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

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Y.K.K. Koh[1], T.Y. Chiu[2], A. Boobis[1], E. Cartmell[2], J.N. Lester[2] and M.D. Scrimshaw[3*]

[1] Faculty of Medicine, Division of Experimental Medicine and Toxicology, Imperial College London, Hammersmith Campus, London, W12 ONN, United Kingdom.

[2] School of Applied Science, Centre for Water Science, Cranfield University, Bedfordshire, MK43 0AL, United Kingdom.

[3] Institute for the Environment, Brunel University, Uxbridge, Middlesex, UB8 3PH, United Kingdom.

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Abstract

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

* corresponding author: mark.scrimshaw@brunel.ac.uk

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

effluents is likely to require intensive clean-up procedures and the use of appropriate internal standards, which allow for correction due to losses during sample preparation and as a result of matrix effects.

Extraction of steroid hormones from wastewater is usually performed by off-line solid phase extraction (SPE) using octadecyl (C_{18})-bonded silica adsorbent [3, 25-28]. Purification of wastewater extracts has been achieved by various means, including liquid-liquid extraction, solid phase purification on C_{18}/NH_2 columns [29, 30], slica gel column chromatography [31, 32], gel permeation on Biobeads SX-3 columns, high performance liquid chromatography (HPLC) fractionation [25, 33], or combinations of all these methods [3, 34, 35].

The objective of this work was to develop a sensitive and robust methodology for the determination of four free steroid estrogens; estrone (E1), E2, estriol (E3), EE2 and the conjugated E1-3S applicable to a range of water and wastewater samples.

2. Experimental

2.1 Reagents and chemicals

All estrogen standards were purchased from Sigma Aldrich (Dorset, UK). Organic solvents, dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), hexane and ethyl acetate (EtOAc) were purchased from Rathburn Chemicals (Walkerburn, UK). Deuterated ($d_{3/4/5}$) labelled internal standards of estrone-2,4,16,16-d_4 (E1-d_4), 17β-estradiol-2,4,16,16,17-d_5 (E2-d_5), estriol-2,4,17-d_3 (E3-d_3), 17α-ethynylestradiol-2,4,16,16-d_4 (EE2-d_4) and sodium estrone-2,4,16,16-d_4 sulfate (E1-3S-d_4) were obtained from C/D/N Isotopes (QMX Laboratories, UK). Stock solutions were prepared in methanol. Two different solid phase extraction cartridges tC18 (500mg/6cc) and aminopropyl (NH_2) anion-exchange (500mg/6cc) were obtained from Waters (Watford, UK) and Varian (Varian Inc, UK) respectively.

2.2 Analytical procedure

Settled sewage and final effluent samples (1L) were filtered through GF/C (VWR International, UK) filters prior to solid phase extraction (SPE). The samples were then loaded onto tC18 cartridges preconditioned with 5ml methanol followed by 5ml MQ water. The flow rate for sample extraction was kept constant between 5-10 ml min^{-1}

under vacuum using vacuum manifold. After the sample was loaded, the cartridge was washed with 3ml of Ultrapure water and then thoroughly dried for half an hour under vacuum prior to elution. The analytes were eluted using 10 ml MeOH followed by 10 ml DCM. A rotary evaporator (Heidolph Instruments, Germany) was employed to concentrate the extracts to 1 ml which was then evaporated to complete dryness under a gentle nitrogen stream. The dry sample was reconstituted with 0.2 ml DCM/MeOH (90:10 v/v). Gel permeation size exclusion chromatography was performed using a PLgel column, 5µm 50Å, 300 x 7.5 mm (Polymer Laboratories, UK). Conjugated and unconjugated steroids were detected at 280nm. A 6ml fraction was collected from the column using an isocratic elution of DCM/MeOH (90:10 v/v) running at 1 ml min^{-1}. All steroids eluted between 5.5 to 11.5 min, and a single fraction corresponding to this time window was collected. This fraction was dried by rotary evaporation to a final volume of approximately 0.2 ml. This was then reconstituted to 2 ml with hexane and loaded onto a conditioned (with 4ml 10% EtOAc/hexane and then 2 ml hexane) NH_2 SPE cartridge at a flow rate between 5-10 ml min^{-1}. The nonpolar steroids E1, E2 and EE2 were then eluted using 6ml EtOAc. The more polar conjugate (E1-3S) and E3 were subsequently eluted in a second fraction using 3% NH_4OH in methanol. The separate eluates were blown to dryness under a gentle stream of nitrogen, reconstituted with 0.2 ml MeOH/H_2O (10:90 v/v) and transferred to autosampler vials prior to analysis using LC/MS/MS.

