.9	1.0	40.0	40.0	4.0
Caffeine	39.9	39.9	4.0	160	160	16.0
Sulfamethoxazole	39.9	39.9	4.0	160	160	16.0
Diphenhydramine	2.0	2.0	0.2	8.0	8.0	0.8
Diltiazem	2.0	2.0	0.2	8.0	8.0	0.8
Carbamazepine	4.0	4.0	0.4	16.0	16.0	1.6
Erythromycin	20.1	20.1	2.0	80.4	80.4	8.0
Fluoxetine	60.0	60.0	6.0	240	240	24.0
Norfluoxetine	30.0	30.0	3.0	120	120	12.0
Sertraline	19.9	19.9	2.0	80.0	80.0	8.0

Table 5 Summary of concentrations of target compounds in reclaimed water and pond water

Compounds	Reclaimed water (ng/L) $n=17$					Pond water (ng/L) $n=9$				
	Mean	SD	Max	Min	Median	Mean	SD	Max	Min	Median
Lincomycin	*ND*		*ND*	*ND*	*ND*	*ND*		*ND*	*ND*	*ND*
Trimethoprim	338	273	920	16	254	1.3	3.7	11	*ND*	*ND*
Caffeine	1,476	1,177	4,315	53	1,217	81	48	172	23	63
Sulfamethoxazole	241	128	409	3.0	263	8.0	5.2	14	*ND*	10
Diphenhydramine	89	48	179	6.3	77	0.67	0.38	1.3	*ND*	0.62
Diltiazem	29	30	111	*ND*	22	*ND*		*ND*	*ND*	*ND*
Carbamazepine	97	55	229	20	83	4.5	1.9	6.6	1.9	5.4
Erythromycin	79	37	141	*ND*	88	*ND*		*ND*	*ND*	*ND*
Fluoxetine	9.7	5.4	24	*ND*	9.7	*ND*		*ND*	*ND*	*ND*
Norfluoxetine	*ND*		*ND*	*ND*	*ND*	*ND*		*ND*	*ND*	*ND*
Sertraline	*ND*		*ND*	*ND*	*ND*	*ND*		*ND*	*ND*	*ND*

ND less than MDL

matter in surface waters has shown to play an important role on matrix effects [32]. The overall results indicate that reclaimed water showed more pronounced effects than pond water for most of the compounds. Signal enhancement was observed on seven compounds in reclaimed water samples while only two compounds showed signal enhancement in the pond water samples.

Analytical performance

Analyte concentrations in fish tissues were determined based on response factors (RFs) of the target compounds relative to the surrogate internal standards. This approach can be used for most trace analysis, as it does not require blank matrix and greatly alleviates the signal suppression or enhancement arising from matrix effects that can affect the sensitivity and response of the mass spectrometer in unpredictable ways.

MDLs were used to evaluate the analytical performance in different matrices and reported in Tables 2 and 3. MDL represents the lowest concentration of the analyte that can be measured and reported with 99 % confidence in a given matrix is greater than zero [39]. It has been discussed and generally assumed that experimentally derived MDLs in a given matrix are appropriate for establishing detection threshold in environmental analysis [4, 5, 21]. Seven replicates of 1 g of clean fish tissues ($n=7$) were spiked with an appropriate amounts of analytes (for spike levels see Table 4) and extracted as described above. Similarly, seven replicates of 250 mL pond water and 250 mL reclaimed water were spiked at the same level and subjected to the SPE-LC-MS/MS analysis. MDLs were then calculated by multiplying the one-side Student's t value at 99 % confidence by the sample standard deviation derived from the concentrations of seven replicate spiked samples [39]. Concentrations below MDLs were reported as "not detected." Compared to the previous studies [5], the protocol demonstrated here offers cleaner fish extracts giving lower MDLs for 10 out of 11 selected compounds. The MDL for diphenhydramine was slightly higher but similar to the value reported by Ramirez et al. [5]. Concentrations of diphenhydramine detected in fish tissues, however, were considerably higher than the calculated MDL. For water samples, MDLs in reclaimed water were significantly higher than those in pond water because of both a more complicated matrix and higher overall concentrations that produced larger standard deviations. The recoveries of norfluoxetine were found at 19 % in fish, 32 % in pond water, and 21 % in reclaimed water, respectively. The low recovery is likely due to the lack of methyl group in norfluoxetine increasing the water solubility compared to fluoxetine.

