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Rapid analysis of pharmaceuticals and personal care products in fish plasma micro-aliquots using liquid chromatography tandem mass spectrometry

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ABSTRACT

A sensitive analytical method based on liquid–liquid extraction (LLE) and liquid chromatography tandem mass spectrometry (LC–MS/MS) was developed for rapid analysis of 11 pharmaceuticals and personal care products (PPCPs) in fish plasma micro-aliquots (~20 μL). Target PPCPs included, bisphenol A, carbamazepine, diclofenac, fluoxetine, gemfibrozil, ibuprofen, naproxen, risperidone, sertraline, simvastatin and triclosan. A relatively quicker and cheaper LLE procedure exhibited comparable analyte recoveries with solid-phase extraction. Rapid separation and analysis of target compounds in fish plasma extracts was achieved by employing a high efficiency C-18 HPLC column (Agilent Poroshell 120 SB-C18, 2.1 mm × 50 mm, 2.7 μm) and fast polarity switching, enabling effective monitoring of positive and negative ions in a single 9 min run. With the exception of bisphenol A, which exhibited relatively high background contamination, method detection limits of individual PPCPs ranged between 0.15 and 0.69 pg/μL, while method quantification limits were between 0.05 and 2.3 pg/μL. Mean matrix effect (ME) values ranged between 65 and 156% for the various target analytes. Isotope dilution quantification using isotopically labelled internal surrogates was utilized to correct for signal suppression or enhancement and analyte losses during sample preparation. The method was evaluated by analysis of 20 μL plasma micro-aliquots collected from zebrafish (*Danio rerio*) from a laboratory bioaccumulation study, which included control group fish (no exposure), as well as fish exposed to environmentally relevant concentrations of PPCPs. Using the developed LC–MS/MS based method, concentrations of the studied PPCPs were consistently detected in the low pg/μL (ppb) range. The method may be useful for investigations requiring fast, reliable concentration measurements of PPCPs in fish plasma. In particular, the method may be applicable for in situ contaminant biomonitoring, as well as bioaccumulation and toxicology studies employing small fishes with low blood compartment volumes.

1. Introduction

The occurrence of pharmaceuticals and personal care products (PPCPs) in the environment has received increasing attention in recent years. PPCPs enter into the environment through individual human activity and as residues from agriculture, manufacturing, veterinary use, as well as community and hospital use [1]. Numerous studies have reported PPCP residues in waste water treatment plant (WWTP) influents and effluents, as well as surface waters, with concentrations generally ranging between 0.001 and 1 μg/L [2–9].

PPCPs can accumulate in tissues of aquatic organisms [10–14] and toxicity tests indicate many of these compounds can cause chronic low-level effects [15–20]. Paroxetine and fluoxetine, commonly prescribed selective serotonin reuptake inhibitor (SSRI) antidepressants, have been detected in fish muscle tissue from Hamilton Harbour, Canada, at concentrations of 0.58 ng/g and 1.02 ng/g, respectively [12]. A recent national pilot study in the United States to assess the accumulation of PPCPs in fish demonstrated the presence of norfluoxetine, sertraline, diphenhydramine, diltiazem, and carbamazepine at ng/g concentrations in fillet composites from effluent-dominated surface waters [21]. The survey showed the anticonvulsant, carbamazepine, was present at a concentration of 2.3 ng/g in fish fillets in Chicago, USA. Sertraline was detected at concentrations as high as 19 and 545 ng/g in fish fillets and liver, respectively.

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While these and other studies have focused on assessing the occurrence and levels of PPCPs in fish muscle and liver tissue, there are relatively few studies of these contaminants of emerging concern in fish plasma. Previously, carbamazepine and ibuprofen residues were detected at 0.3 pg/μL and 102 pg/μL, respectively, in plasma of fish exposed to treated sewage effluents in Sweden [13]. Tanoue et al. [14] reported concentrations of several PPCPs in plasma of cyprinoid fish from an effluent-dominated stream in Japan, with concentrations ranging between 0.03 and 110 pg/μL.

The concentration of xenobiotic compounds in blood plasma is a key parameter for comparative toxicology and pharmacology studies. Plasma concentrations can provide important information regarding recent exposure conditions [21,22]. The "Fish Plasma Model" has been proposed for prioritizing pharmaceuticals for in-depth ecological risk assessment [22,23]. The model utilizes estimated drug concentrations in fish plasma and human therapeutic plasma concentration data to assess exposure risks in fish. Implementation of this approach will require fast, reliable measurements of PPCP residue concentrations in fish plasma.

