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Determination of endocrine-disrupting compounds in environmental samples using gas and liquid chromatography with mass spectrometry

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Abstract

This paper describes certain applications for endocrine-disrupting compounds determination. LC–MS was applied using an electrospray ionization (ESI) technique in positive mode for alcohol polyethoxylates and nonylphenol and octylphenol polyethoxylates ($NPEO_n$ and $OPEO_n$), and in negative mode for 4-nonylphenol (4-NP) and 4-octylphenol (4-OP) to determine targeted compounds in wastewater and sludge. GC–MS and GC–MS–MS were used to determine 4-NP, 4-*tert*.-octylphenol (4-*t*-OP), bisphenol A, estradiol-17β, estriol, estrone, testosterone, 17α-ethynylestradiol, cholesterol, coprostan-3-ol, coprostan and coprostan-3-one in both surface water and wastewater after derivatization with *N*,*O*-bis(trimethyl-silyl)trifluoroacetamide (BSTFA). Extraction from the water samples was by an SPE technique, using either a copolymeric (Oasis HLB) or C_{18} silica sorbents, depending on the target contaminants. Extraction from the sludge samples was by a Soxtec system using methanol. Percentage recoveries for most of the selected compounds, using either a copolymeric (Oasis HLB) or C_{18} silica sorbents, were satisfactory (>60%). Quantification limits for the target compounds were at ppb levels in both water and sludge samples when using LC–ESI–MS in both positive and negative modes. They reached ppt levels in water when using GC–MS (in large volume injection mode) and GC–MS–MS. The results revealed 4-NP, $NPEO_n$ and AEOs in sludge samples at a concentration range of 1.3–8.5 μg/g, and $NPEO_n$, $OPEO_n$ and other compounds, such as coprostan and bisphenol A, in surface water and/or wastewater samples at concentrations ranging from the ppt to ppb levels.

Keywords: Environmental analysis; Endocrine-disrupting compounds

1. Introduction

Large cities are known to be major polluters of aquatic systems in that they release a variety of chemicals. This is in part due to large volumes of domestic and industrial waste discharging into the city sewage systems. A wide variety of pollutants present in this waste has been reported as dangerous for humans and wildlife; due to their biological effect, they are classified as ''endocrine-disrupting compounds'' (EDCs). In 1996, the European Commission defined this class of compounds as ''exogenous substances that cause adverse health effects in an intact organism, or its progeny, consequent to

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changes in endocrine function''. Among the pollutants potentially associated with urban wastewater are: (i) alcohol polyethoxylates (AEOs), alkylphenol polyethoxylates ($APEO_n$, subdivided into nonylphenol [$NPEO_n$] and octyphenol [$OPEO_n$]), and alkylphenols (4-NP, 4-OP, 4-*t*-OP) used in industrial, agriculture and household applications [1,2], (ii) bisphenol A used in the production of polycarbonate, epoxy resins, flame retardant and many other products, (iii) sterols and hormones, either natural or synthetic, used in oral contraceptives. The last group includes estradiol-17β, estriol, estrone, and testosterone, which are derived from the biotransformation of cholesterol, a precursor of mammalian sexual steroids [3]. Cholesterol has also been reported to be reduced to other sterols, like coprostanol, coprostan and coprostane-3-one, by faecal bacteria, when released in municipal effluents and sludge [4,5].

$APEO_n$ and its metabolites were recently restricted or altogether banned in Europe, and there are significant initiatives to reduce their use or phase them out world-wide [6,7]. The European Economic Community (EEC) Directive 86/278 has already proposed setting the maximum limits for 4-nonylphenol (4-NP) and its ethoxylates, $NPEO_1$ and $NPEO_2$, at 50 mg/kg in sludge used on agricultural land. In Canada, the water-quality guidelines have been set for nonylphenol and its ethoxylates at 1 μg/l teq (toxic equivalent), and on June 23rd, 2001 the Canadian Ministers of the Environment and of Health recommended that nonylphenol and its ethoxylates be added to the List of Toxic Substances under the *Canadian Environmental Protection Act* of 1999 (CEPA) [8]. The final decision to declare $NPEO_n$ as toxic followed the publication of a draft of the Priority Substance List assessment report for $NPEO_n$ in which it was concluded that these substances were harmful to the environment. By contrast, no Canadian or European water-quality guidelines have yet been developed for bisphenol A (BPA), sterols and steroids.

Different techniques exist for extracting targeted contaminants from environmental matrices. The most common are liquid–liquid extraction [9], steam distillation [10], solid-phase extraction [11,12], solid-phase micro-extraction [13], immunoextraction [14], Soxhlet extraction [15], steam distillation [9,10], ultrasonication [16], microwave extraction system [17,18], pressurized liquid extraction [19] and supercritical fluid extraction [20].

The analysis of selected contaminants is made difficult by the presence of various polar groups contained in these chemicals, i.e. alcoholic and phenolic hydroxyls, carbonyls and polyethoxylates. Nevertheless, different techniques have been reported for their determination, including radioimmunoassay [21], enzyme-linked immunosorbent assay (ELISA) [22], liquid chromatography and hyphenated gas chromatography [23,24], plus fluorescence and UV detection [25] and mass spectrometry with particle beam [26], electrospray and atmospheric pressure chemical ionization [27]. Gas chromatography has also been applied to the analysis of selected compounds in free and derivatized forms.

Different reagents have been used to derivatize targeted steroids and alkylphenols in order to improve sensitivity and selectivity. These include pentafluorobenzyl (PFBr), *N*,*O*-bis(trimethyl-silyl)-trifluoroacetamide (BSTFA), *N*-methyl-*N*-*tert*.-(butyldimethyl)trifluoroacetamide, hepta-fluorobutyryl, pentafluorobenzoyl and alkyl (e.g. methyl, ethyl, dimethyl, etc.), which have all been reported in the literature [20].

The low levels of selected pollutants (less than ng/l in certain cases) in such complex matrices as sludge and wastewater could seriously affect their efficiency [17,18]. Liquid chromatography with mass spectrometry (LC–MS) enables the determination of steroids and alkylphenols without derivatization. Methods based on mass spectrometry tandem mass spectrometry (MS–MS) detection are reported to be approximately ten times more sensitive than MS detection for treated effluent [28,29]. Several authors have recently reported extremely high sensitivity (<0.1–5.0 ng/l) for estrogenic compounds in environmental samples using LC–MS with electrospray and atmospheric pressure chemical ionization (APCI) detection [25] or LC–MS–MS with electrospray detection [30]. To achieve lower quantitation limits, Ding and Wu [31] suggested using an ion trap GC–MS with large-volume injection (LVI) technique.

The objectives of our work were as follows:

(1) To develop GC–MS and GC–MS–MS methods using BSTFA as the derivatization agent with large-volume and split–splitless injection, re-

spectively, and to detect the four natural steroids (estradiol-17β, estriol, estrone, testosterone) and the synthetic contraceptive additive 17α-ethynyestradiol, 4 sterols (cholesterol, coprostan-3-ol, coprostan, coprostan-3-one), BPA, 4-NP and 4-*t*-OP in surface water and wastewater.

(2) To develop an LC–MS method to determine AEOs (A = aliphatic alcohol groups with C_{12-15}), $NPEO_n$ and $OPEO_n$, including 4-NP and 4-OP, in sludge and wastewater.

