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Analysis of pyrene metabolites in marine snails by liquid chromatography using fluorescence and mass spectrometry detection

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ABSTRACT

As part of a study of the metabolism of aromatic compounds in marine gastropods, a sensitive and selective method was developed to detect, identify and quantify pyrene (PY) and four of its metabolites in tissues: 1-hydroxypyrene (PYOH), pyrene sulfate (PYOS), pyrene glucuronide (PYOG) and pyrenediol disulfate (PYDS). Liquid chromatography (LC) with fluorescence detection was first used to detect the PY derivatives in the visceral mass of whelks exposed to PYOH. The identification of metabolites was accomplished through a combination of retention time and spectral matching with standards, enzymatic hydrolysis, solid phase extraction and LC coupled with electrospray ionization mass spectrometry. In addition to four known PY derivatives, two novel metabolites were identified as pyrenediol glucuronide sulfate and a second isomer of PYDS. The methanol extraction of metabolites from tissue gave excellent mean recoveries, ranging from 67 to 97%, for the available standards PY, PYOH, PYOS and PYOG spiked in both the muscle and visceral mass of *Buccinum* spp. The mean recoveries of a surrogate standard, 2-hydroxyfluorene, spiked in all tissue samples were 100% and 95% for visceral and muscle tissue samples, respectively. The method limits of detection for these compounds were all below 0.2 ng/g of wet tissue, low enough to detect metabolites in reference animals. Results from the application of this method to the quantitative analysis of biotransformation products in the visceral mass of the whelk *Neptunia lyrata* exposed to PYOH contaminated food are also presented. This method will be useful to apply to the analysis of PY metabolites in soft tissues of other animals.

1. Introduction

Polycyclic aromatic compounds are a widely studied class of contaminants, many of which are known to exhibit toxicity to humans and other organisms [1,2]. They are released into the environment both naturally and through human activities as combustion products and components of fossil fuels [3,4]. They are widely distributed throughout the environment and detected in highly variable concentrations in air [5], water [6], sediment and biota [7,8]. In the environment, polycyclic aromatic hydrocarbons (PAH) exhibit a diverse range of reactivities including photochemical oxidation [9], fungal and bacterial catabolism [10,11], biotransformation by organisms [12,13] and hydrolysis of conjugated species [14]. Although smaller PAH with two or three rings are known to degrade relatively easily, larger, more hydrophobic molecules tend to be more persistent. It is the magnitude of production and release into the environment of these chemicals that has led to characterizing them as persistent. Parent compounds and oxidation intermediates can accumulate to concentrations associated with a toxic risk [15,16]. It is because of this potential toxicity that PAH are referred to as priority pollutants and receive international attention.

Pyrene (PY) is a tetracyclic PAH commonly studied as a model compound because it occurs as a major constituent in mixtures with many more harmful PAH. Because of the molecule's high degree of symmetry, oxidation to 1-hydroxypyrene (PYOH) is the predominant initial pathway for PY reactivity in the environment. This makes the study of the compound's fate simpler than that of other PAH which can form numerous oxidation products. In addition to being good model compounds, PY and PYOH are both environmentally relevant and detected in a wide variety of sources and sinks of contaminants including water, sediment and biota [8,17,18].

As is the case with many PAH, the toxicity of PY has been linked to an activated phase I oxidation product [19,20]. These types of inter-

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mediates are also associated, such as in the case of benzo(a)pyrene, with the formation of DNA adducts. The presence of bile metabolites is representative of short-term exposure, while that of DNA-adducts is indicative of chronic exposure and both of these biomarkers are implicated in the genotoxic pathway leading to cancer [21]. These two fates are representative of the reactivity of PAH and occur in addition to the bioaccumulation of hydrophobic contaminants which takes place in many animal species [22,23].

PAH metabolites have been used as biomarkers of PAH exposure in finfish and humans. For example, pyrene metabolites detected in urine are associated with environmental and occupational exposure to PAH [12]. The presence of gall bladder bile metabolites in fish has also been used to assess the state of PAH contamination of a location [24,25]. Work on PAH metabolites in invertebrates is more recent and much of it has been directed towards the study of deposit feeding polychaetes used in risk assessment of contaminated sediments [26]. Biotransformation plays an important role in the environmental fate of a compound, changing the chemical properties of the molecule and altering its reactivity [19]. Phase I oxidation products are typically more reactive and more polar than parent compounds. This also suggests that these compounds are more labile and available in the environment than are parent PAH.

In vertebrates the phase I metabolite PYOH is conjugated to form the phase II metabolites pyrene-1-glucuronide (PYOG) and pyrene-1-sulfate (PYOS) [12]. The formation of these more polar, water soluble molecules facilitates the elimination of the contaminant from the organisms. However, once in the environment they can become a new source of phase I derivatives after hydrolysis [14]. So far, PY metabolites in invertebrates seem to be species specific although the biotransformation products identified in vertebrates as well as pyrene-1-glucoside are also often detected. In clams, terrestrial isopods and the water flea *Daphnia magna*, the metabolites pyrenediol sulfate, pyrene glucose malonate and pyrene glucose sulfate have been identified, respectively [26–29].

For the study of dilute metabolites in urine or those in the gall bladder bile of fish, samples can be concentrated by liquid/liquid or solid phase extraction to enhance their detection [30]. In some studies, samples are hydrolyzed with deconjugating enzymes, converting the phase II metabolites to the phenolic phase I derivative [12]. In the absence of an easily accessible biofluid or when only a small volume of hemolymph is available, the extraction of soft tissue is required. The presence in tissue of natural products covering a range of structures and polarities makes the analysis of PAH metabolites in smaller invertebrates more challenging.

Spectroscopic approaches or hyphenated chromatography–spectroscopy techniques have been used to investigate the presence of metabolites. Because PY is a highly fluorescent compound, fluorescence spectroscopy of PY metabolites gives excellent sensitivity and selectivity as well as characteristic excitation spectra which are comparable to the much less sensitive UV absorbance spectra. Because of the low levels of interfering chemicals in bile, urine or hemolymph, direct spectroscopic measurements using fixed wavelength [31], synchronous [32], or Shpol'skii [33] fluorescence spectroscopy are often also possible. As no chromatographic separation is used, analysis by the first two more frequently used techniques is not specific to the structure of phase II conjugates. In urine, bile and tissue extracts, liquid chromatography with fluorescence detection (LC–FLD) is a commonly used method to measure PYOH, PYOG and other metabolites [34–36]. The non-volatile nature of these metabolites and their sensitive fluorescence detection makes LC–FLD a highly suitable technique to analyze mixtures of PAH and their derivatives. The similar fluorescence response of different conjugates containing the PY moiety makes semi-quantitative analysis possible for novel compounds when reference standards are unavailable. The main drawback of this method is the lack of structural information for identifying new conjugates which requires the use of other instrumental techniques. Nuclear magnetic resonance spectroscopy has also been used to investigate the identity of conjugates formed by macro- [29,37,38] and micro-organisms [10,11] exposed to suitably high levels of PAH.

Both gas and liquid chromatography with mass spectrometry detection (GC–MS and LC–MS) have also been used to characterize and quantify PAH metabolites. The techniques offer the advantages of providing structural information and superior selectivity when compared with fluorescence detection. In GC–MS, only parent PAH can be sensitively analyzed without derivatization, and in most studies polar phase II metabolites must also be hydrolyzed to phase I oxidation products before analysis [39]. LC–MS with electrospray ionization (LC–ESI–MS) detection has recently been used to quantify phase I and phase II metabolites of PY [40] and other PAHs [41]. The strategies used for the detection of common phase II bioconjugates of pharmaceuticals and contaminants have been recently reviewed [42] and are promising for the analysis of a large number of PAH metabolites in complex samples. However, for the sensitive detection of parent and phase I metabolites, this technique can also require derivatization or novel mobile phase additives [43,44].

