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A SPE-HPLC-MS/MS method for the simultaneous determination of prioritised pharmaceuticals and EDCs with high environmental risk potential in freshwater

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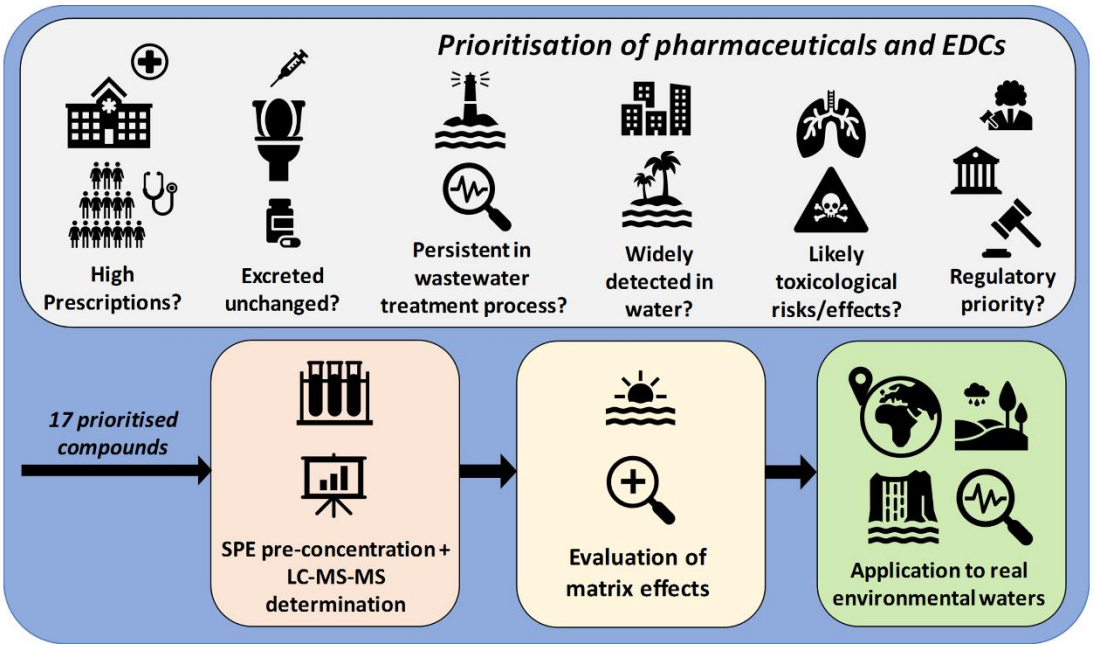

Abstract: This work describes the development, optimisation and validation of an analytical method for the rapid determination of 17 priority pharmaceutical compounds and endocrine disrupting chemicals (EDCs). Rather than studying compounds from the same therapeutic class, the analyses aimed to determine target compounds with the highest risk potential with regard to Scotland, providing a tool for further monitoring in different water matrices. Prioritisation was based on a systematic environmental risk assessment approach, using consumption data; wastewater treatment removal efficiency; environmental occurrence; toxicological effects; and pre-existing regulatory indicators. This process highlighted 17 compounds across various therapeutic classes, which were then quantified, at environmentally relevant concentrations, by a single analytical methodology. Analytical determination was achieved using a single-step solid phase extraction (SPE) procedure followed by high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). The fully optimised method performed well for the majority of target compounds, with recoveries >71% for 15 of 17 analytes. The limits of quantification for most target analytes (14 of 17) ranged from 0.07 $ng \cdot L^{-1}$ to 1.88 $ng \cdot L^{-1}$ in river waters. The utility of this method was then demonstrated using real water samples associated with a rural hospital/setting. Eight compounds were targeted and detected, with the highest levels found for the analgesic, paracetamol (at up to 105910 $ng \cdot L^{-1}$ in the hospital discharge). This method offers a robust tool to monitor high priority pharmaceutical and EDC levels in various aqueous sample matrices.

Keywords:
Pharmaceuticals
Prioritisation
risk assessment
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water quality

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Introduction

The discovery and use of pharmaceuticals is one of society's greatest advances, leading to increased human lifespan and promoting improved health (Johansson, 1998). An unintended consequence of the widespread use of human pharmaceuticals has however been their inadvertent and now ubiquitous introduction into the aquatic environment. This commonly occurs as a result of the excretion (in urine/faeces) of unmetabolised parent compounds and the improper disposal of unused or expired medicinal products – both of which pass into sewage networks (where these are present) and then remain in treated wastewater discharges (Cahill et al., 2004; Charuaud et al., 2019; Fekadu et al., 2019; Kallenborn et al., 2018). Numerous studies have now demonstrated the incomplete removal of pharmaceuticals by sewage treatment systems, with as much as 80% of the total load of any particular pharmaceutical entering the treatment network ultimately being released into the receiving aquatic environment (Botero-Coy et al., 2018; Östman et al., 2018; Ruan et al., 201; Yuan et al., 2014).

The potential effects of pharmaceutical pollution may include the promotion of multi-drug resistant bacterial strains and/or deleterious acute or chronic ecotoxicological impacts on non-target organisms (Brodin et al., 2013; Hernando-Amado et al., 2019; Kumar et al., 2019). For example, fluoxetine has been shown to cause reproductive delay in leopard frogs (Foster et al., 2010; Fursdon et al., 2019; Hellström et al., 2016), while ciprofloxacin can cause genotoxic effects in plankton and algae (Carusso et al., 2018) (Dionísio et al., 2020). Further, certain pharmaceuticals are also endocrine disrupting chemicals (EDCs), and have been shown to exert significant reproductive effects even at trace environmental levels. For example, 17α-ethinylestradiol (a synthetic hormone commonly used in birth control pills) has been extensively studied in fish and shown to cause delays in embryonic development (Almeida et al., 2020; Huff et al., 2018), vitellogenin induction (Zhang et al., 2019; Zhou et al., 2019), intersex development (Jackson et al., 2019; Ujhegyi and Bókony, 2020) and thus reduced reproductive success (Colman et al., 2009; Roy et al., 2018).

The ongoing discharge of pharmaceuticals and EDCs into the wider environment potentially poses a risk to human health and as such there remains a need to evaluate their presence, fate and behaviour in various environmental compartments. This requires the development of robust, sensitive and accurate analytical methods for the simultaneous extraction, detection and quantification of these chemicals at low, environmentally

relevant levels. The most common analytical approach to determine pharmaceuticals and EDC levels in aqueous samples first involves a pre-concentration step (i.e., using solid phase extraction; SPE), and then the use of liquid chromatography with mass spectrometric detection (LC-MS) (Buchberger, 2011;.Hong et al., 2019; Peng et al., 2019). However, many methods focus on compounds that are simply most commonly found (i.e., in water), or, that belong to specific drug classes, i.e., antibiotics (Gurke et al., 2015a, 2015b; Rossmann et al., 2014; Scheurer et al., 2009). As such, there remains a need to develop techniques specifically focussed on those substances thought to pose the greatest risk potential within the aquatic environment. Such methods can be informed by existing prioritisation systems such as those which have led to the creation of "Watch Lists" within the EU Water Framework Directive (WFD, EU) and the UK's Chemical Investigation Program (CIP, UK) (European commission, 2015; UKWIR, 2015). Such regulatory indicators act to highlight those compounds thought to be of most concern and/or requiring more detailed research.

In this study, we describe the development of an SPE protocol combined with subsequent HPLC-MS/MS (high performance liquid chromatography with tandem mass spectrometry) analysis for the routine determination of selected pharmaceuticals and EDCs (first prioritised based on their high environmental risk potential). The work presented involves: (1) prioritisation of compounds across a range of therapeutic classes – all with significant potential to pose risks to the aquatic environment; (2) development of a rapid and sensitive method to measure these compounds at environmentally relevant concentrations ($ng \cdot L^{-1}$); (3) an evaluation of possible matrix effects using different water types; and (4) application of the methodology to real samples collected from a range of sites as part of a hospital discharge focused monitoring study.

