RESIDUES AND TRACE ELEMENTS

Determination of 17β-Estradiol and Its Metabolites in Sewage Effluent by Solid-Phase Extraction and Gas Chromatography/ Mass Spectrometry

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The paper describes a simple and quantitative method for monitoring non-conjugated 17β-estradiol (E_2) and its metabolites estrone (E_1) and estriol (E_3) as environmental contaminants in municipal sewage effluents. Estrogens were preconcentrated and cleaned up by solid-phase extraction using a reversed-phase C_{18} cartridge. They were derivatized with pentafluoropropionic acid anhydride, and the products were analyzed by gas chromatography/mass spectrometry. Recoveries from spiked distilled water and sewage were better than 87% at fortification levels of 100 and 20 ng/L. For a 1 L sewage sample and a concentration factor of 5000, detection limits were 5 ng/L for E_1 and E_2 and 10 ng/L for E_3. In a brief survey of Canadian wastewater, these estrogens were detected in many raw sewage and effluent samples at concentrations ranging from 6 to 109 ng/L for E_1, from <5 to 15 ng/L for E_2, and from <10 to 250 ng/L for E_3.

Many synthetic chemicals can mimic the function of the naturally occurring estrogen, 17β-estradiol (E_2), causing disruption of the endocrine system (1). These chemicals have been hypothetically linked to increased incidence of certain estrogen-dependent cancers in humans, falling sperm counts in men, feminization of fish, and abnormal reproductive organs of wildlife (2–4). Many of these endocrine disruptors are environmental contaminants, with examples including organochlorine insecticides such as DDT as well as its metabolites and derivatives, kepone, some hydroxylated polychlorinated biphenyls, chlorinated dioxins, alkylphenols such as bisphenol A, 4-nonylphenol, and 4-*tert*-octylphenol (5–7). However, data developed from MCF-7 (a human breast cancer cell) bioassay (8) or rainbow trout in vitro hepatocyte bioassay (9, 10) indicate that these xenobiotic compounds are 4 to 6 orders of magnitude weaker than E_2 in relative estrogen potency.

E_2 is the principal hormone that regulates cell proliferation and is responsible for the development and maintenance of the female reproductive system and sex characteristics. In humans, E_2 is metabolized to estrone (E_1) and estriol (E_3) in both conjugated and nonconjugated forms. These steroids are commonly found in human excreta and in urine, serum, and amniotic fluid of pregnant women (11). The synthetic estrogen 17α-ethynylestradiol (EE_2) is the major ingredient of many oral contraceptives that have been used since 1960. Structures of these steroids are shown in Figure 1.

Naturally occurring estrogens enter the environment through excretions of humans, domestic and farm animals, and wildlife. Although the concentrations of these steroids in sewage and environmental samples are very low (at nanogram-per-liter levels), it has been demonstrated that nonconjugated E_2 and EE_2 at concentrations as low as 1 ng/L can induce synthesis of vitellogenin (VG) in male fish (12). VG is a protein precursor of the egg yolk and is normally produced in large quantities by mature female fish. The observation of elevated levels of VG in male fish collected in rivers downstream of sewage treatment plants has led to the belief that estrogenic compounds are present in sewage (13). Although conclusive evidence is still unfolding, E_2 and EE_2 are suspected to be major contributors of estrogenic activities in sewage.

Very few analytical methods for determinating E_2 in environmental samples have been published. Previously, a radioimmunoassay technique was developed for determining E_2 and testosterone in sewage (14). Identification of estrogenic compounds in sewage effluent by gas chromatography/mass spectrometry (GC/MS) was also reported in a British study (15) that involved solid-phase extraction (SPE) of a 20 L sample, liquid chromatographic fractionation, and ion trap GC/MS analysis of underivatized E_1, E_2, and EE_2. To date, no data regarding occurrence of estrogens in the Canadian aquatic environment are available. In this work, we present a simple SPE and GC/MS method for determining free E_2 and its metabolites E_1 and E_3 in sewage effluent. The present study is a continuation of our current research in new analytical methods for detecting other estrogenic compounds such as nonylphenol (16), nonylphenol ethoxylates (17), and carboxylates (18) in sewage effluent and sludge samples.

