Journal of Hazardous Materials 223–224 (2012) 84–93

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

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

Two-stage bile preparation with acetone for recovery of fluorescent aromatic compounds (FACs)

Ali Karami[a,*], Mohd A. Syed[b], Annie Christianus[a], Kristine L. Willett[c], Jeffrey R. Mazzeo[d], Simon C. Courtenay[e]

[a] Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Selangor, Malaysia
[b] Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Selangor, Malaysia
[c] Department of Pharmacology and Environmental Toxicology Research Program, University of Mississippi, University, MS 38677, USA
[d] Pharmaceutical Business Operations, Waters Corporation, 34 Maple Street, Milford, MA 01757, USA
[e] Fisheries and Oceans Canada at the Canadian Rivers Institute, Department of Biology, University of New Brunswick, Fredericton, New Brunswick, Canada

ARTICLE INFO

Article history:
Received 19 December 2011
Received in revised form 19 April 2012
Accepted 23 April 2012
Available online 27 April 2012

Keywords:
Fish bile
Fluorescent aromatic compounds (FACs)
Acetone
Two-stage preparation
Protein

ABSTRACT

In this study we sought to optimize recovery of fluorescent aromatic compounds (FACs) from the bile of African catfish (*Clarias gariepinus*) injected with 10 mg/kg benzo[*a*]pyrene (BaP). Fractions of pooled bile were hydrolyzed, combined with ten volumes of methanol, ethanol, acetonitrile, or acetone, centrifuged and supernatants were analyzed by high-performance liquid chromatography with fluorescent detection (HPLC/FL). As well, to test whether FACs were being lost in solids from the centrifugation, pellets were resuspended, hydrolyzed and mixed with six volumes of the organic solvent that produced best FAC recovery from the supernatant, and subjected to HPLC/FL. Highest FAC concentrations were obtained with 2000 μl and 1250 μl acetone for supernatants and resuspended pellets respectively. FACs concentrations were negatively correlated with biliary protein content but were unaffected by addition of bovine serum albumin (BSA) followed by no incubation indicating that the presence of proteins in the biliary mixture does not simply interfere with detection of FACs. In another experiment, efficiency of acetone addition was compared to two different liquid–liquid extractions (L–LEs). Acetone additions provided significantly higher biliary FACs than the L–LE methods. The new two-stage bile preparation with acetone is an efficient, inexpensive and easily performed method.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Accurate methods of pollutant monitoring are required to address increasing concerns about the adverse effects of xenobiotics discharge into aquatic environments. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous xenobiotics with potential for mutagenicity, carcinogenicity and teratogenicity [1]. Physical and chemical analyses of the sediment and water are unable to estimate the bioavailability of such contaminants in aquatic environments. To address this gap, fish biomarkers have been widely used.

Biliary compounds have been quantified for monitoring of recent and ongoing exposures to many xenobiotics including PAHs [e.g., 2–7]. After biotransformation and conjugation with hydrophilic compounds, PAH metabolites and residual parent compounds accumulate in the gall bladder before being excreted via the intestine. Due to active biotransformation of PAHs in fish, tissue concentrations of PAHs may underestimate the presence of PAHs in the environment [8,9]. As well, PAH concentrations in sediments may not correlate well with concentrations in fish sampled from the same area because many fish species are highly mobile [10,11].

High-performance liquid chromatography with fluorescent detection (HPLC/FL) is commonly used for quantifying PAH parent compounds and metabolites as fluorescent aromatic compounds (FACs) in the bile of fish [12]. Generally bile has undergone liquid–liquid extraction (L–LE) [13–16], or is diluted with organic solvents after hydrolysis [17–35]. Inefficient preparation procedures can underestimate the presence of PAHs in bile and therefore in the aquatic environment.

The first aim of this study was to compare efficiencies of previously applied solvents (acetonitrile, methanol, and ethanol) to a new solvent (acetone) during bile sample preparation and to optimize the volume of organic solvent addition. Secondly, we investigated whether FACs are being lost in the hydrolysis and

Abbreviations: PAHs, polycyclic aromatic hydrocarbons; HPLC/FL, high-performance liquid chromatography with fluorescent detection (HPLC/FL); FACs, fluorescent aromatic compounds; L–LE, liquid–liquid extraction; 7,8-D BaP, 7,8dihydrodiolbenzo[*a*]pyrene; 1-OH BaP, 1-hydroxybenzo[*a*]pyrene; 3-OH BaP, 3-hydroxybenzo[*a*]pyrene; BaP, benzo[*a*]pyrene; BSA, bovine serum albumin.

