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5 # Determination of steroid estrogens in wastewater by high performance liquid
6 # chromatography-tandem mass spectrometry

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8
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11
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18
19 Keywords: estrogen; solid phase extraction; wastewater; liquid chromatography; mass
20 spectrometry
21
22 **Abstract**
23 This paper discusses the requirement for, and presents an analytical procedure for, the
24 determination of four unconjugated steroid hormones and a conjugated steroid
25 (estrone-3-sulfate) in wastewaters. The method quantifies the steroids by LC/MS/MS
26 following solid phase extraction and a two stage clean-up procedure. Samples were
27 extracted using C18 cartridges and eluates were then purified by gel permeation
28 chromatography, followed by a further clean-up step on an aminopropyl cartridge.
29 The limits of detection achieved were 0.2 ng l^{-1} for estriol, 17β-estradiol and 17α-
30 ethinylestradiol, and 0.1 ng l^{-1} for estrone and the conjugate. The robustness of the
31 method was demonstrated by achieving recoveries of >83% for all steroids in settled
32 sewage and final effluent samples with relative standard deviations of 0.5 - 12%. The
33 method was used to analyse a range of samples from a wastewater treatment works in
34 south east England which demonstrated a >80% removal for estrone, estradiol and
35 estriol with little impact on concentrations of ethinylestradiol or the conjugate.

36

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1. Introduction

It has been estimated that over 99% of the estrogenic activity in sewage effluents and surface waters may be attributable to the presence of 17β-estradiol (E2) and 17α-ethynyl estradiol (EE2) at concentrations in the ng l^{-1} range [1]. Exposure studies indicate that fish exposed to such concentrations of these hormones exhibit changes in biomarkers for estrogenicity at environmentally relevant concentrations of 1 ng l^{-1} (E2) and as low as 0.1 ng l^{-1} for EE2 [2-7]. Excretion from humans is the major source of natural and synthetic steroid estrogens to sewage treatment works (STW). The predominant pathway of excretion is as conjugates, either glucuronides or sulfates in urine, with the sulfate conjugate of estrone (E1-3S) being the main urinary excretion product [8, 9]. Due to the activity of β-glucuronidase, gluconuride conjugates are broken down before reaching the STW, however, concentrations of the conjugated steroid, E1-3S, may be important when considering total load reaching STW [10].

Significant attention has, therefore, been given to the possibility of controlling the discharge of steroid estrogens from STW, and within the United Kingdom a research programme, the National Demonstration Programme, has been instigated to investigate the efficiency of a range of treatment processes at reducing concentrations of these compounds [11]. Overall, the work programme will cost up to £40M, with £5M being allocated for sampling and analysis costs. It is therefore important that robust methodology be available to underpin the research output from such studies.

The determination of free and conjugated steroid estrogens has been undertaken by gas chromatography mass spectrometry (GC/MS) or tandem MS [12-16] as well as by liquid chromatography (LC) MS/MS [10, 17-19]. An advantage of using LC/MS/MS is the ability to analyze the estrogens without derivatization, or the need to hydrolyse the conjugated form, which are limiting steps in determining both species [20, 21]. However, using LC/MSMS as an analytical tool is not without difficulties. It is known that electrospray ionisation (ESI) can experience effects related to matrix suppression and isobaric interference when analyzing estrogens as a result of co-eluting compounds during the chromatographic separation [22, 23]. Recent studies using ESI-LC/MS/MS have observed ion suppression that varied by a factor of 8–10 between and within runs for various analytes [24]. Therefore, the need to determine these compounds at trace concentrations in complex matrices such as wastewaters and

