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Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

A new sensitive method based on pressurized liquid extraction (PLE) and purification by gel permeation chromatography (GPC) prior to ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) was developed for the determination in fish homogenate, liver and muscle of twenty pharmaceuticals compounds and metabolites from seven commonly used therapeutic families. An extensive matrix effect evaluation was performed in order to select the best approach when analyzing such complex matrices. Limits of detection (MDLs) for the target compounds were in the range of 0.03–0.50 ng/g for fish homogenate, 0.01–0.42 ng/g for fish muscle, and 0.08–0.98 ng/g for fish liver. The method was applied to fish tissues of eleven fish species from four heavily impacted Mediterranean rivers. Nine compounds from five therapeutic families were measured at concentrations higher than MDLs. Highest levels were found in trout liver, with a maximum concentration of 18 ng/g for carbamazepine, whereas the most ubiquitous compound was diclofenac.

1. Introduction

In recent years, the occurrence, fate, and adverse effects of pharmaceutical residues in aquatic organisms have become a noteworthy issue. In Europe, the legislative proposal for amending the list of priority substances that represent a significant risk to or via the aquatic environment was presented by the European Commission on 31 January 2012, and included for the first time the pharmaceutical substances 17-α-ethinylestradiol (EE2), 17-ß-estradiol (E2) and diclofenac [1]. The U.S. Environmental Protection Agency (EPA) has also included some pharmaceutical substances in the Drinking Water Contaminant Candidate List, such as the antibiotic erythromycin and the estrogenic hormones 17-α-estradiol, estriol and estrone [2]. In addition, many reports have highlighted the potential of pharmaceuticals and/or their metabolites to accumulate in tissues of aquatic organisms, such as crustaceans, molluscs and fish, as a consequence of their chronic exposure in aquatic ecosystems [3–5]. In general, pharmaceutically active compounds (PhACs) are highly hydrophilic, and their bioaccumulation potential might be considered irrelevant, particularly when compared to other pollutants, such as pesticides and persistent organic compounds (POPs). These conventional pollutants have been reported in a vast number of studies to bioaccumulate in different organisms because of their lipophilicity and tendency to bind to organic matter [6–10] and are considered in many priority pollutants lists [11,12]. However, some studies have indicated that the bioaccumulation of PhACs is not only determined by chemical lipophilicity, and other processes should also be considered, such as active transport through biological membranes or uptake and depuration kinetics [13–15].

Analytical techniques used for the detection of PhACs presence at (ultra)trace quantities in environmental matrices have advanced significantly in the last few years and have been summarized in recent reviews [16–24]. Even though an increasing number of analytical procedures have been reported for several therapeutic families in biota in the last years, they are still sparse, probably due to the challenges associated with the complexity of the biological matrices [25]. Groups of PhACs analyzed in biota so far include psychiatric drugs [5,26–29], synthetic hormones [30], and antibiotics [3,29,31,32]. Exhaustive sample preparation followed by sensitive detection techniques is required in these cases, due to the very low concentration of analytes in biological matrices [33]. Both, ultrasonication and pressurized liquid extraction (PLE) have been often used for the extraction of PhACs in aquatic organisms, such as crustaceans, mussels, algae, and fish [27,28,30,34–37]. Regarding the crucial purification step of the sample extract, different

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clean-up procedures have been used: solid-phase extraction (SPE) in Florisil columns [38], Oasis HLB cartridges [39] or techniques like gel-permeation chromatography (GPC) [30,40]. Last step in the analytical process includes the identification and determination of PhACs, usually based on liquid (LC) or gas chromatography (GC) in combination with mass spectrometry (MS) detection, because it provides high selectivity, specificity, and sensitivity required for this type of studies [5,27,29].

This work describes the development, optimization and validation of a method for the determination of 20 multi-class pharmaceuticals and metabolites in fish (Table 1), selected according to their detection frequency in water and sediment in Mediterranean rivers [41,42] as well as to their potential negative effects in aquatic organisms [43–46]. Selected compounds included analgesics and non-steroidal anti-inflammatories (NSAIDs), diuretics, psychiatric drugs, anti-histaminics, antihelmintics, β2-adrenergic receptor agonists and β-blockers. The method developed is based on an extraction step using pressurized liquid extraction (PLE) followed by gel permeation chromatography (GPC) clean-up and ultra performance liquid chromatography–triple quadrupole mass spectrometry (UPLC–MS/MS) for the detection of target compounds. Critical steps in method development involved (i) the selection and optimization of the most appropriate sample pre-treatment step that allowed the simultaneous extraction of selected compounds from fish homogenates, liver and muscle tissues, (ii) lipid removal from the extract to reduce matrix interferences during analysis and (iii) comparison of several strategies to correct the observed matrix effects.

The method was subsequently applied to assess the occurrence of target compounds in fish samples collected from different sites of four Mediterranean rivers in Spain: Ebro, Llobregat, Júcar and Guadalquivir, all subjected to intensive anthropogenic activity. Nine pharmaceutical compounds belonging to five different therapeutic families were detected for the first time in fish from Mediterranean rivers.

2. Materials and methods

2.1. Standards and reagents

High purity grade (>95%) pharmaceutical standards diclofenac, codeine, carbamazepine, citalopram, diazepam, lorazepam, atenolol, sotalol, propanolol, nadolol, carazolol, hydrochlorothiazide, clopidogrel, salbutamol and levamisole were acquire from Sigma–Aldrich. Sertraline and velafaxine were purchased from the European Pharmacopeia (EP). Metabolites 2-hydroxycarbamazepine (2-HydroxyCBZ) and 10, 11-epoxycarbamazepine (10, 11-EpoxyCBZ) were purchased from Toronto Research Chemicals (TRC). Metropolol was obtained from the US Pharmacopeia (USP). Isotopically labeled compounds, used as internal standards, ibuprofen-d_3, diazepam-d_5, ronidazole-d_3 and fluoxetine-d_5 were acquired from Sigma–Aldrich. Atenolol-d_7, carbamazepine-d_{10}, hydrochlorothiazide-d_2, and citalopram-d_4 were purchased from CDN isotopes. Venlafaxine-d_6 was from TRC. Antypirine-d_3 and cimetidine-d_3, used as surrogate standards, were purchased from CDN isotopes. Individual stock standards, isotopically labeled internal standards and surrogate standards were prepared in methanol at a concentration of 1000 mg/L. Stock solutions and 20 mg/L mixtures in methanol were stored at -20 °C. Working standard solutions (1 mg/L) of all pharmaceuticals, mixtures of isotopically labeled internal standards and surrogate were prepared in methanol/water (10:90, v/v) before each analytical run.

