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Analyses of phenolic endocrine disrupting chemicals in marine samples by both gas and liquid chromatography–mass spectrometry

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Abstract

With the renovation of Boston Harbor's Deer Island wastewater treatment plant and the extension of its diffuser pipes 15 km further into Massachusetts Bay, there arose the question whether the increased load of its secondary-treated wastewater contained significant amounts of phenolic endocrine disrupting chemicals (EDCs). Sampling from an oceanographic research vessel during the summers of 2003 and 2004 allowed for a unique opportunity to obtain clam, zooplankton, and bottom sediment samples. The samples were prepared by enhanced organic-solvent microwave digestion, followed by solid-phase extraction (SPE), derivatization and then analyzed by gas chromatography–mass spectrometry (GC–MS) or left un-derivatized and analyzed by LC–UV and liquid chromatography–mass spectrometry (LC–MS). The marine samples, especially parts of the clams, zooplankton and certain bottom sediments were found to contain primarily bisphenol A (BPA) at concentrations of 1–30 ng/g.

Keywords: Phenolic endocrine disrupting chemicals; EDCs; Xenoestrogens; Gas chromatography–mass spectrometry; Liquid chromatography–mass spectrometry; Solid-phase extraction (SPE)

1. Introduction

Pollution of the marine environment caused by the discharge from sewage treatment plants has become an important international topic. Recent scientific reports have documented that a wide variety of both natural and man-made (anthropogenically-generated) chemicals are added to the environment from these allowed wastewater discharges [1–8]. Certain of these chemicals or their by-products have been shown to affect the endocrine system of living organisms and tend to be persistent in the environment [1]. Endocrine disrupting chemicals (EDCs) consists of a wide variety of different chemicals that may either alter, compete and/or displace certain of the important, natural steroids from their receptor sites thereby changing in the body the function of these natural hormones [9]. Studies have shown that EDCs can be absorbed from water and solid sediments into the marine life and then may be bio-accumulated by orders of magnitude up the food chain [3–10]. Increased body loads of EDCs may cause birth defects, altered immune functions, contribute to sexual dysfunction, even cause cancers and possibly heart disease in living species [5,8,11]. In fairness, there are emerging reports in scattered scientific reports that in modern sewage treatment plants with effective longer residence times and enhanced secondary bioremediation methods, that many of the natural and man-made hormones are removed from the wastewater stream [12–16]. Holbrook's et al. [17] data indicate that 51–67% of estrogenic activity contained in influent wastewater was biodegraded during wastewater or biosolid treatment processes or was not available by their extraction/detection procedure. For our Boston area study site, little data is available except that the Deer Island Waste Treatment Plant is reported to have the most modern of secondary wastewater treatment [18]. We are aware of work by R. Siegener and R.F. Chen involving the identification and distribution of

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4-*n*-nonylphenol, 17α-ethynylestradiol and caffeine in Deer Island's wastewater influent and effluent streams and how those three chemicals are distributed in Boston Harbor and Massachusetts Bay [19].

Sources of endocrine disrupting chemicals include both natural and man-made chemicals from food products, household products, pesticides, plastics, pharmaceuticals, industrial chemicals, and metals [9]. Of particular interest in our study are the four representative phenolic compounds: bisphenol A (BPA), 4-cumylphenol (4-CP), 4-(*t*-octyl)phenol (4-OP) and 4-*n*-nonylphenol (4-NP). Bisphenol A is the monomer used in the manufacturer of polycarbonate and epoxy resins and is found in certain flame-retardants. Because of its strong endocrine disrupting ability, bisphenol A has become the standard phenolic EDC compound to which others are compared. Bisphenol A is the subject of a recent molecular biological review [20]. Next, 4-cumylphenol, chosen as a representing alkylated hindered phenols, is widely used by the rubber, adhesive, plastic and cable industries as an effective anti-oxidant. The straight chain alkylphenols, represented by 4-(*t*-octyl)phenol and 4-*n*-nonylphenol are the reported by-products of the widely-used, commercial formulations of alkylphenol polyethoxylates (APEOs) which are non-ionic surfactants often added in soaps, paints, herbicides and pesticide formulations. Each of these four representative phenolic compounds have been reported to be in-general non-biodegradable, to be effective EDCs and have been detected in our region's wastewater, sewage and/or groundwater [4,10].

Recent reports have claimed that LC–UV and/or liquid chromatography–mass spectrometry (LC–MS) may be the more sensitive technique and may provide for more accurate results when quantifying these endocrine disrupting phenols [1,21]. However, most reports have used gas chromatography–mass spectrometry (GC–MS) with prior compound derivatization [1,4,22]. As is often true for most environmental samples, prior sample clean up and preconcentration is needed to successfully analyze the multitude of different chemicals present at very low concentrations. And whether it is in reviews [1] or in various publications, the actual method of analysis is dictated by various factors, including instrumentation availability, operator expertise as well as the requirement of following the mandated, governmental decreed method. Our present study first sought to find an effective solvent extraction and sample clean-up method using solid-phase extraction (SPE) and then to compare the achievable detection levels of both GC–MS, LC–UV and LC–MS for the four phenolic xenoestrogens that we choose to study.

With the year-2000 renovation of Boston Harbor's Deer Island wastewater treatment plant and the extension of its diffuser pipes 15 km further into Massachusetts Bay, there arose the question whether the increased load of the secondary, biologically-treated, wastewater being discharged in large quantity further out into Massachusetts Bay, might contain significant amounts of endocrine disrupting chemicals that might be negatively impacting the marine environment. Sampling from the research vessel (R/V) Connecticut during summers of 2003 and 2004 allowed for the unique opportunity to obtain clam, zooplankton, and sediment samples.

