Journal of Chromatography A, 1217 (2010) 112–118

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Gas–liquid chromatography–tandem mass spectrometry methodology for the quantitation of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents and from wild populations

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ARTICLE INFO

Article history:
Received 6 July 2009
Received in revised form 1 October 2009
Accepted 23 October 2009
Available online 30 October 2009

Keywords:
Bile
Fish
Estrogen
GC–MS/MS
Alkylphenol
Bisphenol

ABSTRACT

Fish can be exposed to a complex mixture of chemical contaminants arising from the exposure to wastewater treatment works (WwTWs) effluents. Some of these contaminants are estrogenic and have been associated with feminisation of male fish and the presence of populations containing intersex individuals. However the detection of trace levels (ng/L) of estrogenic chemicals surface waters can be difficult and does not give information on the exposure of aquatic organisms to these contaminants. In this study we assessed whether the analysis of estrogenic substances that bioconcentrate in fish bile can be used to detect the exposure of fish to feminising contaminants in receiving waters and effluents, and thus facilitate their monitoring of these substances in aquatic environments. Estrogenic metabolites in bile were deconjugated using enzymatic hydrolysis and partially purified by solid phase extraction. Steroidal and xenoestrogens were derivatized to their trimethylsilyl ethers and quantified by gas–liquid chromatography–mass spectrometry (GC–MS/MS) using multiple reaction monitoring. The method was validated using spiked bile samples from immature female rainbow trout (*Oncorhynchus mykiss*) as well as bile from sexually mature roach (*Rutilus rutilus*) that had been exposed to either tap water or an undiluted estrogenic effluent for 10 days or captured from a river site downstream of a WwTWs effluent discharge. The mean recovery of target analytes from spiked bile was between 86 and 99% and the limit of detection was between 0.1 and 0.7 ng/mL bile for bisphenol A (BPA), 17β-estradiol (E2), estrone (E1) and 17α-ethinylestradiol (EE2), and 11, 60 and 327 ng/mL bile for branched nonyl chain isomeric mixtures of 4-nonylphenolethoxylate (NP1EO), 4-nonylphenol (NP) and 4-nonylphenoldiethoxylate (NP2EO), respectively. All target analytes were detected in bile from roach exposed directly to a WwTWs effluent, with concentrations between 6–13 μg/mL bile for NP, 18–21 μg/mL for NP1EO, 75–135 μg/mL for NP2EO, 0.7–2.5 μg/mL for BPA, E2 and E1 and 17–29 ng/mL for EE2. With the exception of NP2EO, all analytes were detected in at least 2 out of the 5 fish sampled from the River Thames. BPA and NP1EO were detected in all three reference fish held in tap water indicating possible contamination from laboratory plastics. The work shows that analysis of 20–100 μL quantities of bile could be a useful approach in detecting exposure to mixtures of estrogenic contaminants taken up by fish from WwTW effluents and has the potential for monitoring the efficacy of remediation strategies that may be adopted for reduction of these endocrine disrupting chemicals in the aquatic environment.

1. Introduction

The detection of estrogenic responses in fish exposed to wastewater treatment works (WwTWs) effluents was first reported in the UK in peer-reviewed literature in 1994 [1]. Since then, sexual development has been shown to be affected in wild fish populations exposed to WwTWs effluent discharges in a number of countries, and in both freshwater and estuarine environments [2]. One of the most widely reported feminised phenotypes is the condition of intersex, where both male and female sex tissues are contained within the same gonad, and this has been shown to be as a result of exposure to endocrine disrupting compounds which are introduced into the aquatic environment predominantly from WwTWs effluents and industrial outputs [3]. Fish captured or held below WwTWs discharges biosynthesize high levels of plasma vitellogenin, a biomarker of estrogen exposure, and bioconcentrate estrogenic substances [3,4] which include the naturally

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doi:10.1016/j.chroma.2009.10.063

produced estrogens estrone (E1) and 17β-estradiol (E2), the pharmaceutical estrogens 17α-ethinylestradiol (EE2), equilenin and 17β-dihydroequilenin, and the industrial compounds nonylphenol (NP), nonylphenol ethoxylates (NPEOs) and bisphenol A (BPA) [5–7]. The most potent of these compounds are the steroidal estrogens, and in comparison, the estrogenic activity of non-steroidal phenolics such as NP, NPEOs and BPA are relatively weak [8]. Exposure to EE2, at environmentally relevant concentrations has been shown to induce intersex in roach, a species indigenous to UK, and to cause complete gonadal sex reversal at an exposure to 4 ngEE2/L (a concentration sometimes recorded in the most polluting effluents) [9]. However, there is evidence that mixtures of chemicals with similar modes of action can act in an additive manner indicating the importance of determining the exposure to all the estrogenic contaminants that could bioconcentrate in effluent-exposed fish [10,11].

The estrogens E1 and E2, as well as the birth control pharmaceutical EE2, are excreted by humans as conjugated metabolites. However, during transport and processing at the WwTWs, these metabolites are hydrolysed back to the parent compounds resulting in concentrations in UK final WwTWs effluents ranging from 0.1 to 90 ng/L for E2, 1 to 80 ng/L for E1 and <0.1 to 4.3 ng/L for EE2 [12–16]. NP and NPEOs were widely used surfactants which have now been included on the EU's list of priority pollutants and have restricted use; however, their concentrations in WwTWs effluents been reported at between 0.2 and 230 μg/L [13–15]. BPA is mainly used in the synthesis of polycarbonate plastics and epoxy resins and its concentrations in German WwTWs effluents have been reported to be between 0.01 and 1 μg/L [17,18]. The concentrations of estrogenic contaminants in river water are expected to be orders of magnitude lower, and a recent survey of EU rivers detected E1 in 16% of samples with a mean concentration of 4 ng/L, BPA in 34% of samples with a mean of 25 ng/L, and NP in 29% of samples with a mean of 134 ng/L, however, E2 and EE2 were not detected in any samples [19]. In other studies, E1 has been detected in river water at concentrations of <0.4–12.2 ng/L, E2 at 0.4–4.3 ng/L and EE2 at 0.4–3.4 ng/L [20,21].

