ANALYSIS OF ESTROGENS IN SEDIMENT FROM A SEWAGE-IMPACTED URBAN ESTUARY USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY/TIME-OF-FLIGHT MASS SPECTROMETRY

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Abstract—We describe a highly selective and sensitive method for determination of estrone (E1) and β-estradiol (E2) in sediments, using high-performance liquid chromatography/time-of-flight (HPLC-ToF) mass spectrometry. The method involved sequential cleanup of sediment extracts over solid phase extraction cartridges, normal phase HPLC, and immunoaffinity extraction, which combine to remove coeluting matrix interferences. Resulting method detection limits (0.03 and 0.04 ng/g for E1 and E2, respectively) are sufficient to determine E1 and E2 in estuarine sediments collected from sewage-impacted Jamaica Bay (New York, NY, USA). The ToF analyzer has a higher resolution (>6,000) than quadrupole mass analyzers and can provide accurate mass estimation to within 2 mDa, which helped in distinguishing steroids from isobaric matrix interferences. The E1 and E2 were internally mass calibrated with respect to their coeluting surrogate standards, and the mass measurement error was between 1.1 and 1.4 mDa. The levels of E1 and E2 ranged between 0.07 to 2.52 and 0.05 to 0.53 ng/g, respectively. The measured concentrations of steroids in sediments correlated closely with other wastewater tracers. Despite the low concentrations of sediment-associated estrogens, their predicted estrogenic potency exceeds that of other measured estrogenic contaminants.

Keywords—High-performance liquid chromatography Mass spectrometry Sediments Steroids Estrogens

INTRODUCTION

The exposure of organisms in the aquatic environment to estrogens and estrogen-like compounds has been linked to endocrine disruption, as observed in the feminization of male and sexually immature fish [1,2]. Chemical analysis of surface waters has revealed the presence of natural estrogens such as 17β-estradiol (E2), estrone (E1), the contraceptive ethynylestradiol, and estrogen mimics such as alkylphenols [3–5]. Estrogens and estrogen mimics are released into the surface waters via domestic, industrial sewage waste and also from agricultural runoff [6].

The estrogens, once released into the aquatic environment, will partition between the water phase and sediment. The log octanol–water partitioning coefficient (Kow) of E1 and E2 is between 3 and 4 [7,8], suggesting a moderate sorption of these steroids to sediment. The sediment can therefore serve as a sink for these steroids by acting as a removal route for the estrogens from the water column. Wastewater-affected sediment could also act as a source of steroids back to surface waters. The steroids in the sediment could potentially cause endocrine disruption in benthic organisms, including fish. It is therefore important to understand the fate and persistence of these steroids in sediment. Studies to date have examined the sorption and degradation of steroids in sediments by amending steroid mixtures into sediments [8–10]. Few studies have actually monitored environmentally realistic concentrations of these steroids in sediments in the aquatic environment [7,11,12]. Steroid estrogen concentrations in sediments have been found as high as 12 ng/g sediment [12], although most measured concentrations have been reported to be below 1 ng/g [7,11,12]. In order to detect such low levels of estrogens, Ternes et al. [7] used several purification steps to isolate the steroids before gas chromatography/tandem mass spectrometry (GC-MS/MS) analysis of the derivatized analytes. Furthermore, reports exist of steroid hormones in sludge, compost, and soils that rely on analytical detection employing enzyme-linked immunosorbent assays (ELISA) [13]. While ELISA can be an extremely sensitive method for the analysis of steroid hormones, Huang et al. [3] have shown that complex environmental matrices such as extracts from wastewater treatment plants had to be extensively purified before ELISA would provide reliable results. The organic matrix present in extracts from most organic matter–enriched soil or sediment samples is generally even more complex and concentrated than for sewage effluent.