Organisms residing in contaminated environments can be exposed to both parent PAH and their oxidation products. Hydroxylated PAH are detected in environmental samples [17,45] and phase II conjugates excreted by humans have been shown to be hydrolyzed back to their phase I products during sewage treatment [14]. Despite this, bioaccumulation and biotransformation in organisms after direct exposure to PYOH has rarely been studied [29].

This paper describes the analytical approaches used to identify and quantify metabolites of PY and PYOH in soft tissues of marine gastropods exposed to these contaminants. This work uses LC–MS as a tool for structural elucidation and LC–FLD for quantitative and semi-quantitative analysis of PY biotransformation products. The present approach was developed as part of a broader study of PY and PYOH metabolism in marine invertebrates. The species studied included the large marine snails, hereafter referred to as whelks, *Neptunea lyrata* and *Buccinum* spp. as well as the small marine snail *Ilyanassa obsoleta* which are presented elsewhere [46]. A subset of results from one chemical exposure of the whelk *N. lyrata* to PYOH through dietary uptake is presented as an example of the application of these methods along with results from reference animals that help display the sensitivity of the analytical approach.

2. Experimental

2.1. Chemicals and reagents

Pyrene (98%) (PY), 1-hydroxypyrene (98%) (PYOH), 2-hydroxyfluorene (98%) (FLOH), ammonium acetate (99.999%), sulfatase from *Helix pomatia* (Type H-1, 20,560 units/g) and β-glucuronidase from limpets (type L-II, 3,000,000 units/g) were purchased from Sigma–Aldrich (Oakville, ON). Pyrene-1-sulfate (PYOS) was purchased as a potassium salt from the National Cancer Institute (Kansas City, MO). Potassium hydroxide (86.9%), sodium acetate (99.6%) and glacial acetic acid (USP) were purchased from Fisher Scientific (Ottawa, ON). All solvents were HPLC grade and were used without additional purification. Ammonium acetate buffer was filtered with a 0.45 μm Milli-Q Millex HV syringe filter. All labware was carefully rinsed with water, acetone and dichloromethane before use.

A qualitative sample of pyrene-1,6-disulfate (1,6-PYDS) was provided by Dr. Haidrun Anke of the Institute of Biotechnology and Drug Research (Kaiserslautern, Germany). This material has been characterized by ^{1}H NMR and high resolution mass spectrometry [10].

Pyrene-1-glucuronide (PYOG) was purchased from EQ Laboratories (Atlanta, GA) as a 10 ng/μL solution in acetonitrile. The retention time of this standard did not match that of PYOG in a fish bile sample from a previous study [35] and was determined by LC–MS to be a methylated derivative of PYOG ($[M-H]^- = m/z$ 407). Stirring 0.95 μg of this material in 10 mL methanol with excess KOH for 21 h gave complete conversion to PYOG, as determined by LC–FLD.

2.2. Animals and laboratory exposure

Whelks were collected in summer 2007 in the Bay of Fundy around the island of Gran-Manan in New Brunswick, Canada and were of the species *Buccinum* spp. and *N. lyrata*. Animals were maintained in a 91 cm × 91 cm × 46 cm holding tank filled 37 cm high with flowing deep sea water obtained from Bedford Basin, Nova Scotia, Canada. The tank contained a layer of sediment from Hantsport Beach, Nova Scotia, also on the Bay of Fundy and an aerator. Animals were fed a diet of white fish for 2 months leading up to the exposure experiment. An investigation of the feeding preference of the whelks indicated that they preferred the soft tissue of the snail *I. obsoleta* over fish [46].

Exposed *N. lyrata* were fed snail tissue spiked with 8 μg/g PYOH. The contaminated food was prepared by applying a 5 mL acetone solution to the tissue of 100 snails (37.9 g) in a Petri dish. After stirring, the solvent was allowed to evaporate and the contaminant to penetrate the tissue for 30 min. This food was stored in a sealed glass jar at −12 °C until needed and thawed before each subsample was removed. Chemical analysis of a subsample of this food showed no measurable biotransformation products of PYOH [46].

The population of exposed whelk, 24 *N. lyrata*, were held in a tank similar to the holding tank but with a layer of 20–30 mesh Ottawa sand (Fisher Scientific) instead of sediment. Animals were transferred to the exposure tank 1 week prior to starting the experiment and starved for that period. Four unexposed animals were collected at time 0, prior to the exposure, as a control. The 20 remaining animals were then fed 5 g of contaminated food equivalent to 2 μg of PYOH per exposed animal. One week after feeding, four animals were sampled. The remaining animals were fed similar doses of contaminated food each week for the remainder of the exposure with 4 animals being collected each week prior to the next feeding (not presented). All animals were frozen in jars at −12 °C immediately after sampling.

2.3. Method development

2.3.1. LC–FLD analysis

A Hewlett Packard 1090 HPLC with a HP 1046A programmable fluorescence detector was used for all quantitative measurements. The chromatographic method was modified from a previous study [47] and used a Waters X-Terra RP18, 3.5 μm particle size, 2.1 mm × 150 mm fitted with a 10 mm guard column of the same phase. Mobile phases consisted of A = H_2O, B = methanol:acetonitrile:water (38:57:5, v/v/v), both with 10 mM ammonium acetate buffer (pH 6.5 for A). The solvent gradient was 35 min in length with 10% B from 0 to 2 min, followed by a linear gradient to 38% B from 2 to 11 min, to 56% B from 11 to 20 min and to 100% B from 20 to 27 min with a 8 min hold at 100% B to 35 min. An injection volume of 5 μL, a flow rate of 200 μL/min and a column temperature of 40 °C were used throughout.

Fluorescence detection was carried out at an excitation/emission wavelength pair of 235/388 nm for PY, PYOH and their metabolites. In the analysis of extracts where the recovery of the surrogate standard 2-hydroxyfluorene (FLOH) was calculated, a period between 25.5 and 28.0 min was programmed at 260/333 nm. Periodic checks were carried out to ensure that no PY related metabolites were eluting in this region of the chromatogram, which remained consistently free of signals at 235/388 nm.

Fluorescence excitation and emission spectra were collected using an Agilent 1100 series HPLC equipped with an Agilent 1200 series fluorescence detector. The same column and chromatographic conditions as above were used and spectra were collected with a 5 nm step size.

2.3.2. Quantitative and semi-quantitative analysis

All chemical standards were made up in methanol from stock solutions. The calibration and extraction recovery standards consisted of a solution of PY, PYOH, PYOS, PYOG and FLOH in methanol. A separate surrogate standard consisting only of FLOH in methanol was used as a spiking solution for extracted tissues. These chemicals were quantified using either a 5 or 7 point calibration curve (Table 1) ranging from near the detection limit to near the saturation point of the fluorescence detector for each compound. In the absence of an appropriate standard, PYDS was measured using a semi-quantitative approach using the molar response of PYOS.

