Global Water Research Coalition

Endocrine Disrupting Compounds

EDC in sewage sludge: analytical method deveplopment

Global Water
Research Coalition

IWA affiliate

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EDC in sewage sludge: analytical method development

Prepared by:

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Global Water Research Coalition

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Global Water Research Coalition

Global cooperation for the generation of water knowledge

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Preface

Endocrine Disrupting Compounds (EDC) is one of the priority issues of the research agenda of the Global Water Research Coalition.

Analytical methods to determine the occurrence and fate of EDC in the water cycle are of vital importance to study the impact of EDC on public health and the aquatic environment. The aim of this specific research was to develop an analytical method to determine endocrine disrupting compounds (EDCs) in sewage sludges and soils.

This study was co-funded by the UK Water Industry Research (UKWIR) and the Water Environment Research Foundation (WERF). The GWRC likes to acknowledge UKWIR and WERF for their joint leadership to organise this study.

ENDOCRINE DISRUPTERS IN SEWAGE SLUDGE : ANALYTICAL METHOD DEVELOPMENT

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ENDOCRINE DISRUPTERS IN SEWAGE SLUDGE : ANALYTICAL METHOD DEVELOPMENT

Executive Summary

An analytical method has been developed to determine the natural oestrogens, oestrone and 17-β-oestradiol and the synthetic oestrogen ethinyl oestradiol in sewage sludge and soil.

Samples were extracted with methanol using accelerated solvent extraction (ASE). The extracts were purified using a simple clean-up procedure using C18 solid phase extraction cartridges. Analysis to determine free steroid oestrogens was undertaken using liquid chromatography mass spectrometry (LCMS) operated in the negative ion electrospray mode.

During method development a number of solvents and extraction techniques were investigated. ASE extraction using methanol as extraction solvent was selected for further optimisation. Accelerated solvent extraction offers a number of advantages over other solvent extraction techniques, these including lower solvent consumption, reduced extraction times, higher throughput through automation and ease of use. Furthermore, when used to extract sewage sludge the resulting extract does not require filtration prior to analysis.

Statistical performance testing of the method shows that the procedure can be used for the routine monitoring of free steroids in sewage sludge and soil. The statistically derived limits of detection of the method to determine oestrone, 17-β-oestradiol and ethinyl oestradiol in sewage sludge and soil were in the range 25-100 ng g^{-1} and were dependent on the type of sludge or soil tested.

The relative simplicity of this method results in reduced analysis times and a high throughput of samples can be achieved. This method can therefore be applied to the assessment of the occurrence of free steroid oestrogens in biosolids applied to land, and for the analysis of sludge amended soils.

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GLOSSARY

GCMS Gas chromatography mass spectrometry

LCMS Liquid chromatography mass spectrometry

HPLC High performance liquid chromatography

WwTW Wastewater treatment works

PSS Primary sedimentation sludge

AS Activated sludge

MAD Mesophilic anaerobic digested sludge

HDS Heat dried sludge

LS Loamy soil

SIM Selected ion monitoring mode (a mode of operation of a mass spectrometer)

ASE Accelerated solvent extraction

RIA Radio immunoassays

SPE Solid phase extraction

LOD Limit of detection

ESI Electrospray ionisation

ELISA Enzyme linked immunoassays

APCI Atmospheric pressure chemical ionisation

TBDMS tert-butyldimethylsilyl

MSTFA N-methyl-N (trimethylsilyl)trifluoroacetamide

TMSI trimethylsilylimidazole

1 Introduction

There has been considerable concern for a number of years over endocrine disrupting chemicals (EDCs), which have the ability to alter or disrupt the body's hormone or endocrine system. There is compelling evidence that aquatic organisms downstream of input from sewage treatment works show endocrine disruption as a result of exposure to substances in wastewater. Evidence for the oestrogenic activity of such effluents comes from the UK (Purdom *et al.*,1994, Montaganani *et al.*,1996, Harries *et al.*,1997)[1 2 3], Germany (Stumpf *et al.*,1996, Ternes *et al.*,1999)[4 5], Italy (Johnson *et al.*,2000)[6], the Netherlands (Baronti *et al.*,2000)[7], Sweden (Larsson *et al.*,1999)[8] and the USA (Snyder *et al.*,1999)[9].

The list of suspected endocrine disrupters includes some natural and synthetic steroid oestrogens. Research has shown that the natural oestrogens, oestrone and 17-β-oestradiol and the synthetic oestrogen ethinyl oestradiol are responsible for most of the oestrogenic activity found in sewage effluents in the UK (Desbrow *et al.*,1998) [10].

Recent work carried out to model the fate of steroid oestrogens, (SOs) during sewage treatment has indicated that some will partition in significant amounts onto sewage sludge (UKWIR, 2001)[11]. However, there is no data available on the actual levels of such residues in sewage sludges, and consequently the model outputs have not been verified.

The aim of this research was firstly to develop methods for monitoring the concentration of SOs in soil and sewage sludges. Once developed, these methods will be applied (through separately funded projects) to investigate the presence of SOs in a range of soils and sewage sludges. The results of this investigation will be an essential precursor to further research on the fate of SOs in sewage sludge following application to agricultural land. They will also be used to improve models of the fate and behaviour of these chemicals in wastewater treatment processes.

1.1 Project Objectives

The aim of this research was to develop analytical methods for monitoring the concentration of free steroid oestrogens in sewage sludges and soils and to determine the oestrogenicity of soils and sewage sludges from different stages of sewage treatment.

The specific objectives of this work programme were:

1. To develop an analytical method to determine the natural oestrogens, oestrone and 17-β-oestradiol and the synthetic oestrogen ethinyl oestradiol in sewage sludge.

2. To validate and report the analytical method as a Standard Operating Procedure (SOP) in a format to be agreed with the steering committee.

1.2 Review of Analytical Methodology

Various methods have been published for the determination of steroid oestrogens (particularly free steroid oestrogens) in aqueous environmental samples including surface waters, ground and potable water as well as wastewater from sewage

treatment works. These methods are based either on immunoassay methods (enzyme linked immunoassays (ELISA) or radio immunoassays (RIA)) or on chemical analysis methods utilising either gas chromatography mass spectrometry (GCMS) or liquid chromatography mass spectrometry (LCMS).

Tanaka *et al.* (2000)[12] used an ELISA kit for the determination of 17β-oestradiol in environmental samples and has reported a limit of detection of 0.2 ng l^{-1} for 17β-oestradiol. Concentrations of this steroid found in river water surveys were in the range ND–27 ng l^{-1}. Huang and Sedlak (2001)[13] have also reported an ELISA method for the determination of 17β-oestradiol and 17α-ethinyl oestradiol (LODs of 0.1 ng l^{-1}). Snyder *et al.* (1999) used a RIA method to determine 17β-oestradiol and 17α-ethinyl oestradiol in aqueous mixtures. The LODs were stated to be 0.1 and 0.05 ng l^{-1} respectively, but no information was provided on how these LODs were established.

Several GCMS and LCMS methods have been published for the analysis of free steroid estrogens in the aquatic environment. The GCMS methods most frequently used involve solid phase extraction of the sample followed by derivatisation and GCMS analysis of the derivatised extracts.

Different types of solid phase extraction cartridges have been used to isolate steroids from samples prior to instrumental analysis e.g. styrene-divinyl benzene (Larsson *et al.* 1999, Mol *et al.* 2000)[8 14] and C18 silica (Lee and Peart 1998; Desbrow *et al.* 1998)[15] . Most of the methods reported have chemically derivatised the resulting extracts to form non-polar derivatives prior to GCMS analysis. Derivatives reported include acetyl derivatives (Larsson *et al.*1999) and pentafluoropropionyl derivatives (Lee and Peart 1998). Silylation is the most frequently used derivatisation technique deployed and a number of methods using different silylation reagents have been reported. Mol *et al.* (2000) used tert-butyldimethylsilyl (TBDMS) derivatives prior to GCMS analysis. Kuch and Ballschmiter (2001)[16] have reported the use of pentafluorobenzoyl derivatives followed by negative ion chemical ionisation GCMS to determine oestrone, 17- and 17--oestradiol and 17-ethinyloestradiol, as well as several other phenolic compounds. Other methods have been reported where the extracted steroids have been analysed by GCMS without derivatisation (Rodgers-Gray *et al.* 2000)[17].

