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

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Abstract

This study aimed at developing an analytical method for the extraction and quantification of 21 pharmaceutical actives compounds (PhACs) present in fish muscle. Using Norwegian Atlantic salmon as matrix, two extraction methods for PhACs were tested: ultrasound extraction (USE) using methanol (MeOH), acetonitrile (MeCN) or a mixture of MeCN:MeOH (1:1, v/v) as extracting solvents, and QuEChERS method using three different extraction salts. After selecting QuEChERS Original as extracting method of the analytes, three different clean-up methods were evaluated with respect to their efficiency to remove coextracted fat and lipids such as Enhanced Matrix Removal (EMR) and HLB prime. The dispersive-SPE EMR yielded the best recoveries for 21 of 27 analytes. PhACs were quantified by UPLC-MS/MS using SWATH acquisition mode. The method was validated in terms of recoveries, accuracy, linearity, precision, matrix effects at three levels of concentration: 25, 200 and ~~500~~500 ng g^{-1} dw of fish muscle. For the majority of the analytes the recoveries were over 70%. Finally, the validated method was applied to natural riverine fish from the Evrotas river (Greece) and the Adige river (Italy) with positive findings for acetaminophen, propranolol, and venlafaxine reaching concentrations as high as ~~80~~80 ng g^{-1} of muscle.

Keywords: Fish muscle; Pharmaceuticals; QuEChERS; LC-HR-MS/MS; SWATH acquisition; Hybrid-QTOF

1 Introduction

The first report on the presence of pharmaceuticals in the wastewater impacted river appeared in the mid-1970s [1–3] followed by the first systematic works in the late 1990s [4]. Studies on the accumulation of pharmaceutically active compounds (PhACs) on aquatic organisms such as fish were not initiated until the beginning of the ~~21~~21st ~~st~~ century [5]. Fish tissues such as muscles can be considered as a bio-indicator of water pollution due to the direct exposure to wastewater borne contaminants in rivers.

The methods for PhAC extraction from fish muscle includes solid-liquid extraction [6–8], enzymatic microwave-assisted extraction [6,9], ultra-sound extraction (USE) [10], pressurized liquid extraction (PLE) [11,12], solid-phase microextraction (SPME) [13], and rotary extraction [5,14]. The clean-up step usually relies on solid phase extraction (SPE) with various types of sorbents: florisil, alumina, silica gel, hydrophilic-lipophilic balance (HLB) and mixed-mode cation-exchange (MCX) [15–17], gel permeation chromatography, washing with hexane, liquid-liquid extraction, and freezing out [15,18]

An alternative and revolutionary method for the simultaneous extraction and clean-up of pesticides in foodstuff [19] which recently has been used for the determination of PhACs in biological matrices is the so-called QuEChERS (for **Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged and **S**afe). Few studies are dealing with the extraction of PhACs using Q~~U~~uEC~~H~~ hERS in different matrices such as earthworms [18], vegetables [20], and bivalves [21]. QuEChERS is based on liquid-liquid partitioning of the analytes between water and acetonitrile (MeCN) using salts to produce the effect of salting out, followed by a dispersive solid phase extraction (d-SPE). This procedure offers many advantages including basic instrumentation for application, small sample amounts, rapid extraction, and low waste volume, which results in a time-efficient method.

The use of QuEChERs for the determination of PhACs from complex biological matrices, such as fish muscle, generates extracts that contain material prone to adversely affect the mass spectrometric analysis (co-extracts),

ultimately compromising the method robustness. In particular fat and lipids, which in fish samples can be as high as 50%, pose problems [22]. Due to the simplicity, speed, and availability of chemically diverse sorbents that afford different retention mechanisms (primary and secondary amines, octadecylsilane, graphitized carbon and zirconia-based sorbent), d-SPE, as the basis of QuEChERS, remains the most widely used tool for the clean-up step of complex matrices. Recently, studies on QuEChERS-based determination of organic pollutants evaluating d-SPE materials such as ~~e~~Captiva ND sorbents [23] or the more recently launched sorbents enhanced matrix removal (EMR) and HLB prime that were specifically designed for lipid removal from high-fat samples as avocado, salmon and pork [24,25]. However, to the best of our knowledge the latter ones have not been applied in studies for the analysis of PhACs in fish muscle.

For a rapid analysis, the development liquid chromatography coupled to high-resolution ~~accurate~~ mass spectrometry (LC-HRAMS) has been demonstrated to be very useful and effective for the analysis, identification, and quantification of a broad range of compounds incluiding PhACs [26–28]. Moreover, the new developed technologies as SWATH acquisition (Sequential Windowed Acquisition of All THeoretical MS), has proved to be very useful and effective for the identification of target and non-target compounds for high and low molecular weight compounds [29,30]. In addition, this HR~~A~~MS emerging technology allows for a comprehensive strategy for the identification and quantitation of analytes complex samples using orthogonal time-of-flight (QqTOF) platforms. The corresponding MS/MS quantification is obtained by matching high quality MS/MS spectra against a commercial HR-MS/MS spectral library (using reference standards).

Therefore, the aim of the present work was to develop an extraction methodology for the determination of 27 PhACs in fish using QuEChERS. The target analytes were selected to cover a broad range of PhACs frequently reported in the aquatic environment and biota [14,28,31–33]. After selection of the best QuEChERS extraction salt, the performance of three types of clean-up sorbents was evaluated of which two were specifically designed for lipid removal (Waters Oasis HLB Prime and Agilent EMR lipid removal). Subsequently the optimization of extraction and clean-up steps, the analytical methodology was validated for a subset of 21 out of 27 initially selected PhACs in muscle from two fish species: *Salmo salar* and the freshwater fish *Leuciscus cephalus*. Quantitative determination was performed by HPLC-(+)ESI-QToF-MS supported data independent acquisition (DIA) method SWATH [29]. Finally, the method was applied to evaluate the occurrence of PhACs in six fish species namely *Squalius keadicus, Salmo trutta fario, Salmo trutta marmoratus, Cottus gobio, Leuciscus cephalus,* and *Thymallus thymallus* from two different European rivers (Evrotas river, Greece, and Adige river, Italy).

2 Materials and ~~Methods~~methods

2.1 Chemicals ~~& Materials~~and materials

Chemical standards used for this methodology, including isotopically labeled standards (IS), were purchased from Sigma-Aldrich (Steinheim, Germany), Fluka (Honeywell International, NJ), Cerilliant (Sigma Aldrich, TX), Jescuder (Rubí, Spain), LGC Promochem (London, UK), CDN Isotopes (Quebec, Canada), Santa Cruz Biotechnology (Dallas, TX). All PhACs and IS were > 99% purity. Information about individual suppliers and the physico-chemical properties of the compounds can be found in ~~Table A.1,~~Table A.1, Supplementary material (~~SM~~SM). HPLC grade solvents were purchased from Merck (Darmstadt, Germany): methanol (MeOH) (purity ≥99.9%), MeCN (purity ≥99.9%), hexane (purity ≥99.0%), acetone (purity ≥99.7%) and HPLC water. Chloroform (purity ≥99.9%) and dichloromethane (DCM) (purity ≥99.8%) were supplied by Carlo Erba (Barcelona, Spain) and Panreac Química SLU (Barcelona, Spain) respectively.

