ANALYSIS OF SOIL, SEDIMENT AND SLUDGE

Steroid estrogen determination in sediment and sewage sludge: a critique of chromatographic / mass spectrometry methods incorporating a case study in method development

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Abstract

Current analytical methods for determining steroids in sewage sludge and sediment are reviewed and experimental data comparing LC/MS ESI and GC/MS quantification of extracts from these matrices is presented. The effect of matrix influence on method analysis and steroid recoveries was investigated and a comparison of GC and LC determination with mass spectrometry performed. Consequently upon this, an environmental analytically robust methodology is presented and using this technique analysis of sediments determined E1 to be the main steroid (<18 ng/g), EE2 and E3 were determined at far lower concentrations with minimal detection of E2.

Keywords: steroid estrogens; solid matrices; liquid chromatography/mass spectrometry; gas chromatography/mass spectrometry; matrix effects

Abbreviations: ACN, Acetonitrile; AP, alkylphenol; APCI, Atmospheric pressure chemical ionisation; AS, Activated sludge; BSTFA, N,O-Bis(trimethysilyl)trifluoroacetamide; DCM, dichloromethane; dw, Dry weight; E1, Estrone; E2, 17β-estradiol; E3, Estriol; EE2, 17α-ethinylestradiol; E2d4, deuterated 17β-estradiol; EC, Electrochemical; EDC, Endocrine disrupting compound; EEq, Estradiol Equivalent; E1, Electron impact; ESI, Electrospray ionisation; GC, Gas chromatography; GC/MS(MS), Gas chromatography/mass spectrometry (tandem); GPC, gel permeation chromatography; HPLC, High performance liquid chromatography; IS, internal standard; LC, Liquid chromatography; LC/MS(MS), Liquid chromatography/mass spectrometry (tandem); LOD, Limit of detection; MAE, microwave-assisted extraction; MS, Mass spectrometry; MeOH, Methanol; $NaSO_4$, sodium sulphate; ND, not detected; NI, negative ionisation; PLE, Pressurised liquid extraction; RAM, Restricted access materials; RP-HPLC, Reversed phase-high performance liquid chromatography; Rt, retention time; SBR, sequencing batch reactor; SIM, selected ion monitoring; SPE, Solid phase extraction; std. dev, Standard deviation; STW, sewage treatment works; TIE, Toxicity identification and evaluation; TMS, Trimethychlorosilane; ToF, time-of-flight; UV, Ultraviolet; ww, Wet weight; YES, Yeast estrogen screen

1 Introduction

Steroid estrogens of anthropogenic origin have been identified as the major contributors to endocrine disrupting activity in both sewage effluent and surface waters. They are

hydrophobic organic compounds of low volatility and fugacity level 1 (sediment phase) output data has shown good correlation with steroid removal from the aqueous phase [1]. Within treatment processes and the environment, the rate of biotransformation increases until log K_{ow} reaches 3 to 3.5, after which sorption processes dominate in determining their fate [2]. Therefore, E1 (log K_{ow} 3.43), E2 (log K_{ow} 3.94), and EE2 (log K_{ow} 4.15), would be expected to demonstrate a preference for partitioning to the solid phase, however, the lower log K_{ow} of 2.81 for E3 infers it is less likely to be associated with the solids [3].

Estrogenic activity has been identified in sediment and sewage solids [4,5] and field work has confirmed that steroid estrogens will partition to the solid phase in sewage treatment and onto sediment in the receiving aquatic environment [6-8]. Even the relatively hydrophilic E3 having been detected in 79% of all analysed sediment samples [9]. The ability to determine concentrations of steroid estrogens within the solid phase allows for a full assessment and understanding of removal processes in wastewater treatment works. While data on their presence in sediments is important to allow for a full environmental risk assessment to be undertaken, as bed sediments may act as environmental reservoirs controlling bioavailability [1,10,11]. Difficulties associated with the determination of steroid estrogens bound to solids in both wastewater treatment processes and the aquatic environment have resulted in the use of approaches that have avoided direct determination of the compounds in these matrices. A number of laboratory studies investigating fate and partitioning behaviour have therefore utilised an indirect approach to assess the interaction of steroids with the solid phase where concentrations have been determined by difference (Table 1).