GCMS/MS analysis has also been reported for the analysis of steroid estrogens (James *et al.* 1997, 1998, Ternes *et al.* 1999)[18 19] . SPE extraction was followed by the formation of trimethylsilyl (TMS) derivatives or TBDMS derivatives. James *et al* derived their reported limits of detection using a statistically based approach. Limits of detection of 0.2 ng l^{-1} for oestrone and 17β-oestradiol, 0.4 ng l^{-1} for 17α-ethinyl oestradiol (for drinking waters) and 0.4 ng l^{-1} for oestrone and 17β-oestradiol, 0.5 ng l^{-1} for 17α-ethinyl oestradiol (for river waters) were reported.

The extract derivatisation step can be avoided by using HPLC (Lopez De Alda and Barcelo 2000)[20] or LCMS (Baronti *et al.* 2000) analysis instead of GCMS. The limits of detection provided by HPLC with fluorescence or UV detection are often too high for detecting steroid oestrogens at environmentally significant levels but LCMS has been used to analyse for steroid estrogens in aqueous environmental samples. Different ionisation techniques including electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) have been applied. Limits of

detection in the range 50-500 ng l^{-1} and between 3000-5000 ng l^{-1} were reported for oestrone, 17β-oestradiol, oestriol and 17α-ethinyl oestradiol when using ESI and APCI respectively (Lopez de Alda and Barcelo 2000).

Recently two methods have been published which describe analytical methodologies for the determination of steroid oestrogens in sewage sludge. The first is a Japanese study (Takigami *et al.* 2000)[21] in which sludge samples were analysed at different stages of treatment using an immunoassay method. Using this method the concentration of 17β-oestradiol in concentrated activated sludge was reported to be 100 μg kg^{-1}.

A recent paper (Ternes *et al.* 2002)[22] describes an analytical method for the determination of oestrogens in sludge and sediments by liquid extraction and GC MS/MS analysis. The method for the analysis of sludge involved freeze drying the sludge and extraction using methanol and acetone in an ultrasonication bath. The slurry was separated using centrifugation and evaporated to dryness using nitrogen blow down. Internal standards were added to the samples and the extract cleaned up to remove high molecular mass compounds using gel permeation chromatography. The collected fractions were reduced to dryness using a rotary evaporator and dissolved in hexane/acetone (65:35). This extract was further cleaned up using a silica gel column (1.5% water deactivated), the extract reduced to dryness and derivatised using a mixture of N-methyl-N (trimethylsilyl)trifluoroacetamide(MSTFA)/ trimethylsilylimidazole (TMSI)/dithioerytrol (1000:2:2; v/v/w) at 60 °C for 1 hour. The samples were analysed using GC MS/MS. In activated sludge recoveries were obtained between 73-104% and between 57-77% in digested sludge. Limits of detection (which were not statistically derived) were estimated to be 2 ng g^{-1} for oestrone, 17-β-oestradiol and ethinyl oestradiol. The described method failed to determine steroid oestrogens in primary sludge.

2 Analytical Method development

2.1 Strategy for method development

Analytical methodology for the analysis of trace organics in environmental samples has a number of components (isolation/extraction, concentration, clean-up, separation and detection/quantification) and each of these aspects was investigated with the aim of developing a method to determine free steroid oestrogens in sewage sludge and soil samples. It was also considered necessary to utilise techniques that could be rapidly deployed and would provide robust and precise methodology which could be operated using automated laboratory equipment.

2.1.1 Extraction

Extraction of trace organics from sludge and soil can be carried out using a number of techniques which include accelerated solvent extraction, Soxhlet extraction and solvent extraction using ultra-sonication or mechanical shaking. In addition a number of solvents may be used for solvent extraction. It was therefore decided, that for the extraction of free steroids all of these options and a range of solvents would be investigated.

2.1.2 Internal standards

The reason for using internal standards is to improve the precision of quantitative analysis. Generally, internal standards can be classified into three types:

i) a stable isotope labelled analogue of the compound(s) to be determined;

ii) a homologous compound (which is assumed to behave similarly to the compound of interest);

iii) an unrelated compound which behaves similarly (in terms of extraction, clean-up and chromatographic behaviour) to the compound to be determined.

Isotopically labelled internal standards are the preferred choice for analyses which utilise mass spectrometry for detection.

2.1.3 Clean-up

Clean-up of environmental extracts is required to ensure potential analytical interferences or compounds that may degrade chromatographic columns (leading to loss of instrument performance) are removed. In addition a cleaner extract can result in lower detection limits (removal of background results in a higher signal to noise ratio) or (in the case of LCMS) compounds which suppress ionisation are removed resulting in higher sensitivity.

A number of clean-up techniques are available, and can either be carried out using automated (e.g. HPLC, GPC or SEC clean-up) or manual methods (e.g. column chromatography, liquid-liquid partition or SPE). Both automated and manual methods were investigated here.

2.1.4 Analysis

Free steroid oestrogens are polar compounds which can be analysed directly by LCMS or LCMSMS and by GCMS or GCMSMS. Analysis using GCMSMS can be carried out directly but is more usually undertaken after derivatisation of the steroids. However, derivatisation is time consuming and complex to carry out. It was therefore decided that a method would be developed which utilised LCMS.

2.2 Sample collection and preparation

Sewage sludges were collected from a wastewater treatment works (WwTW) from Southern England. This WwTW has a primarily domestic input and serves a population of 130,000 (flow 62Ml/day); it is equipped with a standard mesophilic anaerobic digester and a sludge dryer.

The following samples were collected:

1. Primary Sedimentation Sludge

2. Activated Sludge

3. Mesophilic anaerobic digested sludge

4. Heat dried sludge

5. Loamy soil (Kettering Loam)

The loamy soil selected for this work is Kettering loam (from Turf Management Systems, Buckinghamshire, UK) which has previously been used as a standard test soil in a series of national and international projects on terrestrial ecotoxicology. It represents a typical "real" soil (which has been extensively characterised) against which standard ecotoxicological data can be compared.

All sludge samples were collected in pre-washed glass bottles with glass stoppers. Samples were delivered to the laboratory within 1 day of collection. The samples were kept cold in a cool box (2-6°C) during transportation and upon arrival at the analytical laboratory the samples were stored in a fridge at 2-6°C.

The samples collected contained the following amounts of solids: Primary sedimentation sludge (PSS) 3-4%, Activated sludge (AS) 0.5%, Digested Sludge (MAD) 2-3%, Heat dried sludge (HDS) >95% and Loamy soil (LS) >95%.

Sludge and soil samples received in the analytical laboratory were categorised into two groups. The first group were classified as solids (loamy soil, heat dried soil and) and the second group as a slurry (primary sludge, activated sludge and digested sludge). The slurry samples were centrifuged to separate the solids from the aqueous phase. The dry solids were freeze dried and split into eight portions of 5g each (for each extraction experiment described below). Half of the samples were unspiked (to determine levels of natural steroids in the sample) and the remaining half were spiked with a mixed solution of the three free steroids: oestrone, 17-β-oestradiol and ethinyl oestradiol. The spiked samples were mixed using a metal spatula and allowed to stand overnight (to allow equilibrium with the matrix to take place) prior to extraction using one of the following extraction techniques: solvent extraction using sonication, solvent extraction by mechanical shaking, Soxhlet extraction or accelerated solvent extraction.

Pure standards of the steroids were obtained from Sigma (St. Louis, MO, USA). Stock standard solutions of each compound were prepared at 1 mg ml^{-1} in methanol. A working intermediate containing a mixture of all three steroids was prepared and dilutions of this solution prepared using methanol.

The solvents used for extraction were: toluene, methanol, acetone, and a mixture of methanol/ether/hydrochloric acid.

The methodology used for sample extraction is described in detail below.

2.3 Development of sample extraction technique

The overall aim of the extraction experiments was to obtain the highest recovery with the lowest amount of co-extractable and potentially interfering materials. This would ensure that subsequent clean-up of the resulting extract was kept to a minimum.