Individual stock solutions of the target analytes were prepared in MeOH at a concentration of 1000 mg L^{-1} while the level of individual IS stock solutions was 100 mg L^{-1}. For validation, calibration and spiking purposes, working standard solutions containing all target compounds were prepared in MeOH at concentrations of 10 and 1 mg L^{-1}. A separate mixture of the IS used for internal standard calibration as well as the surrogates were prepared in MeOH at the same concentrations. All solutions were stored in the dark at −20 °C. For extraction purposes, two extraction techniques were assessed: USE and QuEChERS. For the first one, three different solvents were tested: MeOH, MeCN, and MeOH:MeCN (1:1, v/v). In the case of QuEChERS, three extraction salts were tested: the ***original*** QuEChERS [19] consisting of 4.0 g anhydrous magnesium sulfate and 1.0 g sodium chloride (BEKOlut GmbH & Co. KG. Hauptstuhl, Germany); QuEChERS citrate ***buffer*** [34] composed of 4.0 g anhydrous magnesium sulfate, 1.0 g sodium chloride, 1.0 g sodium citrate, and 0.5 g disodium citrate sesquihydrate (BEKOlut GmbH & Co. KG) and QuEChERS ***formate*** [35] consisting of 5.0 g ammonium formate (Sigma-Aldrich, Steinheim, Germany).

For the optimization of the clean-up, three methods were compared: (a) clean-up with the QuEChERS kit (BEKOlut GmbH & Co. KG) consisting of ~~150~~150 mg of primary secondary amin~~o~~e (PSA) sorbents, ~~150~~150 mg of octadecyl (C18)-bonded silica, and ~~900~~900 mg anhydrous magnesium sulfate, (b) clean-up with SPE OASIS HLB Prime, specific for lipids and chlorophyll (3 cc, ~~150~~150 mg; Waters, Milford, MA), and (c) clean-up by d-SPE on Bond elut QuEChERS Enhanced Matrix Removal (EMR) Lipid Removal (Agilent Technologies, Santa Clara, CA) consisting of ~~1.0~~1.0 g EMR-lipid material in a first falcon tube, and a second falcon with the polish salt made of ~~400~~400 mg sodium chloride and ~~1600 mg~~ 1600 mg anhydrous magnesium sulfate. The effect of extract filtration on analyte retention was evaluated with polyethilentereftalate (PTFE) filters (Whatman Puradisc 25 TF filters ~~25~~25 mm,~~mm, 0.45~~ 0.45 µm, Buckinghamshire, UK).

2.2 Fish samples

As fish matrices, Norwegian Atlantic salmon, *Salmo salar*, purchased from a local market in Barcelona (Spain), and chub fish, *Leuciscus cephalus*, from the Sava river were used during this study. The Sava river flows through Slovenia, Croatia, Bosnia and Herzegovina, and Serbia. *Salmo salar* was used for development and optimization of the extraction and clean-up method. It was selected for its high lipid content, because one of the main objectives of this study was to optimize the extraction step avoiding as much as possible the coextraction of lipids. Additionally, both species were used for validation of the extraction of PhACs. Finally, the method was applied to wildfish of two European rivers collected in September 2015: i) *Squalius keadicus* collected from the Evrotas river (Greece) at four sampling points: two reference sites, Uskol and Vivari; one drought impacted reach, Dskol; and one pollution-impacted reach termed WWTP; ii) 12 samples corresponding to *Salmo trutta fario, Salmo trutta marmoratus, Cottus gobio, Leuciscus cephalus,* and *Thymallus thymallus* species from the Adige River (Italy) collected in seven sites along the river. More information about the sampling from Sava, Evrotas and Adige river can be found in a previous study from our group [36].

2.3 Sample preparation

2.3.1 Water and fat content

Water content was determined according to the following reference [37]. In summary ten grams of fresh fish was lyophilized (LyoAlfa 6 system, Telstar Technologies, Terrassa, Spain) for 10 days. Afterwards, to assure complete dryness of the sample, the sample was heated in oven at ~~105~~105 °C for ~~5~~5 h. Then, it was placed into a desiccator until complete cool down. In the case of fat content, fat and lipids were extracted using an ultrasound unit ~~(Fisherbrand®~~(Fisherbrand® FB15064, Waltham, Massachusetts, USA) with five different mix solvents DCM:hexane (1:1, v/v); MeOH:hexane (1:1, v/v); acetone:hexane (1:1, v/v); ~~cloroform:MeOH:water~~ cloroform: MeOH:water (5:10:4, v/v/v) and hexane 100%. In summary, ~~3.0~~3.0 g dw of fish matrix was sonicated with ~~15~~15 mL of solvent ~~(5~~(5 mL times 3). Sonication for ~~15~~15 min was applied in each time. Afterwards, samples were centrifuged for ~~5~~5 min at ~~4000~~4000 rpm and supernatants were collected as a pool of extraction. Extraction solvents were evaporated under a gentle stream of nitrogen with a ~~TurboVap®~~TurboVap® LV (Biotage, Uppsala, Sweden) until constant weight [37].

2.3.2 Optimization of extraction of pharmaceuticals from fish muscle

A schematic procedure of the optimization and validation process is presented in Fig. 1. One gram dw of *Salmo salar* muscle was spiked with 100 ng of pharmaceuticals solutions and IS as surrogates (see Table 1) prepared in MeOH. The sample was vortexed for 1 min to assure a better interaction between the matrix and the analytes. Spiked samples were then left in the fume hood for 1 h to allow evaporation of MeOH, and finally placed in a fridge overnight. Two extraction techniques were applied to the samples: QuEChERS and USE. In the case of USE 1 mL of water was added to the sample. After 1 min of vortexing, 5 mL of solvent (MeOH, MeCN, or MeOH:MeCN (1:1, v/v)) were added, the sample was vortexed again for 1 min followed by sonication for 20 min. The sample was centrifuged at 4000 rpm for 20 min and the supernatant collected. This process was repeated a second time and the supernatants were pooled. A 6-mL aliquot of the supernatant was evaporated under gentle stream of nitrogen and then reconstituted in 1 mL of H_2O/MeOH (90:10). In the case of QuEChERS, nine milliliters of water were added to 1 g of sample. After 1 min of vortexing, 10 mL of MeCN were added and the suspension was vortexed again for 1 min. The extraction salt ***(original, buffer, or formate)*** was added and the tube was shaken to avoid the formation of lumps, followed by a 1-min vortex. Finally, the sample was centrifuged at 4000 rpm for 5 min. A 6-mL aliquot of the supernatant was evaporated under a gentle stream of nitrogen, and reconstituted with 1 mL of H_2O/MeOH (90:10). The sample was centrifuged at 14,000 rpm for 10 min to precipitate as much as possible any suspended fine particle if present. Finally, for additional information, the weight of co-extracts was determined for each method evaporating 2.5 mL of each extract. The most suitable extraction method was selected after comparison of chromatographic peak areas in the extracts with the peak areas of analytes in a standard solution at the same concentration (referred to as extraction efficiency, EE). In the optimized approach, extraction with QuEChERS ***original*** was selected.

