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Method Article

Development of a USE/d-SPE and targeted DIA-Orbitrap-MS acquisition methodology for the analysis of wastewater-derived organic pollutants in fish tissues and body fluids

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A B S T R A C T

Pharmaceuticals (PhACs) are partially removed during wastewater treatment and end up in the receiving waters. As a result, aquatic biota is continuously exposed to a wide range of potentially hazardous contaminants such as PhACs. Therefore, fish could be a good bio indicator to give a direct measure of the occurrence of PhACs in the aquatic environment. In this study, a robust analytical method has been optimized and validated for the determination of 81 organic compounds, mainly PhACs, in seven wild fish tissue types (liver, muscle, pancreas, kidney, skin, heart, and brain) and two body fluids (plasma and bile). Solid samples extraction was performed combining a procedure based on bead beating tissue homogenization plus ultrasound extraction followed by dispersive solid-phase extraction (dSPE) clean-up using zirconia and C18 sorbents for solid matrices, whereas bile and plasma were diluted.

The key aspects of this method are:

• The method facilitated the simultaneous extraction of 81 PhACs of a wide range of polarity (logP from -4.9 to 5.6) in tissues with variable lipid content.

• The validation was performed using *Cyprinus carpio* at 20 ng g^{-1} and 200 ng g^{-1} for solid tissues, 50 ng mL^{-1} and 500 ng mL^{-1} for plasma, and 100 ng mL^{-1} and 1000 ng mL^{-1} for bile. Analyte detection was performed in LC-HRMS Q-Exactive Orbitrap system with full scan and targeted data-independent acquisition (DIA) mode for high-confidence and sensitive quantitation in either (+) or (-) ESI mode.

• The majority of compounds presented recoveries between 40% and 70% and relative standard deviations (RSD) below 30%.

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Name and reference of original method	Peña-Herrera J.M., Montemurro N., Barceló D., Pérez S. Combining quantitative and qualitative approaches using Sequential Window Acquisition of All Theoretical Fragment-Ion methodology for the detection of pharmaceuticals and related compounds in river fish extracted using a sample miniaturized method. Journal of Chromatography A, 461009 (2020) [1]. Peña-Herrera J.M., Montemurro N., Barceló D., Pérez S. Analysis of pharmaceuticals in fish using ultrasound extraction and dispersive SPE clean-up on QuE Z-Sep/C18 followed by LC-QToF-MS detection [2]. Aceña, J., Pérez, S., Eichhorn, P. Solé, M., Barceló, D.1. Metabolite profiling of carbamazepine and ibuprofen in *Solea senegalensis* bile using high-resolution mass spectrometry. Anal Bioanal Chem 409, 5441–5450 (2017) [3]. Zhang X., Danaceau J., Chambers E. Quantitative analysis of THC and its metabolites in plasma using Oasis PRiME HLB for toxicology abd forensic laboratories. Waters Corporation (2016) [4].
Resource availability	Thermo Scientific Xcalibur Software v. 4.1.31.9.

*Method details

Currently, there are several analytical methods for the extraction of organic pollutants that mainly focus on their extraction in fish muscle [5–7] or in whole fish homogenate [8–10] because these can be obtained in sufficient amounts even from small species and are easy to produce, respectively. Analyzing a single tissue or homogenate fish does not allow to obtain information on the distribution of the contaminants within the organism. This, however, is important as the analytes may distribute unevenly with lipophilic compounds being more prone to be retained in tissues rich in fat (e.g brain or liver) and hydrophilic ones being more dominant in blood. With this in mind, we present a methodology for the extraction of 81 wastewater-derived organic pollutants, mainly pharmaceuticals and their metabolites, from various fish tissues, plasma, and bile. The extraction was carried out combining bead beating tissue homogenization with ultrasound-solvent assisted extraction solvent followed by a zirconia-based cleanup step (Z-Sep/C18). Separation and quantitation of the analytes were achieved on a Waters Acquity UPLC system coupled with an Orbitrap Q-Exactive™ mass spectrometer. Acquisition was performed in full-scan mode followed by a targeted-DIA-MS where the accurate mass of each compound was fixed in a narrow retention time window. Data analysis and processing were performed with Thermo Scientific Xcalibur v. 4.1.31.9. The proposed extraction method includes easy to follow steps and is efficient in terms of time (preparation of 12 samples in less than two hours) when compared to other techniques for the extraction of PhACs in solid samples such as Solvent Assisted Extraction (ASE) (between 10 and 30 min by sample), Microwave Assisted Extraction (MAE) (between 5 and 20 min) or Soxhlet (360 min) [11].

Chemicals, reagents, and other materials

- *Material for sample preparation:*

BenchMixer XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, U.S.)

Knife mill with stainless steel grinding chamber (Grindomix GM 200, Retsch GmbH, Haan, Germany)

Precision balance (Mettler Toledo)
5810 R centrifuge (Eppendorf AG, Hamburg, Germany)
2-mL Eppendorf tubes (Eppendorf AG, Hamburg, Germany)
TissueLyzer LT Sample Disruptor (QIAGEN GmbH, Germany)
Stainless Steel Beads, 5 mm (QIAGEN GmbH, Germany)
Ultrasound bath (Fisherbrand® FB15064, Waltham, MA, USA)
TurboVap® LV (Biotage. Uppsala, Sweden)

- *Reagents for standards and samples preparation:*

LC-MS grade acetonitrile (MeCN) (≥99.9 %) (Merck Darmstadt, Germany)
LC-MS grade methanol (MeOH) (≥99.9 %) (Merck Darmstadt, Germany)
LC-MS grade dimethyl sulfoxide (DMSO) (≥99.9 %) (Merck Darmstadt, Germany)
LC-MS grade HPLC water (H_2O) (Merck Darmstadt, Germany)

- *Reagents for the extraction procedure and clean up step:*

LC-MS grade isopropanol (iPrOH) (≥ 99.9 %) (Merck Darmstadt, Germany)
Hydrochloric acid 0.1 N (Merck, Darmstadt, Germany)
QuE Z-Sep/C18 Supel™ QuE (Supelco/Sigma-Aldrich, St. Louis, MO, USA)

- *Reagents for mobile phases preparation:*

Optima™ LCMS Grade acetonitrile (Fisher Chemical, Fisher Scientific SL, Madrid, Spain)
Optima™ LCMS Grade water (Fisher Chemical, Fisher Scientific SL, Madrid, Spain)
Acetic and formic acids (98 %) (Sigma-Aldrich, St. Louis, MO, USA)
Ammonium acetate (Sigma-Aldrich, St. Louis, MO, USA)
Ammonium fluoride (Fisher Chemical, Fisher Scientific SL, Madrid, Spain)