The low levels of estrogenic chemicals in river water make measurement and monitoring of these contaminants in the environment challenging. It has, however, been demonstrated that much higher concentrations of estrogenic compounds may accumulate in fish bile, and in laboratory studies the bioconcentration of waterborne alkylphenols in bile was between 20,000- and 70,000-fold [22–26]. In fish exposed to WwTW effluents, the concentration factors in bile were 4000–6000-fold for EE2 and 10,000–13,000-fold for E2 and E1 [5,6]. The dominant metabolites of these phenolic contaminants in fish bile are phase II metabolites which are mainly conjugates of glucuronic acid, and hydrolysis of bile results in release of the parent compounds which can be readily quantified [27,28]. Analysis of fish bile has also been used for the identification and quantitation of polycyclic aromatic hydrocarbons [29,30] chlorinated phenols [31], and resin and fatty acids from pulp and paper mill effluents [32] and represents a snap shot of the recent exposure of the animal to mixtures of bioavailable contaminants in the environment.

The aim of this study was to determine whether analysis of microlitre amounts of fish bile can be used to detect exposure to mixtures of estrogenic compounds originating from WwTWs effluents, and thus enables a new and more sensitive method for the determination of exposure to these environmental contaminants. We refined an analytical method [33], previously validated for the quantitation of estrogenic chemicals in wastewater samples, and used GS–MS/MS instead of GC–MS to increase the selectivity and sensitivity of the analysis. Estrogenic contaminants in bile samples were deconjugated and extracted on an OASIS HLB solid phase extraction (SPE) cartridge. Following derivatisation, samples were analysed by GC–MS/MS using multiple reaction monitoring mode (MRM). The analytical method was tested on bile samples from roach (*Rutilus rutilus*) held in cages downstream of a WwTW effluent and from fish captured from the Temple Lock reach of the River Thames.

2. Experimental

2.1. Chemicals

E1, E2, EE2, technical nonylphenol (NP), BPA, 2,2′-dihydroxybiphenyl, bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), pyridine, 4-nitrophenol, potassium 4-nitrophenyl sulfate, 4-nitrophenol β-D-glucuronide, β-glucuronidase (type VII-A extracted from *Escherichia coli*), sulfatase (VI from *Aerobacter aerogenes*) and all other chemicals were purchased from Sigma–Aldrich (Poole, UK). [2,4,16,16-$^{4}H_{2}$]Estrone (E1-d_4), [2,4,16,16-$^{4}H_{2}$]17β-estradiol (E2-d_4), [2,4,16,16-$^{4}H_{2}$]17α-ethinylestradiol (EE2-d_4) (isotope purity 96%, chemical purity > 98%), [U-$^{13}C_{6}$-ring] n-nonylphenol (^{13}C-NP) (isotope purity 99%), [U-$^{13}C_{6}$-ring] bisphenol A (^{13}C-BPA) (isotope purity 99%), [U-$^{13}C_{6}$-ring] n-nonylphenol monoethoxylate (^{13}C-NP1EO) (isotope purity 99%, chemical purity ≥ 98%), nonylphenol monoethoxylate (NP1EO) (mixture of alkyl isomers, chemical purity ≥ 98%) and nonylphenol diethoxylate (NP2EO) (mixture of alkyl isomers, chemical purity ≥ 98%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All solvents were of HPLC-grade purchased from Rathburn Chemicals (Walkerburn, Scotland, UK).

2.2. Fish exposure and sample collection

Wild roach (*R. rutilus*) of mixed sex were captured by electrofishing from the Temple Lock stretch of the River Thames on 3rd September 2007. The fish were collected as part of the annual fish monitoring survey which is carried out by the Environment Agency in all the major rivers of England and Wales. Temple reach comprises a 0.5 km stretch between grid references SU8380584364 and SU8518286132, and is 6.2 km downstream of Henley WwTWs which has a population equivalent of 11,620. Another population of maturing roach of mixed sex and 3+ years old were purchased from a fishery (Framlingham Fisheries, Suffolk) and subsequently exposed downstream of the discharge from a WwTWs (Co. Durham, UK) for 10 days in heavy-duty galvanised steel-mesh cages. The wastewater influent had a population equivalent of 47,569 with some industrial inputs, with the influent treated by primary sedimentation and secondary trickling filter. Fish were not fed for the duration of the exposure, though they were able to take any natural food available. Additionally, some roach were kept as reference controls in standard stock tanks with a flow-through system of dechlorinated tap water at 15 °C. At harvest, fish were anaesthetised with ethyl 4-aminobenzoate, stunned by a blow to the head and the spinal cord severed. For caged and laboratory-held control fish, bile was obtained by puncturing the gall bladder with a needle and drawing the fluid into a syringe. For wild fish, gall bladders were removed and frozen, and the bile extracted prior to sample preparation. Bile was stored in vials and frozen on dry ice for transport before storage at −70 °C.

2.3. Hydrolysis of estrogenic contaminants in bile samples

Bile (20–100 μL) was added to 200 μL of β-glucuronidase type VII from *E. coli* (1000 units/mL), 200 μL sulfatase type VI from *A. aerogenes* (2 units/mL) and 500 μL 0.1 M phosphate buffer (0.2 M) sodium dihydrogen orthophosphate:0.2 M disodium hydrogen orthophosphate:water (4:1:5, v/v/v) at pH 6.0. The samples