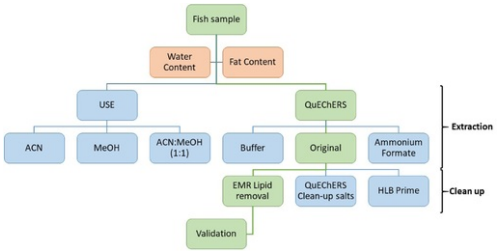

Fig. 1 Workflow of the optimization and validation process. USE: ultrasound extraction, MeOH: Methanol, MeCN Acetonitrile, EMR Enhanced Matrix Removal. Green boxes represent the final methodology validated.

alt-text: Fig. 1

Table 1 Target analytes and deuterated standards with exact mass, transitions and retention time as obtained by UPLC-HR-QToF-MS.

alt-text: Table 1

Compound	Abbreviation	Precursor ion mass $[M-H]^+$	Fragment ion mass $[M-H]^+$	t_R (min)	IS

Acetaminophen	APAP	152.0706	110.0598	0.85	CBZ-d_{10}
Amlodipine	AML	409.1525	238.0626	3.83	N.V.
Atenolol	ATN	267.1703	145.0638	0.74	BZF-d_4
Bezafibrate	BZF	362.1154	138.9944	3.74	BZF-d_4
Bezafibrate-d_4	BZF-d_4	366.1405	143.0195	3.73	–
Carazolol	CRZ	299.1754	116.1071	2.34	VLF-d_6
Carbamazepine	CBZ	237.1022	194.0949	2.97	CBZ-d_{10}
Carbamazepine-d_{10}	CBZ-d_{10}	247.1650	204.1578	2.95	–
Cephalexin	CFX	348.1013	106.0658	1.65	N.V.
Clarithromycin	CLR	748.4842	158.1174	3.20	STL-d_6
Codeine	COD	300.1594	215.1067	1.08	BZF-d_4
Diazepam	DZP	285.0789	154.0413	3.78	STL-d_6
Diclofenac	DCF	296.0240	214.0419	4.27	BZF-d_4
Diltiazem	DTZ	415.1686	178.0305	2.81	VLF-d_6
Fenofibrate	FNB	361.1201	233.0366	5.57	N.V.
Indomethacin	IND	358.0841	138.9939	4.27	BZF-d_4
Ketoprofen	KTP	255.1016	105.0328	3.60	STL-d_6
Loratadine	LRT	383.1521	337.1115	1.88	N.V.
Metoprolol	MTP	268.1907	74.0604	1.89	MTP-d_7
Metoprolol-d_7	MTP-d_7	275.2347	123.1515	1.89	–
Metronidazole	MTR	172.0717	128.0449	0.88	CBZ-d_{10}
Paroxetine	PRX	330.1500	192.1187	2.95	N.V.
Propranolol	PPN	260.1645	155.0845	2.47	STL-d_6
Propyphenazone	PPPN	231.1492	189.1024	3.15	MTP-d_7
Salbutamol	SBT	240.1594	148.0752	0.63	STL-d_6
Sertraline	SRL	306.0811	158.9765	1.68	N.V.
Sotalol	STL	273.1267	133.0760	0.67	STL-d_6
Sotalol-d_6	STL-d_6	279.1644	134.0817	0.66	–
Trimethoprim	TMP	291.1452	230.1160	1.51	STL-d_6
Venlafaxine	VLF	278.2116	58.0656	2.28	VLF-d_6
Venlafaxine-d_6	VLF-d_6	284.2491	64.1026	2.25	–
Verapamil	VPM	455.2904	165.0906	3.11	CBZ-d_{10}

N.V.: Analyte not included in the final validation.

IS: isotopically labeled standards.

2.3.3 Clean-up extracts optimization

After the best extraction method was selected, 3 different clean-up methods were proved: 1) d-SPE clean-up containing PSA and C18, 2) clean-up with SPE cartridge HLB Prime, and 3) d-SPE EMR lipid Removal. In the optimized approach EMR lipid removal was selected. To activate the cleaning component, five milliliter of water were added to the clean-up tube and vortexed for ~~1~~1 min. Then ~~5~~5 mL of the extract was added and the suspension was vigorously hand-shaken and vortexed for ~~1~~1 min. The tube was centrifuged at ~~4000~~4000 rpm for ~~5~~5 min, and ~~5~~5 mL of the supernatant were transferred to the polish tube containing magnesium sulfate to remove the excess of water. This was vortexed for ~~1~~1 min and again centrifuged at ~~4000~~4000 rpm for ~~5~~5 min. Finally, ~~2~~2 mL of the supernatant was evaporated under a gentle stream of nitrogen and the residue dissolved in ~~1~~1 mL of H_2O/MeOH (90:10). As a part of clean-up optimization, the use of PTFE filters with control solutions was tested with ~~1.0~~1.0 mL of standard solution at ~~100~~100 ng mL^{-1}.

Detailed clean-up of extract protocols for the other two methods are described in the SM Appendix 1.

2.4 LC-HR-MS/MS analysis

LC-HR-MS/MS analysis w~~ere~~as performed on ~~a~~the X500R QTOF system (SCIEX, MA), equipped with a TurboV ESI ion source, operated in the positive ion mode. The chromatographic separation of the PhACs was performed by UPLC Exion LC AD system (SCIEX) using a Kinetex EVO C18 packed column ~~(50~~(50 mm × 2.1 mm, ~~mm × 2.1~~ 2.6 µm, Phenomenex, Torrance, CA) thermostated at ~~40~~40 °C. Ammonium formate ~~(5~~(5 mM, pH 3) and MeCN were used as mobile phases at a flow rate of ~~0.4~~0.4 mL min^{-1}. The injection volume was ~~5~~5 µL. Initial conditions (5% MeCN) were maintained for ~~0.1~~0.1 min before the percentage of the organic solvent was increased from ~~5~~5% to 83% in ~~5.9~~5.9 min and then further increased to 98% in the following ~~0.1~~0.1 min. Then, the organic phase was held at 98% for ~~1.4~~1.4 min before returning to the initial conditions in ~~0.1~~0.1 min, which were maintained for ~~1.4~~1.4 min. This gradient provided a ~~9.0~~9.0 min chromatographic run. TOF-MS and TOF-MS/MS data were acquired using the SWATH acquisition mode, based on a data-independent acquisition (DIA) MS strategy that can be applied to collect all MS^2, where every detectable ion from the sample is fragmented [38].