2.3.3. Dissection and chemical extraction of metabolites

Frozen animals were thawed for 30 min prior to dissection. The tissue of each animal was removed from the shell and divided into muscle and visceral mass and each was extracted separately. Muscle mass included the foot and head of the animal as well as the muscle surrounding the proboscis and the penis when present. Visceral mass included the liver, the gonad and the red gut tissue inside the proboscis of the animal. Each tissue was cut up into pieces about 2–3 mm^3 using scissors and a 4 g subsample was transferred to a 50 mL Nalgene fluorinated ethylene propylene centrifuge tube (Fisher Scientific, Ottawa, ON) and spiked with the surrogate standard. In the cases when the total muscle or visceral mass of an animal was <4 g, the entire amount was used in the analysis. The tissue was vortexed and homogenized in 10 mL of methanol using a Brinkmann Polytron homogenizer and then sonicated with a BioLogics 300 V/T ultrasonic homogenizer, centrifuged at 2500 rpm and decanted. Vortex and sonication steps were repeated with three more aliquots of methanol. Throughout these steps, the small amounts of extracted tissue remaining on the homogenization and sonication probes was easily scraped and rinsed back into the tube. The pooled supernatant was concentrated to about 1 mL using rotary evaporation at 45 °C and transferred to a 4 mL sample vial along with methanol and dichloromethane rinses of the round bottom flask. The pooled rinses were reduced to <0.5 mL under a gentle stream of nitrogen at 45 °C. The sample was then made up to a final volume of 4 mL with methanol by weight, assuming an aqueous density for the residue. A subsample of this extract was filtered using a 0.45 μm Millex HV syringe tip filter and analyzed by LC–FLD.

2.3.4. Lipid and moisture content

Subsamples of tissue from a number of animals of each species were collected at the time of dissection. Moisture content values were obtained by drying tissue in a 60 °C oven overnight. Lipid content was determined gravimetrically by exhaustive extraction of the tissue with 1:1 hexane:dichloromethane as reported previously [34]. The material extracted by this method represents the lipid content of a tissue and is useful for intercomparison of tissue samples but does not represent the absolute concentration of any single class of molecules. This tissue was dried at room temperature overnight and then homogenized in 10 mL of solvent with sodium sulfate (reagent grade, Caledon). As in the chemical extraction, sonication and centrifugation followed homogenization and were repeated with three additional aliquots of solvent. The pooled extracts were reduced to 1 mL by rotary evaporation, transferred to a pre-weighed vial and reduced to a thick yellow oil using a gen-

Table 1
LC–FLD calibration data for available standards.

Compound	Molar fluorescence response (peak area/fmol)	R^2	Calibration range (pmol)	Retention time (min)	LC–FLD IDL (fmol)[a]
PY	$y = 0.72x - 0.072$	>0.999	0.03–0.5	32.0	1.8
PYOH	$y = 0.76x - 0.076$	>0.999	0.03–0.5	30.5	3.2
PYOS	$y = 0.81x - 0.0068$	0.999	0.03–0.6	22.6	0.14
PYOG	$y = 1.4x + 0.042$	>0.999	0.02–0.2	19.1	0.75
1,6-PYDS	–	–	–	14.3	–
FLOH	$y = 0.18x - 0.00060$	>0.999	0.1–3	26.7	4.4

[a] Instrumental detection limit (IDL) = 3 S/N.

tle stream of nitrogen. The mass of the extracted material and the moisture content were used to calculate the lipid content of dry tissue.

2.3.5. Extraction recovery

Tissue samples from reference *Buccinum* spp., not exposed to PY or PYOH in the laboratory, were spiked with 50 or 100 μL of a methanol solution of 0.79 μg/mL PY, 0.81 μg/mL PYOH, 3.03 μg/mL FLOH, 1.32 μg/mL PYOS and 0.96 μg/mL PYOG. Samples were extracted using the same extraction procedure described above and the mean percent recovery of each compound was calculated from the LC–FLD chromatograms. Where applicable, the trace amount of each compound measured in unexposed tissue was subtracted from the measured amount (Section 3.4).

Throughout the study, the reproducibility of the extraction procedure was verified by measuring the recovery of a surrogate standard of 2-hydroxyfluorene (FLOH) spiked into each extracted sample. Each tissue sample was spiked with 50 or 100 μL of a 55 μg/mL solution of FLOH in methanol.

2.4. Identification of metabolites

2.4.1. Enzymatic hydrolysis of extracts

Subsamples (500 μL) of visceral tissue extracts of two different exposed animals were blown down to dryness under a gentle stream of nitrogen at 45 °C. To these, either 7 or 0.7 units of sulfatase or 102 units of glucuronidase were added and made up to a final volume of 500 μL with 10 mM acetate buffer (pH 5). The samples were stirred at 37 °C for 2 h, topped with 500 μL of methanol to quench the hydrolysis and analyzed directly by LC–FLD. Controls consisting of only extract and buffer and of only enzyme and buffer were also prepared and analyzed as above.

2.4.2. SPE fractionation of extracts

Fractionation by SPE was carried out using a procedure modified from Singh et al. [36]. Subsamples (2–3 mL) of visceral tissue extracts of selected animals exposed to PYOH were each concentrated to about 0.5 mL by rotary evaporation at 45 °C. Each concentrated extract was loaded onto a 1 g Waters OASIS HLB solid phase extraction cartridge in 10% aqueous acetonitrile (10 mL). Using a Supelco vacuum manifold, samples were rinsed with water and then eluted with 20 mL aliquots of 20, 40 and 80% (v/v) acetonitrile. The content of each fraction was monitored by LC–FLD and the 20% acetonitrile fractions from 5 animals containing metabolites of interest were pooled. This pooled sample was subjected to a second SPE fractionation as described above and the 20% acetonitrile fraction from this second cleanup was used for LC–MS analysis.

2.4.3. LC–MS system

LC–MS analysis was carried out using an Agilent 1200 series LC system and an API4000 QTRAP triple quadrupole linear ion trap mass spectrometer equipped with a Turbo V electrospray ion source (MDS Analytical Technologies, Concord, ON, Canada) operated in negative ion mode. Nitrogen gas at 50 Torr (GS1, GS2) and a spray voltage of −4.5 kV were used in all experiments. Initially a low source temperature of 275 °C was used to ensure that labile compounds such as glucuronide conjugates were not adversely affected. It was later found that increasing the source temperature to 600 °C gave improved sensitivity while not affecting the compounds as feared. This higher temperature was used thereafter.

Initially, for exploratory multiple reaction monitoring (MRM) experiments (not shown) and one enhanced product ion scan (Section 3.3.4) collision energy (CE) was set at −60 eV and declustering potential (DP) to −80, parameters used previously [47]. These parameters were then varied to correspond with those used by Kakimoto et al. [40] which consisted of CE = −38 eV and DP = −60 V. These parameters, with the exception of the varied CE, were used in the other enhanced product ion scans and an optimized MRM experiment (Section 3.3.4). In all cases, the same chromatographic conditions as in the LC–FLD method (Section 2.3.1) were used.

3. Results and discussion

Analysis and extraction methods were developed using tissue from the whelk *Buccinum* spp. The tissues of unexposed animals without a spike were first extracted to examine the matrix material. Then tissues were spiked with available standards that could be expected in the tissue of animals exposed to PY or PYOH to determine the best conditions for optimum recoveries of these compounds. This method was then applied to the study of the metabolism of PY and PYOH in three species of marine gastropods; the whelks *Buccinum* spp. and *N. lyrata* and the snail *I. obsoleta*. The presence of metabolites other than those available as standards was also pursued to determine their structures. Results from one time point of an exposure of *N. lyrata* to PYOH are presented here (Section 3.4) to illustrate a possible application for the described method while the majority of the results of the metabolism studies will be presented elsewhere [46].