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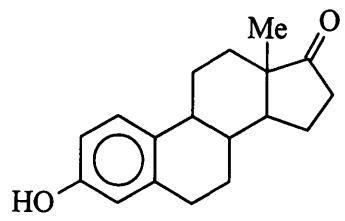
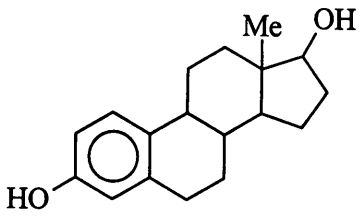
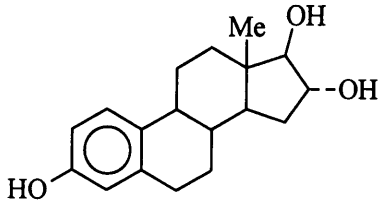


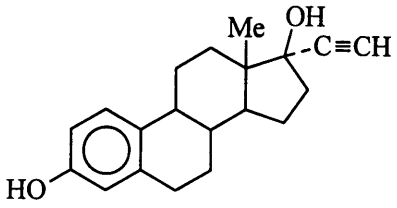
Estrone (E_1)



17ß-Estradiol (E_2)



Estriol (E_3)



17α-ethynylestradiol (EE_2)

Figure 1. Chemical structures of estrogens.

Experimental

Reagents and Chemicals

(**a**) *Solvents.*—Distilled-in-glass grade organic solvents (Burdick & Jackson, Muskegon, WI) were used without further purification.

(**b**) *Estrogens and anhydride.*—E_1, E_2, E_3, EE_2, and pentafluoropropionic acid anhydride (PFPA) were obtained from Sigma-Aldrich-Supelco Canada Ltd. (Oakville, ON, Canada). Stock solutions of each estrogen (1000 µg/mL) were prepared in acetone. Mixtures of the estrogens (100, 10, and 1 µg/mL) for sample fortification and standard preparation were also prepared in acetone. All stock solutions and mixtures were stored in screw-capped centrifuge tubes at –20°C in the dark.

Extraction Apparatus

A home-made system consisting of a 12-port SPE vacuum manifold (Supelco Visiprep DL 5-7044), a 20 L stainless steel tank, and a vacuum pump was used. A 6 mL, reversed-phase SPE cartridge containing 1 g C_{18} sorbent (ENVI-18, Supelco, 5-5706) was used to extract a sewage sample.

Collection of Sewage Samples

Grab or 24 h composite influent, primary, and final effluent samples from various municipal sewage treatment plants were collected. Samples extracted within 48 h after collection were kept at 4°C and no preservative was added. Other samples were preserved with formaldehyde (1%, v/v) and kept at 4°C in the dark until extraction.

Caution: Because of the presence of bacteria, viruses, and parasites that may pose a health hazard to workers, protective clothing, gloves, mask, and safety goggles with full side shields must be used when handling sewage samples as a safety precaution. It is also recommended that workers have proper immunization against diphtheria, tetanus, polio, and hepatitis A. For details, consult your local health authorities.

Extraction of Sewage Samples

Prior to extraction, each sewage sample (1 L) was vacuum filtered through a 47 mm Whatman GF/C filter with a pore size of 1.2 µm installed in an all-glass funnel support assembly (Kontes Ultra-Ware, Vineland, NJ; KT-953825-0000). A filter aid such as Celite 545 was used to minimize plugging of the filter. For recovery experiments, a portion of the free estrogens (e.g., 100 µL of the 1 µg/mL mixture in acetone) was spiked to 1 L distilled water or effluent. The sample was stirred for 15 min prior to SPE.

In preparation for extraction, each C_{18} tube was conditioned with 5 mL acetone, 5 mL methanol, and 10 mL water on an SPE manifold per manufacturer's instruction. The filtered sample was then applied to the extraction tube via a siphon tube and an adaptor (Supelco, 5-7275). An average flow rate of ca 10 mL/min was maintained by adjusting the vacuum to ca –15 in. Hg. As a safety precaution, the vacuum should not exceed –20 in. Hg. When extraction was complete, the tube was dried under vacuum for 5 min. A 5 mL portion of acetone–water (1 + 4, v/v), in 2 equal fractions, was used to rinse the tube, and the washes were discarded. Estrogens were removed from the C_{18} tube by eluting with 10 mL acetone. Because the

