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

3

4 Yuan Li^{1,3}, Mark A. Taggart¹, Craig McKenzie², Zulin Zhang³, Yonglong Lu⁴, Sabolc
5 Pap^{1,5}, Stuart Gibb¹

6

7 1. Environmental Research Institute, North Highland College, University of the
8 Highlands and Islands, Castle Street, Thurso, Caithness, Scotland, KW14 7JD,
9 UK. E-mail: Yuan.Li2@uhi.ac.uk

10 2. Forensic Drug Research Group, Centre for Anatomy and Human Identification,
11 School of Science and Engineering, University of Dundee, Nethergate, Dundee,
12 DD1 4HN, UK.

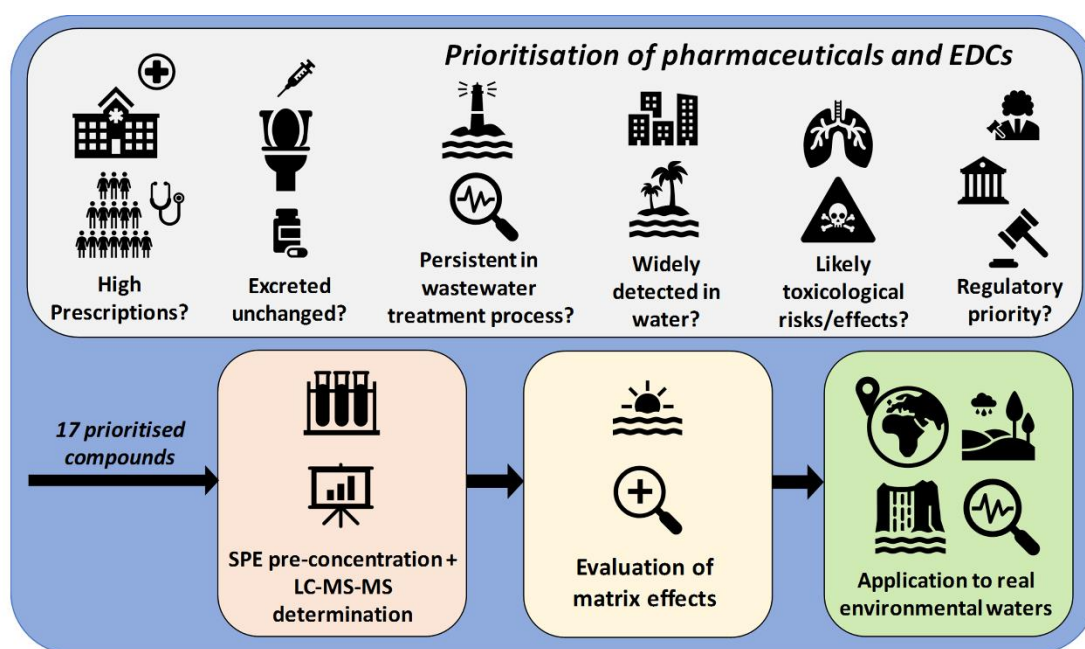
13 3. Environmental and Biochemical Sciences Group, James Hutton Institute,
14 Craigiebuckler, Aberdeen, AB15 8QH, UK.

15 4. Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences,
16 18 Shuangqing Road, Haidian District, Beijing, 100085, China.

17 5. University of Novi Sad, Faculty of Technical Sciences, Department of
18 Environmental Engineering and Occupational Safety and Health, University of
19 Novi Sad, 21000 Novi Sad, Serbia.