* Corresponding author. Present address: Institute of Bioscience, Universiti Putra Malaysia, 43400 Selangor, Malaysia. Tel.: +60 172500449.
E-mail address: alikaramiv@gmail.com (A. Karami).

0304-3894/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jhazmat.2012.04.051

centrifugation step. Almost all the previous studies have discarded the pellet and quantified FACs from only the supernatant. Only in a recent study by Ruczynska et al. [25], pellet was dissolved in methanol omitting the deconjugation step. Therefore, to ensure better recovery of biliary FACs in pellets, deconjugation step was considered in this study. In a further experiment, the efficiency of the best volume and type of organic solvent was compared with L–LE methods. It has been suggested that proteins can interfere with quantification of biliary FACs through binding or other mechanisms [36]. Therefore, for the last experiment we investigated the influence of proteins on quantification of FAC concentration.

2. Materials and methods

2.1. Chemicals

7,8Dihydrodiolbenzo[*a*]pyrene (7,8-D BaP), 1-hydroxybenzo[*a*]pyrene (1-OH BaP), and 3-hydroxybenzo[*a*]pyrene (3-OH BaP) were purchased from the Mid-west Research Institute (USA); β-glucuronidase/arylsulfatase (30/60 U/ml, from *Helix pomatia*) and ethanol (HPLC grade) were from Merck (Germany), bovine serum albumin (BSA) and benzo[*a*]pyrene (BaP) from Sigma Chemical (USA); methanol (HPLC grade) and acetone (HPLC grade) were supplied by JT Baker (USA); acetonitrile (HPLC grade) from Sigma–Aldrich (USA); 2-propanol (min 99.7% purity) from R&M Chemicals (UK); ethyl acetate (99.5% purity) from AnalaR, BDH (UK); methylene chloride (99.99% purity) from Fisher Scientific (USA); low-binding protein membrane filters (0.45 μm) were obtained from Pall Life Sciences (USA); and distilled water (HPLC grade) was produced in the laboratory.

2.2. Fish and sampling

Three and half month old (immature) hatchery-reared African catfish [*Clarias gariepinus*; mean weight (±SE) 167 (±10.3) g] were fasted 48 h prior to starting the experiment. Thirty-five fish were intraperitoneally injected with 10 mg/kg body weight BaP and killed 48 h later with an overdose of clove oil. Gall bladders were carefully excised and put in safe-locked eppendorf tubes, snap-frozen in liquid nitrogen, and stored at −80 °C. Prior to chemical extractions, gall bladders were thawed on ice, punctured to remove bile. Bile from all fish were combined together and the pooled bile was vortexed for 10 min and stored in darkness at 4 °C until analyses for no longer than 10 days.

2.3. Organic solvent addition

2.3.1. Stage one (Experiment I)

Replicates of 20 μl of the pooled bile were added to 460 μl distilled water (DW) and 20 μl β-glucuronidase/arylsulfatase enzyme solution in a falcon tube. The mixture was vortexed for 10 seconds and incubated in darkness at 37 °C for 2 h in a water bath shaker. The temperature was then reduced by placing the mixture on ice for 5 min. Different volumes (250, 500, 1000, 1250, 1500, 2000, 2500, 3500, 4500 or 7500 μl) of organic solvents (methanol, ethanol, acetone or acetonitrile) were added to the mixture. The mixture was vortexed for ten seconds, incubated for 25–30 min on ice on a shaker in darkness, and then centrifuged at 11,000 × *g* at 2 °C for 6 min. The supernatant was divided into two parts and then kept on ice prior to analyses within 12 h. One part of the supernatant was used for FAC determination by HPLC/FL while the other part was tested for protein concentration by the method of Bradford [37], preparing a serial dilution of BSA on a microplate. The details of HPLC analysis have been described in Karami et al. [38]. During protein estimation, all the values were reduced by the values of their respective blanks. Since glutathione conjugated metabolites are generally stored in liver rather than bile [39,40], only the glucuronides and sulfate-conjugated metabolites were quantified in this study.

In this study all the organic solvents were chilled and DW was cooled (2 °C) prior to usage. Prior to loading into the HPLC vials all samples were filtered with 0.45 μm low-binding protein membrane filters. All treatments were done in triplicate.