Extraction methods including Soxhlet extraction, sonication, solvent extraction, steam distillation and accelerated solvent extraction (ASE) were investigated. The

experiments were also designed to determine the optimal extraction solvent for free steroid oestrogen analysis using LCMS.

2.3.1 Solvent extraction using ultrasonication

The freeze dried samples (5g) and samples spiked with free steroids were each placed in a 50 ml glass beaker and the sample extracted in an ultrasonic bath with 25 ml of toluene for 30 minutes. The liquid phase was separated from the solids using filtration. The extraction step was repeated using a further 25 ml aliquot of toluene, the layers separated using filtration and combined with the initial extract in a TurboVap™ concentration tube. The 50 ml extract was concentrated using a TurboVap™ concentrator to an approximate volume of 1-2 ml.

The entire experiment was repeated using other solvents or solvent mixtures. All extracts were then concentrated to dryness using nitrogen blow down apparatus and re-dissolved in methanol (1 ml) prior to LCMS analysis for free steroids.

The extracts obtained using acidic conditions contained very high levels of co-extracted materials (believed to be acids). The extracts were extremely viscous and difficult to concentrate and these extracts were therefore discarded.

2.3.2 Solvent extraction using a mechanical shaker

The freeze dried samples (5g) and samples spiked with free steroids were each placed in a 150 ml screw-top conical flask and the sample extracted on a flat-bed horizontal mechanical shaker with 25 ml of toluene for 1 hour. The liquid phase was separated from the solids using filtration. The extraction step was repeated using a further 25 ml aliquot of toluene, the phases separated using filtration and combined with the first extract in a TurboVap™ concentration tube. The 50 ml extract was concentrated using a TurboVap™ concentrator to an approximate volume of 1-2 ml.

The entire experiment was repeated using other solvents or solvent mixes. All extracts were then concentrated to dryness using nitrogen blow down apparatus and re-dissolved in methanol (1 ml) prior to LCMS analysis of free steroids.

The extracts obtained using acidic conditions contained very high levels of co-extracted materials and were again discarded.

2.3.3 Accelerated solvent extraction

The freeze dried samples (5g) and samples spiked with free steroids were ground and mixed with an equal proportion of Hydromatrix™. The samples were placed into an ASE stainless steel extraction cell and extracted using either acetone, methanol, toluene or a mixture of methanol/ether/hydrochloric acid.

ASE extractions were carried out at 100 °C and 1500 psi using the conditions described in Table 2.1.

The ASE extracts were concentrated using a TurboVap™ concentrator to an approximate volume of 1-2 ml.

All extracts (except the methanol extract) were then concentrated to dryness using nitrogen blow down apparatus and re-dissolved in methanol (1ml) prior to LCMS analysis for free steroids.

Table 2.1 Accelerated solvent extraction conditions used for extraction of sewage sludge and soil

Equipment	ASE 200 Accelerated solvent extractor with 33 ml stainless steel cells
Oven Temperature	150°C
Pressure	14 Mpa (2000 psi)
Oven Heat-up time	5 minutes
Flush Volume	60% of extraction cell volume
Solvent	Either acetone, methanol, toluene or a mixture of a mixture of methanol/ether/hydrochloric acid.
Nitrogen Purge	1 Mpa (150 psi) for 60 seconds

The recovery of free steroids using the various solvents is discussed in section 2.3.5.

2.3.4 Soxhlet extraction

The freeze dried samples (5g) and samples spiked with free steroids were ground and mixed with furnace baked (overnight at 600°C) sodium sulphate. The samples were placed into a Soxhlet thimble and extracted in a Soxhlet apparatus using either acetone, methanol, toluene or a mixture of methanol/ether/hydrochloric acid.

The samples were extracted overnight, the extracts were allowed to cool and concentrated using a TurboVap™ concentrator to an approximate volume of 1-2 ml.

All extracts (except the methanol extract) were then concentrated to dryness using nitrogen blow down apparatus and re-dissolved in methanol (1ml) prior to LCMS analysis of free steroids.

2.3.5 Method performance comparison

Extracts obtained under acidic conditions from the four experiments were discarded as they contained very high levels of co-extractable material (acids). This made the extract difficult to handle or concentrate to a low volume.

Sludge and soil extracts extracted by solvent extraction and by ultrasonication required removal of particulate matter from the extract prior to concentration which was achieved by filtration using silanized glassware.

The filtration procedure required for samples extracted using a shaker or ultrasonication adds another time consuming step to the analytical procedure. Samples extracted by ASE and Soxhlet extraction do not contain large amounts of particulate

matter as they were removed by means of the filters contained in the ASE cells or by retention by the thimbles during Soxhlet extraction.

The results obtained show that all three solvents, toluene, acetone and methanol using all four extraction techniques (solvent extraction by mechanical shaking, ultrasonic extraction, ASE and Soxhlet extraction) were all able to extract free steroids from sewage sludge and soil.

Recent methods for the determination of free steroid oestrogens in solid environmental samples (sediment and sludge) have utilised solvent extraction using ultrasonic (Lopez and Barcelo, 2001) [23] and mechanical shaking or a combination of both (Kanda *et al.,* 2002 and Ternes *et al.* 2002). Although these methods produce acceptable performance the technique is time consuming as it involves a filtration or centrifugation step to remove solids from the final extract.

The preferred extraction techniques were therefore, Soxhlet and ASE extraction. However, the resulting extracts from these two extraction techniques both contained large quantities of dissolved matter (most likely to be high molecular weight compounds such as proteins and carbohydrates) which came out of solution and appeared as solids when the extracts were concentrated to a low volume.

Accelerated Solvent Extraction (ASE) was selected as the extraction method for further method development despite the presence of solids, as it allows automation, faster extractions times and uses less solvent than conventional Soxhlet extraction.

2.4 Sample clean-up

The original approach for sample clean-up was to concentrate the sample to a low volume and carry out clean up using a HPLC method (Kanda *et al.,* 2002 in press). Problems with blockage of the HPLC autosampler occurred due to the presence of solids in the sample extracts. Some of these blockage problems were solved by changing the autosampler needle and seat to “widebore” using a kit available from the manufacturer. Although it was now possible to inject the extracts using an autosampler, the complexity of the extracts still caused major problems with column deterioration. Although this approach has been applied successfully to sediments it was obviously not applicable to sewage sludge.

Two experiments were therefore carried out to obtain a cleaner solvent extract for subsequent analysis. The first experiment was designed to optimise the ASE extraction conditions to obtain an extract with lower levels of co-extractables. The second experiment was designed to remove co-extractables from the ASE extract.

2.4.1 Experiment 1: Optimisation of ASE extraction for selective extraction

An immediate reduction in co-extractable material was achieved by reducing the sample extracted from a 5g sample to a 1g sample.

The temperature and pressure of the extraction process was the next consideration in reducing the amount of co-extracted material.

Method

Freeze dried activated sludge (1g) was spiked with free steroids. The sample was ground and mixed with an equal proportion of Hydromatrix™. The samples were placed into an ASE stainless steel extraction cell and extracted using methanol.

ASE extractions were carried out at 150°C and at various cell pressures ranging from 500, 1000, 1500 and 2000 psi using the conditions described in section 2.3.3. The collected ASE extracts were concentrated using a TurboVap™ concentrator to a final volume of 1 ml prior to LCMS analysis of free steroids.

The same experiment was repeated with the pressure at 1500 psi and cell extraction temperatures of 50°C, 100°C, 150°C and 200°C.

Results

The recovery experiments showed that the free steroids could only be extracted with good recovery (above 70%) with the cell at a minimum temperature of 100°C and a pressure of 1000 psi.

These conditions were therefore used for all subsequent work.

2.4.2 Experiment 2: Extract clean-up using SPE cartridges

The ASE extracts obtained using a 1g sample and extraction at lower cell temperature and pressure still contained a significant amount of co-extractables which caused major problems with HPLC clean-up and LCMS analysis due to blockage of the autosampler needle. It was therefore decided that the extracts should be purified using a further clean-up.