2.3 Instrumental analysis

Concentrations of steroid estrogens were determined using LC/ESI($^-$)/MS/MS consisting of an HPLC (Waters Alliance HPLC system 2695) coupled to a Waters Quattro Premier XE mass spectrometer with a Z-Spray ESI source (Micromass, UK). The steroids were separated on a Gemini C18 column (3µm particle size, 100mm x 2mm i.d., Phenomenex, UK). The mass spectrometer was operated in the negative electrospray ionisation mode using multiple reaction monitoring (MRM). The conditions for detection by the mass spectrometer were as follows: capillary voltage, 3.20kV; multiplier voltage, 650V; desolvation gas flow, 1000 l h^{-1}; cone at -55V; RF lens at 0.2V; cone gas flow at 49 l h^{-1}; desolvation temperature at 350°C and source temperature at 120°C.

3. Results

3.1 Optimisation of LC conditions

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

Please insert Figure 1.

3.2 Optimising MS/MS condition

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

Please insert Table 1.

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

corresponding to a *S/N* ratio of 3 on the chromatogram of actual sample matrices. The MDL were 0.2 – 0.1 ng l^{-1} for settled sewage and sewage effluent samples spiked at 1 ng l^{-1} (Table 2).

Please insert Table 2.

4. Discussion

4.1 Optimisation of methodology

Widely used organic mobile phases in steroid analysis such as ACN and MeOH have been considered. Acetronitrile was previously reported to result in the co-elution between conjugates and free steroids [10]. Methanol gave superior chromatographic resolution with regard to steroid conjugates as well as increased sensitivity compared to ACN. This is in agreement with other studies [36-38]. Therefore methanol was chosen as the organic mobile phase in this study. Sensitivity of LC/ESI($^-$)/MS/MS determination has been previously reported to improve by adding a strong base such as NH_4OH to the mobile phase [37]. It was thus necessary to investigate if this was applicable to this methodology. A concentration range of up to 0.1% NH_4OH was investigated in this study. An initial increase in the signal-to- noise (*S/N*) ratio (5%) was observed when concentrations of less than 0.01% NH_4OH were added. The absolute abundance of ions for all compounds increased significantly when using 0.1% NH_4OH and this was incorporated into the mobile phase used for the analysis of the steroid estrogens.

The steroid estrogen and conjugate steroids were analyzed by tandem MS/MS using ESI^- interface in the negative ion MRM mode. Studies have shown that greater *S/N* ratio when operated in ESI^- thus leading to a lower LOD [39]. Single ion transitions were monitored for all the analytes which were characteristic of the parent compounds. A second transition was also monitored as confirmation (Table 2).

It was demonstrated that high recoveries could be obtained by employing a non-specific SPE method to concentrate the analytes of interest from the complex wastewater matrix. Automated high performance gel permeation chromatography gave high reproducibility and high selectivity for the steroid compounds. The physiochemical nature of the steroid estrogens (particularly the polar nature of E3 and

E1-3S) in this study proved to be a challenge when applied to the PLgel column. PLgel is compatible with an extensive range of organic solvents and over the pH range 7-14. However, in order to maintain the swelling of the resin, addition of polar solvents such as water at concentrations more than 10% by volume was cautioned by the manufacturer. In this study, we endeavoured to find a solvent mixture that dissolved both the nonpolar steroid and the polar conjugate and which also was compatible with the elution solvent used on PLgel column. The results from these experiments indicated that a small volume of MeOH was essential. Poor recoveries were obtained when MeOH was absent in the DCM mobile phase. When MeOH was present in a higher proportion than DCM, the packing material within the PLgel column changed and affected the column performance.

In the final (second) clean-up step, two commercially available SPE cartridges, tC18 and NH_2, were evaluated to assess their ability to remove interferences and provide a clean matrix for LC/MS/MS. Several solvent combinations were also tested. The tC18 was not effective as a clean-up step and hence high noise, ionisation suppression and poor recoveries were observed. Recoveries of less than 43% and 38% were achieved for the unconjugated steroids and conjugated E1-3S respectively (data not shown). In contrast, aminopropyl SPE gave good recoveries and little matrix interference, however, some interference due to ion suppression was observed when both the conjugated and unconjugated steroids were eluted simultaneously from the anion-exchange cartridge. The stepwise wash with 10% EtOAc/hexane and the separate elution of the hydrophilic conjugates and the hydrophobic unconjugated steroids reduced isobaric interferences and ion suppression thus resulting in an increase in *S/N* ratio. These observations and results concurred with those of the findings observed by others [38].