2. Experimental

2.1. Chemicals and reagents

Bisphenol A, caffeine, 4-cumylphenol and 4-(*t*-octyl) phenol were purchased from Sigma-Aldrich (Milwaukee, WI, US). While 4-*n*-nonylphenol was from Supelco (Bellefonte, PA). Biphenyl, used as the internal standard (int. std.), was from Matheson, Coleman and Bell (East Rutherford, NJ, US). The solvents: acetonitrile, methanol and methylene chloride (all HPLC grade) were from Fischer Scientific (Pittsburgh, PA, US) and acetone (HPLC-grade, +99.9%) was from Sigma–Aldrich. The GC derivatizing agent, phenyltrimethylammonium hydroxide (0.5 M in methanol), was from Fluka/Sigma–Aldrich. To deactivate the internal surfaces of laboratory glassware, a pretreatment with a 1% (by volume) solution of Hardsil AP^{TM} (Gelest, Inc., Norrisville, PA, US) in toluene, followed by heating to 200 °C and solvent rinsing is recommended.

2.2. Standard solutions

Primary stock solutions were prepared individually at a concentration of 1.000 mg/mL by weighing 100.0 mg of each compound in a 100 mL volumetric flask (either amber in color or wrapped with aluminum foil to reduce the effect of photooxidation of the phenolic standards). Serially diluted solutions of mixtures of the primary stock solutions were made daily in the appropriate solvent.

2.3. Sample collection

The sampling of the bottom marine sediments utilized a remotely operated vehicle (ROV) called, Phantom III S2, as shown at the following web site: http://ap.nurc.uconn.edu/. This ROV was steered on-board from our oceanographic research boat and was equipped with subsurface illumination and picture taking capabilities. For our sampling, the ROV was equipped with a suction-sampler device consisting of two-approximately 4 L buckets having various mesh screens and compartments that enabled collection of bottom sediment samples. The zooplankton samples were collected by conventional oceanographic, vertically towed plankton net. Fig. 1 presents a map, also available at the above web site, depicting the eight principle marine sampling sites (shown in white boxes) for the summer 2003 and 2004 Aquanaut Programs. The marine sampling areas were generally directly east of the city of Boston, in what is referred to as Massachusetts Bay. Water depth contours for these marine waters are indicated on Fig. 1.

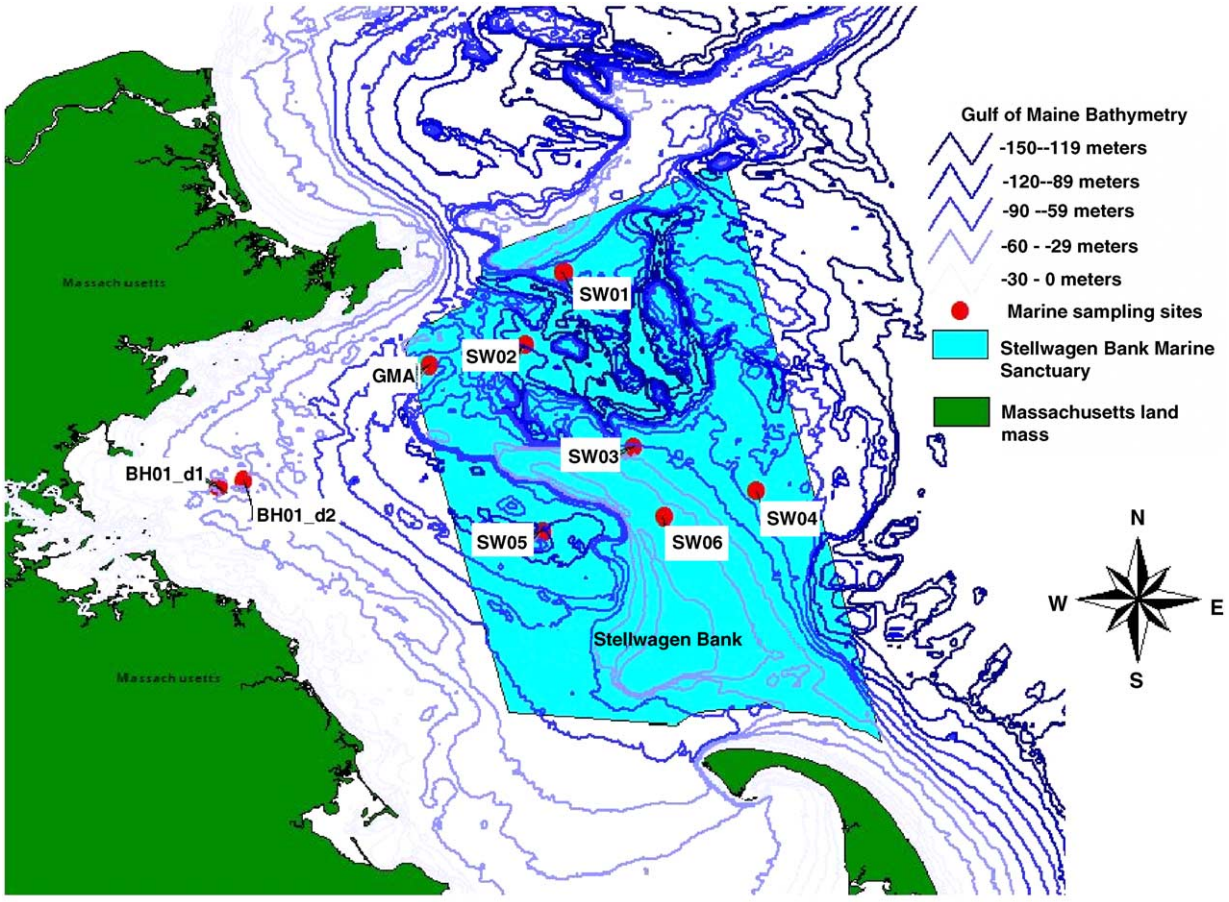

Fig. 1. Map of the marine sampling sites with the ocean depth contours indicated in meters.

