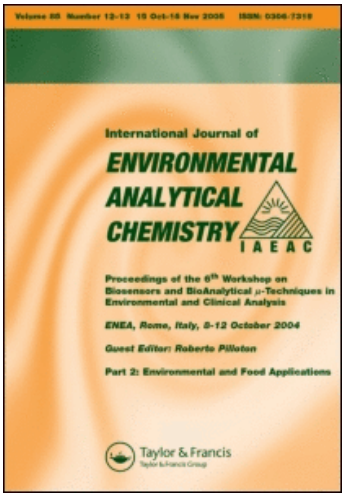

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Simultaneous determination of natural and synthetic steroid estrogens and their conjugates in aqueous matrices by liquid chromatography/mass spectrometry

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An analytical method for the simultaneous determination of nine free and conjugated steroid estrogens was developed with application to environmental aqueous matrices. Solid-phase extraction (SPE) was employed for isolation and concentration, with detection by liquid chromatography/mass spectrometry (LC/MS) using electrospray ionisation (ESI) in the negative mode. Method recoveries for various aqueous matrices (wastewater, lake and drinking water) were determined, recoveries proving to be sample dependent. When spiked at 50 ng/L concentrations in sewage influent, recoveries ranged from 62–89% with relative standard deviations (RSD) $<8.1\%$. In comparison, drinking water spiked at the same concentrations had recoveries between 82–100% with an RSD $<5\%$. Ion suppression is a known phenomenon when using ESI; hence its impact on method recovery was elucidated for raw sewage. Both ion suppression from matrix interferences and the extraction procedure has a bearing on the overall method recovery. Analysis of municipal raw sewage identified several of the analytes of interest at ng/L concentrations, estriol (E3) being the most abundant. Only one conjugate, estrone 3-sulfate (E1-3S), was observed.

Keywords: Conjugated steroid estrogens; Liquid chromatography/mass spectrometry; Wastewater; Drinking water; Humic; Matrix effects

1. Introduction

The presence of endocrine-disrupting compounds (EDCs) in surface waters has been primarily attributed to their incomplete removal from wastewater treatment [1]. Of the EDCs, natural and synthetic steroid estrogens have been implicated as the major contributors to estrogenic activity in sewage effluent [2, 3] and receiving surface waters [4, 5]. The majority of steroid estrogens are excreted from the human body in urine as conjugates, which are largely biologically inactive. However, steroids in the free, deconjugated state have been observed in sewage effluent, implying

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that deconjugation occurs prior to and/or during wastewater treatment. In contrast to other compounds that exhibit estrogenicity, the quantity of natural and synthetic steroids entering sewage treatment works (STWs) is unlikely to decrease due to their origin and use, respectively. Consequently, knowledge of their occurrence and behaviour in the STW is essential in order to assess the potential for endocrine disruption, relating to the use of wastewater as a pathway to the aquatic environment. As precursors to EDCs, conjugate determination and behaviour plays an important part in evaluating wastewater treatment removal efficiency, and environmental risk.

To facilitate removal from the body, steroids are primarily excreted in the polar conjugated form with either a glucuronide or sulphate moiety [6]. Owing to bacterial activity in the intestine, a small percentage are excreted in the biologically active (free steroid) form, via the faeces [7]. Further deconjugation is then mediated by the action of microflora present in the sewers and during wastewater treatment. Urinary excretion favours estriol (E3) > estrone (E1) > 17β-estradiol (E2); however, factors such as sex, diet, age, and the health of the individual can influence the excretory profile, causing E1 to become the most important [8, 9]. Positioning of the conjugate generally favours the carbon 3 (C3) for E1 and E2, and C16 for E3 conjugates (figure 1). The conjugate moiety favoured by E3 and E2 is estriol 16α-glucuronide (E3-16αG) and 17β-estradiol 3-glucuronide (E2-3G) respectively [10, 11]. Estrone excretion has traditionally been as estrone 3-glucuronide (E1-3G) [11], though subsequent studies have since identified estrone 3-sulfate (E1-3S) as the main urinary excretion product [10, 12, 13]. The synthetic steroid estrogen 17α-ethinylestradiol (EE2) is used in contraceptive therapy and is predominately excreted in urine, with 50–90% favouring glucuronide conjugation on the C3, and the remainder mainly as 17α-ethinylestradiol 3-sulfate (EE2-3S) [14].