3.1. LC–FLD

The FLD wavelength pairs were selected by using the excitation and emission maxima of commercial standards of PY, PYOH, PYOS and FLOH and those of PYOG obtained from the hydrolysis of methylated PYOG. To ensure the most sensitive analysis, the wavelengths used in the quantitative analysis method were obtained from spectra acquired using the rough fluorescence scanning capabilities of the detector which would be used for the analysis of extracts (HP 1046A). These spectra varied slightly from better quality spectra obtained for the purpose of identifying unknown metabolites using a more modern instrument (Agilent 1200) (Fig. 1). Despite small differences in the wavelength maxima observed between PY and derivatives, a single wavelength pair was chosen because the method was intended as a tool for detecting any PY related metabolites, not just those used in development. Excitation and emission wavelengths of 235 and 388 nm, respectively, were selected for PY related compounds and of 260 and 333 nm, respectively, for FLOH. These provided superior sensitivity and good selectivity for the target analytes present in the whelk tissue extracts. Compared with

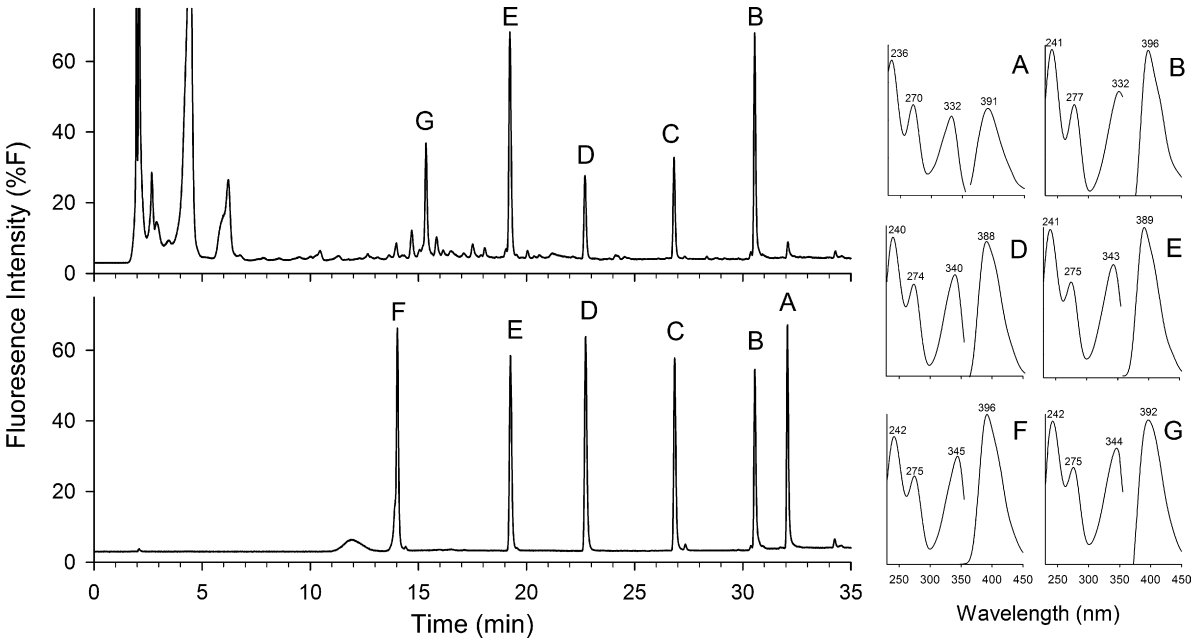

Fig. 1. LC–FLD chromatograms of the visceral tissue extract of *Neptunea lyrata* exposed to PYOH (top) and available chemical standards for PY (A), PYOH (B), FLOH (C), PYOS (D), PYOG (E) and 1,6-PYDS (F) (bottom). Inlays show the fluorescence excitation (388 em) and emission (241 ex) spectra for PY and PY derivatives detected in the whelk tissue extract.

260/380 nm ex/em, a wavelength pair previously used for analysis of PY and its metabolites [34,35], 235/388 shows a general increase in sensitivity of about fourfold for PY, PYOH, PYOS and PYOG without an increase in interfering background from fluorescent natural products present in tissue extracts. By collecting chromatograms at both of these wavelength pairs, the relative fluorescence response can be used as a tool for identifying PY metabolites in complex extracts, especially in the absence of a scanning fluorescence detector. When compared with 335/380, another commonly used wavelength pair [27–29,48,49], 235/388 also showed a significant increase in sensitivity. While low wavelength excitation did result in slightly more interference from fluorescent material in the matrix, the increase in sensitivity allowed for the detection of trace levels of PY metabolites in reference animals (Section 3.4) that was not possible with any other wavelength pair.

The LC column and solvent system were selected to: (a) give good separation selectivity for PY and its known metabolites of varying polarities in the complex matrix of a tissue extract; (b) improve peak shape for sulfated metabolites to improve signal to noise without compromising the sensitivity of less acidic metabolites; and (c) be compatible with ESI–MS as well as FLD detection. Neither methanol nor acetonitrile showed ideal selectivity when used as the only organic modifier. Of the various mixtures investigated, a 60:40 mixture of acetonitrile:methanol was selected to give the best separation of known PY metabolites from each other and from polar and non-polar regions of higher matrix interference in the chromatograms of tissue extracts (Fig. 1). The neutral buffered ammonium acetate mobile phase and the end-capped Xterra C18-silica column were selected to improve peak shape for sulfated metabolites by minimizing interactions between the anionic sulfate and residual silanol groups on the stationary phase. In acidic mobile phases, such as 0.1% trifluoroacetic acid, these effects produce tailing for the peaks of sulfated compounds leading to a reduction in the signal to noise ratio. The absence of ion pairing agents such as trifluoroacetic acid and the use of methanol as a component of the organic modifier make this chromatographic method suitable for use with ESI–MS detection.

Table 2
The recovery of compounds spiked onto *Buccinum* spp. tissue samples.

	PY	PYOH	FLOH	PYOS	PYOG
Visceral tissue[a]	98 ± 1	83 ± 7	98 ± 1	91 ± 11	67 ± 3
Muscle tissue[a]	95 ± 1	78 ± 6	95 ± 1	93 ± 2	79 ± 1
MDL[b] (ng/g wet)	0.07	0.1	0.2	0.01	0.06
MLQ[c] (ng/g wet)	0.4	0.3	0.5	0.03	0.3

[a] Mean percent recovery ± standard deviation $n = 4$.
[b] Method detection limits (MDL), equivalent to S/N = 3 for a 4 g tissue sample.
[c] Method limits of quantification (MLQ), equivalent to S/N = 10 for a 4 g tissue sample.

3.2. Chemical extraction

Extraction parameters were selected to: (a) optimize the recovery of extracted PY and known PY metabolites of varying polarities from whelk tissue; (b) minimize potential degradation of the metabolites during the extraction process and; (c) minimize the extraction of additional matrix material, such as lipids and protein residues from the whelk tissue. Methanol was chosen as the extraction solvent because of the wide range of polarities of the analytes of interest from parent PY to metabolites potentially conjugated with multiple polar groups [27]. When dichloromethane was included as part of the extraction protocol, excessive material that was insoluble in the final methanol solution precipitated in the final stages of the workup. Small masses of precipitate, which were not easily dissolved in small methanol rinses, had the tendency to trap some residue on the glass of the evaporating flask. Both methanol and dichloromethane rinses were necessary to recover all of this material.

Table 3
The mean lipid and moisture content for each of the different types of tissue and species of whelk examined.