2.2. Sample collection and preservation

Fish individuals belonging to 11 different species (*Barbus graellsii*, *Micropterus salmoides*, *Cyprinus carpio*, *Salmo trutta*, *Silurus glanis*, *Anguilla anguilla*, *Lepomis gibbosus*, *Gobio gobio*, *Luciobarbus sclateri*, *Aburnus alburnus*, and *Pseudochondrostoma willkommii*) were collected in five sampling points in each of the four rivers selected (Ebro, Llobregat, Júcar and Guadalquivir) during the summer of 2010. Whole individuals of each class ($n = 3$) were homogenized using a meat grinder, composited into a single sample, freeze-dried and kept at −20 °C until analysis. Fish samples (*S. trutta*) from La Llosa del Cavall reservoir (Llobregat River Basin) were also taken during June of 2011. Liver and muscle tissues were dissected from fish individuals and composited separately. Lipid content was evaluated for fish homogenate of several species, as well as for liver and muscle tissues of Salmo trutta, according to the method developed by Spiric et al. [47].

2.3. Sample extraction optimization

In order to optimize the most suitable extraction method, the following extraction techniques (Fig. 1) were tested for fish homogenate: ultrasonic extraction (USE) (Bandelin), QuEChERS (Agilent Technologies) and pressurized liquid extraction (PLE) with ASE 350® (Thermo Scientific Dionex). Fish homogenate samples were spiked with a mixture of the analytes and subsequently subjected to the chosen extraction methodologies described next to obtain the best recovery results for the target compounds. Ultrasonic extraction was adapted from Schultz et al. [28], using a mixture of 0.1 M aqueous acetic acid/methanol (1:1) as extraction solvent. The extraction included 3 cycles (15 min each) and the supernatant was collected after each cycle and centrifugation at 3500 rpm for 5 min. The second methodology was based on QuEChERS, which involved micro-scale extraction and purification of the extract using dispersive solid-phase extraction (d-SPE). After vortexing with one portion of water for 0.5 min, two portions of ACN were added for the extraction and vortexed again for 1 min. Magnesium sulfate (6 g) and sodium acetate (1.2 g) were used as extraction salts. The mixture was shaken intensively for 1 min and centrifuged (11,000 rpm, 5 min, 4 °C) for the separation of the organic and aqueous phases. An aliquot of the organic phase was purified by dispersive SPE employing sorbent mixture of PSA (0.4 g), C18 (0.4 g) and magnesium sulfate (1.2 g) sorbents for the removal of interfering compounds. Sample preparation using pressurized PLE was finally applied, adapting the method described by Chu et al. [27]. Approximately 1 g of fish homogenate was mixed with hydromatrix (diatomaceous earth, ASE prep DE, Dionex) and placed in a 22 mL stainless steel extraction cell containing a glass-fiber filter (27 mm diameter, type D28, Dionex) in the cell inlet and outlet. The PLE conditions were as follows: oven temperature, 50 °C; pressure, 1500 psi; 5 min heat-up time; three static cycles; static time, 5 min. Additional purification steps were added during PLE method optimization. Three preliminary purification methods were evaluated: (a) addition of approximately 2 g of neutral aluminum oxide (70–230 mesh, Merck) at the bottom of the extraction cell to function as a lipid retainer [37,48]; (b) freezing-lipid technique, by means of redissolving the extract in acetonitrile, which has low solubility for lipids, and storing the extract in the freezer at −20 °C for 30 min. Most of the lipids precipitated and the extract was immediately passed through paper filter to remove them [49]; and (c) a fractionation method by extraction with hexane (1 cycle) to remove nonpolar lipids (defatting step) followed by a second extraction of the sample with methanol (3 cycles) to recover the target analytes [50]. After selecting neutral alumina as the most efficient pre-cleanup step during PLE extraction, other parameters were tested such as the sample size (0.5, 1 and 2 g of fish

Table 1
Chemical structures and precursor ions of selected pharmaceuticals (calculated with MarvinSketch software).

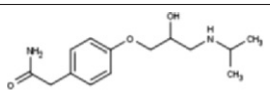
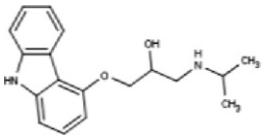
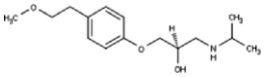
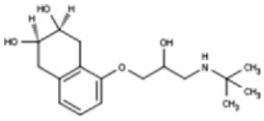
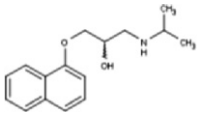
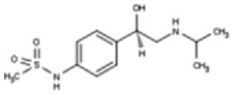
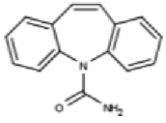
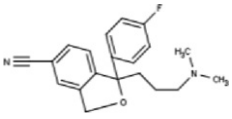
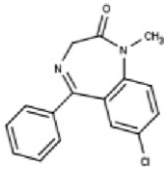
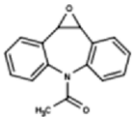
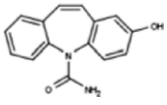
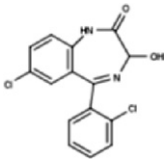
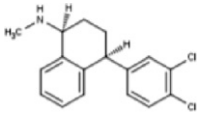
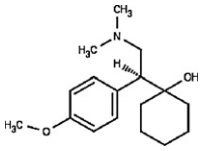
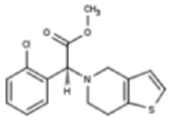
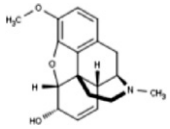
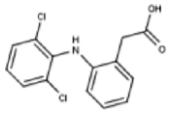
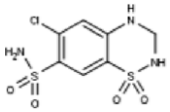
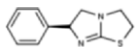
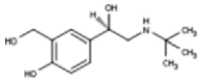
Compound	Therapeutic family	Molecular structure	Precursor ion	Internal standard	pKa	log *P*
Atenolol	β-Blockers		267 $[M+H]^+$	Atenolol-d_7	pKa_1: 9.67 pKa_2: 14.08 pKa_3: 15.95	0.43
Carazolol	β-Blockers		299 $[M+H]^+$	Atenolol-d_7	pKa_1: 9.67 pKa_2: 14.03 pKa_3: 15.00	2.71
Metropolol	β-Blockers		268 $[M+H]^+$	Atenolol-d_7	pKa_1: 9.67 pKa_2: 14.09	1.76
Nadolol	β-Blockers		310 $[M+H]^+$	Atenolol-d_7	pKa_1: 9.76 pKa_2: 13.59 pKa_3: 14.22	0.87
Propanolol	β-Blockers		260 $[M+H]^+$	Atenolol-d_7	pKa_1: 9.67 pKa_2: 14.09	2.58
Sotalol	β-Blockers		273 $[M+H]^+$	Atenolol-d_7	pKa_1: 9.43 pKa_2: 10.07 pKa_3: 14.10	0.05
Carbamazepine	Psychiatric drugs		237 $[M+H]^+$	Carbamazepine-d_{10}	pKa: 13.94	2.77
Citalopram	Psychiatric drugs		325 $[M+H]^+$	Citalopram-d_4	pKa: 9.78	3.76
Diazepam	Psychiatric drugs		285 $[M+H]^+$	Diazepam-d_5	pKa: 2.92	3.08
10,11-EpoxyCBZ	Psychiatric drugs		253 $[M+H]^+$	Carbamazepine-d_{10}	pKa: 19.65	2.31
2-HydroxyCBZ	Psychiatric drugs		253 $[M+H]^+$	Carbamazepine-d_{10}	pKa_1: 9.15 pKa_2: 15.96	2.46
Lorazepam	Psychiatric drugs		321 $[M+H]^+$	Diazepam-d_5	pKa_1: 10.61 pKa_2: 12.46	2.53
Sertraline	Psychiatric drugs		307 $[M+H]^+$	Fluoxetine-d_5	pKa: 9.85	5.15