We report here a method for determining E1 and E2 in sediment/soil samples that relies on high-performance liquid chromatography (HPLC) coupled with time-of-flight (ToF) mass spectrometry and utilizes electrospray ionization (ESI). Salient features of this method include the use of a normal phase cleanup step to reduce sediment matrix interferences, followed by an immunoaffinity extraction step described by Ferguson et al. [14], and the novel application of the ToF mass analyzer. The ToF analyzer has enough resolution to provide an increased level of selectivity above single-quadrupole mass analyzers [15]. More important, the ability of the ToF analysis to provide accurate mass estimation (generally within 2 mDa for small-molecule analysis) provides a powerful tool for analyte confirmation, information that is different and complementary to tandem mass spectrometry (MS-MS) analyses [15]. We have applied this method to analysis of sediments collected from sewage-impacted Jamaica Bay (Fig. 1), for which potentially estrogenic alkylphenol ethoxylate metabolites, polychlorinated biphenyl mixtures, and chlorinated pesticides have been previously measured [4,16].
Fig. 1. Map of Jamaica Bay, Long Island, New York, USA (40°36′N, 73°48′W) showing sediment sampling stations as well as locations and capacities (millions of gallons per day [mgd]) of the four largest wastewater treatment plants discharging into the estuary.

STUDY SITE
Jamaica Bay is situated on the southwestern shore of Long Island (NY, USA) (Fig. 1). It has been a sampling location for previous sediment studies of wastewater-derived contaminants [4,16,17]. The characteristics of this enclosed embayment are summarized elsewhere [4,18]. The freshwater inputs to the bay are dominated by effluents from six wastewater treatment plants discharging approximately 1.1 × 10⁹ L/d. Sedimentation deposition rates have been estimated in the range of 0.92 to 1.4 cm/year in the highly depositional area of Grassy Bay in the northwest corner of the Bay adjacent to John F. Kennedy airport (New York, NY, USA) [17,19].

MATERIALS AND METHODS
Estrone-d⁴ (d⁴-E1), 17β-estradiol-d⁴ (d⁴-E2), and Equilin-d⁴ were obtained from C/D/N Isotopes (Pointe-Claire, PQ, Canada). Monoclonal antibodies to E2 were obtained from Fitzgerald Industries International (Concord, MA, USA), and clone M94150 and monoclonal antibodies to E1 were obtained from Keith Henderson (AgResearch Wallaceville Animal Research Center, Upper Hutt, New Zealand, IgG lot 46).

Sample collection
Sediment samples were collected from Jamaica Bay in the summer of 1998 by the U.S. Environmental Protection Agency (U.S. EPA), Region 2, as part of the Regional Environmental Monitoring and Assessment Program. The samples represent composite surface grab samples from each site. After collection, the samples were air-dried to prevent microbial degradation of the analytes and stored in glass jars at 4°C until further analysis. Other aliquots of the same homogenized samples were analyzed for neutral alkylphenol ethoxylate metabolites [4] and selected semivolatile chlorinated hydrocarbons [16] (Darvine Adams, personal communication for 1998 R-EMAP data, EPA Region 2, NY, USA).

Chemical analysis
Sediment extraction. Dried sediment (2 g) was ground finely in a mortar and pestle and packed in a 150-mm stainless-steel column (4.6-mm i.d.) fitted with 0.2-µm stainless-steel frits (Alltech Chromatography, Deerfield, IL, USA). The packed sediment was spiked with surrogate standards (5 ng each of estrone d₁ (d₁-E1) and estradiol d₁ (d₁-E2). Any remaining volume in the steel cartridge was filled with sea sand that had been baked at 450°C for 6 h. The sediment-filled cartridge was immersed in a heated ultrasonic bath (65°C), and the analytes were extracted by eluting the cartridge with methanol (0.5 ml/min) for 7 min as described previously for the alkylphenol ethoxylate metabolites [4]. Baked sand packed into extraction columns and spiked with surrogates was used as blank. The methanol extracts of the samples were dried under nitrogen and analyzed for steroids using the procedure described by Ferguson et al. [14] with some modifications.