- *Reference standards:*

Highly pure (> 90 %) reference standards of PhACs and deuterated standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), Toronto Research Chemicals (Toronto, Ontario, Canada), or Santa Cruz Biotechnology (Dallas, TX). Depending on the compound solubility, individual stock solutions (5 mg L^{-1} or 10 mg L^{-1}) were prepared in HPLC water, MeOH, or DMSO. A complete list of all the chemical standards used, their physicochemical properties, and the solvent in which they were prepared are reported in Table S1 of the Supplementary Material. Working solution mixtures used for validation were prepared by serial dilution in MeOH at concentrations of 200 ng mL^{-1} and 2000 ng mL^{-1}, whereas the deuterated standards mixture was prepared by serial dilution in MeOH at a concentration of 1000 ng mL^{-1} and added to the samples as surrogate standards at 12.5 ng mL^{-1} for solid tissues and 25 ng mL^{-1} for body fluids. All solutions were stored at −20 °C.

Sampling and sample pre-treatment

The solid tissues and body fluids used for the method validation and matrix-matched calibration curves were obtained from a pool of carp (*Cyprinus carpio*) acquired from a fish farm. In addition, to determine the method applicability a sampling campaign was carried out in Sant Joan Despí, in the lower part of the Llobregat River (Catalonia, Spain) in accordance with the technical requirements established by the Biodiversity and Animal Protection Service of the Department of Agriculture, Livestock, Fisheries, Food and Environment of the Generalitat of Catalonia. Four fish species (*Anguilla Anguilla, Cyprinus carpio, Barbus graellsii*, and *Liza ramada*) were captured downstream of the WWTP outlet. The wild fishes were caught by electro-fishing (200–350 V, 2–3 A, fully rectified triphasic DC). At the sampling location, blood from each individual (2 mL) was extracted with a syringe containing 10 µL of lithium heparin to avoid clotting. Once in the laboratory, blood plasma was prepared by centrifugation of 1 mL aliquots for 10 min at 2500 rpm and 4 °C. The supernatant was transferred to a clean Eppendorf tube and centrifuged again for 10 min at 2500 rpm and 4 °C. Finally, 500 µL of the resulting plasma was stored at -80 °C until analysis. The fish were transported at 4 °C to the laboratory. Bile was immediately treated and stored at -40 °C, to prevent any resorption or rupture

of the gallbladder. Then the fish were dissected and the organs/tissues of interest were carefully separated. They were pre-homogenized using a knife mill with a stainless-steel grinding chamber [M&M] and stored at -40 °C until use.

Analyte extraction protocol

A pool of the samples from the individuals of *Cyprinus carpio* was made for each matrix, they were analyzed to verify the absence of the target analytes and were used for method validation and for the preparation of matrix-matched calibration curves.

Solid matrices (muscle, brain, kidney, pancreas, liver, skin, heart)

1 Put 0.5 g of fresh pre-homogenized sample in a 2 mL Eppendorf tube.
2 Spike the sample with 12.5 µL of deuterated standard mixture (1000 ng mL^{-1}) used as surrogate standards (12.5 ng mL^{-1} in the final extract). The concentration levels at which the reference standards were spiked for validation are specified in Note #1 (see at the end of this section).
3 Vortex the sample for 90 s using the BenchMixer XLQ QuEChERS Vortexer.
4 Let the tube rest for 30 min.
5 Add two stainless steel beads to the tube and homogenize for 90 s (skin: 180 s) in a TissueLyzer Sample Disruptor set at 50 Hz.
6 Vortex the Eppendorf tube again for 60 s using the BenchMixer XLQ QuEChERS Vortexer.
7 Let the tube equilibrate for 15 min.
8 Add 1 mL of the extraction solvent: MeCN/iPrOH (3:1) + 0.1% formic acid.
9 Vortex for 60 s.
10 Sonicate for 10 min.
11 Centrifuge for 12 min at 14000 rpm and -2 °C.
12 Transfer 750 µL of the supernatant to a vial containing 70 mg QuE Z-Sep/C18 sorbent for clean-up.
13 Vortex for 60 s. It is recommended not to exceed the indicated time due to possible hydrolysis of polar compounds.
14 Centrifuge again for 6 min at 14000 rpm and -2 °C.
15 Transfer 500 µL of the supernatant to a 2 mL glass HPLC vial.
16 Evaporate to dryness under a gentle stream of nitrogen.
17 Reconstitute with 500 µL of 5 mM ammonium acetate/MeCN (9:1) and vortex for 60 s prior to injection into the HPLC-ESI-HRMS.

Plasma

1 Put 100 µL of plasma in an Eppendorf tube.
2 Add 387.5 µL of cold MeCN and spike the sample with 12.5 µL of deuterated standard mixture (1000 ng mL^{-1}) for its use as surrogate (corresponding to 25 ng mL^{-1} in the final extract). The concentration levels at which the reference standards were spiked for validation are specified in Note #2.
3 Vortex for 60 s.
4 Centrifuge at for 10 min at 8000 rpm and 4 °C
5 Transfer 400 µL of the supernatant to a glass HPLC vial.
6 Evaporate to dryness under a gentle stream of nitrogen.
7 Reconstitute with 500 µL of 5 mM ammonium acetate /MeCN (9:1) and vortex for 60 s prior to injection into the HPLC-ESI-HRMS.

Bile

1 Put 50 µL of bile in an Eppendorf tube.

2 Add 300 µL of HCl 0.1 N, 137.5 µL of cold MeCN and spike the sample with 12.5 µL of deuterated standard mixture (1000 ng mL^{-1}) for its use as surrogate (corresponding to 25 ng mL^{-1} in the final extract). The concentration levels at which the reference standards were spiked for validation are specified in Note #3.
3 Vortex for 60 s.
4 Centrifuge for 10 min at 14000 rpm and 4 °C.
5 Transfer 300 µL of the supernatant to a glass HPLC vial and add 200 µL of acetone to facility the evaporation.
6 Evaporate to dryness under a gentle stream of nitrogen.
7 Reconstitute with 500 µL of 5 mM ammonium acetate /MeCN (9:1).