Table 1 (*Continued*)

Compound	Therapeutic family	Molecular structure	Precursor ion	Internal standard	pKa	log *P*
Venlafaxine	Psychiatric drugs		278 $[M+H]^+$	Venlafaxine-d_6	pKa_1: 8.91 pKa_2: 14.42	2.74
Clopidrogel	Antiplatelet agent		322 $[M+H]^+$	Diazepam-d_5	pKa: 5.14	4.03
Codeine	Analgesics/anti-inflammatories		300 $[M+H]^+$	Carbamazepine-d_{10}	pKa_1: 9.19 pKa_2: 13.78	1.34
Diclofenac	Analgesics/anti-inflammatories		294 $[M-H]^-$	Ibuprofen-d_3	pKa_1: 4.00 pKa_2: 16.40	4.26
Hydrochlorothiazide	Diuretic		296 $[M-H]^-$	Hydrochlorothiazide-d_2	pKa_1: 9.09 pKa_2: 9.83 pKa_3: 11.31	−0.58
Levamisole	Antihelmintics		205 $[M+H]^+$	Ronidazole-d_3	pKa: 6.98	2.36
Salbutamol	To treat asthma		240 $[M+H]^+$	Atenolol-d_7	pKa_1: 9.40 pKa_2: 10.12 pKa_3: 14.18	0.88

homogenate), extraction solvent (methanol, acetonitrile, water), number of extraction cycles (3 or 4 extraction cycles), and extraction temperature (50, 70, 80 and 90 °C), with the aim to obtain the maximum extraction efficiency with the minimum presence of interfering compounds. Presented results led to the selection of an extraction protocol method based on PLE, with methanol as extraction solvent, 4 extraction cycles of five minutes each at 50 °C for a sample size of 1 g of fish homogenate, 1 g of muscle tissue and 0.5 g of liver tissue. Final extracts were evaporated to dryness under a stream of nitrogen and redissolved in the corresponding organic solvent for the purification step.

2.4. Sample purification optimization

Fish homogenate extracts were spiked with a mixture of the target analytes and a comparison between three purification techniques (Fig. 1) was performed: (a) SPE Florisil cartridges (1 g, 6 ml cartridges); (b) Gel permeation chromatography (GPC) in an Agilent 1260 Infinity high pressure liquid chromatography system in tandem with a diode array detector (HPLC-DAD); (c) SPE Oasis HLB (200 mg, 6 ml cartridges) followed by GPC. For the clean-up method based on SPE Florisil, fish extracts were redissolved in 10 ml of acetonitrile prior SPE, which was performed in a J.T.Baker® system. Florisil cartridges were conditioned with 5 ml hexane followed by 5 ml acetonitrile. Extract was passed through the cartridge and collected for evaporation to dryness. For the purification method based on the use of GPC, 250 μL of 1 ml extracts in methanol were passed through an EnviroPrep, 300 mm × 21.2 mm (10 μm pore size) column coupled to a PLgel Guard column (50 mm × 7.5 mm) (Agilent Technologies). Purified fractions containing target compounds (between minute 13.5 to 26.5) were collected and evaporated to dryness. Finally, for the third clean-up protocol tested fish extracts were redissolved in 250 ml of HPLC-grade water and loaded in Oasis HLB cartridges for SPE. Oasis HLB cartridges were previously conditioned with 6 ml methanol followed by 6 ml HPLC-grade water. After the sample was loaded at 2 mL/min, analytes were eluted with 6 ml of methanol. Eluates were evaporated to final volume of 1 mL of methanol and then injected in GPC for further purification. Final extracts were evaporated to dryness and reconstituted with 1 ml methanol/water (10:90, v/v), and 50 μL of a 1 mg/L mixture containing the internal standards were added.

2.5. Liquid chromatography and mass spectrometry analysis

The chromatographic tandem mass spectrometry method applied for the analysis of fish extracts was adapted from Gros et al. [51]. Briefly, chromatographic separations were carried out with a Waters Acquity Ultra-PerformanceTM liquid chromatography system, using an Acquity HSS T3 colum (50 mm × 2.1 mm i.d., 1.8 μm particle size) for the compounds analyzed in positive electrospray ionization (PI) mode and an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size) for the ones analyzed under negative electrospray ionization (NI) mode. For the analysis in PI mode, separation conditions were as follows: solvent (A) methanol, solvent (B) 10 mM formic acid/ammonium formate (pH 3.2) at a flow rate of 0.5 mL/min. The analysis in NI mode was performed by using acetonitrile (A) and (B) 5 mM ammonium acetate/ammonia (pH = 8) at a flow rate of 0.6 mL/min. The sample volume injected was 5 μL in both cases. The UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems). Source-dependent parameters for compounds analyzed under PI were: curtain gas (CUR), 30 psi; nitrogen collision gas (CAD) medium; source

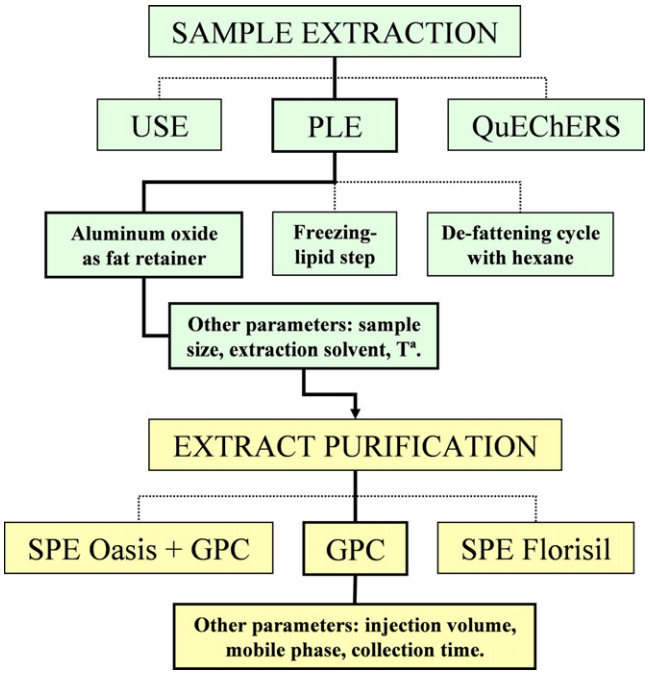


Fig. 1. Sample pretreatment scheme tested during method optimization.