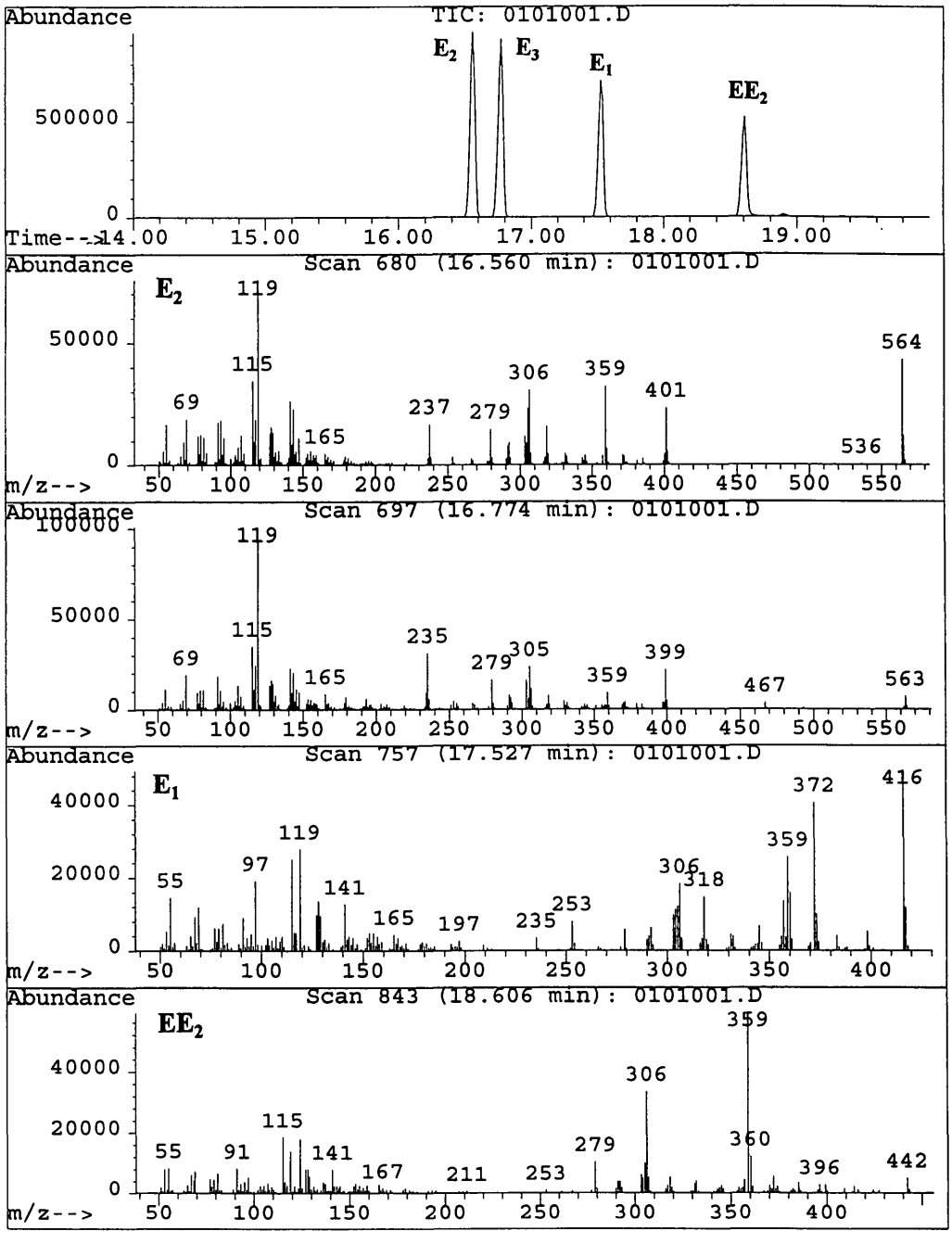


Figure 2. Total ion current chromatogram and mass spectra of PFP derivatives of E_2, E_3, E_1, and EE_2. Concentration, 10 ng/μL for each component.

acetone extract contained a small amount of water, it was gently evaporated to ca 100 μL with nitrogen on a water bath at 40°C. Estrogens were then back-extracted into four 1 mL portions of ethyl acetate, and the extract was dried by passing through a disposable pipet filled with 5 cm anhydrous sodium sulfate. All sewage samples (extracted and unextracted) were discarded down the toilet.

Derivatization of Estrogens

After the ethyl acetate extract was evaporated to 100 μL in a centrifuge tube, the sample was reacted with 50 μL PFPA at ambient temperature (22°C) for 20 min. At the end of reaction, 2 mL petroleum ether (boiling point, 30°–60°C) and 3 mL 1% K_2CO_3 were added and the products were partitioned into the organic layer by agitation on a Vortex mixer for 1 min. After phase separation (centrifugation was required for most sewage samples to break the emulsion), the upper layer was removed and passed through a disposable pipet filled with anhydrous sodium sulfate. The extraction was repeated twice, and the combined petroleum ether fraction was evaporated just to dryness. The residue was redissolved in 200 μL isooctane for GC/MS analysis. For a calibration standard, a mixture of these estrogens (e.g., 50 ng each) was derivatized and worked up as described above.