2.4. Deployment of sacks of marine clams for bioaccumulation studies

For both the summer of 2003 and 2004, native clams (*M. mercineria*) were dug from a local tidal wetland in the Township of Scituate, Massachusetts, which is located on Massachusetts Bay about 50 km south from the central part of the city of Boston. For each sampling at least 60 clams were collected. All were placed in porous burlap bags. The first 15 clams (control-group) were immediately placed in a cooler and transported to our laboratory for subsequent analyses. Later on the same day that the clams were dug, three different sacks, each containing 15 clams, were anchored at the bottom, in about 30 m of water, at selected spots along the new wastewater diffuser pipes of Boston Harbor's Deer Island's wastewater treatment plant (Sites BH01-d1 and BH01-d2 of Fig. 1). Each summer, exactly 30 days later, efforts were made to collect the deployed sack of clams. However, due to snagging of the anchoring ropes to sub-surface obstructions or due to either deliberate or accidental cutting of the rope lines, only one of the three original clam sacks could be retrieved each year. This happened for each of the two summers and for future clam-deployment work, radio-triggered, self-surfacing floatation devices are now planned. But in each case, when the clam sack and its rope were retrieved, a very heavy smell of septic was noted. The retrieved sack containing the clams was refrigerated and similarly delivered to our laboratory for analyses.

2.5. Sample work-up

Immediately after collection, all plankton and sediment samples where either placed in a suitable ice-cooler or in one of the oceanographic boat's refrigerator. Upon return to our laboratory, all samples were placed in deep freezers (−30 °C) or in our laboratory's refrigerator, maintained at −8 °C (freezer section) or +4 °C (refrigerator section). General sample work-up followed the method described by Pedersen and Lindholst [21]. To 1.000 g (wet weight) of the marine or sediment sample was added 20 mL of an organic solvent mixture of methylene chloride:methanol (2:1, v/v), and placed into a special, tight-fitting Teflon® microwave vessel. After setting the CEM Microwave digestion system (model MDS-81D) at 30% power, the sample was extracted for 25 min. The resulting solvent extract was filtered through a filter (Glass Fiber, Type A/E, 0.33 mm thick, pore size 1 μm, 142 mm diameter, Gelman Sciences, Ann Arbor, MI, US), and about 4 mL of an aqueous 0.9% KCl solution added

as described by Pedersen and Lindholst [21]. This resulting mixture was centrifuged for 10 min, and the resulting organic phase decanted to special evaporation tube(s), and the tube(s) placed in a nine-port, Reacti Module 18870 (Pierce Chemical, Rockford, IL, US). The organic solvent was evaporated to dryness using a gentle stream of house nitrogen. The resulting extracted residue was dissolved with 0.50 mL of methanol which was quantitatively transferred and diluted to 100.0 mL with laboratory-quality, deionized-distilled water to which 0.5 g NaCl had been added.

2.6. Crude sample extracts next treated by solid-phase extraction

Special SPE cartridges, recommended for enhanced recovery of polar compounds (Envi-Chrom P, 0.25 g or c.a. 6 mL (1.3 cm in bed-length × 1.3 cm i.d.) of polystyrene-divinylbenzene resin, Supelco, Inc.) were carefully washed and activated according to their manufacturer's suggested procedure. Then the entire 100.0 mL content of the volumetric flask, containing the marine sample extract, known chemical standards, spikes or blanks, was slowly passed through individual SPE cartridge at a flow rate of ∼5 mL/min. The SPE cartridges were supported on a convenient, 12 port holder (12-port. Model 5-7030, Visiprep (Supelco)). Next each SPE cartridge was washed with 6 mL of distilled water and dried under nitrogen. Then the cleaned, concentrated extract on the SPE column was eluted three times with 2.0 mL HPLC-grade acetone, and the resulting eluate collected in a different collection tube. Each tube with its contents was then placed back into the special evaporative tube holder of the same nine-port, Reacti Module. Final solvent evaporation under gentle stream of nitrogen gas followed. The final extract was reconstituted in 0.50 mL of acetone and serially transferred to a special 0.7 mL amber-colored, conical autosampler vial (Part no. 08-0800) and sealed by 8 mm crimp-top having Teflon/Silicone/Teflon septa (Part no. 08-0040A, both from MicroLiter Analytical Supplies, Inc., Suwanee, GA, US).

2.7. GC–MS analysis

GC–MS analysis of all of the samples was performed using a GCQ Gas Chromatograph/ Ion Trap Mass Spectrometer (FinniganMAP/Thermo Inc., San Jose, CA, US). This GC–MS was fitted with an A200S Liquid Autosampler (CTC Analytics/Leap Technologies, Carrboro, NC, US) that allowed for programmed syringe rinsings and reproducible 1.0 μL injections. The GC's capillary column was a 12 m × 0.20 mm i.d. capillary column, with a 0.33 μm liquid film thickness of cross-linked dimethylsilane (HP-1, Agilent Inc., Wilmington, DE, US). The injector port of the GC was set at 280 °C. The liquid samples were automatically injected using the split-injection mode set with a 1:20 split ratio. The transfer line of the GC to the MS was set at 280 °C, and the electron impact (EI) ion source of the MS set at 200 °C. The temperature program was as follows: 40 °C for 2 min, 15 °C/min to 260 °C, 260 °C for 2 min. A fairly long, 7.0 min solvent delay time was used to protect the ion multiplier of the MS instrument from saturation. The carrier gas was high-purity helium with a constant velocity of 35 cm/s. Detection was done by both total-ion and selective-ion mass spectrometry. GC–MS peak assignment was both by comparison of peak retention times and mass spectra compared to those of the known phenolic EDC standards while using biphenyl as the internal standard.

2.8. LC–UV analysis

The LC–UV instrument consisted of a binary solvent pump (Perkin-Elmer, Series 250, Shelton, CT, US), UV-photodiode array detector (Perkin-Elmer Model 235) and an autointegrator (Nelson/Perkin-Elmer LC-1022). The LC column was an Aquasil C-18, 150 mm × 4.6 mm of 5 μm particles (Thermo/Hypersil/Keystone, Bellefonte, PA, US). A reversed-phase, 20 min linear gradient was performed with the weaker mobile phase (solvent A) either acetonitrile:water or methanol:water, both at 60:40% (v/v) to which 0.1% formic acid had been added. The stronger mobile phase (solvent B) was either 100% acetonitrile or methanol, respectively, to which 0.1% formic acid also had been added. The column flow rate was set at 1.0 mL/min, the column temperature held at 30.0 °C, and the diode array detector set to monitor the 275 nm wavelength at an absorbance sensitivity setting of 0.20 AUFS.

