applied sciences

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Pharmaceuticals in Coastal Waters: An UHPLC-TOF-MS Multi-Residue Approach

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Abstract: Anthropogenic chemical contamination represents a key stressor of natural environments with pharmaceuticals comprising a particular group of emerging pollutants with the potential to induce biological responses in non-target organisms. Therefore, an analytical method based on ultra-high-performance liquid chromatography coupled to time-of-flight tandem mass spectrometry (UHPLC-TOF-MS) was developed for estuarine and seawaters, targeting 63 globally used pharmaceuticals (including amoxicillin, ciprofloxacin, sulfamethoxazole, trimethoprim and venlafaxine included in the Surface Water Watch List) from 8 therapeutic groups: antibiotics, analgesic, NSAIDs, antidepressants, β-blockers, lipid regulators, anticonvulsants and antihypertensive drugs. The method presents high selectivity and sensitivity, with the limits of detection ranging from 0.01 to 8.92 ng/L and the limits of quantification from 0.02 to 29.73 ng/L. Considering precision, the highest value was achieved for amoxicillin (20.9%) and the lower for ofloxacin (2.6%), while recoveries ranged from 80.6 to 112.6%. Overall, the quantification method was highly efficient for multi-residues quantification in such complex environmental samples.

Keywords: environmental contamination; emerging contaminants; drugs; seawater; UHPLC-TOF-MS

Citation: Leston, S.; Freitas, A.; Rosa, J.; Vila Pouca, A.S.; Barbosa, J.; Reis-Santos, P.; Fonseca, V.F.; Pardal, M.A.; Ramos, F. Pharmaceuticals in Coastal Waters: An UHPLC-TOF-MS Multi-Residue Approach. *Appl. Sci.* **2023**, *13*, 5975. https://doi.org/10.3390/app13105975

Academic Editor: Ramaraj Boopathy

Received: 18 April 2023
Revised: 3 May 2023
Accepted: 9 May 2023
Published: 12 May 2023

1. Introduction

Coastal pollution has quickly become a global environmental concern. Natural ecosystems worldwide have been struggling with the continuous release of substances and energy that subsequently induce changes in their physical, chemical and biological characteristics, altering functions and processes that often lead to biodiversity and habitats degradation [1–3]. The balance between economic growth and sustainable development pends chronically towards the first, aggravated by the fact that over 50% of the world's population is settled within 200 km of the shoreline [4]. Thus, anthropogenic pressure is even more problematic in coastal ecosystems, with countless forms of pollutants discharged constantly in the water. Pharmaceuticals are among these pollutants, regarded as emerging

contaminants ubiquitous to the aquatic environment, with multiple origins including urban and hospital effluents, veterinary runoffs, pharmaceutical industries and wastewater treatment plants. Although many may be swiftly degraded, due to their continuously release to the environment they are considered pseudo-persistent [2,5–8]. Moreover, they are listed as hazardous substances due to the fact that they retain biological activity capable of inducing effects in non-target organisms even after digestion and excretion [5,7,9], easily permeating cell barriers, which increases the risks of bioaccumulation and biomagnification throughout the food web [7,9]. Therefore, monitoring pharmaceuticals in the environment is paramount.

In this sense, the European Water Framework Directive mandates the surveillance of several substances in all member states to evaluate their risk as contaminants (EU Commission Decision 2020/1161). When a chemical has potential environmental risk but not enough data for adequate assessment, it can be proposed for inclusion as a priority substance in the Surface Water Watch List [10]. When monitoring data sets are enough to establish risk assessment, then substances are excluded from the list [10]. Currently, the revised form of the Watch List mandates the surveillance of eight pharmaceutical substances comprising four antibiotics, one antidepressant and its metabolite and three fungicides, which is a very low number compared to the thousands reaching the aquatic environment daily. Moreover, current legislation is aimed at freshwaters and transitional waters (including estuarine and coastal waters up to 1 nautical mile from the shore). Regarding marine waters, although guidelines with recommendations have been proposed under the OSPAR Commission, legislation is still lacking [5,11]. It comes, then, as no surprise that the majority of surveillance studies available pertain mainly to freshwaters. Another reason for this is the complexity of seawater and estuarine samples due to the presence of salt ions and dissolved organic matter and due to the much lower concentrations as a result of the dilution of contaminants as they are carried away from the coast. All these constraints have pushed advances in the analytical field aiming at the detection and monitoring of a large suite of pharmaceutical compounds at very low concentrations (ng/L). Hence, the development and validation of new analytical tools is essential. In particular, given that the number of contaminants keeps rising and that such compounds are present in mixtures, the use of multi-residue mass spectrometry methods represents a valuable tool to detect and quantify very low concentrations of multiple substances from a single sample analysis. The use of ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry detector time-of-flight (UHPLC-TOF-MS), enables the detection of an unlimited number of compounds with the powerful advantage that it allows reevaluating data to assess the presence of initially untargeted substances.

Therefore, considering the importance of having reliable methods capable of surveying a wide number of pharmaceuticals in seawater, with the possibility of revisiting results for untargeted compounds, the present work describes the development and validation of an UHPLC-TOF-MS method to screen and quantify a large suite of pharmaceuticals in estuarine and coastal waters. Specifically, this method targeted 63 drugs (including amoxicillin, ciprofloxacin, sulfamethoxazole, trimethoprim and venlafaxine from the WFD Watch List) from 8 therapeutic classes of pharmaceuticals: 38 antibiotics, 1 analgesic, 3 non-steroidal anti-inflammatories (NSAIDs), 5 antidepressants, 4 β-blockers, 5 lipid regulators, 3 anticonvulsants and 4 antihypertensive drugs (Table 1). This selection was based on annual reports of the most prescribed and sold human medicines in Portugal [12] and their predicted environmental behavior. The method described will provide an important tool in environmental monitoring of pharmaceuticals in coastal ecosystems with varying salinity gradients, allied to post-analytical survey.

Table 1. List of pharmaceutical compounds monitored with molecular formulas and weight, partition coefficient, exact mass and the summarized results of the method validation.