Acidic compounds are significant components of soil and sludge samples. Acids are largely “unretained” at neutral pH on a C18 columns. This property was used to undertake extract clean-up of the samples.

Method

ASE extracts in methanol (40 ml) were diluted with an equal volume of blank reagent water.

C18 non endcapped cartridges (500 mg, 6ml) were conditioned with 100% methanol followed by 50% aqueous methanol. The sample was passed through the cartridges at less than 10 ml/min. The cartridge was then dried using nitrogen and eluted with 1.5 ml of 85% aqueous methanol (85:15 methanol:water). The elute was collected directly in an autosampler vial and analysed using LCMS.

Results

The SPE clean-up was able to remove a large amount of the co-extracted matter. Visual inspection of the extracts indicated a significant removal of particulate matter and a reduction in the colour of the samples.

2.5 LCMS Analysis

Method

Analysis of free steroids was achieved using LCMS with negative ion electrospray in selected ion monitoring mode (SIM). The equipment used was an Agilent 1100 liquid chromatograph (Agilent, USA) connected to a Micromass Quattro LC mass spectrometer (Manchester, UK).

LCMS analysis was carried out using a Phenomenex LUNA 5μm C18(2)) analytical column. The LC was connected to the mass spectrometer, and the source parameters used were as shown in Table 2.2.

Table 2.2 LCMS analysis of free steroid oestrogens

LC:	**HP 1100 Series**
Column:	Phenomenex LUNA 5 μm C18(2), 150 x 4.60 mm (P/N 00F-4252-E0)
Flow:	0.5 ml/min
LC Initial:	Solvent A: 90% water containing 3% ammonia (28% solution in water) Solvent B: 10% methanol

LC Gradient:

Time	0 min	10 min	18 min	24 min	28 min
%A	90%	50%	10%	10%	90%

Injection volume: 20 ul

MS:	**Micromass Quattro LC**
Source:	Electrospray (negative ion)
Source Temp:	150 °C
Desolvation Temp:	400 °C
Capillary voltage:	3.1 Kv
Cone Voltage:	45 V
Nebuliser gas flow:	75 litres/hour
Drying gas flow:	535 litres/hour
Ions monitored:	269.30 (oestrone) 273.30 (d_4-oestrone) 271.30 (17β-oestradiol) 275.30 (d_4-17β-oestradiol)

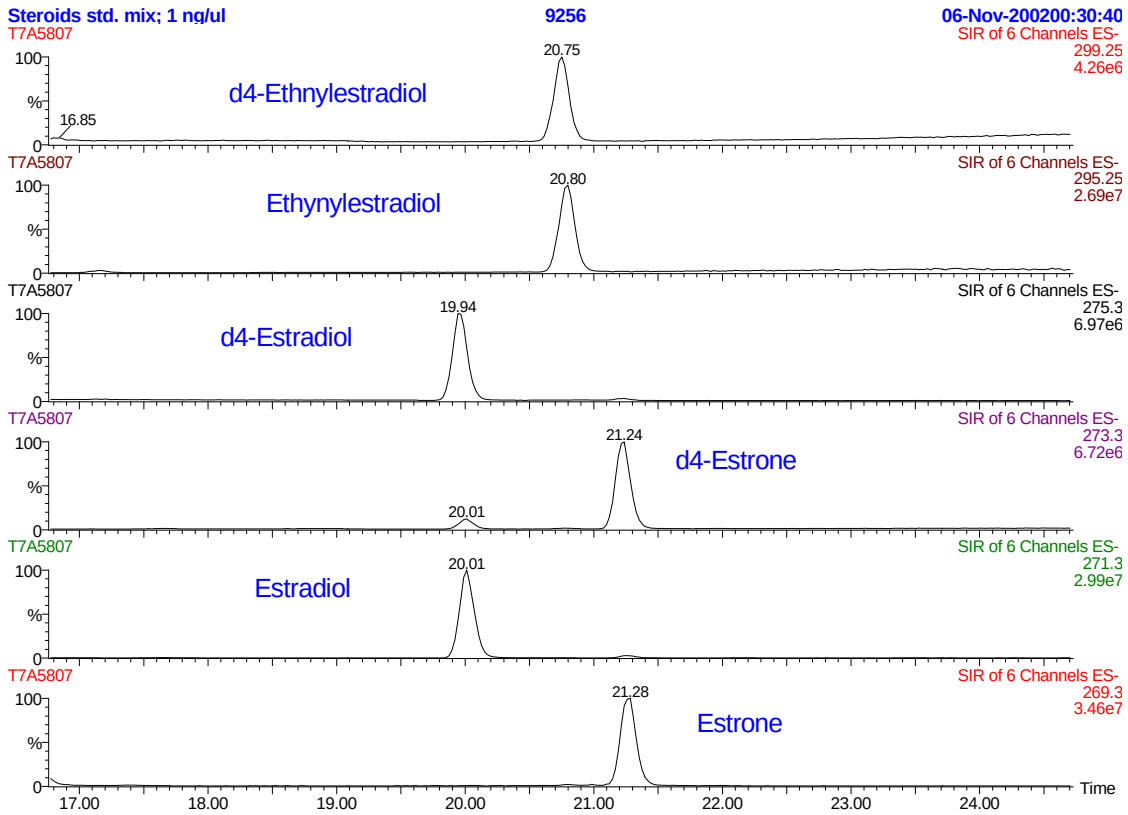
295.25 (17α-ethinyl oestradiol)
299.25 (d_4-17β-ethinyl oestradiol)

The sensitivity of the mass spectrometer was checked by direct injection of a standard solution of the three free steroids in methanol. The analytical column required at least two injections of an analytical standard to condition the column, so the first two injections of standard were not used for calibration.

Sample analysis was carried out following injection of calibration standards in the concentration range 0 to 1 ng µl-1. A typical calibration standard response for the three free steroids and labelled compounds is given in Figure 2.1.

The performance of the LCMS method was evaluated by determining the reproducibility, linearity and sensitivity of the analytical method. Linearity was determined by the analysis of calibration standards containing labelled and unlabelled standards over a calibration range of 0 to 1 ng µl-1. The reproducibility of the analysis was determined by repeat injections of the 0.25 ng µl-1 standard solution.

Figure 2.1 LCMS Chromatogram of a mixed free steroid standard solution containing 1 ng μl^{-1} of oestradiol, oestrone and ethinyl oestradiol and 0.25 ng μl^{-1} of the internal standards d_4-oestradiol, d_4-oestrone and d_4-ethinyl oestradiol.



Results

The results obtained showed excellent linearity of the LCMS method over the calibration range. Reproducibility studies of repeat injection of standard solutions were also good. This data is shown in Table 2.3.

Table 2.3 LCMS linearity and reproducibility of analytical standards

Steroid	Retention time /min	Correlation coefficient (R^2)*	LCMS Precision % (n=24)**
Oestrone	21.29	0.9998	2.4
Oestradiol	20.08	0.9999	2.1
Ethinyl oestradiol	20.82	0.9998	1.4

* Linear regression correlation coefficient (of five data points) for calibration graph over the range 0 to 1 ng/μl

** LCMS precision analysis of repeat injection of a mid-calibration mixed standard

2.6 Statistical Performance testing of the analytical procedure

Method performance testing of the free steroid oestrogens method was carried out in accordance with ISO /TR 13530 (1998).[24] A summary of the methodology is described below. A detailed method description (a standard operating procedure) is provided in Appendix A.

Method

Sewage sludges were collected from the same WwTW as described in section 2.2. All sludge samples were collected in pre-washed glass bottles with glass stoppers. Samples were delivered to the laboratory within 1 day of collection. The samples were kept cold (2-6°C) during transportation in a cool box and upon arrival at the analytical laboratory stored in a fridge at 2-6°C prior to analysis.

Pure standards of the steroids were obtained from Sigma (St. Louis, MO, USA). Stock standard solutions of each compound were prepared at 1 mg ml^{-1} in methanol. A working intermediate containing a mixture of all three solutions and dilutions of this solution were prepared by appropriate dilutions of the stock solutions in methanol.