2.9. LC–MS analysis

In order to accommodate the lower flow rates required for the LC–MS system, the column was changed to an Aquasil C-18, 100 mm × 2.0 mm column, 5.0 μm particles and a 0.3 mL/min flow rate used. The weaker solvent was either acetonitrile:water or methanol:water, in each case 65:35 (v/v) by volume, while the stronger solvent was either acetonitrile or methanol 100%. All of these solvents contained 30 mM ammonium acetate (HPLC-grade) to enhance electrospray ionization (ESI). The following solvent gradient program was used. For 5.0 min isocratic hold on the weak solvent, followed by a 5.0 min linear gradient to the strong solvent. This was followed by a 10.0 min hold on the strong solvent to elute all of the phenolic EDCs and what else might be in the sample.

The same LC instrument that was used for the LC–UV detector was also used with the LC–MS. An appropriate length and internal diameter (30 cm × 0.005 in. i.d.) of PEEK tubing was used to go from the exit of the LC to the MS interface. That interface consisted of a Z-spray, electrospray ionization source on a Quatro II MS/MS (Micromass/Waters, Milford, MA). From preliminary infusion studies, using flow rates of 0.040 mL/min delivered by a syringe pump (Model 11, Harvard Apparatus, Holliston, MA, US), the following optimized

settings were chosen. For the targeted phenolic EDC standards, the electrospray ionization capillary probe was set at −2.75 kV, while for the internal standard, biphenyl and such MS calibrants as caffeine and reserpine a setting of +3.50 kV was used. The optimized cone voltage of −40 V (ESI negative) and +30 V (ESI positive) was used with an ESI source temperature set at 90 °C (ESI negative) and 150 °C (ESI positive), while the desolvation temperatures was 110 °C for both methods. The dual quadrupole mass spectrometer was set to detect masses between 45 and 300 Da with total ion chromatogram (TIC) and/or select ion mode (SIM) chosen.

3. Results and discussion

3.1. Studies first by GC–MS without phenol derivatization followed later by studies using GC–MS with phenol derivatization

The analyses of the Summer 2003 samples were done doing enhanced solvent microwave extraction, followed by SPE sample clean-up, but the GC–MS analyses was performed without doing phenolic derivatization. A review of the results from the first summer's sampling and the literature suggested that additional sensitivity in the analysis method was needed. Comparison studies then showed that derivatization of the four targeted phenolic standards gave considerable improvement in sensitivity, peak shape and peak selectivity for the GC–MS analyses of the four targeted phenolic compounds. The derivatizing reagent chosen because of its reported ability to rapidly derivatize alcohols, phenol and carboxylic acids in wet/aqueous media was a 0.5 M methanolic solution of phenyltrimethylammonium hydroxide (PTA-OH). This is a less familiar derivatizing reagent but has been reported to have the ability to be an effective alkylating agent [23]; leading to the rapid formation of the more volatile, methyl ether derivatives. Experiments were conducted to determine the minimum excess of the PTA-OH derivatizing reagent that would be needed to achieve quantitative conversion of the four phenolic standards to their derivatized form. These experiments showed that a 50-fold by weight excess of the derivatizing reagent, i.e. (1000 μg/mL) of PTA-OH produced 100% derivatization of the four 20 μg/mL phenolic standards. Fig. 2 is a representative total ion chromatogram in the full scan mode of the four derivatized phenolic compounds showing only peaks for each derivatized product, and an early peak for biphenyl, used as the internal standard. This and other chromatograms and mass spectral data were then searched for unreacted phenolic standards.

Table 1 compares the linear calibration plots over the concentration range of 25–250 ng/mL obtained for both the derivatized and the underivatized phenolic compounds when analyzed on the same GC–MS under the very same conditions. For all of the four targeted phenolic standards, enhanced sensitivity was achieved when these phenolic compounds were derivatized prior to GC–MS analyses. Although the coefficients of determination (R^2) for all of the four phenols, whether derivatized or not, were excellent, the GC–MS sensitivities were up to two orders of magnitude higher (reported as the slope of each line's linear calibration plot in Table 1). In addition the derivatized phenols had excellent peak shapes (shown in Fig. 2), not so for the un-derivatized phenols.

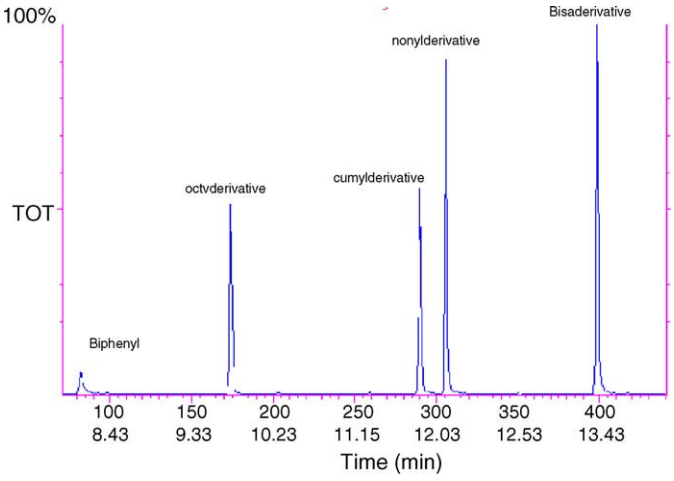

Fig. 2. Representative GC–MS ion chromatogram of the phenyltrimethylammonium hydroxide (PTA-OH) derivatized phenol standards along with biphenyl, used as the internal standard.

It should be noted that the GC–MS calibration plots were obtained by using data taken not in the total ion chromatogram but in selected ion mode of the mass spectrometer. Thus, the ion intensities at the following *m*/*z* ratio were used: 154 for biphenyl, 241 and 256 for the bisphenol A derivative, 211 and 226 for the 4-cumylphenol derivative, 121 and 149 for the 4-(*t*-octyl)phenol derivative and 121 and 234 for the 4-*n*-nonylphenol derivative.