72 effluents is likely to require intensive clean-up procedures and the use of appropriate
73 internal standards, which allow for correction due to losses during sample preparation
74 and as a result of matrix effects.
75
76 Extraction of steroid hormones from wastewater is usually performed by off-line solid
77 phase extraction (SPE) using octadecyl (C_{18})-bonded silica adsorbent [3, 25-28].
78 Purification of wastewater extracts has been achieved by various means, including
79 liquid-liquid extraction, solid phase purification on C_{18}/NH_2 columns [29, 30], slica
80 gel column chromatography [31, 32], gel permeation on Biobeads SX-3 columns,
81 high performance liquid chromatography (HPLC) fractionation [25, 33], or
82 combinations of all these methods [3, 34, 35].
83
84 The objective of this work was to develop a sensitive and robust methodology for the
85 determination of four free steroid estrogens; estrone (E1), E2, estriol (E3), EE2 and
86 the conjugated E1-3S applicable to a range of water and wastewater samples.
87
88 **2. Experimental**
89 *2.1 Reagents and chemicals*
90 All estrogen standards were purchased from Sigma Aldrich (Dorset, UK). Organic
91 solvents, dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), hexane
92 and ethyl acetate (EtOAc) were purchased from Rathburn Chemicals (Walkerburn,
93 UK). Deuterated ($d_{3/4/5}$) labelled internal standards of estrone-2,4,16,16-d_4 (E1-d_4),
94 17β-estradiol-2,4,16,16,17-d_5 (E2-d_5), estriol-2,4,17-d_3 (E3-d_3), 17α-ethynylestradiol-
95 2,4,16,16-d_4 (EE2-d_4) and sodium estrone-2,4,16,16-d_4 sulfate (E1-3S-d_4) were
96 obtained from C/D/N Isotopes (QMX Laboratories, UK). Stock solutions were
97 prepared in methanol. Two different solid phase extraction cartridges tC18
98 (500mg/6cc) and aminopropyl (NH_2) anion-exchange (500mg/6cc) were obtained
99 from Waters (Watford, UK) and Varian (Varian Inc, UK) respectively.
100
101 *2.2 Analytical procedure*
102 Settled sewage and final effluent samples (1L) were filtered through GF/C (VWR
103 International, UK) filters prior to solid phase extraction (SPE). The samples were then
104 loaded onto tC18 cartridges preconditioned with 5ml methanol followed by 5ml MQ
105 water. The flow rate for sample extraction was kept constant between 5-10 ml min^{-1}

106 under vacuum using vacuum manifold. After the sample was loaded, the cartridge was
107 washed with 3ml of Ultrapure water and then thoroughly dried for half an hour under
108 vacuum prior to elution. The analytes were eluted using 10 ml MeOH followed by 10
109 ml DCM. A rotary evaporator (Heidolph Instruments, Germany) was employed to
110 concentrate the extracts to 1 ml which was then evaporated to complete dryness under
111 a gentle nitrogen stream. The dry sample was reconstituted with 0.2 ml DCM/MeOH
112 (90:10 v/v). Gel permeation size exclusion chromatography was performed using a
113 PLgel column, 5μm 50Å, 300 x 7.5 mm (Polymer Laboratories, UK). Conjugated and
114 unconjugated steroids were detected at 280nm. A 6ml fraction was collected from the
115 column using an isocratic elution of DCM/MeOH (90:10 v/v) running at 1 ml min^{-1}.
116 All steroids eluted between 5.5 to 11.5 min, and a single fraction corresponding to this
117 time window was collected. This fraction was dried by rotary evaporation to a final
118 volume of approximately 0.2 ml. This was then reconstituted to 2 ml with hexane and
119 loaded onto a conditioned (with 4ml 10% EtOAc/hexane and then 2 ml hexane) NH_2
120 SPE cartridge at a flow rate between 5-10 ml min^{-1}. The nonpolar steroids E1, E2 and
121 EE2 were then eluted using 6ml EtOAc. The more polar conjugate (E1-3S) and E3
122 were subsequently eluted in a second fraction using 3% NH_4OH in methanol. The
123 separate eluates were blown to dryness under a gentle stream of nitrogen,
124 reconstituted with 0.2 ml MeOH/H_2O (10:90 v/v) and transferred to autosampler vials
125 prior to analysis using LC/MS/MS.
126
127 *2.3 Instrumental analysis*
128 Concentrations of steroid estrogens were determined using LC/ESI($^-$)/MS/MS
129 consisting of an HPLC (Waters Alliance HPLC system 2695) coupled to a Waters
130 Quattro Premier XE mass spectrometer with a Z-Spray ESI source (Micromass, UK).
131 The steroids were separated on a Gemini C18 column (3μm particle size, 100mm x
132 2mm i.d., Phenomenex, UK). The mass spectrometer was operated in the negative
133 electrospray ionisation mode using multiple reaction monitoring (MRM). The
134 conditions for detection by the mass spectrometer were as follows: capillary voltage,
135 3.20kV; multiplier voltage, 650V; desolvation gas flow, 1000 l h^{-1}; cone at -55V; RF
136 lens at 0.2V; cone gas flow at 49 l h^{-1}; desolvation temperature at 350°C and source
137 temperature at 120°C.
138
139