***Note* #1:** For the validation method, solid tissues samples were spiked with 50 µL of 200 ng mL^{-1} or 2000 ng mL^{-1} reference standard mixture (corresponding 20 ng g^{-1} (level 1) or 200 ng g^{-1} (level2) fresh weight, respectively).

Note #2: For plasma validation 100 µL of the sample was added at 375 µL of cold MeCN and 25 µL of 200 ng mL^{-1} or 2000 ng mL^{-1} reference standard mixture for two levels of validation (corresponding to 50 ng mL^{-1} (level 1) and 500 ng mL^{-1} (level 2) respectively).

Note #3: For bile validation 50 µL of the sample was added at 300 µL of HCl 0.1 N, 125 µL of cold MeCN, and 25 µL of 200 ng mL^{-1} or 2000 ng mL^{-1} reference standard mixture (corresponding to 100 ng mL^{-1} (level 1) and 1000 ng mL^{-1} (level 2), respectively).

Instrumental analysis

Chromatographic separation was carried out with a Waters Acquity UPLC system (Water, Milford, MA) under the conditions shown in Table 1. Detection was performed using an Orbitrap Q-Exactive™ mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA) and the acquisition was in full-scan mode followed by a targeted DIA that combines the monoisotopic mass of each compound with short retention time (RT) windows to obtain high-quality confirmation fragment ions (MS2). For this, an inclusion list was prepared containing the *m/z* of all the precursor ions of interest, and the MS2 scanning events were carried out sequentially through selected RT windows associated with each precursor ion. The MS parameters for the selected method are listed in Table 1. Furthermore, an inclusion list with accurate mass and RT window for each target compound was used to generate as many micro-scanning events as shown in Table S2.

Data analysis and processing were performed with Thermo Scientific Xcalibur v. 4.1.31.9. For the quantification, matrix-matched calibration curves were prepared at 8 concentration levels in the range of 0.5 ng mL^{-1} to 500 ng mL^{-1}, including deuterated standard concentration at each point at 12.5 ng mL^{-1} for solids tissues and 25 ng mL^{-1} for body fluids. The calibration curve was constructed by linear weighted least-squares regression (1/x as weighting factor). The deuterated standards associated for each compound are shown in Table S1.

Method validation

According to [12] the performance of the extraction method (n=3) was evaluated in terms of accuracy (recovery study), precision (% RSD), matrix effect (ME), limits of detection (LOD), and limits of quantification (LOQ) for all matrices. Recovery studies were performed in triplicate at 50 ng mL^{-1} and 500 ng mL^{-1} for plasma, 100 ng mL^{-1} and 1000 ng mL^{-1} for bile, and 20 ng g^{-1} and 200 ng g^{-1} for solid matrices.

Extraction efficiency

In order to evaluate the extraction efficiency, relative recoveries were determined by comparing the peak area of the samples spiked before the extraction method, to the peak area of the ones spiked after the extraction method. The blank extract area (sample without spiking) was subtracted, as shown

Table 1
Analytical platform and respective conditions.

Waters Acquity I-Class UPLC®	
Columns	Phenomenex Reversed-phase EVO C18 KINETEX column (100 × 2.1 mm, 2.6 μm) supplied with precolumn Phenomenex p/n: AJO-9000
Mobile phase A	ESI (+): 5 mM of ammonium acetate and 0.1% acetic acid ESI (-): 2 mM ammonium fluoride[1]
Mobile phase B	MeCN
Flow rate	0.2 mL min^{-1}
Run time	19 min
Injection volume	10 μL
Gradient profile	(1) 0.0 min 5% A (2) 10.0 min 30% A (3) 13.3 min 65% A (4) 15.5 min 100% A (5) 17.7 min 5% A (6) 19.0 min 5% A
Auto sample Temp.	10°C
Flow diverted to waste	Flow diverted to waste 18.0 - 19.0 min
Weak Needle wash	H_2O:/MeCN (95:5, v/v)
Strong Needle wash	MeOH 100%
Seal wash	H_2O/MeOH (70:30, v/v)
Thermo Orbitrap Q-Exactive™ mass spectrometer	
Spray voltage	3.5 kV (ESI +), -2.5 kV (ESI -)
Sheath gas (N_2 >95%)	35 (ESI +), 25 (ESI -)
Resolving power	70,000 FWHM
Capillary temperature	350°C
Aux Gas (N_2 >95%)	10
Auxiliary gas heater temp	250°C
S-Lens RF Level	60
Overall method settings	
Chrom. Peak width (FWHM)	12 s
Full MS - SIM	
Microscans	1
Resolution	35,000
AGC target	3e6
Maximum IT	150 ms
Number of scan ranges	1
Scan range	90 to 1000 m/z
Spectrum data type	Profile
DIA	
Microscans	1
Resolution	17.500
AGC target,	2e5
Maximum IT	auto
Loop count	1
MSX count	1
MSX isochronus ITs	On
Isolation window	1.5 m/z
Isolation offset	0.0 m/z
Spectrum data type	Profile
(N) CE / stepped (N) CE	nce: 30

[1] Ammonium fluoride was used because this additive improves the negative ionization. This is due to the high electronegativity of the fluoride ion [27]. Sensitivity is enhanced because in the gas phase fluoride ions capture protons from neutral analytes giving to HF and the formation of $[M+F]^-$ and $[M+FHF]^-$ ions clusters [28]. The use of ammonium fluoride has been previously reported by [29] for PhACs analysis.

