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Analytical methodology for the identification of estrogenic contaminants in fish bile

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Abstract

Effluents from wastewater treatment works (WwTWs) contain estrogenic contaminants that can cause feminised responses in fish. In order to assess the identity of estrogenic contaminants taken up by fish exposed to effluents, an analytical method was developed to detect estrogenic substances in fish bile, where many xenobiotics are excreted and concentrated. Estrogenic metabolites in bile were deconjugated using enzymatic hydrolysis and the estrogenic activity was determined using a yeast estrogen receptor transcription screen (YES). Hydrolysed samples were concentrated by solid-phase extraction (SPE) prior to fractionation by reversed-phase high-performance liquid chromatography (HPLC). Active HPLC fractions were detected by YES assay and analysed by gas chromatography–mass spectrometry (GC–MS) after trimethylsilylation. The method was validated using bile samples from immature female rainbow trout, which had been exposed to either tap water or an undiluted estrogenic effluent for 10 days. Hydrolysis of bile from effluent-exposed fish was complete within 16 h and most of the estrogenic activity in the bile was released by β-glucuronidase rather than sulfatase or β-glucosidase treatment. The estrogenic activity of hydrolysed bile from effluent-exposed fish ranged between 530 and 1440 ng E2eq/mL and was 17–48-fold greater than the activity of bile from reference fish exposed to tap water. The estrogenic activity of bile samples decreased with time in storage (at −70 °C by 7% per month). The recovery of estrogenic activity from SPE was 96 ± 7% (mean ± SD), from HPLC fractionation 87 ± 7% and for the whole method 81 ± 7% ($n = 7$). 17β-Estradiol, estrone, 17α-ethinylestradiol, nonylphenol and short-chain nonylphenol polyethoxylates were all identified from GC–MS analysis of active HPLC fractions of bile from effluent-exposed trout, whereas only 17β-estradiol was detected in bile from fish exposed to tap water. There were also several other minor estrogenic components, at present unidentified, in bile of effluent-exposed fish. The work shows that fractionation of fish bile is a useful approach to identifying mixtures of estrogenic contaminants taken up by fish from WwTW effluents and has the potential for application in the detection of other endocrine disrupting chemicals in fish tissues.

Keywords: Bile; HPLC; GC–MS; Estrogen; Effluent; Nonylphenol

1. Introduction

Effluents from UK wastewater treatment works (WwTWs) were first reported to be estrogenic to fish in 1994 [1], and since then estrogenic contaminants have shown to be widespread in effluents discharging into rivers in England and Wales as well as in Europe and the USA [2–5]. The major estrogenic components in WwTWs effluents have been identified as the natural estrogens 17β-estradiol (E2) and estrone (E1) and the synthetic estrogen 17α-ethinylestradiol (EE2) [6]. In addition, estrogen mimics such as alkylphenols, short-chain alkylphenol polyethoxylates and bisphenol A have also been detected in WwTWs receiving industrial inputs [7,8]. Estrogenic effluents are suspected to be responsible for the high incidence of intersex fish documented in some native fish populations and there is concern about the reproductive capabilities of affected fish [9–11]. However, no conclusive association has been proven between environmentally relevant concentrations of any of the estrogenic contam-

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inants in effluents and the induction of intersexuality in wild fish. For vitellogenin induction, a biomarker response for estrogen exposure, steroid estrogens and steroid estrogens in combination with xenoestrogens have been shown to induce additive effects [12,13]. It is possible that mixtures of estrogenic contaminants accumulate in fish tissues that can act additively to cause intersex. Although there has been much work analysing concentrations of estrogenic compounds in effluents we know very little about the nature of mixtures of estrogenic contaminants in fish tissues [6,14–16]. A wide variety of classes of compounds have been shown to be weakly estrogenic [17], and as typical effluents are complex mixtures which contain many thousands of chemicals from numerous domestic and industrial sources, it is important to identify the most relevant mixture of environmental estrogens that may accumulate to a high degree in fish.

The aim of this study was to develop an analytical method to isolate and identify the estrogenic components present in fish bile. Recent studies have shown that many substances, including estrogens and alkylphenols, concentrate as glucuronide and sulfate conjugates in the fish bile prior to excretion [18–21]. Analysis of bile fluid may be a good indicator of recent exposure to trace amounts of environmental estrogens and to investigate this, fish were exposed to a mixture of estrogenic substances contained within an effluent and their uptake investigated primarily by isolation of contaminants from bile. Bile samples were deconjugated, and estrogenic substances extracted on an OASIS HLB cartridge. The bile extracts were fractionated by reversed-phase liquid chromatography and the individual fractions analysed for estrogenic activity using a yeast estrogen screen (YES) assay. GC–MS was used to identify components present in estrogenically active fractions.