Currently, there are relatively few multi-residue analytical methods for trace quantification of PPCPs in fish plasma. Previous methods [13,14] have employed sample preparation techniques such as ultrasound assisted extraction (UAE), gel-permeation chromatography (GPC) and solid-phase extraction (SPE), prior to analysis by liquid chromatography tandem mass spectrometry (LC–MS/MS). These methods require relatively large amounts of plasma for analysis (0.5–2 mL). In many cases however, only microliter volumes of plasma can be attained from fish. This is especially true for small fish species.

The objective of the present study was to develop a multi-residue analytical method for rapid analysis of PPCPs in fish plasma micro-aliquots (20 μL). Target analytes included 11 key PPCPs that are commonly detected in environmental and biological samples, including bisphenol A (BPA), carbamazepine, diclofenac, fluoxetine, gemfibrozil, ibuprofen, naproxen, risperidone, sertraline, simvastatin and triclosan. The developed method was evaluated and further applied for the analysis of these contaminants of emerging concern in plasma micro-aliquots of zebrafish (*Danio rerio*) subjected to environmentally relevant (parts per billion) levels.

2. Experimental

2.1. Materials

High purity (>97%) standards of diclofenac, naproxen, triclosan, ibuprofen, gemfibrozil, sertraline, risperidone, simvastatin were obtained from Sigma–Aldrich Co. LLC. (St. Louis, USA). Bisphenol A was obtained from Merck (Hohenbrunn, Germany). Carbamazepine was purchased from TCI-EP (Tokyo, Japan). Fluoxetine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Isotopically labelled compounds, propanolol-d_7, ibuprofen-d_3, fluoxetine-d_5, carbamazepine-d_{10} and warfarin-d_5, were purchased from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada). Bisphenol A-d_6, $^{13}C_6$-2,4,5-trichlorophenoxyacetic acid ($^{13}C_6$-TCPAA) and gemfibrozil-d_6 were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). $^{13}C_{12}$-Triclosan was purchased from Wellington Laboratories Inc. (Ontario, Canada). HPLC grade Acetone, methanol and acetonitrile were purchased from Fisher Scientific Inc. (Loughborough, UK). All standards were prepared in methanol and stored at −20 °C.

2.2. Collection of fish plasma micro-aliquots

Adult zebrafish were purchased from a local fish farm in Singapore (Mainland Tropical Fish Farm, Singapore). A flow-through experimental system was employed to expose fish to the studied PPCPs. Fish were separated into three groups, a low dose group, a high dose group and a control group. The low dose fish were exposed to a mixture of the studied PPCPs, with water concentrations ranging between 0.01 and 5 μg/L. Concentrations of individual PPCPs in water for the high dose group ranged between 0.04 and 12 μg/L. Control fish were subjected to the same conditions but without any exposure to the PPCPs. During the exposure period all fish were fed a diet consisting of artemia, twice daily. At the end of exposure, zebrafish were euthanized by submersion in ice water for 5 min. It cannot be submersed for too long otherwise blood clots will occur. The tail of the fish was cut off using sterilized spring scissors to expose caudal vein and blood was collected via pipette (Eppendorf Research, Germany). To maximize blood collection efficiency, we used 200 μL Prot/Elec tips with an elongated capillary tip of 10 μL (#223-9915, Bio-Rad, USA). Tips were fully filled with 18 mg/mL of EDTA (E5134, Sigma–Aldrich) and immersed in the same solution for at least 24 h, completely dried at 60 °C, then cooled to room temperature. The anticoagulant coated tips were prepared within 1 week prior to use. It was found that approximately 10–20 μL of whole blood can be collected from one adult zebrafish. The whole blood was placed in a BD microtainer lithium heparin tube (#422-365971, BD, USA). The plasma was separated by centrifugation at 3000 × *g* for 10 min at 4 °C (Eppendorf Centrifuge 5417R). Then plasma was transferred into a 2 mL centrifuge tube and stored at −80 °C prior to extraction.

2.3. Sample preparation

For extraction of target analytes from the fish plasma micro-aliquots (20 μL), we tested and evaluated two different protocols, including LLE and SPE. To check for background contamination, a procedural blank consisting of 20 μL of Milli-Q water was processed with every batch of 10 samples.