For SWATH acquisition, a single TOF-MS experiment over an *m/z* range from 100 to 850 was set with an accumulation time of 0.1 s followed by nine MS/MS experiments with variable Q1 windows as follows: *m/z* 99.5–171.9, 170.9–206.3, 205.3–235.7, 234.7–275.6, 274.6–324.1, 323.1–391.6, 390.6–508.1, 507.1–545.1, 544.1–800. A collision energy in rampage mode from 20 to 50 eV (35 ± 15 eV) was applied in each mass window, in order to reproduce the same conditions from the MS/MS instrument library. The accumulation time for each MS/MS experiment (window) was set to 30 ms for a total cycle time of 0.45 s including an MS survey scan (0.1 s) acquired at the beginning of each cycle and based on an average LC peak width of 12 s to obtain at least 20 points/peak. The number of variable windows, accumulation times, and total cycle times depended on the chromatography and length of the gradient [39]. Additionally, any drift in the mass accuracy of the Q-ToF-MS was automatically corrected and maintained throughout batch acquisition by infusion of a reserpine solution ($C_{33}H_{40}N_2O_9$, *m/z* 609.28066) with the TwinSprayer making use of the Calibrant Delivery System, an independent path embedded in the analytical sprayer probe. Calibration was run every 10 samples during the batch acquisition. Finally, the source conditions of the system were optimized as follow: ion spray voltage was set to 5500 V; source temperature and nitrogen gas flows (GS1 and GS2) were set to 600 °C and 60 psi, respectively, and the curtain gas was set to 35 psi. For qualitative and quantitative sample processing, SciexOS software v. 1.3 (SCIEX) was used.

2.5 Method validation

The analytical protocol was evaluated with respect to absolute recovery (AR), repeatability intra- and inter-day (RSD), extraction efficiency (EE), matrix effect (ME), sensitivity by method detection limit (MDL) and method quantification limit (MQL), and linearity at three concentration levels (25, 200 and ~~500~~500 ng g^{-1} dw fish) following validation parameters of extraction of pharmaceuticals methods in fish [22], in solid samples [40], in vegetables [20], and validation parameters in LC-MS [41,42].

AR were calculated by comparing the peak areas of fish muscle extracts spiked before extraction and clean-up process ($A_{spiked.fish}$) with peak areas of fish muscle extracts spiked after extraction and clean-up process ($A_{spiked.extract}$), accordingly to *Eq.* (1).

$$AR\% = \left(\frac{A_{spiked.fish}}{A_{spiked.extract}}\right) * 100 \quad \textbf{(1)}$$

Evaluation of the EE of the method was calculated by comparison of ($A_{spiked.fish}$) and peak areas of control solutions in HPLC grade water (A_{HPLC}), according to *Eq.* (2).

$$EE\% = \frac{A_{spiked.fish}}{A_{HPLC}} * 100 \quad \textbf{(2)}$$

ME were evaluated by comparing ($A_{spiked.extract}$) and (A_{HPLC}) according to *Eq.* (3).

$$ME\% = \left(\frac{A_{spiked.extract}}{A_{HPLC}} - 1\right) * 100 \quad \textbf{(3)}$$

Repeatability was calculated as the relative standard deviation of ARs injected in triplicate on the same day. Quantification was performed by the internal standard method with a fixed and known concentration of the IS ~~(100~~(100 ng g^{-1} dw fish) added to the samples and blanks. The calibration curve (CC) was constructed by linear weighted least-squares regression (1/x as the weighting factor) as a result of plotting the ratio of the analyte signal to the IS signal against the analyte concentration. The CC was prepared from matrix-matched standards using the internal standard method [43], spiking the extracts of fish muscles with the appropriate amount of analytes mix in the range of ~~0.5 to 500~~0.5–500 ng mL^{-1} and IS at ~~20~~20 ng mL^{-1} in all levels of the CC. MDL and MQL were determined as the average of three and ten times, respectively, the concentration of each point of the CC divided by the signal to noise (S/N) ratio obt~~e~~ained by the data processing software. Quality controls were prepared as Salmon matrix extracts spiked with standards at a concentration of ~~50~~50 ng mL^{-1}, which were injected every five samples. Blanks consisted of fish from the validation study fortified with surrogate standard mixture after confirm~~at~~ing the absence of the target analytes in the matrix. If detectable, the peak area in the blank sample was subtracted from analyte area. MeOH was injected after each quality control sample to confirm the absence of carryover or memory effects.

3 Results and discussion

3.1 Optimization of UPLC-MS/MS analysis

For the optimization of the chromatographic separation of the PhACs, a buffered acidified aqueous phase and MeCN as organic phase were used. MeCN was selected as extraction solvent recommended for its selectivity to extract organic compounds and low lipid solubility [19,44]. Using SWATH data acquisition mode, the Q1 isolation windows are stepped across the entire mass range on an LC timescale, so that HR product ion spectra are acquired and collected at each step (Q1 windows), obtaining a full MS/MS spectrum of every detectable ion within the same window. Information on fragment ions from the obtained MS/MS SWATH spectra can be used to uniquely confirm specific compounds, typically by matching SWATH data against pre-assembled MS/MS spectral libraries (SCIEX, CA). The width of the isolation window during SWATH acquisition can be considered equivalent to the triple quadrupole SRM selectivity to detect the molecules of interest. While initial approaches used fixed Q1 window widths of *m/z* 20–25 [45], the current SWATH technology used in the present work was improved by a variable window acquisition (SWATH Variable windows calculator, SCIEX, CA). In this way, decreasing the Q1 window size, as well as varying the window width as a function of precursor ion density, the specificity of the system increases, and improves the amount of quantitative data extracted without the risk of missing low abundance peaks [30].

According to SANTE European Commission guideline for pesticides [43], the requirements for the identification in HR-QToF-MS analysis, two ions with mass accuracy equal or mass difference lower than ~~5~~5 ppm are necessary for confirming a positive finding, in this case, exact mass and one ion fragment. The compounds characterized by HR-MS in the present study, are listed with precursors and fragments ion pairs in Table 1 alongside the chromatographic elution times. Under the optimized LC conditions, the separation of the target analytes yielded the extracted ion chromatogram depicted in Fig. 2.