Table 1. Mass number and relative abundance of molecular and major ions of PFP derivatives of estrogens

PFP derivative	M^+, *m/z* (relative abundance, %)	Major ions, *m/z* (relative abundance, %)
E_1	416 (100)	372 (82), 359 (52), 306 (37), 119 (56)
E_2	564 (57)	401 (31), 359 (43), 306 (41), 119 (100)
E_3	726 (5)	563 (7), 399 (22), 359 (9), 119 (100)
EE_2	442 (9)	359 (100), 306 (57), 279 (18), 119 (24)

Analysis of Estrogen Derivatives by GC/MS

A Hewlett-Packard 5890 Series II GC equipped with a 5972 mass-selective detector and a 30 m × 0.25 mm id × 0.25 μm film thickness HP-5-MS column was used for quantitation of estrogens. GC conditions were as follows: injection port, 250°C; interface, 280°C; initial oven temperature, 70°C with a 1 min hold; programming rates, 30°C/min (from 70° to 180°C); and 5°C/min (from 180° to 290°C). Carrier gas (helium) linear velocity was held constant at 36.9 cm/s. Splitless injections (1 μL) were made by a HP7673 autosampler with a splitless time of 1 min. The electron energy and electron multiplier voltage were 70 eV and 400 V above autotune value, respectively. The detector was tuned each day by using perfluorotributylamine (PFTBA) and the standard spectra autotune program. Full-scan mass spectral data were collected from *m/z* 50 to 600. For quantitation of estrogens in a sample extract, selected ion monitoring (SIM) was used. Response factors for the estrogens were generated by injecting mixtures of their pentafluoropropionyl (PFP) derivatives at 2 levels (1000 and 200 pg/μL). The following quantitation and confirmation ions, respectively, were used in SIM work: *m/z* 372 and 416 (E_1), *m/z* 564 and 401 (E_2), *m/z* 563 and 399 (E_3), and *m/z* 359 and 306 (EE_2). Using the external standard method, we calculated the concentration of each estrogen in a sample by multiplying its area (for the quantitation ion) by the average response factor generated from the above 2 standards and dividing by an appropriate concentration factor (i.e., 5000 in this case).

Results and Discussion

Extraction of Estrogens from Sewage

Estrogens and other steroidal hormones in plasma and urine of a small sample size (i.e., a few milliliters) have been extracted successfully by solvents such as ethyl acetate (19), as well as a mixture of diethyl ether and dichloromethane (20). Extraction of estrogens and estrogen conjugates in late-pregnancy fluids has been reported by using a graphitized carbon black cartridge (11). Our extraction procedure was based on a previously published SPE method for estrogen and testosterone in sewage, using a C_{18} column (14). For convenience and consistency with our previous SPE procedures, suspended particulates in the sewage sample were removed by vacuum filtration through a layer of Celite and a glass fiber filter of 1.2 μm pore size. However, this process should be performed only immediately before the preconcentration step. Otherwise, reduction in flow rates or even plugging of the adsorption media may still occur if the filtered sample is stored for an extended period.

Prior to elution of estrogens, the C_{18} cartridge was washed with 5 mL acetone–water (1 + 4, v/v) and the washing was discarded. This cleanup step was very useful because it removed coextractives that were much more polar than the estrogens. Its effectiveness was best illustrated in the extraction of some sewage samples with known inputs from the textile industry: After the dark-colored dyes were removed by this cleanup step, the fraction containing the estrogens was nearly colorless.

In our experience, a small amount of water (100 to 200 μL) always remains on the C_{18} cartridge even if the cartridge is dried under vacuum for 30 to 60 min. The water, which is removed alongside the estrogen in the acetone elution step, must be removed before chemical derivatization. Rather than use a prolonged nitrogen evaporation step that causes loss of analytes to eliminate residual water, we stopped evaporation when the volume was about 100 μL, or when the solvent was almost entirely water. Then we back-extracted the estrogens into ethyl acetate, which was subsequently evaporated to about 100 μL for derivatization. A small volume of ethyl acetate was necessary to keep the sewage extract in solution, and because ethyl acetate is miscible with PFPA, its presence made derivatization efficient.