1. Methodologies and chemicals

1.1 Chemicals and reagents

All prioritised compound standards were of the highest purity available (>98%) and supplied by Sigma-Aldrich (UK). Isotopically labelled internal standards were purchased from Qmx. Both individual compound stock standards and isotopically labelled internal standards (ILIS) were prepared in methanol, except for ciprofloxacin, which was dissolved in methanol containing 1 µM NaOH to enhance solubility. Mixed compound standards and calibration standards were prepared using appropriate dilutions of

individual stock solutions, in 50:50 v/v methanol:Milli-Q® water. All solutions were stored in amber glass vials at −20℃ in the dark.

HPLC-grade acetonitrile, ethyl acetate, acetone and methanol were provided by VWR Chemicals (Poole, England). Formic acid, acetic acid, ammonium acetate and ammonium hydroxide were all analytical grade and supplied by Sigma–Aldrich. Oasis HLB 6cc (200 mg) and Oasis HLB Prime 6cc (200 mg) SPE cartridges were obtained from Waters Corporation (Milford, MA, USA).

1.2 Instrumentation

The quantification of target analytes was performed using a HPLC-MS/MS system, consisting of an Agilent 1100 HPLC with a CTC PAL auto-sampler coupled to a Micromass Quattro Ultima Platinum mass spectrometer (Manchester, UK) equipped with an electrospray ionisation source (ESI). Ions were acquired in multiple reaction monitoring (MRM) mode. Precursor ions for each compound were determined by direct infusion of individual compound standards whilst in full-scan mode (at m/z 50-1000). During infusion the optimum cone voltage (CV) to achieve maximum signal response for each ion was selected. Product ion scanning was then performed to obtain product ions, and collision energy (CE) was optimised for each individual analyte. The highest intensity characteristic precursor to product ion MRM transition was used for quantification (quantifier), while a second was used for confirmation (qualifier). To sustain an adequate signal response for every compound, analytes were measured within optimised time windows. Data acquisition and analysis were carried out using MassLynx 4.1 software (Micromass, Manchester, UK).

1.3 Sample preparation

SPE was employed for sample enrichment and clean-up, and several stationary phases were tested under a range of elution conditions to optimise compound recovery (see **Fig. S1** for schematic of the process). All SPE experiments were conducted in triplicate, using 20 mL of Milli-Q water spiked to a starting concentration of 10 $\mu g \cdot L^{-1}$ for each analyte (ultimately 500 $\mu g \cdot L^{-1}$ in final extract/following the SPE process, assuming 100% recovery). For the final protocol, SPE cartridges were preconditioned with methanol (6 mL) and then Milli-Q water (6 mL), both at a flow rate of 1 $mL \cdot min^{-1}$. 20 mL spiked water samples were passed through the cartridges at a flow rate of 1 $mL \cdot min^{-1}$ and then cartridges were rinsed with Milli-Q water once (1 mL). Cartridges were then dried under

vacuum for >30 min to remove excess water. Then, the analytes were eluted with two consecutive 6 mL elution's using methanol (MEOH), or, acetone (ACE) and ethyl acetate (EAC) at 50:50 v/v (depending on desired recoveries for certain compounds), at 1 $mL \cdot min^{-1}$. The eluates were then evaporated under a gentle stream of high purity nitrogen at 40℃ until they were almost dry, then reconstituted with 0.4 mL of 50:50 *v/v* MEOH:Milli-Q. Absolute recoveries were determined compared to quality-control (QC) standards of 500 $\mu g \cdot L^{-1}$.

1.4 Method quantification

Compound selectivity was verified by measuring two MRM transitions per analyte. Calibration linearity was studied by analysing standards in triplicate at nine concentrations in the range from 2 to 500 $\mu g \cdot L^{-1}$. Satisfactory linearity using weighed (1/x) least squares regression was assumed when the correlation coefficient (R^2) was > 0.99. Method accuracy and precision (expressed as recovery and repeatability, using relative standard deviation) were studied with recovery experiments (using Milli-Q water spiked with analytes). Instrumental limits of detection (LOD) for each compound were determined as the minimum detectable amount of analyte giving a signal-to-noise (S/N) ratio of 3 (using the quantification transition).

For the investigation regarding matrix effects, a known amount of analyte (10 $\mu g \cdot L^{-1}$) and ILIS (1 $\mu g \cdot L^{-1}$) was added to tap water and river water (filtered and unfiltered). Taking into account an enrichment factor of 50 (whereby 20 mL of water sample was reconstituted into 0.4 mL for analysis following SPE), quality-control (QC) standards of 500 $\mu g \cdot L^{-1}$ (for the analytes) and 50 $\mu g \cdot L^{-1}$ (for the ILIS) were then used for quantification.

1.5 Application to real samples

A range of water samples were collected from sites associated with/in the vicinity of a rural UK hospital (in Caithness, Scotland). These were (1) the local potable untreated surface water source, (2) the hospital water inflow, (3) the hospital combined wastewater effluent discharge, (4) the combined local municipal WWTP influent and (5) the combined effluent from the same municipal WWTP (for Wick town, Caithness). A sub-set of 8 target compounds were monitored over 4 weeks at these sites. Water samples (2 L) were collected in amber glass bottles and 1 L was filtered through 0.7 µm glass microfiber filters (47 mm, MF300, Fisher Scientific, UK). Filtrates were spiked with 0.25 mL of ILIS mixed standard working solution (at 100 $\mu g \cdot L^{-1}$; equivalent to a 25 $ng \cdot L^{-1}$

concentration in 1 L of sample). SPE cartridges were preconditioned with MEOH and Milli-Q water, then 1L water samples were passed through the cartridges at a flow rate of 1 mL·min^{-1}. The SPE extract was eluted with 2×6 mL MEOH and reconstituted with 0.5 mL of 50:50 v/v MEOH:Milli-Q, leading to an enrichment factor of 2,000 and a final concentration of 100 µg·L^{-1} ILIS in the analysed sample. Quantification was made using external QC standards and calibration standards, with recovery assessed based on relative responses. To ensure the precision and accuracy of the data required, all targeted compounds in real water analysis have been assigned with their own ILIS standards to correct possible quantification errors. All samples were stored at 4℃ in the dark until SPE extraction, which was performed within 48 hr of sample collection.

2. Results and Discussion

2.1 Prioritisation of target compounds

As there are > 3,000 pharmaceuticals registered for use in the European Union (EU), it is necessary to prioritise these whilst accounting for risk (Boxall et al., 2012). Many prioritisation schemes have been proposed in recent years, commonly based on consumption data, environmental occurrence and/or toxicological effects (Kötke et al., 2019; Li et al., 2020; Mansour et al., 2016; Pereira et al., 2016; Roos et al., 2012). However, many field monitoring studies still focus on compounds that are most commonly found in water (López-Serna et al., 2011; Rossmann et al., 2014) – many of which may (or may not) be likely to elicit toxicological effects.

Here, a systematic prioritisation approach was first used to identify compounds that may pose the greatest risk in the aquatic environment. For the evaluation of the environmental risk of pharmaceuticals and EDCs, it is difficult to estimate if adverse effects (both acute and chronic toxicity as well as other potentially more subtle biological and behavioural effects) on non-target organisms occur at environmentally relevant concentrations. In this study, a risk score was used as a primary prioritisation parameter to characterize substances that pose potential ecological risks to the aqueous environment by comparing their environmental occurrence with their known toxicologically relevant concentrations. The risk score - hazard quotient (HQ) value was calculated as the ratio between measured environmental concentration (MEC) and the predicted no effect concentration (PNEC; i.e., the environmental level at which no adverse effect on relevant non-target

organisms/ecosystem function is expected) (Booth et al., 2020). When the HQ ≥ 1, a high risk of adverse effects is expected (De Souza et al., 2009; Ccanccapa et al., 2016).