Until recently, conjugate determination in wastewater and the receiving aquatic environment employed gas chromatography/mass spectrometry (GC/MS) and to a lesser extent, bioassays [15–17]. Both approaches require the hydrolysis of the conjugate to form the free steroid, with GC methodology entailing subsequent derivatisation to enable analysis [15, 18]. Levels of the conjugates are thereby deduced by subtracting the total (conjugate plus free) steroids after hydrolysis, from the free-only concentration sequentially determined in a similar sample. This indirect approach compounds errors from the inefficiency of the hydrolysis method and the recoveries of the extraction and quantification procedures [16, 19–21]. A U.S. study determined

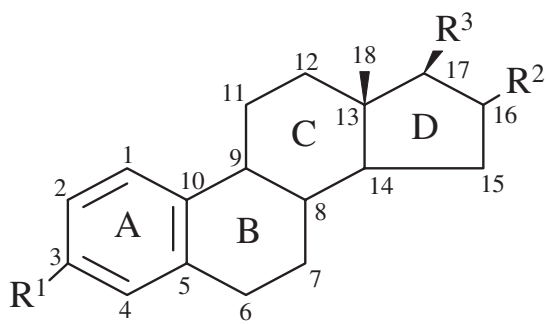


Figure 1. Basic steroid structure with carbon numbering (C1–18) and ring lettering (A–D). R1 = OH, CH_3O, or conjugate group; R2 = H, OH, or conjugate group; R3 = O, OH, or OH(C $\equiv$ CH).

the conjugate contribution in municipal effluent to be $<2\%$ of total steroids in the sample. However, the authors highlighted that the enzymatic hydrolysis step deconjugated only 30% of the sulfated conjugates and recoveries were $67 \pm 11\%$ for sulfated estradiol and $76 \pm 25\%$ for glucuronide estradiol [22]. In addition, the indirect approach is unable to elucidate the conjugate moiety and carbon positioning on the steroid, so the exact conjugate cannot be determined.

Liquid chromatography (LC) coupled with mass spectrometry (MS) allows for direct determination of the steroid conjugate, negating modification of the analyte structure to render it suitable for analysis. Recently, extrapolation from the clinical field has utilised detection by LC tandem MS (LC/MS/MS) with electrospray ionisation (ESI) [23–25] for conjugates in environmental aqueous matrices. The use of LC/MS(/MS) has the following advantages:

(i) Direct determination of the conjugate rather than a modified derivative ensures greater accuracy and allows moiety contribution and carbon positioning of the conjugate moiety to be deduced;
(ii) Reduction in time and analyte losses during sample preparation as hydrolysis and derivatisation procedures are not required; and
(iii) The opportunity to view the relationship between the free steroid and the conjugated precursors within the same analytical run, eliminating any variation or compounding of errors between different analytical runs/methods and allowing the assessment of any transformation processes between the two groups.

However, ionisation suppression can occur with LC/MS(/MS) ESI, eliciting a decrease in the MS signal response of the analyte of interest. There are several possible causes of this, a common one being matrix interferences caused by co-eluting compounds [26]. Ion suppression between a factor of 8–10 has been observed for steroid glucuronides in clinical matrices analysed by LC/MS/MS ESI, which was found to be both sample- and retention time dependent [27]. Though several analytical approaches are recognised for assessing potential matrix interferences [28, 29], they may not always achieve the goal of eliminating the suppression [30].

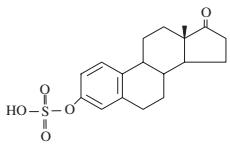
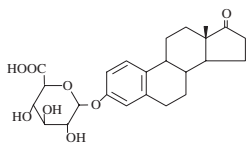
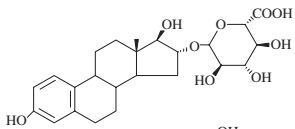
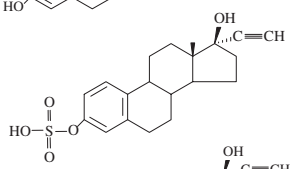
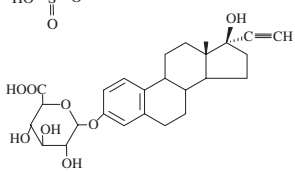
The aim of this study was to develop a methodology for free and conjugated steroid detection with application to environmental aqueous matrices. To achieve this, solid-phase extraction (SPE) was employed for isolation and concentration, followed by direct determination using LC/MS ESI. Method recoveries were evaluated for several aqueous matrices spiked at ng/L levels and the ion-suppression contribution to the overall method recovery was elucidated for municipal raw sewage. The free and conjugated steroids investigated for the study are presented in tables 1 and 2. Conjugate selection was based on the anthropogenic excretory profile summarised earlier.

2. Experimental

2.1 *Reagents*

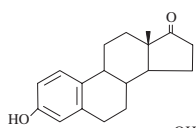
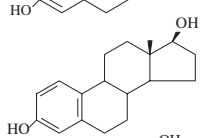
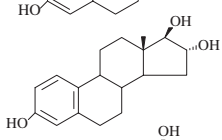
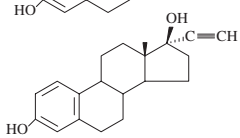
The free steroid estrogens, E1-3S, E1-3G, and E3-16αG were obtained from Sigma, UK and the EE2 conjugates (EE2-3S, EE2-3G) from Steraloids, U.S. Primary free and conjugated steroid stock solutions were each prepared at nominal

Table 1. Properties and structures of the conjugated steroid estrogens used in this study.