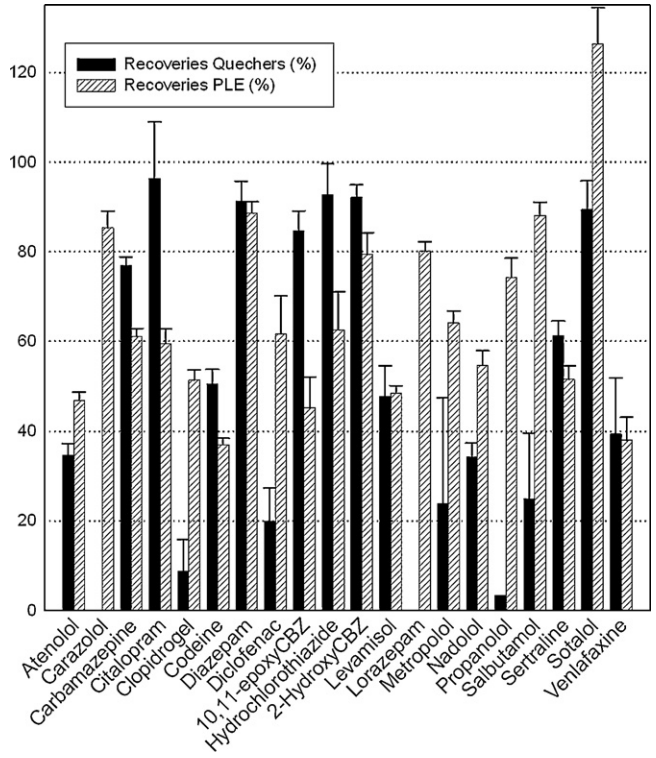


Fig. 2. Comparison of extraction efficiencies (%) between QuEChERS and PLE for the target analytes ($n = 3$).

temperature (TEM) was 650 °C; ion spray voltage was 5500 V; ion source gases GS1 and GS2 were set 60 and 50 psi, respectively. For compounds analyzed under NI, such parameters were: curtain gas (CUR), 30 psi; nitrogen collision gas (CAD) medium; source temperature (TEM) was 650 °C; ion spray voltage was −3500 V; ion source gases GS1 and GS2 were set 60 and 70 psi. Two SRM transitions between the precursor ion and the two most abundant fragment ions were monitored for each compound. The first transition was used for quantification purposes, whereas the second one was used to confirm the identity of the target compounds. Besides the monitoring of the SRM transitions, the relative abundance of the two SRM transitions in the sample were compared with those in the standards, and the relative abundances in the samples must be within ±20% of the two SRM ratios in the analytical standards [51].

3. Results and discussion

3.1. Extraction and purification of the analytes from fish tissues

After the comparison of the three preliminary extraction techniques described in the previous section (USE, QuEChERS and PLE), USE was discarded as a viable option, as only five out of twenty compounds were efficiently recovered. Results obtained with QuEChERS, which had before been successfully applied to the analysis of polar compounds in biological matrices, such as pesticides in food matrices [52] and antibiotics in bovine liver [53], were quite satisfactory, with recoveries surpassing 40% for most of compounds (Fig. 2). However, PLE was finally selected as the most appropriate extraction technique, due to the overall better recoveries (Fig. 2) of relevant compounds (diclofenac, propanolol), the lower relative standard deviation (RSD) (%), and the significantly lesser matrix interferences observed during analysis when compared to QuEChERS. PLE allows the use of conventional solvents at high temperature and pressure to improve the extraction of pollutants from solid samples, but it may still co-extract a high proportion of matrix interferences [33,54]. Therefore, the possibility of a simultaneous purification step (pre clean-up) during PLE extraction to reduce the coextraction of other matrix components was assessed. An intra-cell clean-up with neutral alumina (2 g) was selected among the tested methods as the best sorbent, since the extraction efficiency for target compounds did not decrease while the lipid content in the extracts was reduced (results not shown). Further refinement of the PLE procedure was accomplished by optimizing several critical parameters, such as extraction solvent and its volume, cycle time and number of extraction cycles, extraction temperature and amount of biomass. Among all these parameters, extraction solvent appeared to be the most relevant. At 50 °C, methanol provided the highest recoveries when compared to other solvents (acetonitrile), solvent mixtures (methanol/water, 1:1; methanol/acetonitrile, 1:1) or solvent with additive (acidified methanol) (Fig. S1). The rest of the parameters did not significantly improve the recoveries. Sample size was set at 1 g for fish homogenate and muscle tissues, whereas 0.5 g was selected in the case of liver tissue to minimize the lipid content in the extract and thus the potential interferences during analysis. Temperature was set at 50 °C to avoid analyte degradation, as pharmaceuticals are often thermolabile [20]. Four cycles of 5 min each was established as extraction time and 100% flush volume and 90 s of nitrogen purge were the final parameters selected.

Regarding clean-up procedures, Fig. 3 presents the recoveries for the target analytes obtained with the three purification techniques tested. Florisil cartridges showed generally low recoveries, and thus it was discarded. SPE with Oasis HLB followed by GPC caused decrease of overall recoveries, particularly in the case of β-blocker compounds and, in consequence, a single GPC purification step was selected as clean-up method, as it provided satisfactory results for most of the target compounds. Several parameters were additionally optimized: mobile phase (dichloromethane (DCM), DCM/MeOH mixtures), flow rate (3, 4 and 5 ml/min) and injection volume (100, 250, 500, 750 μL). The final selected parameters were the following: DCM/MeOH (90:10, v/v) as mobile phase, at 5 mL/min flow rate, injection volume of 250 μL for homogenate samples, 500 μL for liver samples and 750 μL for muscle samples.

