The significance of sample mass in the analysis of steroid estrogens in sewage sludges and the derivation of partition coefficients in wastewaters.

T.Y. Chiu[a], Y.K.K. Koh[b], N. Paterakis[a], A.R. Boobis[b], E. Cartmell[a], K.H. Richards[a], J.N. Lester[a] and M.D. Scrimshaw[c*]

[a] Centre for Water Science, School of Applied Science, Cranfield University, Bedfordshire, MK43 0AL, UK.
[b] Faculty of Medicine, Division of Experimental Medicine and Toxicology, Imperial College London, Hammersmith Campus, London, W12 ONN, UK.
[c] Institute for the Environment, Brunel University, Uxbridge, Middlesex, UB8 3PH, UK.
* corresponding author mark.scrimshaw@brunel.ac.uk
tel. +44 (0)1895 267299 fax: +44 (0)1895 269761

ABSTRACT

Optimization of an analytical methd for determination of steroid estrogens, through minimizing sample size, resulted in recoveries >84%, with relative standard deviations <3% and demonstrated the significance of sample size on method performance. Limits of detection were 2.1 to 5.3 ng/g. Primary sludges had estrogen concentrations of up to one order of magnitude less than those found in biological sludges (up to 994 ng/g). However, partition coefficients were higher in primary sludges (except estriol), with the most hydrophobic compound (ethinylestradiol) exhibiting the highest Kp value, information which may be of value to those involved in modeling removal during wastewater treatment.

Keywords: Sewage sludge; steroid estrogens; removal; modeling.

1. Introduction

For many years it has been known that a wide rage of organic micropollutants of anthropogenic origin are present in wastewater [1] and recently those with endocrine disrupting ability have become the focus of attention. It has been estimated that over 99% of the estrogenic activity in sewage effluents and surface waters may be attributable to the presence of free steriod estrogens [2]. Steroids are excreted in the

conjugated form and estrone-3-sulfate (E1-3S), which is relatively slow to deconjugate, may contribute to the load arriving at sewage treatment works (STW) [3]. As presently operated, the ability of biological wastewater treatment to remove steroid estrogens is limited [4,5]. Once in receiving waters, the compounds are likely to undergo biotransformation, although they have the potential to bio-concentrate [6] and accumulate in organisms [7]. Such complex behavior, which is not fully understood, leads to uncertainty in determining the significance of their occurrence in the environment [8].

It has been demonstrated that the sorption of steroid estrogens onto sediment correlates with the total organic carbon content of the sediment, although the presence of organic carbon was not a prerequisite for sorption [9]. Similarly, antibiotics have been shown to exhibit sorption, unrelated to soil organic carbon content, type even though some had relatively low lipophilicity [10]. Although the potential sorption of steroid estrogens is generally regarded as weak, numerous studies have demonstrated that sediments and sewage solids act as sinks for these compounds [11,12].

The ability to determine estrogens within the solid phase allows for a full assessment and understanding of removal processes in the environment and during wastewater treatment processes, however, analytical methods to detect these compounds have predominantly focused on the aqueous phase [13], in samples such as surface waters or sewage effluents, whilst sludges, sediments and soils have received considerably less attention [14]. This has been primarily because of the difficulties associated with the extraction and clean-up of these types of sample [15,16]. A quantitative LC/MS/MS method to analyse these compounds in water has previously been reported [17]. This study develops, evaluates and applies this approach to generate a robust methodology for the determination of four unconjugated steroid estrogens; E1, E2, E3, EE2 and the conjugated E1-3S in sewage sludge samples.

2. Experimental

2.1. Sewage samples

Sewage sludge samples were obtained from four STWs, three were conventional activated sludge works, where primary sludge was obtained from two locations and waste activated sludge (WAS) from three. The fourth was operating as a biological

nutrient removal (BNR) plant where primary sludge passed through a drum thickener prior to fermentation to produce volatile fatty acids. At this site the primary sludge, WAS and fermented, drum thickened sludge were sampled.

2.1 Analytical procedure

The standards, reagents and analytical method used have been reported elsewhere [17], however, a brief description of the handling of samples and spiking for recovery is given below as it is of significance in relation to the results reported in this work. For method recovery work freeze-dried sludges were spiked with mixed estrogen standards to give final concentrations of 10 ng/g (low recovery, LR) or 75 ng/g (high recovery, HR) samples. Steroid estrogens were solvent extracted from freeze-dried sludge on a Multi-Reax system (Heidolph Instruments, Schwabach, Germany) using 10ml ethyl acetate in a 25 ml Teflon tube with mechanical shaking for 1 hour followed by centrifugation at 1500 g for 10 min. The extraction was repeated twice and combined supernatants were evaporated to approximately 0.2 ml then made to 2 ml with hexane. This solution was subjected to clean up by passing through a 500 mg/3ml silica solid phase extraction cartridge (Wateres Ltd., Watford, UK) pre-conditioned with hexane (6 ml), eluted with ethyl acetate (3ml) and then with methanol (2ml). The flow rate for sample extraction and elution was kept constant between 5-10 ml/min using a vacuum manifold. The combined eluates were evaporated to dryness on a rotary evaporator then re-constituted in 2 ml of DCM/MeOH (90:10). This purified sample was then subjected to further clean up by GPC, anion-exchange SPE and finally LC/MS/MS quantification all as described previously [17].