Conjugated estrogen	Type of steroid	Molecular formula	Molecular weight (g/mol)	Structure
Estrone 3-sulfate (E1-3S)	Natural	$C_{18}H_{22}O_5S$	350.5	
Estrone 3-glucuronide (E1-3G)	Natural	$C_{24}H_{30}O_8$	446.5	
Estriol 16α-glucuronide (E3-16αG)	Natural	$C_{24}H_{32}O_9$	464.5	
17α-Ethinylestradiol 3-sulfate (EE2-3S)	Synthetic	$C_{20}H_{24}O_5S$	376.5	
17α-Ethinylestradiol 3-glucuronide (EE2-3G)	Synthetic	$C_{26}H_{32}O_8$	472.5	

Note: Molecular weight is now a deprecated term.

Table 2. Properties and structure of the free steroid estrogens used in this study.

Free estrogen	Type of steroid	Molecular formula	Molecular weight (g/mol)	Structure
Estrone(E1)	Natural	$C_{18}H_{22}O_2$	270.4	
17β-Estradiol (E2)	Natural	$C_{18}H_{24}O_2$	272.4	
Estriol (E3)	Natural	$C_{18}H_{24}O_3$	288.4	
17α-Ethinylestradiol (EE2)	Synthetic	$C_{20}H_{24}O_2$	296.4	

concentrations of 1000 μg/mL in methanol (MeOH). These 1000 μg/mL solutions were diluted to 10 μg/mL solutions of individual conjugates in water/MeOH (90:10 v/v). All solvents were HPLC grade, obtained from Rathburns, U.K. Stock solutions and calibration standards were stored at $4 \pm 0.5^{\circ}C$ when not in use. Glassware was cleaned in 5% Decon 90 detergent, East Sussex, U.K., followed by 10% dilute nitric acid and then rinsed with ultrapure water obtained from a Maxima Ultrapure water generator (USF Elga, U.K.). Steroid adsorption and degradation on glassware has previously been reported [31]. Therefore after cleaning, the glassware was silanised with 1% dichlorodimethylsilane (Sigma, U.K.) in toluene and then rinsed with toluene followed by MeOH [9], before being allowed to dry prior to use.

2.2 *Sample preparation*

Solid-phase extraction (SPE) was undertaken using Waters™ Sep-Pak Vac 3cc reversed-phase tC18 cartridges (Watford, U.K.) and a Waters™ Sep-Pak Vacuum Manifold (Watford, U.K.). Cartridges were conditioned at a flow rate of 2.5 mL/min with 5 mL of MeOH followed by 5 mL of ultrapure water. Prior to extraction, 1 L aqueous samples were filtered through 0.45 μm GFC (VWR International, U.K.) and the filter paper was washed with 10 mL of MeOH, which was then added to the filtered sample [32]. After loading at a flow rate of 5 mL/min, the cartridge was washed with 1 mL of ultrapure water and then dried for 1 hour under vacuum prior to elution with 2 mL of acetonitrile (ACN)/water (7:3). The eluate was blown to dryness under nitrogen and then reconstituted with 250 μL of MeOH/water (8:2, v/v). For raw sewage, to avoid cartridge blocking and possible saturation, two cartridges in parallel were utilised, each receiving 500 mL of a 1 L sample. After washing and drying, the eluate from each cartridge was collected in the same vial and then dried, prior to reconstitution as above.

2.3 *Instrumentation*

The LC/MS system utilised has been previously described [33]. Ultrapure water and HPLC-grade MeOH mobile phases were delivered at 200 μL/min under gradient conditions using two Perkin–Elmer Series 200 LC pumps. Prior to use on the LC system, the mobile phases were filtered through 0.22 μm Durapore membrane filters (Millipore, U.K.). Samples were injected using a Perkin–Elmer Series 200 Autosampler with an injection volume of 20 μL. Chromatographic separation was achieved using a Synergi 4 μ Hydro-RP column (75×2 mm; 4 μm) with guard column (4×2 mm). Analysis was in single ion monitoring (SIM) and LC/MS operating conditions are summarised in table 3.

2.4 *Method detection limits, recoveries and repeatability*

Calibration standards containing all nine analytes in MeOH/water (80:20, v/v) were used to produce six-point calibration curves from 0.001 to 1 μg/mL. Each curve was fitted with a linear trendline through zero using TurboQuan v.1.0 software, the correlation coefficients of which were consistently 0.98 or above.

Table 3. LC/MS operating conditions for the analysis of free and conjugated steroids.