For LLE, 20 μL of thawed plasma (pooled from five individual fish) was added to a 2 mL centrifuge tube and spiked with eight isotopically labelled internal surrogate (IS) compounds (propanolol-d_7, ibuprofen-d_3, fluoxetine-d_5, carbamazepine-d_{10}, warfarin-d_5, bisphenol A-d_6, gemfibrozil-d_6 and $^{13}C_{12}$-triclosan). For six of the 11 target analytes, a matching mass labelled compound was available, while five of the target compounds were assigned a labelled compound exhibiting similar physicochemical properties, matrix effect and method recovery. A list of the corresponding internal surrogate compounds used to quantify the various target analytes is shown in Table 1. The amount of internal surrogate compounds spiked varied between 7.5 and 256.8 ng, depending on analyte sensitivity (Table 1). 1 mL of ice-cold acetone was added to extract target compounds and to precipitate proteins. Centrifuge tubes were sonicated for 3 min, then centrifuged at 12,000 rpm under 4 °C for 5 min. Extraction was repeated a second time with an additional 1 mL of acetone. The combined supernatant was evaporated under a gentle nitrogen stream to dryness. The test tube was reconstituted with 120 μL of methanol:water (1:4, v/v). Final extracts were transferred into LC vials and spiked with 5 μL of the injection internal standard, $^{13}C_6$-TCPAA (5 mg/L). The main purpose of the injection internal standard, $^{13}C_6$-TCPAA, is to determine the absolute recovery of internal surrogate compounds spiked prior to extraction [24]. All extracts were stored in darkness at 4 °C prior to LC–MS/MS analysis.

For SPE, 1.5 mL of 1% formic acid was added to 20 μL plasma micro-aliquots, then spiked with internal surrogate standards. The mixture was diluted to 5 mL with Milli-Q water before SPE. SPE was performed using Phenomenex X-33u 200 mg SPE cartridges. The conditioning of the SPE cartridges was performed with 5 mL of methanol followed by 5 mL of Milli-Q water. A vacuum manifold system was employed. The 5 mL samples were passed through the cartridges at a flow rate of 5 mL/min. The cartridges were

Table 1
List of target PPCP compounds, along with the compound class, molecular structure, acid dissociation constant (pK_a), octanol–water distribution coefficient ($\log D_{ow}$), name and spiking amount (ng) of internal surrogate compounds (IS) used for isotope dilution quantification.

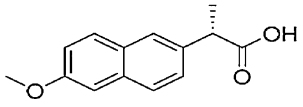
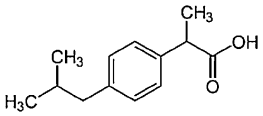
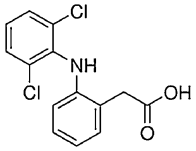
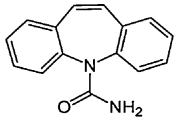
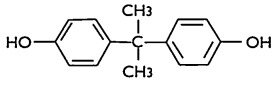
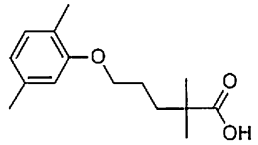
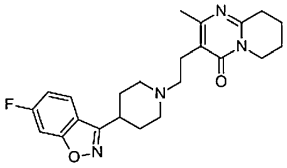
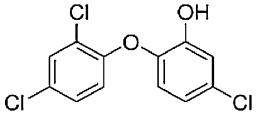
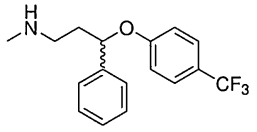
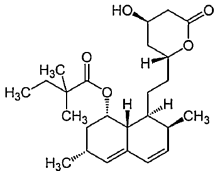
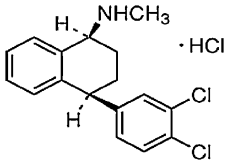
Compound	Class	Structure	pK_a [a]	$\log D_{ow}$ [a] (pH 7)	Internal surrogate compound (IS)	Amount of IS spiked (ng)
Naproxen	Non-steroidal anti-inflammatory drug (NSAID)		4.4[b]	0.76	Wafarin-d_5	99.1
Ibuprofen	NSAID		4.2[b]	1.09	Ibuprofen-d_3	233.2
Diclofenac	NSAID		4.0[b]	1.26	Ibuprofen-d_3	233.2
Carbamazepine	Anti-seizure		15.4[c]	3.52	Carbamazepine-d_{10}	25.1
Bisphenol A	Plasticizer		9.5[c]	4.25	Bisphenol A-d_6	248.7
Gemfibrozil	Fibrates		4.7[b]	1.93	Gemfibrozil-d_6	7.5
Risperidone	Atypical anti-psychotic		8.2[d]	1.04	Proponolol-d_7	249.6
Triclosan	Antibacterial and antifungal		7.8[c]	5.31	$^{13}C_{12}$-Triclosan	12.5
Fluoxetine	Antidepressant		10.0[d]	1.59	Fluoxetine-d_5	256.8
Simvastatin	Antilipidemic		13.5[c]	4.17	$^{13}C_{12}$-Triclosan	12.5