Although there are (as yet) no legally binding discharge limits set in the EU for pharmaceuticals and EDCs, multiple compounds have been highlighted as 'priority substances' for further investigation by EU and UK regulatory frameworks, i.e., through 'Watch Lists' created as part of the EU Water Framework Directive (WFD) and the priority lists created through the UK's Chemical Investigation Programme (CIP) (European commission, 2018; UKWIR, 2019). These result in increased monitoring and research and may ultimately lead to statutory discharge limits for certain compounds (Brack et al., 2017; Miarov et al., 2020; Nijsingh et al., 2019; Petrie et al., 2015; Voulvoulis et al., 2017). As such, these regulatory indicators have also been taken into account here.

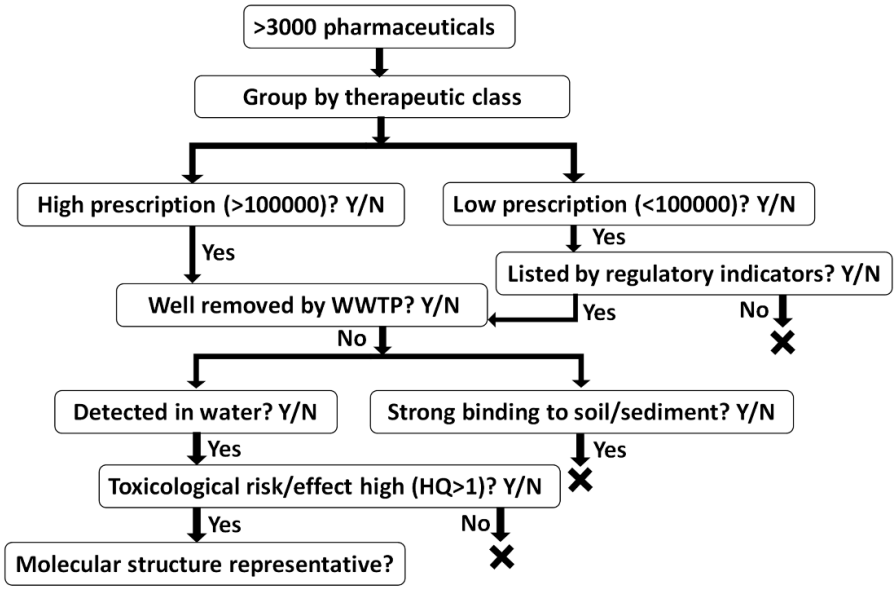


Fig.1. Decision tree of compound prioritisation

Here, the first step in our prioritisation process was to consider prescription rates within Scotland. As shown in **Fig.1**, pharmaceuticals were first grouped by therapeutic class, and within each class, compounds prescribed >100,000 times per year (ISD Scotland, 2016) were highlighted. Substances prescribed below this value were only highlighted if they had been listed as existing priorities within the EU's WFD Watch List(s) and/or the UK's CIP Programme. Analytes highlighted were then evaluated further by considering WWTP removal efficiency, reported environmental concentrations (in water) and toxicological risk. Combining the reviewed range of pharmaceutical and EDC monitoring data (MEC) and their PNEC values, the risk scores were calculated to characterize

substances that have pose potential adverse effects to the aqueous environment at current detection levels. All preliminarily prioritised substances were then considered in terms of their physico-chemical properties – and where these had very similar molecular structures (which may then result in similar environmental fate), a single substance was selected as representative of a certain group.

The prioritisation selection criteria applied here were, in summary: a) prescription statistics (ISD Scotland, 2016); b) legislative indicators, i.e., WFD 'Watch List' and/or UK CIP listed; c) removal efficiency in WWTP; d) environmental occurrence in water; e) biological toxic effects (informed by HQ calculated with PNEC and MEC); and f) physico-chemical properties. **Table 1** shows a summary regarding the prioritised list of compounds targeted in this study (Li et al., 2019).

Table 1. Prioritised compounds in this study and the criteria and data used

Class	**Compounds**	**Use statistics**[1] (items)	**Legislative indicator**	**WWTP removal efficiency** (%)	**Environmental occurrence** ($ng \cdot L^{-1}$)	**PNEC**[2] ($ng \cdot L^{-1}$)	**HQ**[3] (>1?)	**Log K_{ow}**	**pK_a**
Antibiotic (anti-infective)	**Trimethoprim**	487128	No	0-50	10-28000	500	56	0.9	7.1
Antibiotic (macrolide)	**Clarithromycin**	268489	EU[a,b]+UK[4]	0-24	3.5-621	250	2.5	3.2	8.9
Antibiotic (Fluoroquinolone)	**Ciprofloxacin**	99441	EU[b]+UK	45-78	6–2500	100	25	0.3-0.7	5.9; 8.9
Antimicrobial	**Triclosan**	10-1000 t/yr[5]	UK	45-89	3.9-434	50	8.68	4.2-4.8	7.9
Analgesic	**Paracetamol**	5482031	No	0-90	160-65000	1000	65	0.5-0.9	9.5
NSAID[6]	**Ibuprofen**	915788	UK	72-90	44–990000	1650	600	4.0	4.9
NSAID	**Diclofenac**	595709	EU[a]+UK	9-60	10–510000	3310	154	4.5	4.2
SSRI[7]	**Fluoxetine**	816346	UK	3-60	2.1-2000	110	18.2	1.2	10.1
Antiepileptic	**Carbamazepine**	223601	UK	0-53	290–4596	420	10.9	2.47	13.9
Metabolite	**Carbamazepine-10-11-epoxide**	N/A[8]	UK	N/A	8-2100	N/A	N/A	1.26	13.9
β-blocker	**Propranolol**	557628	UK	34-80	108-1130	244	4.63	0.78	9.5
Blood lipid regulator	**Atorvastatin**	1637000	UK	40-80	10-210	86	2.44	6.36	4.46
Anti-diabetic	**Metformin**	1140162	UK	0-85	100-47000	13450	3.49	1.3	12.4
Steroid hormone (Natural)	**Estrone (E1)**	N/A	EU[ab]+UK	0-61	1.8-60	6	10	2.45-3.43	10.5
Steroid hormone (Natural)	**17β-Estradiol (E2)**	N/A	EU[ab]+UK	0-87	0.72-51	2	25.5	3.94-4.01	10.7
Steroid hormone (Synthetic)	**17α-ethynyl estradiol (EE2)**	298045	EU[ab]+UK	0-85	0.36-4.3	0.35	12.3	3.67–4.15	10.4
Steroid hormone (Natural)[9]	**Estriol (E3)**	N/A	No	0-90	0.11-18	60	0.3	2.55-2.81	10.4

1. Prescription statistics for Scotland 2014-15 (ISD Scotland, 2016); 2. Predicted no-effect concentration (PNEC); 3. Hazard quotient (HQ) incorporating MEC with PNEC; 4. EU a. Commission Implementing Decision 2015/495 (European commission, 2015); b. 2018/840 (European commission, 2018); UK, Chemical Investigation Programme (UKWIR, 2019); 5. Triclosan usage in EU per year; 6. Nonsteroidal anti-inflammatory drugs (NSAIDs); 7. Selective serotonin reuptake inhibitors (SSRIs); 8. N/A, not applicable/available. 9. References used for the prioritisation data are listed in Table. S1 (supporting information).