Solvent profile	Time (min)	Flow rate (μL/min)	Gradient profile	H_2O: MeOH (%)
	−10	200	0	90:10
	0	200	1	70:30
	10	200	1	50:50
	35	200	0	50:50
	65	200	1	10:90
	66	200	0	10:90
Ion selection	Conjugated steroid	$[M-H]^-$ (m/z)	Free steroid	$[M-H]^-$ (m/z)
	E1-3S	349.5	E1	269.4
	EE2-3S	375.5	E2	271.4
	E1-3G	445.5	E3	287.4
	E3-16αG	463.5	EE2	295.4
	EE2-3G	471.5		
MS parameters	Mode	$[M-H]^-$		
	Nebuliser gas	8		
	Curtain gas	8		
	TurboIon spray	−3.5 kV		
	Temperature	375°C		
	Orifice	Period 1, −30; Period 2, −50; Period 3, −40		
	Ring	Period 1, −170; Period 2, −210; Period 3, −180		
	Dwell (ms)	Period 1, 200; Period 2, 500; Period 3, 300		

Quality controls and blanks of MeOH/water (80:20, v/v) were incorporated into each analytical run. The limits of detection (LOD) and quantification (LOQ) were calculated from spiked environmental samples [23]. The analyte peak height (X_H), analyte concentration (X_C) and baseline variability near the analyte peak (B_V) was used to determine the method detection limit (MDL) which can be cited as the LOD or LOQ, when the signal (S) in the signal-to-noise ratio is 3 and 9, respectively [equation (1)].

$$MDL = SB_V\left(\frac{X_C}{X_H}\right) \tag{1}$$

Several aqueous matrices were utilised for recovery determination by spiking with the analyte mix at 50 ng/L. Wastewater was used to evaluate the influence of complex matrices on method stability as well as analyte recovery. Blank wastewater samples were extracted and analysed and any environmental steroid concentrations were subtracted from the spiked samples to allow an accurate assessment of method recovery. Analysis of drinking water allowed for method recoveries not influenced by interfering compounds from the matrix, whilst lake water and reagent water spiked to 5 mg/L with humic acid assessed the effect of organic matter on recovery. Recoveries were calculated by dividing the concentration obtained at the fortification level by the spiked standard solution, adjusting to a percentage. Recovery studies were repeated four times to obtain RSD.

As recoveries proved to be matrix dependent, the contributions of the extraction procedure and matrix-dependent ion suppression were assessed by relating the responses to standard solutions and samples spiked pre- and post-extraction [34]

Table 4. Approach for assessing contribution of matrix-dependent ion suppression and sample preparation on method recoveries (modified from [26]).

Approach	Explanation
Extraction yield:	Percentage recovery for the extraction procedure alone, discounting any matrix effects on ionisation
Matrix effect:	Effect of matrix extract on ionisation yield determined by comparing the standards response against the extracted matrix spiked prior to LC/MS ESI detection
Method recovery:	Effect of the extraction procedure on the absolute signal abundance is evaluated by comparing the response of standards with that for spiked, extracted samples [Extraction yield (%) × matrix effect (%) = method recovery (%)]

(table 4). This approach was assessed using raw sewage, the most complex of aqueous samples, and therefore the sample likely to contribute the greatest matrix interference.

3. Results and discussion

3.1 *LC/MS method development*

Single standard solutions were used to identify $[M-H]^-$ ions and peak-retention times. Several solvent methods were tested in developing a LC method to separate the five conjugated steroids. Each solvent programme incorporated a 10 min equilibration period prior to sample injection. Conjugate separation utilised a gradual but continuous gradient, allowing resolution of all five conjugates. Separation of free steroids has previously been achieved using an isocratic period of 50:50 v/v aqueous–organic solvent [33]. This was therefore applied to the LC method after conjugate elution, accounting for the hydrophobic nature of the free steroids. The time necessary to elute all four free steroids was experimentally determined using a standard mix of both free and conjugated steroids. Separation between free and conjugated steroids during the same LC/MS analytical run is of particular benefit when evaluating transformation processes that occur between them. Methanol was chosen over ACN as the organic mobile phase, as co-elution between the conjugates and free steroids occurred when using ACN. In addition, MeOH has previously been found to give superior chromatographic resolution with regard to steroid conjugates [17], as well as increased sensitivity [23]. To improve sensitivity and allow greater manipulation of MS settings, chromatographic separation was divided into three acquisition periods. The retention times of free and conjugated steroids are illustrated in figure 2.