2.3.2. Stage two (Experiment II)

Seventy-two bile fractions were added to DW, hydrolyzed, and 2000 μl of acetone (the treatment that produced highest FAC concentrations in Experiment I) were added to the mixtures as described above. After centrifugation the supernatants were discarded carefully and falcon tubes containing pellets were kept at 4 °C for 30 min to evaporate the residues of organic solvents that could interfere with enzymatic activities. Pellets were resuspended in 470 μl DW and vortexed for 5 min. Thereafter, 10 μl of β-glucuronidase/arylsulfatase enzyme solution was added, incubated in a water bath shaker for 2 h at 37 °C and then put on ice for 5 min. Organic solvents (methanol, ethanol, acetone or acetonitrile) were added at six volumes (250, 500, 1000, 1250, 1500 or 2000 μl). The mixtures were vortexed, incubated on ice on a shaker for 25–30 min, and centrifuged at 11,000 × *g* for 6 min at 2 °C. Similar to Experiment I, supernatants were divided into two parts for quantification of protein and FAC contents.

As a supplementary stage, after the addition of 2000 μl acetone to the hydrolyzed bile samples, pellets were resuspended, hydrolyzed and 1250 μl acetone (the treatment producing highest biliary FACs in Experiment II) was added to the mixtures. Pellets were resuspended in DW as described above, and after hydrolysis six volumes (250, 500, 1000, 1250, 1500 or 2000 μl) of the organic solvents were added to the mixtures. After centrifugation, supernatants were quantified for proteins and FACs.

2.4. Liquid–liquid extractions (Experiment III)

Two L–LE procedures generally based on the method of Steward et al. [14] were used in this experiment. Twenty μl of the bile pool was diluted with 500 μl 0.2 M sodium acetate buffer, pH 5. Unconjugated metabolites were extracted in a three-step procedure: first with ethyl acetate:acetone (2:1, v/v) and then twice with ethyl acetate. Organic solvents were combined and reduced under nitrogen gas stream. Thereafter, the residue was dissolved in 800 μl methanol. The aqueous phase was blown down under nitrogen gas stream to remove the traces of organic solvents. Twenty microliters of β-glucuronidase/arylsulfatase enzyme solution was added to the aqueous phase, vortexed and incubated in darkness at 37 °C for 2 h in a water bath shaker. Thereafter, deconjugated metabolites during the hydrolysis procedure were extracted with ethyl acetate:acetone (2:1, v/v) and then twice with ethyl acetate. The organic fractions were recombined and analyzed for conjugated FACs as described above. To compare the influence of different organic solvents on extraction of FACs during L–LE, the method of Chen and Chang [41] was modified and adapted to the method of Steward et al. [14]. Extraction was done by 2-propanol:methylene chloride (10%, v/v) for the first step followed twice by methylene chloride. Different volumes (680, 1360, 2040 or 3400 μl) of organic solvents (ethyl acetate:acetone and ethyl acetate, 2-propanol:methylene chloride and methylene chloride) were used during the extraction processes.

2.5. BSA addition (Experiment IV)

To test the impact of proteins on detection of FACs, different BSA dosages were added to the bile mixtures. Briefly, 480 μl