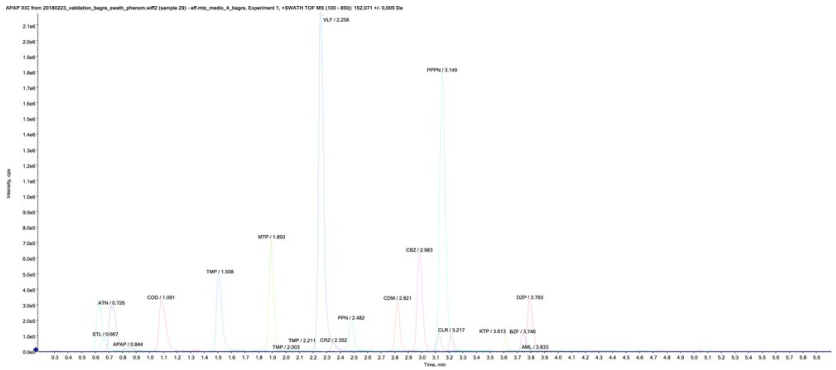

Fig. 2 Extracted Ion Chromatogram of target analytes in Leuciscus cephalus matrix at mid level concentration of validation ~~(200~~(200 ng g^{-1} dw). Over each chromatogram peak ~~"abreviation~~"abbreviation name/retention time". See Table 1 for abbreviation names.

alt-text: Fig. 2

3.2 Water and fat determination

The water content in *Salmo salar* and *Squalius keadicus* was 57% and 77%, respectively. The determination of the fat content was evaluated by extraction with different solvents: hexane, acetone/hexane (1:1) and DCM/hexane (1:1) yielded values of 35.4%, 36.2% and 37.8% dw in *Salmo salar* (22.6%, 23.1% and 24.1% in fw respectively, RSD 3.6–5.8%). In case of MeOH/hexane (1:1) and chloroform/MeOH/water (5:10:4), the fraction of fat was higher (57.7%

and 45.8% respectively, corresponding to 36.8% and 29.2% in fw respectively). The apparently higher fat content of the methanolic USE extracts was attributed to more efficient coextraction of other matrix components. Regardless of these considerations, the fat content reported here for *Salmo salar* is similar those reported in other studies (9.0–23.2%) [46,47]. Given the high fat content, we considered the use of *Salmon salar* for method development. More information about the fat content results in *Salmo salar* is provided in the SM Table A.2.

3.3 Optimization of the extraction

As the extracts after USE and QuEChERS extraction appeared turbid, we considered filtration as a strategy for removing suspended matter. The suitability of this step was first evaluated by filtering ~~1~~1 mL of standard solutions (~~100~~(100 ng mL^{-1}) through PTFE filter membranes. While 19 of them were recovered in the filtrate higher than 70% of the initial concentration, AML, CLR, DTZ, FNB, LRT, PRX, SRL and VPM presented losses of more than 30%. For this reason, the further use of filters was discarded. The problem with suspended fine particles was overcome by centrifuging the reconstituted extracts.

In the case of USE three different solvents were used for extraction. In case of QuEChERS, three salts were tested. The comparison was evaluated according the EEs (see ~~2.6 Method validation~~Section 2.5).

Results of extraction for both methods are presented in Fig. 3. As general trend, extractions with USE (Part "A" in Fig. 3) were very similar regardless of solvent used, except for a few components like BZF, DCF, DTZ, and KTP. In the case of DCF, each solvent derived in a different EE. On the other hand, using QuEChERS ***original, buffer,*** or ***formate***, (Part "B" in Fig. 3) EE presented slight differences, but the high variability (RSD) was recorded using formate extraction. In many cases, this extraction salt presented the highest efficiency during extraction, but its high variability was a problem to be addressed. Two out of three variants tested for USE and QUECHERS, respectively, have shown recoveries of over 30%. The number of recovered compounds was 21 and 20 respectively. Furthermore, some compounds were not definitively extracted by USE neither by QuEChERS as AML, FNB, LRT, PRX and SRL (results no shown). It could be probably explained by its high pKa for majority of them or because of its high affinity to fat and lipid matrix that stay retained in this part of the muscle.

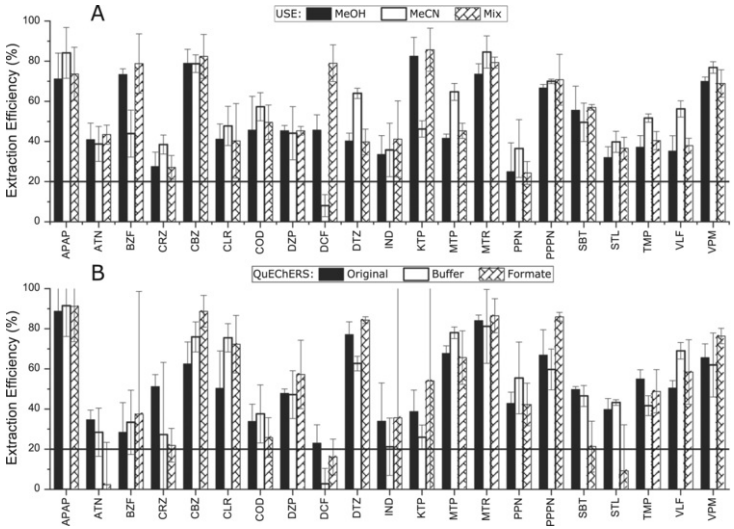

Fig. 3 Comparison of extraction efficiency (EE) for USE and QuEChERS. EE calculated as Eq. (2) in Section 2.5~~2.4. Method validation.~~ , Lines in bars indicate RSD.

alt-text: Fig. 3

Additionally, there are nine PhACs that presented EE very similar using USE and QuEChERS extractions methods (difference ≤10%, calculated as the average of extraction technique), e.g. CRZ, CBZ, IND, PPPN, or VPM. Despite some PhACs have been extracted in more proportion using USE like ATN, BZF, COD or SBT (19, 32, 18, 15% more respectively), other PhACs presented better results when extracted with QuEChERS as CLR, DTZ, PPN or VLF (23, 27, 18, 16% more respectively). Variation of EE are connected with differences in polarity, solubility, dissociation octanol-water, and some other physicochemical properties that can affect adsorption and solubility processes of each family of PhACs (table of physical chemical ~~propierties~~properties in SM Table A.1).

We also evaluated the weight of coextracts for each USE and QuEChERS extraction. When MeOH was used for PhACs extraction from the fish samples, higher coextracted mass (CM) was obtained. In case of USE with MeOH, the CM was 10.11 mg mL^{-1} as compared to 3.96 mg mL^{-1} for MeCN. On the other hand, during QuEChERS extraction with MeCN as solvent, the weight of coextracts was 5.59, 3.89, and 7.6 mg mL^{-1} for the *original, buffer, and formate*,

respectively. A series of photos of the extracts obtained with USE and QuEChERS are presented in the SM (Fig. A.2).

The initial criteria for the selection of the best extraction method were the best EE as well as the lowest variability (RDS <20%). As the extraction methods were comparable in efficiency and RSD (except formate), some other practical aspects (for example, “quick and easy”), fewer coextractions and ecological factors (for example, lower amount of solvent used) were also considered. For these reasons, QuEChERS original was selected as extracting method.