To further improve sensitivity, a 0.1 μg/mL standard of each steroid at 200 μL/min was infused and the operating parameters affecting MS detection for $[M-H]^-$ were sequentially optimised by taking into account the intensity differences observed in the sample signal. In this way, temperature, ion spray voltage, ion spray positioning, nebuliser gas, heater gas, and curtain gas settings were optimised in that order. In each case, the optimum setting was judged to be that producing the greatest signal measured in counts per second (cps). The orifice and ring settings were optimised by automatically ramping in increments of 5 and 10, respectively, throughout their operating ranges. Optimum dwell on each ion was considered

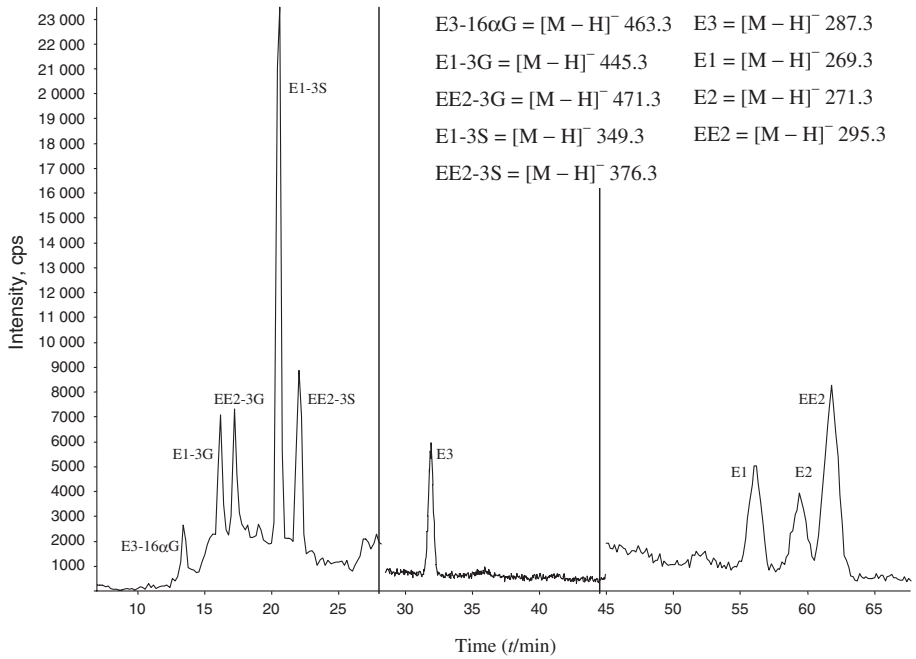


Figure 2. Chromatogram of conjugated and free steroids spiked to 150 ng/L in sewage influent prior to extraction.

that which resulted in superior signal-to-noise (S/N) resolution, an example of dwell influence can be observed in figure 3. Using the optimised MS settings, greater sensitivity was achieved for all steroids (table 3).

When using LC/MS, analyte identification by one quantification ion rather than two (quantification and confirmation) ions increases sensitivity but may reduce specificity as matrix interferences may have the potential to elute at an identical retention time and $[M-H]^-$ as the analyte of interest. The ratio between the quantification and confirmation ion can be used to confirm that the compound detected in the sample is the analyte of interest. Indeed, using confirmation ions is not a problem for most compounds when analysing by LC/MS, as their environmental presence is sufficiently high that sensitivity does not become compromised. Two approaches to overcoming any uncertainty are to rerun either in full scan [35] or using different MS settings to achieve dominant fragmentation ions other than $[M-H]^-$ [26]. Such an approach is not required when utilising LC/MS/MS, as further fragmenting the chosen ion to give the daughter ions retains sensitivity and confirms quantification [24]. However, as sensitivity is less compromised with tandem MS, two ions may be used and then each further fragmented to produce daughter ions, giving unequivocal confirmation [23].

3.2 *Sample preparation*

Solid-phase extraction procedures are often inadequate, only removing a proportion of the matrix interferences, and have limited success in negating ion suppression [36]. Detection at ng/L levels necessitates sample preconcentration but will also concentrate