2. Experimental

2.1. Chemicals and reagents

Estradiol-17β, estriol, estrone, 17α-ethynyestradiol, cholesterol, coprostanol, coprostan, coprostan-3-ol, coprostan-3-one, bisphenol A, bisphenol A-d_{16} (internal standard, I.S.), 4-NP and 4-*t*-OP were purchased from Sigma–Aldrich Canada (Oakville, Ontario, Canada), and 4-OP was purchased from Aldrich (L'Isle d'Abeau, France). Hexaethylene glycol hexadecyl ether ($C_{16}EO_6$) and hexaethylene glycol octadecyl ether ($C_{18}EO_6$) were obtained from Fluka (Buchs, Switzerland). The Neodol 25-9, a gift from Shell Chemical (Houston, TX, USA), was made of polyethylene glycol dodecylether ($C_{12}EO_n$: 21%), polyethylene glycol tridecylether ($C_{13}EO_n$: 20%), polyethylene glycol tetradecylether ($C_{14}EO_n$: 28%), polyethylene glycol pentadecylether ($C_{15}EO_n$: 31%). A 4-*tert*.-octylphenol polyethoxylated ($OPEO_n$: Triton X-100), obtained from Aldrich (L'Isle d'Abeau, France), was used for the quantification of octylphenol oligomer. Nodinet P40, made of nonylphenol polyethoxylated ($NPEO_n$), was purchased from Fluka. The testosterone was purchased from Steraloids (Wilton, NH, USA) and the BSTFA was from Supelco (Oakville, Ontario, Canada). Solvents, including dichloromethane, isooctane, acetone and hexane, were all distilled-in-glass grade and purchased from Caledon Laboratories (Georgetown, Ontario, Canada). Methanol optima was from Fisher Scientific (Nepean, Ontario, Canada). Diethylether and ammonia were purchased from Carlo Erba (Val de Reuil, France) and triethylamine and acetic acid (analytical quality grade) were from Fluka (Buchs, Switzerland).

2.2. Standard solutions

Primary stock solutions of all targeted chemicals were prepared individually at a concentration of 1.0 g/l by weighing about 10 mg of each substance in a 10-ml volumetric flask and diluting to volume with methanol. The group 1 spiked solution, containing estradiol-17β, estriol, estrone, 17-α-ethynyestradiol, cholesterol, coprostanol, coprostan, coprostan-3-ol, coprostan-3-one, bisphenol A, 4-NP and 4-*t*-OP, was prepared in methanol at a concentration of 1.0 mg/l. The group 2 spiked solution, containing a mixture of AEOs ($C_{12}EO_n$, $C_{13}EO_n$, $C_{14}EO_n$, $C_{15}EO_n$, $C_{16}EO_n$, $C_{18}EO_n$), $OPEO_n$ and $NPEO_n$ was prepared at a concentration of 10 mg/l in methanol for sludge samples and at a concentration of 100 mg/l in methanol for wastewater samples. The 4-NP and 4-OP were prepared in methanol at a concentration of 10 mg/l. A solution of bisphenol A-d_{16} was prepared in methanol at a concentration of around 120 mg/l then diluted 100 times in methanol to give the internal standard solution (I.S.). Working solutions were prepared in methanol for group 1 (including the I.S.) and group 2 at concentrations ranging from 1.0–100 and 0.005–1.0 mg/l, respectively.

2.3. Sample collection and preservation

Five discrete samples of effluent were collected in Pyrex borosilicate glass containers from two sewage treatment plants, and one sample of surface water was collected downstream from one of the two wastewater effluents. The effluent samples were filtered, and formaldehyde (1–3%, v/v) was added to prevent changes in composition due to biodegradation. The samples were then stored in the dark at 4 °C before extraction and analysis; the time between collection and extraction was less than 48 h. Five samples of sludge, from a domestic wastewater treatment plant and available for use in agricultural fields, were also collected, oven dried at 40 °C, sieved and stored at −20 °C until analysis. Only fractions below 250 μm were analysed.

2.4. Sludge sample spiking

Two hundred microlitres (200 μl) of a methanolic solution containing AEOs, $NPEO_n$, $OPEO_n$, 4-NP and 4-OP were added to a slurry made of 10 g of sludge dissolved in 10 ml of methanol. The spiking level was 200 ng/g for each compound. After a 24-h period of equilibration, the solvent was evaporated off by a gentle stream of nitrogen and left to stand for at least 1 week at 4 °C. Prior to extraction, the water content was adjusted to 10% (w/w) and the material was left to absorb for 1 day. Blank samples were prepared in the same way, using pure methanol as the spiking agent.

2.5. Wastewater sample spiking

Four hundred microlitres (400 μl) of a methanolic solution containing AEOs, $NPEO_n$, $OPEO_n$, 4-NP and 4-OP were added to 2 l of a filtered wastewater sample. The spiking level was 20 μg/l for each compound. SPE extraction was performed after homogenization.

2.6. Sample preparation and pre-concentration

2.6.1. Sludge sample

Sludge samples were Soxtec-extracted prior to clean up. A 10-g aliquot of the homogenized sample was Soxtec-extracted with 50 ml of methanol. The sample was submerged in methanol for 45 min, then rinsed for a minimum of 4 h. The methanolic extract was diluted with water to obtain a matrix of water–methanol (70:30, v/v), and then passed through C_{18} cartridges (1 g, 6 ml, obtained from Supelco, Bellefonte, USA). Cartridge conditioning and elution procedures were similar to those applied to water analysis (see below). The extract was reconstituted to a final volume of 1 ml in the appropriate HPLC mobile phase prior to analysis.

2.6.2. Wastewater samples

2.6.2.1. SPE with C_{18} cartridges

Optimization of the SPE extraction of 4-OP, NP, AEOs, $OPEO_n$ and $NPEO_n$ from the wastewater samples using C_{18} cartridges was performed according to a work published by Castillo et al. [32]. The C_{18} cartridges (1 g, 6 ml) were first conditioned with 5 ml each of methanol and water. Then 250 ml of influent or effluent samples were filtered through a 0.45-μm cellulose acetate membrane filter, and passed through the cartridges at a flow-rate of 5 ml/min. After drying by vacuum pressure, the cartridges were eluted with 2×5 ml of a hexane–dichloromethane (90:10, v/v) solution, and then with 2×5 ml of a methanol–dichloromethane (90:10, v/v) solution. The compounds of interest are found in the last fraction. After evaporation of the methanol–dichloromethane solution, the extract was reconstituted to a final volume of 1 ml in the appropriate mobile phase prior to analysis.

2.6.2.2. SPE with polymeric cartridges

Optimization of the SPE extraction of 4-t-OP, NP, BPA, steroids and sterols from the wastewater samples using Oasis HLB cartridges was performed according to the Waters Oasis applications for endocrine disruptors analysis [33].

First the sorbent was conditioned with 6 ml diethylether, 5 ml of methanol and 5 ml of water. Then 1 l of surface water, or 250 ml of effluent were filtered with a 0.45-μm cellulose acetate membrane filter and passed through the Oasis HLB cartridges (6 ml) filled with 500 mg of poly(divinylbenzene-co-*N*-vinypyrrolidone sorbent (Waters, Saint-Quentin-en-Yvelines, France). After drying by vacuum pressure, the cartridges were rinsed with 5 ml of a methanol–water (40:60, v/v) solution, followed by 5 ml of water and 5 ml of a methanol–ammonia–water (10:2:88, v/v) solution. The cartridges were then eluted with 10 ml of a methanol–diethylether (10:90, v/v) solution. After the evaporation of the methanol–diethylether solution, the extract was derivatized.

2.7. Chromatographic analysis

2.7.1. Derivatization for GC–MS and GC–MS–MS analysis

Standard solutions and extracts were derivatized in a test tube by the addition of BSTFA, as follows: 500 μl of the standard solution or sample extract with 50 μl of the internal standard solution (I.S.) were evaporated to dryness at 30 °C under a gentle nitrogen gas flow; 50 μl of BSTFA was added; the

test tube was closed and placed in a water bath at 60 °C for 15 min; 500 μl of an isooctane–acetone (99:1, v/v) solution was added and the tube was then placed in an ultrasonic bath for 10 min. After derivatization, the extract was ready for injection into the GC–MS or the GC–MS–MS system.

2.7.2. GC–MS analysis

The GC–MS analyses were performed using a Varian (Les Ulis, France) system consisting of a 3800 CX gas chromatograph equipped with a Varian model 1079 split–splitless temperature-programmed injector, a 8200 CX autosampler and a Saturn 2000 ion-trap mass spectrometer (Varian, Les Ulis, France). Saturn 2000 software; Varian, Les Ulis, France) was used for data processing. The injector was equipped with a treated Siltek 2.0 mm I.D. liner without glass wool. Large-volume injections (40 μl) of a derivatized extract or derivatized standard solution were used. The injector temperature was initially set at 85 °C, then increased at a rate of 200 °C/min to 300 °C, where it was maintained. The split–splitless valve operated in split mode from 0 to 1.5 min, in splitless mode from 1.5 to 3.0 min, then back in split mode from 3.0 to 50.0 min.