3.2. Recoveries of the four standard phenolic compounds when carried though the entire analysis procedure

Table 2 reports overall that good-to-excellent percent recoveries, some of the highest reported so far in the literature

Table 1
Summary of the GC–MS linear calibration plots over the concentration ranges 2–40 μg/mL for the underivatized and 25–250 ng/mL for the derivatized phenols

Phenolic compounds	Coefficient of determination (R^2)	Equation of the line
Octylphenol	0.9999	$y = 0.0554x - 0.0277$
Octylphenol derivative	0.9988	$y = 2.4306x + 0.0229$
Nonylphenol	0.9994	$y = 0.044x - 0.0309$
Nonylphenol derivative	0.9944	$y = 4.0079x - 0.1297$
Cumylphenol	0.9984	$y = 0.0639x - 0.0935$
Cumylphenol derivative	0.9994	$y = 2.5874x + 0.0222$
Bisphenol A	0.9883	$y = 0.0472x - 0.1694$
Bisphenol A derivative	0.9988	$y = 5.3277x - 0.0193$

Table 2
Percent recoveries for the four phenol EDCs standards at the 100 μg/L concentration when carried through the entire analysis procedure: microwave extraction, SPE clean up and concentration, pre-derivatization followed by GC–MS analysis

Phenolic compounds	% Recovery
Bisphenol A	95
4-Cumylphenol	86
4-(*t*-octyl)phenol	86
4-*n*-Nonylphenol	60

[1,3,4,6,21,22], when the four phenolic compounds, at the 100 ng/mL concentration level, were subjected to the entire analysis procedure, i.e. through sample microwave extraction, SPE clean-up followed by derivatization and GC–MS analyses.

3.3. LC–UV analyses for the targeted phenolic compounds underivatized

It was found that LC–UV, with the absorbance monitored at 275 nm, provided for a convenient analysis method for the various, underivatized phenolic compounds at higher concentrations, in the range from 1 to 100 μg/mL. Fig. 3a and b show the separation of the four phenolic standard compounds, along with caffeine and biphenyl, obtained using an Aquasil C-18 column, 150 mm × 4.6 mm i.d. packed with 5 μm particles and a 1.0 mL/min flow rate.

The chromatograms were obtained using identical 20 min linear gradients starting with 60:40% (v/v) either in acetonitrile:water (Fig. 3a) or methanol:water (Fig. 3b) while the final solvent was 100% in either acetonitrile or methanol, respectively. All solvents contained 0.10% formic acid. It should be noted that all of the compound peaks eluted under gradient conditions and excellent peak shapes were achieved. Table 3 summarizes the calibrations plots for the four target phenolic standards over the concentration range of 1–100 μg/mL. In all cases both for the usual least squares and weighted least squares linear regression methods, the data for 7–10 replicate runs at each concentration range were recorded. The advantage of using weighted linear regression as a statistical method to do linear regressions is

Table 3
Summary of the LC–UV calibration plots when linear least squares and weighted linear least squared are compared over the concentration range of 1–100 μg/mL

Phenolic compounds	Coefficient of determination (R^2)	Equation of the line
Octylphenol	0.9961	$y = 7697x - 25853$
Octylphenol (weighted)	–	$y = 7810x + 1894$
Nonylphenol	0.9955	$y = 2286x + 32395$
Nonylphenol (weighted)	–	$y = 2328x + 59$
Cumylphenol	0.9994	$y = 9148x - 5711$
Cumylphenol (weighted)	–	$y = 9286x + 2458$
Bisphenol A	0.9982	$y = 14912x + 96969$
Bisphenol A (weighted)	–	$y = 17837x - 2635$

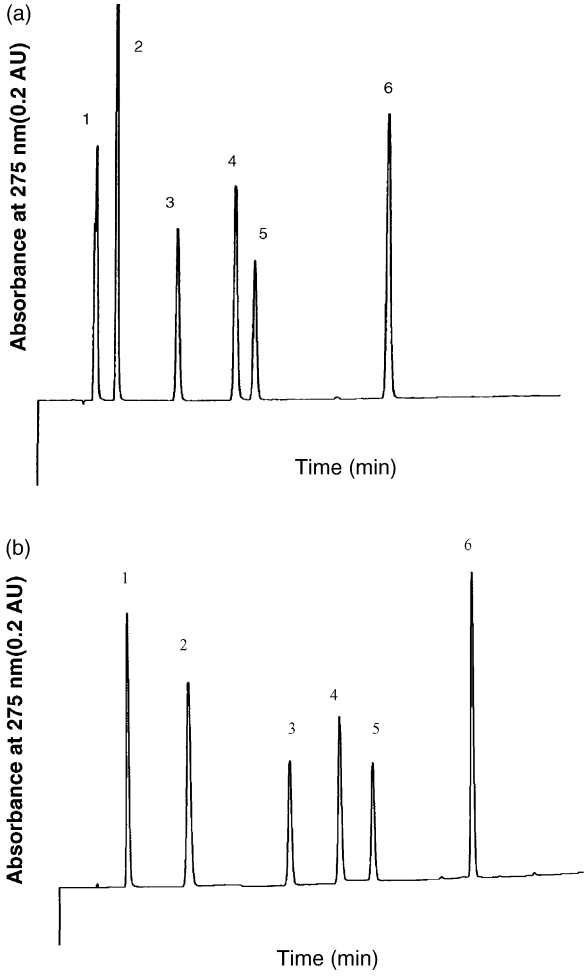

Fig. 3. (a) HPLC–UV, 20 min linear gradient chromatogram of a standard mixture using the acetonitrile-based solvent method. (b) HPLC–UV, 20 min linear gradient chromatogram of a standard mixture using the methanol-based solvent method. Peak Identity (amount of material injected) for both chromatograms: 1—caffeine (125 ng), 2—bisphenol A (500 ng), 3—4-cumylphenol (500 ng), 4—biphenyl (250 ng), 5—4-(*t*-octyl)phenol (500 ng), 6—4-*n*-nonylphenol (1000 ng).

that it gives proportionally higher weight to the individual calibration concentration having the lower relative standard deviations. As Table 3 reports, in all four cases the weighted least squares linear regression method gave higher slope values (increase sensitivity) and lower *y*-intercept values than did the usual least squares linear regression method.