140 **3. Results**

141 *3.1 Optimisation of LC conditions*

142 LC optimisation was carried out on Gemini C18 column (Phenomenex, UK). A
143 gradient separation was achieved using two solvents, water containing 0.1% NH_4OH
144 (A) and MeOH containing 0.1% NH_4OH (B). Gradient conditions were initiated with
145 20% B followed by an increase to 50% B (over 3.5 min). The proportion of solvent B
146 was then increased to 60% maintained for 9 min before the column was returned to
147 starting conditions 20% B (over 3 min) and held for 2.5 min to equilibration. The total
148 run time was 18 min and a sample volume of 20µl of was injected into the HPLC
149 (Figure 1). Eight point calibration curves were made for each of the steroids within
150 the linear range of the instrument (1 – 100 ng ml^{-1}). The concentrations of the steroid
151 estrogens in the samples were calculated relative to the deuterated standards using the
152 MassLynx software.

153

154 Please insert Figure 1.

155

156 *3.2 Optimising MS/MS condition*

157 The optimal MS/MS conditions for the analysis of conjugated and unconjugated
158 steroid estrogens were examined. The optimisation was carried out in the negative
159 mode using electrospray ionization. Single standard solutions were used to identify
160 W^- ions and peak retention times (Table 1). The optimisation of operating parameters
161 affecting MS detection such as dwell time, cone voltages and collision energy on each
162 ion were carried out by the direct infusion of 100 ng ml^{-1} standard of each steroid at
163 flow rate of 10 µl min^{-1}. The optimum conditions were reached when the highest
164 intensities or superior signal-to-noise (S/N) resolution were achieved for each
165 conjugate and unconjugated steroid estrogens. For greater manipulation of MS
166 settings and to improve sensitivity, chromatographic separation was divided into two
167 acquisition periods. In the first period between 0 and 11 min, intensities of ions for
168 E1-3S, E1-3S- d_4, E3 and E3- d_3 were monitored, while in the second acquisition
169 period between 11and 18 min, intensities of ions for E1, E1-d_4, E2, E2- d_5, EE2 and
170 EE2- d_4 were detected (Figure 2).

171

172 Please insert Table 1.

173

174 Please insert Figure 2.

175

176 *3.3 Concentration/extraction and clean-up*

177 Several SPE procedures were assessed using one litre samples spiked with the steroid
178 estrogens at 15 ng l^{-1} each. Utilising any selective elution for this first step, to begin a
179 sample clean-up, resulted in poor recoveries, and the cartridges were therefore eluted
180 with 10ml methanol followed by 10 ml DCM. The use of gel permeation as a
181 subsequent preparation step was a challenge due to the relatively high polarity of E1-
182 3S and E3. Therefore initial work focussed on finding a solvent system which would
183 dissolve the range of estrogens, but which was also compatible with the PLgel column.
184 The optimal compromise in adjusting polarity of the solvent mix and achieving
185 desired swelling of the gel within the GPC column was achieved with 10% MeOH in
186 DCM.

187

188 The anion exchange SPE was used as a final clean-up step to remove interferences
189 that may otherwise affect the LC/MS/MS analysis of the steroids. Employing a Varian
190 NH_2 weak anion exchange cartridge, recoveries of more than 83% were achieved for
191 all of the steroid estrogens including the conjugated steroid E1-3S. The scheme for the
192 analytical procedure developed in this study is shown in Figure 3.

193

194 Please insert Figure 3.

195

196 *3.4 Evaluation of method performance*

197 The calibration curves for the determination of the analytes were obtained by
198 performing a linear regression analysis on the standard solutions using the ratio of
199 standard area to internal standard area. The calibrations were all linear with r^2 values
200 greater than 0.998. The instrument detection limits (IDL) were 9 – 20 pg based on the
201 extraction of reagent grade water samples spiked at 1 ng l^{-1}. The recoveries of the
202 analytes were evaluated by spiking at both low (2 ng l^{-1}) and high (15 ng l^{-1})
203 concentrations in settled sewage and final effluent. The recoveries for three replicate
204 samples spiked in samples of settled sewage and final effluent ranged from 83 - 100%,
205 with relative standard deviations of 0.3 - 12% (Table 2). Recoveries obtained in this
206 study were calculated by the subtraction of concentrations observed in unspiked
207 samples. The method detection limit (MDL) is reported as concentrations