3.4 Clean-up optimization

Three clean-up methods using SPE and d-SPE were tested: clean-up from QuEChERS extraction kit, HLB Prime, and EMR- Lipid Removal. The analysis of the EE results revealed that i) EE of PhACs are almost the same comparing the best clean-up and without clean-up, i.e. ATN, COD, SBT (difference of extraction ≤10% for all 3 clean-up tested); ii) a reduction of EE for some compounds when the clean-up step was applied. This is the case of APAP, DTZ or MTR with a reduction of EE ranging between ~~4.5 to~~4.5% and 56.2% understanding that recoveries vary in each clean-up type. Reduction of the EE with the clean-up step can be explained because part of the compound continues retained in the sorbent salts and additionally, because the inclusion of the clean-up means more steps and manipulation of the sample, that lead to lose more analyte during the process [48,49]. Finally, from the results of the present study, it can be concluded that (iii) the cleaning process also improves the signal of the extracted analyte, e.g. DZP, DCF, or VPM with an increase in EE up to 25% comparing the three cleaning steps tested and without clean-up. Using the criteria as improvement of EE, reduction of ME and practical factors, EMR lipid removal was selected as clean-up step. With this clean-up, at least seven PhACs presented better EE with no affectation of another characteristics as the RSD, and any of the selected pharmaceutical presented a reduction of the EE more than 10%, as presented in Fig. 4 where EE is compared without or with d-SPE-EMR clean-up. The selection of this clean-up allows to present a complete method based on QuEChERS extraction and dispersive clean-up process, an easy use and low cost (that even can by lower in the near future, with increment of use, as it happened with another clean-up methods [24]).

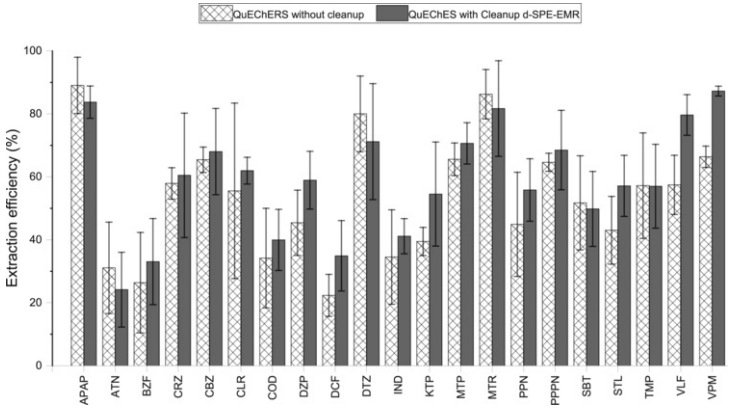

Fig. 4 Comparison of extraction efficiency (EE) for QuEChERS original without clean-up and with clean-up d-SPE-EMR in Salmo salar matrix. EE calculated as Eq. (2) in Section 2.5~~2.4. Method validation.~~. Lines in bars indicate RSD.

alt-text: Fig. 4

3.5 Validation method

After the selection of the extraction and clean-up method, the analytical methodology was evaluated with the parameters described in section 2.6, *method validation*, for two different matrix *Salmo salar* and *Leuciscus cephalus*. Each validation was prepared by quintuplicate at three different concentration levels. Due to the low recoveries obtained during the selection of the extraction method, AML, CFX, FNB, LRT, PRX, and SRL were excluded from validation. For recovery studies, blank samples of both matrices were spiked with analytes after the extraction. Eventually, any target PhACs in the blank samples were below MDL. The results for the method validation for PhACs in *Salmo salar* and *Leuciscus cephalus* at 200 ng g^{-1} fish dw (mid-level) are showed in Tables 2 and 3, respectively. The informations of the validation method for low and high level of concentrations (25 and 500 ng g^{-1} dw respectively) are reported as SM (Tables A.3 and A.4).

Table 2 Performance of the method for the target PhACs evaluated in Salmo salar fish matrix at mid-level concentration, ~~200~~200 ng g^{-1} dw fish.

alt-text: Table 2

	Absolute ~~Recovery~~recovery[a]	Repetatibility		Extraction ~~Efficiency~~efficiency[b]	Mattrix ~~Effect~~effect[c]	MDL[d]	MQL[d]	Linearity
Unit	(%)	$RSD_{intra\text{-}day}$ (%)	$RSD_{inter\text{-}day}$ (%)	(%)	(%)	~~ng g~~ng g^{-1} dw	~~ng g~~ng g^{-1} dw	r^2

APAP	80.8	6.3	7.7	86.5	7.1	25.43	84.79	0.99897
ATN	31.1	16.1	18.2	25.2	−18.9	2.41	8.04	0.99626
BZF	43.1	15.4	18.6	38.5	−10.7	5.52	18.38	0.99960
BZF-d4	42.3	14.5	16.7	37.4	−11.5	–	–	–
CRZ	84.1	21.3	23.6	62.6	−25.6	3.79	12.63	0.99469
CBZ	82.0	9.2	8.1	70.4	−14.2	0.59	1.97	0.99723
CBZ-d10	89.8	5.7	7.9	79.1	−11.9	–	–	–
CLR	75.1	7.3	8.2	58.4	−22.2	1.69	5.63	0.99928
COD	46.3	7.7	9.0	38.6	−16.7	4.52	15.06	0.99575
DZP	86.7	9.5	13.1	53.6	−38.2	1.33	4.42	0.99799
DCF	54.1	11.6	14.3	37.4	−30.8	23.74	79.14	0.99656
DTZ	99.4	19.2	21.9	73.5	−26.0	0.25	0.82	0.99580
IND	66.8	4.8	6.0	41.2	−38.3	14.68	48.93	0.99893
KTP	59.6	13.0	16.6	53.6	−10.1	4.27	14.22	0.99352
MTP	84.2	5.6	6.7	70.9	−15.8	7.13	23.76	0.99651
MTP-d7	83.0	12.6	16.5	70.0	−15.7	–	–	–
MTR	85.0	15.6	17.8	83.0	−2.4	22.00	73.33	0.99812
PPN	85.2	9.1	11.7	59.6	−30.1	6.99	23.29	0.99076
PPPN	77.7	11.8	13.8	71.8	−7.6	0.90	3.00	0.99573
SBT	54.4	11.5	15.9	47.7	−12.3	1.06	3.54	0.99966
STL	72.0	9.3	11.2	59.6	−17.2	4.14	13.79	0.99797
STL-d6	66.4	11.4	12.8	55.0	−17.2	–	–	–
TMP	74.6	13.7	15.8	58.7	−21.3	2.82	9.41	0.99923
VLF	95.5	6.4	7.2	81.9	−14.2	0.31	1.04	0.99556
VLF-d6	96.2	4.3	4.9	69.8	−27.4	–	–	–
VPM	118.8	0.2	4.1	81.9	−31.0	0.45	1.49	0.99787

MDL Method Detection Limit, **MQL** Method Quantification Limit.

[a] Calculated by comparing peak areas of fish extracts spiked before extraction/clean-up process and peak areas of fish extracts spiked after extraction/clean-up process, spiked both at mid-level (200 ng g^{-1} dw of fish) *Eq.* (1) in Section 2.5.

[b] Calculated by comparing peak areas of fish muscle extracts spiked before extraction/clean-up process and peak areas of control solutions in HPLC grade water spiked both at mid-level (200 ng g^{-1} dw of fish). *Eq.* (2) in Section 2.5.

[c] Calculated by comparison of peak areas of extracts from fish muscle spiked after extraction and clean-up process and areas of control solutions in HPLC grade water spiked both at mid-level (200 ng g^{-1} dw of fish) *Eq.* (3) in Section 2.5.