Compound	Molecular Formula	LogP	Molecular Weight	$[M + H]^+$	ΔM (ppm)	LoD (ng/L)	LoQ (ng/L)	Repeatability (%)	Reproducibility (%)	Recovery (%)	RT (min)
Analgesic											
Acetaminophen	$C_8H_9NO_2$	0.51	151.0633	152.0706	−0.8	1.89	6.30	3.0%	4.5%	99.8%	2.21
Antibiotics											
Amoxicillin	$C_{16}H_{19}N_3O_5S$	0.75	365.1045	366.1118	2.3	0.11	0.38	16.2%	20.9%	107.2%	6.91
Azithromycin	$C_{38}H7_2N_2O_{12}$	3.03	748.5085	749.5158	−0.9	0.01	0.04	11.5%	14.4%	96.0%	5.04
Benzylpenicillin	$C_{16}H_{18}N_2O_4S$	1.83	334.0987	335.1060	0.1	0.75	2.52	3.1%	4.7%	88.7%	4.49
Ceftiofur	$C_{19}H_{17}N_5O_7S_3$	1.22	523.0290	524.0363	1.3	0.03	0.09	10.5%	16.8%	94.7%	5.85
Cephalexin	$C_{16}H_{17}N_3O_4S$	0.55	347.0939	348.1013	0.5	0.04	0.10	16.6%	18.5%	98.6%	4.85
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	−0.13	478.1142	479.1216	0.7	1.73	5.77	2.2%	3.2%	99.5%	4.78
Cinoxacin	$C_{12}H_{10}N_2O_5$	1.25	262.0589	263.0663	−0.8	0.03	0.09	14.9%	16.9%	92.5%	5.25
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	−0.57	331.1332	332.1405	0.6	3.47	11.56	3.2%	4.7%	110.5%	4.47
Danofloxacin	$C_{19}H_{20}FN_3O_3$	0.33	357.1488	358.1562	0.5	3.25	10.82	6.5%	9.7%	110.1%	4.59
Doxycyclin	$C_{22}H_{24}N_2O_8$	−0.72	444.1532	445.1605	−0.6	0.28	0.94	6.7%	9.5%	101.5%	5.23
Enoxacin	$C_{15}H_{17}FN_4O_3$	−0.97	320.1284	321.1358	0.9	3.33	11.09	3.9%	5.8%	109.5%	4.33
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	0.58	359.1645	360.1718	0.6	2.27	7.58	2.5%	3.8%	101.4%	4.66
Epi-Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	−0.13	478.1142	479.1216	0.6	1.00	3.33	2.2%	3.3%	99.0%	4.54
epi-Tetracycline	$C_{22}H_{24}N_2O_8$	−0.56	444.1532	445.1605	1.0	0.40	1.32	6.5%	7.2%	104.7%	4.27
Flumequine	$C_{14}H_{12}FNO_3$	1.62	261.0801	262.0874	−0.3	0.01	0.04	8.2%	10.1%	80.6%	6.18
Marbofloxacin	$C_{17}H_{19}FN_4O_4$	−0.53	362.1390	363.1463	0.6	2.51	8.35	3.1%	4.6%	108.6%	4.29
Nalidixic acid	$C_{12}H_{12}N_2O_3$	0.95	232.0847	233.0921	−0.9	0.92	3.07	4.2%	6.3%	106.0%	6.07
Norfloxacin	$C_{16}H_{18}FN_3O_3$	−0.47	319.1332	320.1405	−0.4	1.81	6.04	9.0%	10.2%	109.6%	4.41
Ofloxacin	$C_{18}H_{20}FN_3O_4$	−0.02	361.1437	362.1511	0.6	0.99	3.31	1.4%	2.6%	91.5%	4.43
Oxolinic acid	$C_{13}H_{11}NO_5$	0.86	261.0637	262.0710	0.6	3.08	10.26	3.4%	7.1%	92.3%	5.50
Oxytetracycline	$C_{22}H_{24}N_2O_9$	−0.99	460.1481	461.1555	0.3	0.24	0.79	7.6%	11.4%	104.3%	4.40
Spiramycin	$C_{43}H_{74}N_2O_{14}$	2.99	842.5140	843.5213	−0.2	0.01	0.04	5.6%	7.1%	87.7%	5.02
Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	0.97	284.0134	285.0208	−1.0	0.43	1.43	6.0%	7.0%	103.9%	4.95
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	0.25	250.0524	251.0597	−1.1	0.01	0.02	5.5%	8.2%	107.5%	3.81
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	1.08	310.0735	311.0809	−0.9	0.30	0.98	2.7%	4.1%	100.3%	5.70

Table 1. *Cont.*

Compound	Molecular Formula	LogP	Molecular Weight	$[M + H]^+$	ΔM (ppm)	LoD (ng/L)	LoQ (ng/L)	Repeatability (%)	Reproducibility (%)	Recovery (%)	RT (min)
Sulfadimidin	$C_{12}H_{14}N_4O_2S$	0.43	278.0837	279.091	−0.9	0.14	0.47	1.9%	2.5%	100.9%	4.51
Sulfadoxine	$C_{12}H_{14}N_4O_4S$	0.72	310.0735	311.0809	−0.7	0.03	0.10	6.6%	7.7%	98.3%	4.94
Sulfamethizole	$C_9H_{10}N_4O_2S_2$	0.53	270.0245	271.0318	−1.3	0.32	1.06	12.7%	15.2%	100.0%	4.52
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	0.79	253.0521	254.0594	−0.7	0.03	0.09	2.5%	3.7%	99.5%	5.12
Sulfapyridine	$C_{11}H_{11}N_3O_2S$	0.84	249.0572	250.0645	−0.7	0.46	1.52	3.3%	3.3%	104.4%	3.80
Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	1.24	300.0681	301.0754	−0.7	0.03	0.09	4.5%	6.7%	88.3%	5.71
Sulfathiazole	$C_9H_9N_3O_2S_2$	0.88	255.0136	256.0209	−1.3	0.56	1.87	6.6%	10.6%	108.6%	3.65
Sulfisomidine	$C_{12}H_{14}N_4O_2S$	0.84	278.0837	279.091	−0.2	0.10	0.32	9.8%	14.6%	87.0%	3.42
Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	1.14	267.0677	268.075	0.1	0.02	0.07	9.8%	13.5%	101.1%	5.04
Tetracycline	$C_{22}H_{24}N_2O_8$	−0.56	444.1532	445.1605	0.2	0.48	1.61	5.5%	8.2%	112.6%	4.57
Tilmicosin	$C_{46}H_{80}N_2O_{13}$	3.34	868.5660	869.5733	−0.9	0.01	0.05	3.7%	4.5%	95.9%	5.40
Trimethoprim	$C_{14}H_{18}N_4O_3$	1.26	290.1378	291.1452	0.3	0.80	2.68	4.3%	6.4%	109.5%	4.24
Tylosin A	$C_{46}H_{77}NO_{17}$	1.46	915.5191	916.5264	−0.9	0.36	1.21	4.6%	6.1%	86.7%	5.90
Anticonvulsants											
Carbamazepine	$C_{15}H_{12}N_2O$	2.10	237.1022	237.1022	−0.8	0.01	0.05	12.5%	18.7%	102.9%	6.08
Gabapentin	$C_9H_{17}NO_2$	−1.90	172.1332	172.1332	−0.5	0.81	2.69	4.1%	6.1%	80.6%	3.61
Topiramate	$C_{12}H_{21}NO_8S$	1.29	340.1061	340.1061	0.6	0.03	0.11	2.9%	3.7%	104.7%	5.82
Antidepressants											
Alpha-Hydroxyalprazolam	$C_{17}H_{13}ClN_4O$	1.53	324.0777	325.0851	−0.3	0.02	0.08	5.6%	8.3%	84.9%	6.12
Fluoxetine	$C_{17}H_{18}F_3NO$	4.09	309.1340	310.1413	−0.2	0.01	0.03	5.2%	7.8%	109.3%	4.88
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	2.98	320.0119	321.0192	3.1	2.98	9.94	7.6%	8.7%	108.9%	4.53
Sertraline	$C_{17}H_{17}Cl_2N$	5.06	305.0738	306.0811	0.4	0.03	0.09	3.2%	4.0%	109.6%	6.13
Venlafaxine	$C_{17}H_{27}NO_2$	2.69	277.2041	278.2115	−0.9	0.02	0.08	6.0%	7.3%	99.6%	5.24
Antihypertensives											
Furosemide	$C_{12}H_{11}ClN_2O_5S$	2.71	331.015	331.015	−1.0	0.62	2.07	7.7%	11.6%	109.3%	6.04
Indapamide	$C_{16}H_{16}ClN_3O_3S$	2.52	366.0674	366.0674	−0.3	0.03	0.09	3.6%	4.0%	100.4%	6.27
Irbesartan	$C_{25}H_{28}N_6O$	4.51	429.2397	429.2397	−0.7	0.03	0.17	10.5%	15.7%	96.0%	6.31
Losartan	$C_{22}H_{23}ClN_6O$	4.50	423.1695	423.1695	−0.5	0.05	0.09	12.3%	18.4%	107.1%	5.77