	% moisture ± SD	% lipid ± SD
Buccinum spp. muscle ($n = 6$)	72 ± 2	2 ± 1
Buccinum spp. visceral ($n = 5$)	60 ± 5	8 ± 1
Neptunea lyrata muscle ($n = 4$)	74.6 ± 0.4	0.8 ± 0.4
Neptunea lyrata visceral ($n = 5$)	70 ± 5	10 ± 3

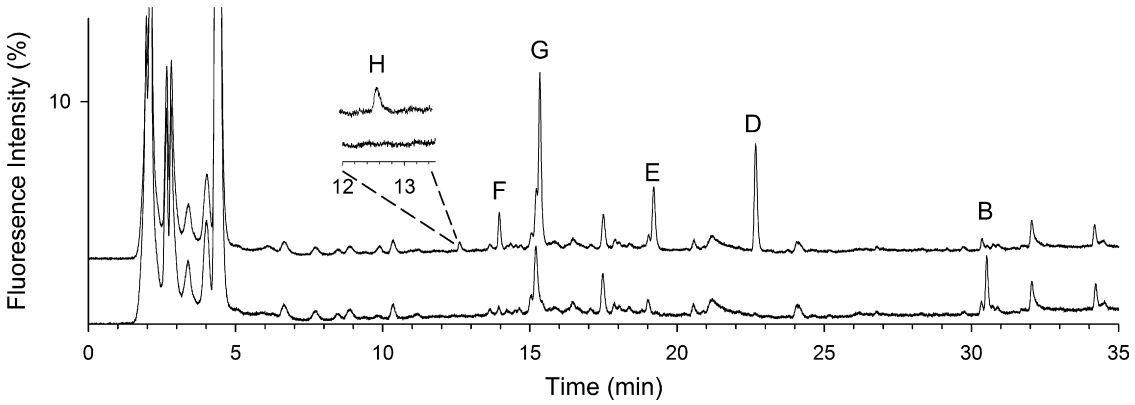

Fig. 2. LC–FLD chromatograms of the visceral tissue extract from a PYOH exposed whelk (top) and the same extract treated with sulfatase (bottom).

In preliminary extraction attempts it was observed that spiked tissue left to dry overnight showed much lower recoveries than tissue processed wet. This can be attributed to degradation of the compounds in the tissue after dissection and was particularly evident in samples of visceral mass where recoveries were in some cases <50% for tissue dried overnight. For this reason all tissue samples were processed wet as soon as possible after dissection. In cases when extraction was not possible immediately following dissection, tissue samples were immediately frozen until they could be processed.

The chosen parameters gave excellent mean recoveries for PY and the metabolites PYOH and PYOS while lower but reasonable mean recoveries were obtained for PYOG (Table 2). Recovery values were also excellent for the surrogate standard FLOH. While no correction of results was made for these recovery values, it should be noted that they indicate that final results will be generally weighted against PYOH and PYOG.

In the absence of stable isotope labelled standards for each PY derivative, FLOH was selected as a surrogate spike and included in each tissue extraction to ensure extraction consistency and to monitor for potential sample loss. The recovery of FLOH was $100 \pm 9\%$ from visceral tissue and $95 \pm 6\%$ from muscle tissue for 44 samples of each type extracted during the subsequent metabolism studies [46]. Using the student's *t*-test, FLOH recovery values for muscle and visceral tissue were found to be significantly different ($p = 0.003$). However, these recoveries were not significantly different between *N. lyrata* and *Buccinum* spp. tissue ($p = 0.5$). This result, as well as a similar lipid and moisture content for the tissues from each species (Table 3) leads us to expect the same quality of analysis in tissues from the two similar whelk species. Since FLOH recoveries were mostly >90%, the values were not used to adjust the results for each sample. However, in two cases where significant sample loss occurred during sample manipulation, the determined FLOH recovery value was used to adjust the levels of PY derivatives.

3.3. Identification of metabolites

3.3.1. Chromatographic and spectroscopic identification of metabolites

A number of signals were significantly amplified in the chromatograms of visceral tissue extracts from animals exposed to PYOH compared with unexposed animals. A number of compounds were identified as known PY metabolites by matching retention times and fluorescence spectra to that of available standards (Fig. 1). Peaks B, D and E were identified as PYOH, PYOS and PYOG, respectively. The fluorescence spectrum of peak G matched that of the other PY conjugates however the compound's retention time did not match that of any of the available metabolites.

3.3.2. Enzymatic hydrolysis

Two deconjugating enzymes, sulfatase and glucuronidase, were used to characterize phase II conjugates of PY in the extracts of the visceral mass of *N. lyrata* exposed to PYOH. This experiment was carried out to confirm the identity of the compounds determined by LC–FLD and to help identify other compounds which might be conjugates of PY with sulfuric or glucuronic acid.

Treatment with sulfatase showed hydrolysis of PYOS, PYOG and 1,6-PYDS peaks (Fig. 2). Similarly poor selectivity between sulfate and glucuronide conjugates was also observed with the use of glucuronidase and with the use of lower levels of sulfatase (not shown). These experiments supported the identification of peak F as 1,6-PYDS which had not been identified by LC–FLD alone. In addition to known metabolites, two other compounds, peaks G and H, were also affected by the enzymatic hydrolysis (Fig. 2) indicating they are likely PY conjugates containing sulfuric or glucuronic acid. Since poor selectivity of hydrolysis was observed, it was also possible that these unknowns would be conjugates of some other endogenous molecule such as glucose, which have been observed in many invertebrates [26]. The PYOH peak was the only product which increased in intensity in the hydrolyzed extracts. Since 1,6-PYDS was present in the extracts, the absence of a signal from 1,6-pyrenediol in the hydrolyzed extracts was surprising. The absence of this compound could be due to its lack of sensitivity at the wavelength pairs examined (235/388 and 260/380) or to a lack of stability under the conditions of the hydrolysis. Pyrenediol has previously shown poor stability under laboratory conditions, quickly degrading to form pyrenequinone [50] which shows a different fluorescence response than PY phenols and conjugates [11]. This instability could explain the lack of detection of a new peak. It also suggests that if pyrenediol were present in the extracted tissue it might not be detectable by our methods. This reactivity might also indicate the formation of additional unpredicted metabolites that could result from a reaction with other biogenic molecules. These could be pursued in future work.

3.3.3. Solid phase extraction

In order to be able to concentrate peaks G and H (Fig. 2) for LC–MS analysis, extracts from visceral tissues of *N. lyrata* exposed to PYOH were subjected to a reversed phase Oasis HLB SPE fractionation. The use of acetonitrile as the organic modifier gave better selectivity than methanol in the step gradient elution. Peaks G and H, as well as PYOG were isolated in the 20% acetonitrile fraction, PYOS was isolated in the 40% acetonitrile fraction and PYOH was isolated in the 80% acetonitrile fraction.

The goal of the fractionation was to prepare a concentrated sample of the unidentified metabolites for qualitative LC–MS analysis and not to develop an optimized quantitative separation. This

Table 4
MRM transitions used to detect conjugates of PY in whelk tissue extracts.