Ultimately, 17 compounds were identified as priority substances here, belonging to a wide range of compound classes (11), i.e., antibiotics, antimicrobials, analgesics, non-steroidal anti-inflammatory drugs, psychoactive drugs, β-blockers, blood lipid regulators, antidiabetics, anti-ulcer agents and estrogens (as well as associated metabolites). Fifteen compounds were associated with high potential risk (HQ>1) within the aqueous environment, including ibuprofen, diclofenac, paracetamol, trimethoprim, E2, ciprofloxacin, fluoxetine, EE2, carbamazepine, E1, propranolol, metformin, clarithromycin, atorvastatin and triclosan (in HQ value order from high to low). This largely aligns with key legislative indicators (given these were also one of our criteria), with the only pharmaceutical compounds in addition to CIP/WFD indicators being trimethoprim and paracetamol. These two compounds have been highlighted as their current occurrence levels outstrip its known toxicologically relevant concentrations (PNEC) as shown in **Table 1**, the high HQ scores of trimethoprim (56) and paracetamol (65) indicated that the adverse effects on non-target organisms may occur in the aquatic environment. Trimethoprim is the second most commonly prescribed antibiotic in Scotland, and reports show that up to 80% of this is excreted unmetabolised by the human body (De Liguoro et al., 2012; Kasprzyk-Hordern et al., 2009). It has been found to be resistant to the biological wastewater treatment (Lindberg *et al.* 2006), one of the most frequently occurring antibiotics found in UK wastewaters, being detected in 65% of effluent samples with a maximum concentration of 1,300 $ng \cdot L^{-1}$ (Ashton, Hilton & Thomas 2004). Similarly, paracetamol is one of the most commonly prescribed drugs globally, due to its antipyretic and analgesic properties. Even though the reported removal efficiencies in WWTPs are relatively high (up to 90%), it is often found at high levels in the aquatic environment (e.g., maximum 10,000 $ng \cdot L^{-1}$ in US natural waters and at 65,000 $ng \cdot L^{-1}$ in the River Tyne, UK) (Kolpin et al., 2002; Roberts and Thomas, 2006). Such high levels of paracetamol continuously introduced into the aquatic environment have been found to cause negative ecological effects in various wild organisms (Nunes et al., 2014), the high HQ scores of these compounds in this study reinforced the necessity of further investigation of such pollutants.

UK CIP (UKWIR, 2019) identified a wide range of substances that may pose a significant risk to the environment in the UK. Following the prioritisation procedure used here, fourteen compounds on CIP were prioritised for investigation. At EU level, priority substances were first introduced under the WFD Commission Implementing Decision (EU) 2015/495, which listed ten watch list substances, and required this list to be updated every two years according to Commission Implementing Decision (EU) 2008/105 (European commission, 2008).

Accordingly, diclofenac was originally prioritised in the first WFD watch list (European commission, 2015) and monitored intensively. On the basis of sufficient high-quality monitoring data available for this compound, diclofenac has since been removed from the watch list in June 2018 (European commission, 2018). Meanwhile, the antibiotic ciprofloxacin has been added due to its potential to drive antimicrobial resistance in the environment. Macrolide antibiotics (clarithromycin, erythromycin and azithromycin) have been retained in the watch list, while, clarithromycin, the highest prescribed macrolide, was chosen as the representative compound, based on the fact that these substances have similar molecular structures and physico-chemical properties.

As well as 'parent' pharmaceutical compounds, one of the 17 compounds listed here is a metabolite. While most studies tend to focus on primary pharmaceuticals, there is now increased recognition that excreted metabolites may also pose risks in the environment (Roberts and Thomas, 2006). Carbamazepine, one of the most prominent anti-epileptic drugs with annual worldwide usage of 1,014 tons and 223,601 prescription in Scotland has been targeted in this study due to the poor removal in WWTP, high detection levels and potential risks in the environment (ISD Scotland, 2016; Radjenović, Petrović & Barceló 2009). As well as 'parent' pharmaceutical compound, the metabolite of carbamazepine, carbamazepine-10-11-epoxide has been found to be biologically active and shows similar or higher toxicity relative to its parent compound (Calisto and Esteves, 2009; Miao and Metcalfe, 2003). Therefore, carbamazepine-10-11-epoxide has been included as a representative metabolite. Moreover, there are several potent natural estrogens of concern (estrone (E1), 17 β-estradiol (E2) and estriol (E3)), which are not dissimilar to the synthetic xenoestrogen - 17α-ethynyl estradiol (EE2) which has been of concern for many years (Burkhardt-Holm, 2010; Czarny et al., 2019; Qin et al., 2020; Yu et al., 2019). Three of these four EDCs (E1, E2 and EE2) have also been highlighted by both the EU's WFD Watch List schemes and the UK's CIP system. As estriol (E3) poses ecotoxicological effects similar to E1, E2, and EE2, this estrogen has also been targeted for investigation here.

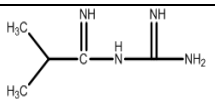
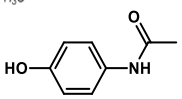
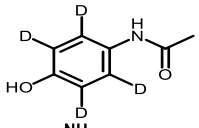
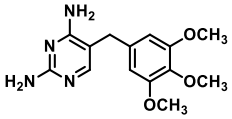
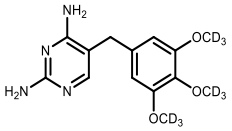
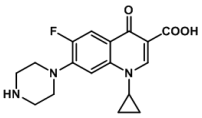
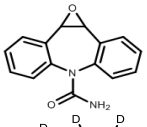
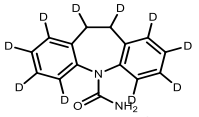
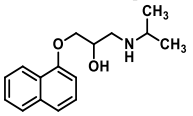
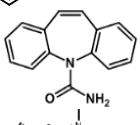
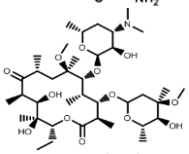
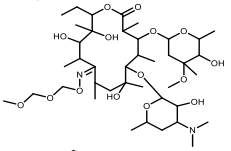
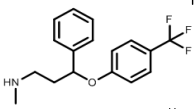
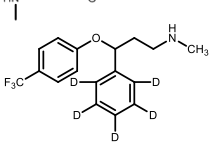
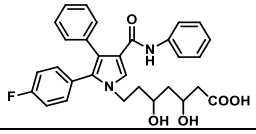
2.2 Detection method development

2.2.1 HPLC separation and MS/MS optimisation

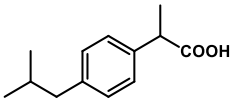
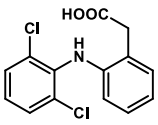
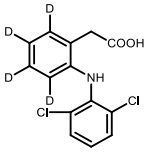
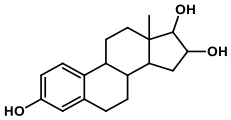
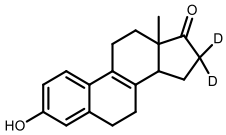
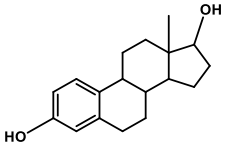
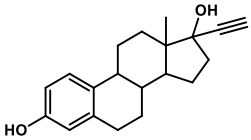
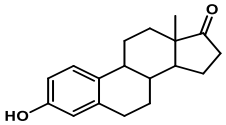
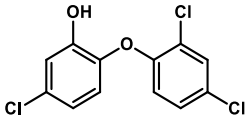
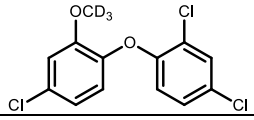
To optimise compound separation and sensitivity, methanol and acetonitrile along with different buffers (ammonium acetate, ammonium hydroxide, formic acid and acetic acid at

various concentrations) were tested as mobile phases. MS parameters were optimised to attain maximum sensitivity and selectivity. Of the 17 substances, 10 showed a higher response using the protonated $[M+H]^+$ ions and positive ion (PI) mode while 7 were better using negative mode (detecting the deprotonated $[M-H]^-$ ions). For both modes, several HPLC columns and various operational parameters/gradient designs (i.e., different flow rates and slopes) were tested in order to optimise peak separation, signal response and minimise run time. Good peak shape and sensitivity were achieved in PI mode using a reverse-phase Waters XBridge BEH C18 column (2.1 mm I.D. x 100 mm, 2.5 µm) with 0.1% formic acid as the aqueous phase and acetonitrile at 45℃. For the 7 NI compounds, sufficient separation was obtained using 0.025% ammonium hydroxide (in water) and acetonitrile and a Phenomenex Kinetex EVO C18 column (3.0 mm I.D x 100 mm, 2.6 µm) at 25℃. The optimised gradient elution programs used are shown in **Table S2** (Supporting Information) alongside representative chromatograms for pure standard mixtures monitored in both modes (**Fig. S2**). Optimised mass spectrometry parameters, precursor and product ions, retention times (RT) and instrumental LODs in both PI and NI modes are summarised in **Table 2** and **3**, respectively.