Table 1. *Cont.*

Compound	Molecular Formula	LogP	Molecular Weight	$[M + H]^+$	ΔM (ppm)	LoD (ng/L)	LoQ (ng/L)	Repeatability (%)	Reproducibility (%)	Recovery (%)	RT (min)
β-Blockers											
Atenolol	$C_{14}H_{22}N_2O_3$	0.57	266.1630	267.1703	−0.7	0.01	0.03	3.0%	4.2%	104.4%	3.47
Bisoprolol	$C_{18}H_{31}NO_4$	2.30	325.2253	326.2326	−1.0	0.14	0.46	8.3%	9.1%	94.7%	5.29
Carvedilol	$C_{24}H_{26}N_2O_4$	3.05	406.1892	407.1965	−0.9	0.83	2.76	13.4%	19.7%	108.7%	5.87
Propranolol	$C_{16}H_{21}NO_2$	3.03	259.1572	260.1645	−0.6	0.06	0.21	4.5%	6.5%	100.9%	4.39
Lipid regulators											
Atorvastatin	$C_{33}H_{35}FN_2O_5$	4.24	559.2603	559.2603	0.6	8.92	29.73	7.9%	10.2%	88.6%	8.46
Bezafibrate	$C_{19}H_{20}ClNO_4$	3.97	362.1154	362.1154	−0.8	0.07	0.24	7.4%	8.6%	85.7%	6.91
Fenofibrate	$C_{20}H_{21}ClO_4$	4.86	361.1201	361.1201	0.7	0.02	0.06	17.2%	18.1%	103.5%	9.41
Gemfibrozil	$C_{15}H_{22}O_3$	3.61	251.1642	251.1642	−0.8	0.21	0.71	4.5%	6.8%	110.4%	4.99
Simvastatin	$C_{25}H_{38}O_5$	4.51	419.2792	419.2792	−1.6	2.80	9.34	11.7%	12.2%	110.3%	6.20
NSAID's											
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	4.98	295.0166	296.024	−0.5	0.02	0.05	5.9%	8.8%	106.0%	7.70
Ibuprofen	$C_{13}H_{18}O_2$	3.50	206.1306	207.138	−0.4	2.72	9.08	3.5%	5.3%	108.9%	6.22
Nimesulide	$C_{13}H_{12}N_2O_5S$	2.56	308.0466	309.054	0.3	0.03	0.08	7.4%	11.1%	104.5%	7.23

2. Materials and Methods

2.1. Standards and Reagents

All reagents and solvents used for sample preparation were of analytical grade with the exception of mobile phase solvents, which were of LC-MS grade. Methanol, acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany), while pharmaceutical standards were acquired from Sigma-Aldrich (Madrid, Spain) with the highest purity grade available (>99%). The internal standard sulfameter was also from Merck. The target analytes, sorted by classes together with molecular formulas and weight, partition coefficients (logP), exact mass and summarized results of method validation, are presented in Table 1.

Ultrapure water was obtained daily from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Nitrogen was produced in-house with a generator from Peak Scientific Instruments Ltd. (Chicago, IL, USA). Solvents and water were filtered through 0.45 µm Whatman nylon membrane filters (Whatman, Maidstone, VT, USA) prior to degassing in an ultrasonic bath.

Individual stock solutions (1 mg/mL) were prepared by accurately weighing each standard and internal standard, adjusting for purity and salt forms and dilution in methanol, except for beta-lactams that were prepared in water. Solutions were vortex mixed and sonicated, when necessary, to assure complete dissolution. All stock solutions were stored at −20 °C, and beta-lactams were stored in small and disposable aliquots to avoid several freeze/unfreeze steps that can rapidly degrade them. Suitable working dilutions were prepared with convenient concentrations for the quantification of target compounds.

2.2. Sample Treatment

Sample extraction, purification and concentration were adapted from Sousa and colleagues [13] and Pereira and colleagues [14]. The method was optimized with estuarine and seawaters collected in the Tejo Estuary (Portugal) and transported in high-density polyethylene bottles (pre-washed with 10% nitric acid and triple rinsed with ultrapure water). Monitoring samples were collected in triplicate in 31 sites covering the whole estuary, including sites near effluents discharge from wastewater treatment plants. These samples were acidified to pH 2 with formic acid, transported on ice and frozen at −20 °C until analysis.