Solid phase extraction cleanup. The sediment extracts were
reconstituted in 1.5 ml of methanol and further diluted to a large volume of MilliQ® (Millipore, Bedford, MA, USA) water (1 L) to ensure dissolution of the extracts. Subsequently, the sediment extract was loaded on a layered-bed Lichrolut EN (0.2 g) and C₈ (0.5 g of Varian BondElut) solid phase extraction cartridge (Varian, Palo Alto, CA, USA). Prior to sample loading, the cartridges were preconditioned in sequence with hexane, acetone, methanol, and MilliQ water as described previously [14]. After loading the sample, to reduce levels of interfering contaminants, the cartridges were washed with 25% acetone/water (3 × 2 ml) and the analytes eluted with 80% acetone/water (3 × 2 ml). The extracts were dried in a Savant SpeedVac centrifugal vacuum evaporator (Savant Instruments Inc., Farmingdale, NY, USA). Direct cleanup of these sediment extracts over the immunoaffinity column followed by liquid chromatography/mass spectrometry analysis as described previously [14] for sewage effluents resulted in >90% ion suppression of E2 and E1. The increased ion suppression observed in the sediment extracts, compared to treated sewage effluent, is most likely related to greater amounts of extracted organic matter. We therefore introduced an extra normal phase HPLC cleanup step for the sediment extracts before extraction over the immunoaffinity column.

**Normal phase HPLC and immunoaffinity cleanup.** The steroids E2 and E1 were separated on a Supelcosil diol column (25 cm × 10 mm, 5-µm particle size; Supelco, Bellefonte, PA, USA), using a Shimadzu pump (LC-600) (Kyoto, Japan), with a gradient using solvents A (10% solvent B contained in hexane) and solvent B (10% MeOH in ethyl acetate). The steroids in standard solution were detected with an online Shimadzu ultraviolet-visible spectrophotometer (Shimadzu SPD-6AV) with wavelength set at 284 nm. The HPLC conditions involved a linear gradient from 10% B to 25% B in 10 min, at a flow rate of 5 ml/min, followed by a linear gradient from 25 to 70% B for the next 5 min and a 5-min hold at 70% B. Elution of E1 and E2 were observed at 5.2 and 6.5 min, respectively. Sediment extracts were loaded on the diol column and fractions collected in the region of elution of E2 and E1. The fractions were dried, reconstituted in 25 ml of 5% methanol. The extracts were purified over the synthesized immunoaffinity column following the procedure of Ferguson et al. [14] with minor modifications. After loading the sample on the immunoaffinity column, the column was washed with 20% MeOH (5 ml), and the steroids were eluted with 70% MeOH (4 ml), followed by 1 ml of MilliQ water. The column was reconditioned with phosphate-buffered saline (10 ml) between samples. The immunoaffinity column eluents were evaporated to dryness and reconstituted in 200 µl of 25% acetonitrile in MilliQ water. Internal standard (6 ng of Equilin-d₄) was added to each sample before analysis by liquid chromatography/mass spectrometry.