Compounds were separated on a 30 m×0.25 mm I.D. low bleed-MS column coated with a 0.25 μm film of CP-Sil 8 CB (95% dimethyl–5% phenyl polysiloxane; Varian–Chrompack, Les Ulis, France) connected to a 5 m×0.53 mm I.D. deactivated Siltek guard column (Restek, Evry, France). The column temperature was initially set at 85 °C for a period of 3 min, then increased at a rate of 10 °C/min to 130 °C. Once at 130 °C, the rate of increase slowed to 3 °C/min until it reached the final temperature of 300 °C, which was maintained for 3 min. Helium was used as the carrier gas at a constant flow of 1.2 ml/min. Data acquisition was performed in full scan mode from 50 to 650 u at 1 s/scan. The transfer line was set at 280 °C. The general conditions used for GC–MS analysis are shown in Table 1. An example of a chromatogram in total ion current (TIC) mode for the target compounds is shown in Fig. 1.

2.7.3. GC–MS–MS analysis

The GC–MS–MS analyses were performed using a Thermoquest (Les Ulis, France) system consisting of a Trace GC 2000 gas chromatograph equipped with a PTV split–splitless temperature injector, an AS 2000 autosampler and a Polaris ion-trap mass spectrometer (Thermofinnigan, Les Ulis, France). Excalibur software from Thermofinnigan was used for data processing. The injector was equipped with a 12 cm×2 mm I.D. Silcoseeve liner (Thermofinnigan). Injection volumes of 2 μl of a derivatized extract or standard solution were used. The PTV split–splitless valve was operated in splitless mode up to a temperature of 300 °C. Once this temperature stabilized, it was maintained in splitless mode for a period of 1.5 min. Compounds were separated on a 30 m×0.25 mm I.D. column, coated with 0.25 μm of 95% dimethyl–5% phenyl polysiloxane phase

Table 1
General conditions used for GC–MS analysis

Compound number	Name	Retention time (min)	Molecular mass	*m/z* used for quantification
1	4-*tert*.-Octylphenol	19.14	206.3	207
2	4-Nonylphenol	28.14	220.4	179, 292
3	Bisphenol A	38.94	228.3	358
4	Bisphenol A-d_{16}	38.68	244.3	369
5	Estrone	48.08	270.4	218, 258, 342
6	Estradiol-17β	48.94	288.4	286, 416
7	Testosterone	49.13	288.4	227, 258, 360
8	17-α-Ethynylestradiol	50.17	296.4	268, 368
9	Coprostan	51.97	372.7	217, 358
10	Estriol	53.58	272.4	312, 387, 415
11	Coprostan-3-ol	57.13	388.7	216, 356, 370
12	Coprostn-3-one	58.76	386.7	161, 317, 386
13	Cholesterol	59.07	386.7	330, 354, 369

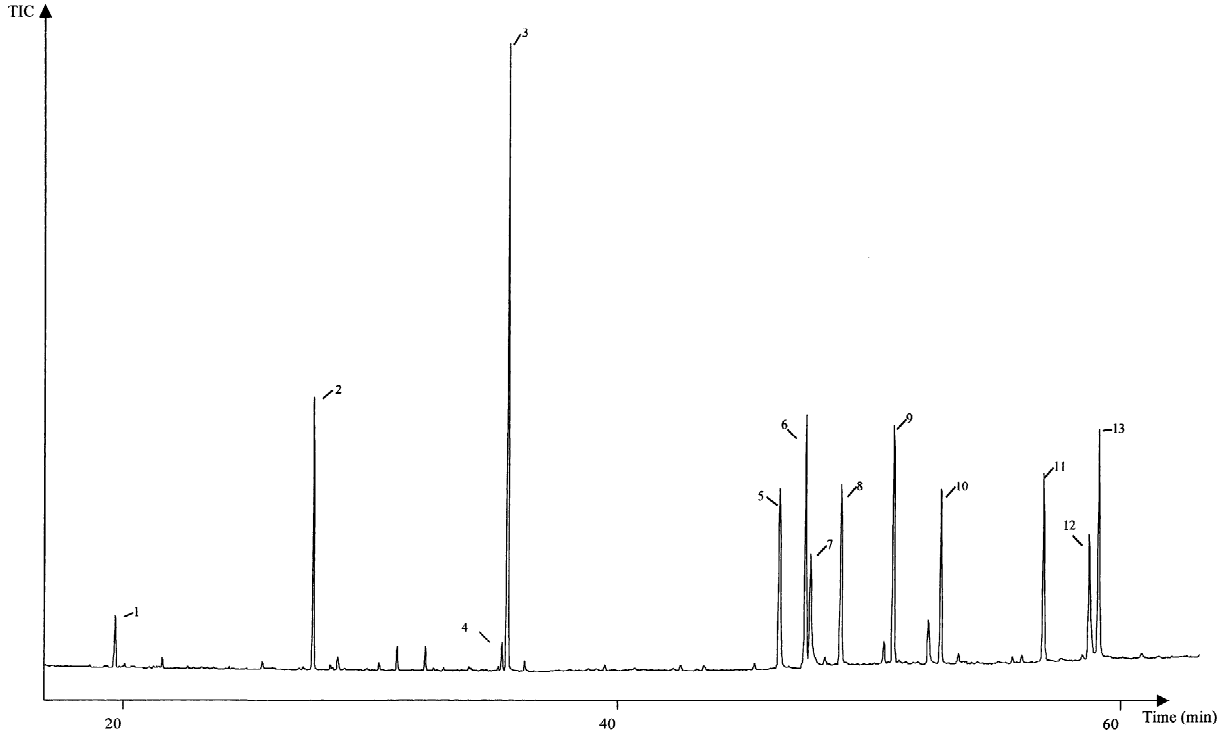

Fig. 1. LVI–GC–MS full scan chromatogram of alkylphenols, bisphenol A and a steroid mixture of approximately 400 ng/μl of each compound in derivatized form with BSTFA; injected volume: 40 μl. Peak assignments: 1=4-*t*-OP, 2=4-NP, 3=bisphenol A, 4=bisphenol A-d_{16}, 5=estrone, 6=β-estradiol, 7=testosterone, 8=17-α-ethynylestradiol, 9=coprostan, 10=estriol, 11=coprostan-3-ol, 12=coprostan-3-one, 13=cholesterol.

(BPX-5, SGE, Courtaboeuf, France). The injector and column temperatures were the same as for GC–MS. Helium was used as the carrier gas at a constant flow of 1 ml/min. The transfer line was set at 320 °C with the external ion source at 280 °C.

For GC–MS–MS, the ions in EI for the target compounds were selected and fragmented with CID helium gas collision in the ion trap using a collision excitation voltage at 1 V. The mass spectra resulting from these fragments were scanned from *m/z* ion 50 to the mass of the selected ions. Selection of the ions was arranged according to different segments. The general conditions used for GC–MS–MS analysis are shown in Table 2.

2.7.4. LC–MS analysis

The LC mobile phase was delivered by a Varian 9012 elution gradient pump. The mobile phase consisted of a mixture of A (methanol–acetonitrile 50:50, v/v) and B (water), both acidified with 0.5% (v/v) acetic acid and 0.1% (v/v) triethylamine for running in positive and negative ionization mode, respectively. The mobile phase composition was 70% A at the beginning of the gradient, which was then linearly increased to 100% in 28 min, where it remained isocratic for 2 min. AEOs, $NPEO_n$, $OPEO_n$, 4-NP and 4-OP were separated by means of a Hypersyl Green Env column (150×4.6 mm I.D., 5 μm particle size) equipped with a guard column (both from Interchim, Montluçon, France). The flow-rate was 0.8 ml/min and the eluent was split so that 0.3 ml/min entered the mass spectrometer source. Twenty microlitres (20 μl) of the SPE extracts were injected into the LC system.