The first step of sample treatment consisted in the addition of the internal standard sulfameter to 500 mL water samples before the sequential filtering through glass microfiber filters (110 mm), cellulose nitrate filters (0.45 µm) and Sartolon polyamide filters (0.2 µm). Purification was achieved with solid phase extraction (SPE) using OASIS HLB cartridges (200 mg) without pre-conditioning. Subsequently, cartridges were washed with 5 mL of methanol:water (10:90), dried for 15 min at low vacuum pressure and then eluted with 6 mL of methanol. The final extract was dried under N2 flow at 40 °C. The residue was re-dissolved with 500 µL of mobile phase (0.1% of formic acid in water), filtered through a PVDF Mini-uniprep TM filter (0.45 µm) and injected in the UHPLC-TOF-MS for detection and quantification.

2.3. Instrumentation

During method development, the following equipment was used: analytical balances Toledo PC200 and AE100 (Greifensee, Switzerland), Heidolph Reax 2 overhead mixer (Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), Turbovap Zymark evaporator connected to a nitrogen generator (Hopkinton, MA, USA) and Whatman Mini-Uniprep PVDF (polyvinylidene fluoride) 0.45 µm filters (Clifton, NJ, USA) for final extract filtration before UHPLC injection.

The chromatographic separation and mass spectrometry detection were performed with an UHPLC Nexera X2 Shimadzu coupled with a Triple TOFTM 5600+ from AB Sciex (UHPLC-ToF-MS). The system consisted of a vacuum degasser, autosampler with controlled temperature, binary pump and an oven for the chromatographic column. This system was equipped with an analytical reverse-phase column Zorbax Eclipse Plus C18—2.1 × 50 mm, 1.8 µm (Agilent) maintained at 40 °C, after testing a temperature range from 25 to 45 °C.

The flow rate was 500 µL/min and the mobile phases were [A] formic acid 0.1% (*v/v*) in water and [B] acetonitrile with the following gradient program: 0–5 min from 97% to 40% [A]; 5–9 min from 40% to 0% [A]; 9–10 min from 0% back to 97% [A]; 11–12 min 97% [A]. The autosampler was set at 10 °C to maintain samples queued for injection refrigerated to prevent degradation. The injection volume selected was 10 µL, based on previous studies.

The TOF-MS ionization was performed with positive electrospray ion source (ESI+) mode with full-scan data acquisition from 100 to 920 Da and using the Analyst® TF 1.7 Identification and quantification were performed with PeakViewTM 2.2, LibraryViewTM and MultiQuantTM 3.0.2. The identification criteria followed were based on the exact mass with an error below 5 ppm, variation of relative retention time to a maximum of 2.5% and the isotope ratio difference lower than 10% (Commission Regulation (EU) 2021/808). The TOF-MS detector was calibrated between every 10 injections to guarantee the accurate mass resolution.

2.4. Validation Procedure

Given the lack of specific legislation setting maximum residue levels (MRL) for pharmaceuticals in environmental waters, with the exception of those included in the Water Framework Directive [15], validation followed the Commission Regulation 2021/808 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results [16]. The optimized method was validated quantitatively with the following parameters: specificity, selectivity, precision and linearity. However, since the WFD sets the maximum acceptable method detection limit, the limit of detection (LOD) and limit of quantification (LOQ) were calculated instead of the decision limit (CC_α) and the detection capability (CC_β).

Identification criteria were based on the accurate mass measurement of the base ion with an error < 5 ppm (Δm), a maximum variation of 2.5% in the relative retention time (RRT) of the analyte compared with the standard and an isotope ratio difference lower than 10% when compared with the theoretical pattern. Figure 1 presents the example of the identification of oxytetracycline. The following equations were used to calculate the Δm and RRT [17].

Equation (1): relative retention time (RRT)

$$\mathrm{RRT} = \frac{\mathrm{RT_{analyte}}}{\mathrm{RT_{internal\ standard}}} \quad (1)$$

where $\mathrm{RT_{analyte}}$ is the retention time of the analyte, and $\mathrm{RT_{internal\ standard}}$ is the retention time of the internal standard.

Equation (2): Deviation of RRT (ΔRRT)

$$\Delta\mathrm{RRT}\ (\%) = \left(\frac{\mathrm{RRT_{spiked\ samples}} - \mathrm{RRT_{standard}}}{\mathrm{RRT_{standard}}}\right) \times 100 \quad (2)$$

Equation (3): Deviation of exact mass (Δm)

$$\Delta\mathrm{m}\ (\mathrm{ppm}) = \left(\frac{\text{Exact mass} - \text{Detected mass}}{\text{Exact mass}}\right) \times 10^6 \quad (3)$$

The isotope ratio difference by the overlap of spectrums is automatically calculated by the software PeakViewTM 2.2 and LibraryViewTM 2.2.

Validation procedure and quality control samples (spiked samples) were performed using coastal water samples, collected and previously analyzed to prove they were blank samples. For the selectivity and specificity study, 20 blank coastal water samples were analyzed to assess if any interference could compromise the accurate identification of the target compounds. Additionally, the same samples were spiked with all compounds to verify the detection capability through the identification criteria, which also allowed the evaluation of precision (repeatability and reproducibility) in terms of relative standard deviation (RSD) and the recovery. The linearity was evaluated in a concentration range between 0.8 and 6 ng/L,

and the LOD and LOQ were estimated in accordance with the observed signal-to-noise ratio in the spiked samples and blank samples, respectively 3:1 and 10:1.