Derivatization and GC/MS Analysis

Several derivatization procedures have been published for GC determination of estrogens. For example, formation of trimethylsilyl (TMS; 19, 21, 22) and *tert*-butyldimethylsilyl derivatives of E_1, E_2, and E_3 (22), dimethylethylsilyl derivatives of EE_2 and other steroids used in oral contraceptive formulations (22), and PFP derivatives of testosterone acetate (20) have been demonstrated. In the beginning of this study, we evaluated several estrogen derivatives for ease of formation, stability of products, and chromatographic and mass spectral properties. In addition to TMS and PFP derivatives, we also studied acetyl and heptafluorobutyryl (HFB) derivatives. We selected PFP derivatives for this work because their formation produced very few side products and they were stable for weeks at –20°C. This stability made it possible to save sample extracts for further analysis. In addition, PFP derivatives were formed in the shortest time under the least vigorous conditions, requiring only 20 min of reaction at room temperature. Although we did not determine the absolute yield of PFP derivatives, we assumed the reaction to be complete and quantitative because longer reaction times (e.g., 60 and 120 min) or higher reaction temperatures (e.g., 50° and 65°C) did not improve yield. Pentafluoroacylation of estrogens also was reproducible. For 6 replicate reactions with 50 ng of each estrogen, coefficients of variation for the yields of the products ranged from 3.8% (for E_2) to 6.6% (for E_3).

As illustrated by the chromatogram shown in Figure 2, all PFP derivatives were baseline resolved and their order of elu-

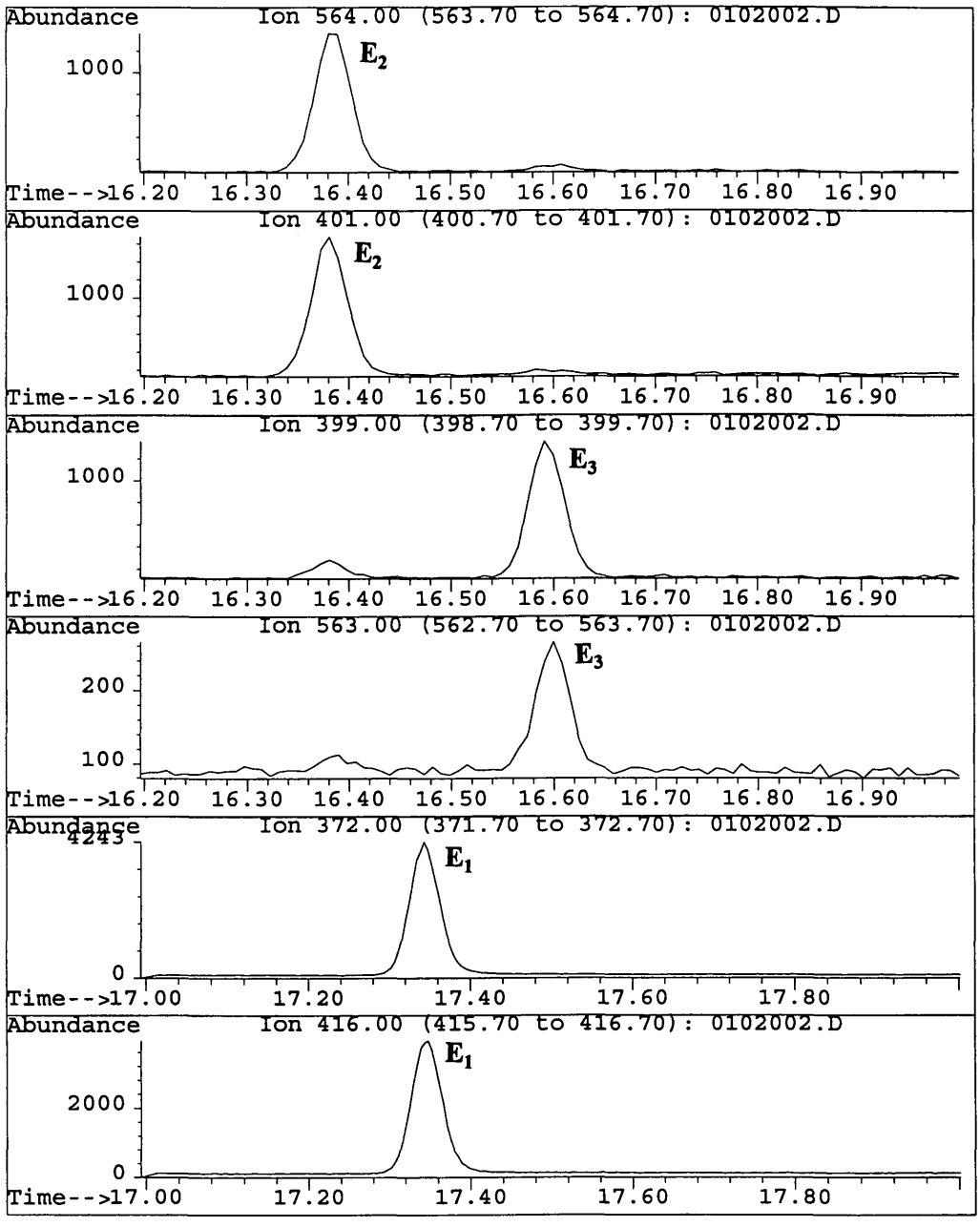


Figure 3. Extracted ion chromatograms for quantitation and confirmation ions of E_2, E_3, and E_1 from a 200 pg/μL standard.