Compound	Precursor > product	Assignment	Peak
1-Hydroxypyrene	217 > 217[a]	$[PYO]^-$ > $[PYO]^-$	B
Pyrene-1-sulfate [42]	297 > 217[a]	$[M-H]^-$ > $[PYO]^-$	D
	297 > 189[a]	$[M-H]^-$ > $[M-H-SO_3-CO]^-$	D
Pyrene-1-glucuronide [42]	393 > 217[a]	$[M-H]^-$ > $[PYO]^-$	E
	393 > 189[a]	$[M-H]^-$ > $[M-H-C_6H_8O_6-CO]^-$	E
Pyrene-1-glucoside [42]	379 > 217[a]	$[M-H]^-$ > $[PYO]^-$	–
	439 > 217[a]	$[M+CH_3CO_2]^-$ > $[PYO]^-$	–
Pyrene glucoside sulfate [27]	459 > 217	$[M-H]^-$ > $[PYO]^-$	–
	459 > 241[a]	$[M-H]^-$ > $[M-H-PYO]^-$	–
	459 > 97[a]	$[M-H]^-$ > $[HO_3SO]^-$	–
	459 > 379	$[M-H]^-$ > $[M-H-SO_3]^-$	–
Pyrenediol sulfate [29]	313 > 233[a]	$[M-H]^-$ > $[M-H-SO_3]^-$	–
	313 > 81[a]	$[M-H]^-$ > $[SO_3H]^-$	–
Pyrene glucuronide sulfate	533 > 473[a]	$[M-H]^-$ > $[M-H-SO_3]^-$	–
	533 > 217[a]	$[M-H]^-$ > $[PYO]^-$	–
Pyrenediol disulfate	393 > 233[a]	$[M-H]^-$ > $[M-H-(SO_3)_2]^-$	F+G
	393 > 313[a]	$[M-H]^-$ > $[M-H-SO_3]^-$	F+G
	313 > 81[a]	$[M-H-SO_3]^-$ > $[SO_3H]^-$	F+G
	313 > 233[a]	$[M-H-SO_3]^-$ > $[M-H-(SO_3)_2]^-$	–
Pyrenediol glucuronide sulfate	489 > 313	$[M-H]^-$ > $[M-H-C_6H_8O_6]^-$	H
	489 > 233[a]	$[M-H]^-$ > $[M-H-SO_3-C_6H_8O_6]^-$	H
	489 > 409	$[M-H]^-$ > $[M-H-SO_3]^-$	H
	409 > 233[a]	$[M-H-SO_3]^-$ > $[M-H-SO_3-C_6H_8O_6]^-$	–
Pyrenediol glucuronide	409 > 233	$[M-H]^-$ > $[M-H-C_6H_8O_6]^-$	–
Pyrenediol diglucuronide	585 > 409[a]	$[M-H]^-$ > $[M-H-C_6H_8O_6]^-$	–
	585 > 233	$[M-H]^-$ > $[M-H-(C_6H_8O_6)_2]^-$	–

[a] Transitions included in the final optimized MRM method.

cleanup was necessary as the crude extracts could only be concentrated to about 1 mL/g of extracted tissue before excessive precipitation and high viscosity made injection on an analytical LC column inappropriate. Several 20% fractions from exposed animals were pooled and concentrated for LC–MS identification of new metabolites. In this sample, peak G was at a level about 50 times more concentrated than in a typical extract.

3.3.4. LC–MS/MS identification of metabolites

Using information from the literature pertaining to the biotransformation of PY [27,29,40] and the detection of bioconjugates by tandem mass spectrometry [42], a list of target metabolites and their MS/MS fragmentations was constructed (Table 4). These consisted of mono and di-conjugated species including conjugates with sulfuric acid, glucuronic acid and glucose. Using LC–MS/MS in MRM mode, crude extracts of visceral mass from PYOH exposed animals and the pooled 20% acetonitrile fractions from the SPE fractionation were screened for known and hypothesized transitions of these metabolites in ESI negative ion mode.

MRM signals for transitions of the known PY metabolites identified by LC–FLD were detected and their identities confirmed in the unconcentrated extracts (Fig. 3). Because ESI is a soft ionization technique and PYO^- is stable enough to resist collision induced dissociation (CID), the transition 217 > 217 gave a pseudo single ion monitoring trace (Fig. 3a) showing a peak at the retention time of PYOH. Weak signals were also observed in this trace due to the *m*/*z* 217 fragment that formed in the ion source from PYOS and PYOG. These two metabolites were detected specifically with transitions corresponding to the loss of sulfur trioxide (SO_3) (297 > 217) or anhydroglucuronic acid (393 > 217) in the q2 collision chamber to give the PYO^- ion at *m*/*z* 217 (Fig. 3b and c, respectively). Three transitions corresponding to predicted losses were detected for PYDS (Fig. 3d–f). The transition 393 > 313 corresponded to the loss of SO_3, 393 > 233 to the loss of two SO_3 and 313 > 81 to the loss of PYO from $[M-H-SO_3]^-$ ion to give a bisulfite ion (HSO_3^-). These transitions were detected weakly at a retention time matching that of 1,6-PYDS (Fig. 1) and strongly at the retention time of peak G (Fig. 2). This suggests that peak G represents an isomer of PYDS other than 1,6-PYDS, and therefore a novel biotransformation product. In all cases in the literature where pyrenediol or its conjugates were detected as metabolites of PY they were identified as either the 1,6 or the 1,8 isomers [10,11,51]. It is therefore likely that peak G represents 1,8-PYDS. Confirmation of this assignment could be achieved by NMR characterization or with the availability of a synthetic standard.

The signals for the two isomers of PYDS were detected in the concentrated 20% acetonitrile SPE fraction. In addition, three MRM transitions predicted for pyrenediol conjugated with one sulfuric and one glucuronic acid (Table 4) showed a peak at 11.7 min (Fig. 4). This peak matched that of the smallest peak hydrolyzed by sulfatase (Fig. 2). The signal for 489 > 409 corresponded to the loss of SO_3, 489 > 313 to the loss of anhydroglucuronic acid ($C_6H_8O_6$) and 489 > 233 to the loss of both from pyrenediol glucuronide sulfate. This compound also represents a novel metabolite previously unreported in the literature for PY. A similar conjugate of another, more carcinogenic PAH, benzo[a]pyrene (BaP), has been reported in rats exposed to BaP [52].

Enhanced product ion (EPI) spectra for the novel PYDS metabolite (Fig. 5, panel G) were obtained at various collision energies. This was done to better understand the CID fragmentation of di-conjugated derivatives of PY to optimize sensitive MRM detection. These spectra show that the transition 393 > 313 is strongest at CE = −20 eV while 393 > 233 and 313 > 233 are strongest at CE = −38 eV. Also of interest is the additional loss of a hydrogen radical at higher collision energies. This did not however yield a sensitive MRM transition.