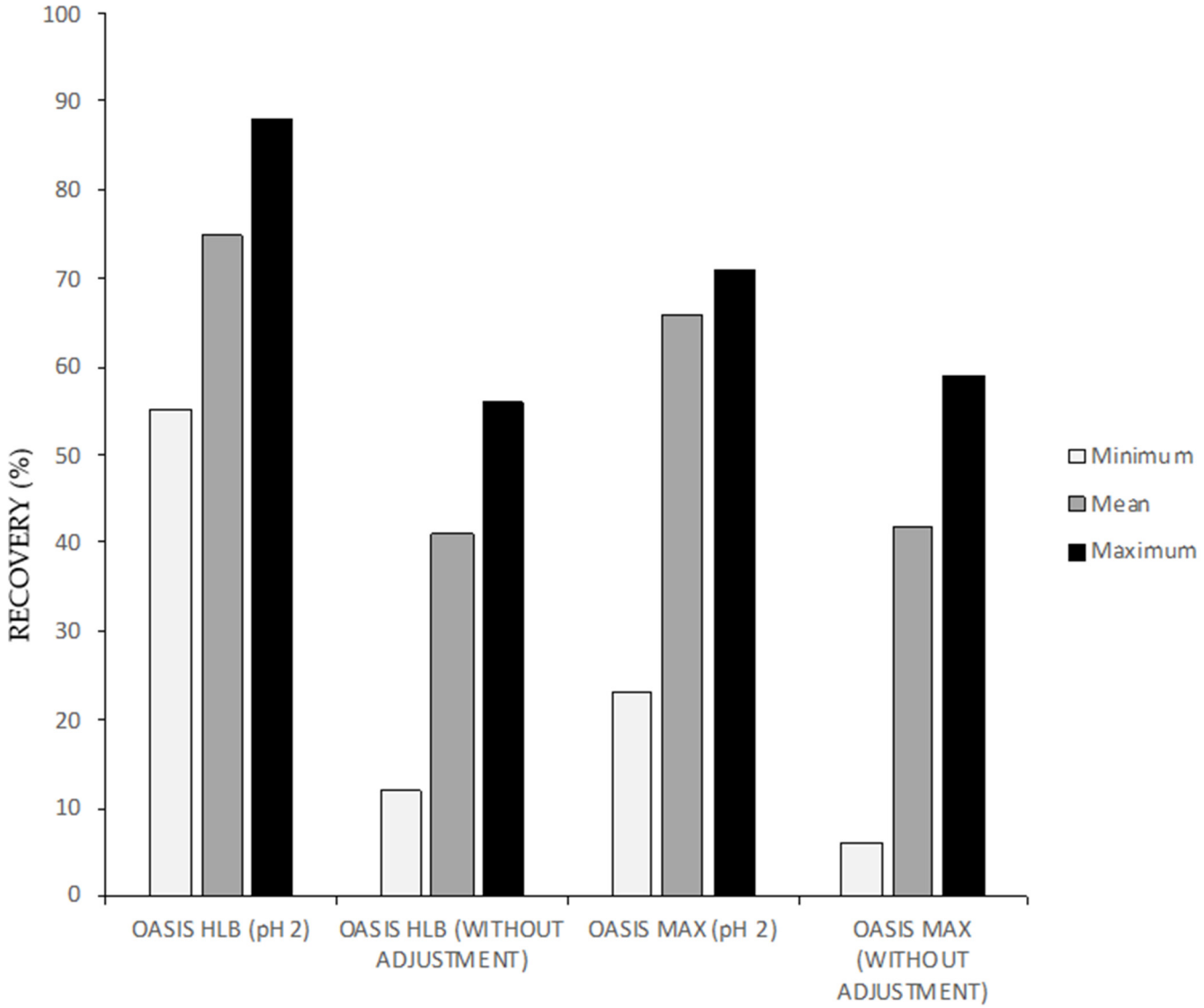

Figure 1. Minimum, mean and maximum recoveries obtained in sample extraction variations (SPE cartridge and initial pH adjustment of the sample).

3. Results and Discussion

3.1. Method Development

The monitoring project BIOPHARMA was set to establish baseline data of estuarine contamination dictating the need for an UHPLC-TOF-MS method capable of detecting and quantifying up to 63 pharmaceuticals (belonging to 8 therapeutic classes) in a single run. As it is widely known, the continuous release of such contaminants poses severe risks to such highly productive systems, potentially affecting the key functions and services they provide [18]. Within the monitoring program that BIOPHARMA comprised, 31 sampling sites were strategically selected to cover the whole Tejo estuarine area, including areas adjacent to wastewater treatment plant effluents (WWTP) discharging into the system [18,19]. Thus, having a reliable and fast validated method that could deal with a high number of samples with varying salinities and the possibility of revisiting the results post-analyses was key.

3.1.1. Solid Phase Extraction

The starting point for sample extraction, purification and concentration of water samples was an LC-MS/MS method published by Pereira and colleagues [14], which in turn was based on the work of Sousa and co-authors [13]. The procedures described for LC-MS-MS were tested and optimized for UHPLC-TOF-MS aiming to ultimately achieve the best recovery rates possible for all the pharmaceuticals of interest.

Since estuarine, coastal and wastewater samples are complex matrices, clean-up is a key step in ensuring a clear final extract. Thus, spiked 500 mL samples (with the 63 compounds of interest and the internal standard) were submitted to a 3-step filtration sequence starting with 100 mm glass microfiber filter, followed by a 0.45 µm cellulose nitrate filter and finally a 0.2 µm polyamide filter. This ensured the removal of most of the co-extracted matter, including salt ions and dissolved organic matter that otherwise would be in the final extract and would lower greatly the recoveries due to ionization suppression effects and even compound losses [13]. This sequential filtration was compared with 1- and 2-step sequences and proved to produce the best recoveries when compared with spiked ultrapure water samples (to guarantee that no other interferent was present in the water) at the same levels. In addition, the use of internal standard can overcome the possible matrix effects that can compromise the accurate identification and quantification of the target compounds by correcting fluctuations due to sample extraction, chromatographic behavior and the ionization efficiency. The internal standard selection was based on the linearity and recovery studies.

The physico-chemical differences among the 63 compounds, particularly regarding polarity, are presented in the Table 1 in terms of the coefficient of partition, logP. This parameter is given as an indicator of the polarity of the compounds targeted in the present method and is defined as the ratio of soluble concentration between two immiscible solvents, namely octanol and water. In practice, the more polar and hydrophilic the compound is, the lower is the logP. The presented logP in Table 1 comprises theoretical predicted values obtained in the database of https://go.drugbank.com; accessed on 1 February 2023 and calculated with the virtual computational Chemistry Laboratory, http://www.vcclab.org; accessed on 1 February 2023. Considering the range of polarities of the target compounds, ensuring the cartridges for clean-up and extraction is paramount. The previous methods were performed with Oasis MAX cartridges preconditioned with methanol and water. However, in the present work a comparison with Oasis HLB (200 mg) was performed since these reversed-phase cartridges are filled with polymeric sorbent which provides hydrophilic and lipophilic retention characteristics enhancing results for both polar and non-polar compounds. Another important feature of Oasis HLB is that preconditioning is not necessary in this type of SPE, reducing the volume of organic solvents needed and, thus, achieving a greener method. In addition, and most relevant in terms of saving time in sample processing, there is the sorbent being water wettable, which means that it can run dry without compromising retention and recovery. Given all these features, it became important to compare clean-up and extraction with both cartridges types, with and without acidifying the samples to pH 2. The minimum, maximum and mean recoveries are presented in Figure 1 and clearly show that Oasis HLB performed better with than Oasis Max, and that pH must be adjusted to 2 to ensure the best recoveries. Although Waters™ clearly states preconditioning is not required, Oasis HLB were tested with and without this step, and results clearly show that it is, in fact, unnecessary (Figure 2).