208 corresponding to a *S/N* ratio of 3 on the chromatogram of actual sample matrices. The
209 MDL were 0.2 – 0.1 ng l^{-1} for settled sewage and sewage effluent samples spiked at 1
210 ng l^{-1} (Table 2).
211
212 Please insert Table 2.
213
214 **4. Discussion**
215 *4.1 Optimisation of methodology*
216 Widely used organic mobile phases in steroid analysis such as ACN and MeOH have
217 been considered. Acetronitrile was previously reported to result in the co-elution
218 between conjugates and free steroids [10]. Methanol gave superior chromatographic
219 resolution with regard to steroid conjugates as well as increased sensitivity compared
220 to ACN. This is in agreement with other studies [36-38]. Therefore methanol was
221 chosen as the organic mobile phase in this study. Sensitivity of LC/ESI($^{-}$)/MS/MS
222 determination has been previously reported to improve by adding a strong base such
223 as NH_4OH to the mobile phase [37]. It was thus necessary to investigate if this was
224 applicable to this methodology. A concentration range of up to 0.1% NH_4OH was
225 investigated in this study. An initial increase in the signal-to- noise (*S/N*) ratio (5%)
226 was observed when concentrations of less than 0.01% NH_4OH were added. The
227 absolute abundance of ions for all compounds increased significantly when using
228 0.1% NH_4OH and this was incorporated into the mobile phase used for the analysis of
229 the steroid estrogens.
230
231 The steroid estrogen and conjugate steroids were analyzed by tandem MS/MS using
232 ESI^{-} interface in the negative ion MRM mode. Studies have shown that greater *S/N*
233 ratio when operated in ESI^{-} thus leading to a lower LOD [39]. Single ion transitions
234 were monitored for all the analytes which were characteristic of the parent compounds.
235 A second transition was also monitored as confirmation (Table 2).
236
237 It was demonstrated that high recoveries could be obtained by employing a non-
238 specific SPE method to concentrate the analytes of interest from the complex
239 wastewater matrix. Automated high performance gel permeation chromatography
240 gave high reproducibility and high selectivity for the steroid compounds. The
241 physiochemical nature of the steroid estrogens (particularly the polar nature of E3 and

242 E1-3S) in this study proved to be a challenge when applied to the PLgel column.
243 PLgel is compatible with an extensive range of organic solvents and over the pH
244 range 7-14. However, in order to maintain the swelling of the resin, addition of polar
245 solvents such as water at concentrations more than 10% by volume was cautioned by
246 the manufacturer. In this study, we endeavoured to find a solvent mixture that
247 dissolved both the nonpolar steroid and the polar conjugate and which also was
248 compatible with the elution solvent used on PLgel column. The results from these
249 experiments indicated that a small volume of MeOH was essential. Poor recoveries
250 were obtained when MeOH was absent in the DCM mobile phase. When MeOH was
251 present in a higher proportion than DCM, the packing material within the PLgel
252 column changed and affected the column performance.
253
254 In the final (second) clean-up step, two commercially available SPE cartridges, tC18
255 and NH_2, were evaluated to assess their ability to remove interferences and provide a
256 clean matrix for LC/MS/MS. Several solvent combinations were also tested. The tC18
257 was not effective as a clean-up step and hence high noise, ionisation suppression and
258 poor recoveries were observed. Recoveries of less than 43% and 38% were achieved
259 for the unconjugated steroids and conjugated E1-3S respectively (data not shown). In
260 contrast, aminopropyl SPE gave good recoveries and little matrix interference,
261 however, some interference due to ion suppression was observed when both the
262 conjugated and unconjugated steroids were eluted simultaneously from the anion-
263 exchange cartridge. The stepwise wash with 10% EtOAc/hexane and the separate
264 elution of the hydrophilic conjugates and the hydrophobic unconjugated steroids
265 reduced isobaric interferences and ion suppression thus resulting in an increase in *S/N*
266 ratio. These observations and results concurred with those of the findings observed by
267 others [38].
268
269 *4.2 Method performance*
270 The methodology described here obtained higher recovery of E1-3S compared to
271 other studies; 10% influent and 49% for effluent [40]; 89% influent, 87% effluent and
272 93% for river water [37]; 87% laboratory water and 97% for surface water [30]. The
273 method detection limit obtained was similar to that of Isobe et al. [19] (one step clean-
274 up using Florisil) for E1, E1-3S and EE2 of 0.1 ng l^{-1}, 0.1 ng l^{-1} and 0.2 ng l^{-1}
275 respectively. An advantage of the method described here, however, is an improved