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

43 **Keywords:**

44 Pharmaceuticals

45 Prioritisation

46 risk assessment

47 trace level determination

48 water quality

49

50 ***Corresponding author.** E-mail: Yuan.Li2@uhi.ac.uk (Yuan Li, Environmental

51 Research Institute, Castle Street, Thurso, Scotland, UK, KW14 7JD

52 **Introduction**

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

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

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

85 relevant levels. The most common analytical approach to determine pharmaceuticals and
86 EDC levels in aqueous samples first involves a pre-concentration step (i.e., using solid
87 phase extraction; SPE), and then the use of liquid chromatography with mass
88 spectrometric detection (LC-MS) (Buchberger, 2011; Hong et al., 2019; Peng et al., 2019).
89 However, many methods focus on compounds that are simply most commonly found (i.e.,
90 in water), or, that belong to specific drug classes, i.e., antibiotics (Gurke et al., 2015a,
91 2015b; Rossmann et al., 2014; Scheurer et al., 2009). As such, there remains a need to
92 develop techniques specifically focussed on those substances thought to pose the greatest
93 risk potential within the aquatic environment. Such methods can be informed by existing
94 prioritisation systems such as those which have led to the creation of “Watch Lists” within
95 the EU Water Framework Directive (WFD, EU) and the UK’s Chemical Investigation
96 Program (CIP, UK) (European commission, 2015; UKWIR, 2015). Such regulatory
97 indicators act to highlight those compounds thought to be of most concern and/or
98 requiring more detailed research.

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

109 **1. Methodologies and chemicals**

110 **1.1 Chemicals and reagents**

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

117 individual stock solutions, in 50:50 v/v methanol:Milli-Q[®] water. All solutions were
118 stored in amber glass vials at -20°C in the dark.

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

124 **1.2 Instrumentation**

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

139 **1.3 Sample preparation**

140 SPE was employed for sample enrichment and clean-up, and several stationary phases
141 were tested under a range of elution conditions to optimise compound recovery (see [Fig.
142 S1 for schematic of the process](#)). All SPE experiments were conducted in triplicate, using
143 20 mL of Milli-Q water spiked to a starting concentration of 10 µg·L⁻¹ for each analyte
144 (ultimately 500 µg·L⁻¹ in final extract/following the SPE process, assuming 100%
145 recovery). For the final protocol, SPE cartridges were preconditioned with methanol (6
146 mL) and then Milli-Q water (6 mL), both at a flow rate of 1 mL·min⁻¹. 20 mL spiked
147 water samples were passed through the cartridges at a flow rate of 1 mL·min⁻¹ and then
148 cartridges were rinsed with Milli-Q water once (1 mL). Cartridges were then dried under

149 vacuum for >30 min to remove excess water. Then, the analytes were eluted with two
150 consecutive 6 mL elution's using methanol (MEOH), or, acetone (ACE) and ethyl acetate
151 (EAC) at 50:50 v/v (depending on desired recoveries for certain compounds), at 1
152 mL·min⁻¹. The eluates were then evaporated under a gentle stream of high purity nitrogen
153 at 40°C until they were almost dry, then reconstituted with 0.4 mL of 50:50 v/v
154 MEOH:Milli-Q. Absolute recoveries were determined compared to quality-control (QC)
155 standards of 500 µg·L⁻¹.

156 **1.4 Method quantification**

157 Compound selectivity was verified by measuring two MRM transitions per analyte.
158 Calibration linearity was studied by analysing standards in triplicate at nine
159 concentrations in the range from 2 to 500 µg·L⁻¹. Satisfactory linearity using weighed
160 (1/x) least squares regression was assumed when the correlation coefficient (R^2) was >
161 0.99. Method accuracy and precision (expressed as recovery and repeatability, using
162 relative standard deviation) were studied with recovery experiments (using Milli-Q water
163 spiked with analytes). Instrumental limits of detection (LOD) for each compound were
164 determined as the minimum detectable amount of analyte giving a signal-to-noise (S/N)
165 ratio of 3 (using the quantification transition).

166 For the investigation regarding matrix effects, a known amount of analyte (10 µg·L⁻¹) and
167 ILIS (1 µg·L⁻¹) was added to tap water and river water (filtered and unfiltered). Taking
168 into account an enrichment factor of 50 (whereby 20 mL of water sample was
169 reconstituted into 0.4 mL for analysis following SPE), quality-control (QC) standards of
170 500 µg·L⁻¹ (for the analytes) and 50 µg·L⁻¹ (for the ILIS) were then used for quantification.

171 **1.5 Application to real samples**

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

181 concentration in 1 L of sample). SPE cartridges were preconditioned with MEOH and
182 Milli-Q water, then 1L water samples were passed through the cartridges at a flow rate of
183 1 mL·min⁻¹. The SPE extract was eluted with 2×6 mL MEOH and reconstituted with 0.5
184 mL of 50:50 v/v MEOH:Milli-Q, leading to an enrichment factor of 2,000 and a final
185 concentration of 100 µg·L⁻¹ ILIS in the analysed sample. Quantification was made using
186 external QC standards and calibration standards, with recovery assessed based on relative
187 responses. To ensure the precision and accuracy of the data required, all targeted
188 compounds in real water analysis have been assigned with their own ILIS standards to
189 correct possible quantification errors. All samples were stored at 4°C in the dark until SPE
190 extraction, which was performed within 48 hr of sample collection.