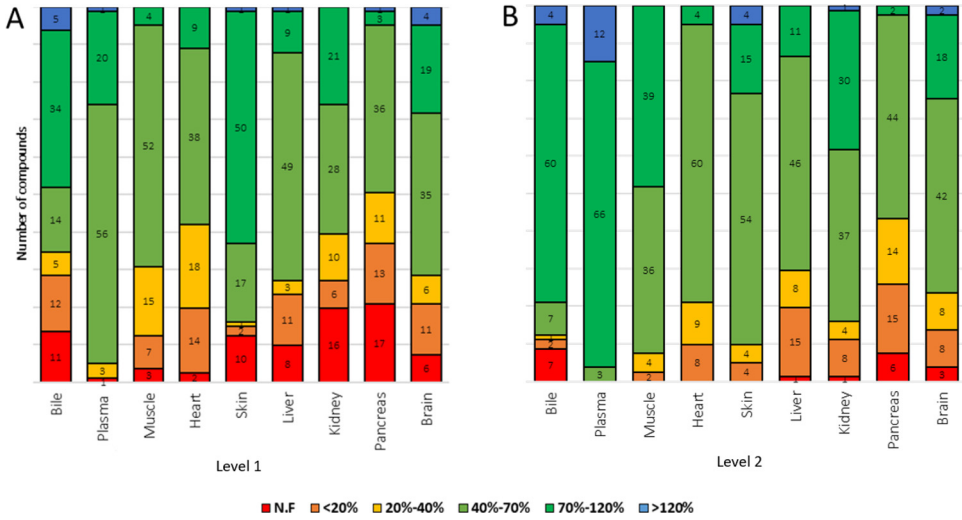

Fig. 1. Recoveries obtained for all matrices at the two validation levels. **A**: Level 1 (100 ng mL^{-1} for bile, 50 ng mL^{-1} for plasma and 20 ng g^{-1} for solid tissues). **B**: Level 2 (1000 ng mL^{-1} for bile, 500 ng mL^{-1} for plasma and 200 ng g^{-1} for solid tissues).

in Eq. (1).

$$RR\ (\%) = \left(\frac{Area_{\ pre-spiked} - Blank\ extract}{Area_{\ post-spiked} - Blank\ extract} \right) x100 \quad (1)$$

Fig. 1 shows the recovery ranges obtained for the 81 compounds. Most compounds displayed recoveries between 40% and 70% for all matrices, except for skin, bile, and plasma where the majority of compounds recovered in the range of 70% to 120%. The high recoveries obtained in bile and plasma can be attributed to the fact that are fewer complex matrices (see Fig. 2). Additionally, the validation levels for these two matrices are greatest (100 ng mL^{-1} and 50 ng mL^{-1} for the level 1 respectively).

At level 2, the number of compounds with recoveries between 70 to 120% increases substantially in bile, plasma, and muscle (see Fig. 1), while the number of compounds not found is smaller. On the other hand, the liver, kidney, and pancreas are more complex matrices where for approximately 30% of the compounds recoveries below 40% were obtained, especially for negative ionization compounds.

Overall, the compounds that obtained the highest average recoveries and were recovered in a percentage higher than 70% in most matrices were sotalol, caffeine, fluconazole, hydrochlorothiazide, and pentobarbital for the first validation level and trimethoprim, acridone, bromazepam, sitagliptin, N-acethyl-smx, CBZ-10,11-epoxide, hydrochlorothiazide, chloramphenicol, pentobarbital, and fipronil for the second validation level.

Flufenamic acid was the only compound not recovered in any matrix for the first level of validation, however, for the second level it was possible to recover it in bile, plasma, muscle, skin, and kidney in a percentage higher than 40%. Atorvastatin was only recovered in bile and plasma for the first level of validation, while in the second level it was recovered in all matrices except the pancreas. Ciprofloxacin and omeprazole only presented acceptable recovery values in plasma for the first level of validation, but at the second level, ciprofloxacin was recovered in all matrices except the brain, and omeprazole was recovered in muscle, heart, skin, and brain with values higher than 45%. In general, oxytetracycline and norfluoxetine were the compounds that presented the lowest recoveries in the two levels of validation for all matrices except in the plasma and skin.

In relation to the compounds with the best recoveries (>90%) obtained in each of the matrices evidenced that in the first validation level in bile are sertraline (99%), ketoprofen (93%), sulfamethazine (99%), nalidixic acid (93%), flumequine (93%), oxazepam (103%), propyphenazone (91%),

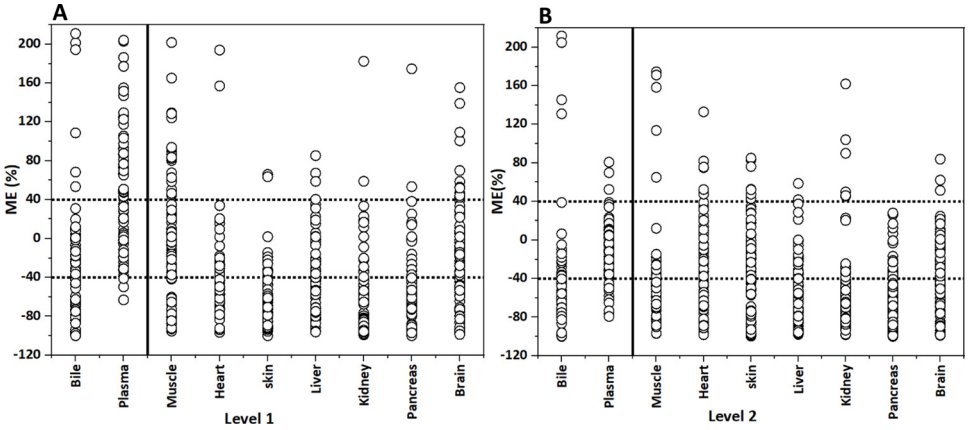

Fig. 2. Matrix effect obtained by all tissues and body fluids for the two validation levels. **A**: Level 1 (100 ng mL^{-1} for bile, 50 ng mL^{-1} for plasma and 20 ng g^{-1} for solid tissues). **B**: Level 2 (1000 ng mL^{-1} for bile, 500 ng mL^{-1} for plasma and 200 ng g^{-1} for solid tissues).

temazepam (94%), pentobarbital (110%), bisphenol A (100%), and atorvastatin (92%). While in plasma, compounds such as sertraline (105%), paroxetine (91%), and norfluoxetine (93%) stand out. The number of compounds with recoveries greater than 90% increased to 49% for the second validation level in bile and to 77% for plasma.

In solid tissues for the first validation level, sertraline and fipronil sulfide stand out in muscle (99%) (95%) and skin (102%) (95%) respectively. In the kidney, the highest recoveries were obtained for temazepam (96%) and diazepam (109%); in the pancreas, acetaminophen (110%) and, in the brain, sulfamethoxazole (101%) and bromazepam (106%). For the second validation level, furazolidone (97%) and erythromycin (105%) stand out in the muscle; fluoxetine (118%), sertraline (106%), loratadine (91%), bromazepam (100%), paroxetine (96%) and norfluoxetine (99%) in the skin and; clarithromycin (110%), diazepam (93%), verapamil (110%), desmethylcitalopram (102%), alprazolam (91%) and atorvastatin (95%) for the kidney. In the heart and in the liver, no compound was recovered a percentage greater than 90% for any of the validation levels. In the case of the liver, the low recoveries may be associated with the fact that this organ, which is the main metabolic organ of fish [13,14], has a higher lipid content than in the whole body homogenates [15,16] and retains a greater amount of blood [17]. The heart also has a high blood content which interferes with the suppression of the analyte signal, as can be seen in Fig. 2.