3. Results and discussion

3.1. Method development

Several solvents were tested for the extraction of estrogens from sludge with ethyl acetate producing the highest recoveries. There are precedents for the use of this solvent for the extraction of estrogenic steroids in marine sediments [18] and alkylphenolic surfactants in sewage sludge [19]. Silica cartridges were evaluated for clean-up, although due to the relatively high polarity of E1-3S and E3, selecting a solvent compatible with the cartridge was a challenge, however, selectivity in elution from the cartridges was achieved with 3 ml ethyl acetate followed by 2 ml methanol.

3.2. Impact of sample size on recoveries

Extraction and clean-up steps are undertaken prior to quantification and the extent of manipulation required depends on the quantity of analyte present in the sample, the amount of contamination (co-extractives) from the solid matrix, and for the final quantification, the analytical tool to be utilized. Using a sample preparation protocol involving solvent extraction, GPC and silica gel cleanup, mean recoveries of estrogens from 0.5 g (dry weight) sludge of greater than 70% have been obtained [20].

Therefore, 0.5 g of primary sludge was initially selected for evaluating the performance of the analytical method. Recoveries for the steroid estrogens in both high and low spiked sludge samples were poor, all being <5% (Table 1). It was suspected that the sample size was impacting recoveries, and to investigate this hypothesis, the effects of using different dry weights (0.2 and 0.1g) of sample on method performance was evaluated. Recoveries of >84% with excellent repeatability were achieved for both low and high spikes from samples of 0.1g sludge (Table 1). Whilst good recoveries for the majority of steroid estrogens (E1, E2 and E1-3S) were observed using 0.2g sludge, the high %RSD and the low recoveries for E3 and EE2 at low spikes exhibited no improvement over the 0.5 g sample size. It is assumed that interferences still occurred and hindered the determination of the estrogens.

3.3. The evaluation of matrix effects

The matrix interference was evaluated using the smallest (0.1 g) sample and a blank which were unspiked or spiked with the steroids (low and high spike of 50 ng/g and 75 ng/g respectively). The signal suppression was derived using the approach described in [21]. For the blank, signal suppression of 5-8% was observed for all analytes in both low and high spikes (50 or 75 ng/g). However, analysis of sewage sludge demonstrated an increase in suppression due to matrix effects, with more polar compounds (E1, E3 and E1-3S) exhibiting least suppression (8-18%). Both E2 and EE2 signals were suppressed to a greater extent in the more complex solid matrix (10-28%). The impact of sample size and concentration factors on matrix effects has previously been observed to be of significance in relation to reducing matrix effects in the analysis of alklyphenols [22].

Ion suppression is commonly encountered in LC/MS/MS where the ESI^- interface is utilized [23]. This effect, if not well characterized, may lead to erroneous quantification of an analyte of interest. Several analytical approaches to reduce matrix interferences have been discussed elsewhere [14]. In this study, a three-step clean-up procedure to reduce the impact of interferences inherent in the wastewater matrices has been utilized, together with the addition of deuterated internal standards. Due to the complex composition of the sludge, the reliability of the data was confirmed precisely using the ratio of spiked standards in various matrices. Although the current clean-up procedure involves multiple manual steps, automated clean-up procedures incorporating on-line SPE coupled to LC/MS/MS at present lack the flexibility to incorporate such complex clean-up steps into their protocols [19], and are more likely to suffer from matrix effects. They may, however, incorporate the use of internal standards [24].

3.4. Method performance

Evaluation of method performance involved undertaking recovery tests on samples of primary sludge and matrix free-filter paper. The LR and HR recovery studies (Table 1) were performed using sewage sludge spiked at 10 ng/g and 75 ng/g of mixed steroid standards respectively along with deuterated internal standards (75 ng/L) each of E1-d_4, E2-d_5, E3-d_3, EE2-d_4 and E1-3S-d_4). Determination of the limit of detection (LOD) was performed with matrix free-filter paper spiked at 5 ng/g and 50 ng/g of mixed steroid standards respectively and the internal standards. The reproducibility of this method for primary sludge samples of 0.1 g, represented as relative standard deviation, ranged from 1 to 3% (Table 1).