Table 2. Mass spectrometry parameters for target compounds analysed in positive ionisation (PI)

Compound	Mol. Weight ($g \cdot mol^{-1}$)	Precursor ion	CV[1] (V)	Product ions	CE[2] (eV)	RT[3] (min)	Corresponding ILIS	Molecular structure	LODs[4] ($\mu g \cdot L^{-1}$)
Metformin	129	130	45	60 71	8 10	1.22	Paracetamol -D4		0.072
Paracetamol	151	152	45	110 93	10 16	3.78	Paracetamol -D4		0.213
Parcetamol-D4	155	156	45	114 97	10 16	3.81	-		-
Trimethoprim	290	291	60	230 261	20 20	7.69	Trimethoprim -D9		0.049
Trimethoprim-D9	299	300	60	264 234	20 20	7.64	-		-
Ciprofloxacin	331	332	55	288 245	14 20	7.99	Trimethoprim -D9		0.107
Carbamazepine-10-11-epoxide	252	253	40	236 180	6 16	11.29	Carbamazepine -D10		0.054
Carbamazepine -D10	246	247	60	204 201	14 16	13.34	-		-
Propranolol	259	260	50	116 183	14 14	11.81	Carbamazepine -D10		0.143
Carbamazepine	236	237	60	194 192	14 16	13.39	Carbamazepine -D10		0.057
Clarithromycin	748	749	60	158 590	26 16	15.11	Roxithromycin		0.039
Roxithromycin	837	838	60	679 158	26 16	15.16	-		-
Fluoxetine	309	310	35	44 148	8 6	15.42	FLX-D5		0.052
Fluoxetine-D5	314	315	35	44 153	8 6	15.37	-		-
Atorvastatin	559	560	50	440 466	18 14	19.71	Fluoxetine-D5		0.059

328 **Table 3.** Mass spectrometric parameters for target compounds analysed in negative ionisation (NI)

Compound	Mol. Weight ($g \cdot mol^{-1}$)	Precursor ion	CV[1] (V)	Product ion	CE[2] (eV)	RT[3] (min)	Corresponding ILIS	Molecular structure	LODs[4] ($\mu g \cdot L^{-1}$)
Ibuprofen	206	205	35	161	4	2.21	Diclofenac-D4		0.272
Diclofenac	296	294	35	250 214	8 18	4.14	Diclofenac-D4		0.428
Diclofenac-D4	300	298	35	254 217	8 18	4.15	-		-
Estriol	288	287	115	171 145	30 30	5.64	E1-D2		2.177
Estrone-D2	272	271	105	145 159	28 28	11.49	-		-
17β-Estradiol	272	271	125	145 183	28 30	10.46	E1-D2		0.771
17α-ethynylestradiol	296	295	125	145 159	28 30	11.17	E1-D2		1.082
Estrone	270	269	125	145 159	28 28	11.44	E1-D2		0.376
Triclosan	289	289 287	35	35 35	4 4	13.72	Triclosan-D3		0.891
Triclosan-D3	292	290	35	35 37	4 4	13.72	-		-

329 1. CV - cone voltage; 2. CE - collision energy; 3. RT - retention time; 4. LOD - limit of detection.

330 All compounds had two abundant product ions, except ibuprofen, for which only one was
331 monitored due to poor fragmentation. Transitions identified here are in agreement with those
332 from other studies (Löffler and Ternes, 2003; Jelić et al., 2009; Ferrer et al., 2010; Golet et al.,
333 2001).

334 **2.2.2 Optimisation of Solid Phase Extraction (SPE) procedure**

A number of SPE protocols (different cartridges, elution solvents, pH conditions, etc.) were evaluated for pharmaceutical and EDC recovery. The choice of SPE stationary phase can play a crucial role in enhancing recovery of analytes and SPE selection is frequently based on the physico-chemical properties of target compounds. Here, the lipophilic-hydrophilic-balanced, reverse-phase polymeric sorbent Oasis HLB cartridge was used to accommodate the wide range of physico-chemical characteristics exhibited by the prioritised pharmaceuticals and EDCs (with pK_a ranging from 4.2 to 13.9, and Log K_{ow} from 0.28-6.36). This cartridge has also been shown to be less susceptible to matrix effects than other media (Gorga et al., 2013; Van De Steene et al., 2006; Vazquez-Roig et al., 2010). Two HLB cartridges (Oasis HLB and Oasis HLB Prime) were evaluated using methanol and acetone:ethyl acetate at 50:50 *v/v* as solvents. To study any pH related recovery effects, different solution pH values were tested (i.e., no pH adjustment or pH = 2). The average absolute recoveries (and relative standard deviations (SD)) for each target compound are shown in **Table 4**.

To evaluate possible quantification errors introduced by analyte loss during sample processing and fluctuations in instrument sensitivity, 1 $\mu g \cdot L^{-1}$ of ILIS was added as a surrogate to samples prior to extraction (ILIS = 50 $\mu g \cdot L^{-1}$ post-SPE, assuming 100% recovery). The ILIS compounds applied in this study were selected based on the following criteria: (i) a 2H-isotope or a 13C labelled isotope compound - which shared the same (or very similar) physico-chemical properties to the analyte; (ii) with a chromatographic retention time close to that of the analyte; (iii) and similar SPE recovery and ionisation response to the analyte. Given the large number of compounds targeted here it was unfeasible to correct each analyte with its own individual ILIS, hence, ILIS analogues were used for certain groups (i.e., E1-D2 for the four estrogens) on the basis of compound similarity, retention time and recovery. Relative recoveries (calculated using the recovery data for the ILIS compounds), and the ILIS compounds used, are presented in **Table 5**.

Table 4. Absolute mean SPE recoveries of prioritised pharmaceuticals and EDCs using different SPE protocols