3.4. LC–MS analyses for the targeted phenolic compounds underivatized

Comparison of the LC–MS analyses of the targeted phenolic compounds using the two different solvents systems, acetonitrile or methanol with additives such as 0.1% by volume formic acid or 30 mM ammonium acetate showed that the methanol-based solvents yielded the cleaner mass spectral results. Acetonitrile, although a stronger eluent for

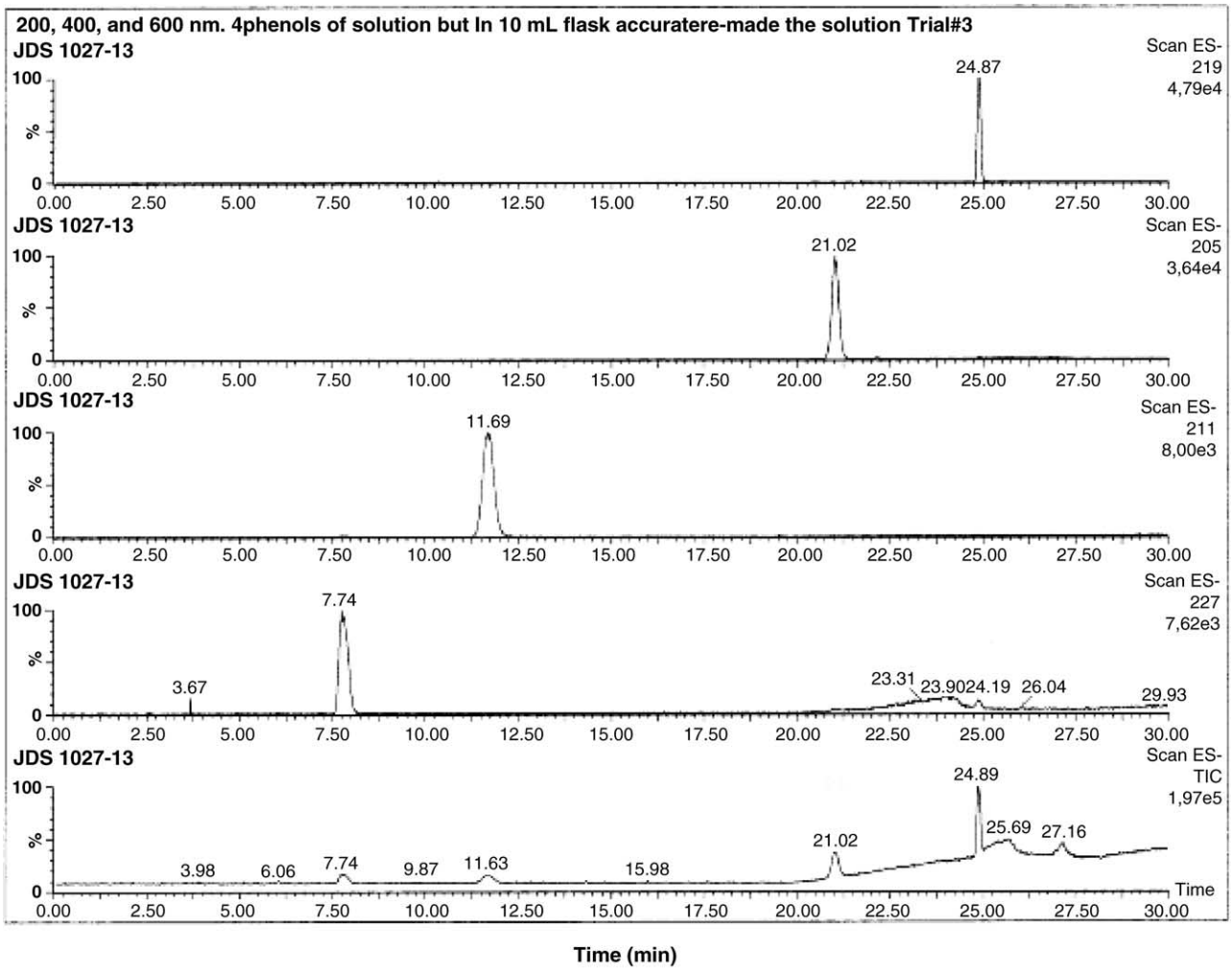

Fig. 4. LC–MS runs of the four phenolic standards in the methanol-based gradient method. Bottom trace is the total ion chromatogram of a scan from *m*/*z* of 45–300 Da. And going up in sequence, the selected ion trace at *m*/*z* of 227 (for bisphenol A), at *m*/*z* of 211 (for 4-cumylphenol), at *m*/*z* of 205 (for 4-(*t*-octyl)phenol) and at *m*/*z* of 219 (for 4-*n*-nonylphenol).

LC–UV analysis, tended to leave carbon deposits on the negatively charged tip of the stainless steel ESI capillary of the mass spectrometer, while the methanol solvent did not. With the shorter Aquasil column and with flow rates of 0.1–0.2 mL/min to be more compatible with the MS, the retention times of the phenolic EDCs standards were found to have increased to 25 min. Published studies also have reported using methanol for the LC–MS analysis of the same or similar phenolic compounds [21]. The lowest trace in Fig. 4 shows a representative LC–MS total ion chromatogram obtained with the four phenolic EDCs standards with the methanol/waters gradient system with 30 mM ammonium acetate added. Using the extracted mass spectral ion option on the total ion chromatograph (going up in the traces of Fig. 4) yielded the specific extracted ion chromatograms for the four phenols. It should be noted that negative electrospray ionization was used and in each case the $[M - H]^-$ ion gave the strongest ion: bisphenol A (*m*/*z* = 227), 4-cumylphenol (211), 4-(*t*-octyl)phenol (205) and 4-*n*-nonylphenol (219).