4.2 Method performance

The methodology described here obtained higher recovery of E1-3S compared to other studies; 10% influent and 49% for effluent [40]; 89% influent, 87% effluent and 93% for river water [37]; 87% laboratory water and 97% for surface water [30]. The method detection limit obtained was similar to that of Isobe et al. [19] (one step clean-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} respectively. An advantage of the method described here, however, is an improved

MDL for E2 and E3 of 0.3 ng l^{-1} and 1.5 ng l^{-1} respectively. Other published works have reported MDL for E1-3S at 0.16 ng l^{-1} [38] and 0.2 ng l^{-1} [37]. Although similar MDL for E1-3S have been obtained with methodology employing two clean-up steps, detection limits for non-conjugated steroids were compromised (E1, E2, E3 and EE2 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 described here is thus more robust in comparison to other works that have included conjugated steroids into their analysis with either a one or two step clean-up regime [30, 37, 38, 40].

4.3 Application to wastewater samples

The concentrations of the steroids detected in the wastewater are summarized in Table 3. All compounds were detected in the settled sewage. The treatment at the works was a trickling filter, and there was little or no removal of either the synthetic estrogen (EE2) or of the conjugate, E1-3S. However, removal of the naturally occurring, free steroids (E1, E2, and E3) was between 80 - 98%. Estrone was least efficiently removed, which may be a result of the biological transformation of E2 to E1 in the filter. It is intended to apply the method developed here to more extensive studies of biological treatment processes to understand the impact of process variables on removal of estrogens from wastewaters. Data presented here corroborates with that from other studies which have demonstrated the occurrence of E1-3S in wastewaters [10, 37, 41] and receiving waters [30].

Please insert Table 3.

5. Conclusions

A sensitive and selective analytical method based on SPE, GPC, aminopropyl anion exchange and LC/MS/MS has been developed for the determination of polar conjugated and nonpolar unconjugated steroid estrogens in complex wastewater samples at sub-ng l^{-1} concentrations. To analyse estrogens at such concentrations in wastewater is challenging due to matrix effects and the range of interferences likely to be present. Therefore, a series of purifications steps have been optimised which result in the removal of many matrix interferences. The technique is relatively rapid, semi-automated and hence not as time consuming as other extraction and cleanup approaches such as liquid-liquid extraction, or where derivatization is required, thus

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

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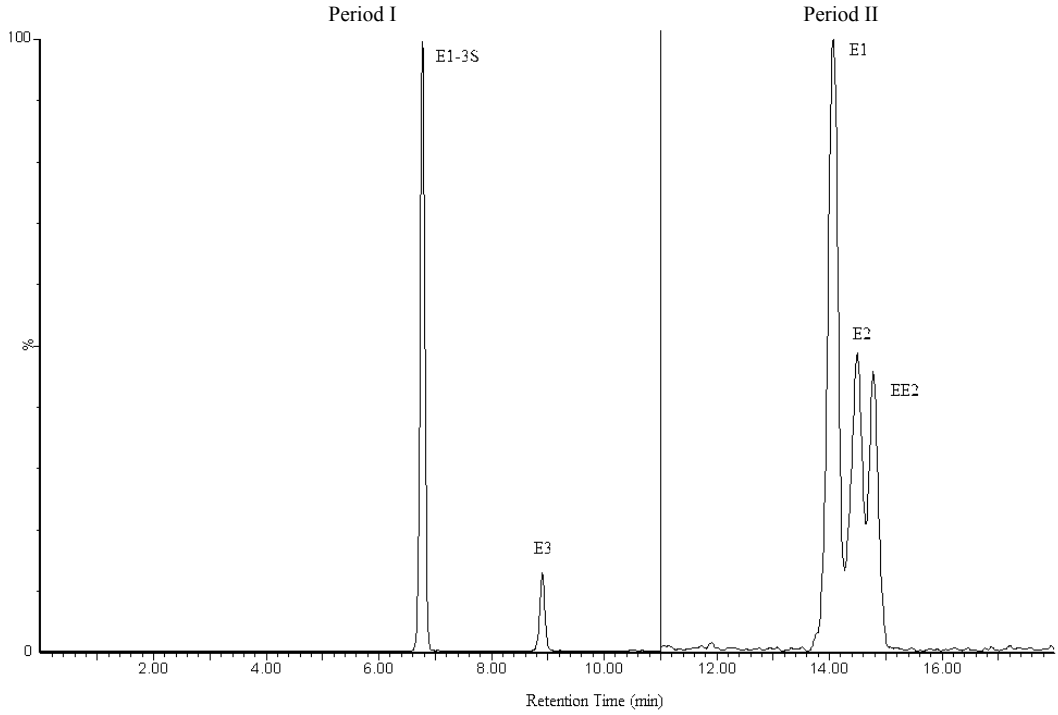

Figure 1. Total ion chromatogram of conjugated and unconjugated steroids standard solutions at 20 ng l^{-1}.