Although an indirect approach is viable for use in such studies, it does not give direct information on concentrations in the solid phase. In addition, a full directly measured mass balance also allows for the evaluation of degradation, as the assumption must otherwise be that all the difference in concentrations must be a result of sorption. From an analytical viewpoint, however, techniques used in Table 1 also suffer from several drawbacks when analysis is undertaken for quantification of residues in environmental samples. Quantification by HPLC with UV and fluorescence lacks the sensitivity and specificity for samples such as sediments, where steroids have been detected at ng/kg levels [6], while radio-labelled materials can only be used under laboratory conditions, and field data is frequently required to back up such studies. Mass balances cannot be accurately produced if only one compartment is being determined in a multi-compartmental (e.g. solid/water) system. As a

result, environmental analysis requires a direct approach involving extensive sample pre-treatment prior to chromatographic separation and then linking to MS to achieve the required sensitivity and greater selectivity.

[Insert Table 1]

Prior to final quantification, extraction and clean up are undertaken, the extent of manipulation required to isolate steroid estrogens from sediment or sewage sludge being dependent on the quantity of analyte present in the sample; contamination of the solid matrix with co-extractives; and the analytical tool to be utilised [19]. Steroid estrogens enter sewage treatment and resulting watercourses at ng/l concentrations, of which only a proportion will bind to the solid phases. In order to detect at these levels, sample pre-concentration is necessary but will also concentrate any potentially interfering contaminants, which are often present in far greater quantities than the steroid estrogens. Though sample preparation is able to remove matrix interferences, the more intense the extraction and clean up procedures, the greater the potential for analyte losses resulting in lower recoveries. A compromise must therefore be made between the need for detection limits of environmental relevance; dependent on sample amount and volume injected onto the analytical instrument, whilst limiting the amount of matrix interference present in the sample to be analysed. For 'dirtier' sludge samples, 0.5g were used compared to 5g of sediment and recoveries were >70% and >90% respectively due to recovery losses from the more thorough sludge clean up required to remove as many interferences as possible [7]. Analyte recovery can also be concentration dependent giving lower recoveries when spiked at smaller concentrations [20]. Hence, it is prudent to spike at concentrations similar to those found environmentally when determining method recoveries. The choice of analytical tool can influence such decisions; GC/MS/MS used an injection volume of 2 µl, which also had the advantage of introducing less co-eluting interferences, compared to 40µl required for GC/MS to obtain similar sensitivity [21]. Single MS may give rise to false positives due to the more limited selectivity and it has been hypothesised that environmental concentrations may be overestimated when analysed by GC/MS [22]. The study found an unidentified impurity to have a similar Rt and m/z values as EE2 and concluded that MS/MS spectra is required to distinguish between the two. Though Rt and m/z values identified by GC/MS were similar, the ratio between the precursor and confirmation ions for the two contaminants were different and could be utilised for distinguishing between analytes of interest and interferences giving similar responses.

Detector choice can also influence sensitivity, quadrupole MS has been predominately used though ion trap can increase sensitivity by 'trapping' a larger number of molecules for ionisation.

Analysis has predominately utilised GC/MS for steroid determination in solid matrices [6,7,23,24] although LC/MS is becoming more popular [9,25-27] and may become the analytical tool of choice similar to other environmental contaminants and drug determination in the clinical field. A comparison of sensitivity between different analytical tools favoured LC/MS/MS > GC/MS/MS > LC/MS [28]. Matrix effects can be observed with any analytical tool due to insufficient co-extractive removal during the sample preparation stage. Ionisation suppression is a known phenomenon in LC/MS ESI [29], occurring in the ionisation chamber and modifying the ionisation yield, as a result of the presence of co-eluting compounds, and may cause a decrease in response in the MS signal produced by the analyte of interest. This paper presents the results obtained from both LC/MS and GC/MS quantification during the method development for the determination of steroid estrogens in sediment and sewage sludge samples. The results are discussed with reference to issues related to sample preparation and the use of LC/MS systems with ESI to highlight issues related to problems encountered with reproducibility during method development.

2 Materials and Methods

2.1 Reagents

Steroids (powder form, purity of >99%) and the derivatising reagent BSTFA + 1% TMS were obtained from Sigma-Aldrich (Poole, UK). The deuterated estradiol standard, E2d4, was a gift from LGC (Teddington, UK). All solvents were HPLC grade (Rathburn, Walkerburn, UK). Solutions and standards were stored at 4 ± 0.5 °C when not in use. Glassware were cleaned in 5% detergent (Decon 90, East Sussex, UK) followed by 10% dilute nitric acid and rinsed with ultrapure water. Prior to use, dried glassware was silanised with 1% dimethyldichlorosilane (Sigma-Aldrich, Poole, UK) in toluene, then rinsed with toluene and MeOH [30].