After elution and evaporation to dryness, the final eluate was resuspended with 500 µL of mobile phase (0.1% of formic acid in water). This increase in volume when compared with the previous methods (200 µL) is due to a final filtration step with PVDF Mini-uniprep filter (0.45 µm) before injection. The extraction efficiency was tested with the spiked samples run in triplicates with ESI(+)-TOF-MS as all the analytes ionize with positive polarity. The recovery rates obtained with the optimized method ranged from 80.6% for both flumequine and gabapentin and 112.6% for tetracycline. Simultaneously extracting substances within such a polarity range can be a real challenge, and one step that helped increase the recovery

rates was acidification with formic acid to pH 2. This increased the solubility of more basic drugs such as ciprofloxacin, azithromycin or fluoxetine by increasing their ionized forms and their retention by ionic interactions. Acidification also favored the extraction of neutral compounds containing carboxylic acid and phenolic groups [20].

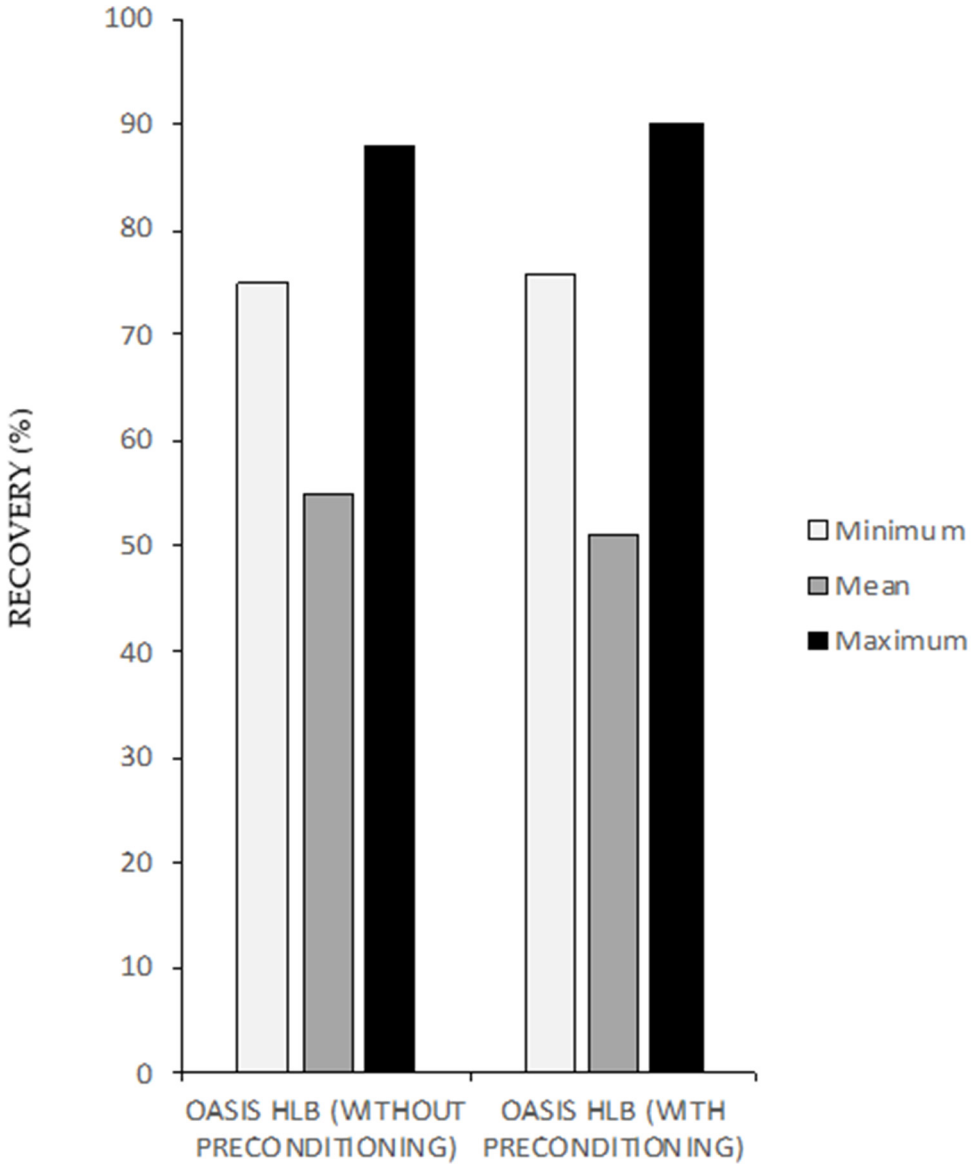

Figure 2. Minimum, mean and maximum recoveries achieved with SPE with and without preconditioning the OASIS HLB.

3.1.2. UHPLC-TOF-MS Performance

Optimizing the chromatographic separation of a high number of analytes with different chemical properties in one single run can be challenging. The resulting final method will reflect a compromise between several key steps including ionization efficiency, peaks shape, resolution and time of analyses [21]. The process started with the selection of the most suitable analytical column and, therefore, four columns with varying length and pore diameter were tested, namely Acquity BEH C18 (2.1 × 100 mm and 1.7 µm), Acquity HSS C18 (2.1 × 100 mm and 1.8 µm), Kinetex Biphenyl (2.1 × 50 mm and 1.7 µm) and Zorbax Eclipse Plus C18 (2.1 × 50 mm and 1.8 µm). The full comparisons of results obtained for the four columns tested are presented in Figure 3. Based on the number of compounds detected, the reverse-phase Zorbax Eclipse Plus C18 column (2.1 × 50 mm and 1.8 µm) was selected. One known advantage of using smaller particles packed columns, in this case 1.8 µm, is the

elution of analytes in a narrower, more concentrated band that increases resolution, peak capacity (efficiency) and linear velocity [22]. Additionally, to enhance the separation of target analytes, a width of 2.1 mm was preferred with a flow rate of 500 µL/min. As for the length, and considering the gradient time, the 50 mm column produced the highest peak capacity. Given the high number of analytes, it is necessary to ensure maximum resolution to decrease matrix suppression caused by their simultaneous elution [21].