3.5. Comparison of the method detection limits (MDLs) of the four targeted phenol compounds by both GC–MS and LC–MS

Table 4 compares our laboratory's method detection limits for the four targeted phenolic compounds by the four, different analytical modes used. Note the MDLs reported are those obtained following US EPA's specified method which gives the minimum concentration of a substance that can be measures and reported to a certainty of 99% as being found in an actual sample [24,25]. From a study of Table 4 it is clear that phenol pre-derivatization followed by GC–MS affords the best sensitivity and lowest method detection levels. This is especially true for bisphenol A, whose MDL was found to be over two orders of magnitude lower for GC–MS with derivatization (0.004 ng) to what our laboratory was able to achieve by LC–MS (1.0 ng), both cases using the extracted ion of the total ion chromatogram method.

Table 4
Comparison of method detection limits of phenolic EDC compounds by different analytical methods used in our laboratory

Phenolic compounds	GC–MS (non-derivatized) (ng)	GC–MS (derivatized w/phenyltrimethyl-NH_4OH) (ng)	LC–UV (275 nm) (ng)	LC–MS negative ESI (TIC/extracted ion) (ng)
Bisphenol A	0.80	0.004	1.6	1.0
4-Cumylphenol	0.30	0.010	3.4	2.4
4-(*t*-octyl)phenol	0.20	0.010	3.4	2.2
4-*n*-nonylphenol	0.20	0.010	4.7	1.3

3.6. Analyses of marine samples from both the summer 2003 and summer 2004 aquanaut cruises

Table 5 reports the results of the analyses of the various marine samples that were collected from the 2003 and 2004 summer cruises. For the summer 2003 samples, referring to Fig. 1. to locate the sampling location, at sampling location BH01_d1, the site closest to the MWRA wastewater diffuser pipes, none of the targeted phenolic compounds were detected in the plankton samples, while 4-(*t*-octyl)phenol was detected at 12.5 ng/g in the sediment sample. For the sampling location SW01, the most northerly sampling site of the cruise and closest to Gloucester Harbor, 4-(*t*-octyl)phenol was detected at 11.4 ng/g in the sediments, again with no-detection found in the plankton sample. It is suggested that the detection of 4-(*t*-octyl)phenol at sampling location SW01 may be coming from a secondary source of sewage discharge to the north of Stellwagen National Marine Sanctuary, perhaps from sewage plants or rivers further north of Massachusetts Bay. The report of a concentration of 22.9 ng/g for *n*-nonylphenol in the sediments, and 3.7 ng/g of 4-cumylphenol in the plankton at sampling location SW06, one of the most southerly sampling sites and deep in the Stellwagen Bank Marine Sanctuary became a concern and would need to be reinvestigated in the following year's sampling. It should be emphasized that these results from the summer 2003 sampling were deemed to be significant because for each of the positive detections reported in Table 5 had been repeated and fully quantitated using the method of standard addition to not only confirm the identity but also to the concentration of the reported peak. Of the remaining five sampling sites visited by the Aquanaut 2003 cruise, no detectable concentrations of either bisphenol A or of the other targeted phenols were found in either the plankton or sediment samples.

An important part of the overall research plan was to expose clams to the expected diluted wastewater being discharged in high quantities from the Deer Island wastewater effluent pipes. It was expected that that clams might be used to bioaccumulate and hence aid in the detection of the targeted phenols. On 6/5/03, 60 clams were collected in the vicinity of Scituate, MA. Of the set of 60 clams, 15 were designated as controls. These animals were frozen while alive, returned to the laboratory and stored at −80 °C. Upon analysis of certain of the clam parts of this control group, the gills of one of the three (controlled) clams analyzed were found to have bisphenol A at 17.3 ng/g (gill dry weight). The remaining 45 clams dug on that day were divided and placed in three different sacks. Those sacks were sunk to the water bottom, water depth of approximately 30 m at three sites along the wastewater diffuser pipes of Boston's Deer Island wastewater treatment plant. After a month of exposure to the effects of the wastewater effluent, only one sack of the clams was located and retrieved. Clams from this lone sack were analyzed, and no-detectable levels of the targeted phenolic EDCs were found.

Table 5
Results of the analysis of the phenolic compounds for both summer samplings at the locations as indicated on the site map (Fig. 1)

Sampling site	Sample type	Concentration of phenols in sample (ng/g), AP2003	Concentration of phenols in sample (ng/g), AP2004
BH01_d1	Sediment	12.5 (octylphenol)	1.5 (bisphenol A)
BH01_d2	Sediment	Not sampled	5.0 (bisphenol A)
SW01	Sediment	11.4 (octylphenol)	3.5 (bisphenol A)
SW02	Sediment	N.D.	N.D.
SW04	Sediment	N.D.	N.D.
SW05	Sediment	N.D.	N.D.
SW06	Sediment	22.9 (nonylphenol)	N.D.
GMA	Sediment	Not sampled	N.D.
BH01_d1	Plankton	N.D.	5.0 (bisphenol A)
SW01	Plankton	N.D.	11 (bisphenol A)
SW02	Plankton	N.D.	8.6 (bisphenol A)
SW03	Plankton	N.D.	8.3 (bisphenol A)
SW04	Plankton	N.D.	13 (bisphenol A)
SW06	Plankton	3.7 (cumylphenol)	N.D.
BH01_d2	Clam gills	N.D.	28.3 (bisphenol A) SD = 2.0, RSD = 6.9, $n = 3$
Control	Clam gills	17.3 (bisphenol A)	15.4, (bisphenol A) SD = 1.0, RSD = 7.0, $n = 2$

N.D. is below MDL as given in table.

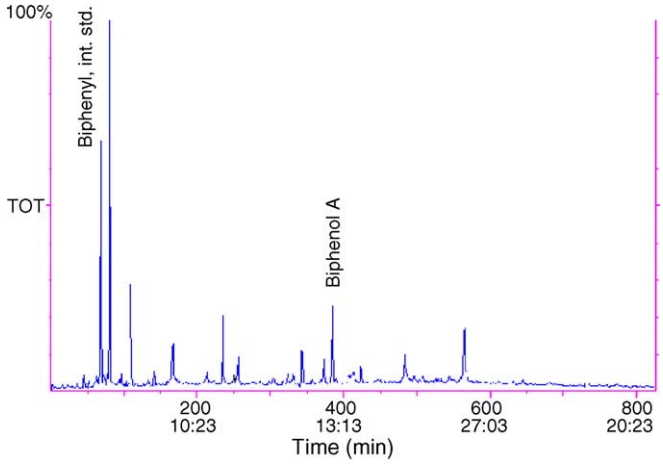

Fig. 5. Total ion chromatogram (GC–MS with derivatization) of the extract of a sediment sample taken close to the wastewater diffuser outfall pipes showing a peak for bisphenol A.