2.2 Extraction and Clean up Procedures

In 25 ml capped Teflon tubes, 5 g dried sediment or 0.5 g dried sludge were extracted on a rotary shaker (Jouan S.A. C3*i*, Saint-Herblain, France) with 10 ml of hexane/acetone (1:1) for

1 hour. The samples were then centrifuged for 10 minutes at 2500 rpm and the liquid phase collected. Samples were further extracted with diethyl ether/hexane (10:1) for one hour, centrifuged as previously and supernatant decanted. The combined supernatants were evaporated to dryness by rotary evaporation (40^{o}C) and blown to dryness with nitrogen.

Samples were reconstituted to 1 ml with DCM: hexane (1:1), for open column GPC on a 10mm diameter glass column packed to a bed depth of 200mm with Bio Beads SX-3, 200-400 mesh (Bio-Rad Laboratories, Hercules, California, USA). Samples were quantitatively loaded onto the column in 1 ml of solvent, followed by a wash of 10 ml DCM: hexane (1:1). Subsequently, the column was eluted with 18 ml of solvent, which was collected and evaporated as before. The column was then further washed with 15 ml of eluent prior to re-use. After evaporation of the eluent samples were reconstituted in water/methanol (10:1) prior to cleanup on C18 solid phase extraction cartridges (3cc Sep-Pak C18 Waters, Watford, UK). Cartridges were conditioned at a flow rate of 2.5 ml/min with 20 ml of MeOH followed by 10 ml of ultra pure water. The sample was then loaded onto the cartridge, which was dried for 30 minutes under vacuum prior to elution with 2.0 ml of ACN/ water (7:3). The eluate was blown to dryness under nitrogen. For LC/MS analysis, sample reconstitution was either with 250 µl of ACN: water (8:2) or MeOH: water (8:2, v:v). For GC/MS analysis, the sample was derivatised with 30µl of the BSTFA/TMS mixture at 70^{o}C, blown down and then reconstituted with 200µl of hexane for quantification.

2.3 Operating Conditions for LC/MS and GC/MS

The LC system utilised has been previously described [31]. Two separations were developed to allow for the impact of a longer run time, and therefore theoretically improved separation from co-eluting compounds, to be investigated. A run time of 24 minutes was developed using ACN: water mobile phase with a RP Hypersil BDS C18 column (100 × 2.1mm, 5µm), with a gradient 10 to 80% ACN over 24 minutes (200µl/min) and a 60 minute separation used MeOH:water mobile phase with an RP Synergi column (100 × 2.1mm, 5µm) with 10 to 90% methanol again at 200 µl/min. Detection utilised time scheduled SIM conditions, with an electrospray interface operating in NI mode $[M–H]^{-}$. Gas chromatography MS determination was carried out according to [1]. Identification and quantification was by SIM, with peak identity confirmation using a secondary ion.

2.4 Experimental

Samples for spiking and analysis were air dried for 72 hours and ground to prior to analysis. Initial studies involved spiking of bulk wet sediment (concentrations of 3.5-5.1 μg/g dry weight) which was allowed to air dry. However for subsequent determination of method recoveries and matrix experiments 5 g of (air) dried sediment or 0.5 g of dried sludge were spiked with the addition of 5 ml acetone to aid mixing [7] and left for several hours to allow sorption processes to occur. The effects of the sediment matrix and sample preparation on analyte recovery were made by comparing the responses of the instrument to standard solutions and spiked samples (Table 2) [32].