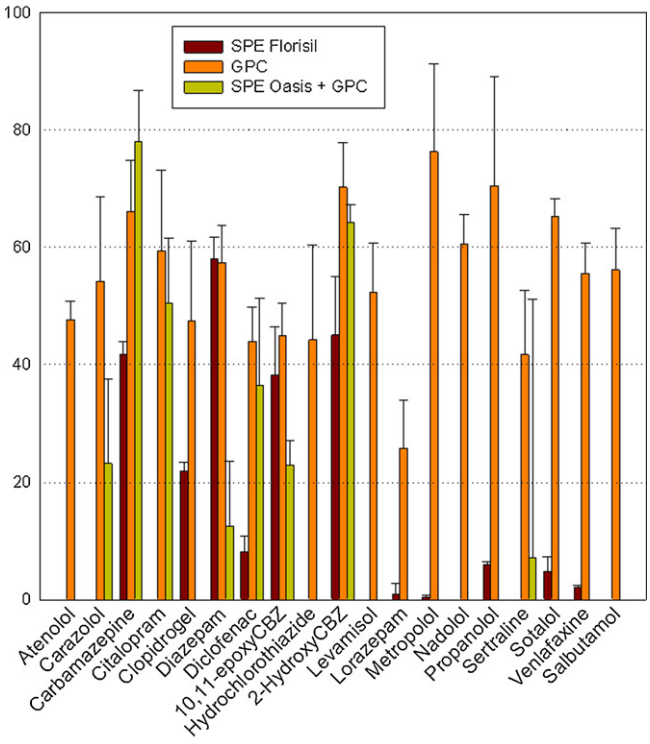


Fig. 3. Comparison of cleanup recoveries (%) between three purification techniques for the selected compounds ($n = 3$).

Collection time interval for the pharmaceuticals was established from min. 13.5 to 26.5 of the 40 min chromatographic cycle.

Final method consisted in PLE extraction using methanol as solvent at 50 °T during 4 cycles of 5 min, followed by an extensive GPC purification, with DCM/MeOH (90:10, v/v) as mobile phase at 5 ml/min flow rate. Total recoveries were determined for the final sample treatment method and for different fish tissues (fish homogenate, liver and muscle) by comparing concentrations obtained after the whole analytical procedure, calculated by internal sample calibration. Results from each matrix are presented for fish homogenate of three representative species (*Cyprinus carpio*, *Barbus graellsii* and *Silurus glanis*) (Table 2) and for liver and muscle tissues (Table 3). Two spiking levels (20 and 100 ng/g) were considered, the lowest level selected according to concentrations found in previous studies found in literature [26,55]. Recoveries were considered acceptable when they were in the range 40–140%, due to (a) the analytical challenge that poses the development of a multianalyte method for such diverse PhACs (with different lipophilicity and pKa) and (b) to the intensive sample pretreatment demanded in biota matrices [4,5]. Surrogate addition was applied to monitor for recovery differences and problems during the extraction phase of the analysis. Two isotopically labeled compounds were selected: antipyrine-d_3 and cimetidine-d_3, which presented recoveries of 108.3 ± 1.3 and 78.8 ± 3.0 (%), respectively.

3.2. Matrix effects

Ion suppression or enhancement by the presence of coextracted matrix components is a major problem for mass spectrometry detectors when ionization is performed by electrospray interfaces, particularly with complex matrices such as biota [56]. To evaluate matrix effects on the analysis of target compounds in the different fish tissues, peak areas of fish extract (previous subtraction of the analytes peak areas corresponding to the native analytes present in the sample) spiked at 5 ng/g were compared to those of the analytes in the solvent (methanol/water 10:90, v/v) spiked at the same concentration. Calculations were performed in triplicate for fish homogenate, muscle and liver tissues.

Percentage of signal reduction or enhancement for all the compounds is presented in Table S1. The MS signal of the majority of the compounds analyzed in the different biota extracts was suppressed. The highest signal suppression was found in liver tissues (up to 83%), which may be attributed to the higher lipid content of liver (48%) compared to fish homogenate (15%). Matrix effects calculated for various tissues and fish species were contrasted with the lipid content, as presented in Fig. 4 for selected compounds. As expected, ion suppresion (carbamazepine, clopidogrel and venlafaxine) or enhancement (diclofenac) was more obvious as the percentage of lipids increased. Further evaluation of the matrix effects was performed in fish homogenates from three representative fish species considering that lipid percentage for *C. carpio* and *B. graellsii* was approximately 10–15%, while for *Silurus glanis* was

Table 2
Mean percent recoveries ($n = 3$) at two spiking levels of the target compounds in fish homogenates.

	% Recovery ± RSD					
	Spiking level: 20 ng/g			Spiking level: 100 ng/g		
	Barbus graellsii	*Cyprinus carpio*	*Silurus glanis*	*Barbus graellsii*	*Cyprinus carpio*	*Silurus glanis*
Atenolol	46.2 ± 2.0	48.3 ± 6.8	47.7 ± 3.1	47.4 ± 10	60.8 ± 5.3	47.5 ± 8.7
Carazolol	39.7 ± 5.9	27.4 ± 2.9	54.2 ± 14	52.1 ± 9.2	35.3 ± 8.2	38.2 ± 4.7
Carbamazepine	66.2 ± 3.3	71.5 ± 4.2	66.1 ± 8.7	64.1 ± 11	78.7 ± 3.1	74.9 ± 7.0
Citalopram	60.8 ± 4.7	63.9 ± 0.6	59.4 ± 14	107.8 ± 0.4	59.2 ± 23	43.3 ± 18
Clopidrogel	36.9 ± 2.4	31.2 ± 2.0	47.4 ± 14	40.6 ± 11	51.3 ± 2.9	48.4 ± 9.0
Codeine	44.9 ± 7.9	51.8 ± 5.1	42.6 ± 7.5	31.2 ± 4.4	35.3 ± 8.2	36.7 ± 2.3
Diazepam	53.4 ± 3.1	55.8 ± 3.9	57.3 ± 6.3	59.3 ± 11	64.2 ± 4.0	60.1 ± 10
Diclofenac	56.9 ± 12	56.3 ± 24	43.9 ± 5.9	61.3 ± 11	41.6 ± 8.2	44.4 ± 1.0
10,11-epoxyCBZ	45.1 ± 20	33.4 ± 4.3	44.8 ± 5.6	33.9 ± 3.9	34.7 ± 0.6	44.8 ± 4.4
Hydrochlorothiazide	40.3 ± 11	19.3 ± 5.8	44.2 ± 16	68.1 ± 8.1	60.4 ± 12	65.6 ± 11
2-HydroxyCBZ	61.7 ± 21	56.2 ± 2.8	70.2 ± 7.5	64.9 ± 6.2	59.2 ± 2.8	71.4 ± 6.8
Levamisol	44.6 ± 7.6	40.3 ± 4.0	52.4 ± 8.4	45.2 ± 2.5	46.6 ± 1.6	50.8 ± 8.2
Lorazepam	39.6 ± 12	41.8 ± 16	25.8 ± 8.2	58.9 ± 15	35.4 ± 4.5	55.6 ± 5.1
Metropolol	66.9 ± 2.9	48.2 ± 2.4	76.2 ± 15	98.6 ± 19	76.4 ± 4.7	61.7 ± 12
Nadolol	46.9 ± 19	53.0 ± 4.3	60.6 ± 5.0	53.7 ± 9.7	46.2 ± 3.0	37.2 ± 7.3
Propanolol	55.8 ± 8.8	41.2 ± 3.4	70.5 ± 18	97.1 ± 18	62.5 ± 15	42 ± 0.6
Salbutamol	50.5 ± 3.6	27.4 ± 13	56.1 ± 7.1	46.0 ± 9.2	60.5 ± 4.6	55.1 ± 1.4
Sertraline	38.0 ± 11	29.2 ± 9.5	41.6 ± 11	37.3 ± 6.8	54.8 ± 5.1	34.0 ± 8.0
Sotalol	56.1 ± 2.2	66.0 ± 4.3	41.6 ± 11	54.1 ± 8.6	42.8 ± 3.2	34.0 ± 8.0
Venlafaxine	57.0 ± 2.2	48.3 ± 5.9	55.5 ± 5.1	41.8 ± 10	57.2 ± 1.6	55.7 ± 7.2