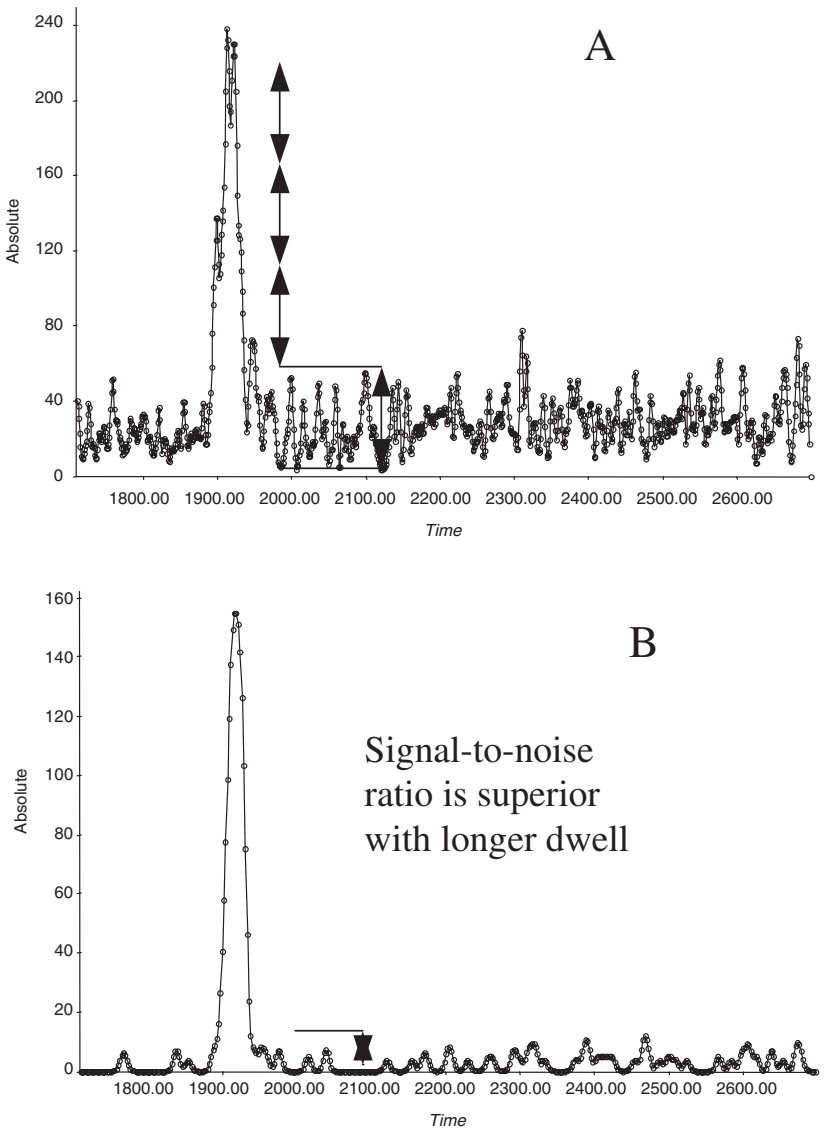


Figure 3. Influence of dwell parameters on peak resolution A, 50 ms dwell on $[M-H]^-$ ion ±0.25 amu; B, 200 ms dwell on $[M-H]^-$ ion ±0.25 amu.

any potentially interfering contaminants, which are often present in far greater quantities than the steroids. Therefore, a balance must be achieved between using a sample volume which when concentrated allows detection limits of environmental relevance whilst limiting the amount of matrix interference in the sample to be analysed [26].

Several SPE procedures were assessed using 1 L of ultrapure water spiked to 50 ng. The use of ultrapure water for determining extraction recoveries allowed for an assessment of the extraction recovery alone and also avoided signal suppression by co-extracted humics which are identified as the main cause of ion suppression [37]. When eluting with 10 mL of MeOH followed by reconstitution after drying [31],

recoveries of 70–103 ± 6% were achieved. Repeating with sewage effluent led to decreased recoveries due to a culmination of losses from the extraction procedure and ion suppression observed on the LC/MS. The increase in ion suppression observed was likely a result of organic interferences co-eluting from the SPE cartridge. Their presence may also have led to breakthrough of the steroids due to saturation of the adsorption sites on the cartridge.

A dual-fraction procedure analysed without further drying or processing was also assessed, resulting in recoveries between 95–105 ± 8%. The first fraction was designed as a wash {2.5 mL of 24 mM ammonium acetate [NH_4Ac], followed by 5mL of MeOH/24 mM NH_4Ac 45/55 v:v and then 7.5 mL of water} whilst the second would elute the analytes of interest using 5 mL of MeOH/water (40:60, v:v). Contrary to the findings from the original study [19], the conjugates were predominately eluted during the washing stage with the exception of E1-3S, which eluted in both fractions. As analysis of the fractions was without subsequent drying, which allows sample concentration, obtaining detection limits of environmental relevance would have been unachievable using this approach.

A 2 mL mixture of ACN/water (7:3, v/v) was finally used for SPE elution and, after drying by nitrogen and reconstitution, gave recoveries for the nine analytes between 82–100 ± 5%. It is likely that sample drying and reconstitution may result in some loss of steroids; however, if sensitivity was not an issue, the extracted sample could be injected directly onto the analytical tool without the need for drying and reconstitution [38, 39]. It must be noted that raw sewage utilised two SPE cartridges in series whereas all other aqueous matrices used just one cartridge, a decision made as loading of 1 L onto one cartridge sometimes led to reduced flow likely due to blockages. This different approach will have a bearing on the resultant recoveries, as has been observed for a similar set-up [40].

3.3 *Method recoveries, matrix contribution and detection limits*

It can be hypothesised that cleaner matrices such as drinking water will contain less interference, and hence are less likely to saturate the extraction cartridges and cause suppression on LC/MS. Saturation of the SPE cartridge will allow both steroids and other organic compounds present in the matrix to pass through at both the loading and eluting stages. Method recoveries for several aqueous matrices are shown in table 5.