[Insert Table 2]

3 Results

3.1 Spiking of wet and dry sediments

Initial method development for quantifying the steroid estrogens in sediments involved spiking wet sediment with a standard mixture in acetone and air drying over 72 hours. Recoveries for this work were E1 238.5%, E2 0.9%, EE2 75.3% and for E3 27.1%, with the value for E2 being much less than expected and not readily explicable through consideration of the technique in chemical terms. To further study the poor recovery of E2, a subsequent batch of sediment was spiked with only this steroid (at 3.5 μg/g dw) and again allowed to air dry. The sample was quantified with a mixed standard and recovery of E2 was 4.7%. However, E1 was also detected in the sample at a concentration of 1.8 μg/g. To determine a recovery figure for E1, a further sample was spiked with this compound and a figure of 54% was obtained. Correcting the concentration of E1 detected when spiking sediment with E2 for this recovery, the actual concentration measured was 3.3 μg/g (dw), which is in good agreement with the original spike concentration of E2 (3.5μg/g). It is apparent that the E1 present was formed through transformation of E2 during the preparation (air drying) of the sediment after spiking. Repeating the experiment with autoclaved sediment confirmed that E2 transformation to E1 was a biotic process. This rapid transformation of E2 to E1 following spiking has implications for the determination of E2 in terms of sample handing and processing prior to extraction and in situations where air drying is employed, consideration should be given to the transformation processes that may occur. As a result, spiking of the solid matrix for recovery determination was carried out after drying of the sample to assess extraction efficiency only and avoid analyte loss being attributed to transformation processes.

3.2 Ionisation suppression in LC/MS analysis

To compare the performance of the two chromatographic separations, extracted samples were run on both the shorter run time, ACN/water, mobile phase system and then on the longer, higher resolution separation which utilised MeOH and water. There was poor agreement of quantification between the methods, despite good external standard calibration in both systems (correlation co efficient >0.99), and there was no response observed for E3 on the shorter run (Table 3). Subsequent replacement of the Hypersil BDS column utilised in the short run with a new column led to improved recoveries with a response being observed for E3 (57.1%). It is therefore possible that highly retained compounds from previous injections were eluting from the column and affecting the ionisation process in the ESI source in addition to the possible impact of co-eluting compounds from each injection and that historical use of the LC column may impact on quantification. Both the gradient programs (ACN and MeOH) used did include a final wash step with high organic content (90%) for 10 minutes, and extension of this wash is currently being assessed to determine if method robustness can be improved.

[Insert Table 3]

As the separation with longer run time (MeOH/water gradient on the RP Synergi column) appeared to be more robust, this was utilised to study the relative impacts of matrix effects and sample preparation procedures on quantification. Method recovery was adversely influenced from both sample losses from the extraction/clean up procedure and from matrix interferences (ionisation suppression effects in the ESI source). Evaluation of losses from sample preparation effects alone (extraction yield) resulted in recoveries of 60 – 70% (Table 4). However, as a result of further signal loss, with contributions ranging from 17 – 26% within the LC/MS system due to matrix effects, overall method recoveries were found to be below this as shown in Table 4.

[Insert Table 4]

3.3 Use of E2d4 to study matrix effects

Further study of the possible impact of matrix induced ionisation suppression were undertaken through the addition of E2d4 as an instrumental internal standard to samples prior to LC/MS analysis utilising the 60 minute separation, previously shown to be more robust in

response to matrix effects. Theoretically, the use of such internal standards should improve precision, however, in this instance external standard calibration resulted in greater reproducibility (standard deviations: E1 2.3%, E2 0.3%, E3 3.6% and EE2 2.8%) than using an internal standard calibration (E1 29.5%, E2 7.5%, E3 11.1% and EE2 11.5%). Calibration curves for both external and internal standard calibration were both linear, however, variation in the instrument response to the internal standard (E2d4) added to sample extracts was noticeable with responses for samples up to 50% below those obtained from standard solutions (Figure 1). It was also apparent that a significant amount of noise was present in the SIM from the sample extract in Figure 1 and the selectivity of the single quadruple as a detection method is more limiting than tandem for the complex nature of environmental samples. It is apparent from the above results that suppression effects for the co-eluting E2 and E2d4 are different, as the reproducibility was not improved when using the internal standard calibration method.

[Insert Figure 1]

3.4 Comparison of LC/MS and GC/MS results

As a result of the issues related to ionisation suppression observed during quantification by LC/MS, a comparison with GC/MS was undertaken by extracting spiked sediment (native concentrations of all analytes below detection limits). After SPE cleanup, each sample was split into two fractions, one to be used for quantification by LC/MS and ~~also~~ the second fraction undergoing derivatisation prior to GC/MS quantification. The LC/MS separation again utilised an MeOH/water gradient shown to be less prone to influence by ionisation suppression in the ESI source. There were significant differences in the results obtained for the same samples by both techniques, in terms of both total recoveries and the standard deviations in results based on 6 samples (Table 5). For sediment samples, analysis by LC/MS gave better recoveries, though generally with greater standard deviations than that obtained by GC/MS. Comparing activated sludge data, LC/MS recoveries were low though standard deviations were better than when analysing sediment samples. Recoveries for E1 had the greatest variation between GC/MS and LC/MS analysis. Whether analysed by GC/MS or LC/MS, recoveries for each steroid varied between sediment and sludge samples, one reason being due to larger matrix interference when analysing sludge samples.