Table 3
Mean percent recoveries ($n = 3$) in fish liver and muscle tissues (spiking level: 20 & 100 ng/g).

	% Recovery ± RSD			
	Spiking level: 20 ng/g		Spiking level: 100 ng/g	
	Liver	Muscle	Liver	Muscle
Atenolol	47.4 ± 0.6	59.9 ± 2.7	68.4 ± 12	46.9 ± 7.2
Carazolol	57.9 ± 2.7	67.9 ± 8.3	74.8 ± 3.1	85.3 ± 15
Carbamazepine	85.5 ± 3.3	61.2 ± 8.8	102.3 ± 8.7	74.8 ± 4.8
Citalopram	52.3 ± 0.3	33.5 ± 0.6	125.7 ± 12	59.5 ± 13
Clopidrogel	44.0 ± 11	60.3 ± 2.3	45.0 ± 9.5	51.3 ± 9.2
Codeine	38.7 ± 0.3	53.0 ± 0.7	62.3 ± 3.2	50.1 ± 5.3
Diazepam	31.5 ± 3.1	44.2 ± 5.5	110.0 ± 10	88.7 ± 6.6
Diclofenac	38.0 ± 2.8	66.8 ± 5.9	43.9 ± 3.4	63.7 ± 12
10,11-epoxyCBZ	27.5 ± 4.5	27.5 ± 7.3	49.3 ± 3.6	45.2 ± 7.9
Hydrochlorothiazide	68.2 ± 0.9	79.7 ± 7.4	91.9 ± 16	67.2 ± 19
2-HydroxyCBZ	45.1 ± 0.2	49.9 ± 2.6	95.5 ± 4.4	79.3 ± 13
Levamisol	35.0 ± 2.3	84.1 ± 4.1	28.1 ± 13	48.5 ± 15
Lorazepam	54.8 ± 1.8	86.2 ± 8.1	21.3 ± 8.0	42.8 ± 2.2
Metropolol	45.1 ± 0.3	79.7 ± 7.4	91.9 ± 7.9	64.1 ± 5.6
Nadolol	55.8 ± 1.2	60.3 ± 2.3	79.3 ± 5.8	47.3 ± 10
Propanolol	60.8 ± 9.8	84.6 ± 2.1	92.8 ± 5.7	74.3 ± 17
Sertraline	85.0 ± 0.6	92.0 ± 5.7	150.6 ± 8.2	88.1 ± 11
Sotalol	50.0 ± 7.7	87.7 ± 2.1	86.3 ± 13	48.5 ± 12
Venlafaxine	63.1 ± 0.1	68.8 ± 2.7	124.4 ± 6.6	126.4 ± 11.
Salbutamol	47.1 ± 4.2	49.1 ± 3.2	52.9 ± 4.4	38.0 ± 1.2

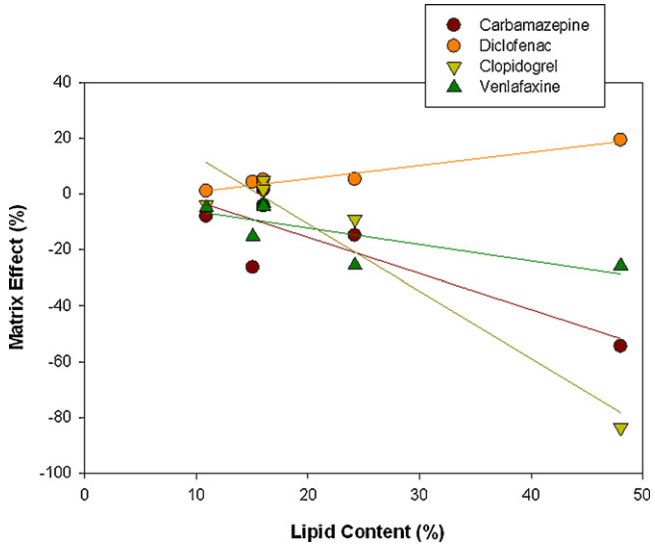


Fig. 4. Matrix effects vs lipid content represented for carbamazepine (fit curve $R^2 = 0.74$), clopidogrel ($R^2 = 0.92$), diclofenac ($R^2 = 0.95$), and venlafaxine ($R^2 = 0.77$).

up to 25%. The results presented in Table S1 show a great similarity in the response in (a) *C. carpio* and (b) *B. graellsii*, and opposite to (c) *S. glanis*, where the majority of the compounds presented greater ion suppression and a very low deviation compared to the other species, which could be a direct consequence of the higher lipid content of *S. glanis*. These results highlighted the relevance of using a suitable approach to compensate for matrix effects during analysis of the same biota matrix, considering the variability in composition that different species might have.