tion is E_2, E_3, E_1, and EE_2. Tailing of the EE_2 derivative occurred, and its response to the mass-selective detector decreased as contaminants accumulated in the splitless liner. We think this derivative, which has one active OH group, became adsorbed to the polar coextractives, such as surfactants, that are common in sewage samples. The EE_2 response can be easily restored by replacing the liner and cutting a few centimeters of the capillary column in the injector end. By contrast, responses of the PFP derivatives of E_1, E_2, and E_3 were not greatly affected by cleanliness of the liner.

All hydroxyl groups in naturally occurring, endogenous estrogens—3-OH of E_1, 3- and 17-OH of E_2, and 3-, 16-, and 17-OH of E_3—reacted readily with PFPA to yield the respective mono-, di-, and tri-PFP derivatives, as evidenced by molecular ions at *m/z* 416, 564, and 726 (Table 1). The molecular ion (M^+) for the E_3 derivative was observed on a Hewlett-Packard MS Engine because its molecular weight is beyond the upper mass range of the mass-selective detector. Under the reaction conditions used, no partially derivatized E_2 and E_3 (i.e., a monosubstituted E_2 and either a mono- or disubstituted E_3) were observed in the chromatogram. The absence of such intermediates enabled straightforward quantitation of the estrogens. By contrast, a monosubstituted derivative of the synthetic estrogen EE_2 was formed (M^+ at *m/z* 442). Because mestranol, the 3-methyl ether derivative of EE_2, did not react with PFPA under such conditions, we think that the hydroxyl group at position 17 was protected from being derivatized by the presence of the ethynyl group at the same location.

Table 2. Accuracy and precision of recovery data of estrogens from fortified water and sewage samples ($n = 6$)

	Mean recovery ± standard deviation, %			
	From distilled water at indicated spiking level (ng/L)		From sewage at indicated spiking level (ng/L)	
Estrogen	100	20	100[a]	20[b]
E_1	101 ± 3	107 ± 5	96 ± 4	102 ± 8
E_2	95 ± 4	91 ± 5	105 ± 5	98 ± 9
E_3	93 ± 5	98 ± 6	101 ± 8	127 ± 13
EE_2	89 ± 6	102 ± 8	88 ± 8	87 ± 11

[a] A composite sewage final effluent (mixture of 3 samples from different treatment plants) was spiked. As all sewage final effluents are naturally contaminated with detectable amounts of E_1 and E_3, the results for these 2 estrogens in the spiking experiments have to be corrected (subtracted) for the blanks.

[b] A composite sewage final effluent that had been extracted previously by this procedure was spiked. Results were not corrected because the concentrations of estrogens in blanks were below detection limits.

All PFP derivatives displayed ions at *m/z* 69 (CF_3^+) and 119 ($C_2F_5^+$), which are characteristic of the PFP group (Figure 2). Molecular ions for the E_1 and E_2 derivatives were either the base peak or very strong. Those for E_3 and EE_2 were relatively weak. All the estrogens gave a prominent peak at *m/z* 359. This ion is likely due to loss of carbons at positions 15, 16, and 17 of the 5-member ring (ring D), their substituents, and a hydrogen from the molecular ion of each derivative. With the E_2 derivative for example, the ion at *m/z* 359 or $(M - 205)^+$ arises from loss of C_3H_5O, C_2F_5CO, and H (presumably from the carbon at position 14) from M^+. For EE_2, the ion corresponding to $(M - 83)^+$ arises from loss of C_3H_5O, the ethynyl group, and a hydrogen from M^+. The fragmentation pattern of EE_2 also provided direct evidence for the fact that the hydroxyl group at position 17 of this synthetic steroid was not derivatized.

The mass numbers and relative abundance of the major ions for the PFP derivatives are listed in Table 1. Their mass spectra are shown in Figure 2. Extracted ion chromatograms of the quantitation and confirmation ions derived from a calibration standard are depicted in Figure 3.