Table 1 (*Continued*)

Compound	Class	Structure	pK_a [a]	Log D_{ow} [a] (pH 7)	Internal surrogate compound (IS)	Amount of IS spiked (ng)
Sertraline	SSRI anti-depressant		9.5[d]	2.88	Fluoxetine-d_5	256.8

[a] Estimated values, obtained from SPARC.
[b] Weak acid.
[c] Neutral compound.
[d] Weak base.

then washed with 5 mL of 5% methanol in water and then eluted with 3 mL of acetone, followed by 5 mL of methanol. The extracts were evaporated to dryness under a gentle nitrogen stream. The test tubes were reconstituted in 120 μL of methanol:water (1:4, v/v), transferred to LC vials and spiked with the injection internal standard (25 ng of $^{13}C_6$-TCPAA).

2.4. Identification and quantification of target analytes using LC–MS/MS

Liquid chromatography was performed with a DIONEX Ultimate 3000 HPLC system. Compounds were separated chromatographically using a high efficiency Agilent Poroshell 120 SB-C18 column (2.1 mm × 50 mm, 2.7 μm). The high-efficiency Poroshell 120 column can provide high efficiency at lower pressures, thus avoiding the need for ultra-high performance liquid chromatography and sub 2 μm columns. The column was maintained at a temperature of 40 °C for the entire run. The mobile phase used for chromatographic separations consisted of a binary mixture of solvents A (5 mM ammonium acetate in Milli-Q water) and B (methanol:acetonitrile, 1:1, v/v), at a flow rate of 0.3 mL/min. The gradient elution was as follows: initial conditions, 20% B, 0.5–4.0 min, increase linearly to 100% B; 4.0–6.5 min, 100% B; 6.5–6.7, return to initial conditions, 6.7–9 min, 20% B. The sample volume injected was 10 μL.

The HPLC was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbo Ion Spray source. Fast polarity switching was employed for operation in both electro-spray ionization (ESI) positive and negative modes with multiple reaction monitoring (MRM). Source-dependent parameters for compounds analyzed in ESI positive mode were: curtain gas, 30 psi; nitrogen collision gas, high; source temperature was 550 °C; ion spray voltage was 5500 V; ion source gases GS1 and GS2 were set at 55 and 60 psi, respectively. For compounds analyzed in negative mode, parameters were as follows: curtain gas, 15 psi; ion spray voltage was −4500 V; while all other parameters were the same as in ESI positive mode. Identification and quantification of all target analytes and internal surrogate compounds were conducted via tandem mass spectrometry. Ions were acquired in multiple reaction monitoring (MRM) mode, with a dwell time of 20 ms. Optimum ion transitions and MS operating conditions were determined by infusion with a syringe pump. A summary of the monitored ion transitions and corresponding operating parameters is shown in Table 2. For tandem MS, the overall number of MRM transitions monitored influences the degree of data points acquired. For the

Table 2
MRM transitions and MS parameters for target PPCP compounds and isotope labelled surrogate standards.