An evaluation of the best approach to compensate for the observed matrix effects was therefore performed. In the first place, standard addition was considered, as it is, by definition, the most effective approach for compensating matrix effects. However, it was difficult and time-consuming due to the great of samples to processes. Other approaches include matrix-matched calibration and internal calibration with isotopically labeled standards. Matrix-matched calibration requires a control matrix similar to the real sample and that does not contain the target analytes, which was not available in this case. Internal standard calibration entails the addition to the extract of isotopically labeled standards that are structurally similar to the target analytes to compare the instrument response during the analysis. Since only ten (out of 20 target compounds) isotopically labeled standards were available to use as internal standard during method optimization, an alternative strategy for the correction of matrix effect were explored, namely internal sample calibration [57,58]. Two calibration curves in the range of 0.1–25 ng/mL were prepared in both, a solvent mixture and fish extract, and internal standards were added to the two of them at the same concentration. Fig. 5 presents the internal standard calibration (solvent curve) and the internal sample calibration (curve prepared in fish extract) for two compounds, carbamazepine and clopidogrel. In the case of carbamazepine (Fig. 5a), whose quantification was corrected with its analogue labeled compound (carbamazapine-d_{10}), both calibration curves were very similar, which indicates that the internal standard was compensating the observed matrix effects. In the case of clopidogrel (Fig. 5b) whose corresponding analogue labeled compound was not available, calibration curves were noticeably different, as the internal standard (diazepam-d_5) used during quantification with the solvent curve was not correcting completely the ion suppression observed in the real sample. Consequently, the most advantageous approach for an accurate determination of target compounds seemed to be the quantification using a calibration curve prepared with spiked fish extracts and internal standard addition (internal sample calibration), which was able to correct the matrix effects for all the compounds. Internal sample calibration is thus recommended especially in those cases when the analogue internal standard is not available.

Due to the differences observed in lipid content between fish species, it was important to assess if quantification of analytes was affected by the use of the different fish matrices to build the calibration curves. The accuracy of the measurements when preparing the internal sample calibration in three representative species (*C. carpio*, *B. graellsii* and *S. glanis*) was evaluated. An extract of *C. carpio* was spiked (25 ng/g) with a mixture of the analytes and quantified using internal standard calibration and internal sample calibration prepared in extracts of the three fish species. As expected, the most accurate measurement corresponds to the internal sample calibration with *C. carpio* extracts, since the same fish sample was used for building the internal sample calibration curve. Internal sample calibration curve built in *B. graellsii* extracts, with similar amount

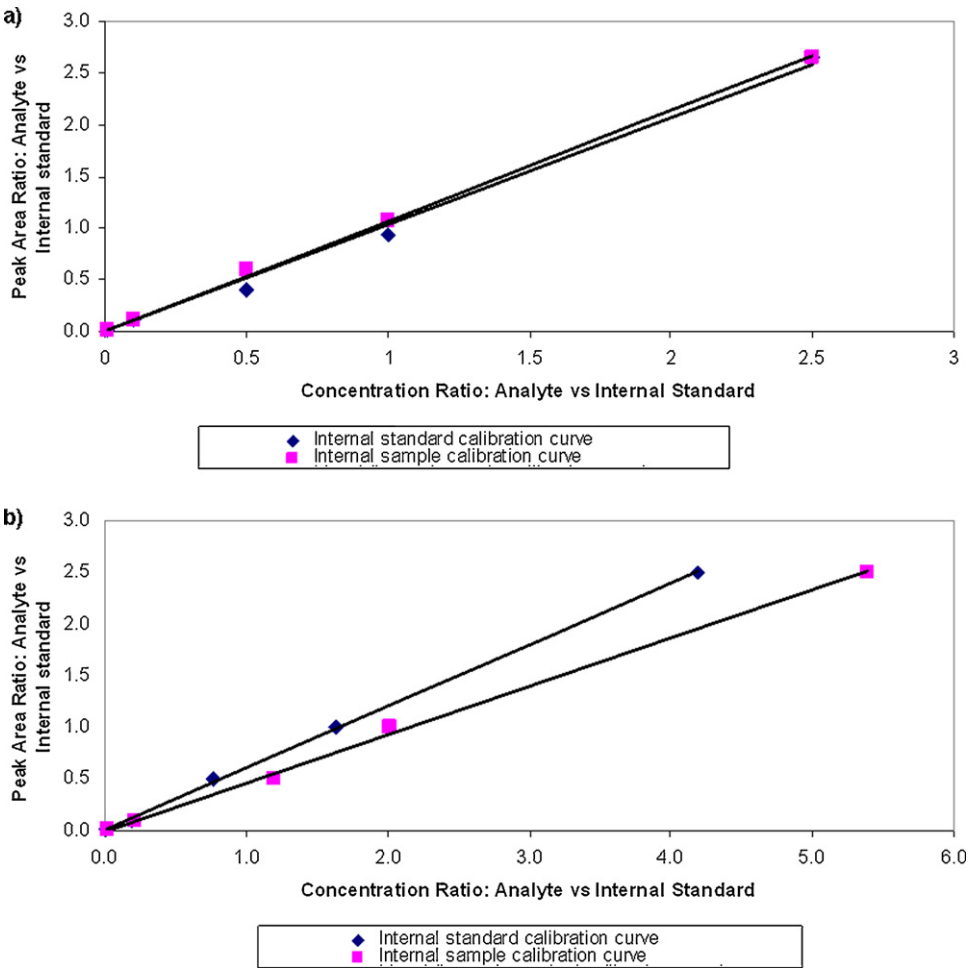


Fig. 5. Comparison between calibration curves in the solvent, and in real sample with internal standard for the pharmaceuticals (a) carbamazepine (carbamazepine-d_{10} as internal standard) and (b) clopidogrel (diazepam-d_5 as internal standard). Calibration curves were prepared in *C. carpio* extracts.

of fat content than *C. carpio*, (Fig S2) was quite optimal at the time of quantifying target compounds in *C. carpio* samples, except for some slight deviation for particular compounds. On the contrary *S. glanis*, with higher fat content that the other two species studied, exhibited great discrepancy in the results between calibration methods, particularly in the case of some β-blockers compounds. These results reiterated the significance of considering the characteristics of the different species, particularly lipid content, when working with internal or external sample calibration. The preparation of internal sample calibration curves in an appropriate matrix, namely extracts of the same specie or at least species with similar characteristics, is suggested as the best approach for an accurate determination of pharmaceutical compounds in this type of biota samples.

3.3. Method validation parameters

Method detection limits (MDL) and method quantification limits (MQL) for fish homogenate, liver and muscle are presented in Table 4. Both, MDL and MQL, were determined in spiked samples ($n = 3$) of the three matrices considered as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. MDLs for the target compounds were in the range of 0.03–0.50 ng/g for fish homogenate, 0.01–0.42 ng/g for fish muscle, and 0.08–0.98 ng/g for fish liver, and were in general similar or lower than those currently reported [5,29,59].

Accuracy and precision were calculated from five repeated injections of a spiked sample at 25 ng/g in the same day. Accuracy was defined as the deviation of the measured mean concentration from the spiked concentration, expressed in percentage, as described by Bogialli et al. [60]. Precision was expressed as the relative standard deviation of the measured concentration. Both values were lower than 20% for the three matrices considered (Table 5). Calibration curves were generated using linear regression analysis ($r^2 > 0.98$) in the concentration range from 0.1 to 50 ng/g.