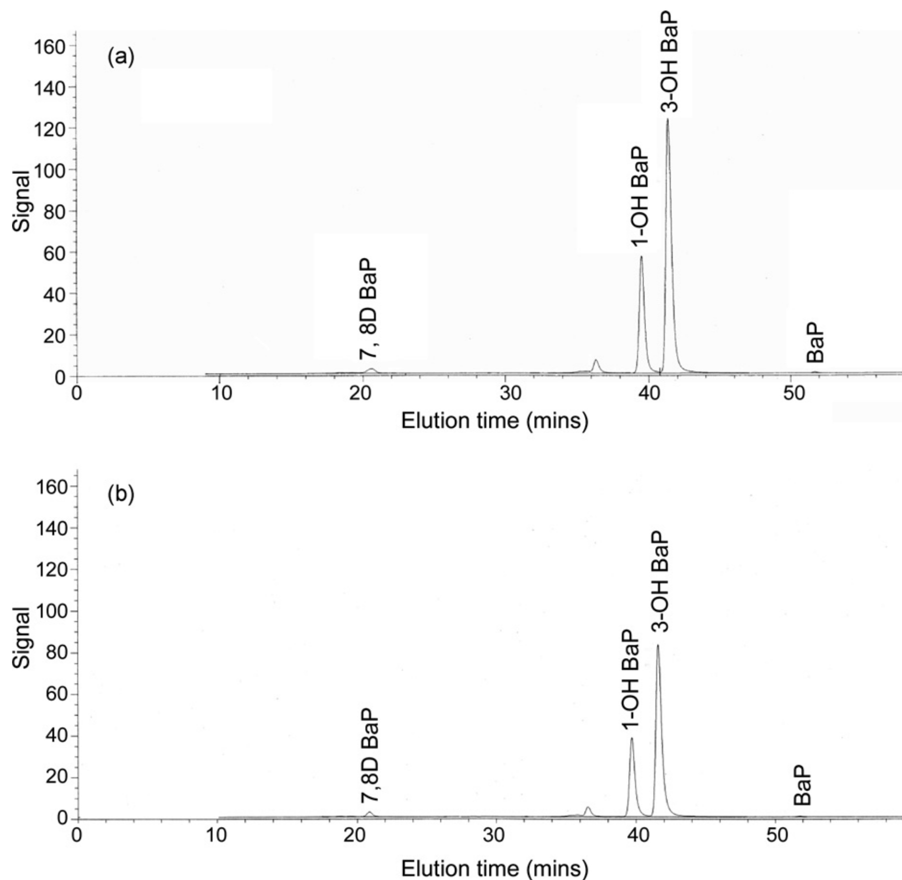

Fig. 1. High performance liquid chromatography (HPLC) chromatograms of biliary FACs after addition of 2000 μl of: (a) acetone, and (b) methanol in Experiment I.

of the pooled bile was mixed with 11,040 μl DW and 480 μl β-glucuronidase/arylsulfatase enzyme solution. Thereafter, the mixture was vortexed and incubated in darkness at 37 °C in a water bath shaker for 2 h. After hydrolysis, the mixture was divided into two parts: half was divided into six 1-ml solutions to which 0, 0.5, 1, 2, 5 or 7 mg of BSA was added before vortexing, the other half was divided into six 1-ml solutions to which 4000 μl acetone was added followed by centrifuging as described above. Afterwards, 8000 μl DW and 0, 0.5, 1, 2, 5 or 7 mg of BSA was added to 1-ml of solutions followed by vortexing and quantification of biliary FACs by HPLC/FL. The experiment was done in triplicate.

2.6. Statistics

In Experiments I, II and III separate two-way ANOVAs were applied on each FAC to test the differences between the type, and volume of organic solvents, and their interaction (solvent × volume). Duncan's multiple range tests were run when significant differences were detected. Separate Student's *t*-tests were applied on each FACs to test the differences between the selected treatment, the treatment which resulted in the highest biliary FACs, of the organic solvent experiments and the selected treatment of L–LE experiment (Experiments I + II vs. Experiment III). Differences among biliary protein concentrations in Experiments I and II were tested by two-way ANOVAs with factors: organic solvent, volume, and solvent × volume interaction. In addition, the strength and significance of relationships between the protein and the FAC concentrations in Experiments I, II, and IV were tested by Pearson's correlation. For Experiment IV, one-way ANOVAs were used to test the influence of BSA additions on each biliary FAC concentration. Post hoc Duncan's multiple range tests were run when significant differences were detected.

2.7. Ethics statement

All experiments were done in accordance with Malaysian legislation and the Code of Practice and accreditation criteria of the University Federation of Animal Welfare, UK (UFAW) [42].

3. Results

3.1. Experiment I: optimizing recovery of FACs from supernatants

HPLC chromatograms of biliary FACs after the addition of 2000 μl acetone and methanol are depicted in Fig. 1.

The effect of solvent volume on recovered biliary FAC concentrations differed with solvent type (solvent × volume interaction, Table 1). This interaction was driven by 1-OH BaP, 3-OH BaP and BaP rather than by 7,8-D BaP (Table 1). The main objective of this study was to select the best treatment combination (solvent × volume); therefore, Duncan's multiple range tests were run on the concentrations of 1-OH BaP, 3-OH BaP and BaP over treatment combination (solvent × volume, Table 2). Due to the non-significant solvent × volume interaction separate Duncan's multiple range tests were run on 7,8-D BaP concentrations over solvent, and volume (Fig. 2a and b, respectively). The three organic solvents acetone, ethanol and acetonitrile produced relatively similar concentrations of 7,8-D BaP (Fig. 2a) and recovery was increased up to volume of 2000 μl and then declined with the higher solvent volumes (Duncan's multiple range tests, Fig. 2b).