Recoveries of three replicate analyses for each compound from the spiked filter paper were greater than 87% (Table 1). Method recoveries obtained for the analytes under study were 98%, 95% and 105% for E1, E2 and EE2 respectively from the primary sludge, which are comparable to those obtained elsewhere for E1 (119%), E2 (83%), EE2 (113%) in activated sludge [20]. The method detection limit (MDL) defined as the analyte concentration corresponding to that giving a *S/N* ratio of 3 were 2.1 – 5.3 ng/g for the primary sludge samples spiked at 10 ng/g (Table 2).

3.5. Concentrations of steroid estrogens in sewage sludges

To understand the behavior of steroid estrogens during wastewater treatment processes, and to elucidate the significance of mechanisms responsible for their removal, it is necessary to determine their concentrations in sludges. Therefore the method was applied to a range of primary sludges, WAS and a sample of fermented, drum thickened sludge were utilized to elucidate partitioning behavior at a number of STW.

All compounds were detected in the sludges analysed. In general, E1, E2 and EE2 were found in greater amounts compared to E3 and E1-3S (Fig. 1), which is probably a result of the more hydrophobic compounds exhibiting a greater affinity for the solids. The presence of the more hydrophilic compounds E1-3S and E3 to levels of as high as 59 ng/g and 27 ng/g respectively demonstrates that it is important to include the hydrophilic compounds in the determination of estrogens in solid matrices. Concentrations in the WAS were almost an order of magnitude above those observed in primary sludges, which indicate that adsorption to the biosolids occurs during the biological treatment stage. It is also apparent that the fermentation of primary sludge has little impact on the concentrations of estrogens.

3.6. Partitioning of steroid estrogens to sewage sludges

The distribution of the estrogens between the solid and liquid phase can be described by the partition coefficients, Kp, which may be calculated from the concentrations in the aqueous and dissolved phase, along with total suspended solids concentrations. The logKp values for the estrogens calculated at the sites studied are presented in table 3. the data indicate that there are differences in sorption behavior between the two sludge types (primary and WAS) with logKp values decreasing in the order EE2> E1> E1-3S> E2> E3 in primary sludge, and EE2> E1≈ E3≈ E2> E1-3S in the WAS samples. The observed behavior in these sludge samples agrees with experimental data which indictaed logKp for E1 > E2 (2.37 and 1.98) [25], although differs from observations which report EE2 being less than E2 (logKoc 3.45 – 3.85 and 3.71 – 4.12) [26].

The average Kp values for primary sludges were above those for the biological WAS for all compounds except E3 (Table 3). Biological sludges have a greater carbon content than the primary sludge, which may contain a significant amount of inorganic

matter. The use of Kd values in evaluating the significance of sorption in the removal of estrogens has been reported [27], who utilized KD values derived from a study on nitrifying activated sludge [28]. Values for E1, E2 and EE2 of 2.60, 2.67 and 2.76 [28], exhibit good agreement with average values reported for WAS in Table 3, however, it is evident that some variation exists between sites, and that logKd values are higher in primary sludges.

Conclusions

A sensitive and selective method utilizing a three stage sample clean-up and LC-MS-MS analysis has been developed for the determination of free estrogens and a conjugated estrone sewage sludge solids, detection limits of between 2.1 and 5.3 ng/g were achieved. Partitioning coefficients indicated that logKow values are not an entirely reliable predictor of sorption behavior and the field data presented in this work may be of use to those modeling removal of estrogens in wastewater treatment processes

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Figure legends and table headers

Figure 1. Concentrations of steroid estrogens on primary sludges, WAS and fermented solids from the drum thickener at the BNR works.

Table 1. The influence of sample size (0.5 – 0.1g dry weight) of primary sludge on percentage recoveries and %RSD (*n*=3)

Table 2. Method detection limits in primary sludge (*n*=3).

Table 3. LogKp values calculated for the steroid estrogens in primary sludges, WAS and fermented solids from the drum thickener.