Compound	Ion transitions (m/z)[a]	Retention time (min)	Dwell time (ms)	Polarity	Declustering potential (V)	Entrance potential (V)	Collision energy (eV)	Collision cell exit potential (V)
Naproxen	229.2 > 169.2	2.51	20	–	−50	−6	−41	−6
	229.2 > 170	2.51	20	–	−50	−6	−19	−9
Warfarin-d_5	312.1 > 160.9	2.59	20	–	−236	−12	−34	−9
$^{13}C_6$-TCPAA[b]	259 > 201	2.76	20	–	−34	−10	−19	−7
Ibuprofen	205 > 159	3.39	20	–	−37	−3	−9	−5
Ibuprofen-d_3	208.2 > 163.8	3.37	20	–	−45	−7	−12	−12
Diclofenac	294 > 250	3.39	20	–	−34	−10	−15	−5
	294 > 34.9	3.39	20	–	−34	−10	−54	−4
Carbamazepine	237.1 > 194	3.61	20	+	187	7	27	11
Carbamazepine-d_{10}	247.1 > 204.1	3.61	20	+	156	9	27	12
Bisphenol A	227.1 > 133	3.86	20	–	−130	−7	−31	−8
Bisphenol A-d_6	233.1 > 138	3.86	20	–	−120	−11	−33	−7
Gemfibrozil	249.1 > 121	4.08	20	–	−60	−4	−20	−6
Gemfibrozil-d_6	255.3 > 121.1	4.08	20	–	−258	−10	−22	−6
Risperidone	411.5 > 190.8	4.27	20	+	238	5	28	14
	411.5 > 110	4.27	20	+	238	5	69	7
Proponolol-d_7	267.2 > 116	4.34	20	+	209	11	25	6
Triclosan	286.8 > 35	4.84	20	–	−80	−11	−39	−9
$^{13}C_{12}$-Triclosan	299 > 35	4.84	20	–	−57	−10	−39	−6
Fluoxetine	310.2 > 44.1	5.11	20	+	61	8	54	11
Fluoxetine-d_5	315.4 > 44	5.11	20	+	151	7	57	11
Simvastatin	399 > 115	5.16	20	–	−55	−3	−31	−2
Sertraline	306.2 > 159	5.37	20	+	45	5	36	9
	306.2 > 275.2	5.37	20	+	45	5	17	51

[a] For compounds with two ion transitions, the first MRM transition was used for quantification and the second for confirmation. Only one MRM transition was monitored for internal standards.
[b] $^{13}C_6$-2,4,5-trichlorophenoxyacetic acid used as injection internal standard for determining recovery of internal surrogate compounds spiked.

purpose of this study, only one MRM transition was monitored for labelled internal standards, as well as analytes which structurally similar isotopically labelled internal surrogates were available (e.g., ibuprofen, carbamazepine, BPA, gemfibrozil, triclosan, fluoxetine). For analytes that were assigned an alternative internal surrogate compound (e.g., diclofenac, naproxen, risperidone, sertraline and simvastatin), two MRM transitions were monitored.

Following Method 1694 by US EPA [24], we utilized an isotope dilution calibration approach. Specifically, a series of five calibration standard solutions (CS1–CS5) was prepared, with varied concentrations of the target analytes (range: 0.5–300 pg/μL) and a fixed concentration of the internal surrogate compounds. The relative response (RR) for each target analyte was computed for the five calibration solutions. Mean RR values were used for quantification only if the relative standard deviation (RSD) of the five observed RR values was below 20%, thereby indicating good linearity [24].

2.5. Method performance assessment

Method validation experiments involved extraction and analysis of plasma samples fortified with target analytes at the anticipated concentration level (10–100 ppb in plasma), (Table 1). Relative and absolute recoveries were determined and assessed according to US EPA protocols [24].

To evaluate matrix effects for the target compounds we employed the approach proposed by Matuszewski et al. [25]. Specifically, 40 μL of matrix (zebrafish plasma from control fish) was spiked with 5 μL of target analyte mixture, then extracted and reconstituted in 80 μL of methanol. This extract was divided into two sub-samples, A and B. Sub-sample A (40 μL) was spiked with 10 μL of the target analyte mixture, along with 50 μL of Milli-Q water. Sub-sample B (40 μL) was diluted with 10 μL of methanol, along with 50 μL of Milli-Q water. A separate analytical standard (S) was prepared by combining 40 μL of methanol with 10 μL of the target analyte mixture and 50 μL of Mill-Q water. By comparing analyte peak areas of sub-samples and the analytical standard, a matrix effect (ME) value was calculated as:

$$\mathrm{ME}\,(\%) = 100 \times \frac{A_i - B_i}{S_i} \quad (1)$$

where: A_i, B_i and S_i are the peak areas of the analytes (i) in subsample A, subsample B and the analytical standard (S), respectively. An ME of 100% indicates no matrix effect. Signal suppression or enhancement are indicated by ME values <100% or >100%, respectively [12].

3. Results and discussion

3.1. Recovery of analytes by LLE and SPE

Both the Phenomenex X-33u SPE cartridge and LLE extraction protocols performed relatively well. Absolute recoveries of spiked native target analytes were above 50% (Fig. 1). The highest recoveries were observed for simvastatin, while triclosan exhibited the lowest. For example, using LLE the absolute recoveries of spiked simvastatin and triclosan were 137 ± 16% and 55 ± 1.89%, respectively. Using isotope dilution quantification to correct for losses, relative recoveries of nearly 100% (85–120%) were achieved for all compounds, demonstrating good accuracy when using the assigned internal surrogate compounds.