2. Experimental

2.1. Chemicals

E1, E2, EE2, technical 4-nonylphenol (NP), bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), β-glucuronidase (type VII-A extracted from *Escherichia coli*), β-glucosidase (type H-1 extracted from almonds), sulfatase (type VI from *Aerobacter aerogenes*) and all other chemicals were obtained from Sigma-Aldrich (Poole, UK). [2,4,16,16-$^4H_2$]E1 (E1-d_4), [2,4,16,16-$^4H_2$]E2 (E2-d_4) and [2,4,16,16-$^4H_2$]EE2 (EE2-d_4) (isotope purity 96%, chemical purity >98%) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). All solvents were of HPLC-grade purchased from Rathburn Chemicals (Walkerburn, UK).

2.2. Exposure of fish to WwTW effluent

Immature (1–2-year-old) female rainbow trout (*Oncorhynchus mykiss*), 250–320 g body weight, were obtained from Houghton Springs Farm, Dorset. Trout were exposed for 10 days in continuous flow through tanks containing either tap water or effluent from a WwTW. The WwTW site chosen for the study received an influent load of 138,000 population equivalents, and was primarily domestic, with only 6% of the load from industrial sources. The influent had been subjected to primary treatment, activated sludge and trickling filter secondary treatments. The final WwTW effluent has been shown to be estrogenic, and the estrogenic contaminants in the effluent have been well characterised from previous studies [14]. The volume of tanks containing the fish was approximately 1 m^3 and flow rate approximately 3–5 L/min. Fish were fed once daily with commercial trout pellets until 48 h prior to sampling when food was withheld to increase the bile volume. At the end of exposure the fish were sacrificed, and the gall bladder removed and stored immediately in dry ice then at −70 °C on return to the laboratory. All glassware used for sample storage and subsequent analysis was washed with detergent, thoroughly rinsed with water, washed with acetone, then baked at 500 °C for 2 h before use.

2.3. Enzymatic hydrolysis of estrogenic contaminants in the bile samples

Lyophilised enzyme material was dissolved in HPLC grade water to the following activities; β-glucuronidase 1000 units/mL, sulfatase 2 units/mL, and β-glucosidase 20 units/mL. The enzymes (200 μL of each solution) were added to 0.1 M phosphate buffer at pH 6.0 (1500 μL) and water (800 μL) containing bile (100 μL). A pH of 6.0 was found to give sufficient activity for all three enzymes. The solution was incubated for up to 16 h at 37 °C, then 300 μL of glacial acetic acid added. An aliquot of each hydrolysed sample was kept aside for direct determination of the total estrogenic activity and the remainder concentrated by solid-phase extraction. The activity and specificity of the individual enzymes was monitored separately by incubation with standard substrates (10 μg in 100 μL water) of nitrophenol glucuronide, nitrophenol sulfate and salicin. Deconjugation of the standards was determined by following the formation of 4-nitrophenol and 2-hydroxymethylphenol by HPLC with ultraviolet detection at 280 nm [22].

2.4. Solid-phase extraction of hydrolysed estrogenic components from bile

The hydrolysed bile was diluted with water (2 mL) and passed through an OASIS HLB cartridge (200 mg; Waters), which had been conditioned with methanol (5 mL) followed by water acidified with 1% acetic acid (5 mL). After sample loading, the cartridge was washed with water (2 mL), dried under vacuum, and the bile extract eluted with methanol (5 mL), ethyl acetate (3 mL) and hexane (3 mL). The ethyl acetate and hexane fractions were combined, evaporated to dryness, reconstituted in 500 μL of methanol then combined with the initial methanol fraction. The combined

methanol was gently evaporated under nitrogen to approximately 60 μL, and 60 μL of water then added prior to HPLC fractionation.

2.5. HPLC fractionation

Samples were fractionated on a Waters HPLC system comprising a model 600 pump and controller, model 717 autosampler and model 996 photodiode array detector. The following standards were used to monitor HPLC separation; 6-α-hydroxyestradiol, β-estriol, 16-α-hydroxyestrone, bisphenol-A, E2, EE2, E1, and technical NP. An aliquot of the sample (100 μL) was injected onto a Novapak C_{18} column (5 μm particle size; 250 mm × 4.6 mm; Waters). Mobile phase solvents were water acidified with 0.2% acetic acid (A) and acetonitrile (B) in an initial ratio (A:B) of 69:31. Separation was achieved at room temperature using a flow rate of 1.0 mL/min with the following gradient programme: 0 min (69:31); 35 min (65:35); 50 min (0:100); 60 min (0:100). Fractions were collected at 1 min intervals.