3.4. Application to environmental samples

The developed method was applied for the determination of pharmaceuticals in fish from four Mediterranean rivers and one reservoir, including fish homogenates, muscle and liver tissues. Nine compounds (carazolol, carbamazepine, citalopram, clopidogrel, diclofenac, propanolol, salbutamol, and venlafaxine) from five therapeutic families were determined at concentrations higher than MDLs (Table 6). Pharmaceutical levels were lower than 10 ng/g in fish homogenates, which is consistent with published scientific literature on the topic [5,26,27]. Highest levels were found in trout liver, where the concentration of carbamazepine was 18 ng/g. Most ubiquitous and recurring compound was diclofenac, detected in 9% of the total number of samples.

Table 4
Method detection and quantification limits (MDL, MQL) in fish homogenate, liver and muscle tissues (ng/g, dry weight).

	MDL (ng/g)			MQL (ng/g)		
	Homogenate	Liver	Muscle	Homogenate	Liver	Muscle
Atenolol	0.05	0.12	0.10	0.17	0.41	0.32
Carazolol	0.04	0.09	0.03	0.15	0.31	0.10
Carbamazepine	0.04	0.08	0.01	0.13	0.25	0.04
Citalopram	0.12	0.09	0.05	0.41	0.29	0.16
Clopidrogel	0.04	0.26	0.08	0.13	0.87	0.26
Codeine	0.06	0.13	0.06	0.18	0.43	0.20
Diazepam	0.08	0.12	0.08	0.25	0.41	0.25
Diclofenac	0.50	0.65	0.19	1.66	2.16	0.62
Epoxycarbamazepine	0.09	0.19	0.11	0.30	0.62	0.38
Hydrochlorothiazide	0.05	0.11	0.17	0.17	0.35	0.57
Hydroxycarbamazepine	0.03	0.25	0.08	0.09	0.83	0.26
Levamisol	0.04	0.29	0.02	0.13	0.97	0.08
Lorazepam	0.49	0.77	0.42	1.62	2.58	1.42
Metropolol	0.20	0.36	0.18	0.67	1.19	0.60
Nadolol	0.03	0.13	0.09	0.10	0.42	0.30
Propanolol	0.09	0.36	0.18	0.29	1.21	0.60
Sertraline	0.32	0.98	0.18	1.08	3.28	0.61
Sotalol	0.26	0.20	0.07	0.88	0.66	0.24
Venlafaxine	0.04	0.40	0.16	0.15	1.33	0.55
Salbutamol	0.07	0.24	0.11	0.25	0.78	0.38

Table 5
Accuracy[a] and precision[b] data of selected pharmaceuticals in fish homogenate, liver and muscle (spiking level 25 ppb).

	Homogenate		Liver		Muscle	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Atenolol	−0.1	2.1	−1.6	14	−1.6	6.6
Carazolol	11	7.3	−12	4.4	−10.9	17
Carbamazepine	−14	4.0	−17	13	−18	5.9
Citalopram	7.1	1.1	−19	14	6.2	1.1
Clopidrogel	−2.5	14.	0.1	12	0.3	9.2
Codeine	−17	15	−0.7	3.9	−17	15
Diazepam	2.3	6.7	−0.4	13	−19	8.6
Diclofenac	0.9	2.7	1.3	18	−20	14
10,11-epoxyCBZ	−5.9	2.0	0.3	4.5	−20	12
Hydrochlorothiazide	−4.2	12	−0.1	20	−6.9	12
2-HydroxyCBZ	−18.9	4.9	−0.1	5.4	−17	9.1
Levamisol	14	3.8	0.2	16	−5.9	6.1
Lorazepam	16	21	8.2	13	4.1	20
Metropolol	0.0	6.3	−0.1	9.7	−9.1	11
Nadolol	1.4	6.4	0.1	7.1	1.0	13
Propanolol	−11	21	0.0	6.9	−0.1	17
Salbutamol	−1.5	3.9	0.0	16	0.1	11
Sertraline	2.9	6.9	3.4	11	−2.6	10
Sotalol	−15	2.9	−19	7.7	−14	7.2
Venlafaxine	13	7.5	−15	10	−11	20

[a] Calculate as [(mean calculated concentration − spiked concentration)/spiked concentration] ×100.
[b] Expressed as relative standard deviation (RSD (%)).

Table 6
Concentration of pharmaceuticals (ng/g, dry weight) in fish homogenates and tissues from fish collected in Mediterranean rivers (Spain).

Therapeutic family	Compound	Species	Matrix	Mean concentration (ng/g) (n = 3)	Standard deviation	Detection frequency (%)
Anti-inflamatory	Diclofenac	*Barbus graellsii*	Homogenate	8.8	±0.5	9
		Micropterus salmoides	Homogenate	4.1	±0.9	
Psychiatric drug	Citalopram	*Cyprinus carpio*	Homogenate	0.8	±0.1	3.5
	Carbamazepine	*Salmo trutta*	Liver	17.9	±2.4	–
	Venlafaxine	*Cyprinus carpio*	Homogenate	0.6	±0.02	2
Antiplatelet agent	Clopidogrel	*Cyprinus carpio*	Homogenate	<MLQ		2
β-blockers	Carazolol	*Silurus glanis*	Homogenate	3.8	±0.7	3.5
		Anguilla anguilla	Homogenate	<MLQ		
	Propanolol	*Silurus glanis*	Homogenate	4.2	±1.0	3.5
	Sotalol	*Pseudochondrostoma willkommii*	Homogenate	<MLQ		2
To treat asthma	Salbutamol	*Silurus glanis*	Homogenate	2.6	±0.3	2

4. Conclusions

The multi-residue analytical method described in this paper, based on pressurized liquid extraction, gel permeation chromatography purification and UPLC–MS/MS analysis, provides the necessary sensitivity for the simultaneous trace-level detection of 20 multi-class pharmaceuticals. Recoveries obtained for most of the target compounds were higher than 50%. The application of UPLC–MS/MS operated in the MRM mode, with two transitions monitored for each compound, provided good sensitivity, selectivity and confirmation of positive findings. A thorough evaluation of the matrix effects was performed and lipid content in fish species and tissues was considered to select the best approach to compensate observed matrix effects and to provide an accurate determination of pharmaceutical compounds. Internal sample calibrations in an appropriate matrix were selected as the best strategy in this type of sample. The method presented detection limits in the low ng/g range for the several fish species, thus providing a sensitive, reliable and robust tool that can be used for routine analysis of multi-class pharmaceuticals in different fish tissues. Anti-inflammatories, psychiatric drugs and β-blockers were detected in fish samples from the most polluted sites of Mediterranean rivers.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.03.001.

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