For electrospray MS experiments, an SSQ 7000 mass spectrometer (Finnigan, San Jose, CA, USA) equipped with a standard atmospheric pressure ionization source was used. The electrospray voltage and

Table 2
General conditions used for GC–MS–MS analysis

Segment [time (min)]	Retention time (min)	Compound	Selected ions	Daughter ions	MS–MS full scan interval
8.0–18.0			50–650		
18.1–26.0	18.23	4-*tert*.-Octylphenol	207	151, 163, 179	50–207
26.1–35.5	27.66	4-Nonylphenol	179	73	50–180
35.6–47.0	36.50	Bisphenol A	357	191, 267, 357	50–360
	36.27	Bisphenol A-d_{16}	368	197, 368	50–370
47.1–51.0	47.63	Estrone	342	242, 257	50–350
	48.14	Estradiol-17β	285	229, 256, 269	50–300
	48.52	Testosterone	226	198, 211	50–230
	50.82	17-α-Ethynylestradiol	425	193, 231, 407	150–440
51.1–52.2	51.25	Coprostan	217	121, 147, 161	50–220
52.3–56.0	52.59	Estriol	414	295, 311, 324	50–420
56.1–59.0	56.23	Coprostan-3-ol	215	133, 145, 159	50–220
	58.24	Coprostan-3-one	161	119, 133, 145	50–165
	58.24	Cholesterol	353	171, 185, 199	120–360

the collision voltage on quadrupole were set to 4.5 kV and 10 V, respectively. The temperature of the heated capillary was 250 °C. Nitrogen was used as a nebulizing gas at a pressure of 5 bar. The m/z interval between 200 and 1200 in positive ionization mode, and the two ions at m/z 205 and 219 in negative ionization mode, were monitored. Examples of chromatograms acquired under these conditions for AEOs, $NPEO_n$, $OPEO_n$, 4-NP and 4-OP are shown in Fig. 2.

3. Results and discussion

3.1. GC–MS analysis

All alkylphenols, bisphenol A, steroids and sterols selected for this study contain hydroxyl groups (except for coprostan), and therefore required a derivatization step (in this case, a silylation reaction) prior to GC analysis. The objective of the derivatization was to better separate selected compounds in the capillary column by increasing their volatility and decreasing their interaction with the stationary phase or with the material in the injection chamber, thereby enhancing the selectivity and sensitivity of the analysis [34,35]. Another benefit of this reaction is that ions of much higher m/z are formed compared to the parent compound [34], allowing for improved identification of the compounds by MS. BSTFA was selected as the silylation agent because of its fast reactivity with compounds containing hydroxyl groups, its high volatility resulting in non-coelution of early eluting peaks, and the high volatility, stability and good solubility of the derivatized compounds.

3.1.1. Repeatability of large-volume injection

Five standard solutions of the studied compounds, at concentrations ranging from 10.3 to 1030 μg/l, were derivatized and injected (40 μl) five times in GC–MS in full scan mode to determine detector repeatability. Relative standard deviation (RSD) was $<10\%$, showing the good repeatability of LVI mode.

3.1.2. Calibration curve and linearity

An internal calibration was used with a bisphenol A-d_{16} standard. The linearity of the responses, obtained by injecting 40 μl of standard solutions of the derivatized compounds, was studied in relation to concentrations ranging from 10.3 to 1030 μg/l according to the ISO 8466-1 standard method and using the variance homogeneity test (F-test). Five calibration levels were prepared and injected five times using the LVI mode. A statistical study of the results demonstrated the concordance of the responses with the linear model for each compound. The response of the MS detector was linear for all the derivatized compounds with correlation coefficients $r^2>0.99$.

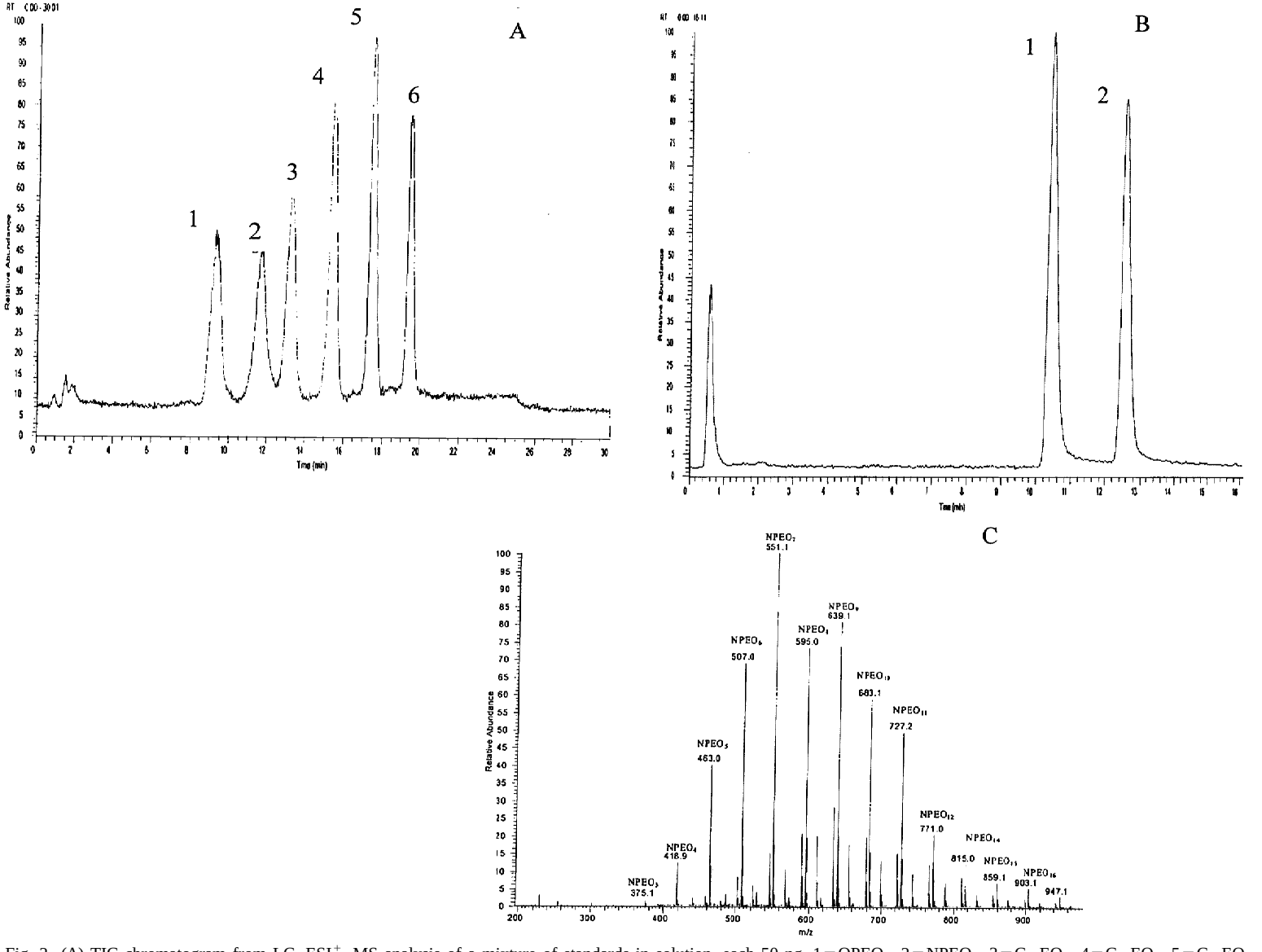

Fig. 2. (A) TIC chromatogram from LC–ESI$^+$–MS analysis of a mixture of standards in solution, each 50 ng. 1=OPEO$_n$, 2=NPEO$_n$, 3=C$_{12}$EO$_n$, 4=C$_{13}$EO$_n$, 5=C$_{14}$EO$_n$, 6=C$_{15}$EO$_n$. (B) SIM chromatogram from LC–ESI$^-$–MS analysis of a mixture of 1=4-octylphenol, 2=4-nonylphenol. (C) Mass spectrum of NPEO$_n$.