191 **2. Results and Discussion**

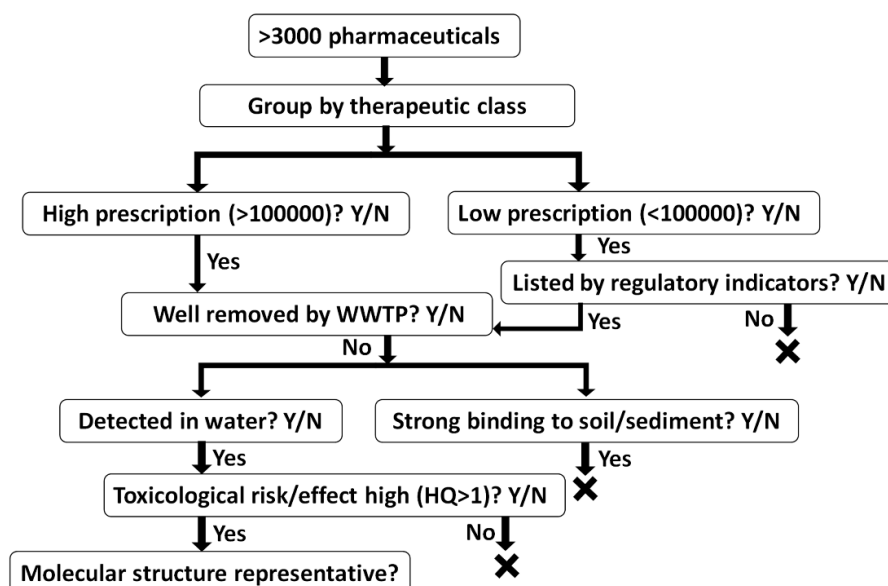
192 **2.1 Prioritisation of target compounds**

193 As there are > 3,000 pharmaceuticals registered for use in the European Union (EU), it is
194 necessary to prioritise these whilst accounting for risk (Boxall et al., 2012). Many
195 prioritisation schemes have been proposed in recent years, commonly based on
196 consumption data, environmental occurrence and/or toxicological effects (Kötke et al.,
197 2019; Li et al., 2020; Mansour et al., 2016; Pereira et al., 2016; Roos et al., 2012).
198 However, many field monitoring studies still focus on compounds that are most
199 commonly found in water (López-Serna et al., 2011; Rossmann et al., 2014) – many of
200 which may (or may not) be likely to elicit toxicological effects.

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

212 organisms/ecosystem function is expected) (Booth et al., 2020). When the $HQ \geq 1$, a high
213 risk of adverse effects is expected (De Souza et al., 2009; Ccaccapa et al., 2016).

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



224

225

Fig.1. Decision tree of compound prioritisation

226 Here, the first step in our prioritisation process was to consider prescription rates within
227 Scotland. As shown in Fig.1, pharmaceuticals were first grouped by therapeutic class,
228 and within each class, compounds prescribed >100,000 times per year (ISD Scotland,
229 2016) were highlighted. Substances prescribed below this value were only highlighted if
230 they had been listed as existing priorities within the EU’s WFD Watch List(s) and/or the
231 UK’s CIP Programme. Analytes highlighted were then evaluated further by considering
232 WWTP removal efficiency, reported environmental concentrations (in water) and
233 toxicological risk. Combining the reviewed range of pharmaceutical and EDC monitoring
234 data (MEC) and their PNEC values, the risk scores were calculated to characterize

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

240 The prioritisation selection criteria applied here were, in summary: a) prescription
241 statistics ([ISD Scotland, 2016](#)); b) legislative indicators, i.e., WFD ‘Watch List’ and/or
242 UK CIP listed; c) removal efficiency in WWTP; d) environmental occurrence in water;
243 e) biological toxic effects (informed by HQ calculated with PNEC and MEC); and f)
244 physico-chemical properties. **Table 1** shows a summary regarding the prioritised list of
245 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·L ⁻¹)	PNEC ² (ng·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 \(supporting information\)](#).