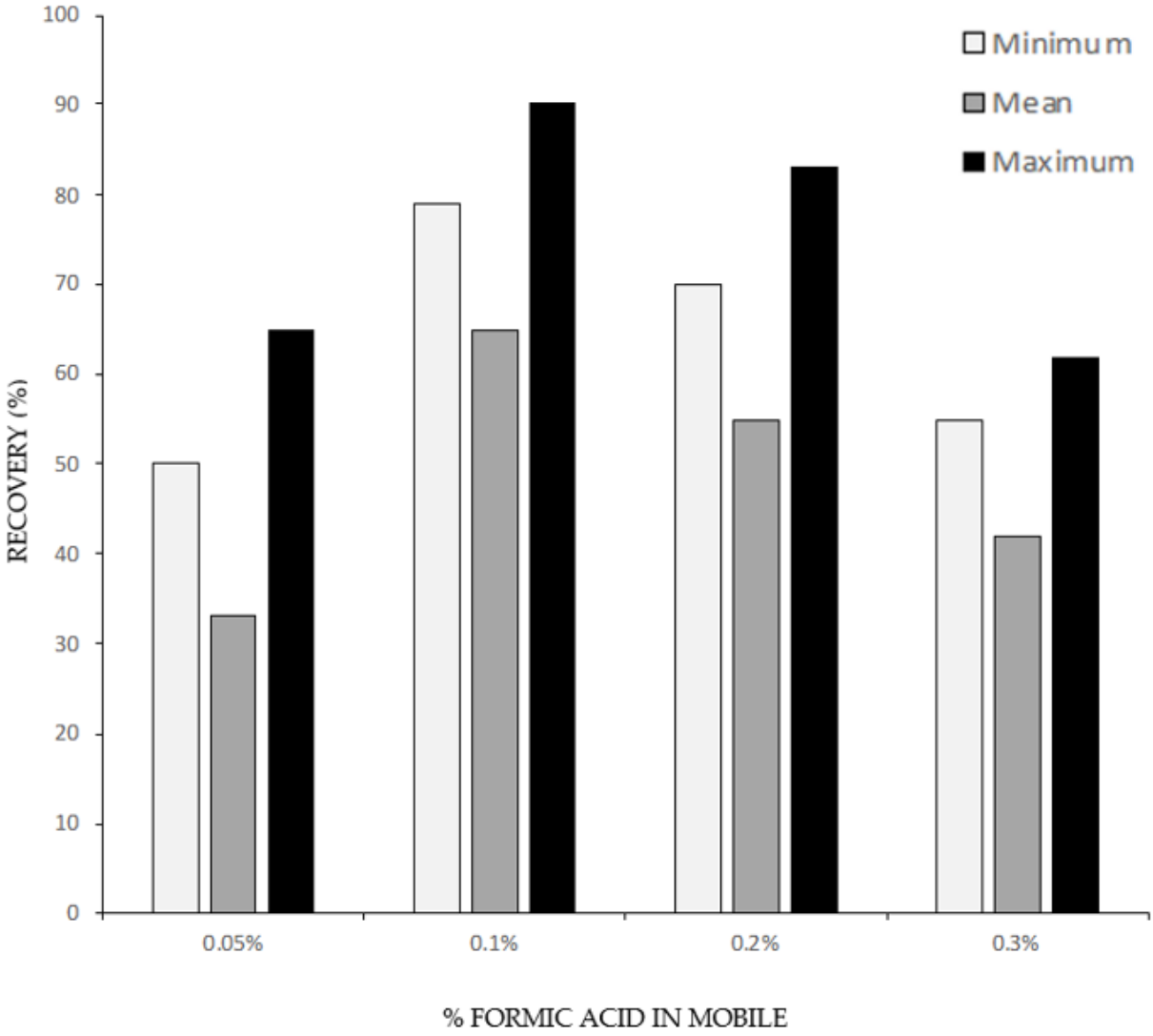

Figure 3. Minimum, mean and maximum recoveries obtained for the variation of mobile phase composition, in percentage of formic acid.

Electrospray ionization is greatly influenced by analytes chargeability and is particularly suited for polar compounds. When using the positive ion mode (ESI+), a pH modifier is usually added to the solvent to enhance the signal intensities of the target analytes, in particular of weak bases which are more extensively protonated in acidic medium [23]. In this work, formic acid was selected with the aqueous phase (A) consisting of water acidified with formic acid 0.1% to avoid signal suppression effects. The ideal concentration of formic acid was selected in previous laboratory tests by performing a set of analyses with variation of formic acid in water ranging from 0.05% to 0.3%. Results are presented in Figure 4 in which it is clear that the best recoveries were achieved with 0.1% of formic acid. As for the organic phase, acetonitrile was selected since it reduced the background noise. Overall, the resolution was improved with the mobile phases chosen, with a total run time of 12 min.

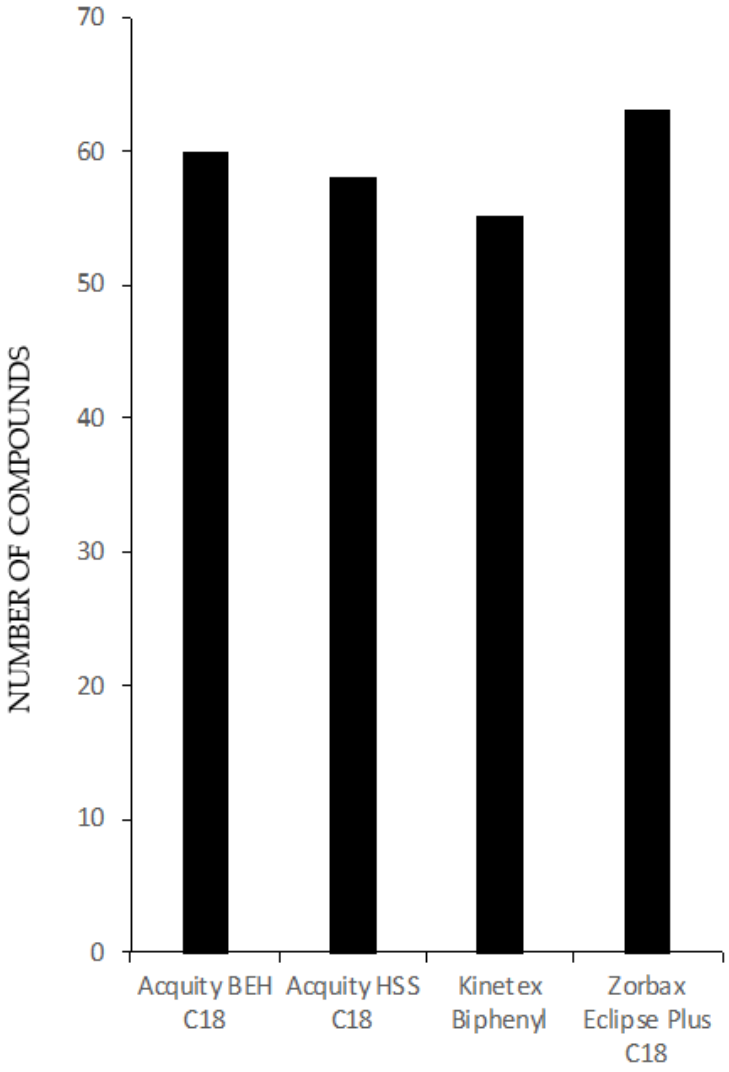

Figure 4. Number of compounds detected for each chromatographic column tested.