Sludge and soils samples received in the analytical laboratory were categorised into two groups, the first were classified as solids (loamy soil, heat dried soil and digested sludge) and the second group as a slurries (primary sludge and activated sludge). The slurry samples were centrifuged to separate the solids from the aqueous phase.

The dry solids were freeze dried and five 1g portions taken. One portion was unspiked to determine levels of natural steroids in the sample and labelled as the blank. The other four samples were spiked with a mixed solution of the three free steroids: oestrone, 17-β-oestradiol and ethinyl oestradiol. Two samples were spiked at a “low”

concentration and two samples were spiked at a "high" concentration. The low spikes were spiked at 50 ng g^{-1} and the high spikes were spiked at 500 ng g^{-1}.

The spiked samples were mixed using a metal spatula and allowed to stand overnight to allow equilibrium with the matrix to take place.

Freeze dried samples (1g) were ground and mixed with an equal proportion of Hydromatrix™. The samples were placed into an ASE stainless steel extraction cell and spiked with a mixture of labelled internal standards. The void at the top of the extraction cell was filled using more Hydromatrix™.

ASE extractions were carried out using the conditions shown in Table 2.4.

The collected ASE extracts in methanol (40 ml) were diluted with an equal volume of blank reagent water.

Table 2.4 Final Accelerated solvent extraction conditions to determine method performance

Equipment	ASE 200 Accelerated solvent extractor with 33 ml stainless steel cells
Oven Temperature	100°C
Pressure	10 Mpa (1000 psi)
Oven Heat-up time	5 minutes
Flush Volume	60% of extraction cell volume
Solvent	Methanol
Nitrogen Purge	1 Mpa (150 psi) for 60 seconds

C18 non endcapped cartridges (500 mg, 6 ml) were conditioned with 100% methanol followed by 50% aqueous methanol. The sample was passed through the cartridge at less than 10 ml/min. The cartridge was dried using nitrogen and eluted with 1.5 ml of 85% aqueous methanol (85:15 methanol:water). A portion (500 µl) of each extract was concentrated to 100 µl prior to LCMS analysis. LCMS analysis was undertaken using the conditions described in section 2.5.

Results

A method for the analysis of free steroid oestrogens in sewage sludge and soil samples has been developed and validated. Performance testing of the analytical procedure at a low spike of 500 ng g^{-1} has provided relative percentage recoveries for each of the analytes: oestrone, 17-β-oestradiol and ethinyl oestradiol of 79-107%, 75-105% and 88-105% respectively over the range of sludge and soil samples tested. The absolute recovery and response of individual steroids in primary sludge was low

compared to the other samples. The drop in sensitivity could have been due to suppression of the mass spectral signal by other organics in the sample or losses during sample preparation.

Performance testing of the analytical procedure at a concentration of 50 ng g^{-1} showed that high and reproducible recovery could be obtained for all three free steroids in all soil and sludge samples tested. Performance testing of soil and sludge samples spiked at 50 ng g^{-1} has provided relative percentage recoveries for each of the analytes as follows: oestrone, 17-β-oestradiol and ethinyl oestradiol of 63-124%, 69-114% and 75-122% respectively over the range of sludge and soil samples tested.

A summary of the performance testing of the analytical method and limits of detection achieved is provided in Table 2.5.

Method Benefits

The developed method presented has a number of distinct advantages. Up to 24 freeze dried sewage sludge samples can be analysed using an automated procedure (accelerated solvent extraction). Methanol extracts do not need filtration or centrifugation prior to analysis and they do not require lengthy concentration steps (rotary evaporation or TurboVap™ concentration). The use of LCMS also eliminates the need to derivatise sample extracts. The relative simplicity of this method results in reduced analysis times and a high throughput of samples. This method can therefore be used by routine analytical laboratories for the assessment of the occurrence of free steroid oestrogens in biosolids applied to land and in the analysis of sludge applied soils.

Table 2.5 Summary of the analytical performance of the developed method to determine free steroids in sewage sludge and soil samples. Samples extracted utilising accelerated solvent extraction , clean-up using SPE and analysis using LCMS operated in the negative ion electrospray mode

Sample	High Spike (spiked at 500 ng/g dw)			Low Spike (spiked at 50 ng/g dw)			*Limit of detection ng/g dw
	Concentration ng/g	% Recovery	%RSD (n=12)	Concentration ng/g	% Recovery	%RSD (n=12)	
Oestrone							
Loamy soil	481.7	96.3%	38.4%	54.1	108.2%	15.2%	**38.2**
OECD synthetic soil	396.7	79.3%	32.4%	53	106.0%	18.1%	**44.6**
Mesophilic anaerobic digested sludge	433	86.6%	45.6%	31.5	63.0%	42.3%	**62.0**
Heat dried sludge	409.2	81.8%	35.1%	61.8	123.0%	27.3%	**78.5**
Activated Sludge	419.1	83.8%	38.0%	62	124.0%	23.1%	**66.6**
Primary Sludge	533.3	106.7%	39.9%	32.3	64.6%	45.5%	**68.3**
Oestradiol							
Loamy soil	510.8	102.2%	27.9%	48.2	96.4%	19.0%	**42.6**
OECD synthetic soil	453.3	90.7%	23.3%	51.5	103.0%	22.0%	**52.7**
Mesophilic anaerobic digested sludge	388.3	77.7%	35.1%	53.7	107.4%	36.2%	**90.4**
Heat dried sludge	470.1	94.2%	19.2%	57.3	114.6%	30.4%	**81.0**
Activated Sludge	376.7	75.3%	40.7%	51.6	103.2%	35.4%	**84.9**
Primary Sludge	527.5	105.5%	20.6%	34.5	69.0%	47.7%	**76.5**
Ethinyl oestradiol							
Loamy soil	476.3	95.2%	33.9%	38.6	77.2%	18.4%	**33.0**
OECD synthetic soil	444.2	88.8%	11.3%	44.3	88.6%	25.5%	**52.5**
Mesophilic anaerobic digested sludge	473.3	94.7%	32.1%	60.8	121.6%	23.4%	**66.2**
Heat dried sludge	485	97.0%	26.6%	38.3	76.6%	28.6%	**50.9**
Activated Sludge	526.7	105.3%	31.0%	51.6	103.2%	30.2%	**72.5**
Primary Sludge	505.2	101.0%	18.8%	37.6	75.2%	48.5%	**84.8**

3 Discussion

A number of investigators have reported levels of alkylphenols, APEOs, phthalates, organotins, bisphenol A, polybrominated diphenyl ethers and PAH to be present in sludge at concentrations varying by several orders of magnitude. The majority of work undertaken on levels of target EDCs (or on the determination of total oestrogenicity) and in particular the fate of steroid oestrogens in wastewater treatment works have been directed at sewage effluents. One reason for this has been due to the lack of analytical methodology to determine these compounds in sludge.

The aim of this work was therefore to develop a performance tested analytical method to determine the natural oestrogens, oestrone and 17-β-oestradiol and the synthetic oestrogen ethinyl oestradiol in sewage sludge and sludge amended soil. A further aim was to validate the developed procedure (to ISO/TR 13530) and report the analytical method as a standard operating procedure (SOP).

During method development a number of solvents and extraction techniques were investigated. ASE extraction using methanol as extraction solvent was selected for further optimisation. Accelerated solvent extraction offered a number of advantages over other solvent extraction techniques, these include lower solvent consumption, reduced extraction times, higher throughput through automation and ease of use. Furthermore, when used to extract sewage sludge the resulting extracts did not require filtration prior to cleanup or analysis.

However, the resulting extracts from ASE extraction contained large quantities of dissolved matter (most likely to be high molecular weight compounds such as proteins and carbohydrates) and acids which came out of solution and appeared as solids when the extracts were concentrated to a low volume. A cleanup technique was therefore required to remove these solids from the extracts. The original approach for sample clean-up was to concentrate the sample to a low volume and carry out clean up using HPLC fractionation. Although this approach has been applied successfully to sediments it was not applicable to sewage sludge.

An effective cleanup of the extracts was achieved using C18 solid phase extraction cartridges. The SPE clean-up was able to remove a large amount of the co-extracted matter. Visual inspection of the extracts indicated a significant removal of particulate matter and a reduction in the colour of the samples. This simple cleanup procedure allowed analysis of the extract to proceed without any further problems.