3.1.3. Quantification limits and recovery studies

Recovery studies were performed by extracting the target compounds from 1-l of Milli-Q water that had been spiked with a mixture of standard solutions at three concentration levels (50, 200 and 1000 ng/l) by SPE on Oasis HLB, followed by GC–MS analysis with 40-μl injections in LVI mode. Oasis HLB cartridges gave higher percentage recoveries (>60%) for the majority of selected compounds (steroids, sterols and BPA), except for alkylphenols, for which percentage recoveries were some 25% for 4-*t*-OP and roughly 50% for 4-NP. Note that the two alkylphenol products are phenolic compounds and so are probably better extracted at pH levels below 5—the pH of both the surface water and wastewater was about 7. Future experiments will be conducted on Oasis HLB cartridges at lower pH levels in order to improve percentage recoveries of alkylphenols.

Quantification limits (QL) for the GC–MS technique using the large-volume injection mode (40 μl) were estimated based on injections of the lowest concentrations of the standard solutions. They were determined by the SD values calculated from ten injections (QL = 10 SD). Table 3 shows the quantification limits and the percentage recoveries obtained for each compound using Oasis HLB adsorbent. Use of the large-volume injection method allowed us to obtain lower quantification limits than with other procedures, which use larger volume samples and conventional GC-injection. It should be noted that QL were between 0.5 and 40 ng/l, depending on the compound, when using large-volume injection combined with SPE extraction

3.2. GC–MS–MS

3.2.1. Calibration curve, linearity and quantification limits

An internal calibration was used with a bisphenol A-d_{16} standard. The linearity of the responses was obtained by injecting 2 μl of standard solutions of the derivatized compounds in split–splitless mode, and studying them in relation to concentrations ranging from 10.3 to 1030 μg/l. A statistical analysis of the results demonstrated the concordance of the responses with the linear model for each compound. The response of the MS–MS detector was linear for all the derivatized compounds with correlation coefficients $r^2 > 0.99$. Quantification limits, estimated with the S/N ratio ($S/N = 10$), are similar to those obtained with GC–MS using large-volume injection.

Table 3
Mean recovery values (R1, R2, R3) and relative standard deviations (RSD(1), RSD(2), RSD(3) $n = 3$ replicates, three levels) of alkylphenols, bisphenol A, and steroids determined from spiked distilled water using SPE with Oasis HLB cartridges followed by derivatization with BSTFA and GC–MS analysis

Compound	Recoveries (%) and RSD						QL (ng/l)	
	R1 (%) ($n=3$)	RSD(1) (%)	R2 (%) ($n=3$)	RSD(2) (%)	R3 (%) ($n=3$)	RSD(3) (%)	LVI–GC–MS (injected vol: 40 μl)	GC–MS–MS (injected vol: 2 μl)
4-*tert.*-Octylphenol	25	5	26	4	24	3	5	1
4-*n*-Nonylphenol	52	8	48	12	50	10	2	1
Bisphenol A	74	5	82	4	78	3	0.5	0.5
Estrone	94	5	96	3	98	4	10	2
Estradiol-17β	99	5	109	4	104	4	2	3
Testosterone	96	4	99	6	95	2	20	1
17-α-Ethynylestradiol	96	2	98	3	100	2	5	20
Coprostan	40	20	48	18	44	16	10	5
Estriol	82	22	90	19	98	16	10	12
Coprostan-3-ol	88	24	95	20	102	22	10	30
Coprostan-3-one	87	19	96	16	99	13	15	5
Cholesterol	60	6	66	4	72	2	15	40

Spiking levels: 50, 200, and 1000 μg/l; ND, not determined.
R1 (%), RSD(1) (%): recovery and RSD for level 50 μg/l; R2 (%), RSD (2)(%): recovery (%) and RSD (%) for level 200 μg/l,; R3 (%), RSD (3) (%): recovery (%) and RSD (%) for level 1000 μg/l (R1, R2, R3 = mean of three replicate for each level).
Limits of quantification (QL) calculated from ten injections of the lowest concentrations of the standard solutions.

3.3. LC–MS analysis

Using LC chromatography in reversed-phase mode allowed us to elute all the oligomer constituents of each homolog component of non-ionic surfactants ($OPEO_n$, $NPEO_n$, $C_{12}EO_n$, $C_{13}EO_n$, $C_{14}EO_n$, and $C_{15}EO_n$) into a single peak. Information on the oligomer distribution (n varying from 3 to 17) could be obtained by extracting selected m/z ions from the TIC for each peak in the chromatograms. Fig. 2A shows an example of an LC–MS chromatogram for non-ionic surfactants containing six commercial standards in positive ESP ionization mode. Fig. 2B shows the separation of 4-NP and 4-OP in ESI negative ionization mode. Nonionic polyethoxylated surfactants were identified in the positive ionization mode, with oligomers distributed in correspondence with the following expressions [12]:

$NPEO_n$: $C_8H_{17}–C_6H_4(OCH_2CH_2)_nOH$;
$M_w = 229 + 44n$

$NPEO_n$: $C_9H_{19}–C_6H_4(OCH_2CH_2)_nOH$;
$M_w = 243 + 44n$

APEO: $C_xH_{2x+1}(OCH_2CH_2)_nOH$;
$M_w = 14x + 18 + 44n$

Fig. 2C gives an example of an $NPEO_n$ mass spectra with n values of 3–16.

In addition, 4-OP and 4-NP were detected and quantified in the negative ionization mode using the pseudo-molecular ions m/z 205 and 219.

3.3.1. Calibration curve and quantification limits

External calibration was used for AEOs, $NPEO_n$, $OPEO_n$, 4-OP and 4-NP. The calibration curves were obtained by injecting 20 μl of the standard solutions at concentrations ranging from 1 to 100 mg/l. The response of the ESI–MS was linear, with correlation coefficients $r^2 > 0.994$. Quantification limits, obtained by spiking a wastewater sample and a sludge sample in full-scan mode for AEOs, $NPEO_n$ and $OPEO_n$, and in SIM mode for 4-NP and 4-OP, are shown in Table 4. After preconcentration of 250 ml of wastewater and LC–ESI–MS analysis of AEOs, $NPEO_n$ and $OPEO_n$ in full scan mode and LC–ESI–MS analysis of 4-NP and 4-OP in SIM mode, quantification limits ranged from 0.1 to 0.5 μg/l, depending on the compound. For the sludge sample, after Soxtec extraction of 10 g of sample, quantification limits ranged from 1 to 20 ng/g, depending on the compound. The low quantification limits for 4-NP and 4-OP were a result of their quantification in SIM mode.

3.3.2. Recovery studies

Recovery studies were performed by extracting, in five replicates, the target compounds from 250 ml of

Table 4
Mean recovery values (R) and relative standard deviations (RSD, n = five replicates) of AEOs, $OPEO_n$, $NPEO_n$, nonylphenol and octylphenol determined from spiked wastewater using SPE with C_{18} cartridges and spiked sludge samples using Soxtec extraction, followed by LC–MS analysis

Compound	Number of replicates	Wastewater			Sludge		
		R (%)	RSD (%)	QL (μg/l)	R (%)	RSD (%)	QL (ng/g)
$C_{12}EO_n$	5	75	8	0.1	90	15	10
$C_{13}EO_n$	5	82	11	0.1	88	14	10
$C_{14}EO_n$	5	84	9	0.1	92	11	10
$C_{15}EO_n$	5	88	8	0.1	85	15	10
4-Nonylphenol	5	102	12	0.1[a]	72	16	1[a]
4-Octylphenol	5	98	11	0.1[a]	74	16	1[a]
$OPEO_n$	5	92	7	0.5	69	12	20
$NPEO_n$	5	94	8	0.5	71	12	20

Spiking levels: 20 μg/l in wastewater samples and 200 ng/g in sludge samples. Limits of quantification (QL) estimated from spiked wastewater and sludge samples ($S/N = 10$).

[a] QL calculated using SIM mode of detection with LC–MS (m/z 219, 205).