**HPLC-ToF-MS analysis.** Steroids were quantified in the sediment extracts using HPLC-ToF-MS in negative-ion mode. The steroids were separated on a Betasil C18 column (3-µm particle size, 15-cm × 2.1-mm i.d.; Keystone Scientific, Bellefonte, PA, USA) using a Waters 2695 LC pump (Milford, MA, USA) with similar HPLC conditions described previously [14]. The steroids were detected using a Micromass (Manchester, UK) ToF-MS (LCT™ mass spectrometer, equipped with a 4.6-GHz time-to-digital converter) with a Z-SPRAY® ESI source. The ESI source parameters were optimized by infusing standard steroid solutions. The electrospray conditions optimized at a capillary voltage of −2,200 V, sample cone at −60°C, and source temperature at 120°C. The desolvation gas (nitrogen) was set at a flow rate of approximately 400 L/h, and desolvation temperature was 200°C. The microchannel plate detectors were operated at 2,750 V. The instrument was externally calibrated using polyalanine. Four-point calibration curves were made for the steroids within the linear range of the instrument (0.5–10 ng/ml, r² > 0.999). The E1 and E2 in the samples were measured using isotope dilution methods using the deuterated surrogate standards. The E1 and E2 were also internally mass calibrated with respect to their coeluting surrogate standards, using the manufacturer’s All File Accurate Mass Measure software process. As a quality control measure, an estimate of surrogate recovery was calculated relative to the internal standard, Equilin-d₄. Concentrations of E1 and E2 were not corrected for recovery because of the isotope dilution approach. Estimated recoveries (± relative standard deviation) varied between samples and averaged 64 ± 14% and 71 ± 14% for d₄ E1 and d₄ E2, respectively. Reduced recoveries in some samples were due to a combination of sample matrix–dependent differences in ionization suppression between surrogate and internal standards and the extensive amount of sample purification conducted. The efficiency of extracting native E1 and E2 in samples was assessed by comparing measured concentrations in Jamaica Bay sediments with more exhaustive extractions that either used twice as much methanol solvent or followed methanol extraction with a stronger methylene chloride:methanol solvent. No additional steroid hormone was detected with more rigorous extraction in this work in agreement with results from a prior study with structurally related alkylphenols [4]. The precision of the method was determined based on the analysis of replicate extractions of the same sediment sample.

**RESULTS AND DISCUSSION**

**Sensitivity and selectivity of analysis by HPLC-ToF-MS**

Measuring steroids in complex matrices such as sediments can be very difficult since it involves removing impurities that interfere with the analysis. Ternes et al. [7] used extensive cleanup steps for steroid analysis in sludge and sediment by the GC-MS/MS and obtained method detection limits (MDLs; defined as minimum amount of steroids present in a sample that produced a signal-to-noise [S/N] ratio of 3) in the range of 0.06 to 0.12 ng/g (derived from reported limit of quantitation). By contrast, the less comprehensive sample purification approaches were employed by Petrovic et al. [12] to determine multiple classes of endocrine-disrupting chemicals in sediment and water samples. Application of that method resulted in higher MDLs in the range of 0.5 to 1 ng/g by HPLC-MS equipped with a quadrupole mass analyzer. Our method involved sequential cleaning of the sediment extracts over solid-phase extraction cartridges and normal phase HPLC followed by immunoextraction to remove matrix-associated interferences subsequent to analysis by HPLC-ToF-MS. The MDLs (defined as S/N = 3) varied between samples and were determined as 0.03 ng/g (standard deviation [SD] = 0.015, n = 8) and 0.04 ng/g (SD = 0.012, n = 8) for E1 and E2, respectively. We were able to achieve similar MDLs compared to Ternes et al. [7] and 10- to 17-fold greater sensitivity than Petrovic et al. [12]. Variation in MDLs between the different methods may also result from differences in instruments used, in sample preparation, and from sample-dependent coeluting interferences.

The ToF mass spectrometer provides different and com-
plementary information than GC-MS/MS analysis using triple-

quadrupole mass analyzers. The superior resolving power of
ToF and mass measurement accuracy provide greater ability
to resolve some isobaric interferences, and the ability to es-
timate accurate mass and elemental composition provides a
powerful tool for analyte confirmation. These distinct advan-
tages of the ToF instrument helped in enhancing the selectivity
of steroid analysis in this work. The identities of E2 and E1
in the samples were confirmed by retention time, mass ac-
curacy measurements, and elemental composition calculated
from the instrument software. In the samples, E2 and E1 were
mass calibrated with respect to their coeluting deuterated sur-
rrogates. The representative mass spectra shown of E2 and E1
(Fig. 2) illustrate both the resolving power of the ToF analyzer
and the good agreement between measured and actual accurate
masses target chemicals (mass errors of 1.4 and 1.1 mDa for
E2 and E1, respectively). From the estimated accurate masses,
the instrument software can calculate possible elemental com-
position. Limiting possible elements to a combination of C,
H, O, and N in this analysis, typically two matches were found
that fell within an instrument-specified tolerance of 2.0 mDa.
One match was for the elemental formula corresponding to E1
or E2 and the other for structures containing six nitrogen at-
oms; an unlikely possibility for such low-molecular-weight
compounds. The inset in Figure 2 shows that the ToF instru-
ment was capable of resolving the mass of E2 (calculated
accurate, mass to charge, \(m/z = 271.1712\)) and a contaminant ion (\(m/z = 271.2265\)) in the
region of elution of E2. (A) and (B) represent reconstructed ion
chromatograms of E2 in a sediment sample (S4), with varying mass win-