Matrix effects

To evaluate the matrix effect (suppression or enhancement of the analyte signal), the ratio of the peak area of the analyte spiked into the final extracts (*Area post-spiked*) is compared to the peak area of the analyte in a solvent (*Area in solvent*) at the same concentration. The blank extract area was subtracted according to Eq. (2):

$$ME\ (\%) = 100\ x\left(\frac{Area_{\ post-spiked} - Blank\ extract}{Area\ in\ solvent} - 1\right) \quad (2)$$

For most of the compounds, a strong signal suppression was observed, especially in the heart, kidney, liver, and pancreas, where more than 40% of the compounds presented a suppression of less than -40%. However, for the other matrices, the ME was calculated within an acceptable range, -40% to +40%, with a larger number of compounds within this range for the higher level. Plasma was the only matrix that displayed a strong signal enhancement (>40%) for most of the compounds, but only for the first validation level, since at the second level, more than 70% of the compounds had an acceptable

Table 2
LOD and LOQ estimated for each matrix.

Matrix	LOD (ng g^{-1})	LOQ (ng g^{-1})
Bile[1]	0.02–28.71	0.08-95.69
Plasma[1]	0.01–1.56	0.03–5.20
Muscle	0.01–9.27	0.02–30.90
Heart	0.0004–3.85	0.001–12.82
Skin	0.004–3.46	0.01–11.54
Liver	0.01–2.66	0.03–8.87
Kidney	0.01–14.91	0.02–49.70
Pancreas	0.001–8.55	0.004–28.49
Brain	0.004–14.10	0.01–47.00

[1] Units in ng mL^{-1}.

ME. Most compounds with negative ionization had strong signal suppression (close to -100 %) in the most complex matrices (liver, kidney and, pancreas).

In general, the recoveries obtained for most compounds were between 40% and 70% and the ME was signal suppression of the analytes. Similar recoveries and ME values were obtained by [9] and [15] for liver; by [18] and [19] for muscle and plasma. Matrices such as heart, skin, and pancreas were validated here for the first time.

Detection and quantification limits

The detection and quantification limits were estimated according to [20] based on the average of the signal-to-noise ratio (*S/N*) that the analytes showed in the pre-spiked samples and the spiked samples concentration (*C*), according to Eq. (3) and Eq. (4). Table 2 shows the LOD and LOQ ranges of the extraction method.

$$LOD = \frac{3\ x\ C}{S/N} \tag{3}$$

$$LOQ = \frac{10\ x\ C}{S/N} \tag{4}$$

The highest LOD and LOQ were calculated for oxytetracycline compound. Muscle 9.27 ng g^{-1} and 30.90 ng g^{-1}, heart 3.85 ng g^{-1} and 12.82 ng g^{-1}, kidney 14.91 ng g^{-1} and 49.70 ng g^{-1}, brain 14.10 ng g^{-1} and 47.00 ng g^{-1}, and in bile 10.90 ng mL^{-1} and 36.35 ng mL^{-1}, coinciding with its low percentage recovery in most matrices.

The recoveries, RSDs, ME, LOD and LOQ for the two validation levels for each compound and matrix, are reported in Tables S3, S4, and S5.

Method robustness

According to [12] the method robustness can be expressed as the relative standard deviation (RSD%) of the data obtained. In this study, the robustness was expressed as a function of the calculated RSD of the recoveries (samples analyzed in triplicate) for each validation level. In addition, RSD $<$30% and recovery $>$40% for the two validation levels were established as acceptability criteria to define the compounds that can be reliably determined in each matrix. Table 3 shows the compounds that met the range of acceptability for each of the matrices.

For the plasma 78 of the compounds satisfy the established criteria, while in the pancreas the number of compounds within the acceptable range was 33. However, it is important to remark that since our multi-matrix method, it is valid to expect that these values fluctuate from one matrix to another, as a consequence of the intrinsic characteristics of each one, without compromising the robustness of the method.

Table 3
PhACs within acceptable range.[1]

PhACs	Muscle	Heart	Skin	Liver	Kidney	Pancreas	Brain	Plasma	Bile
Benzotriazole	√	√	√	x	x	x	√	√	√
5-methyl-Benzotriazole	√	√	√	√	√	x	√	√	√
Acetaminophen	x	x	√	x	x	x	x	√	x
Acridone	√	√	√	√	√	x	√	√	√
Alprazolam	√	√	√	x	√	x	x	√	√
Amantadine	√	√	√	√	√	√	√	√	√
Atenolol	√	√	√	√	√	√	√	√	√
Atorvastatin	x	x	x	x	x	x	x	√	√
Benzoylecgonine	√	√	√	√	√	√	√	√	√
Bezafibrate	√	√	√	√	√	x	√	√	√
Bisphenol-A	x	x	x	√	x	x	x	√	√
Bromazepam	x	√	√	√	√	√	√	√	√
Caffeine	√	√	√	√	√	√	√	√	√
Carazolol	x	x	√	√	√	x	√	√	x
Carbamazepine	√	√	√	√	√	√	x	√	√
CBZ-10,11-epoxide	√	√	√	√	√	√	√	√	x
Clarithromycin	√	√	√	x	√	√	√	√	x
Chloramphenicol	√	√	√	√	√	x	√	√	√
Ciprofloxacin	x	x	x	x	x	x	x	√	√
Clofibric_Acid	√	√	√	√	√	√	√	√	√
Coca-ethylene	√	√	√	x	√	√	x	√	x
Cocaine	√	√	√	x	√	x	√	√	x
Codeine	√	√	√	√	√	x	√	√	√
Cotinine	√	√	√	√	x	x	√	√	√
Desmethylcitalopram	√	x	x	x	√	x	x	√	x
Diazepam	√	√	√	x	√	x	x	√	√
Diclofenac	x	x	x	√	x	x	√	√	x
Diltiazem	√	√	√	√	√	√	x	√	x
Erythromycin	√	√	√	√	√	x	√	√	x
Fipronil	√	√	√	x	√	x	x	√	√
Fipronil_Desulfinyl	x	x	x	x	√	x	x	√	x
Fipronil_Sulfide	x	x	√	x	x	x	x	√	x
Fipronil_sulfone	√	x	x	x	x	x	x	√	x
Fluconazole	√	√	√	√	√	√	√	√	√
Flufenamic_Acid	x	x	x	x	x	x	x	x	x
Flumequine	x	√	x	x	x	√	x	√	√
Fluoxetine	√	x	√	√	√	x	√	√	x
Norfluoxetine	x	x	x	x	x	x	x	√	x
Furazolidone	√	√	√	x	√	√	√	√	√
Furosemide	x	x	√	√	x	x	√	√	√
Hydrochlorothiazide	√	√	√	√	√	√	√	√	√
Ibuprofen	√	√	√	√	x	√	x	√	√
Irgasan	x	x	√	x	x	x	x	√	x
Ketoprofen	√	√	√	√	√	√	√	√	√
Lamotrigine	√	√	√	√	x	x	x	√	√
Loratadine	x	x	√	x	x	x	x	√	√
Lorazepam	√	√	√	√	√	x	√	√	x
Losartan	x	x	x	x	x	x	x	√	√
Mefenamic acid	x	x	x	x	x	x	√	√	x
Metformin	x	√	√	x	x	x	√	x	x
Metoprolol	√	√	√	√	√	√	√	√	√
Midazolam	x	x	√	x	√	x	x	√	x
Morphine	x	x	x	x	x	x	x	√	√
Nadilixic Acid	x	x	x	x	x	√	x	√	√
Omeprazole	x	x	x	x	x	x	x	√	x
Oseltamivir_phospate	√	x	√	√	√	x	√	√	x
Oseltamivir-CBX	√	√	x	√	√	√	x	√	√
Oxazepam	x	√	√	√	√	√	√	√	√
Oxytetracycline	x	x	x	x	x	x	x	x	x