2.6. YES bioassay

The estrogenic activities of hydrolysed bile samples and HPLC fractions were determined by a YES assay. This bioassay has been validated in the detection of a wide range of estrogen receptor agonists including E2, E1, EE2 as well as xenoestrogens such as alkylphenols, alkylphenol polyethoxylates and bisphenol A [23,24]. Briefly, the human estrogen receptor (hER) gene has been stably integrated into the yeast genome together with an expression plasmid containing the estrogen-response element (ERE), which controls expression of the reporter gene Lac-Z (encoding the enzyme β-galactosidase). Activation of the receptor hER, by binding of a ligand, causes binding to the ERE and consequently the production of β-galactosidase. The enzyme is then secreted into the medium and metabolises the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG) (normally yellow) into a chlorophenol that can be measured by absorbance at 540 nm.

Samples of bile extracts and blanks in either ethanol or methanol were serially diluted with ethanol, and 20 μL volumes transferred to 96-well flat-bottomed multi-well plates. The ethanol was evaporated at room temperature followed by the addition of yeast and assay medium containing the chromogenic substrate to the wells, and the plates incubated for 3–5 days. The absorbance of each sample at 540 nm was determined after subtraction of absorbance at 620 nm to correct for differences in yeast growth. The estrogenic activity of the bile sample was determined by comparing the linear range of concentration–response curve of the sample with that of an E2 standard curve included on each plate. The estrogenicity of each sample was expressed in terms of ng E2 equivalents/mL (ng E2eq/mL) of bile. In agreement with published values [23,24] the median effect concentration for E2 was typically around 100 pM.

2.7. GC–MS

The identities of E1, E2, EE2, and NP components were determined by GC–MS after derivatisation to their trimethylsilyl ethers. Target HPLC fractions were evaporated to dryness, 20 μL of pyridine and 20 μL of BSTFA added and the sample heated for 15 min at 65 °C. The analytical instrument used was an HP 5890 gas chromatograph, fitted with a 30 m HP5-MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness), and connected to a Kratos MS80 mass selective detector. The carrier gas was helium at a constant pressure of 51.7 kPa, injection port temperature was 250 °C and GC interface temperature was 280 °C. The sample (1 μL) was introduced using a splitless injection. The MS detector was used in selected ion mode (SIM) for analysis of E1, E2, and EE2 and in full scan mode for the determination of nonylphenols and nonylphenol polyethoxylates. The source temperature was 280 °C with electron energy of 70 eV.

The oven temperature programme was as follows: 100 °C for 1 min, 10 °C/min to 280 °C, 280 °C for 11 min. The ions monitored were **342.1**, 257.1 (E1); **416.2**, 285.1 (E2); **425.2**, 440.2 (EE2) and for deuterated internal standards **346.1**, 261.1 (E1-d_4), **420.2**, 287.1 (E2-d_4); **429.2**, 444.2 (EE2-d_4); the ions marked in bold were used for quantification and the others for confirmation. Full scan data was used for quantification of branched *para*-alkyl isomers of NP by comparison with technical NP standard using an external calibration method. Calibration standards for E1, E2, and EE2 covered the concentration range 0.1–10 ng/μL; for NP the range was 1–20 ng/μL

3. Results and discussion

3.1. Hydrolysis of bile samples

The rate of deconjugation of estrogenic contaminants in bile samples isolated from effluent-exposed fish was determined by incubation with a mixture of three hydrolytic enzymes, β-glucuronidase, sulfatase and β-glycosidase for up to 24 h. A mixture of three hydrolytic enzymes was used to deconjugate contaminants in the bile as both glucuronide and sulfate conjugates of xenobiotics are reported to be formed in fish, and glucose as well as glucuronide conjugates of estrogenic alkylphenols have been detected in the cyprinid fish *Scardinius erythrophthalmus* [19]. Aliquots of the incubating solution were analysed for estrogenic activity by the YES assay and the data showed that although the rate of deconjugation varied considerably between bile samples, it was complete for all samples by 16 h (Fig. 1). Thereafter, the incubation time allowed for deconjugation of contaminants in the bile samples was a minimum of 16 h. The nature of the conjugation was determined by incubation of bile samples with individual enzymes (Table 1). Previous studies with standards had shown that the enzymes were specific and showed no