dows resulting in improvement of the signal-to-noise (S/N) ratio.

Fig. 3. Enhanced selectivity and sensitivity of estrone (E1) analysis
by high-performance liquid chromatography/time-of-flight mass spec-
trometry analysis. Inset shows the spectrum of E1 (mass to charge,
\(m/z = 269.1553\)) and a contaminant ion (\(m/z = 269.2481\)) in the
region of elution of E1. (A) and (B) correspond to reconstructed ion
cromatograms of E1 in a sediment sample, with varying mass win-
dows resulting in a varying signal-to-noise (S/N) ratio.

Fig. 2. Enhanced selectivity and sensitivity of estradiol (E2) analysis
by high-performance liquid chromatography/time-of-flight mass spec-
trometry analysis. Inset shows the spectrum of E2 (mass to charge,
\(m/z = 271.1712\)) and a contaminant ion (\(m/z = 271.2265\)) in the
region of elution of E2. \((A)\) and \((B)\) are reconstructed ion
cromatograms of E2 in a sediment sample (S4), with varying mass win-
dows resulting in an improvement of the signal-to-noise (S/N) ratio.
out the upper reaches of Jamaica Bay. In all sediments analyzed (other than the sample from station S1, where the measured concentrations were near the detection limit), the levels of E1 measured were four to seven times higher than the levels of E2 (Table 1). Jamaica Bay receives discharges from sewage treatment plants that may contain higher concentrations of E1 compared to E2, typical of sewage effluent discharges as observed by other groups [14,20,21]. Therefore, assuming the affinity of E1 and E2 for sediment particles to be approximately equal [9], the concentrations of E1 versus E2 in the sediment may reflect the concentration levels of the individual steroids in the Jamaica Bay waters. Alternately, increased concentration of E1 versus E2 may be caused by oxidation of E2 to E1 before or after incorporation in the sediment bed [7].

The sediments in Jamaica Bay are most likely accumulating steroids from wastewater sources that discharge into the bay. The concentrations of E1 and E2 were highest in sediments S4 and S9, which are located within a depositional basin [17] close to a large wastewater discharge treatment plant (see Fig. 1). A significant correlation ($p < 0.01$ based on a correlation coefficient of 0.991 and four degrees of freedom [22]) was observed between steroids levels and previously reported total organic carbon concentrations [4,19]. The correlations of steroid hormones with sediment organic matter and other stable particle-reactive wastewater contaminants are consistent with the hypothesis that sediment-associated E1 and E2 are also relatively stable to postdepositional transformation. The depositional properties of Jamaica Bay may contribute to relative stability of estrogenic organic compounds in sediments. For example, sediments from the bay are subjected to substantial organic enrichment from both sewage-derived organic matter and enhanced autochthonous production from the eutrophic water column. Aerobic microbial degradation is also limited to the very surface of Jamaica Bay sediments, and rapid sediment burial rates occurring at some of the sites sampled may protect organic contaminants from transformations occurring near the sediment–water interface.