Variation in method recoveries for different samples has been observed for conjugated steroids in environmental samples, with the authors citing uncertainty as to the cause [24]. Recoveries for E1-16αG from 250 mL and 100 mL of sewage influent were 27 and 68%, respectively [23] and those authors proposed that saturation of adsorption sites during SPE by organic compounds present in the matrix may partially or completely contribute. Results from this study infer that the matrix will also be responsible for eliciting varying degrees of ionisation suppression on the LC/MS(/MS) and, coupled with the extraction procedure, will decrease method recoveries (table 6). Hence, assessing the potential matrix influence on the method recovery when using LC/MS(/MS) for quantification is an important consideration and it must not be assumed that method recoveries are dependent on the extraction procedure alone.

Table 5. Percentage method recoveries for 1 L aqueous matrices spiked to 50 ng/L [RSD from 4 samples].

Aqueous matrix		E1	E2	E3	EE2	E1-3G	E1-3S	E3-16αG	EE2-3G	EE2-3S
Drinking water	Mean	97.4	99.3	100.2	99.1	84.5	93.6	82.4	81.9	97.2
	RSD	3.9	4.3	4.1	3.9	4.3	4.9	3.3	3.4	3.4
Reagent water + humic 5 mg/L	Mean	102.7	90.3	87.3	91.9	80.7	78.8	76.0	76.9	94.3
	RSD	2.2	2.8	3.6	1.6	2.6	3.0	2.5	1.9	2.0
Lake water	Mean	81.3	91.1	97.8	89.3	86.3	88.9	73.7	79.7	85.9
	RSD	3.9	3.2	2.9	3.1	2.2	4.1	1.8	3.1	2.8
Sewage effluent	Mean	74.6	74.3	78.4	87.2	74.8	83.3	70.5	74.6	84.5
	RSD	2.4	2.4	3.8	2.4	3.2	5.1	4.2	2.1	3.1
Sewage influent	Mean	67.8	66.5	71.7	88.5	63.3	80.3	61.8	62.8	79.6
	RSD	3.0	2.8	7.5	3.9	3.7	5.4	7.5	3.5	7.1

Table 6. Influence of sample preparation and LC/MS quantification on method recovery for steroid estrogens spiked to 50 ng/L in sewage influent (%) [* = 100% equates to no ion suppression occurring on LC/MS; $n = 4$].

Approach		E1	E2	E3	EE2	E1-3G	E1-3S	E3-16αG	EE2-3G	EE2-3S
Extraction yield	Mean	72.2	75.4	82.8	95.6	71.0	83.9	69.4	88.3	86.5
Matrix effect*	Mean	93.9	88.2	86.6	92.6	89.5	95.7	89.0	71.0	92.0
Overall recovery	Mean	67.8	66.5	71.7	88.5	63.3	80.3	61.8	62.8	79.6
	RSD	3.0	2.8	7.5	3.9	3.7	5.4	4.2	3.6	8.1

Whether recoveries are matrix related has been investigated in several studies. Recoveries for SPE-LC/MS/MS of pesticides in tap and river water observed a matrix-related recovery [40]. In another study, no matrix influence was observed on method recoveries when assessed using deionised water and effluent [41]. This study also analysed influent for steroid levels, though no reference is made to method recoveries obtained using this matrix. However, the authors state that matrix interferences were observed during the derivatisation stage. Another study concluded that recoveries were not matrix dependent as long as sample volumes were not exceeded (100 and 250 mL for influent and effluent, respectively) [25]. Recoveries were obtained by analyte addition to previously analysed samples which is a recognised method for assessing ion suppression [29], although it is more accurate to infuse post-column to discount losses from chromatography [42, 43] so any steroidal losses occurring from the extraction stage (whether due to saturation of adsorption sites or incomplete elution) are not taken into account. Rather than determining the overall method recovery, as well as losses from extraction and machine recoveries (ion suppression and machine variability), only the recoveries relating to the machine have been evaluated. In fact, as machine variability was compensated for with the use of an internal standard (I.S.), these recoveries correlate to the ion suppression observed on the LC/MS/MS and thus the variation in recoveries can be attributed to the different matrices.

Using the TurboQuan software and equation (1), the LOD for each environmental aqueous matrix were calculated (table 7). Limits of detection were the poorest for glucuronides, and thus is likely to be due to their being both less acidic and less polar than the sulfated conjugates [23]. The LOD appears to be

Table 7. Limits of detection (ng/L) as signal-to-noise ratio 3:1 calculated from 1-litre environmental samples spiked at 50 ng/L with steroids.