246 Ultimately, 17 compounds were identified as priority substances here, belonging to a wide
247 range of compound classes (11), i.e., antibiotics, antimicrobials, analgesics, non-steroidal anti-
248 inflammatory drugs, psychoactive drugs, β -blockers, blood lipid regulators, antidiabetics, anti-
249 ulcer agents and estrogens (as well as associated metabolites). Fifteen compounds were
250 associated with high potential risk ($HQ > 1$) within the aqueous environment, including
251 ibuprofen, diclofenac, paracetamol, trimethoprim, E2, ciprofloxacin, fluoxetine, EE2,
252 carbamazepine, E1, propranolol, metformin, clarithromycin, atorvastatin and triclosan (in HQ
253 value order from high to low). This largely aligns with key legislative indicators (given these
254 were also one of our criteria), with the only pharmaceutical compounds in addition to CIP/WFD
255 indicators being trimethoprim and paracetamol. These two compounds have been highlighted
256 as their current occurrence levels outstrip its known toxicologically relevant concentrations
257 (PNEC) as shown in **Table 1**, the high HQ scores of trimethoprim (56) and paracetamol (65)
258 indicated that the adverse effects on non-target organisms may occur in the aquatic environment.
259 Trimethoprim is the second most commonly prescribed antibiotic in Scotland, and reports show
260 that up to 80% of this is excreted unmetabolised by the human body ([De Liguoro et al., 2012](#);
261 [Kasprzyk-Hordern et al., 2009](#)). It has been found to be resistant to the biological wastewater
262 treatment ([Lindberg et al. 2006](#)), one of the most frequently occurring antibiotics found in UK
263 wastewaters, being detected in 65% of effluent samples with a maximum concentration of 1,300
264 $\text{ng}\cdot\text{L}^{-1}$ ([Ashton, Hilton & Thomas 2004](#)). Similarly, paracetamol is one of the most commonly
265 prescribed drugs globally, due to its antipyretic and analgesic properties. Even though the
266 reported removal efficiencies in WWTPs are relatively high (up to 90%), it is often found at
267 high levels in the aquatic environment (e.g., maximum 10,000 $\text{ng}\cdot\text{L}^{-1}$ in US natural waters and
268 at 65,000 $\text{ng}\cdot\text{L}^{-1}$ in the River Tyne, UK) ([Kolpin et al., 2002](#); [Roberts and Thomas, 2006](#)). Such
269 high levels of paracetamol continuously introduced into the aquatic environment have been
270 found to cause negative ecological effects in various wild organisms ([Nunes et al., 2014](#)), the
271 high HQ scores of these compounds in this study reinforced the necessity of further
272 investigation of such pollutants.

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

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

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

306 **2.2 Detection method development**

307 **2.2.1 HPLC separation and MS/MS optimisation**

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

310 various concentrations) were tested as mobile phases. MS parameters were optimised to attain
311 maximum sensitivity and selectivity. Of the 17 substances, 10 showed a higher response using
312 the protonated $[M+H]^+$ ions and positive ion (PI) mode while 7 were better using negative mode
313 (detecting the deprotonated $[M-H]^-$ ions). For both modes, several HPLC columns and various
314 operational parameters/gradient designs (i.e., different flow rates and slopes) were tested in
315 order to optimise peak separation, signal response and minimise run time. Good peak shape and
316 sensitivity were achieved in PI mode using a reverse-phase Waters XBridge BEH C18 column
317 (2.1 mm I.D. x 100 mm, 2.5 μ m) with 0.1% formic acid as the aqueous phase and acetonitrile
318 at 45°C. For the 7 NI compounds, sufficient separation was obtained using 0.025% ammonium
319 hydroxide (in water) and acetonitrile and a Phenomenex Kinetex EVO C18 column (3.0 mm
320 I.D x 100 mm, 2.6 μ m) at 25°C. The optimised gradient elution programs used are shown in
321 **Table S2 (Supporting Information)** alongside representative chromatograms for pure standard
322 mixtures monitored in both modes (**Fig. S2**). Optimised mass spectrometry parameters,
323 precursor and product ions, retention times (RT) and instrumental LODs in both PI and NI
324 modes are summarised in **Table 2 and 3**, respectively.