Analysis of the extracts was achieved using LCMS with negative ion electrospray ionisation in selected ion monitoring mode (SIM). The performance of the LCMS method was evaluated by determining the reproducibility, linearity and sensitivity of the analytical method. Linearity was determined by the analysis of calibration standards containing labelled and unlabelled standards over a calibration range of 0 to 1 ng μl^{-1}. The reproducibility of the analysis was determined by repeat injections of the 0.25 ng μl^{-1} standard solution. The results obtained showed excellent linearity of the LCMS method over the calibration range. Reproducibility studies of repeat injection of standard solutions were also good.

Statistical performance testing of the method shows that the procedure can be used for the routine monitoring of free steroids in sewage sludge and soil. The statistically derived limits of detection of the method to determine oestrone, 17-β-oestradiol and ethinyl oestradiol in sewage sludge and soil were in the range of 25-100 ng g^{-1} and were dependent on the type of sludge or soil tested.

4 Conclusion

A performance tested method for the determination of free steroid oestrogens in sludge and soil has been developed. Limits of detection lower than 100 ng g^{-1} were achieved for all three target compounds (oestrone and 17-β-oestradiol and the synthetic oestrogen ethinyl oestradiol) in loamy and synthetic soil as well as in a primary sludge, activated sludge, heat dried sludge and mesophilic anaerobic sludge.

The relative simplicity of this method results in reduced analysis times and a high throughput of samples can be achieved. This method can therefore be applied to the assessment of the occurrence of free steroid oestrogens in biosolids applied to land, and for the analysis of sludge amended soils.

One of the limitations of the developed methodology is the limits of detection achieved. In order to determine the fate of free steroids in sewage sludge, further work is required to improve the detection limit to allow lower levels to be determined (<10 ng g^{-1}). In addition, this work was carried out on sludge from a single sewage treatment works and it would be prudent to assess the performance of the method in a range of sludges from other WwTWs.

5 References

1. Purdom, C. E.; Hardiman, P. A. and Bye, V. J (1994) Estrogenic Effects of Effluents from Sewage Treatment Works. *Chem. Ecol*., **8**, 275-285.

2. Montagnani, D. B.; Puddefoot, J.; Davie, T. J. A.; Vinson, G. P. J. (1996) Environmentally Persistent Oestrogen-like Substances in UK River Systems, *Chart. Inst. Water. Environ. Management*, **10**, 399-406.

3. Harries, J. E.; Sheahan, D.A.; Jobling, S.; Matthiessen, P.; Neall, P.; Routledge, E. J.; Rycroft, R.; Sumpter, J. P.; Taylor, T. (1996) A Survey of Estrogenic Activity in United Kingdom Inland Waters, *Environ. Toxicol. Chem*., **15**, 1993-2002.

4. Stumpf, M.; Ternes, T. A.; Haberer, K.; Baumann, (1996) Determination of Natural and synthetic Estrogens in Sewage Plants and River Water, *W. Vom. Wasser*., **87**, 251-261.

5. Ternes, T. A.; Stumpf, M.; Müller, J.; Haberer, K.; Wilken, R. D.; Servos, M. (1999) Behavior and occurrence of estrogens in municipal sewage treatment plants — I. Investigations in Germany, Canada and Brazil, *Sci. Total. Environ*., **225**, 81-90.

6. Johnson, A. C.; Belfroid, A.; Di Corcia, A. (2000) Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. *Sci. Total. Environ*., **256**, 163-173.

7. Baronti, C.; Curini R.; D'Ascenzo, G.; Di Corca, A.; Gentili, A.; Samperi, R. (2000) Monitoring Natural and Synthetic Estrogens at Activated Sludge Sewage Treatment Plants and in a Receiving River Water, *Environ. Sci. Technol*., **34**, 5059-5066.

8. Larsson, D. G. J.; Adolfsson-Erici, M.; Parkkonen, J.; Pettersson, M.; Olsson, P-E.; Förlin, L. (1999) Ethinyloestradiol - an undesired fish contraceptive? *Aquat. Toxicol*., **45**, 91-97.

9. Snyder, S. A.; Keith, T. L.; Verbrugge, D. A.; Snyder, E. M.; Gross, T. S.; Kannan, K.; Giesy, J. P. (1999) Analytical Methods for Detection of Selected Estrogenic Compounds in Aqueous Mixtures, *Environ. Sci. Technol*., **33**, 2814-2820

10. Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P. and Waldock, M. (1998) Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol*, **32**, 1549-1558

11. Rogers H.R., Watts C.D. and Palfrey L. (2001) QSAR Studies on Fate and behaviour of steroid endocrines in sewage treatment, UKWIR Report Ref. No. 01/TX/04/3

12. Tanaka, H., Yamamoto, M., Koresawa, Y., Komori, K., Sonoda, K., Higashitani, T. and Nozoe, J. (2000) State of pollution caused by endocrine disruptors in rivers in Japan. Paper presented at 1st World Congress of the International Water Association held in Paris 3-7 July 2000.

13. Huang, C.H. and Sedlak, D.L. (2001) Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. *Environ. Toxicol. Chem*, **20**, 133-139.

14. Mol, H.G.J., Sunarto, S and Steijer, O.M. (2000) Determination of endocrine disruptors in water after derivatization with N-methyl-N-(tert-butyldimethyltrifluoroacetamide) using gas chromatography with mass spectrometric detection. *Journal of Chromatography A*, **879**, 97-112.

15. Lee, H.B. and Peart, T.E. (1998) Determination of 17β-oestradiol and its metabolites in sewage effluent by solid-phase extraction and gas chromatography/mass spectrometry. *Journal of AOAC International*, **81**, 1209-1216.

16. Kuch, H.M. and Ballschmiter, K. (2001) Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. *Environ. Sci. Technol*, **35**, 3201-3206.

17. Rodgers-Gray, T.P., Jobling, S., Morris, S., Kelly, C., Kirby, S., Janbakhsh, A., Harries, J., Waldock, M., Sumpter, J.P. and Tyler, C.R. (2000) Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environ. Sci. Technol*, **34**, 1521-1528.

18. James, H.A., Fielding, M., Franklin, O., Williams, D. and Lunt., D. (1997) Steroid concentrations in treated sewage effluents and water courses - implications for water supplies. Final Report to the Department of the Environment and UK Water Industry Research Limited. Report No. DWI 4323.

19. James, H.A., Waldock, M.J., Kelly, C., Sutton, A. and Franklin, O. (1998) Unifying analytical methods for the determination of oestrone, oestradiol and ethinyloestradiol in water. Final Report to the Department of the Environment. Report No. DWI 4445/1.

20. López de Alda, M.J. and Barceló, D. (2000) Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by liquid chromatography-diode array detection-mass spectrometry. *Journal of Chromatography A*, **892**, 391-406.

21. Takigami, H., Taniguchi, N., Matsuda, T., Yamada, M., Shimizu, Y. and Matsui S. (2000) The fate and behaviour of human estrogens in a night soil treatment process. *Water Science and Technology*, **42**(7-8), 45-51.

22. Ternes, T. A. Andersen, H. Gilberg, D. Bonerz M (2002) Determination of Estrogens in Sludge and Sediments by Liquid extraction GC/MS/MS. *Analytical Chemistry*, **74** (14), 3498-3504

23. Lopez de Alda M.J. and Barcelo D. (2001) Use of solid phase extraction in various of its modalities in the determination of estrogens and progestogens in sediment and water, *Journal of Chromatography A.*, **938**, 145-153

24. ISO /TR 13530 (1998) Water Quality - Guide to Analytical Quality Control for Water Analysis

Appendix A: Standard Operating Procedure for the determination of free steroid oestrogens in sewage sludge and Soil

1 SCOPE

This document outlines the procedure for the determination of oestrone, 17β-oestradiol and 17α-ethinyl oestradiol in sewage sludge and soil using Accelerated Solvent Extraction (ASE™) and liquid chromatography mass spectrometry (LCMS)

2 PRINCIPLE

Samples are centrifuged (only required if the sample consists of liquid and solid phases) and the solids freeze dried prior to extraction. The solids are extracted using ASE and the liquids extracted using solid phase extraction (SPE) cartridges.