(*continued on next page*)

Table 3 (*continued*)

PhACs	Muscle	Heart	Skin	Liver	Kidney	Pancreas	Brain	Plasma	Bile
Paroxetine	*x*	*x*	√	*x*	*x*	*x*	*x*	√	*x*
Pentobarbital	√	√	√	√	√	√	√	√	√
Propyphenazone	√	√	√	√	√	√	√	√	√
Salbutamol	*x*	√	√	√	√	√	√	√	√
Sertraline	√	√	√	√	√	√	√	√	√
Sitagliptin	√	√	√	√	√	*x*	√	√	*x*
Sotalol	√	√	√	√	√	√	√	√	√
Sulfadimethoxine	√	*x*	√	√	*x*	*x*	√	√	√
Sulfamethazine	√	*x*	√	√	*x*	*x*	√	√	√
Sulfamethoxazole	√	*x*	√	√	*x*	*x*	√	√	√
N-acethyl_SMX	√	√	√	√	√	√	√	√	√
Sulfapyridine	√	*x*	√	√	*x*	*x*	√	√	√
Temazepam	√	√	√	√	√	√	*x*	√	√
Tramadol	√	√	√	√	√	√	√	√	*x*
Triclocarban	*x*	*x*	√	*x*	*x*	*x*	*x*	√	*x*
Trimethoprim	√	√	√	√	√	√	√	√	√
Valsartan	√	*x*	√	√	*x*	*x*	*x*	√	√
Valsartan Acid	*x*	*x*	*x*	*x*	*x*	*x*	*x*	√	√
Venlafaxine	√	√	√	√	√	√	√	√	*x*
O-Desmethylvenlafaxine	√	√	√	√	√	√	√	√	√
Verapamil	*x*	*x*	√	*x*	√	√	*x*	√	√
Warfarin	√	√	√	√	√	*x*	√	√	√
Total of compounds with acceptable range (√)	**52**	**48**	**63**	**49**	**49**	**33**	**47**	**78**	**52**

[1] The parameters for which an acceptable range was determined were that the compound obtained a recovery greater than 40% and an RSD less than 30% for the two validation levels. (√) Meets acceptable range. (*x*) Does not meet acceptable range.

Occurrence of the target analytes in fish samples

In order to evaluate the applicability of the validated method, tissues and body fluids from 4 wild fish (*Cyprinus carpio, Liza ramada, Barbus graellsii, and Anguilla Anguilla*) were analyzed. Quantification was performed by the internal standard method with matrix-matched calibration standards. The present method compensates for recovery losses and ionization suppression/enhancement effects [21,22]. Therefore, the deuterated standards were spiked as surrogates at the beginning of the extraction at a concentration of 12.5 ng mL^{-1} for solid tissues and 25 ng mL^{-1} for body fluids as previously indicated.

As a result, 11 PhACs were detected. The kidney and pancreas presented the higher detection frequency with 12 and 10 positive detections, respectively. Sertraline was detected in all individuals and in all matrices except bile, with a concentration range between 5.7 ng g^{-1} (heart) and 1010.2 ng g^{-1} (pancreas). Similar concentrations of sertraline in fish tissues have been reported previously. A maximum concentration (cmax) of 110 ng g^{-1} in the brown trout liver collected downstream of a WWTP in the USA has been reported by [23]. A cmax of 44 ng g^{-1} in the kidney of brown trout caught in a river in the southern Czech Republic was reported by [24]. Diltiazem and venlafaxine were detected in most matrices although only in *Liza ramada*. Diltiazem concentrations were less than 10 n g^{-1} in all matrices (brain, kidney, liver, pancreas and plasma), whereas venlafaxine was quantified in a range from 27.4 ng g^{-1} (skin) to 153.8 ng g^{-1} (brain). The presence of diltiazem has been reported by other authors [25] in the brain of fish with a Cmax of 1.5 ng g^{-1} and a Cmax of 34 ng g^{-1} in the muscle of *Leuciscus cephalus* captured from the Sava river in Slovenia has been reported by [5]. Othe authors [26] reported a Cmax of 20.8 ng g^{-1} for venlafaxine in the liver of rainbow trout exposed to undiluted effluent from a Swedish municipal WWTP for 13 days.

Acridone (643.5 ng mL^{-1}), caffeine (50.2 ng g^{-1}), ketoprofen (1.1 ng g^{-1}), and loratadine (3.0 ng mL^{-1}) were only detected in one matrix (bile, brain, kidney, heart, and plasma) respectively. Fluoxetine was detected in the brain (5.2 ng g^{-1}) which was also detected in the kidney (22.9 ng g^{-1}).