325

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

Compound	Mol. Weight (g·mol ⁻¹)	Precursor ion	CV ¹ (V)	Product ions	CE ² (eV)	RT ³ (min)	Corresponding ILIS	Molecular structure	LODs ⁴ (µg·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
Paracetamol-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·mol ⁻¹)	Precursor ion	CV ¹ (V)	Product ion	CE ² (eV)	RT ³ (min)	Corresponding ILIS	Molecular structure	LODs ⁴ (µg·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**

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

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

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

No.	Recoveries % and (\pm %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 (\pm 17)	67 (\pm 4)	38 (\pm 10)	36 (\pm 6)	41 (\pm 5)	44 (\pm 6)	34 (\pm 2)	45 (\pm 16)	90 (\pm 6)	2 (\pm 0)	38 (\pm 1)	59 (\pm 1)	39 (\pm 3)	51 (\pm 1)	49 (\pm 6)	35 (\pm 0)	10 (\pm 1)	1
		HLB Prime	71 (\pm 10)	83 (\pm 3)	65 (\pm 4)	75 (\pm 4)	87 (\pm 0)	56 (\pm 10)	67 (\pm 11)	74 (\pm 6)	93 (\pm 2)	2 (\pm 0)	56 (\pm 1)	90 (\pm 1)	68 (\pm 4)	83 (\pm 2)	45 (\pm 1)	45 (\pm 5)	12 (\pm 2)	6
2	ACE:EAC	HLB	56 (\pm 5)	77 (\pm 2)	37 (\pm 10)	33 (\pm 14)	40 (\pm 13)	49 (\pm 3)	59 (\pm 2)	11 (\pm 12)	90 (\pm 4)	3 (\pm 3)	38 (\pm 2)	65 (\pm 7)	6 (\pm 1)	52 (\pm 3)	26 (\pm 5)	1 (\pm 0)	11 (\pm 3)	2
		HLB Prime	90 (\pm 6)	91 (\pm 2)	97 (\pm 3)	111 (\pm 9)	101 (\pm 3)	107 (\pm 4)	98 (\pm 8)	11 (\pm 3)	99 (\pm 2)	1 (\pm 0)	42 (\pm 1)	98 (\pm 0)	28 (\pm 6)	102 (\pm 4)	26 (\pm 7)	0 (\pm 0)	27 (\pm 5)	10
3	pH 2 MEOH	HLB	29 (\pm 2)	30 (\pm 3)	29 (\pm 72)	30 (\pm 1)	38 (\pm 1)	53 (\pm 0)	53 (\pm 3)	3 (\pm 1)	72 (\pm 2)	195 (\pm 21)	34 (\pm 1)	5 (\pm 1)	47 (\pm 1)	49 (\pm 0)	26 (\pm 3)	45 (\pm 0)	10 (\pm 4)	0
		HLB Prime	43 (\pm 7)	46 (\pm 10)	63 (\pm 7)	66 (\pm 8)	63 (\pm 3)	104 (\pm 7)	77 (\pm 4)	3 (\pm 0)	96 (\pm 4)	145 (\pm 6)	74 (\pm 9)	22 (\pm 2)	81 (\pm 1)	85 (\pm 0)	27 (\pm 6)	84 (\pm 5)	11 (\pm 4)	6
4	pH 2 ACE:EAC	HLB	57 (\pm 1)	36 (\pm 1)	27 (\pm 3)	29 (\pm 4)	37 (\pm 5)	49 (\pm 0)	62 (\pm 10)	2 (\pm 0)	81 (\pm 1)	36 (\pm 11)	42 (\pm 8)	20 (\pm 1)	40 (\pm 8)	54 (\pm 6)	9 (\pm 1)	32 (\pm 2)	6 (\pm 1)	1
		HLB Prime	58 (\pm 6)	63 (\pm 0)	73 (\pm 1)	77 (\pm 4)	75 (\pm 3)	121 (\pm 19)	91 (\pm 8)	1 (\pm 1)	88 (\pm 6)	144 (\pm 2)	56 (\pm 0)	25 (\pm 1)	91 (\pm 4)	94 (\pm 11)	14 (\pm 1)	46 (\pm 1)	14 (\pm 3)	7