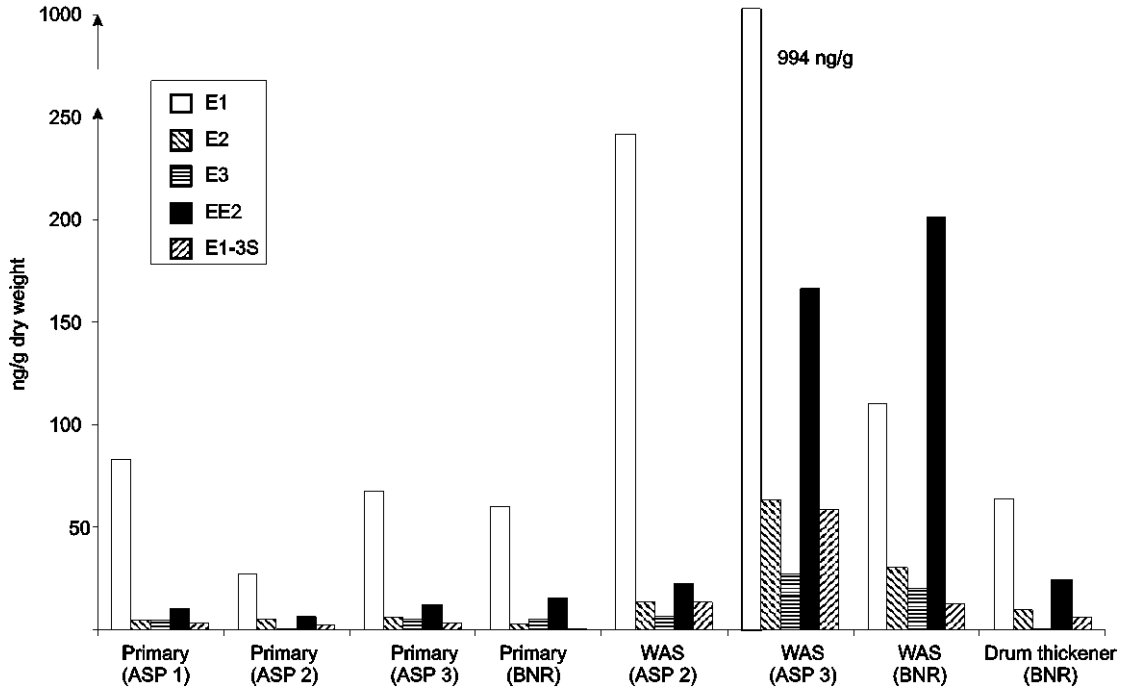

Figure 1. Concentrations of steroid estrogens on primary sludges, WAS and fermented solids from the drum thickener at the BNR works.

Table 1. The influence of sample size (0.5 – 0.1g dry weight) of primary sludge on percentage recoveries and %RSD (*n*=3)

Steroid estrogens	0.5 g sample		0.2 g sample (% RSD)		0.1 g sample (% RSD)		blank matrix (%RSD)	
	LR[a]	HR[b]	LR[a]	HR[b]	LR[a]	HR[b]	LR[c]	HR[d]
E1	<5	<5	84 (22)	84 (21)	98 (2)	100 (2)	100 (5)	100 (2)
E2	<5	<5	100 (16)	95 (11)	95 (1)	99 (1)	87 (10)	99 (3)
E3	<5	<5	<5	84 (21)	99 (2)	84 (2)	98 (8)	97 (3)
EE2	<5	<5	<5	83 (7)	105 (1)	100 (1)	99 (5)	98 (6)
E1-3S	<5	<5	82 (30)	82 (16)	109 (2)	100 (3)	100 (11)	99 (7)

[a]10 ng/g or [b]75 ng/g was spiked to sludge (75 ng/g of deuterated internal standard); [c]5 ng/g or [d]50 ng/g standard was spiked (75 ng/g of deuterated internal standard).

Table 2. Method detection limits in primary sludge (*n*=3).

Steroid estrogens	MDL[a] (ng/g) in sludge
E1	2.1
E2	4.9
E3	4.5
EE2	5.3
E1-3S	2.6

[a]10 ng/g was spiked to solid matrices of sludge (75 ng/g of deuterated internal standard); 0.1g Dry weight of sludge was used to derive the MDL

Table 3. LogKp values calculated for the steroid estrogens in primary sludges, WAS and fermented solids from the drum thickener.

	E1	E2	E3	EE2	E1-3S
Primary (ASP 1)	n/a	n/a	n/a	n/a	n/a
Primary (ASP 2)	2.85	2.84	1.78	3.81	3.10
Primary (ASP 3)	3.22	2.17	1.61	3.76	2.36
Primary BNR	3.14	2.07	2.65	3.40	2.45
Primary (mean)	3.07	2.36	2.01	3.66	2.64
WAS (ASP 2)	2.53	2.78	2.79	2.93	2.05
WAS (ASP 3)	2.40	2.66	2.35	3.35	1.52
WAS (BNR)	1.99	1.11	1.45	2.00	1.60
WAS (mean)	2.31	2.18	2.20	2.76	1.72
Drum thickener (BNR)	1.98	1.22	1.31	2.89	2.57

n/a. could not be calculated as data on suspended solids content was unavailable.