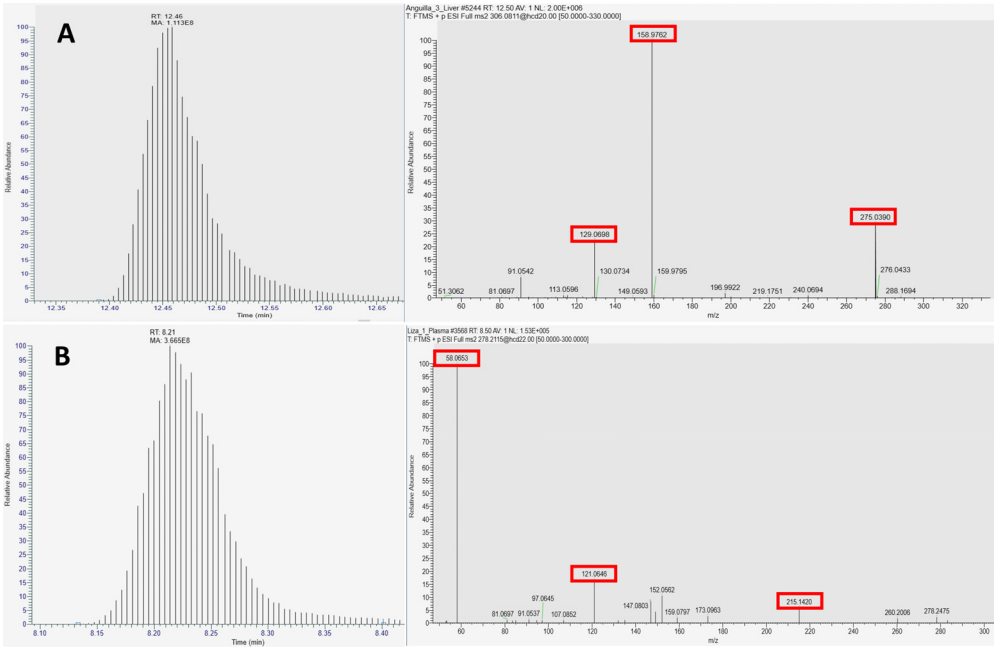

Fig. 3. Chromatograms and their corresponding MS2 spectra for two of the compounds detected in fish tissues and body fluids. Characteristics product ions are highlighted in a red box. **A**: Chromatogram and MS2 spectra for venlafaxine detected in *Anguilla anguilla* liver. **B**: Chromatogram and MS2 spectra for sertraline detected in *Liza ramada* plasma**.**

Clarithromycin was detected in the kidney (2.5 ng g^{-1}) and pancreas (6.8 ng g^{-1}). Metoprolol was detected in kidney (105.5 ng g^{-1}), skin (13.6 ng g^{-1}) and heart (1.3 ng g^{-1}) and sotalol was detected in skin (4.3 ng g^{-1}), liver (5.4 ng g^{-1} to 7.9 ng g^{-1}), pancreas (3.1 ng g^{-1}) and plasma (<LOQ). Fig. 3 shows chromatograms and their corresponding MS2 spectra for two of the compounds detected in fish tissues and body fluids.

Conclusions

Overall, the extraction method developed showed recoveries between 40% and 70 % and satisfactory analytical parameters for most of the validated fish tissues/organs and for a wide range of compounds with different physicochemical properties. This method will allow the evaluation of the distribution of PhACs in fish exposed to wastewater and select a subset of matrices that provide sensitive detection of these compounds in unprotected fish.

Tissues and body fluids from four fish species were analyzed. Due to the complexity of the matrices, especially the pancreas, liver, and heart (which presented the lowest recovery values and highest analyte suppression), it was decided to perform the quantification with the internal standard method with deuterated standards as surrogates at the beginning of the extraction and to use matrix-matched calibration to ensure the reliability of the calculated data. The application of this approach together with the extraction method developed allowed the detection and quantification of 11 PhACs in similar concentrations to previous studies.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2022.101705.