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

No	Recoveries % and (\pm %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			CBZ-D10			RTM	FLX-D5			
1	MEOH	HLB	48 (\pm 21)	77 (\pm 3)	66 (\pm 13)	60 (\pm 5)	56 (\pm 4)	88 (\pm 2)	101 (\pm 6)	49 (\pm 5)	64 (\pm 1)	1 (\pm 0)	51 (\pm 6)	94 (\pm 10)	60 (\pm 2)	85 (\pm 4)	129 (\pm 4)	45 (\pm 1)	39 (\pm 3)	5
		HLB Prime	64 (\pm 6)	92 (\pm 3)	112 (\pm 8)	103 (\pm 6)	96 (\pm 5)	97 (\pm 3)	92 (\pm 4)	102 (\pm 26)	210 (\pm 19)	4 (\pm 1)	117 (\pm 3)	95 (\pm 1)	63 (\pm 0)	88 (\pm 1)	126 (\pm 4)	85 (\pm 1)	113 (\pm 5)	12
2	ACE:EAC	HLB	69 (\pm 5)	77 (\pm 1)	77 (\pm 4)	91 (\pm 10)	104 (\pm 7)	91 (\pm 10)	97 (\pm 1)	3 (\pm 0)	85 (\pm 18)	3 (\pm 2)	45 (\pm 9)	126 (\pm 7)	3 (\pm 0)	89 (\pm 0)	127 (\pm 11)	83 (\pm 24)	80 (\pm 17)	10
		HLB Prime	80 (\pm 2)	94 (\pm 1)	96 (\pm 6)	99 (\pm 4)	93 (\pm 3)	96 (\pm 3)	99 (\pm 3)	31 (\pm 7)	227 (\pm 6)	2 (\pm 1)	113 (\pm 2)	99 (\pm 1)	29 (\pm 7)	90 (\pm 0)	135 (\pm 16)	82 (\pm 5)	123 (\pm 7)	12
3	pH 2 MEOH	HLB	56 (\pm 3)	77 (\pm 0)	46 (\pm 2)	57 (\pm 2)	83 (\pm 11)	71 (\pm 4)	93 (\pm 2)	1 (\pm 0)	40 (\pm 2)	62 (\pm 4)	95 (\pm 2)	3 (\pm 1)	77 (\pm 8)	80 (\pm 3)	104 (\pm 4)	95 (\pm 1)	9 (\pm 0)	8
		HLB Prime	147 (\pm 28)	101 (\pm 2)	66 (\pm 6)	83 (\pm 4)	92 (\pm 1)	103 (\pm 5)	110 (\pm 1)	4 (\pm 2)	126 (\pm 18)	221 (\pm 5)	134 (\pm 6)	33 (\pm 3)	116 (\pm 3)	120 (\pm 3)	133 (\pm 2)	95 (\pm 0)	67 (\pm 16)	8
4	pH 2 ACE:EAC	HLB	79 (\pm 6)	80 (\pm 1)	64 (\pm 7)	74 (\pm 9)	100 (\pm 3)	67 (\pm 17)	103 (\pm 4)	1 (\pm 0)	63 (\pm 4)	61 (\pm 9)	92 (\pm 7)	21 (\pm 0)	79 (\pm 1)	86 (\pm 2)	111 (\pm 0)	88 (\pm 2)	10 (\pm 1)	9
		HLB Prime	161 (\pm 13)	101 (\pm 1)	98 (\pm 25)	117 (\pm 46)	116 (\pm 36)	99 (\pm 14)	107 (\pm 2)	2 (\pm 1)	98 (\pm 5)	186 (\pm 11)	110 (\pm 1)	28 (\pm 4)	109 (\pm 2)	102 (\pm 1)	130 (\pm 8)	95 (\pm 2)	114 (\pm 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; Estradiol E3; 17 β -Estradiol E2; 17 α -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.

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

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

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

384 Regarding the optimal SPE conditions, 12 of 17 compounds were recovered at >75% and
385 <125% in tested conditions 1, 2 and 4 based on the ILIS correction (**Table 5**). Using
386 absolute recoveries (**Table 4**), condition 2 was found to be most effective (>75% recovery
387 for 10 compounds with HLB Prime). The ILIS corrected values (**Table 5**) were generally
388 in agreement with the absolute recoveries (**Table 4**), with the enhancement of recoveries
389 (**Table 5**) in conditions 1 and 4 suggesting the ILIS correction appropriately ensured
390 successful quantification by compensating for losses of compounds.