[d] Calculated as reported in section 2.5 ~~*equation 4*~~ *Eq.* ~~(4) in text~~.

Table 3 Performance of the method for the target PhACs evaluated in Leuciscus cephalus fish matrix at mid-level concentration, ~~200~~200 ng g^{-1} dw fish.

alt-text: Table 3

	Absolute ~~Recovery~~recovery[a]	Repetatibility		Extraction ~~Efficiency~~efficiency[b]	Mattrix ~~Effect~~effect[c]	MDL[d]	MQL[d]	Linearity
Unit	(%)	$RSD_{intra\text{-}day}$ (%)	$RSD_{inter\text{-}day}$ (%)	(%)	(%)	~~ng g~~ng g^{-1} dw	~~ng g~~ng g^{-1} dw	r^2
APAP	58.4	22.1	29.4	41.2	−29.5	31.57	105.22	0.97468
ATN	32.3	18.3	22.3	20.9	−35.3	7.14	23.79	0.99288
BZF	64.9	6.7	9.8	48.1	−25.8	7.42	7.42	0.99879
BZF-d4	62.3	6.2	10.5	52.4	−15.8	–	–	–
CRZ	N.V.	N.V.	N.V.	N.V.	N.V.	N.V.	N.V.	N.V.
CBZ	86.2	2.1	3.6	48.1	−44.2	1.92	1.92	0.99478
CBZ-d10	78.3	5.1	3.4	43.1	−44.9	–	–	–
CLR	69.8	9.0	14.0	21.0	−70.0	4.79	15.95	0.99926
COD	40.3	15.1	18.9	22.9	−43.1	18.78	62.61	0.99429
DZP	81.5	12.0	17.3	15.1	−81.4	7.02	23.41	0.99000
DCF	96.6	10.8	19.3	12.2	−87.4	91.01	303.36	0.99421
DTZ	63.6	3.6	5.9	13.3	−79.1	53.72	179.06	0.99817
IND	102.6	33.1	38.1	8.7	−91.5	7.77	25.89	0.99733
KTP	85.9	8.0	12.1	53.1	−38.1	10.39	34.64	0.98188
MTP	80.4	8.8	12.3	49.1	−38.9	7.56	26.19	0.99900
MTP-d7	76.5	7.9	11.1	52.3	−31.7	–		–
MTR	64.3	16.5	19.6	43.1	−33.0	25.61	85.37	0.99130
PPN	122.3	23.9	26.8	26.0	−78.8	26.82	89.41	0.99157
PPPN	99.5	2.9	4.0	63.5	−36.2	3.29	10.95	0.99734
SBT	54.2	9.7	17.0	42.4	−21.8	1.46	4.86	0.99663
STL	62.2	11.9	19.4	47.0	−24.4	3.34	11.12	0.99179
STL-d6	59.6	7.0	10.9	42.4	−28.9	–		–
TMP	65.7	5.2	6.9	37.7	−42.6	8.49	28.30	0.99119
VLF	91.1	4.4	7.2	67.7	−25.7	0.55	1.82	0.99905
VLF-d6	74.8	4.7	6.4	41.4	−44.6	–	–	–
VPM	88.8	8.4	13.4	11.7	−86.9	7.26	24.19	0.99287

MDL Method Detection Limit, **MQL** Method Quantification Limit. N.V.: Analyte no validated.

[a] Calculated by comparing peak areas of fish extracts spiked before extraction/clean-up process and peak areas of fish extracts spiked after extraction/clean-up process, spiked both at mid-level (200 ng g^{-1} dw of fish) *Eq.* (1) in Section 2.5.

[b] Calculated by comparing peak areas of fish muscle extracts spiked before extraction/clean-up process and peak areas of control solutions in HPLC grade water spiked both at mid-level (200 ng g^{-1} dw of fish). *Eq.*

(2) in Section 2.5.

[c] Calculated by comparison of peak areas of extracts from fish muscle spiked after extraction and clean-up process and areas of control solutions in HPLC grade water spiked both at mid-level (200 ng g^{-1} dw of fish) *Eq.* (3) in Section 2.5.

[d] Calculated as reported in section 2.5 ~~*equation 4*~~ *Eq.* ~~(4) in text.~~

Calibration curves were constructed by linear weighted least-squares regression with a factor of 1/*x* and including at least 5 concentration points, which calculated concentration did not differ more than 20% from theoretical concentration. In the case of *Salmo salar*, the linearity was $r^2 > 0.99$ for all PhACs. Additionally, ARs were calculated following the Eq. (1) (Section 2.5 ~~Validation Method~~) and were lower than 50% for ATN (31%), BZF (43%), COD (46%), due to the low EE and also the ME. Nevertheless, these values are corrected using BZF-d4 as isotopically labeled surrogates during quantification. In fact, BZF-d4 is affected similarly in AR, EE and ME (Table 2) and can minimize this reduction during analysis and quantification [50]. Ionization suppression effect was present in all cases but APAP that presented an enhancement of the signal. Coextracted components from biological matrices such as salts (e.g. sulphates and phosphates), carbohydrates (e.g. glucose and nucleotide sugars), amines (e.g. aminoacids and urea), lipids (e.g. cholesterol and triglycerides), among others (vitamins, and bilirubin, insulin and non target contaminants) increases the matrix effect during the analysis of organic compounds in biological matrices [40,51]. This effect is due to the possible competition with the target analytes for the charges available in the ms source. The result is an ionic suppression that is fairly known in the positive ionization mode using ESI [51,52].

The method repeatability was satisfactory for all studied compounds, below 20% in all cases but CRZ ($RSD_{intra\text{-}day}$ 21.3%, $RSD_{inter\text{-}day}$ 23.6%) and DTZ ($RSD_{inter\text{-}day}$ 21.9%). Regarding the sensitivity, the method developed allows to detect most of PhACs from 1.0 to 10.0 ng g^{-1} dw of salmon. Five compounds present MDLs from 0.25 ng g^{-1} to 1.0 ng g^{-1} dw of salmon (CBZ, DTZ, PPPN, VLF, and VPM). However, four compounds presented values close to 20 ng g^{-1} dw, e.g. APAP (25.43 ng g^{-1} dw), DCF (23.74 ng g^{-1} dw), IND (14.68 ng g^{-1} dw), and MTR (22 ng g^{-1} dw). Compared with the salmon, *Leuciscus cephalus* presented some significant differences in term of AR, EE and ME. The recoveries for ATN and COD were lower than 50% (32% and 40%, respectively). All others compounds presented good AR between 52% and 122%. Except few cases, EE was lower compared with the salmon matrix. In particular, 8 compounds (ATN, CLR, COD, DZP, DCF, DTZ, IND, and PPN) had EE lower than 30%. In spite of the reduced amount in fat content compared with the salmon, *Leuciscus cephalus* presented a higher suppression of analytes signal. *Leuciscus cephalus,* as member of the Cypriniformes, is more tolerant to the lack of oxygen in the water and, consequently, to the pollution. This means some additional compounds present in the water (organic and inorganic molecules) could accumulate in fish and be subsequently co-extracted with the matrix, interfering during analysis of PhACs. In fact, the Sava river, where the fish samples are from, is a river significantly impacted by wastewater contaminants [36,53,54] that can be accumulated in fish and interfere with a clear extraction and detection of the analyte [55–58]. In addition to, there are some studies with the same species (*Leuciscus cephalus*) in the same river (Sava) detected accumulation of metals in different part of the fish [54] that can interact with PhACs and affect the extraction, means that the possibility of having conjugates or chelates of the PhACs is quite high [59]. Anyway, linearity response for 18 PhACs presented r^2 higher than 0.99 in calibration curves that were constructed with the same conditions of the previous matrix, but APAP, ($r^2 = 0.97$), KTP ($r^2 = 0.98$), and CRZ that was no possible to validate in this matrix.