Prior to extraction of the solids, a mixture of deuterated internal standards (d_4-oestradiol, d_4-17β-oestradiol and d_4-17α-ethinyl oestradiol) are added, and the sample ground and mixed with Hydromatrix™. The samples are placed into ASE stainless steel extraction cells and extracted using methanol at 100 °C and 1500 psi. The collected ASE extracts are diluted with an equal portion of organic free water prior to re-extraction using SPE.

The liquid portion of the sample is spiked with deuterium labelled internal standards (as above) and mixed with an equal portion of methanol.

SPE extraction of the liquid samples or ASE extracts (both 50% aqueous methanol) is undertaken using C_{18} solid phase extraction cartridges.

The cartridges are conditioned with 100% methanol followed by 50% aqueous methanol. The sample is passed through the SPE cartridge at less than 10 ml/min. The cartridge is dried using nitrogen and eluted with 85% aqueous methanol (85:15 methanol:water).

The extracts are analysed using LCMS operated in negative ion electrospray mode using selected ion monitoring.

3 TERMS, DEFINITIONS AND SYMBOLS

For the purposes of this standard operating procedure, the following terms, definitions and symbols apply:

3.1 Analyte

The three free steroid oestrogens determined using this method: oestradiol, 17β-oestradiol and 17α-ethinyl oestradiol)

3.2 Calibration standard

A solution prepared from a secondary standard and/or stock solutions and used to calibrate the response of the instrument with respect to the analyte concentration.

3.3 Calibration verification standard or continuing calibration check

A mid point calibration standard that is used to verify calibration.

3.4 Spiking

The addition of d_4-labelled steroid oestrogens. The recovery of these standards is used to correct values of native analytes of interest.

3.5 Statistical Performance Characteristics

Quantification for measured values of the possible deviations resulting from the random part of the measuring process; e.g. repeatability or instability [ISO 6879:1995]

3.6 Method Blank

A blank soil sample that has been treated exactly as a sample through the complete analytical procedure including extraction, clean-up, identification and quantification including all the relevant reagents and materials.

3.7 ASE

Accelerated Solvent Extraction.

3.8 LCMS

Liquid Chromatography Mass Spectrometry.

3.9 MDL

Method detection limit.

4 PERFORMANCE CHARACTERISTICS

See Annex A.

5 HAZARDS

Hazard assessments should be carried out for all of the chemicals and procedures used and should be consulted prior to carrying out any work with the chemicals involved.

All of the steroids determined are oestrogenically active, and it must be assumed that the internal standards are also active. Appropriate precautions should be taken when handling the pure compounds and standard solutions of these compounds.

Several of the reagents used are potentially hazardous. Methanol and acetonitrile are toxic and flammable.

6 APPARATUS

6.1 Reagent preparation:

Pasteur pipette,

Analytical balance (4 place),

Volumetric Flasks (range between 5 ml and 50 ml)

Range of glass syringes (between 10 µl and 1 ml)

6.2 Freeze drying apparatus

6.3 Accelerated solvent extraction

Dionex ASE 200 Accelerated Solvent extractor with 33 ml stainless cells.

Dionex vials for collection of extracts (60 ml)

Dionex ASE cell cellulose filter (P/N 049458)

6.4 Solid Phase extraction apparatus

Vacuum manifold to which several solid phase extraction cartridges can be attached via 2 litre conical flasks. The flow rate through each individual cartridge is controlled by adjusting the vacuum applied to each one.

6.5 Extract Concentration Equipment

Test Tubes (10ml)

TurboVap™ concentrator with thermostatically controlled water bath.

6.6 LCMS

Liquid Chromatograph (LC) conditions:

LC:	Agilent 1100 system with autosampler, binary pump, degasser and column heater.
Column:	Phenomenex LUNA 5u C18(2), 150 x 4.60 mm (P/N 00F-4252-E0)
Flow:	0.5 ml/min
LC Initial:	Solvent A: 90% Water containing 3% ammonia ,
	Solvent B: 10% Methanol

LC Gradient:

Time	0 min	10 min	18 min	24 min	28 min
%A	90%	50%	10%	10%	90%

Injection volume: 20 μl

Mass Spectrometer (MS): Micromass Quattro LC

Source: Electrospray (negative ion)

Source Temp: 150 C

Desolvation Temp: 400 C

Capillary voltage: 3.1 Kv

Cone Voltage: 45 V

Nebuliser: 75 litres/hour

Drying: 535 litres/hour

Acquisition mode : Selected ion monitoring (SIM)

The system performance should be optimized by injecting a mixed tuning solution containing the three free steroids in methanol. The mixed standard (500 μl of 10 ng μl^{-1}) is introduced using a direct injection loop.

Ions monitored:

269.30 (oestrone)

273.30 (d_4-oestrone)

271.30 (17β-oestradiol)

275.30 (d_4-17β-oestradiol)

295.25 (17α-ethinyl oestradiol)

299.25 (d_4-17α-ethinyl oestradiol)

The retention times of the free steroids are about 19-21 minutes and the data system is programmed to monitor over the time period 15-30 minutes following the injection of the extract.

7 REAGENTS

All reagents must be of sufficient purity that they do not give rise to significant interfering peaks in the analysis. Purity must be checked for each batch of materials by the running of procedural blanks with each batch of samples analysed.

7.1 Standards and internal standards.

Oestrone, 17β-oestradiol and 17α-ethinyl oestradiol are available as crystalline solids from various sources. The internal standards used are 2,4,16,16-d_4-oestrone, 2,4,16,16-d_4-17β-oestradiol and 2,4,16,16-d_4-17α-ethinyl oestradiol. These are available from CDN isotopes (UK agents Univar plc, K&K-Greeff Ltd, Suffolk House, George Street, Croydon CR9 3QL).

7.2 Silanising solution

A solution (10% v/v) of dimethyldichlorosilane in methylene chloride is used to deactivate the surface of all glassware which comes into contact with the samples and extracts.

Silanising solution should preferable be made up as required, but excess solution can be stored for short periods (up to 1 week).

7.3 Solvents

Methanol - Rathburns HPLC grade or equivalent

Acetonitrile – Rathburn silylation grade

Deionised water

7.4 Hydromatrix

7.5 Solid phase cartridges

500 mg/6 ml BondElute C_{18} non-end-capped solid phase extraction cartridges, 20 ml capacity.

8 STANDARD SOLUTIONS

8.1 Internal Standard Stock Solutions, 1mg/ml

Dissolve 10mg of each compound into a 10ml volumetric flask containing acetone, and then make up to the mark. The solution codes are given below.

Compound	Code
d_4-oestrone	d_4-ESTN-ST-Ax
d_4-17β-oestradiol	d_4-ESTRA-ST-Ax
d_4-17α-ethinyl oestradiol	d_4-EEST-ST-Ax

These solutions are stable for one year when stored in a freezer at –18 °C.

8.2 Internal Standard Intermediate solution, 10ng/ul (d_4-STR-INT-Ax)

Add 100µl of each stock solution into a 10ml volumetric flask containing acetone, then make up to the mark.

This solution is stable for 1 year if stored in a freezer –18 °C.

8.3 Steroid Standard Stock Solutions, 1 mg/ml

Dissolve 10 mg of each compound into a 10 ml volumetric flask containing acetone, then make up to the mark. The solution codes are given below.

Compound	Code
oestrone	ESTN-ST-Ax
17β-oestradiol	ESTRA-ST-Ax
17α-ethinyl oestradiol	EEST-ST-Ax

These solutions are stable for one year when stored in a freezer.

8.4 Steroid Standard Intermediate solution, 10 ng/ul (STR-INT-Ax)

Add 100 µl of each stock solution into a 10 ml volumetric flask containing acetone, then make up to the mark.

This solution is stable for 1 year if stored in the freezer.

9 SAMPLING

Sample bottles should be made of glass. Plastic screw tops are suitable provided they are fitted with PTFE® or PTFE®-faced liners. Alternatively, ground-glass stoppered glass bottles may be used. Prior to use, the bottles should be cleaned with a suitable proprietary cleaning agent (e.g. Decon 90) and following thorough rinsing, with deionised water, and dried. The bottles should be deactivated with 20-30ml of silanising solution.