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

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

411 **2.3 Matrix effect study**

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

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

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

456 electrosprayed droplets (Gómez et al., 2006). This is consistent with other studies and may
457 suggest that analysing samples without filtration may sometimes be more appropriate
458 (depending on the analytes concerned and aims of the study) (Berset and Ochsenbein,
459 2012; Tran et al., 2013). For filtered river samples, methanol elution provided better
460 recoveries for most target compounds in this study. In terms of limits of quantification
461 (LOQs) calculated when processing 1 L of water - these were in the range of $0.07 \text{ ng}\cdot\text{L}^{-1}$
462 to $9.07 \text{ ng}\cdot\text{L}^{-1}$ (as shown in **Table 6**). For 14 out of 17 compounds (excluding ibuprofen,
463 ciprofloxacin and E3), method LOQs were $0.07 \text{ ng}\cdot\text{L}^{-1}$ to $1.88 \text{ ng}\cdot\text{L}^{-1}$, which is somewhat
464 lower than those previously reported in other studies (Choi et al., 2007; Ding et al., 2009;
465 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·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; Estrone 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.

466 2.4 Analysis of real water samples

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

473 **Table 7.** Summary of the field monitoring results obtained for 8 target compounds (ng·L⁻¹) in
 474 real water samples. Samples collected from a combined rural hospital discharge (Wick General,
 475 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

476 Beyond those sites shown in **Table 7**, no target pharmaceuticals were detected (>LOQ)
 477 in the surface source water or the treated hospital drinking water supply tested. Likewise,
 478 EE2 was never found (< LOQ = 1.88 ng·L⁻¹), the method LOD standard of which has
 479 been updated to 0.035 ng·L⁻¹ based on the Commission Implementing Decision
 480 (European Commission, 2018), suggesting the challenge and necessity of improving the
 481 analytical methodology to monitor such compounds at lower concentrations. In the
 482 hospital discharge, all the targeted pharmaceuticals were detected except EE2, with
 483 paracetamol and carbamazepine detected in every sample. The highest concentrations
 484 were recorded for paracetamol, with a maximum of 105,910 ng·L⁻¹, followed by
 485 trimethoprim (9,111 ng·L⁻¹) and clarithromycin (7,940 ng·L⁻¹). Regarding the WWTP
 486 wastewater influent tested, the highest levels were noted for paracetamol (105,780 ng·L⁻¹)
 487 and ibuprofen (6,018 ng·L⁻¹). The increased mean detection level of paracetamol
 488 (33267 ng·L⁻¹ to 67483 ng·L⁻¹) and ibuprofen (139 ng·L⁻¹ to 471 ng·L⁻¹) between the

489 hospital discharge and wastewater influent indicated the possible presence of other
490 inputting sources of such pharmaceuticals besides the hospital discharge. Lower levels of
491 trimethoprim (818 ng·L⁻¹ to 621 ng·L⁻¹) and clarithromycin (1271 ng·L⁻¹ to 246 ng·L⁻¹)
492 in WWTP influent versus the hospital discharge may be attributed to the degradation
493 and/or dilution in the aquatic environment between those two sites (Gracia-Lor et al.,
494 2011). Higher levels of carbamazepine and ibuprofen may reflect greater (human) intakes
495 in the community versus the hospital. In terms of the final WWTP effluent, all the
496 previously detected pharmaceuticals remained detectable – albeit at reduced levels in
497 some cases. Five of the pharmaceuticals monitored were at lower mean levels in discharge
498 versus influent – but, two (carbamazepine, clarithromycin) were more elevated in
499 discharge water. These results reinforce the need to apply multiclass pharmaceutical
500 monitoring methods in order to gain a better understanding of the fate/behaviour of these
501 compounds at the catchment scale. Likewise, they highlight the ongoing need to create
502 WWTP processes that can efficiently eliminate these bioactive pollutants of concern.

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

512 **3. Conclusion**

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

521 Application of the method to ‘real’ environmental samples from a rural catchment in
522 Scotland, illustrated the occurrence of pharmaceuticals in various wastewater matrices.
523 The highest concentrations found were for paracetamol, with a mean level of 67,483 ng·L⁻¹
524 in municipal WWTP influent. The successful application of this method to real water
525 matrices validated its applicability within routine monitoring studies regarding these
526 priority pharmaceutical and EDC contaminants.

527 **Appendix A. Supplementary material**

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

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