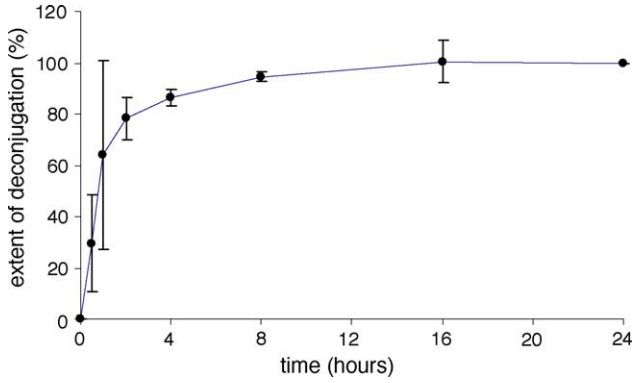

Fig. 1. The effect of the incubation time with a mixture of hydrolysis enzymes (β-glucuronidase, sulfatase and β-glucosidase) on the release of estrogenic activity from bile of effluent-exposed rainbow trout. Results are expressed as a percentage of the value at 24 h and are a mean ± SD of three bile samples.

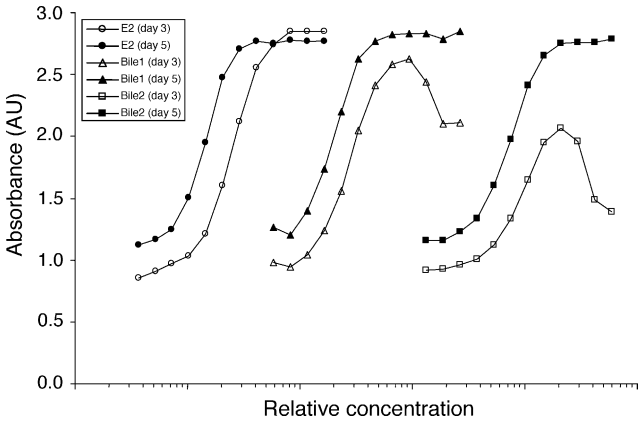

Fig. 2. The relative response in the yeast estrogen screen (YES) for 17β-estradiol and two (hydrolysed) bile samples of rainbow trout exposed to effluent: comparison of the YES response after 3 and 5 days of incubation in the assay. The volume of 17β-estradiol starts at 20 μL of a 1×10^{-7} M solution and the volume of the bile extract starts at 20 μL. Successive points are a twofold dilution of the previous point. The *x*-axis is a log scale.

cross activity between the three hydrolytic activities [22]. A small proportion of the estrogenic activity in trout bile (23% of the total activity of hydrolysed bile) was released by incubation in acidic buffer alone, and there was no further increase in estrogenic activity of the bile when hydrolysed by sulfatase or β-glucosidase enzymes. The majority of estrogenic activity was released after incubation with β-glucuronidase enzyme confirming that most of the estrogenic residues in trout bile were in the form of inactive glucuronide conjugates.

3.2. Measurement of the estrogenic activity of trout bile in the YES assay

It was noticed that response curves for the estrogenic activity of many hydrolysed bile samples differed when compared with the standard curve of E2. After a 3-day incubation period in the YES assay, which is the incubation time routinely used to assay the activity of single compounds, incomplete concentration–response curves were obtained for the majority of the hydrolysed bile samples, which was in contrast to the complete response curve obtained for the E2 standard (see Fig. 2). However, a full response curve was obtained for all the bile samples after further 2 days incubation. The suppressed responses at the lower dilutions of bile samples resulted in a difference of up to 40% in the measured estrogenic activity at day 3 compared to day 5, and therefore all samples were quantified after incubation in the YES assay for 5 days. This difference in the YES assay response between the two incubations times could be due to the presence of anti-estrogenic or other chemicals in the bile sample which either interfered with the estrogen receptor–ligand interaction or other elements of the yeast response. The capacity of yeast to metabolise xenobiotics is limited [25] but it is possible that after 5 days incubation of the bile sample, the concentrations of any anti-estrogenic or other chemicals in the bile mixture were reduced by abiotic transformations, thus allowing a full estrogenic response in the YES assay.

The estrogenic activity of hydrolysed bile from trout exposed to WwTW effluent ranged from 530 to 1440 ng E2eq/mL (mean ± SD: 820 ± 363 ng E2eq/mL, $n = 5$). The activity of bile from reference trout exposed to tap water was 27-fold lower, between 12 and 55 ng E2eq/mL bile (mean ± SD: 30 ± 20 ng E2eq/mL, $n = 5$). The limit of detection (LOD) of the YES assay, determined from three times the SD of the baseline noise, was 1.0 ng E2eq/mL. The activity of blank samples, comprising the incubation buffer and hydrolytic enzymes, was less than the LOD of the YES assay.