[Insert Table 5]

3.5 Influence of sample origin on method recovery

It would appear from Table 5 that method recoveries are dependent on the sample matrix, irrespective of analytical technique utilised. Sludge samples from two STWs were used to determine method recoveries and assess whether sample origin affected similar matrices. Biological treatment in the two STWs was by different processes, one utilised SBRs and the other an AS process and analysis was undertaken by LC/MS. Recoveries for each of the four steroid estrogens was lower in SBR sludges compared to sludges obtained from the AS process.

[Insert Figure 2]

4 Discussion

Irrespective of the analytical technique utilised in the environmental analysis of steroid estrogens, an important consideration are the potential problems that may arise due to matrix interference especially when dealing with complex matrices such as sediment or sludge. When analysing by LC/MS ESI the potential for matrix interference should be fundamental to method development, as this approach is prone to ion suppression. There are several recognised analytical approaches that can be utilised to account for potential matrix interferences [33,34], which include:

1. The use of stable isotopic internal standards;
2. Thorough extraction/clean up procedure for removal of interfering contaminants;
3. Use of eluent additives to increase ionisation for LC/MS;
4. Calibration standards made up in the matrix material, and
5. Calibration based on standard additions

Studies on the occurrence of matrix effects in the determination of drugs from biological fluids have also indicated that exogenous materials leached from sample handling during analysis may also play a role in causing matrix effects in LC/MS [35], and consideration should be given to such sources in evaluating methods used for environmental samples.

The extent of clean up is important in eliminating matrix interferences and methods for the determination of estrogens have utilised silica gel which is an efficient procedure for removal of sulphur and humic acid interferences whilst still retaining high steroid recoveries [7,20]. However, the use of silica gives poor recoveries for E3, which binds strongly to the material,

hence other clean up methods must be utilised and have included SPE [36] and GPC followed by SPE (this study. A balance must be struck between lower recoveries from sample loss during the extraction/clean up procedure and elimination many possible co-eluting compounds that may interfere with steroid determination removal. The determination of polar pesticides in water was an area where LC/MS made an initial impact in the environmental sector. However, in a matrix which is relatively clean compared to sediments and sludges, problems with matrix enhancement have been observed [37] although more commonly suppression, particularly attributed to humic substances, has been observed [38-40].

The use of an isotopically labelled IS can limit the impact of matrix effects, although it has been noted that in some cases extraction conditions can allow isotope exchange to take place [41]. However, in this study matrix effects have been demonstrated to have an increased impact on reproducibility when comparing an external calibration against internal calibration with E2d4, indicating that underlying problems may not be resolved even in situations where the ideal, isotopically labelled standards are available. Such standards co-elute with the analyte of interest along with any associated matrix interferences, however, should the IS not co-elute, an evaluation of suppression effects in different areas of the chromatogram utilising an infusion-based approach may be required [42]. One study used equilin 2,4,16,16-d4 as its IS, spiked at 250µl/L [43] and it is possible that spiking in this study was not at high enough concentrations.

Source design, with the incorporation of a nano-splitting device which reduced flow rates to the ESI interface to as low as 0.1 µl/min, has been demonstrated to improve performance in regard to signal suppression [44], and further developments to instruments may help in resolving such issues. Matrix interference can affect single MS, though is decreased when using tandem MS due to the use of daughter ions for definitive quantification. The increased sensitivity of tandem MS allows for smaller sample amounts, which will therefore contain fewer potentially interfering contaminants. The use of tandem MS also allowing greater sensitivity (hence smaller sample amounts can be used) and selectivity. Column switching and waste diversion from the source have also been used to analyse steroid estrogens by LC/MS in sediments and environmental waters respectively [25,45], both practices limiting the amount of potential matrix interferences entering the analytical tool.