The investigated target analytes are from multiple compound classes and exhibit a variety of functional groups and range widely in physicochemical properties such as acid dissociation constant (pK_a) and octanol–water distribution coefficient (D_{ow}). It is often difficult to find a single extraction protocol that can universally work to capture multi-class PPCPs. With the exception of sertraline and fluoxetine, absolute recoveries observed with LLE approach were comparable to SPE. The LLE method provides satisfactory recoveries of multi-class PPCPs, spanning a range of pK_a between 4 and 13 and log D_{ow}'s between 0.7 and 5.3. The results indicate the relatively faster and cheaper LLE protocol is an effective strategy for extraction of these target PPCPs from fish plasma micro-aliquots.

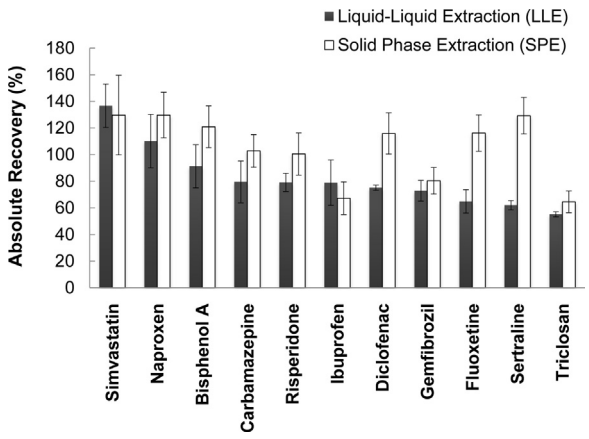


Fig. 1. Absolute recovery (%) of individual PPCPs for extraction of 20 μL fish plasma micro-aliquot using LLE and SPE. Data are presented as mean recovery, with error bars representing standard deviation of triplicate samples ($n = 3$).

3.2. Matrix effects

LC–MS/MS is widely used for identification and quantification of PPCPs in environmental and biological samples. It is well-established that this analytical technique suffers from ion suppression or enhancement related to sample matrix interference. The average ME values ($n = 3$) for target analytes are shown in Table 3. The average ME values of diclofenac, gemfibrozil, ibuprofen, naproxen, BPA were around 100%, indicating negligible matrix effects. The average ME values of simvastatin, fluoxetine and sertraline were 74.4, 78.9 and 65.4%, respectively, indicating moderate signal suppression. The average ME value was 28.21% for triclosan which indicates significant ion suppression. The average ME values of carbamazepine and risperidone were 156 and 136%, respectively, indicating signal enhancement. The relatively high mean ME for carbamazepine (156%) also exhibited high variability among triplicate samples (RSD = 42.84%), indicating a potential ME range of 113–198%. It is important to note that ME values in this range are relatively common using LC–MS/MS. Moreover, the observed matrix effect for carbamazepine will likely have negligible effect on measurement accuracy, as this method utilizes deuterium labelled carbamazepine-d_{10} for isotope dilution quantification of this compound.

Table 3
Matrix effect (ME, %), method detection limits (MDL) and method quantification limits (MQL) for target PPCPs in fish plasma.

Compound	Mean matrix effect (ME, %) ± SD	MDL (pg/μL)	MQL (pg/μL)
Naproxen	100.3 ± 6.3	0.69	2.30
Ibuprofen	118.9 ± 2.7	0.63	2.10
Diclofenac	104.8 ± 5.8	0.48	1.60
Carbamazepine	156.0 ± 42.8	0.19	0.64
Bisphenol A	102.8 ± 11.4	8.11	27.00
Gemfibrozil	105.1 ± 6.2	0.09	0.30
Risperidone	136.3 ± 13.1	0.015	0.05
Triclosan	28.2 ± 4.9	0.39	1.31
Fluoxetine	78.9 ± 4.9	1.30	4.33
Simvastatin	74.4 ± 8.2	0.12	0.38
Sertraline	65.4 ± 10.2	0.42	1.40

Table 4
Measured concentrations (mean ± SD, pg/μL) of PPCPs in zebrafish plasma micro-aliquots for control, low-dose and high dose exposure groups.