3.3. Solid-phase extraction of bile samples

The hydrolysed bile samples were concentrated on the OASIS cartridge prior to HPLC analysis and aliquots of the sample eluting from the OASIS cartridges in methanol, ethyl acetate and hexane were analysed by YES assay. The recovery of estrogenic activity from the deconjugated bile of fish exposed to effluent on the OASIS cartridge was 96 ± 7% (Table 2). Almost all of the activity was associated with the

Table 1
The estrogenic activity of bile from effluent-exposed rainbow trout after deconjugation with individual enzymes

Sex of fish	Number of samples	Estrogenic activity of bile, mean ± SD[a]			
		Buffer only	Sulfatase	β-Glucosidase	β-Glucuronidase
Immature female	3	23 ± 10	24 ± 10	26 ± 10	101 ± 8

[a] Results expressed as a percentage of the estrogenic activity of the bile, measured by YES assay, after hydrolysis with a combination of all three enzymes.

Table 2
Recoveries for different stages of the isolation of estrogenic contaminants from trout bile

Stage	Number of samples	Recovery (%) (±SD)
SPE isolation on OASIS	10	96 ± 7
Evaporation and addition of methanol: water (50:50) prior to HPLC	7	97 ± 7
Recovery from HPLC fractionation	10	87 ± 7
Total recovery from both SPE and HPLC procedures	7	81 ± 7

methanol fraction, less than 2% being associated in the ethyl acetate or hexane elutions (data not shown). The recovery for the final evaporation of methanol to a small volume (60 μL) and addition of water (60 μL) prior to HPLC analysis was 97 ± 7% (Table 2). Care was taken to avoid complete evaporation of methanol as this led to some loss of estrogenic activity, due either to volatility or incomplete resolubilisation of some of the estrogenic components.

3.4. HPLC fractionation of extracts of hydrolysed bile

A reversed phase liquid chromatography programme was developed to separate E1, E2, and EE2 as well as other xenoestrogens (Fig. 3). A gentle solvent gradient was required to effectively separate E1, E2, and EE2. All sixty fractions produced from HPLC separation of bile extracts were screened for estrogenic activity by analysis of 20 μL of solution (from 1.0 mL total) in duplicate using the YES assay. Any active fractions that were too estrogenic for quantification were diluted and re-analysed.

The HPLC-UV chromatogram of bile extracts from effluent-exposed trout contained additional peaks not detected in the chromatogram of bile extracts from reference fish., including compounds eluting at 36, 37 and 56 min (Fig. 4a and b). When bile extracts from reference trout were fractionated on HPLC and the fractions analysed by YES assay, only one major fraction (retention time 27 min) with estrogenic activity was observed (Fig. 5a). The retention time

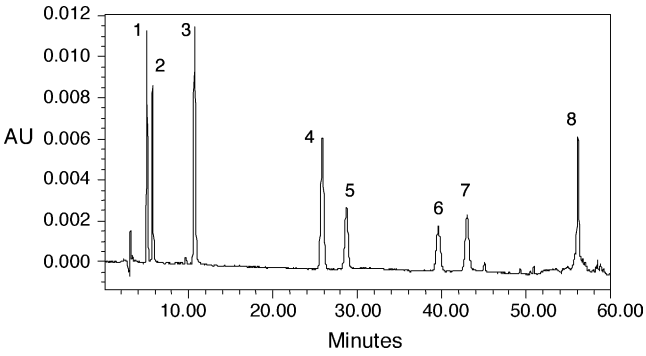

Fig. 3. Reverse phase HPLC-UV (280 nm) chromatogram of the separation of a mixture (250 ng) of estrogens and xenoestrogens: 6-α-hydroxyestradiol (1), β-estriol (2), 16-α-hydroxyestrone (3), bisphenol-A (4), 17β-estradiol (5), ethinylestradiol (6), estrone (7), and technical nonylphenol (8).