276 MDL for E2 and E3 of 0.3 ng l^{-1} and 1.5 ng l^{-1} respectively. Other published works
277 have reported MDL for E1-3S at 0.16 ng l^{-1} [38] and 0.2 ng l^{-1} [37]. Although similar
278 MDL for E1-3S have been obtained with methodology employing two clean-up steps,
279 detection limits for non-conjugated steroids were compromised (E1, E2, E3 and EE2
280 at 0.8 ng l^{-1}, 0.5 ng l^{-1}, 1.4 ng l^{-1} and 1.2 ng l^{-1} respectively) [43] . The procedure
281 described here is thus more robust in comparison to other works that have included
282 conjugated steroids into their analysis with either a one or two step clean-up regime
283 [30, 37, 38, 40].
284
285 *4.3 Application to wastewater samples*
286 The concentrations of the steroids detected in the wastewater are summarized in Table
287 3. All compounds were detected in the settled sewage. The treatment at the works was
288 a trickling filter, and there was little or no removal of either the synthetic estrogen
289 (EE2) or of the conjugate, E1-3S. However, removal of the naturally occurring, free
290 steroids (E1, E2, and E3) was between 80 - 98%. Estrone was least efficiently
291 removed, which may be a result of the biological transformation of E2 to E1 in the
292 filter. It is intended to apply the method developed here to more extensive studies of
293 biological treatment processes to understand the impact of process variables on
294 removal of estrogens from wastewaters. Data presented here corroborates with that
295 from other studies which have demonstrated the occurrence of E1-3S in wastewaters
296 [10, 37, 41] and receiving waters [30].
297
298 Please insert Table 3.
299
300 **5. Conclusions**
301 A sensitive and selective analytical method based on SPE, GPC, aminopropyl anion
302 exchange and LC/MS/MS has been developed for the determination of polar
303 conjugated and nonpolar unconjugated steroid estrogens in complex wastewater
304 samples at sub-ng l^{-1} concentrations. To analyse estrogens at such concentrations in
305 wastewater is challenging due to matrix effects and the range of interferences likely to
306 be present. Therefore, a series of purifications steps have been optimised which result
307 in the removal of many matrix interferences. The technique is relatively rapid, semi-
308 automated and hence not as time consuming as other extraction and cleanup
309 approaches such as liquid-liquid extraction, or where derivatization is required, thus

310 allowing for rapid, selective and sensitive analysis of both conjugate and
311 unconjugated steroids in wastewater.
312

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317 Plc and Yorkshire Water (Kelda Group Plc) for providing their support and funding.

LIST OF TABLES

Table 1. Optimised LC/MS/MS conditions for MRM chromatographic acquisition of steroid estrogens[a]

Table 2. Method recoveries (%) and relative standard deviations (RSD %) and method detection limit (MDL) from settled sewage and final effluent (*n*=3).

Table 3. Concentrations of conjugated and unconjugated steroid estrogens in samples from a wastewater treatment works (January 2007).

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Figure 1. Total ion chromatogram of conjugated and unconjugated steroids standard solutions at 20 ng l^{-1}.

Figure 2. (a) MRM chromatograms of E1-3S-d_4, E1-3S, E3-d_3 and E3. (b) MRM chromatograms of EE2-d_4, EE2, E2-d_5, E1-d_4, E2 and E1 (settled sewage spiked at 2 ng l^{-1}).

Figure 3. Diagram summarising the analytical procedure for the determination of the steroid estrogens.