3.2. Validation

Once separation and optimization of the UHPLC-TOF conditions were achieved, the quality parameters were studied to ensure suitable identification, confirmation and quantification of the target analytes. Therefore, validation was based on specificity, selectivity, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ). The summarized results of the validation are detailed in Table 1. Prior to validation protocol, several samples were analyzed to select blank samples to be used during the process. For that, coastal waters were collected far from urbanization or industrial regions. Those samples were then used for spiking purposes and for blank interferences analysis.

Specificity and selectivity—that is, the capacity of the method to discriminate between the target analytes and interferences that might have similar behavior—were confirmed by analyzing 20 blank samples of coastal water to verify the absence of interference above a signal-to-noise ratio of 3 at the retention time of the target analytes that could compromise their detection and identification. Additionally, in the spiked blank samples, all identification criteria were fulfilled without false-negative results, proving once more the specificity of the method.

Linearity was determined in a concentration range of 0.8 to 6 ng/L through direct injection of standard solutions. However, for some compounds where the LOD and the LOQ were found to be out of the validation concentration range (ciprofloxacin, danofloxacin, enoxacin, enrofloxacin, marbofloxacin, oxolinic acid and ibuprofen), extra calibration

concentration points were added from 6 ng/L until 12 ng/L. Repeatability (within-day precision) and reproducibility (inter-day precision) were established for each analyte as relative standard deviation (RSD). Repeatability ranged from 1.4% for ofloxacin and 17.2% for fenofibrate whereas reproducibility was between 2.5% for sulfadimin and 20.9% for amoxicillin. Regarding the LOD of the method, values were between 0.01 ng/L for flumequine, spiramycin, tilmicosin, azithromycin, fluoxetine and atenolol and 8.92 ng/L corresponding to atorvastatin. Taking a closer look at the LOD of the 5 substances from the EU Watch List, the values were much lower than requested by the legislation. Specifically, the LOD were 0.11 ng/L for amoxicillin, 3.47 ng/L for ciprofloxacin, 0.03 ng/L for sulfamethoxazole, 0.80 ng/L for trimethoprim and 0.02 ng/L for venlafaxine, while the maximum acceptable LOD regarded in the EU 2020/1161 are, respectively, 70 ng/L, 89 ng/L, 100 ng/L (both sulfamethoxazole and trimethoprim) and 6 ng/L.

As for the LOQ, fluoxetine and atenolol presented 0.03 ng/L while atorvastatin had the highest value of 29.73 ng/L. However, all the other analytes presented values below 12 ng/L for both LOD and LOQ.

Looking at recoveries, values were between 80.6% and 112.6%, well within the acceptable range for a multi-residue method and in accordance with specific guidelines for validation of analytical methods as stated in the Commission Implementing Regulation (EU) 2021/808.

3.3. Application to Field Samples

The driving force for optimization of the present method was, as previously stated, the monitoring project BIOPHARMA. With 31 sampling sites and over 60 compounds to monitor, a reliable multi-residue method was fundamental. Therefore, once it was fully optimized and validated, it was applied to the analyses of samples collected in triplicate at the Tejo estuary. Results reflected the presence of pharmaceuticals throughout the system, with their detection in all collected samples (n = 93). All 8 therapeutic groups were present with a total of 32 analytes confirmed. The most represented groups were antibiotics and β-blockers, with sulfathiazole and bisoprolol detected at all sites [18]. Regarding the recently updated EU Watch List [15], 4 out of the 5 pharmaceuticals included in the method were consistently detected in the Tejo Estuary [18]. Venlafaxine presented a detection frequency of 87.3%, followed by trimethoprim (32.3%), ciprofloxacin (29%) and sulfamethoxazole (3.2%).

For full details of the results of the environmental analyses, see the works of Reis-Santos and colleagues [18] and Fonseca and co-authors [19].

4. Conclusions

The UHPLC-TOF-MS multi-residue analytical method here described provides a powerful ally in environmental water monitoring which is further reinforced by the possibility of revisiting results since one of the main advantages of the full scan acquisition mode is to keep a digital print in the selected range of masses. In that sense, untargeted compounds can be reanalyzed in the future without new sample extraction. The lower extraction volumes are also an advantage, especially considering field sampling management and the reduction in time and costs in clean-up procedures. Due to the high number of analytes detected and the reduced run time, it will boost the analytical power of laboratories and studies that have to process a high number of samples and compounds. The validated method was then applied to real samples collected in the Tejo estuary, and in all 93 samples, residues of pharmaceutical compounds were detected with incidence of sulfathiazole, bisoprolol, venlafaxine, trimethoprim, ciprofloxacin and sulfamethoxazole.

Author Contributions: Conceptualization, S.L., A.F., J.B., P.R.-S., V.F.F. and F.R.; Methodology, S.L., A.F. and J.R.; Validation, S.L., A.F., J.R. and A.S.V.P.; Formal analysis, S.L., A.F., J.R. and A.S.V.P.; Investigation, S.L., A.F., J.R. and P.R.-S.; Resources, M.A.P. and F.R.; Writing—original draft, S.L. and A.F.; Writing—review & editing, S.L., A.F., J.R., A.S.V.P., J.B., P.R.-S., V.F.F., M.A.P. and F.R.; Visualization, A.F. and V.F.F.; Supervision, S.L.; Project administration, S.L.; Funding acquisition, S.L. and V.F.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available upon request.

Acknowledgments: The authors would like to thank Fundação para a Ciência e a Tecnologia (FCT) for funding the research via project grants PTDC/MAR-EST/3048/2014 (BIOPHARMA), UIDB/04292/202, MARE (Marine and Environmental Sciences Centre, UIDB/04292/2020 and UIDP/04292/2020), ARNET (Aquatic Research Network Associated Laboratory, LA/P/0069/2020). and through the strategic plan of the Centre for Functional Ecology—Science for People and the Planet (CFE; UIDB/04004/2021), financed by FCT/MCTES through national funds (PIDDAC). Additional support was obtained via project "RENATURE—Valorization of the Natural Endogenous Resources of the Centro Region" (CENTRO-01-0145-FEDER-000007), funded by the Comissão de Coordenação da Região Centro. Sara Leston is supported by a researcher contract at the University of Coimbra (DL57/2016/IT057-18-7255).

Conflicts of Interest: The authors declare no conflict of interest.

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