For the Summer 2004 sampling cruise, when the extracted marine samples were analyzed using the more sensitive pre-derivatization of the phenolic compounds followed by GC–MS, the results were as follows and are also summarized in Table 5. For the sediment samples taken from the important sampling site, BH01_d1, directly around the wastewater discharge pipes, and from site BH01_d2, slightly down gradient, were found to contain bisphenol A at concentrations of 1.5 and at 5.0 ng/g, respectively. While for site SW01, the most northerly site, bisphenol A was found at a concentration of 3.5 ng/g. Fig. 5 is the total ion chromatogram for the extract of the sediment from that site. It should be noted that the chromatogram is relatively clean and shows a clear peak (later confirmed by spiking experiments) for bisphenol A. Bisphenol A was not found in any of the other sediment samples taken from the other sites. The zooplankton samples at five of the six sites were found to range from 5.0 to 13 ng/g in bisphenol A concentration, which indicated perhaps diffuse dispersal and the beginning of bioaccumalation of bisphenol A in detectable concentration throughout the entire water column into the zooplankton. For the 2004 year sampling the sediment and plankton sample of site SW06 were found not to any of the targeted phenolic EDCs, which did not confirm the detection of 4-nonylphenol nor the 4-cumylphenol that had been detected in the previous summer's sampling. Since none of the targeted phenols where found in either the sediment nor the plankton samples from site SW06, it is deemed that this site, which is well into the middle of the Stellwagon Bank Marine Sanctuary, may indeed be clear of the four targeted phenols examined.

The summer of 2004 clam dispersal bioaccumulation experiments confirmed that bisphenol A at 15.4 ng/g (SD = 1.0 ng/g, RSD = 7.0, $n = 2$) was present in the gills of the control clam group having been just dug from the marshy wetland area around Scituate harbor. Hence, in two summers, reproducible concentrations of bisphenol A, 17.3 ng/g in the summer of 2003 and 15.4 ng/g in the summer of 2004 were found in the gills of clams taken from tidal waters near Scituate harbor. In late June 2004, three sacks each containing 15 clams again were dispersed on the bottom in about 30 m of water about 90 m apart in the vicinity of the wastewater diffusers. A month later, it was a great disappointment when only one sack out of the three of the dispersed clams was retrieved. In the gills of those clams bisphenol A was found at concentrations of 28.3 ng/g (SD = 2.0, RSD = 6.9, $n = 3$). Fig. 6 shows the GC–MS total ion chromatogram of one of those extracts. Not only is there a definite peak for bisphenol A, but the presence in the total ion chromatogram of the broad, partially resolved band at higher molecular weight is indicative of heavy weight (marine oil) pollution. That such pollution is present is not surprising as the site around the wastewater diffusion pipes is closer to the coast and lies near a busy large-cargo boat lane directly leading into Boston Harbor.

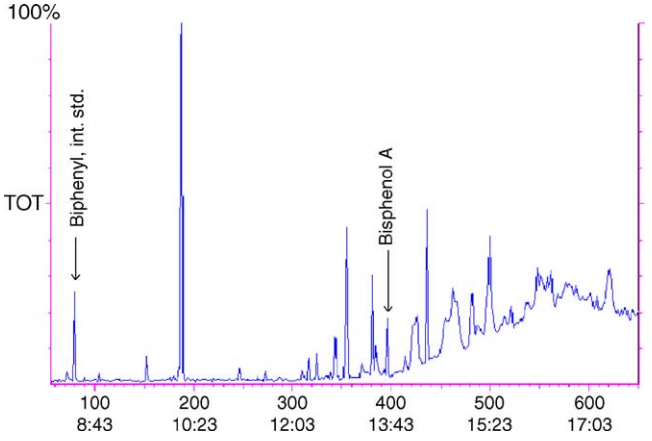

Fig. 6. Total ion chromatogram (GC–MS with derivatization) of the extract of the gills of a clam deployed for 30-days in the summer of 2004 near the Deer Island wastewater diffuser outfall pipes showing a peak for Bisphenol A and an un-resolved complex mixture at higher molecular weight is often due to heavy oil contamination.

4. Conclusions

Analyses of marine samples (bottom sediment samples) collected by a remotely operated vehicle (ROV) submersible, zooplankton samples and from the gills of clams indicated the presence of certain phenolic EDCs. Careful sample pre-treatment involving enhanced organic-solvent extraction by microwave heating, followed by an effective solid-phase extraction clean-up and pre-concentration allowed for the detection by either GC–MS or LC–MS. Four different analysis methods were compared, i.e. GC–MS (non-derivatized), GC–MS (derivatized), LC–UV (non-derivatized) and LC–MS (non-derivatized) in terms of their method detection limits for the four phenolic compounds: bisphenol A, 4-cumylphenol, 4-(*t*-octyl)phenol and 4-*n*-nonyl phenol. Pre-derivatization of the phenols followed by

GC–MS provided for the highest sensitivity of the four analysis methods used. Our method detection limits as presented in Table 4 are very similar to the detection limits reported by Rudel et al. [4], who reported detection limits of 1–20 ng/mL for bisphenol A and 4-*n*-nonylphenol.

Our analyses of the bottom sediment, plankton and clam extracts taken from the same eight sites located near, downgradient from the Boston Harbor Deer Island wastewater diffuser outfall pipes and around Stellwagen Bank National Marine Sanctuary showed that primarily bisphenol A was present at concentration levels of 1–30 ng/g. This study agrees with studies by others that document that the dispersal of large amounts of secondary wastewater into the marine environment continues to be of environment concern.

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