No.	Recoveries % and (±%RSD)		Analytes detected in -ve mode							Analytes detected in +ve mode										No. >75% and <125%
			IBU	DCF	E3	E2	EE2	E1	TCS	MET	PARA	CFX	TMP	CBZE	PPL	CBZ	CTM	FLX	ATV	
1	MEOH	HLB	35 (±17)	67 (±4)	38 (±10)	36 (±6)	41 (±5)	44 (±6)	34 (±2)	45 (±16)	90 (±6)	2 (±0)	38 (±1)	59 (±1)	39 (±3)	51 (±1)	49 (±6)	35 (±0)	10 (±1)	1
		HLB Prime	71 (±10)	83 (±3)	65 (±4)	75 (±4)	87 (±0)	56 (±10)	67 (±11)	74 (±6)	93 (±2)	2 (±0)	56 (±1)	90 (±1)	68 (±4)	83 (±2)	45 (±1)	45 (±5)	12 (±2)	6
2	ACE:EAC	HLB	56 (±5)	77 (±2)	37 (±10)	33 (±14)	40 (±13)	49 (±3)	59 (±2)	11 (±12)	90 (±4)	3 (±3)	38 (±2)	65 (±7)	6 (±1)	52 (±3)	26 (±5)	1 (±0)	11 (±3)	2
		HLB Prime	90 (±6)	91 (±2)	97 (±3)	111 (±9)	101 (±3)	107 (±4)	98 (±8)	11 (±3)	99 (±2)	1 (±0)	42 (±1)	98 (±0)	28 (±6)	102 (±4)	26 (±7)	0 (±0)	27 (±5)	10
3	pH 2 MEOH	HLB	29 (±2)	30 (±3)	29 (±72)	30 (±1)	38 (±1)	53 (±0)	53 (±3)	3 (±1)	72 (±2)	195 (±21)	34 (±1)	5 (±1)	47 (±1)	49 (±0)	26 (±3)	45 (±0)	10 (±4)	0
		HLB Prime	43 (±7)	46 (±10)	63 (±7)	66 (±8)	63 (±3)	104 (±7)	77 (±4)	3 (±0)	96 (±4)	145 (±6)	74 (±9)	22 (±2)	81 (±1)	85 (±0)	27 (±6)	84 (±5)	11 (±4)	6
4	pH 2 ACE:EAC	HLB	57 (±1)	36 (±1)	27 (±3)	29 (±4)	37 (±5)	49 (±0)	62 (±10)	2 (±0)	81 (±1)	36 (±11)	42 (±8)	20 (±1)	40 (±8)	54 (±6)	9 (±1)	32 (±2)	6 (±1)	1
		HLB Prime	58 (±6)	63 (±0)	73 (±1)	77 (±4)	75 (±3)	121 (±19)	91 (±8)	1 (±1)	88 (±6)	144 (±2)	56 (±0)	25 (±1)	91 (±4)	94 (±11)	14 (±1)	46 (±1)	14 (±3)	7

Table 5. Mean SPE recoveries of prioritised pharmaceuticals and EDCs calculated using the ILIS recovery data to correct responses

No	Recoveries % and (±%RSD)		Analytes detected in -ve mode							Analytes detected in +ve mode										No. >75% and <125%
	Applied ILIS		IBU	DCF	E3	E2	EE2	E1	TCS	MET	PARA	CFX	TMP	CBZE	PPL	CBZ	CTM	FLX	ATV	
			DCF-D4		E1-D2				TCS-D3	TMP-D9				CBZ-D10			RTM	FLX-D5		
1	MEOH	HLB	48 (±21)	77 (±3)	66 (±13)	60 (±5)	56 (±4)	88 (±2)	101 (±6)	49 (±5)	64 (±1)	1 (±0)	51 (±6)	94 (±10)	60 (±2)	85 (±4)	129 (±4)	45 (±1)	39 (±3)	5
		HLB Prime	64 (±6)	92 (±3)	112 (±8)	103 (±6)	96 (±5)	97 (±3)	92 (±4)	102 (±26)	210 (±19)	4 (±1)	117 (±3)	95 (±1)	63 (±0)	88 (±1)	126 (±4)	85 (±1)	113 (±5)	12
2	ACE:EAC	HLB	69 (±5)	77 (±1)	77 (±4)	91 (±10)	104 (±7)	91 (±10)	97 (±1)	3 (±0)	85 (±18)	3 (±2)	45 (±9)	126 (±7)	3 (±0)	89 (±0)	127 (±11)	83 (±24)	80 (±17)	10
		HLB Prime	80 (±2)	94 (±1)	96 (±6)	99 (±4)	93 (±3)	96 (±3)	99 (±3)	31 (±7)	227 (±6)	2 (±1)	113 (±2)	99 (±1)	29 (±7)	90 (±0)	135 (±16)	82 (±5)	123 (±7)	12
3	pH 2 MEOH	HLB	56 (±3)	77 (±0)	46 (±2)	57 (±2)	83 (±11)	71 (±4)	93 (±2)	1 (±0)	40 (±2)	62 (±4)	95 (±2)	3 (±1)	77 (±8)	80 (±3)	104 (±4)	95 (±1)	9 (±0)	8
		HLB Prime	147 (±28)	101 (±2)	66 (±6)	83 (±4)	92 (±1)	103 (±5)	110 (±1)	4 (±2)	126 (±18)	221 (±5)	134 (±6)	33 (±3)	116 (±3)	120 (±3)	133 (±2)	95 (±0)	67 (±16)	8
4	pH 2 ACE:EAC	HLB	79 (±6)	80 (±1)	64 (±7)	74 (±9)	100 (±3)	67 (±17)	103 (±4)	1 (±0)	63 (±4)	61 (±9)	92 (±7)	21 (±0)	79 (±1)	86 (±2)	111 (±0)	88 (±2)	10 (±1)	9
		HLB Prime	161 (±13)	101 (±1)	98 (±25)	117 (±46)	116 (±36)	99 (±14)	107 (±2)	2 (±1)	98 (±5)	186 (±11)	110 (±1)	28 (±4)	109 (±2)	102 (±1)	130 (±8)	95 (±2)	114 (±56)	12

Where particular low/high recoveries have been observed, these are shaded grey for ease of noting (<35% / >135% - dark grey; <75%/ >125% - light grey). Metformin MET; Paracetamol PARA; Trimethoprim TMP; Ciprofloxacin CFX; Carbamazepine-10-11-epoxide CBZ; Propranolol PPL; Carbamazepine CBZ; Clarithromycin CTM; Fluoxetine FLX; Atorvastatin ATV; Ibuprofen IBU; Diclofenac DCF; Estriol E3; 17β-Estradiol E2; 17α-ethynylestradiol EE2; Estrone E1; Triclosan TCS. Trimethoprim-D9 TMP-D9; Carbamazepine-D10 CBZ-D10; Roxythromycin RTM; Fluoxetine-D5 FLX-D5; Diclofenac-D4 DCF-D4; Estrone-D2 E1-D2; Triclosan-D3 TCS-D3. Methanol MEOH; acetone ACE; ethyl acetate EAC.

Recoveries obtained varied markedly between compounds and SPE conditions used (as may be expected given the physico-chemical diversity of the prioritised compounds). It is evident that data corrected for ILIS recovery (**Table 5**) provided better results for most target compounds (as compared to absolute recovery data; **Table 4**). This was most evident for the analytes clarithromycin, fluoxetine, trimethoprim and the estrogens. This indicated that analyte losses occurred throughout the analytical procedure and that ILIS correction helped ensure better quantification (compensating for any losses).

In terms of SPE, higher recovery values were achieved using the Oasis HLB Prime cartridges under the tested conditions. The Oasis HLB Prime provided satisfactory recoveries (>75% and <125%) for more analytes (**Table 4** and **5**), which may be attributed to the strong hydrophobic interaction between analytes and retention sorbent of HLB prime cartridges (Beltran et al., 2010). For the extremely polar compound metformin, which was previously reported as not recoverable using an SPE procedure, satisfactory recoveries (102% ± 26%) were observed in condition 1 (Cahill et al., 2004).

A dependency on SPE pH was observed for certain substances. For instance, the ILIS corrected recovery for propranolol and trimethoprim was enhanced at pH 2, while for carbamazepine-epoxide and metformin it was reduced. Notably, ciprofloxacin was overestimated when using acidified conditions, which may be attributed to pH-induced molecular conformation changes. Ciprofloxacin has a zwitterionic nature and exists in cation, zwitterion, and/or anion species under different pH conditions (see **Fig. S3**). We postulate that the acidification of the SPE process to pH 2 charged the cationic amine moiety positively, resulting in an increased number of ions entering the MS. The dependency of substances with a zwitterionic nature on pH has also been reported by other authors (Rossmann et al., 2014).

Regarding the optimal SPE conditions, 12 of 17 compounds were recovered at >75% and <125% in tested conditions 1, 2 and 4 based on the ILIS correction (**Table 5**). Using absolute recoveries (**Table 4**), condition 2 was found to be most effective (>75% recovery for 10 compounds with HLB Prime). The ILIS corrected values (**Table 5**) were generally in agreement with the absolute recoveries (**Table 4**), with the enhancement of recoveries (**Table 5**) in conditions 1 and 4 suggesting the ILIS correction appropriately ensured successful quantification by compensating for losses of compounds.