The results reported here indicate that there are a range of possible effects that may influence the quantification of steroid estrogens in sediments and sewage sludges utilising LC/MS as a final quantification technique. The aim of LC/MS is to separate individual analytes by HPLC such that the eluent delivered to the electrospray ionisation source contains analytes that are resolved from other matrix components. It has been proposed that longer run times and multi-step clean up procedures may minimise matrix effects, and also recognised that although improving resolution may reduce matrix effects, problems related to hydrophobic components with retention times which overlap analysis are perhaps most difficult to eliminate [35]. Such effects were observed in this work, the extended run programme using MeOH allowing greater separation between the analytes of interest and any interfering compounds and where the replacement of the column with a new one appeared to resolve the lack of response for E3. The importance of obtaining adequate chromatographic separation has been highlighted [46], and although the use of high resolution mass detectors, such as ToF, may avoid false positives [47], it does not solve issues related to the introduction of sample to the detector where the cause of ion suppression occurs.

Another consideration in method development and vital prior to any field work undertaken is that the recovery of spiked samples should be determined for each sample origin, be that geographical, type of STW or solid matrix (sediment, activated sludge, primary). This then accounts for the variation in chemical composition and functionality of the solid sample and allows for a complete assessment of robustness [9,23,27]. Results presented herein demonstrate that recoveries and standard deviations were dependent on sample type (sediment, sludge) and STW process for similar sludges (SRB, AS) and variation in recoveries according to solid sample type from a STW has also been identified [27]. This may be the impact of treatment process type or sewage composition on the matrix produced. This also has repercussions of countering matrix effects by performing a calibration from standards made up in the sample matrix [33].

Sample preparation can also impact method recoveries as demonstrated in this study. Spiking onto wet sediment led to transformation of E2 with partial conversion to E1 and as a result, spiking was undertaken after air drying of the sample (freeze drying not an economical option and oven drying at high temperatures would adversely affect steroid recovery). Wet sediment also led to problems with extraction using solvent immiscible with water, even with the addition of $NaSO_4$ (results not shown). To allow sorption processes to occur and an accurate

assessment of recoveries, sample should be left for a minimum of several hours prior to extraction.

Despite a number of issues of concern that have been raised regarding the use of LC/MS for the determination of contaminants in complex environmental matrices, there has been a significant increase in its application since the turn of the century [48]. However, the soft ionisation techniques utilised in the ESI and APCI sources put pressure on selectivity and the use of MS/MS or other high resolution instruments is considered by some obligatory for confirmation purposes [47]. The use of GC/MS and tandem MS is still routine for the determination of polar compounds such as steroid estrogens with derivatisation being undertaken where required [7] and standard methods for the determination of APs also utilise GC, though the analysis is limited to parent APs and short chain ethoxylates [49,50]. Such approaches may reflect the fact that LC/MS techniques have not yet become incorporated into routine methods for environmental analysis and that further understanding of factors influencing robustness of the technique is required.

5 Conclusions

- The complex nature of sediments and sewage sludges has been demonstrated to give rise to a number of issues which need to be considered in developing robust methods for the determination of trace contaminants by LC/MS.
- Evaluate the matrix contribution as part of the method recovery, especially when utilising LC/MS for analysis
- Solid composition varies considerably between sample type (sediment, primary, activated sludge) and origin and can affect the method recovery likely due to varying contribution of matrix influence
- The selection of solvent mobile phase and its influence on the length of the liquid chromatography programme affects the resolution of analytes from the matrix and therefore the degree of ionisation suppression due to matrix interferences.
- A comparison of GC/MS and LC/MS analysis gave variable recoveries for each steroid estrogen. Recoveries were better by LC/MS but generally at the cost of greater standard deviation comparative to GC/MS

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Table 1 Indirect approaches utilised for determining steroid estrogens in solid matrices

Matrix	Steroids	Method of Analysis	Reference
Sediment	E1; E2; EE2	Initial conc. – aqueous conc. = solid conc. HPLC with UV and florescence detection	[12]
Activated sludge	[^{3}H] EE2	Only the aqueous phase measured. HPLC / UV detection, liquid scintillation counter, radio-chromatography	[13]
Wastewater biosolids	[C^{14}] E2; [C^{14}] EE2	Biomass oxidized to obtain radioactivity Liquid scintillation counter	[14]
Sediment	E1; E2; EE2	Initial conc. – aqueous conc. = solid conc. GC/MS EI+ mode SIM	[1]
Activated sludge	E1; E2; EE2	Diluted with drinking water to 10% activated sludge to facilitate analysis GC/MS SIM	[15]
Sediment	[C^{14}] E2; [C^{14}] EE2	Initial conc. – aqueous conc. = solid conc. Liquid scintillation counter	[16]
Soil	[C^{14}] E2	Liquid scintillation counter Combustion analysis for [C^{14}] E2 sorbed to soil	[17]
Activated sludge	E1; E2; E3	Supernatant of filtered activated sludge used GC/MS full scan	[18]