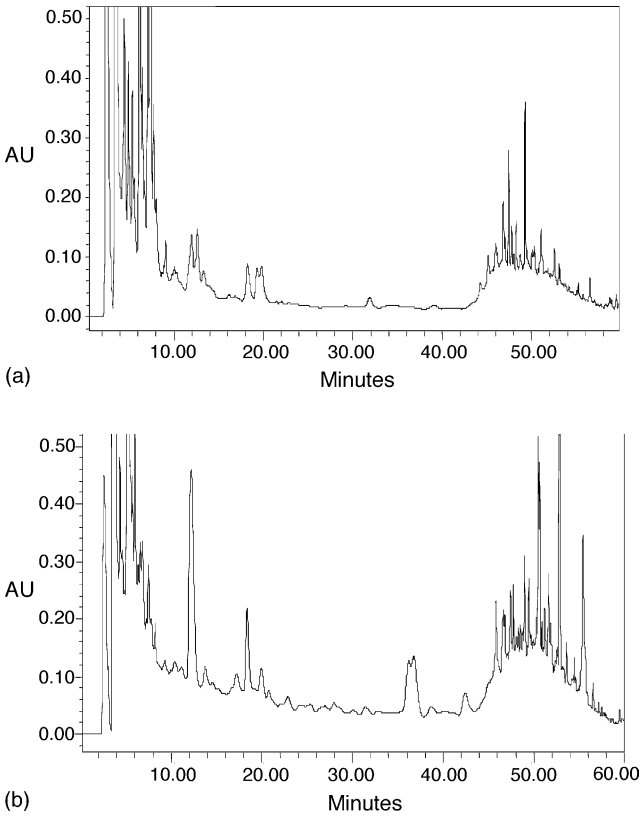

Fig. 4. Representative HPLC-UV profiles of extracts of 100 μL bile of juvenile rainbow trout (a) from trout exposed to tap water, (b) from trout exposed to WwTW effluent; detection by UV at 280 nm.

of the estrogenic fraction on HPLC corresponded with that of the E2 standard when analysed immediately after the bile sample, and the presence of E2 in the estrogenic fraction was confirmed by GC–MS analysis. There were a number of estrogenic fractions in the bile extracts from effluent-exposed fish (Fig. 5b). The fractions eluting at 28–29 min, 39–40 min and 42–43 min corresponded to retention times of E2, EE2 and E1, respectively. The concentrations of E1 and EE2 in their respective fractions were determined by comparing the response of standard E1 and EE2 to that of E2 in the YES assay and also by quantitative GC–MS analysis of an aliquot of each fraction. A comparison of the concentrations of the steroids by GC–MS and YES analysis confirmed that the concentrations of E2, E1 and EE2 most likely accounted for all of the estrogenic activity in their respective fractions (Table 3). Clusters of branched-chain nonyl isomers corresponding to technical NP, as well as short-chain NP ethoxylates containing between 1 and 4 ethoxy units ($NP_{1–4}EO$), were detected from GC–MS analysis of the HPLC fraction eluting at 56 min (Fig. 6). However, most of the estrogenic activity of this fraction could be attributed to NP, as in the YES assay short-chain NPEOs are 10-fold less active than the parent alkylphenol [24]. Quantitation of NP by GC–MS also revealed that the majority of the estrogenic activity in this fraction could be attributed to NP rather than the NPEOs (see Table 3). In addition to the presence of estrogenic steroids and NP/NPEOs