References

[1] J.M. Peña-Herrera, N. Montemurro, D. Barceló, S. Pérez, Combining quantitative and qualitative approaches using Sequential Window Acquisition of All Theoretical Fragment-Ion methodology for the detection of pharmaceuticals and related compounds in river fish extracted using a sample miniaturized method, J. Chromatogr. A 1620 (2020) 461009, doi:10.1016/j.chroma.2020.461009.
[2] J.M. Peña-Herrera, N. Montemurro, D. Barceló, S. Pérez, Analysis of pharmaceuticals in fish using ultrasound extraction and dispersive spe clean-up on que Z-Sep/C18 followed by LC-QToF-MS detection, MethodsX 7 (2020) 101010, doi:10.1016/J.MEX.2020.101010.
[3] J. Aceña, S. Pérez, P. Eichhorn, M. Solé, D. Barceló, Metabolite profiling of carbamazepine and ibuprofen in Solea senegalensis bile using high-resolution mass spectrometry, Anal. Bioanal. Chem. 409 (23) (2017) 5441–5450, doi:10.1007/s00216-017-0467-7.
[4] "Quantitative Analysis of THC and its Metabolites in Plasma Using Oasis PRiME HLB for Toxicology and Forensic Laboratories | Waters." https://www.waters.com/waters/library.htm?locale=en_US&lid=134916779 (accessed Nov. 04, 2021).
[5] J.M. Peña-Herrera, N. Montemurro, D. Barceló, S. Pérez, Combining quantitative and qualitative approaches using Sequential Window Acquisition of All Theoretical Fragment-Ion methodology for the detection of pharmaceuticals and related compounds in river fish extracted using a sample miniaturized method, J. Chromatogr. A 1620 (2020) 461009, doi:10.1016/j.chroma.2020.461009.
[6] B. Huerta, et al., Presence of pharmaceuticals in fish collected from urban rivers in the U.S. EPA 2008–2009 National Rivers and Streams Assessment, Sci. Total Environ. 634 (2018) 542–549, doi:10.1016/j.scitotenv.2018.03.387.
[7] J.M. Peña-Herrera, N. Montemurro, D. Barceló, S. Pérez, Development and validation of an analytical method for determination of pharmaceuticals in fish muscle based on QuEChERS extraction and SWATH acquisition using LC-QTOF-MS/MS system, Talanta 199 (2019) 370–379, doi:10.1016/j.talanta.2019.01.119.
[8] K. Grabicová, et al., Water reuse and aquaculture: pharmaceutical bioaccumulation by fish during tertiary treatment in a wastewater stabilization pond, Environ. Pollut. 267 (2020) 115593, doi:10.1016/j.envpol.2020.115593.
[9] B. Huerta, A. Jakimska, M. Gros, S. Rodríguez-Mozaz, D. Barceló, Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry, J. Chromatogr. A 1288 (2013) 63–72, doi:10.1016/J.CHROMA.2013.03.001.
[10] A. Serra-Compte, D. Álvarez-Muñoz, S. Rodríguez-Mozaz, D. Barceló, Multi-residue method for the determination of antibiotics and some of their metabolites in seafood, Food Chem. Toxicol. 104 (2017) 3–13, doi:10.1016/J.FCT.2016.11.031.
[11] R. Manasfi, F. Labad, N. Montemurro, Development of methods for the determination of PhACs in soil/earthworm/crop system irrigated with reclaimed water Pérez Solsona, S., Montemurro, N., Chiron, S., Barceló, D., Interaction and Fate of Pharmaceuticals in Soil-Crop Systems. The Handbook of Environmental Chemistry, 103, Springer, Cham, 2020, doi:10.1007/698_2020_650.
[12] A. Kruve, et al., Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part I, Anal. Chim. Acta 870 (1) (2015) 29–44, doi:10.1016/J.ACA.2015.02.017.
[13] P.M. Ondarza, S.P. Haddad, E. Avigliano, K.S.B. Miglioranza, B.W. Brooks, Pharmaceuticals, illicit drugs and their metabolites in fish from Argentina: Implications for protected areas influenced by urbanization, Sci. Total Environ. 649 (2019) 1029–1037, doi:10.1016/J.SCITOTENV.2018.08.383.
[14] X. Yin, et al., Tissue-specific accumulation, elimination, and toxicokinetics of illicit drugs in adult zebrafish (Danio rerio, Sci. Total Environ. 792 (2021) 148153, doi:10.1016/J.SCITOTENV.2021.148153.
[15] K. Grabicova, A. Vojs Staňová, O. Koba Ucun, A. Borik, T. Randak, R. Grabic, Development of a robust extraction procedure for the HPLC-ESI-HRPS determination of multi-residual pharmaceuticals in biota samples, Anal. Chim. Acta 1022 (2018) 53–60, doi:10.1016/J.ACA.2018.04.011.
[16] C. Pietsch, C. Schulz, P. Rovira, W. Kloas, P. Burkhardt-Holm, Organ damage and hepatic lipid accumulation in carp (cyprinus carpio L.) after feed-borne exposure to the mycotoxin, deoxynivalenol (DON), Toxins (Basel) 6 (2014) 756–778, doi:10.3390/toxins6020756.
[17] Z. Xie, G. Lu, S. Li, Y. Nie, B. Ma, J. Liu, Behavioral and biochemical responses in freshwater fish Carassius auratus exposed to sertraline, Chemosphere 135 (2015) 146–155, doi:10.1016/J.CHEMOSPHERE.2015.04.031.
[18] K. Grabicová, et al., Water reuse and aquaculture: Pharmaceutical bioaccumulation by fish during tertiary treatment in a wastewater stabilization pond, Environ. Pollut. 267 (2020) 115593, doi:10.1016/j.envpol.2020.115593.

[19] P. Arnnok, R.R. Singh, R. Burakham, A. Pérez-Fuentetaja, D.S. Aga, Selective Uptake and Bioaccumulation of Antidepressants in Fish from Effluent-Impacted Niagara River, Environ. Sci. Technol. 51 (18) (2017) 10652–10662, doi:10.1021/ACS.EST.7B02912.
[20] N. Montemurro, C. Postigo, A. Lonigro, S. Perez, D. Barceló, Development and validation of an analytical method based on liquid chromatography–tandem mass spectrometry detection for the simultaneous determination of 13 relevant wastewater-derived contaminants in lettuce, Anal. Bioanal. Chem. 409 (23) (2017) 5375–5387, doi:10.1007/S00216-017-0363-1/FIGURES/3.
[21] A. Kruve, et al., Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part II, Anal. Chim. Acta 870 (1) (2015) 8–28, doi:10.1016/J.ACA.2015.02.016.
[22] M. Cortese, M. R. Gigliobianco, F. Magnoni, R. Censi, and P. Di Martino, "molecules Compensate for or Minimize Matrix Effects? Strategies for Overcoming Matrix Effects in Liquid Chromatography-Mass Spectrometry Technique: A Tutorial Review," 2022 doi:10.3390/molecules25133047.
[23] B. Du, P. Perez-Hurtado, B.W. Brooks, C.K. Chambliss, Evaluation of an isotope dilution liquid chromatography tandem mass spectrometry method for pharmaceuticals in fish, J. Chromatogr. A 1253 (2012) 177–183, doi:10.1016/J.CHROMA.2012.07.026.
[24] K. Grabicova, et al., Bioaccumulation of psychoactive pharmaceuticals in fish in an effluent dominated stream, Water Res 124 (2017) 654–662, doi:10.1016/j.watres.2017.08.018.
[25] E.S. McCallum, E. Krutzelmann, T. Brodin, J. Fick, A. Sundelin, S. Balshine, Exposure to wastewater effluent affects fish behaviour and tissue-specific uptake of pharmaceuticals, Sci. Total Environ. 605–606 (2017) 578–588, doi:10.1016/J.SCITOTENV.2017.06.073.
[26] K. Grabicova, et al., Tissue-specific bioconcentration of antidepressants in fish exposed to effluent from a municipal sewage treatment plant, Sci. Total Environ. 488–489 (1) (2014) 46–50, doi:10.1016/J.SCITOTENV.2014.04.052.
[27] L. Narduzzi et al., "metabolites Ammonium Fluoride as Suitable Additive for HILIC-Based LC-HRMS Metabolomics," doi:10.3390/metabo9120292.
[28] K. Takkis, et al., Signal Enhancement in the HPLC-ESI-MS/MS analysis of spironolactone and its metabolites using HFIP and NH4F as eluent additives, Anal. Bioanal. Chem. 409 (12) (2017) 3145, doi:10.1007/S00216-017-0255-4.
[29] N. Montemurro, A. Orfanioti, R. Manasfi, N.S. Thomaidis, S. Pérez, Comparison of high resolution mrm and sequential window acquisition of all theoretical fragment-ion acquisition modes for the quantitation of 48 wastewater-borne pollutants in lettuce, J. Chromatogr. A 1631 (2020) 461566, doi:10.1016/J.CHROMA.2020.461566.