	Plasma Concentration (pg/μL)						
	Control group (n = 20)	Low dose (n = 3) (4 h)	Low dose (n = 3) (24 h)	Low dose (n = 3) (3 days)	High dose (n = 3) (4 h)	High dose (n = 3) (24 h)	High dose (n = 3) (3 days)
Naproxen	<MDL	25.80 ± 2.37	50.30 ± 1.34	15.00 ± 0.78	131.00 ± 3.67	279.00 ± 17.40	82.70 ± 6.42
Ibuprofen	0.94 ± 0.31	13.00 ± 0.10	16.30 ± 0.65	9.09 ± 0.62	68.30 ± 9.57	77.40 ± 6.69	49.70 ± 4.14
Diclofenac	1.74 ±1.48	1.52 ± 0.38	2.68 ± 0.28	0.99 ± 0.46	11.20 ± 0.67	25.20 ± 1.96	17.50 ± 1.79
Carbamazepine	0.40 ± 0.38	0.80 ± 0.04	2.30 ± 0.28	1.10 ± 0.17	7.65 ± 0.01	13.20 ±1.26	7.32 ± 0.75
Bisphenol A	<MDL	<MDL	<MDL	<MDL	17.60 ± 2.20	10.50 ± 1.60	10.40 ± 0.48
Gemfibrozil	0.35 ± 0.31	32.10 ± 1.90	46.60 ± 3.50	28.50 ± 1.09	174.00 ± 13.40	235.00 ± 38.00	179.00 ± 8.60
Risperidone	<MDL	<MDL	0.07 ± 0.01	0.093 ± 0.003	0.21 ± 0.02	0.65 ± 0.01	0.88 ± 0.03
Triclosan	0.98 ± 0.04	0.90 ± 0.04	0.95 ± 0.25	0.93 ± 0.03	2.08 ± 0.43	3.21 ± 0.24	4.41 ± 0.37
Fluoxetine	<MDL	<MDL	<MDL	1.32 ± 0.14	2.20 ± 0.43	7.13 ± 0.93	6.90 ± 0.70
Simvastatin	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Sertraline	<MDL	<MDL	<MDL	0.44 ± 0.39	1.83 ± 0.73	2.20 ±0.50	2.26 ± 0.17

Triclosan and sertraline exhibited significant matrix effects, with a relatively high degree of signal suppression. Isotope dilution quantification of triclosan residues using $^{13}C_{12}$-triclosan provided effective resolution of this effect. We did not utilize an isotopically labelled sertraline internal surrogate compound in the present study. However, use of the structurally similar labelled internal surrogate, fluoxetine-d_5, an SSRI antidepressant, was sufficient, as the recovery and matrix effects were comparable. This was also the case for the four other target analytes that were assigned an alternative labelled compound, including diclofenac (IS; ibuprofen-d_3), simvastatin (IS; $^{13}C_{12}$-triclosan), naproxen (IS; wafarin-d_5) and risperidone (IS; proponolol-d_7).

3.2.1. Detection and quantification limits

For LC–MS/MS analyses, analyte responses were consistently linear over the concentration range of 0.5–300 ng/mL, with correlation coefficients (r^2) ranging between 0.991 and 0.999. Relative responses were very reproducible over this concentration range, with relative standard deviations (RSD) between 2 and 20%. Thus, mean RR values (n = 5) were used for quantification of target analytes.

For each target analyte, a method detection limit (MDL) was defined as a signal-to-noise (S/N) ratio of 3:1 in sample matrix and the method quantification limit (MQL) as S/N of 10:1. Specifically, MDLs and MQLs were determined by monitoring the signal of target analytes that yielded a signal-to-noise of 3 and 10 respectively in zebrafish plasma extract.

For some target analytes (BPA, triclosan, diclofenac, gemfibrozil, ibuprofen, naproxen and fluoxetine), trace residues were observed in procedural blanks. In such cases, a value equal to 3 times and 10 times the standard deviation of observed blank concentrations was used for MDL and MQL, respectively. A summary of the obtained MDLs and MQLs for the target PPCPs is shown in Table 3.

In general, the method performed very well, typically achieving detection of target analytes in the low to sub parts per billion (ppb) range. BPA exhibited comparatively higher detection limits, due to more pronounced background contamination in procedural blanks. For other compounds MDLs ranged between 0.015 and 1.30 pg/μL plasma. Similarly, MQLs of the target compounds were generally low, ranging between 0.05 and 4.33 pg/μL plasma. The developed LC–MS/MS based method for analysis of multi-class PPCPs achieved MDLs and MQLs comparable to previously reported methods, which typically utilized larger sample volume (0.5–2 mL) and more comprehensive extraction and cleanup steps [13,14,22]. For example, in the present study utilizing LLE of fish plasma micro-aliquots, MDLs for ibuprofen and triclosan, commonly targeted contaminants in biomonitoring studies, were 0.63 pg/μL and 0.39 pg/μL, respectively. These are equivalent to previously reported MDLs for ibuprofen (0.28 pg/μL) and triclosan (0.57 pg/μL) following extraction of large sample volumes and conventional SPE cleanup [14].