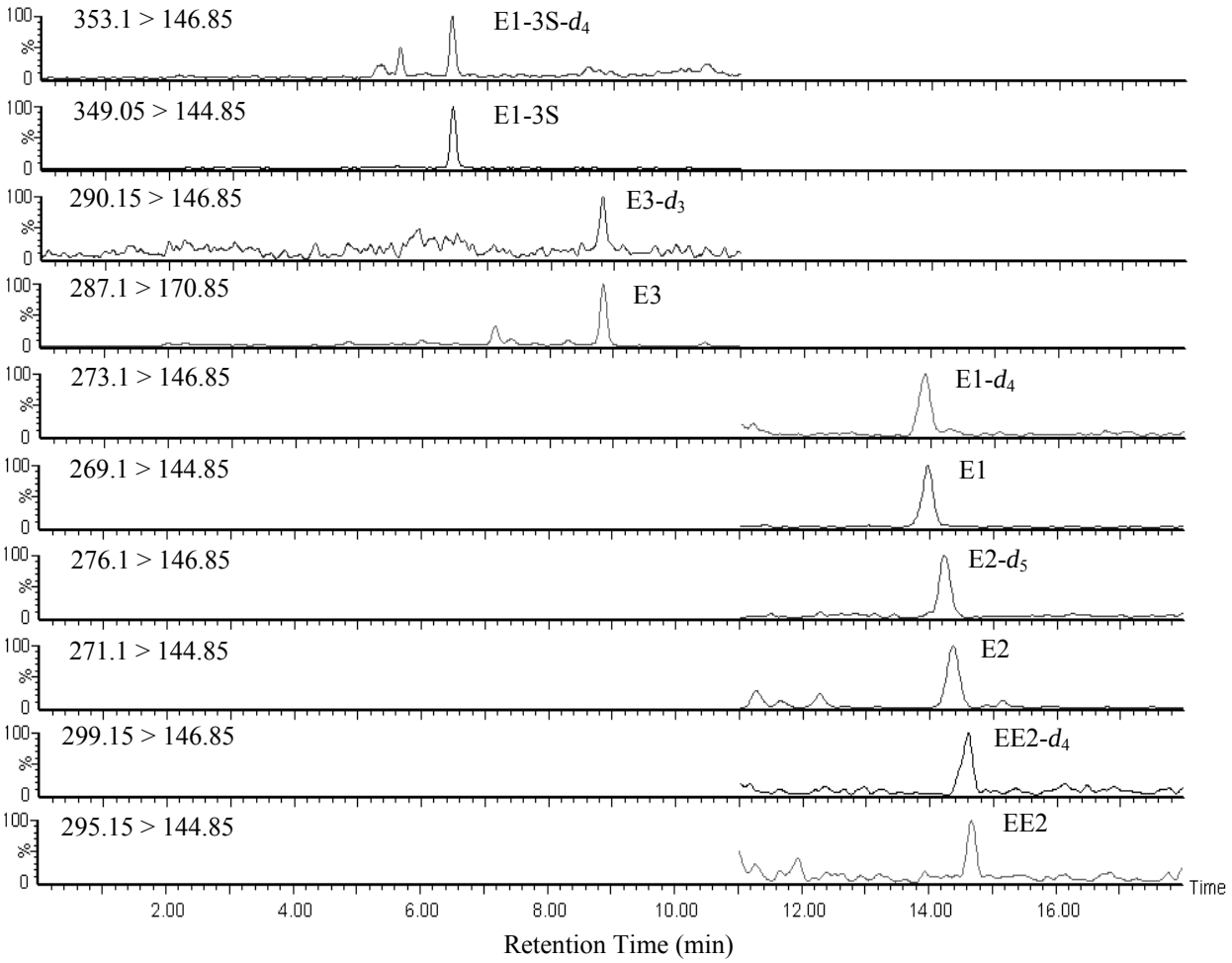

Figure 2. (a) MRM chromatograms of E1-3S-d_4, E1-3S, E3-d_3 and E3. (b) MRM chromatograms of EE2-d_4, EE2, E2-d_5, E1-d_4, E2 and E1 (settled sewage spiked at 2 ng l^{-1}).