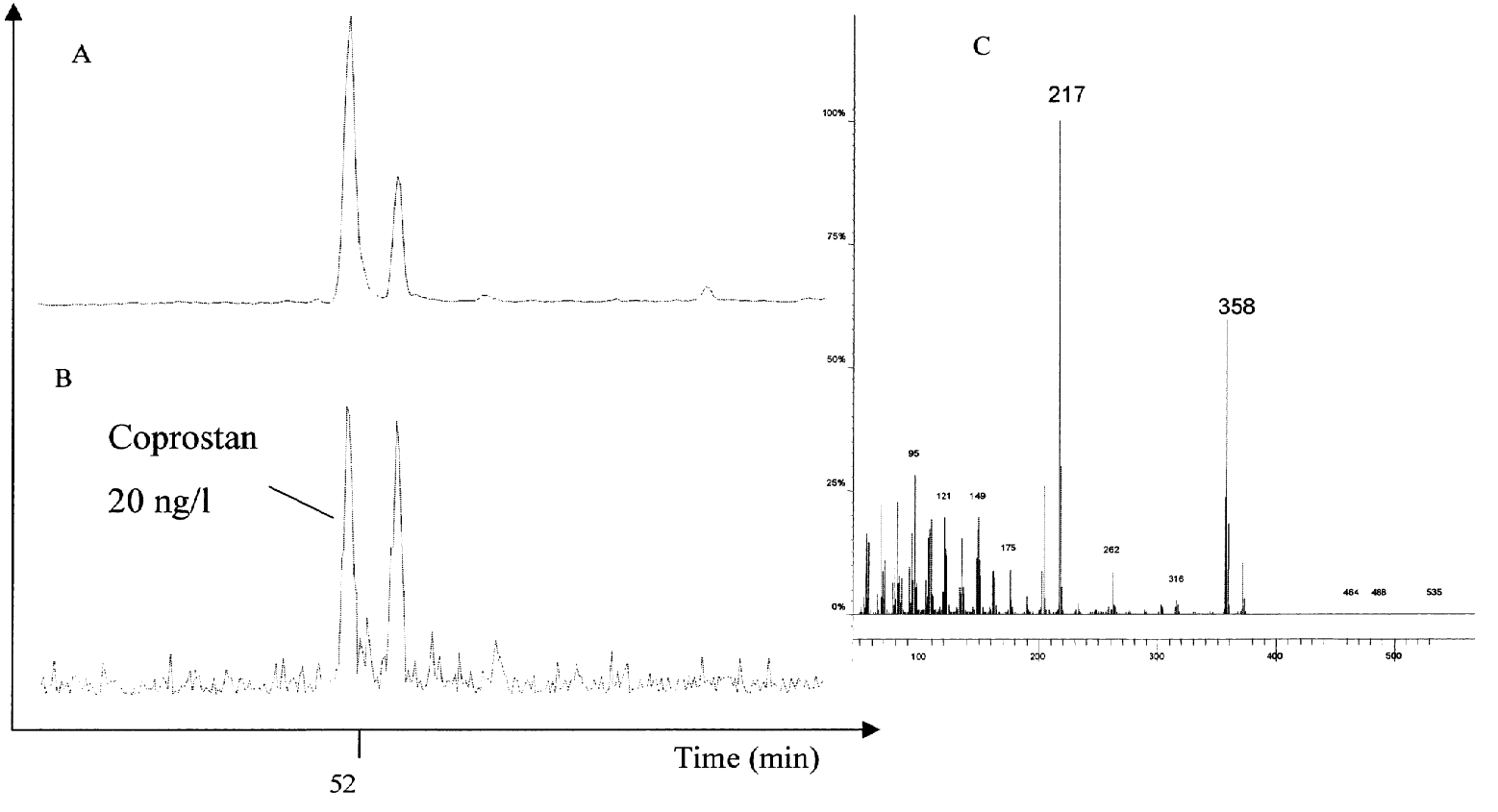

Fig. 3. Identification of coprostan in a derivatized river sample extract using LVI–GC–MS (40 μl). (A) GC–MS chromatogram in TIC mode; (B) reconstructed ion chromatogram (m/z: 217+358); (C) identification of coprostan by its mass spectra.

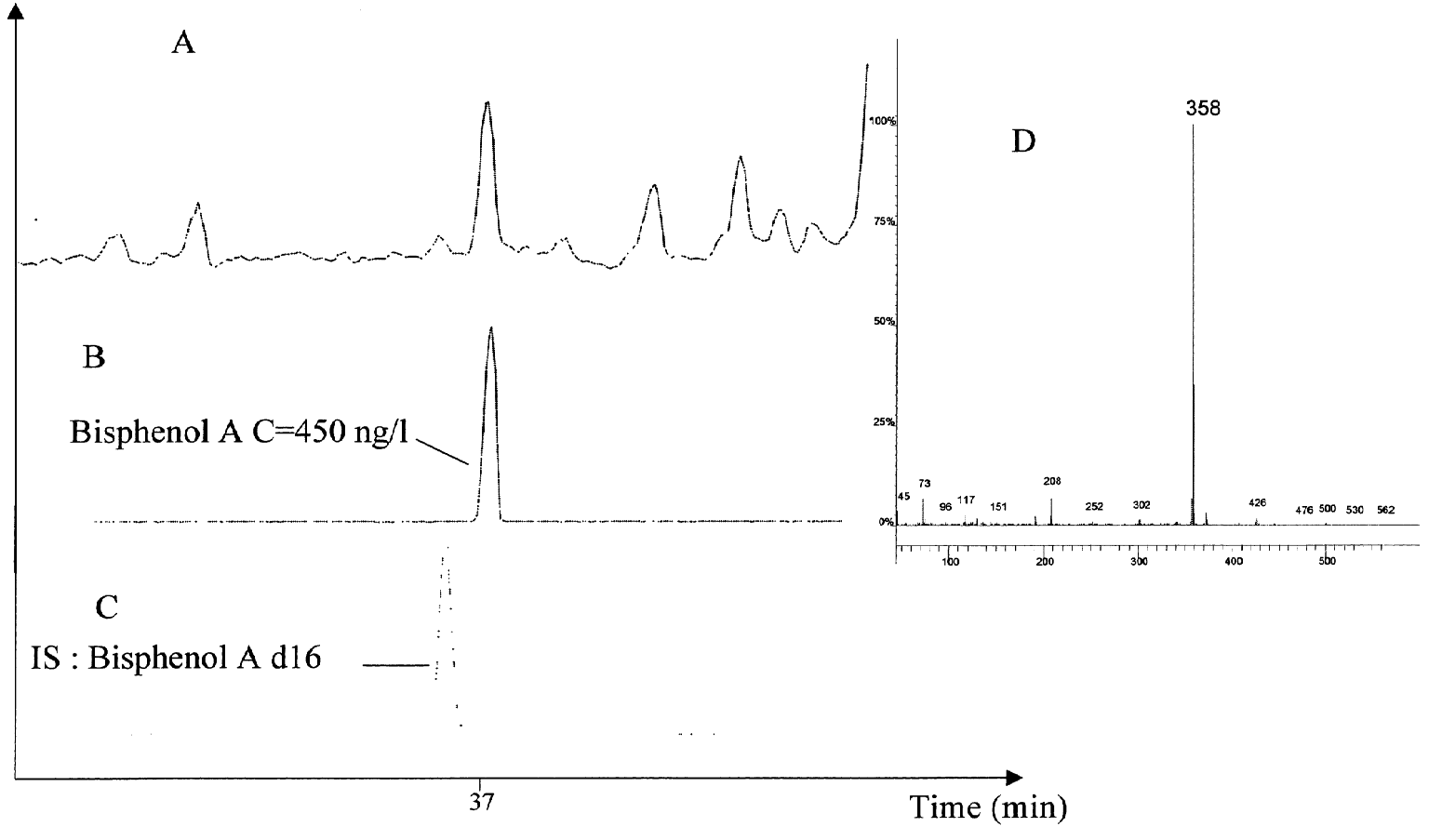

Fig. 4. Identification of bisphenol A in a derivatized domestic wastewater extract using LVI–GC–MS (40 μl). (A) GC–MS chromatogram in TIC mode; (B) reconstructed chromatogram for m/z 358, bisphenol A; (C) reconstructed chromatogram for m/z 369, bisphenol A-d_{16}; (D) identification of bisphenol A by its mass spectra.