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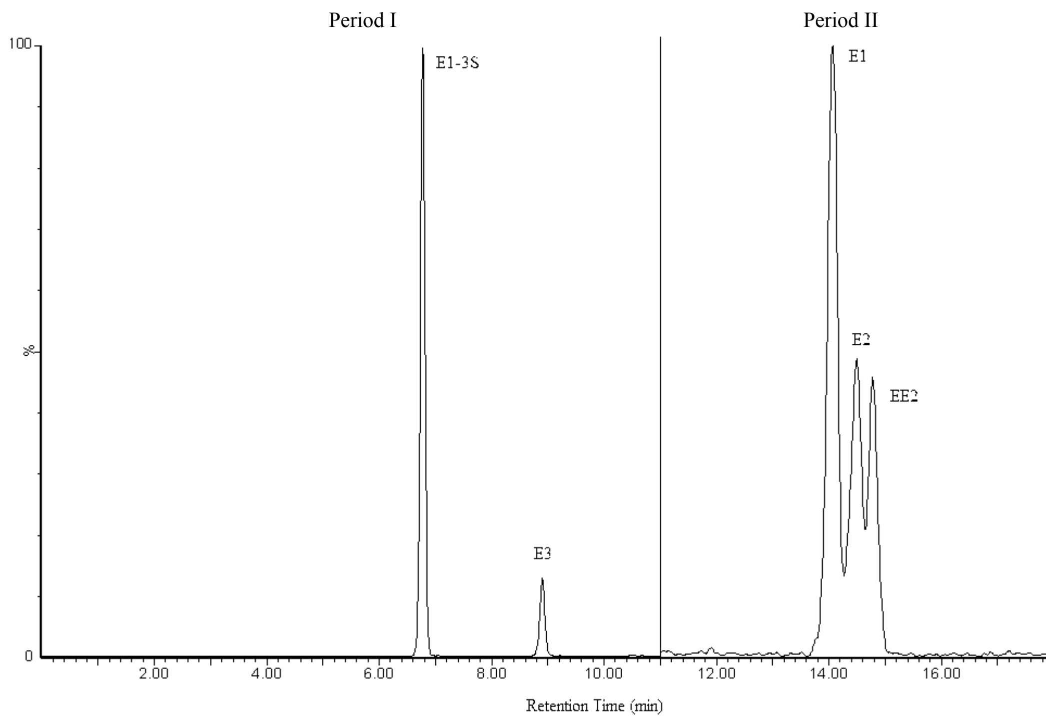
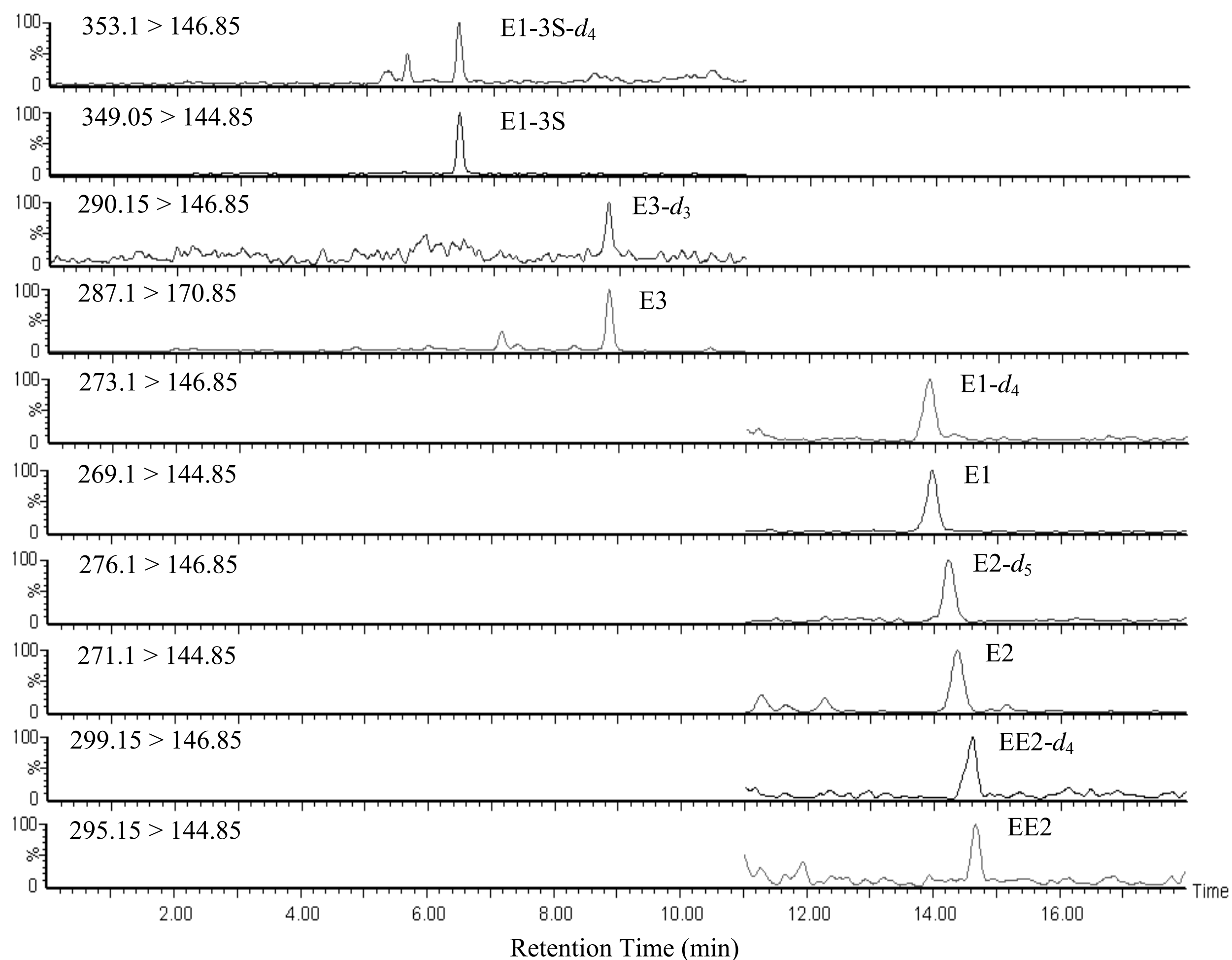
1 List of figures
2