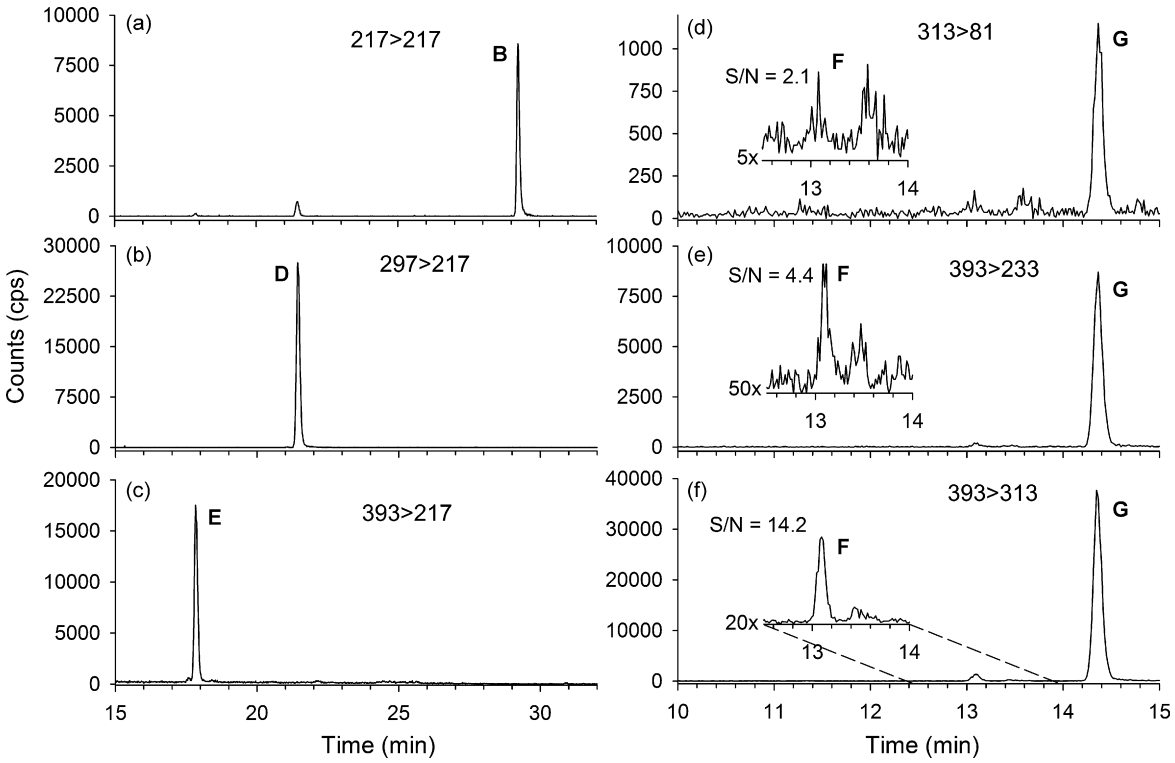

Fig. 3. MRM analysis of the visceral extract from *Neptunea lyrata* exposed to PYOH. Compounds identified include PYOH (B), PYOS (D), PYOG (E), 1,6-PYDS (F) and an isomer of PYDS (G) other than 1,6-PYDS.

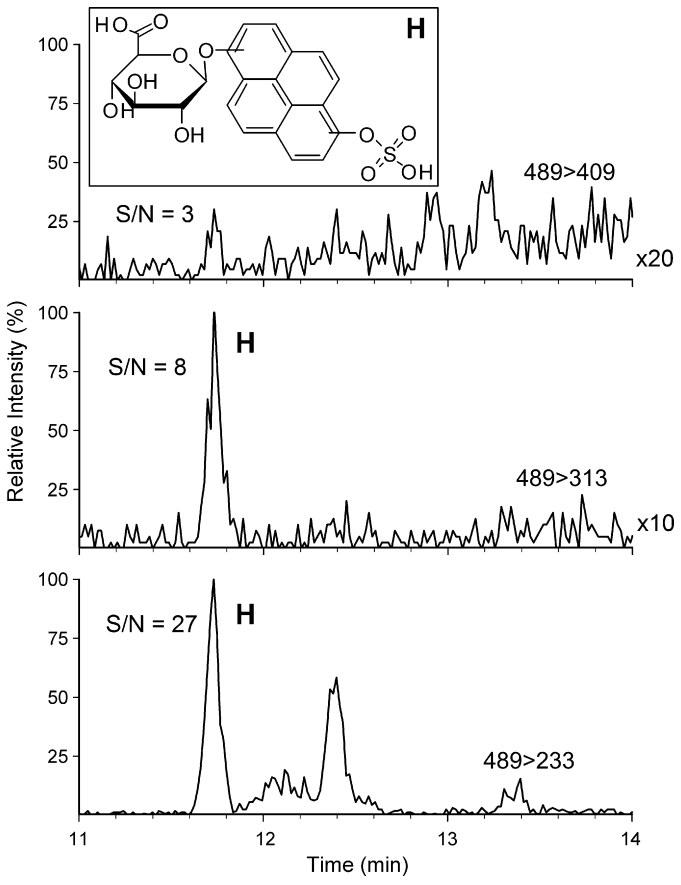

Fig. 4. MRM transitions detected for peak H, pyrenediol glucuronide sulfate, in the 20% acetonitrile SPE fraction of exposed extracts.

The $[M-H]^-$ ions of PYDS and PYOG have the same nominal mass, m/z 393 ($C_{16}H_9O_8S_2^-$ and $C_{22}H_{17}O_7^-$), but can easily distinguished by retention time and product ion spectrum. The EPI spectra for PYOG at various CE (Fig. 5, panel E) show that loss of anhydroglucuronic acid was the predominant transition. At CE of −80 eV, the loss of CO from PYO^- was also observed. This product ion was also observed for PYOS (not shown) and might be useful for the detection of other novel PY biotransformation products. This transition and the absence of product ions characteristic of the glucuronic acid moiety at m/z 175 and m/z 131 indicate a somewhat different gas phase fragmentation of PYOG than has been previously reported [40,53]. This difference was verified using PYOG from a laboratory reference fish bile obtained in a previous study [35]. This difference reinforces the importance of verifying MS system specific fragmentation of a compound before beginning MRM measurements.

Using a standard of PYOH, PYOS and PYOG the change in MRM sensitivity when the source temperature was changed from 250 to 600 °C was examined. This showed an increase in sensitivity of 50–150% depending on the transition. A new MRM method was developed using the more sensitive source temperature and limiting the number of transitions to the two most sensitive pairs for those compounds which were detected (Table 4). Using this method, the method detection limits by MRM were found to be close to 0.04 and 0.2 ng/g for PYOS and PYOG and >40 ng/g for PYOH. The method detection limits for these compounds by LC–FLD (Table 2) were 0.01, 0.06 and 0.1 ng/g for PYOS, PYOG and PYOH, respectively. This illustrates the wide range of ESI–MS sensitivities for metabolites with similar FLD response (Fig. 1). It is only for the acidic phase II metabolites that LC–MS analysis offers similar sensitivity to LC–FLD. To obtain comparable detection limits for phase I metabolites by LC–MS it would be necessary to first fractionate the extracts and derivatize the fraction containing PYOH before analysis. This highlights the advantage of analyzing samples using the strengths of multiple techniques.