The 'optimal' SPE condition that provided the best recovery for each compound varied due to the variety of physico-chemical properties represented in the priority list. For most target compounds, condition 2 was found the most effective based on the high values of both absolute and ILIS corrected recoveries, therefore was selected for further study. Meanwhile, low recovery was noted for certain substances (metformin, ciprofloxacin and propranolol <35%) in this condition. To reach a compromise, that gives an acceptable recovery for most compounds with the least loss, condition 1, retaining 16 out of 17 compounds, with the exception of ciprofloxacin, was also selected for further investigation.

Although quantitation with ILIS assured sufficient recoveries, under certain circumstances, the use of ILIS can be a complicated approach for analytes from a diverse range of chemical classes (Gracia-Lor et al., 2011). Quantitation with ILIS needs to be well characterised when it does not ensure an adequate correction. For instance, undesirable enhancement of ILIS recovery was observed for paracetamol while satisfactory absolute values (72-99%) were obtained under tested conditions. Similar inadequate ILIS recovery was found for ibuprofen. This was attributed to the mass loss of its ILIS analogue not coinciding with the analyte under the same conditions so that the ILIS calculation exaggerated the process efficiency, making the ILIS correction unnecessary (Marín et al., 2009; Renew and Huang, 2004). Therefore, the absolute recoveries of paracetamol and ibuprofen have been adopted for evaluation.

2.3 Matrix effect study

The influence of environmental matrix on accurate quantitative LC-MS/MS analysis has been widely discussed (Frigerio et al., 2019; Fu et al., 2018; Huang et al., 2020). Non-target components present in samples can have a significant impact on analyte recovery and ionisation which may deplete or enhance MS signal intensity and thus affect accurate quantification (Irlam et al., 2019; Meerpoel et al., 2018; Tran et al., 2020). The assessment of matrix effect has been conducted in a number of approaches during the development of quantitative analytical method, the most commonly used one may refer to the "absolute" matrix effect, comparing the signal response of a standard present in an extract containing co-eluting components to the response of a standard in a "not contaminated" neat solvent (Matuszewski et al., 2003). Although the presence of this absolute matrix effect (which is often obtained by a comparison of the response of analyte spiked after extraction to the

response in the neat solution) is of some concern, the more important parameter in the evaluation of an analytical method is the demonstration of the absence of a "relative" matrix effect in different sources of environmental water matrices. To validate the overall performance of the analytical method in this study, the effects of water matrices were evaluated by comparing recoveries of analytes in different water matrices (spiked before extraction). The suppression or enhancement of recoveries in **Table 6** demonstrated the overall effects of matrices (undetected coeluting components reacting with primary ions formed in the HPLC−MS/MS interface) and recoveries (competition with matrix components, which can largely be compensated by isotope-labeled internal standards) from different water sources. All values presented were corrected using ILIS, except for paracetamol and ibuprofen, where absolute recoveries are given (due to inadequate ILIS correction as discussed above).

With a number of exceptions, fairly limited effects of matrix were observed for many of these pharmaceuticals and EDCs, which is consistent with previous findings (Cha et al., 2006; Tong et al., 2009; Tuc Dinh et al., 2011). Some effects were noted for 6 compounds, with >50% recovery suppression for two (atorvastatin and ibuprofen), ~20-40% suppression for three (metformin, paracetamol and clarithromycin) and <20% enhancement for trimethoprim. This was likely due to ion suppression in the MS ESI source due to matrix components (Gómez et al., 2006; Kasprzyk-Hordern et al., 2008). The lack of ILIS correction for paracetamol and ibuprofen likely made these effects more obvious and meant effective correction could not be achieved. For atorvastatin, its high Log K_{ow} (6.36) suggests the compound would tend to bind with organic matter present in water – and the SPE process presumably failed to overcome this. For several analytes (e.g., E3, paracetamol, trimethoprim, clarithromycin), a filtered river water matrix resulted in lower recovery versus unfiltered, indicating no filtration is beneficial to remain pharmaceutical compounds when recovering them from environmental water matrices. This may be attributed to the pharmaceutical analytes sorbed onto suspended particular matter present in the river samples, which was then removed during membrane filtration, causing the concentrations of freely dissolved analytes to be lower for further detection. The co-extracting components in river water matrix may also mask the analyte peaks by raising the chromatogram baseline, leading to underestimated integrated peak areas. Meanwhile, the co-extracting matrix may reduce ionisation efficiency of the analytes by taking up some of the limited number of excess charged sites on the surfaces of

electrosprayed droplets (Gómez et al., 2006).This is consistent with other studies and may suggest that analysing samples without filtration may sometimes be more appropriate (depending on the analytes concerned and aims of the study) (Berset and Ochsenbein, 2012; Tran et al., 2013). For filtered river samples, methanol elution provided better recoveries for most target compounds in this study. In terms of limits of quantification (LOQs) calculated when processing 1 L of water - these were in the range of 0.07 $ng \cdot L^{-1}$ to 9.07 $ng \cdot L^{-1}$ (as shown in **Table 6**). For 14 out of 17 compounds (excluding ibuprofen, ciprofloxacin and E3), method LOQs were 0.07 $ng \cdot L^{-1}$ to 1.88 $ng \cdot L^{-1}$, which is somewhat lower than those previously reported in other studies (Choi et al., 2007; Ding et al., 2009; Tuc Dinh et al., 2011).

Table 6. Recoveries of prioritised pharmaceuticals and EDCs in different water matrices (using ILIS correction, except for paracetamol and ibuprofen)

Recoveries % and (±%RSD)		Analytes detected in -ve mode							Analytes detected in +ve mode										No. >75% and <125%
		IBU	DCF	E3	E2	EE2	E1	TCS	MET	PARA	CFX	TMP	CBZE	PPL	CBZ	CTM	FLX	ATV	
Applied ILIS			DCF-D4	E1-D2				TCS-D3	TMP-D9		TMP-D9		CBZ-D10			RTM	FLX-D5		
MEOH	Milli-Q	71 (±10)	92 (±3)	112 (±8)	103 (±6)	96 (±5)	97 (±3)	92 (±4)	102 (±26)	93 (±2)	4 (±1)	117 (±3)	95 (±1)	63 (±0)	88 (±1)	126 (±4)	85 (±1)	113 (±5)	13
	Tap Water	32 (±3)	100 (±1)	129 (±9)	116 (±5)	115 (±21)	95 (±1)	96 (±0)	47 (±11)	51 (±5)	26 (±10)	137 (±2)	112 (±3)	60 (±4)	101 (±2)	105 (±9)	100 (±3)	43 (±0)	9
	River water Unfiltered	5 (±4)	99 (±2)	112 (±14)	98 (±10)	102 (±15)	96 (±3)	109 (±1)	44 (±2)	62 (±3)	6 (±5)	131 (±8)	110 (±1)	65 (±6)	97 (±3)	108 (±3)	93 (±4)	14 (±5)	10
	River water Filtered	14 (±5)	100 (±6)	81 (±2)	103 (±24)	104 (±20)	96 (±0)	107 (±2)	60 (±1)	55 (±14)	10 (±3)	124 (±1)	111 (±1)	73 (±1)	97 (±1)	89 (±4)	95 (±1)	55 (±4)	11
ACE:EAC	Milli-Q	90 (±6)	94 (±1)	96 (±6)	99 (±4)	93 (±3)	96 (±3)	99 (±3)	31 (±7)	99 (±2)	2 (±1)	113 (±2)	99 (±1)	29 (±7)	90 (±0)	135 (±16)	82 (±5)	123 (±7)	13
	Tap Water	40 (±2)	97 (±1)	96 (±4)	99 (±2)	92 (±12)	90 (±4)	105 (±4)	2 (±1)	67 (±5)	1 (±0)	130 (±7)	101 (±2)	21 (±5)	96 (±2)	103 (±5)	94 (±5)	6 (±3)	10
	River water Unfiltered	13 (±4)	97 (±1)	96 (±23)	98 (±6)	93 (±4)	98 (±3)	106 (±2)	3 (±0)	57 (±9)	1 (±2)	121 (±8)	107 (±3)	33 (±9)	88 (±0)	107 (±5)	97 (±2)	4 (±2)	11
	River water Filtered	13 (±1)	105 (±2)	81 (±6)	97 (±1)	98 (±5)	95 (±1)	100 (±4)	3 (±1)	56 (±6)	1 (±0)	119 (±32)	112 (±8)	39 (±11)	92 (±4)	104 (±2)	103 (±6)	57 (±0)	11
Method LOQ ($ng \cdot L^{-1}$)		9.07	0.78	4.48	1.31	1.88	0.66	1.61	0.27	0.69	4.46	0.08	0.09	0.39	0.11	0.07	0.10	0.70	