3
4
5 Figure 1. Total ion chromatogram of conjugated and unconjugated steroids standard
6 solutions at 20 ng l^{-1}.
7

1



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3 Figure 2. (a) MRM chromatograms of E1-3S-d_4, E1-3S, E3-d_3 and E3. (b) MRM
4 chromatograms of EE2-d_4, EE2, E2-d_5, E1-d_4, E2 and E1 (settled sewage spiked at 2
5 ng l^{-1}).
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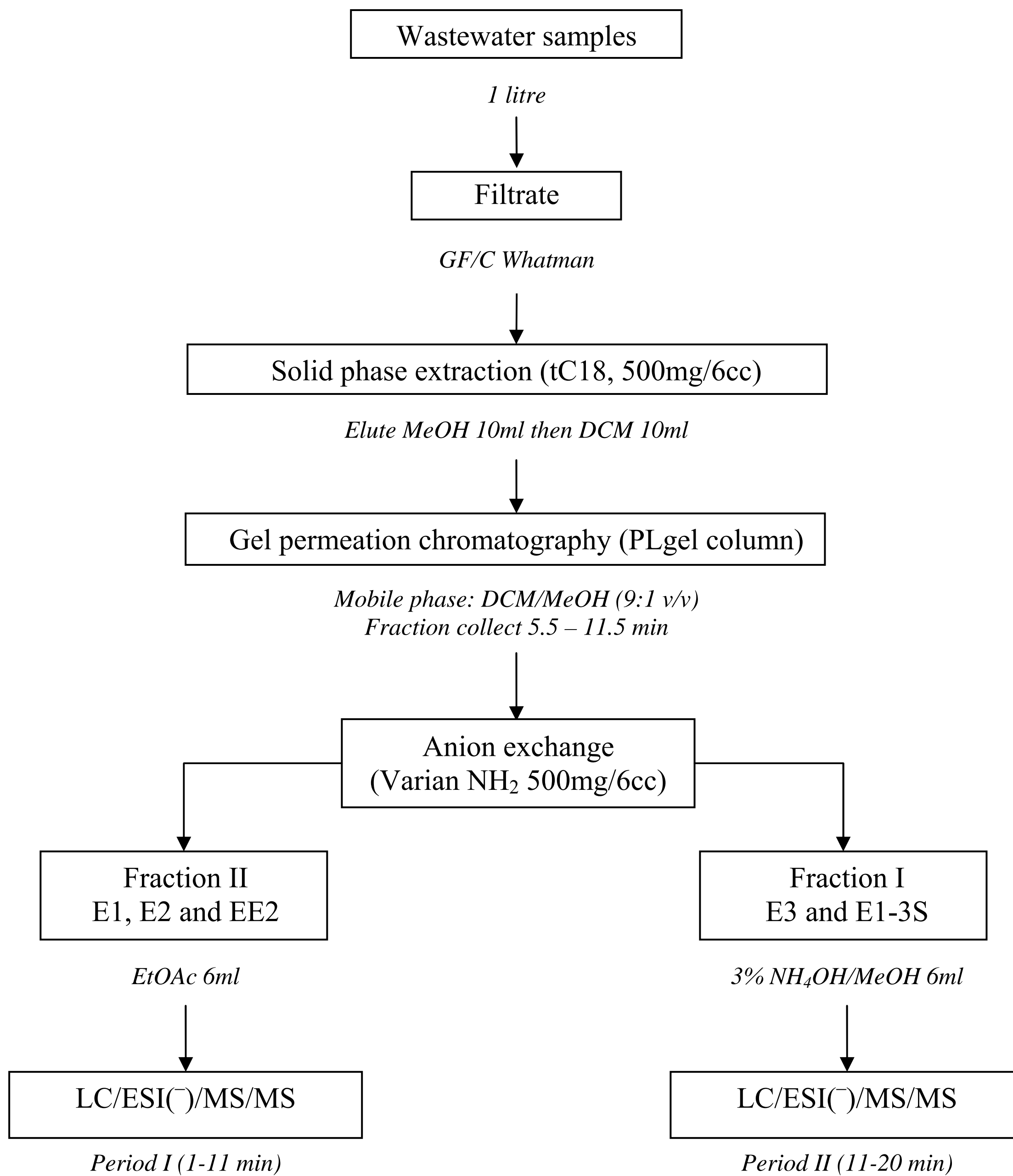