The endocrine-disrupting ability of the steroid hormones in bed sediments will depend on the concentration, bioavailability, and persistence of E2 and E1 in the sediments. The persistence and fate of these steroids will depend on the oxygenation conditions present in the bay. As discussed previously, aerobic degradation is probably very limited in Jamaica Bay sediments. Under anaerobic conditions, E2 is converted to E1 [8] and can persist as E1. The E1 is an endocrine disrupter as evaluated by the yeast-based, estrogen receptor–mediated ß-galactosidase gene expression assay (YES assay). However, E1 is less estrogenic than E2 by a factor of 10 [23]. Although E1 and E2 are present in far lower concentrations compared to some of the other endocrine-disrupting compounds detected in Jamaica Bay, they can be highly estrogenic. We have tried to predict the estrogenic activity of E1 and E2 relative to some of the compounds detected in Jamaica Bay sediments (Table 2). The predictions do not take into account the persistence and bioavailability of the compounds. The estrogenic potency of many of these compounds relative to E2 based on the YES assay were obtained from Fang et al. [23], who computed a potency value using YES assay data sets derived by two different groups [24,25]. The estrogenic potency for polychlorinated biphenyls was obtained using the YES assay by Layton et al. [26]. However, the estrogenic potency of NP and NP ethoxylates were obtained from Legler et al. [6], who used the estrogen receptor–mediated luciferase reporter gene assay (ER-CALUX assay) to assess estrogenicity. Since the potency of NP assessed by the ER-CALUX assay [6] was similar to the potency determined by the YES assay [23], we included...
Environmental matrices require mass spectrometric approaches that provide a high degree of selectivity given the abundance of isobaric interferences. The results from this work show that concentrations of E1 and E2 in sediments from wastewater-impacted Jamaica Bay are comparable to levels found in highly populated rivers in Europe [7,12] and that although the levels are in the low-ng/g range, they cannot necessarily be ignored in environmental assessment of the causes of endocrine disruption observed in wild fish.

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### REFERENCES

### Table 2. Predicted estrogen activity of some of the contaminants detected in Jamaica Bay (New York, NY, USA) sediments

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (nmol/g)</th>
<th>Estrogenic activity (estrogenic potency × concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum</td>
<td>Minimum</td>
</tr>
<tr>
<td>E2</td>
<td>$1.95 \times 10^{-3}$</td>
<td>$1.84 \times 10^{-4}$</td>
</tr>
<tr>
<td>E1</td>
<td>$9.36 \times 10^{-3}$</td>
<td>$2.58 \times 10^{-4}$</td>
</tr>
<tr>
<td>$p,p'$-DDE$^b$</td>
<td>$8.7 \times 10^{-3}$</td>
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</tr>
<tr>
<td>$p,p'$-DDT$^c$</td>
<td>0.027</td>
<td>0.0</td>
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<tr>
<td>NP$^d$</td>
<td>62.5</td>
<td>0.14</td>
</tr>
<tr>
<td>OP$^e$</td>
<td>0.19</td>
<td>0.014</td>
</tr>
<tr>
<td>NP1EO$^f$</td>
<td>46.34</td>
<td>0.41</td>
</tr>
<tr>
<td>NP2EO$^g$</td>
<td>10.82</td>
<td>0.19</td>
</tr>
<tr>
<td>Total PCBs$^h$</td>
<td>2.78</td>
<td>$4.8 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

$^a$E2 = estradiol; E1 = estrone; DDT = $p,p'$ dichloro-diphenyl-trichloroethane; DDE = $p,p'$ dichloro-diphenyl-trichloroethylene; NP = nonylphenol; OP = octylphenol; NP1EO = nonylphenol 1 ethoxylate; NP2EO = nonylphenol 2 ethoxylate; PCBs = polychlorinated benzenes.

$^b$Data from Fang et al. [23], which combined yeast estrogen screen assay data from two different data sets by Gaido et al. [25] and Coldham et al. [24].

$^c$Ferguson et al. [4].

$^d$Estrogen receptor–mediated luciferase reporter gene assay data from Legler et al. [6].

$^e$Adams et al. [16].

$^f$Yeast estrogen screen assay data from Layton et al. [26] for Aroclor 1248.