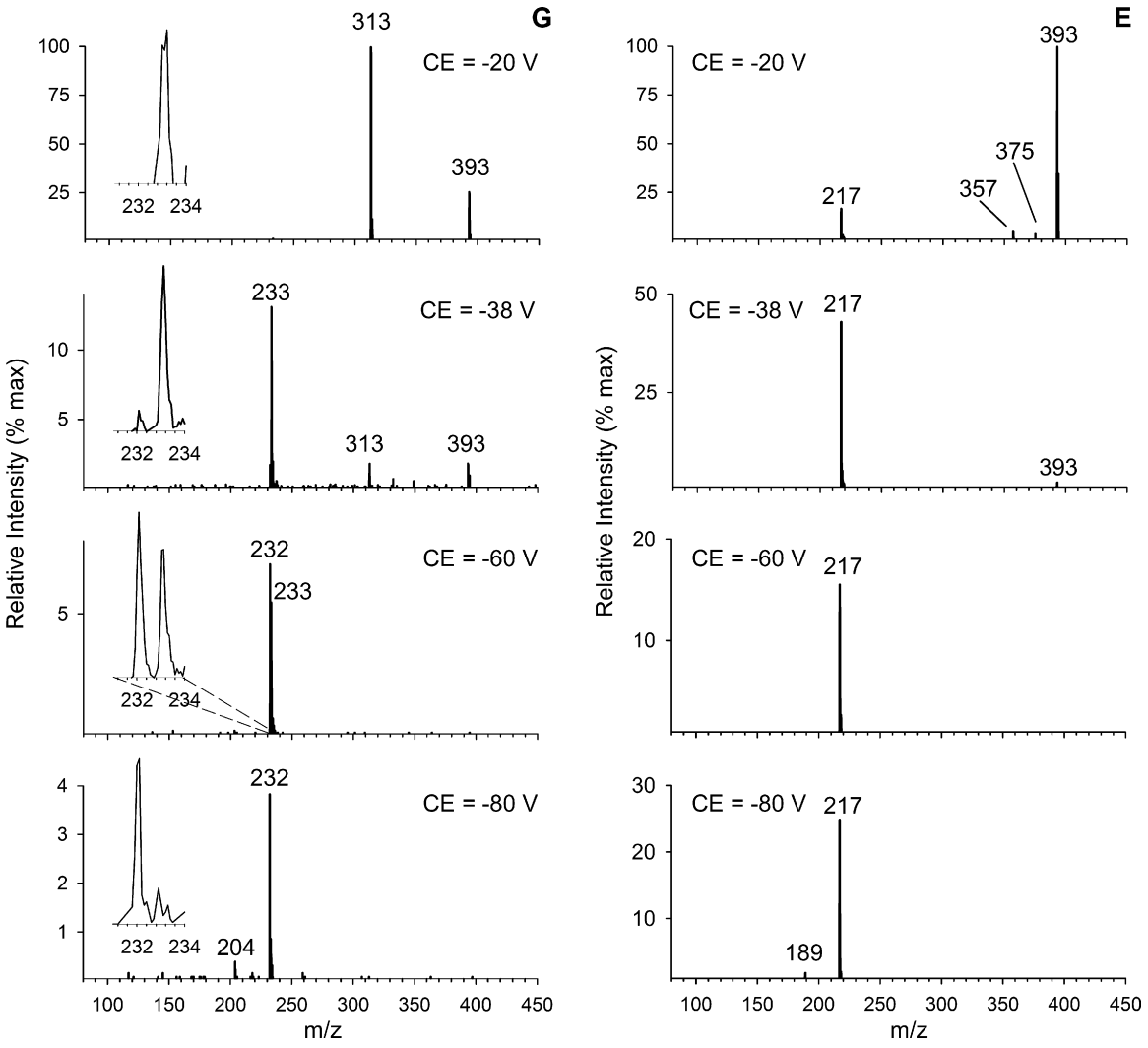

Fig. 5. Enhanced product ion spectra of *m*/*z* 393 in pooled 20% acetonitrile SPE fraction of PYOH exposed *Neptunea lyrata*. Panel G shows spectra of the new PYDS peak and panel E shows spectra of PYOG, both at increasing collision energies of −20, −38, −60 and −80 V.

3.4. Laboratory exposure

The methods described for extraction and analysis of PY metabolites have so far been applied to the study of the biotransformation of both PY and PYOH in three species of marine gastropods *Buccinum* spp., *N. lyrata* and *I. obsoleta*. Laboratory exposures with contaminant uptake through feeding, sediment and seawater were performed. Changes in the bioaccumulation and biotransformation of PY and PYOH over time, dose and between species have been investigated [46]. As an example of one such experiment, the levels of PY metabolites in the visceral mass of *N. lyrata* which were fed one dose of food spiked with PYOH were compared to those of laboratory control animals. Control *N. lyrata* collected at time 0 in the PYOH exposure showed trace levels of PY metabolites (Table 5 and Fig. 6). This background can be attributed to the sediment used in the holding tanks before the exposure, where a few ng/g of PY and no PYOH were detected. When compared to other published PY concentrations in clean sediment, which have varied from <0.001 ng/g [54] to 601 ng/g [55], the detection of metabolites in these whelk illustrates the sensitivity of the analytical methods. It is also of interest to note that of the PY derivatives detected in the control animals, the novel isomer of PYDS identified in this study represented the major constituent. Furthermore, as the control animals were exposed to trace levels of PY and not PYOH, the novel isomer of PYDS is shown to be a metabolite of PY as well as PYOH.

Comparison of tissue extracts from exposed animals to control animals (Table 4) shows bioaccumulation and biotransformation of PYOH from the contaminated food source. The primary metabolites produced from PYOH were the mono- and di-sulfated conjugates.

The presence of PYDS as a major metabolite of PY and PYOH illustrates the importance of investigating the identity of the biotransformation products of contaminants in organisms. In this case, the use of hydrolysis followed by measurement of PYOH would not give an accurate representation of the tissue burdens of PY biotransformation products. It is also important to note that di-conjugated

Table 5
Mean PYOH and metabolite concentrations for the visceral mass of control and PYOH exposed *Neptunea lyrata*.

Compound	Control[a]	Exposed[a]
PYOH	0.7 ± 0.9	14 ± 10
PYOS	1.6 ± 0.9	26 ± 27
PYOG	0.5 ± 0.7	3 ± 4
PYDS	7 ± 3	20 ± 8
Σ PYOH	9 ± 4	62 ± 38

[a] Mean ± standard deviation for $n = 4$ (ng PYOH/g wet tissue).

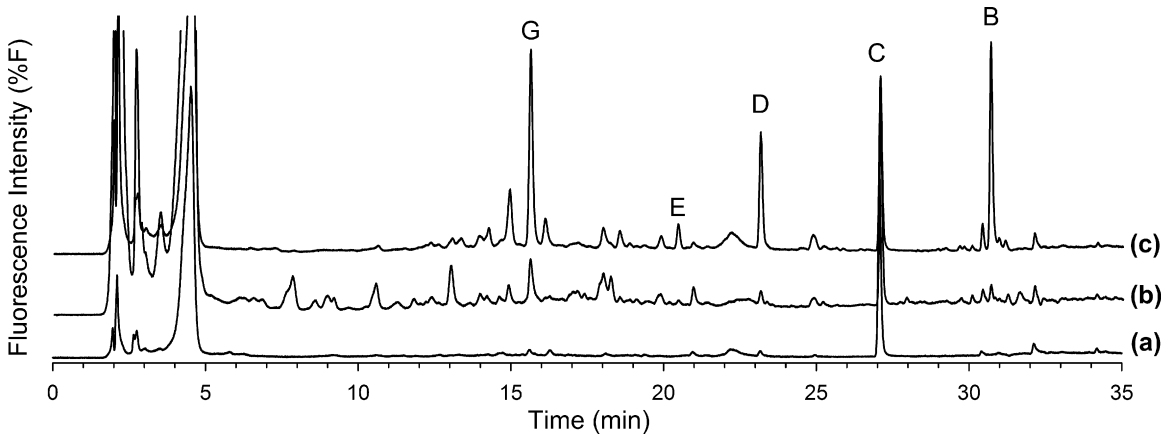

Fig. 6. LC–FLD chromatograms of tissue extracts from the visceral tissue of a control *Neptunea lyrata* (b) and the muscle (a) and visceral (c) tissue of *Neptunea lyrata* exposed to PYOH. The labelled peaks represent the quantified compounds PYOH (B), PYOS (D), PYOG (E), PYDS (G) and the spike FLOH (C).

biotransformation products of pyrene have also been identified in other organisms, including humans [56], rats [51] and clams [29] but so far not in finfish.

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