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2 Figure 3. Diagram summarising the analytical procedure for the determination of the
3 steroid estrogens.
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1 Table 1. Optimised LC/MS/MS conditions for determination of the steroid estrogens[a]
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Estrogen	Period (min)	[a]MRM *m/z*	Dwell time (msec)	Collision energy (V)	Cone (V)	Retention time (min)	IDL[b] (pg)
E1	II (11-20)	269.10>144.85	85	40	70	13.97	12
		269.10>158.80	85	45	70		
E2	II (11-20)	271.10>144.85	85	45	60	14.37	20
		271.10>158.80	85	40	60		
E3	I (0-11)	287.10>170.85	95	50	55	8.90	18
		287.10>144.85	95	50	55		
EE2	II (11-20)	295.15>144.85	85	40	60	14.67	16
		295.15>158.80	85	40	60		
E1-3S	I (0-11)	349.05>144.85	60	65	50	6.77	9
		349.05>269.00	60	40	50		
E1-d_4	II (11-20)	273.10>146.85	85	45	60	13.91	
E2-d_5	II (11-20)	276.10>146.85	85	50	55	14.23	
E3-d_3	I (0-11)	290.15>146.85	90	65	50	8.86	
EE2-d_4	II (11-20)	299.15>146.85	85	50	60	14.60	
E1-3S-d_4	I (0-11)	353.10>146.85	60	65	50	6.75	

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4 [a] MRM transitions: the first for quantification, the second for confirmation.
5 [b] Instrument detection limit at 1 ng l^{-1} replicate measurement (n=7).
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2 Table 2. Method recoveries (%) and relative standard deviations (RSD %) and method
3 detection limit (MDL) from settled sewage and final effluent (n=3).

Compound	Settled sewage (% RSD)		Final effluent (% RSD)		MDL[c] ($ng\ l^{-1}$)	
	Low spike[a]	High spike[b]	Low spike[a]	High spike[b]	Settled sewage[a]	Final effluent[a]
E1	98 (1.5)	95 (4)	100 (2)	88 (3)	0.1	0.1
E2	100 (0.6)	88 (1.6)	100 (7)	88 (4)	0.2	0.2
E3	100 (0.7)	98 (0.3)	83 (12)	86 (6)	0.2	0.2
EE2	90 (10)	88 (5)	100 (2)	83 (5)	0.2	0.2
E1-3S	97 (1)	95 (4)	96 (2)	99 (1)	0.1	0.1

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5 [a]2 ng of standard or [b]15 ng of standard was spiked to 1 litre of settled sewage/final
6 effluent (15 $ng\ l^{-1}$ of deuterated internal standard).
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1 Table 3. Concentrations of conjugated and unconjugated steroid estrogens in samples
2 from a wastewater treatment works (January 2007).
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Compound	Settled sewage (ng l^{-1})	Final effluent (ng l^{-1})	Removal (%)
E1	15	3.0	80
E2	5.0	0.7	86
E3	50	1.0	98
EE2	1.2	1.0	17
E1-3S	10	12	-

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