Table 2 Approach for assessing influence of matrix effects and sample preparation on steroid estrogen recovery

Approach	Explanation
Method Recovery:	Comparing response of standards with that for spiked, extracted samples the effect of the extraction and clean up procedure on the absolute signal abundance is evaluated
Matrix Effect:	Comparing response of standards with that for extracted blank matrix spiked prior to instrumental determination, the effect of matrix extract on yield of ionisation when analysed by LC/MS ESI
Extraction Yield:	The effect of extraction and clean up alone, discounting matrix effects on ionisation on steroid estrogen recovery [Extraction yield (%) x matrix effect (%) = method recovery (%)]

Table 3 Comparison of the recoveries obtained by running samples on two chromatographic separations with different run times (%).

Separation		E1	E2	E3	EE2
Short run (25 min) (ACN/H_2O)	mean	74.7	95.6	0.0	80.3
	std. dev	5.2	8.2	-	1.2
Long run (60 min) ($MeOH/ H_2O$)	mean	66.7	55.0	58.6	74.0
	std. dev	0.9	0.6	0.7	8.9

Table 4 Influence of extraction / clean up and matrix effects (ionisation suppression) on the overall method recovery (%)

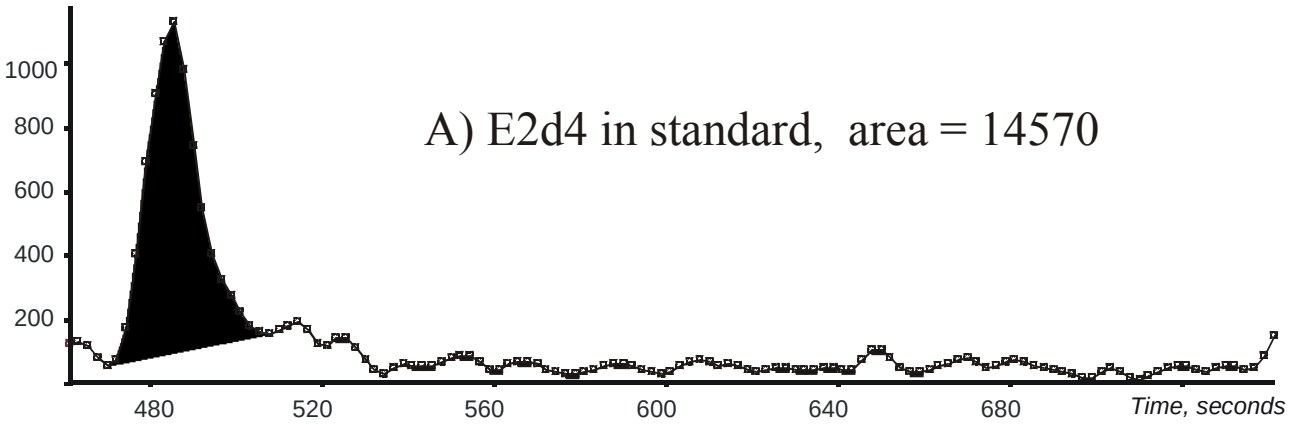
Approach		E1	E2	E3	EE2
Extraction yield	mean	64.5	61.1	70.7	67.8
	std. dev	9.8	7.5	5.0	5.4
Matrix effect	mean	75.9	75.0	74.1	82.9
	std. dev	10.1	5.2	7.3	3.9
Overall recovery	mean	48.9	44.0	52.4	56.3
	std. dev	4.3	2.9	4.9	7.1