Table 6 Concentrations (ng/g) of target compounds detected in fish tissue ($n=7$) from the freshwater pond

Compounds	Mean	SD	Max	Min	Median
Lincomycin	*ND*		*ND*	*ND*	*ND*
Trimethoprim	*ND*		*ND*	*ND*	*ND*
Caffeine	1.3	1.6	4.5	*ND*	1.2
Sulfamethoxazole	*ND*		*ND*	*ND*	*ND*
Diphenhydramine	0.55	0.27	0.97	0.08	0.59
Diltiazem	*ND*		*ND*	*ND*	*ND*
Carbamazepine	0.20	0.25	0.66	*ND*	0.10
Erythromycin	*ND*		*ND*	*ND*	*ND*
Fluoxetine	*ND*		*ND*	*ND*	*ND*
Norfluoxetine	*ND*		*ND*	*ND*	*ND*
Sertraline	*ND*		*ND*	*ND*	*ND*

ND less than MDL

Analysis of environmental samples

Mosquito fish (*G. holbrooki*) sampled from a saltwater marsh not influenced by reclaimed water and a freshwater pond affected by reclaimed water influence were analyzed for target analytes. Reclaimed water and pond water samples collected over a period of 2 months were also concurrently analyzed. Concentrations of target analytes in water samples are summarized in Table 5. 73 % of target compounds were consistently detected in reclaimed water samples. However, fluoxetine, diltiazem, and erythromycin were not detected in the pond water influenced by reclaimed water. The possible explanation is that these compounds can be rapidly dissipated from the water phase as a result of adsorption to sediment where they seem to be persistent [40–43]. Therefore, it is not surprising that neither fluoxetine nor diltiazem was detected in fish tissues in this study even though they have been reported to accumulate in fish [5, 7, 9]. As shown in Table 6, caffeine, diphenhydramine, and carbamazepine were detected in fish tissues from the freshwater pond but no target compounds were detected in those from the saltwater marsh. Bioaccumulation factors for caffeine, diphenhydramine, and carbamazepine in mosquito fish (*G. holbrooki*) were calculated accordingly and found at 29±26, 821±422, and 108±144, respectively. The calculated bioaccumulation factor (BAF) for carbamazepine was consistent with literature value [44]. All the resulting BAFs were higher than those calculated based on log K_{ow} (octanol/water partition coefficient) [45] and EPA K_{ow} based Aquatic BioAccumulation Model (KABAM; 1 for caffeine, 93 for diphenhydramine, and 14 for carbamazepine) [46]. Upon plotting a graph of BAF vs log K_{ow}, a positive relationship was observed (R^2=0.5665) which was consistent with previously proposed theory [47]. While diphenhydramine and carbamazepine have been previously observed in fish tissues [5], potential accumulation of caffeine in fish is reported here for the first time.

Conclusions

This study presents the development of a new method for the analysis of selected pharmaceuticals in fish tissue, reclaimed water, and surface water directly affected by reclaimed water. Compared to previous methods, accelerated solvent extraction followed by MCX mixed-mode SPE cleanup provided a better alternative due to cleaner extracts giving lower method detection limits. 73 % and 45 % of all target compounds were consistently detected in reclaimed water and surface water, respectively. Caffeine, diphenhydramine, and carbamazepine were detected in mosquito fish from the freshwater pond directly affected by reclaimed water influence but they were not detected in those from the saltwater mash. Bioaccumulation factors for caffeine, diphenhydramine, and carbamazepine in mosquito fish were also calculated and found at 29±26, 821±422, and 108±144, respectively.

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