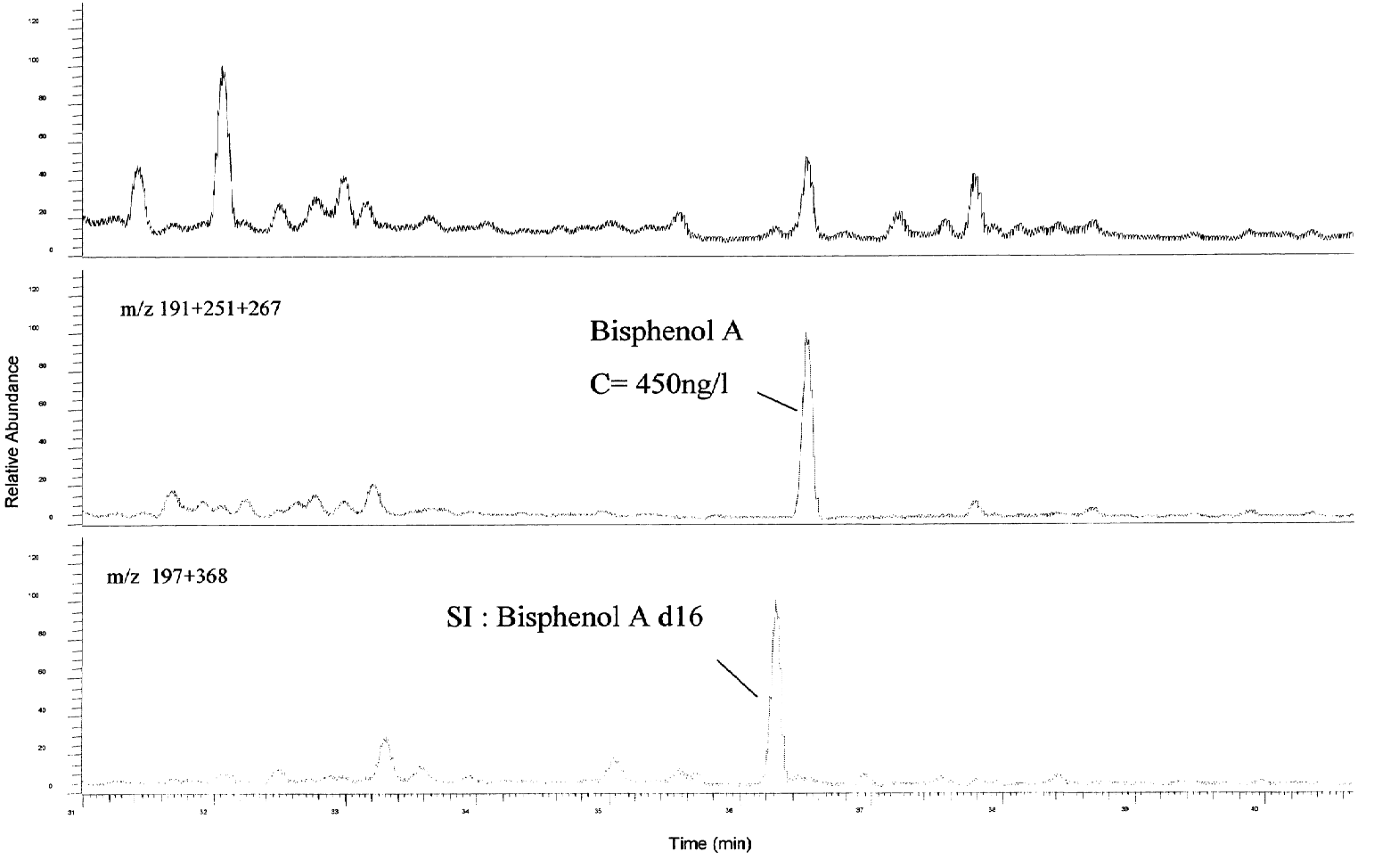

Fig. 5. Confirmation by GC–MS–MS of bisphenol A in a derivatized extract from a wastewater sample.

wastewater from a domestic wastewater treatment plant. The filtered sample was spiked at 20 μg/l level then extracted by SPE on C_{18} cartridges, followed by LC–MS analysis with 20 μl injection as described previously.

For sludge samples, recoveries were performed by extracting, again in five replicates, the same compounds from 10 g of sludge sample that had been spiked at 200 ng/g level and extracted by the soxtec technique, followed by LC–MS analysis with 20 μl injection as described previously.

C_{18} cartridges and the Soxtec method gave recoveries in wastewater and in sludge higher than 70% for 4-nonylphenol, 4-octylphenol, $C_{12}EO_n$, $C_{13}EO_n$, $C_{14}EO_n$, $C_{15}EO_n$, $OPEO_n$ and $NPEO_n$ (Table 4).

The recoveries of 4-nonyphenol and 4-octylphenol SPE extraction were better with C_{18} cartridges (98 and 102%) than with polymeric sorbent Oasis HLB (25 and 50%). Nevertheless, polymeric sorbent was selected for the extraction of steroids and sterols because of its better performances with these compounds.

3.4. Environmental levels

GC–MS, GC–MS–MS and LC–MS with ESI in both the negative and positive ionization mode were applied to the analysis of the target compounds in surface water, wastewater and sludge samples.

Figs. 3–6 show the GC–MS and GC–MS–MS chromatograms obtained from surface water and wastewater samples. As can be seen in Fig. 3, based on retention time and mass spectra, coprostan was determined in the surface water downstream from one of the two wastewater effluents at 20 ng/l using GC–MS with large-volume injection. BPA was detected and quantified in a wastewater sample at 450 ng/l using GC–MS in LVI mode (Fig. 4). These results were confirmed by GC–MS–MS for BPA with ions m/z 191, 267, 357 produced from the parent ion m/z 357 (Fig. 5), and for coprostan with daughter ions m/z 121, 147, 161 produced from the parent ion m/z 217 (Fig. 6). The S/N ratio in GC–MS–MS and GC–MS for coprostan, and for BPA in environmental samples, demonstrates the high degree of sensitivity and the specificity of both