Regarding the sensitivity of the method, *Leuciscus cephalus* presented higher detection and quantification limits compared *Salmo* ~~*Salar*~~*salar* (Tables 2, 3). Most of PhACs presented MDL between 1.0 and 10 ng g^{-1} dw, while VLF showed the lowest MDL with this matrix (0.55 ng g^{-1} dw). Seven compounds (APAP, COD, DCF, DTZ, KTP, MTR, PPN) presented MDL higher than 10 ng g^{-1} dw. In the case of DCF, the MDL and MQL are considerably high, 91.01 and 303.36 ng g^{-1} dw, respectively. For this reason, although it is shown in Table 3, we decided to exclude it from the validation of the Leuciscus. Repeatability intra-day of the method in this matrix has a good reproducibility except for APAP, IND, and PPN that presented RSD over 20% and similar results are present in inter-day repeatability incluiding ATN, that presented 22.3% of RSD.

3.6 Test of the validated method in environmental samples

In order to test the validated method, PhACs were analyzed in six fish species namely *Squalius keadicus, Salmo trutta fario, Salmo trutta marmoratus, Cottus gobio, Leuciscus cephalus,* and *Thymallus thymallus* from Evrotas river (Greece) and from Adige river (Italy). In total, 16 samples were analyzed from eleven different sample points. Calibration curve was performed with the matrix of *Salmon salar* due that the majority of samples were belonging to a same species, *Salmo trutta fario* and *Salmo trutta marmoratus*. When samples do not come from the same matrix, a pool of all matrices is recommend to simplify the quantification procedure [60]. Because of the lack of contaminant-free matrices to prepare this pool of matrices, the choice fell on salmon for similarity of species.

According to the results, the presence of PhACs were below limit of detection for samples from the Evrotas river. It is important to notice that the main impact of pollution on the river, are the agricultural activities including agricultural and industrial waste and over-exploitation of water for irrigation [36]. In fact, studies on the same river reported the presence of 11 out of 62 PhACs investigated in water samples from the same sampling points of the fish of the present study. In particular, the higher values were found for STL, CBZ, and TMP, respectively below ~~10~~10 ng L^{-1}, and KTP around ~~40~~40 ng L^{-1} in the WWTP sampling point (close to a wastewater emissary) [60].

In case of the Adige river, APAP was detected in the concentration of ~~24.01~~24.01 ng g^{-1} dw and PPN in the concentration of ~~79.6~~79.6 ng g^{-1} dw in samples from two different sampling points. Additionally, APAP, PPN, and VLF were detected below limit of quantitation in fish muscle of the last three sampling points along the river. In the case of the Adige River, the main pollutants activities are connected with the hydro-peaking effects, agricultural activities and

pollutants accumulated from in glaciers [36]. In both cases, in the Evrotas and in the Adige rivers, the affectations for PhACs are not predominant.

4 Conclusions

An analytical method for the determination of 21 PhACs in fish muscle was developed and validated at three concentrations levels. The developed method, based on QuEChERS extraction, presented advantages as low-time consumption and economic factors because of the simple use of materials, low volumes required including low fat and matrix co-extraction compared to the USE technique. Additionally, the use of EMR lipid removal as a clean-up in a dispersive format following the QuEChERS protocol provided an enhancement of analytical signals affected by lipid matrix effect, of fish muscle during pharmaceutical extractions. The EMR lipid removal performance was better compared to the other two clean-up methodologies tested, improving notoriously the extraction efficiency compared to the extraction efficiency without clean-up.

The analysis of PhACs by HRMS-QToF-MS system, supported by the SWATH acquisition mode, is an effective analytical tool that allows detection of analytes without settings steps of ion mass, ion fragment, retention time or energy values. The SWATH acquisition mode allows to obtain an ~~MS / MS~~ MS/MS spectrum of all analytes in a sample, that in case of the fish detection limit was in the low range up to ~~0.25~~0.25 ng g^{-1} dw for Salmo salar and ~~0.55~~0.55 ng g^{-1} dw in Leuciscus cephalus with high precision and over a wide linearity range.

Furthermore, an additional advantage of SWATH acquisition is that MS/MS data are still able to identify non-targeted compounds thanks to a retrospective analysis that can be carried out at any time. ~~Declaration of interestNone.~~

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Declaration of interest

None.

Appendix A. ~~Supporting information~~Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.01.119.

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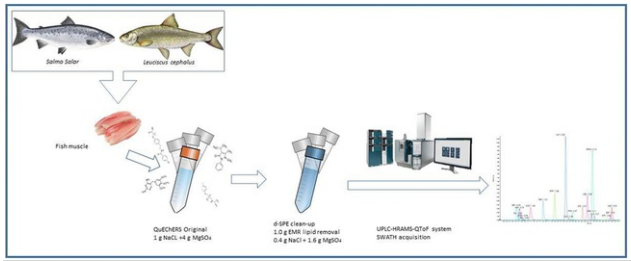
Appendix A. Supplementary material

Multimedia Component 1

Supplementary material.

Graphical abstract



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Highlights

- ~~A new rapid method for the determination of 21 Pharmaceutical actives compounds in fish muscle~~Development of a rapid method for the determination of 21 PhACs in fish muscle.~~.~~

- ~~The optimized method was selected after comparing two extraction methods, three different extracting salts, and three different clean-up of extracts.~~Two extraction methods, 3 extracting salts, and 3 clean-up of extracts were compared.
- The ~~developed procedure~~method is based on QuEChERS extraction and clean-up, followed by UPLC–HRMS.
- ~~Validated method was applied to riverine European fish~~The validated method was applied to riverine European fish.

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Answer: [3] I. Wilson, Steroids as Water Pollutants: A Literature Survey of Synthetic and Natural Hormonal Steroids Analytical Methods for Their Determination, 1976, Water Research Centre, Great Britain
[15] B. Huerta, S. Rodríguez-Mozaz and D. Barceló, Chapter 6 analysis of pharmaceutical compounds biota, In M. Petrovic, D. Barceló and S. Pérez, (Eds.), Comprehensive Analytical Chemistry, 2013, Vol. 62, pp. 169–193. Elsevier. Printed in Poland. https://doi.org/10.1016/B978-0-444-62657-8.00006-9.

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