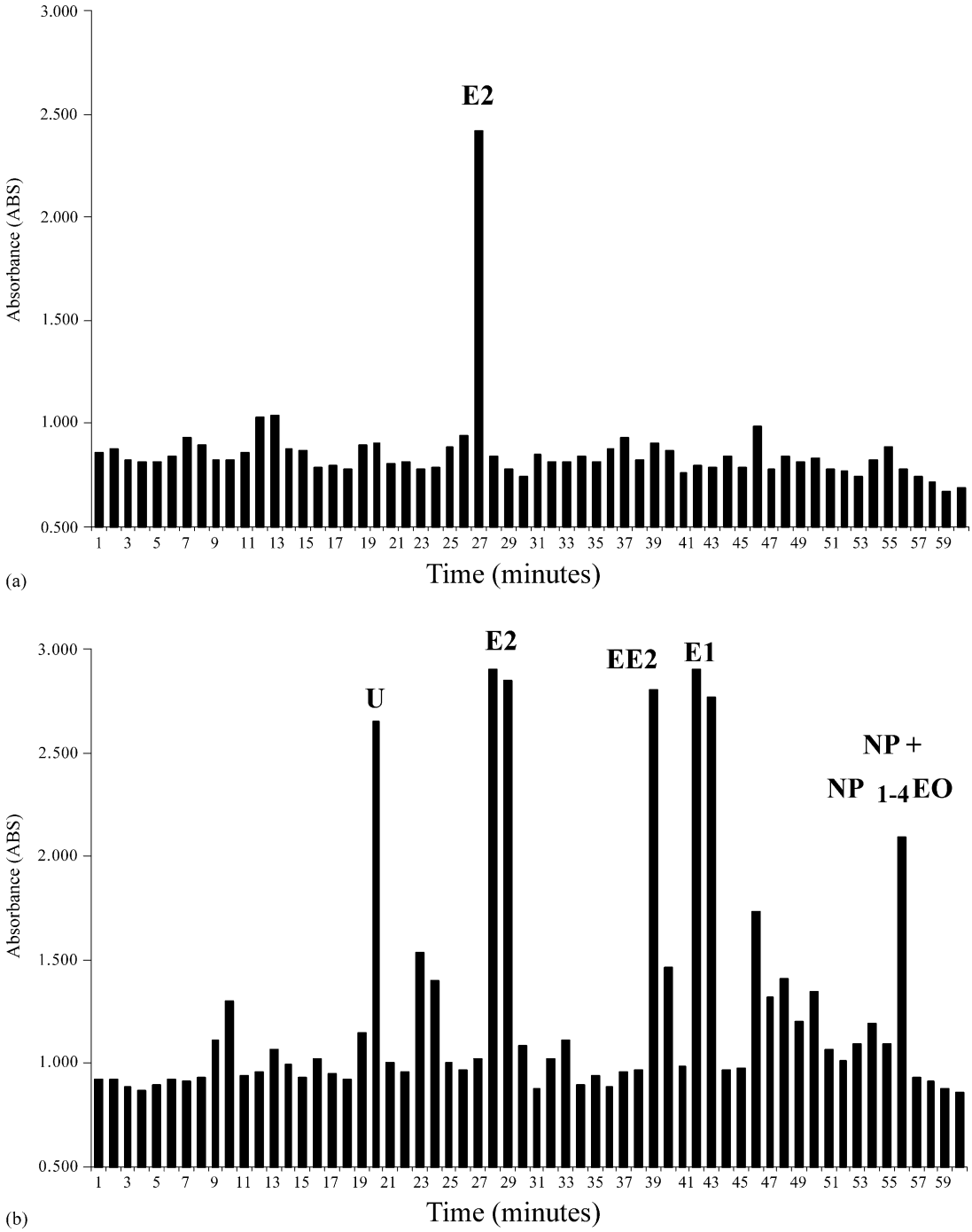

Fig. 5. Representative HPLC profiles of the estrogenic activity of extracts of 100 μL bile of juvenile rainbow trout (a) from trout exposed to tap water, (b) from trout exposed to WwTW effluent; detection by measuring the response (absorbance) of 20 μL of each HPLC fraction in the YES assay. U: unidentified component.

in bile of effluent- exposed fish, several other estrogenic fractions were detected, the most prominent of which eluted after 19–20 min on HPLC (labelled U in Fig. 5b). The identity of the estrogenic substances in these fractions is currently being investigated.

Quantitation by GC–MS of the active bile fractions revealed that the most predominant estrogenic contaminants in terms of concentration were NP (43.0 ± 6.7 μg/mL) followed by E1 (1.1 ± 0.4 μg/mL), E2 (342 ± 81 ng/mL) and EE2 (17 ± 4 ng/mL) (Table 3). The concentrations of the steroids, E2, E1 and EE2, in 100 μL bile would give a response on HPLC-UV of less than 0.005 AU at 280 nm and would not be detected amongst the other co-eluting components in the bile (Fig. 4b). In contrast, using HPLC-UV analyses, NP (and NPEOs) were easily detected in the bile of effluent-exposed fish reflecting

Table 3
GC–MS analysis of estrogenic HPLC fractions in bile extracts from effluent-exposed trout

HPLC fraction (min)	Identity of estrogenic component (by GC–MS)	Concentration in bile determined by YES assay (ng/mL)[a]	Concentration in bile determined by GC–MS (ng/mL)	Proportion of estrogenic (%) activity in fraction determined by GC–MS
Fractions 28/29	E2			
Sample 1		412	345	84
Sample 2		328	259	79
Sample 3		513	421	82
Mean ± SD E2		418 ± 93	342 ± 81	82 ± 3
Fractions 39/40	EE2			
Sample 1		20	21	103
Sample 2		12	13	108
Sample 3		21	17	81
Mean ± SD EE2		18 ± 5	17 ± 4	98 ± 15
Fractions 42/43	E1			
Sample 1		1012	966	95
Sample 2		883	779	88
Sample 3		1674	1739	104
Sample 4		1030	971	94
Mean ± SD E1		1150 ± 356	1114 ± 426	95 ± 7
Fraction 56	NP, $NP_{1–4}EO$			
Sample 1		40,897	37,953	93
Sample 2		47,959	40,508	84
Sample 3		50,312	50,655	101
Mean ± SD NP		46,389 ± 4900[b]	43,039 ± 6719[b]	93 ± 9[b]

[a] Calculated from the response of standard estrogens, compared with that of E2 in the YES assay: E1 gave a response of 26% of the E2 response, EE2 gave a response of 98% of the E2 response and NP gave a relative response of 4000 times less than E2.
[b] The contribution of $NP_{1–4}EO$s were not included in the calculation of estrogenic activity of the fraction.

the high concentrations of these xenobiotics in the bile samples.