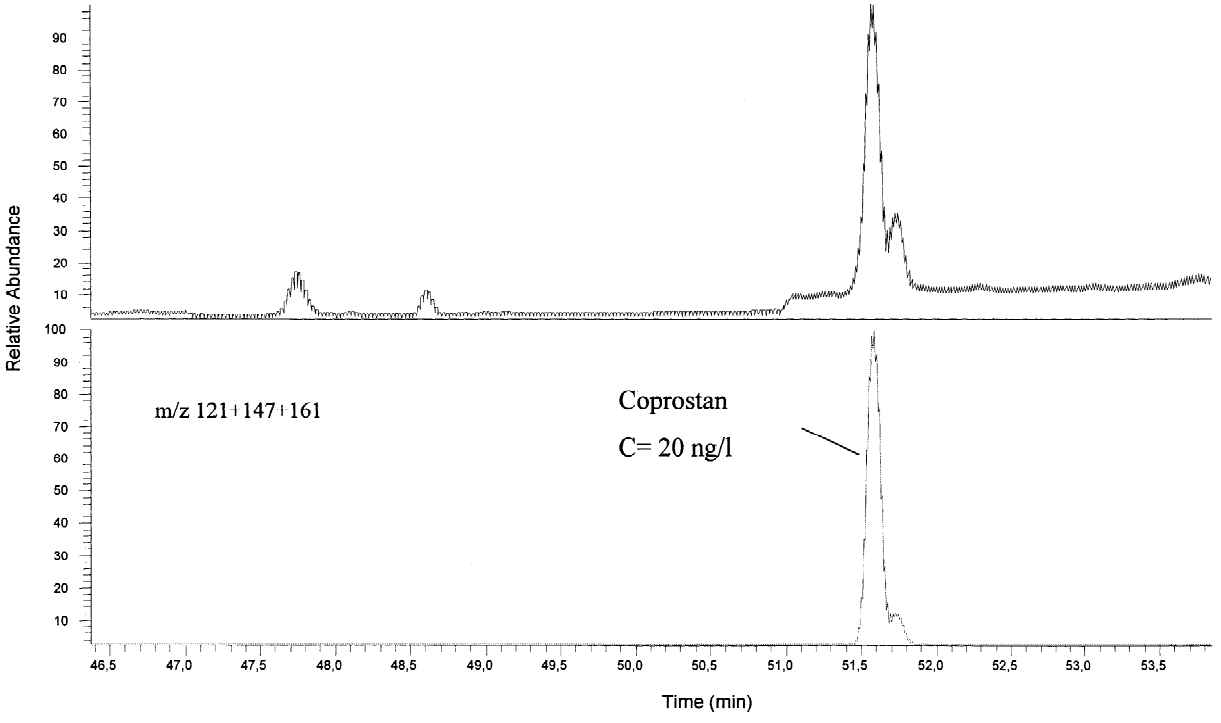

Fig. 6. Confirmation by GC–MS–MS of coprostan in a derivatized river sample extract.

analytical techniques. Fig. 7 shows the analysis of $NPEO_n$ and AEOs in a sludge extract obtained by Soxtec extraction. The LC–MS in positive ionization mode enabled the identification and quantification of (a) $NPEO_n$ and $OPEO_n$ in wastewater at 31 and 138 μg/l, respectively, (b) $NPEO_n$ in sludge at a concentration level of 1.3 μg/g, and (c) $C_{12}EOs$, $C_{13}EOs$, $C_{14}EOs$, and $C_{15}EOs$ in sludge at concentration levels of 2.8, 4.1, 5.4, 8.5 μg/g, respectively. LC–MS in the negative ionization mode enabled the determination of 4-NP in sludge at 1.5 μg/g; 4-OP was not detected. The results for en-

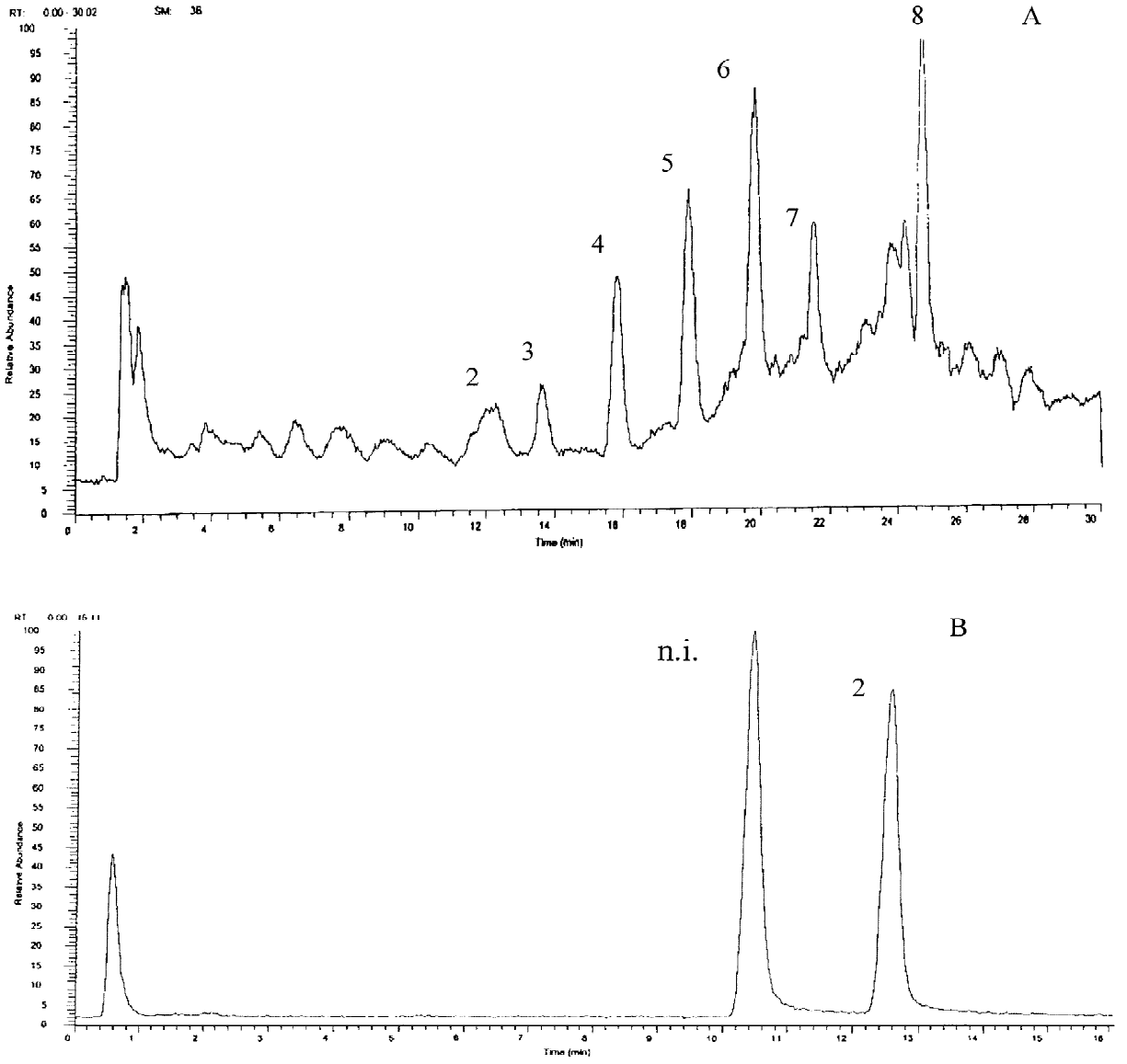

Fig. 7. (A) TIC chromatogram from LC–ESI–MS analysis of a Soxtec-extracted sludge sample in methanol: 1 = not detected, 2 = $NPEO_n$ (1.3 μg/g), 3 = $C_{12}EO_n$ (2.8 μg/g), 4 = $C_{13}EO_n$ (4.1 μg/g), 5 = $C_{14}EO_n$ (5.4 μg/g), 6 = $C_{15}EO_n$ (8.5 μg/g), 7 = $C_{16}EO_n$ (not quantified), 8 = $C_{19}EO_n$ (not quantified). (B) SIM chromatogram from LC–ESI^-–MS analysis of a Soxtec-extracted sludge sample in methanol: 1 = not detected, 2 = 4-nonylphenol (1.5 μg/g), n.i. = not identified.

vironmental samples agree with those cited in the literature for similar matrices [34,35].

4. Conclusion

Endocrine-disrupting compounds (EDC) ($OPEO_n$, $NPEO_n$, AEOs, 4-NP, 4-OP, 4-*t*-OP, BPA, steroids and sterols) can be determined in environmental samples using GC–MS, GC–MS–MS and LC–MS. LC–MS combined with the SPE technique using cartridges filled with C_{18} and Soxtec extraction enables non-ionic surfactants to be determined in water and sludge samples at ppt and ppb levels, respectively. LC–MS in the positive ionization mode enables the detection of $OPEO_n$, $NPEO_n$, $C_{12}EO_n$, $C_{13}EO_n$, $C_{14}EO_n$, and $C_{15}EO_n$, whereas ESI in the negative mode enables the detection of alkylphenols. GC–MS combined with large-volume injection (40 μl) and SPE Oasis HLB can be used to determine 4-NP, 4-*t*-OP, BPA, steroids and sterols, after derivatization with a BSTFA agent, at ppt levels in water samples. GC–MS–MS with a split–splitless injector mode (2 μl) shows a sensitivity similar to that of GC–MS with large-volume injection (40 μl) for the studied compounds. $NPEO_n$, 4-NP, and BPA have all been detected in water and sludge samples at high concentrations. The performances of these methods will make it possible to determine various contaminants in environmental samples at concentration levels that can induce endocrine effects.

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