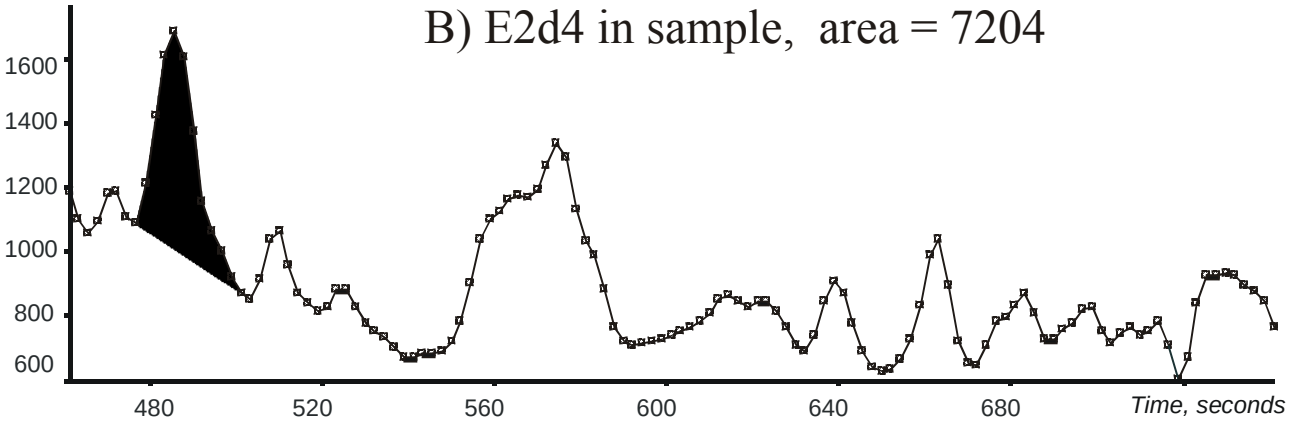
Table 5 Comparison of recoveries obtained by LC/MS and GC/MS quantification of sediment and sludge samples

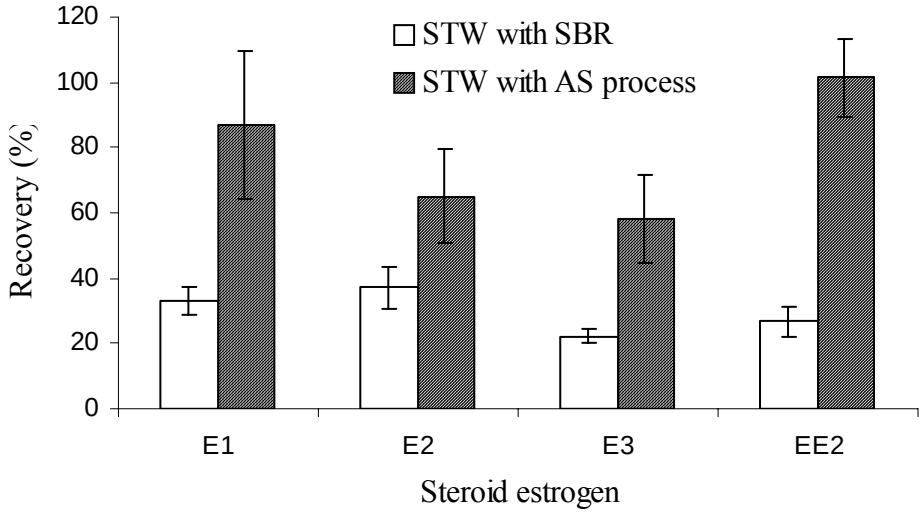
Matrix	Technique		E1	E2	E3	EE2
Sediment	LC/MS	mean	76.2	65.5	52.4	78.6
		std. dev	15.2	13.4	3.8	15.7
	GC/MS	mean	23.7	43.8	38.9	71.3
		std. dev	3.8	5.7	4.6	13.9
Sludge	LC/MS	mean	33.1	37.2	22.2	26.8
		std. dev	4.5	6.4	2.0	4.5
	GC/MS	mean	105.7	83.1	17.8	43.6
		std. dev	14.2	9.1	6.9	0.9

Figure 1 SIM chromatograms of relevant time period showing the effect of matrix on the response obtained from addition of E2d4 (0.074 μg/ml) shown by comparison of a standard solution (A) and a sediment extract (B).

Figure 2 Method recoveries for sludge samples obtained from two STWs with different biological treatment processes


A) E2d4 in standard, area = 14570
1000
800
600
400
200
480
520
560
600
640
680
Time, seconds


B) E2d4 in sample, area = 7204
1600
1400
1200
1000
800
600
480
520
560
600
640
680
Time, seconds


120
100
80
60
40
20
0
Recovery (%)
STW with SBR
STW with AS process
E1
E2
E3
EE2
Steroid estrogen