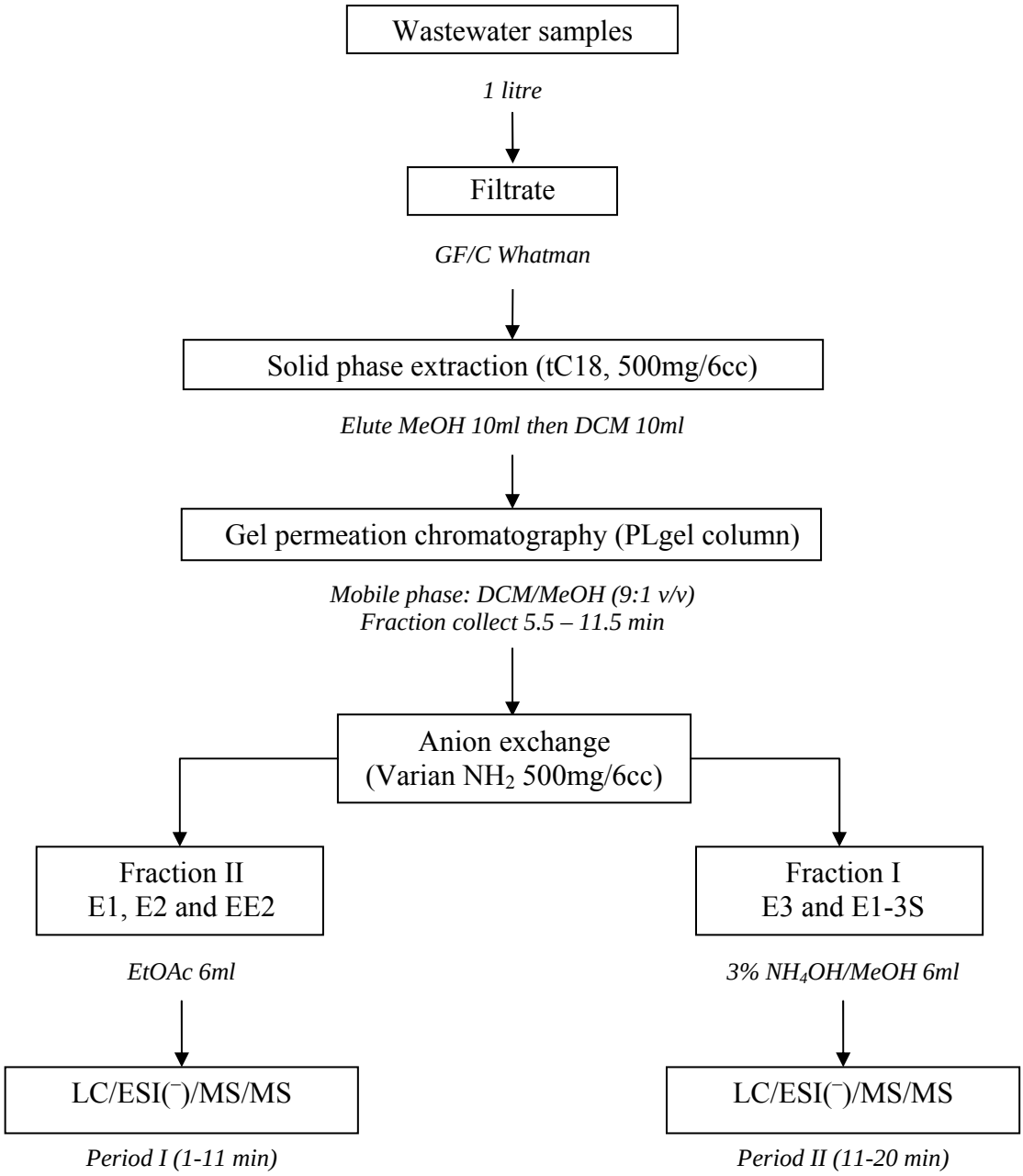

Figure 3. Diagram summarising the analytical procedure for the determination of the steroid estrogens.

Table 1. Optimised LC/MS/MS conditions for determination of the steroid estrogens[a]

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	

[a] MRM transitions: the first for quantification, the second for confirmation.
[b] Instrument detection limit at 1 ng l^{-1} replicate measurement (n=7).

Table 2. Method recoveries (%) and relative standard deviations (RSD %) and method 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

[a]2 ng of standard or [b]15 ng of standard was spiked to 1 litre of settled sewage/final effluent (15 ng l^{-1} of deuterated internal standard).

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

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	-