The relative concentrations of estrogenic contaminants in the bile of effluent-exposed fish reflected the relative concentrations of these contaminants in many domestic WwTW effluents including the one used in this study [10]. For instance NP, the most predominant contaminant in the bile, has been identified in domestic sewage effluents at concentrations between 0.2 and 10 μg/L whereas the steroidal estrogens, E1, E2 and EE2 are present at much lower concentrations of 1–200, 1–100 and 0.1–10 ng/L effluent, respectively [6,15,16].

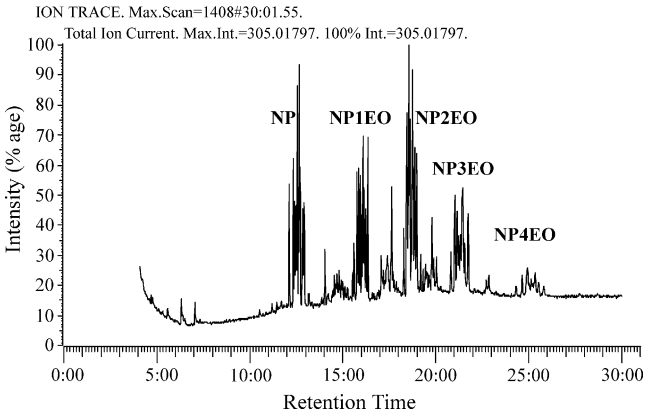

Fig. 6. GC–MS total ion chromatogram of nonylphenol contaminants in bile of rainbow trout exposed to effluent. Bile extracts were fractionated by HPLC and components in an estrogenic fraction eluting at 56 min were derivatised to their trimethylsilyl ethers prior to analysis by mass spectrometry.

When the active bile fractions were quantified in terms of estrogenic activity by YES analysis, E2 and E1 were the most predominant contaminants (418 ± 93 and 299 ± 93 ng E2eq/mL, respectively) whereas NP and EE2 comprised 12 ± 1 and 18 ± 5 ng E2eq/mL of estrogenic activity. EE2 comprised less than 3% of the total estrogenic activity due to its low concentrations in the bile of effluent-exposed fish, however in vivo EE2 has been shown to be between 5- and 66-fold more potent than E2 and so is likely to be a predominant estrogenic contaminant in the fish bile [13,26].

Analysis of the estrogenic activity in HPLC fractions of bile from reference juvenile female trout exposed to tap water revealed that it contained E2 at concentrations of 30 ng/mL, which indicated that although endogenous estrogens are present in bile of juvenile trout bile, they do not contribute significantly to the amounts of E2 detected in the bile of effluent-exposed fish. The use of juvenile rather than sexually mature fish to detect exogenous estrogenic contaminants in the bile is an advantage, as mature fish containing fully developed gonads would synthesise much greater amounts of endogenous estrogens, which could significantly contribute to the estrogenic activity in the bile.

The recoveries from HPLC, calculated as the sum of the individual components compared with the total estrogenic activity of the injected mixture, were greater than 87 ± 7%. The recovery for the whole analytical procedure, from extraction of hydrolysed bile to quantitation of individual compounds after HPLC fractionation was highly acceptable at 81 ± 7% (Table 2).

3.5. Estrogenic activity of bile samples during long-term storage

Bile samples from effluent-exposed fish were stored at −70 °C for up to a year after the initial analysis for estrogenic activity by YES assay. During this time aliquots of bile were periodically hydrolysed and their estrogenic activity determined in the YES assay. The estrogenic activity of five bile samples stored between July 2002 and May 2003 declined with time at an average rate of loss of 7.0 ± 1.2% per month. It is not clear which of the estrogenic components in the bile were degrading, however one possibility is that oxidation of E2 to the less estrogenic E1 occurs during long-term storage of bile samples. The bile should be analysed as soon as possible after collection as even during storage at ultra low temperatures, significant losses of estrogenic components were detected.

4. Conclusions

A method has been developed and validated to detect estrogenic contaminants present in fish using an estrogen receptor transcription screen to assay HPLC fractions of hydrolysed fish bile for estrogenic activity. The method allowed efficient recovery of range of estrogenic contaminants from bile of immature rainbow trout exposed to estrogenic effluents, providing the bile samples were analysed soon after collection. The results indicate that a mixture of steroidal estrogens, E2, E1 and EE2, as well as the nonylphenolics, NP and $NP_{1–4}EO$s were present in bile of effluent-exposed trout whereas only low concentrations of E2 were detected in bile of reference fish exposed to tap water. The high bioconcentration of estrogenic contaminants in trout bile indicates that this method could be used to detect and isolate other potential estrogenic contaminants present in WwTW effluents. In addition, using appropriate receptor screens, this analytical approach could also be used to identify other bioavailable xenobiotics present in effluents, such as environmental (anti-)androgens, that could contribute to endocrine dysfunction in fish.

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