Method Performance

The accuracy and precision of this procedure was determined by analysis of distilled water and sewage effluent samples fortified with the estrogens at environmentally relevant levels (Table 2). At spiking levels of 100 and 20 ng/L, method precision was very good, with standard deviations of less than 10% for most samples. Recoveries of estrogens, ranging from 87 to 107% for all except one case, were close to quantitative.

Table 3. Levels of E_1, E_2, and E_3 in sewage influent, primary effluent, and final effluent[a]

				Estrogen found, mean ± standard deviation, ng/L		
Location	Sewage type	Sampling date	No. of replicates	E_1	E_2	E_3
Burlington	Primary	12/16/97	3	26 ± 1	7 ± 0.6	128 ± 10
Burlington	Final	12/16/97	3	6 ± 0.5	<5	18 ± 1
Burlington	Primary	1/20/98	3	53 ± 4	14 ± 2	220 ± 20
Burlington	Final	1/20/98	3	8 ± 1	<5	33 ± 1
Dundas	Primary	1/13/98	2	70, 68[d]	9, 8[d]	243, 203[d]
Dundas	Final	1/13/98	2	8, 10[d]	<5, <5	<10, <10
Edmonton	Primary, preserved[b]	8/20/97	3	109 ± 5	<5	209 ± 14
Edmonton	Final, preserved[b]	8/20/97	3	72 ± 2	<5	<10
Guelph	Influent, preserved[b]	12/18/97	2	58, 75[d]	<5	164, 158[d]
Guelph	Final, preserved[b]	12/18/97	2	18, 16[d]	<5	37, 30[d]
Guelph	Influent	1/22/98	3	41 ± 4	15 ± 2	250 ± 32
Guelph	Final	1/22/98	3	14 ± 1	<5	30 ± 2
Montreal, north	Influent[c]	11/17/97	3	28 ± 6	6 ± 1	72 ± 6
Montreal, south	Influent[c]	11/17/97	3	15 ± 2	7 ± 0.7	53 ± 3
Montreal	Primary[c]	11/17/97	3	19 ± 3	6 ± 0.7	68 ± 7

[a] All samples were grab and unpreserved except where noted.

[b] With formaldehyde (1%, v/v), 4°C in the dark.

[c] 24 h composite sample.

[d] For $n = 2$, individual results are given.