Where clearest reductions in recovery are evident (i.e., matrix effects most likely), these have been shaded grey for ease of noting (>50% dark grey; 20-40% light grey; reduction owing to suspended particulate matter- medium grey). .Metformin MET; Paracetamol PARA; Trimethoprim TMP; Ciprofloxacin CFX; Carbamazepine-10-11-epoxide CBZ; Propranolol PPL; Carbamazepine CBZ; Clarithromycin CTM; Fluoxetine FLX; Atorvastatin ATV; Ibuprofen IBU; Diclofenac DCF; Estriol E3; 17β-Estradiol E2; 17α-ethynylestradiol EE2; Estrone E1; Triclosan TCS. Trimethoprim-D9 TMP-D9; Carbamazepine-D10 CBZ-D10; Roxythromycin RTM; Fluoxetine-D5 FLX-D5; Diclofenac-D4 DCF-D4; Estrone-D2 E1-D2; Triclosan-D3 TCS-D3. Methanol MEOH; acetone ACE; ethyl acetate EAC.

2.4 Analysis of real water samples

To validate the applicability of this method, it was applied to identify and quantify 8 priority pharmaceuticals and EDCs in various real water samples (to fit in the target of the hospital monitoring project – possible detected analytes based on local description data). Given the matrix effects observed in testing, an additional ILIS (paracetamol-D4) was applied (relevant recovery data was provided in Supporting information **Table S3**). Monitoring results are presented in **Table 7**.

Table 7. Summary of the field monitoring results obtained for 8 target compounds (ng·L^{-1}) in real water samples. Samples collected from a combined rural hospital discharge (Wick General, Scotland), and, the influent and effluent from Wick municipal WWTP

Compound	Hospital discharge (n = 20)		Wastewater influent (n = 20)		Wastewater effluent (n = 20)	
	Detection frequency (%)	Mean (range)	Detection frequency (%)	Mean (range)	Detection frequency (%)	Mean (range)
Paracetamol	100	33,267 (7,959-105,910)	100	67,483 (5,849-105,780)	100	8,567 (516-36,201)
Trimethoprim	85	818 (<LOD-9,111)	100	621 (155-2,170)	84	440 (<LOD-634)
Carbamazepine	100	13 (3-47)	100	306 (40-684)	100	459 (212-709)
Clarithromycin	45	1,271 (<LOD-7,940)	57	246 (<LOD-830)	100	371 (60-836)
Fluoxetine	32	16 (<LOD-37)	26	19 (<LOD-46)	15	16 (<LOD-29)
Ibuprofen	45	139 (<LOD-675)	100	471 (5-6,018)	73	73 (<LOD-178)
Diclofenac	75	77 (<LOD-593)	63	196 (<LOD-392)	36	102 (<LOD-250)
EE2	0	<LOD	0	<LOD	0	<LOD

Beyond those sites shown in **Table 7**, no target pharmaceuticals were detected (>LOQ) in the surface source water or the treated hospital drinking water supply tested. Likewise, EE2 was never found (< LOQ = 1.88 ng·L^{-1}), the method LOD standard of which has been updated to 0.035 ng·L^{-1} based on the Commission Implementing Decision (European Commission, 2018), suggesting the challenge and necessity of improving the analytical methodology to monitor such compounds at lower concentrations. In the hospital discharge, all the targeted pharmaceuticals were detected except EE2, with paracetamol and carbamazepine detected in every sample. The highest concentrations were recorded for paracetamol, with a maximum of 105,910 ng·L^{-1}, followed by trimethoprim (9,111 ng·L^{-1}) and clarithromycin (7,940 ng·L^{-1}). Regarding the WWTP wastewater influent tested, the highest levels were noted for paracetamol (105,780 ng·L^{-1}) and ibuprofen (6,018 ng·L^{-1}). The increased mean detection level of paracetamol (33267 ng·L^{-1} to 67483 ng·L^{-1}) and ibuprofen (139 ng·L^{-1} to 471 ng·L^{-1}) between the

hospital discharge and wastewater influent indicated the possible presence of other inputting sources of such pharmaceuticals besides the hospital discharge. Lower levels of trimethoprim (818 $ng \cdot L^{-1}$ to 621 $ng \cdot L^{-1}$) and clarithromycin (1271 $ng \cdot L^{-1}$ to 246 $ng \cdot L^{-1}$) in WWTP influent versus the hospital discharge may be attributed to the degradation and/or dilution in the aquatic environment between those two sites (Gracia-Lor et al., 2011). Higher levels of carbamazepine and ibuprofen may reflect greater (human) intakes in the community versus the hospital. In terms of the final WWTP effluent, all the previously detected pharmaceuticals remained detectable – albeit at reduced levels in some cases. Five of the pharmaceuticals monitored were at lower mean levels in discharge versus influent – but, two (carbamazepine, clarithromycin) were more elevated in discharge water. These results reinforce the need to apply multiclass pharmaceutical monitoring methods in order to gain a better understanding of the fate/behaviour of these compounds at the catchment scale. Likewise, they highlight the ongoing need to create WWTP processes that can efficiently eliminate these bioactive pollutants of concern.

Compared to levels reported in other European countries for these target compounds (Gros et al., 2010; Gros et al., 2007; López-Serna et al., 2011), the surface water data collected here demonstrated how relatively 'pristine' source water can be in the Scottish Highlands (in a remote inland lake, currently entirely 'free' of these contaminants). However, the WWTP concentrations seen here (both influent and effluent) were highly comparable with data from Germany, Belgium and the US (Cahill et al., 2004; Gurke et al., 2015a; Rossmann et al., 2014; Vergeynst et al., 2015). This clearly highlights the impact that pharmaceutical consumption is and can have – even in remote and otherwise pristine hydrological systems.

3. Conclusion

A sensitive analytical methodology for the simultaneous determination of up to 17 priority pharmaceuticals and EDCs was developed and validated using an optimised SPE protocol and HPLC-ESI-MS/MS detection. A risk-based approach was applied to identify compounds that may pose the greatest environmental concern. The diversity of analytes selected meant that some compromises were needed when applying this analysis (i.e., accepting reduced recovery for certain compounds). The optimal SPE protocol used Oasis HLB Prime cartridges with no pH adjustment and elution with methanol. The use of ILIS improved the reliability of the entire process and helped evaluation of matrix effects.

Application of the method to 'real' environmental samples from a rural catchment in Scotland, illustrated the occurrence of pharmaceuticals in various wastewater matrices. The highest concentrations found were for paracetamol, with a mean level of 67,483 $ng \cdot L^{-1}$ in municipal WWTP influent. The successful application of this method to real water matrices validated its applicability within routine monitoring studies regarding these priority pharmaceutical and EDC contaminants.

Appendix A. Supplementary material

Supplementary data associated with this article is present in the Supporting Information.

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