3.3. Quantitative determination of PPCP residues in fish plasma micro-aliquots

Measured concentrations of PPCPs in plasma of zebrafish are shown in Table 4. Ten compounds were determined at concentrations higher than MDLs. Simvastatin residues were not detectable in these plasma samples, thus indicating concentrations below the MDL (<0.12 pg/μL). For the high dose group, all target analytes were detected well above the quantification limits. For the low dose group, PPCPs were typically detected at lower concentrations. For some PPCPs, concentrations in the low dose samples were below the MDL (BPA, fluoxetine, sertraline). However, several PPCPs were observed in plasma samples of fish from the unexposed control group. For example, residues of ibuprofen (0.94 ± 0.31 pg/μL), diclofenac (1.74 ± 1.48 pg/μL), gemfibrozil (0.35 ± 0.31 pg/μL) and

Table 5
Method detection limits (MDLs) and method quantification limits (MQLs) of PPCPs in fish plasma micro-aliquots (this study) compared with observed concentrations of those compounds previously reported in biomonitoring studies utilizing fish plasma.

	PPCP concentration in fish plasma (pg/μL)					
	MDL (this study)	MQL (this study)	Umea, Sweden[a]	Stockholm, Sweden[a]	Gothenburg, Sweden[a]	Matsuyama, Japan[b]
Naproxen	0.69	2.30	36	33	46	NA
Ibuprofen	0.63	2.10	13	5.5	102	2.5–3.8
Diclofenac	0.48	1.60	20	2.2	7.4	0.15–0.83
Carbamazepine	0.19	0.64	0.9	0.3	1	0.03–0.055
Risperidone	0.015	0.05	0.4	0.3	2.4	NA
Triclosan	0.39	1.31	NA	NA	NA	11–110
Sertraline	0.42	1.40	1.2	1.1	< 0.5	0.14–0.51

[a] Data are mean concentrations reported by Fick et al. [13].
[b] Data are Min–Max range of concentrations reported by Tanoue et al. [14].

triclosan (0.98 ± 0.04 pg/μL) were quantifiable in plasma of control fish.

A common contaminant biomonitoring approach is to conduct in situ exposure experiments with wastewater effluent or receiving water samples. In particular, the recently proposed "Fish Plasma Model" requires accurate determination of accumulated PPCP residues in fish plasma following exposure to WWTP effluents [22,23]. Table 5 shows concentrations of several PPCPs monitored in fish plasma following in situ exposure to WWTP effluents [13,14]. Concentrations in fish plasma in those studies typically ranged between 0.1 and 100 pg/μL. The MDLs and/or MQLs achieved in the present study (also shown in Table 5) were in most cases lower than the reported concentrations. For example, the MQL of ibuprofen in the present study (2.1 pg/μL) is sufficiently low enough to quantify ibuprofen residues previously observed in fish plasma samples, which ranged between 2.5 and 102 pg/μL. It is important to note that PPCP concentrations in our low dose group exposure experimental system, were in many cases, lower than actual concentrations in WWTP effluent [13].

In addition to biomonitoring initiatives, the developed method may also be applicable for use in laboratory-based bioaccumulation and toxicological studies of PPCPs using small laboratory fishes. Pharmacokinetic and toxicology studies of contaminants of emerging concern in fish often require analysis of plasma [26–30]. Common test organisms used in such studies include zebrafish (*D. rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnows (*Pimephales promelas*). These species are relatively small in size (1–5 g) and exhibit low blood compartment volumes. The developed method can provide quantification of low levels of PPCP residues in as little as 20 μL of fish plasma.

4. Conclusions

We developed a method for fast, reliable analysis of multiple PPCP residues in fish plasma micro-aliquots using LC–MS/MS. The key advantages of the method include (i) low sample size requirement (20 μL plasma), (ii) a simple LLE sample preparation step using minimal solvent and (iii) use of a high efficiency HPLC column and fast polarity switching for rapid analyte separation and effective monitoring positive and negative ions in a single 9 min run. Quantification limits of the target PPCPs in fish plasma were in the low to sub pg/μL (ppb) range, which is sufficiently low for in situ contaminant biomonitoring and laboratory studies investigating pharmacokinetics and toxicology of these compounds.

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