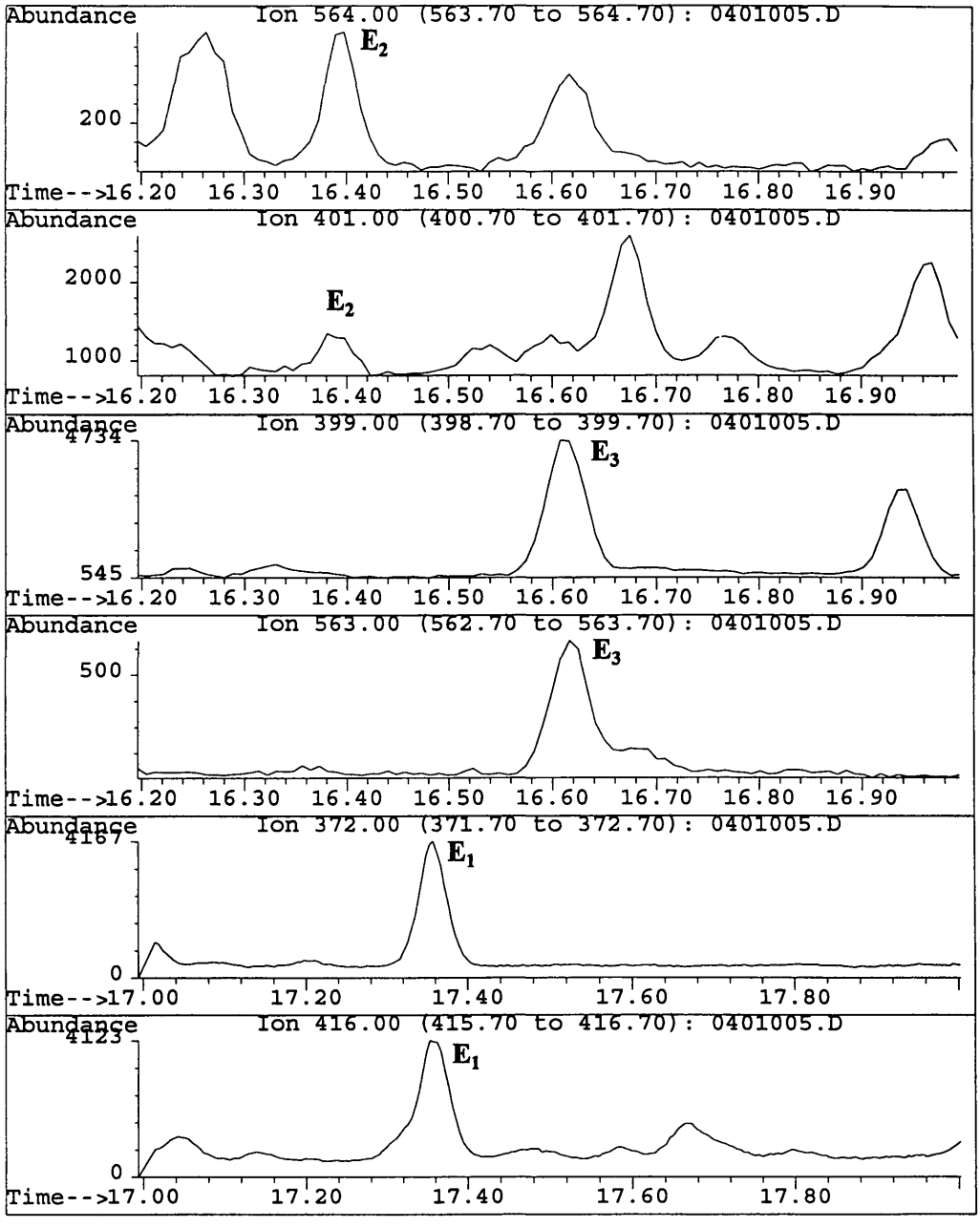


Figure 4. Extracted ion chromatograms of a derivatized, primary sewage effluent extract indicating the presence of E_2, E_3, and E_1 in the sample.

With a concentration factor of 5000 (1 L sample extracted and a final volume of 200 μL), detection limits (based on a signal-to-noise ratio of 10:1) for these estrogens in sewage samples were estimated to be 5 ng/L for E_2, E_1, and EE_2 and 10 ng/L for E_3. Detection limits could be reduced by a factor of 2 to 3 for distilled water samples where interference is minimal.

Application

This method has been used to determine steroidal estrogens in the sewage treatment plants of a few Canadian cities. E_1, E_2, and E_3 were found in all sewage influent and primary effluents that were extracted within 48 h after collection. In these samples, concentrations of E_1 and E_3 were relatively high, varying from 14 to 109 ng/L and from 53 to 250 ng/L, respectively (Table 3). The levels of E_2, the most potent endogenous estrogen, in the influent or primary effluent were much lower, ranging from 6 to 15 ng/L. These concentrations are substantially lower than those reported by Shore et al. (48–141 ng/L; 14) for raw sewage samples collected in Israel. Shore et al. used a radioimmunoassay technique, which might have measured other cross-reactive estrogens at the same time. To a smaller extent, discrepancy of the results could also be related to differences in weather (i.e., amount of precipitation) and patterns of water usage in the 2 countries.

The occurrence of E_1, E_2, and E_3 in a primary sewage effluent collected in Burlington is illustrated in Figure 4. The identity of each estrogen was confirmed by the presence of both characteristic ions at the expected retention time (± 0.04 min)

and in a similar area ratio (± 35%) compared with a standard (Figure 3). Although E_1 and E_3 were found in 2 preserved primary effluent samples (which were analyzed 1–3 months after collection), E_2 was not detected in those samples. Therefore the stability of E_2 and other estrogens in preserved sewage should be investigated.

As indicated in Table 3, the concentrations of the estrogens in the final effluents were substantially lower. In fact, in all cases, the concentration of E_2 was below the detection limit of 5 ng/L. E_1 and E_3 levels decreased by a factor of 4 between the primary and the final effluents collected on the same day from the same sewage treatment plant. The reduction is probably due to degradation of E_2 and its metabolites by activated sludge in the secondary sewage treatment processes (24). In the final effluents, concentrations varied from 5 to 19 ng/L for E_1 and from <10 to 34 ng/L for E_3. These levels are about 3 orders of magnitude lower than those found for nonylphenol ethoxylates and their degradation products in the same samples (16, 18, and unpublished results). None of the sewage effluents tested had any detectable amounts of EE_2.

The results suggest that this method is adequate for determining E_1 and E_3 in most sewage samples, as well as E_2 in the raw influent and primary sewage effluent, but it may not be sensitive enough for determining E_2 in final effluent. This procedure is not likely to be applicable to determination of EE_2 in sewage samples because the estimated environmental concentration of this estrogen is much lower than the method detection limit of 5 ng/L (15). A survey of the occurrence of E_2 and its metabolites in sewage samples collected across Canada is underway.

Acknowledgments

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