Samples should be kept at 4 °C and extracted as soon as possible after sampling.

10 PROCEDURE

10.1 Glassware pre-treatment

A solution of a dimethyldichlorosilane in methylene chloride is used to deactivate the surface of all of the glassware which comes into contact with the samples and extracts. Sufficient of this solution (0.5 - 30 ml, depending on the surface area of the glassware involved) is poured into all of the sample bottles, ASE vials and conical flasks and swirled over the surface, ensuring complete coverage. Excess silanising solution is then allowed to drain and disposed of. Finally, the glassware is rinsed with dichloromethane.

10.2 Sample collection and preservation

Samples should be collected in suitable glass bottles (1 litre). Since the steroids to be monitored are not stable in sludge for more than a few days, samples should be analyzed as soon as possible following collection. If storage is unavoidable, samples should be kept in a refrigerator at 4°C.

10.3 Sample pretreatment

If a sample contains visible liquid and solid phases (e.g. primary and activated sludge), the sample should be centrifuged and the solid and liquid phases placed into separate containers.

Freeze dry the solids prior to extraction.

Solid samples are extracted using the procedure given in Section 10.4.

To each liquid sample 100µl of the internal standard solution (d_4-STR-INT-Ax) is added, giving a concentration of 250 ngl^{-1}.

Liquid samples are extracted using the procedure given in Section 10.5

10.4 ASE Extraction of solids

10.4.1 Addition of dispersing agent

An aliquot of the freeze-dried sample (1g) is mixed with approximately 7-8 g of Hydromatrix in a small beaker using a spatula (do not use sodium sulfate as this will dissolve in methanol using the applied ASE conditions).

10.4.2 Filling the ASE cell

Insert a disposable cellulose filter in the bottom of the 33 ml stainless steel ASE cell using the cellulose filter insertion tool. Transfer the entire contents of the beaker containing the sample and Hydromatrix into the ASE extraction cell. Press down the contents using the insertion tool.

Add 25 µl of the internal standard solution (d_4-STR-INT-Ax) giving a concentration of 250 ng g^{-1} to each cell. Add 25 µl of the matrix spike (STR-INT-Ax) to the control sample.

Fill the void volume in the cells with Hydromatrix and place another cellulose filter at the outlet of the cell. Screw the top cap onto the cell body and hand tighten. Check the end of each cap to verify that the O-rings are in place and in good condition; replace if necessary.

10.4.3 Load the cell tray

Load the ASE cells into the tray slots. Hang the cells vertically from their top caps. Also, load the collection tray with the appropriate number of 60 ml pre-cleaned and silanized sample vials and rinse tubes in the required positions.

10.4.4 Rinsing/Priming the system

Fill the solvent reservoir with methanol and set it inside the ASE 200 sample compartment. Press the rinse button to prime and rinse the ASE.

10.4.5 Sample extraction

The samples should be extracted using the following conditions:

Oven Temperature:	100°C
Pressure:	10 Mpa (1500 psi)
Oven heat-up time:	5 min
Static time:	5 min
Flush volume:	60% of extraction cell volume
Solvent:	Methanol

10.4.6 Post-extraction procedure

Allow the cells to cool for at least 15 minutes, discard the contents and clean the cells.

Remove sample extracts from the ASE and transfer the contents to 150 ml pre-cleaned, silanized conical flasks.

10.5 SPE Extraction of liquids and ASE extracts

Pre-treatment of liquids and ASE extracts.

Add 40 ml blank water to the ASE extract to obtain a 50% aqueous methanol solution.

Add an equal portion of methanol to the liquid sample to produce a 50% aqueous methanol solution.

The liquid and ASE extracts are now ready for SPE extraction.

10.5.2 SPE cartridge conditioning

An Isolute C_{18} cartridge (500 mg; non-end-capped) is conditioned by passing 100% methanol (5 ml), then 50% aqueous methanol (5 ml) through it at a flow rate of less than 10 ml min^{-1}. The flow rate of the sample is controlled by application of a vacuum. Ensure that the cartridge does not dry out during this process or prior to passage of a sample through the cartridge.

10.5.3 Extract sample

The sample is passed through the cartridge at a flow rate of less than 10 ml min^{-1}, the flow rate again being controlled by application of a vacuum.

10.5.4 Dry SPE cartridge

After all of the sample has passed through the cartridge, it is left under vacuum for a further 5 minutes, then further dried for 10 minutes using clean nitrogen gas.

10.5.5 Elute steroids

The steroids are eluted from the cartridge with an 85:15 mixture of methanol/water (1.5 ml) at a flow rate of 2 ml min^{-1} and collected in a 2 ml Agilent autosampler vial. Concentrate the contents to a final volume of 100 µl using a nitrogen-blow down apparatus. Cap the vial and analyse the contents using LCMS.

10.6 LCMS analysis

Optimize the operating conditions of the LCMS system e.g. according to the manufacturers instructions. Typical conditions for LCMS using a Micromass Quattro are provided in section 6.6.

11 RESULTS

Using the mass spectrometer software the area of each specific peak can be measured. For each determinand the response ratio is then calculated.

$$[\text{Response}] = \frac{\text{Pk Area (D)}}{\text{Pk Area (I.S.)}} \qquad \text{(a)}$$

where:

Pk Area (D) is the peak area of the determinand

Pk Area(I.S.) is the peak area of the d_4-labelled corresponding internal standard

Using the data system attached to the analytical instrument plot the response ratio against the concentration for the standards. From the plotted calibration curve the slope and intercept are determined by linear regression.

By determining the response ratio in the unknown samples, AQC blanks and controls, described above, this can then be applied to the following equation and the concentration of each determinand calculated.

Concentration = [Response - Intercept] / [Slope]

12 QUALITY CONTROL

The quality of the analysis is assured through reproducible calibration and testing of the extraction, clean-up and LCMS systems. A series of quality control samples should be analyzed with each batch of samples and monitored through control charting and other quality review procedures. It is recommended that with every batch of samples extracted, a blank and a spiked control sample (spiked with oestrone, 17β-oestradiol and 17α-ethinyl oestradiol each at 250 ng g^{-1}) are produced.

Annex A

PERFORMANCE CHARACTERISTICS

A.1. Type of sample: Sewage sludge (biosolids) and soils

A.2. Range of application: Between the "MDL" to 1000 ng g^{-1}

A.3. Linear range: This method is linear between 0 – 1000 ng g^{-1}

A.4. Standard Deviation:

Sludge and soil - Determined by analysis of eleven batches of duplicates of spiked sludge and soil samples

Steroid	% Relative standard deviation (n=22) at 50 ng g^{-1}				
	LS	MAD	HDS	AS	PSS
oestrone	15.2%	42.3%	27.3%	23.1%	45.5%
17β-oestradiol	19.0%	36.2%	30.4%	35.4%	47.7%
17α-ethinyl oestradiol	18.4%	23.4%	28.6%	30.2%	48.5%

Where LS=Loamy soil, MAD = mesophilic anaerobic digested sludge, HDS = Heat dried sludge, AS=Activated sludge and PSS=Primary settled sludge

A.5 Limit of detection:

Steroid	Limit of detection ng g^{-1}				
	LS	MAD	HDS	AS	PSS
oestrone	38.2	62	78.5	66.6	68.3
17β-oestradiol	42.6	90.4	81	84.9	76.5
17α-ethinyl oestradiol	33	66.2	50.9	72.5	84.8

A.6 % Recovery

Free steroids spiked at 50 ng g^{-1}

Steroid	% Recovery (n=22) at 50 ng g^{-1}				
	LS	MAD	HDS	AS	PSS
oestrone	108.2%	63.0%	123.0%	124.0%	64.6%
17β-oestradiol	96.4%	107.4%	114.6%	103.2%	69.0%
17α-ethinyl oestradiol	77.2%	121.6%	76.6%	103.2%	75.2%

A.7 Interferences

None identified but any compounds that produce the same mass fragments and elute at the same retention time as the determinands could result in false positives.