Aqueous matrix	E1	E2	E3	EE2	E1-3G	E1-3S	E3-16αG	EE2-3G	EE2-3S
Drinking water	1.0	1.1	0.8	0.6	2.3	0.8	4.5	2.6	1.6
Lake water	1.2	1.5	1.1	0.8	2.8	0.9	5.1	3.1	1.6
Sewage effluent	1.1	2.0	1.5	0.9	3.3	1.0	5.3	3.2	2.2
Sewage influent	2.4	3.9	2.1	1.8	6.1	1.9	7.1	5.7	3.5

matrix dependent, with little difference observed between the lake and sewage effluent samples.

Regarding spiked concentrations, LOQ determination in one study was by analyte addition with the criterion being to at least quadruplicate the original environmental concentrations [25]. For the same steroids analysed in this study, spiking would have been between 17.2–288 ng/L in influent and 6.4–68 ng/L in effluent (assuming analyte addition is only quadruplied). However, for samples that did not initially contain any of the analyte(s) of interest, no reference is made as to what concentration was added in order to determine the LOQ.

Another approach was to spike each matrix at different concentrations, 10 ng/L for effluent and 20 ng/L for influent [23]. In this study, similar spiking in all matrices allowed comparison between the recoveries and LODs. However, a further improvement could be to determine at several concentrations (*e.g.*, 5–50 ng/L) and use the gradient of the plot between analyte concentration and corresponding peak height, which would account for any impact lower concentrations may have on the recoveries.

3.4 *Application to environmental samples*

To test the validity of the method, duplicate drinking water, lake water, and raw sewage samples from two STWs were analysed, one of the STWs receiving predominantly municipal influent, the other a large percentage from industry. To negate the addition of any preservatives that may affect the analytes within, immediately after being sampled the 1-litre samples were filtered through 0.45 μm GFC filters and loaded onto pre-conditioned SPE cartridges. The cartridges were then washed, dried for one hour, and frozen before being transported to the main lab where they were then de-thawed, re-dried to remove any condensation, and then the steroids were eluted from the cartridge.

No conjugated or free steroids were observed in the drinking water or lake samples. Though free and conjugated steroids have been detected in receiving aquatic waters [16, 25], it is likely that these are eliminated during retention in the surface waters and drinking water treatment as a result of transformation and sorption processes. However, free steroids have been identified in drinking water in Germany [44], highlighting potential concerns for water reuse [45]. In the treatment works receiving predominately municipal waste, several of the analytes were identified, with E1 at 21.07 ± 0.18 ng/L. Concentrations for the nine free and conjugated steroids in raw municipal sewage are depicted in figure 4. Estrone 3-sulfate was the only conjugate detected, its presence being due to the recalcitrant nature of the sulfate moiety [25]. No EE2, either in the free or conjugated form, was observed in any samples. In contrast, from the sewage treatment works receiving a high proportion

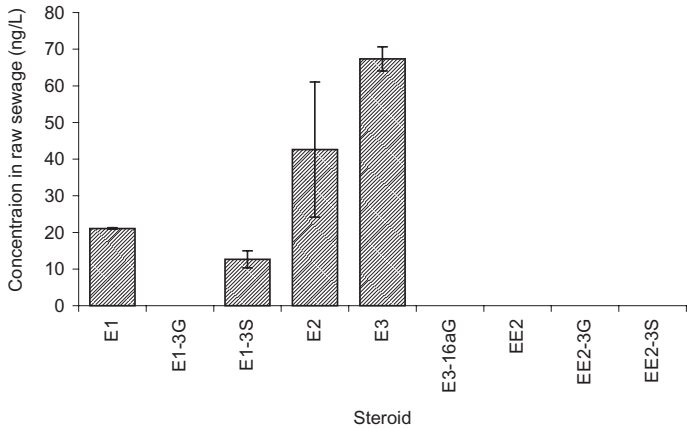


Figure 4. Free and conjugated steroid estrogens identified in the raw sewage of a municipal wastewater treatment plant from spot duplicate samples with ± standard deviation expressed as error bars.

of industrial waste, only E1 was detected in the influent at 3.70 ± 0.64 (standard deviation) ng/L.

4. Conclusions

Liquid chromatography/mass spectrometry ESI preceded by solid-phase extraction allows for simultaneous direct determination of free and conjugated steroids in aqueous matrices. The major advantage of this method over GC/MS(/MS) quantification is that conjugated steroids can be analysed intact, without hydrolysis and derivatisation which are required prior to GC analysis. In addition, conjugate moiety, positioning on the carbon skeleton, and quantification of each free and conjugated steroid can be deduced within the same analytical run. The sample matrix had some influence on both the method recovery and LODs. An evaluation of matrix effects and extraction procedure on overall method recovery show that both will decrease steroidal recoveries and are analyte dependent. The method proved to be robust upon application to several environmental samples. No steroids were identified in lake or drinking water samples. For sewage influent receiving a large proportion of industrial waste only E1 was observed, whilst in municipal raw sewage several of the analytes were detected, only one of which was conjugated (E1-3S).

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