Table 1
Two-way ANOVAs testing for effect of organic solvent (solvent) and volume of organic solvent (volume) on the selected biliary FACs (7,8-D BaP, 1-OH BaP, 3-OH BaP, BaP) in Experiment I; df error = 80.

Biliary FAC	Source of variation	df	F	P
7,8-D BaP	Solvent	3	3.29	0.025*
	Volume	9	12.01	<0.001*
	Solvent × volume	27	1	0.47
1-OH BaP	Solvent	3	86.22	<0.001*
	Volume	9	199.78	<0.001*
	Solvent × volume	27	9.98	<0.001*
3-OH BaP	Solvent	3	103.32	<0.001*
	Volume	9	245.28	<0.001*
	Solvent × volume	27	9.7	<0.001*
BaP	Solvent	3	6.99	<0.001*
	Volume	9	81.42	<0.001*
	Solvent × volume	27	3.25	<0.001*

* Significant difference at $P < 0.05$ level.

Concentrations of BaP parent compound and metabolites generally increased with increased volumes of acetone solvent up to 2000 μl but then decreased with higher acetone volumes (Duncan's multiple range tests; Table 2).

3.2. Experiment II: optimizing recovery of FACs from centrifuged pellets

The effect of solvent volume on recovery of FACs from pellets differed with solvent (Two-way ANOVA, solvent × volume interaction term, $P < 0.001$). Duncan's multiple range tests showed the highest FACs concentrations were obtained by the addition of 1250 μl acetone (Table 3). Similar to the previous experiment, higher replications may be required in subsequent studies to significantly exhibit differences.

Biliary FAC concentrations recovered in Experiments I and II were strongly and negatively correlated with protein concentration of the mixture injected into the HPLC (Table 4). Due to influence of solvent volume on protein concentration with some solvents (Experiments I and II, Two-way ANOVAs, solvent × volume interaction term, $P < 0.001$), protein concentrations were examined by solvent volume. In 7 volumes of Experiment I and 4 volumes of Experiment II, acetone caused the lowest protein concentrations in the mixture (data not shown).

Repeating the extractions of centrifuged pellets carried out in Experiment II produced negligible FAC concentrations (non-detectable in most cases). Therefore, no statistical analysis was run on the results.

Table 2
Mean (±SE) concentrations (μM) of biliary 7,8-D BaP, 1-OH BaP, 3-OH BaP and BaP over treatment combinations in Experiment I. Values with different letters are significantly different (Duncan's multiple range tests, $P < 0.05$), $n = 3$.

Solvent	Volume (μl)	Biliary FAC			
		7,8-D BaP	1-OH BaP	3-OH BaP	BaP
Acetone	250	10.16 ± 0.31	4.96 ± 0.25^{a}	7.49 ± 0.4^{a}	0.45 ± 0.01^{a}
	500	21.34 ± 1.37	205.88 ± 7.47^{d}	270.1 ± 14.93^{def}	1.15 ± 0.06^{ab}
	1000	27.56 ± 3.1	301.08 ± 8.48$^{f–j}$	380.55 ± 12.1$^{h–l}$	2.52 ± 0.13$^{c–f}$
	1250	31.61 ± 4.08	321.76 ± 7.36^{ij}	421.15 ± 8.54^{lm}	3.46 ± 0.16$^{e–i}$
	1500	28.08 ± 2.46	323.05 ± 4.4^{j}	414.64 ± 3.7$^{j–m}$	3.57 ± 0.33$^{f–j}$
	2000	38.77 ± 2.73	382.62 ± 10.43^{k}	456.79 ± 12.17^{m}	5.44 ± 0.31^{m}
	2500	30.14 ± 1.28	304.11 ± 16.26$^{f–j}$	405.47 ± 24.75$^{i–l}$	2.75 ± 0.1$^{c–g}$
	3500	27.98 ± 1.07	317.32 ± 14.95^{hij}	414.07 ± 22.88$^{j–m}$	3.69 ± 0.32$^{g–j}$
	4500	23.28 ± 2.06	296.39 ± 10.33$^{f–j}$	401.89 ± 16$^{i–l}$	4.12 ± 0.25$^{h–k}$
	7500	27.45 ± 1.34	297.23 ± 16.44$^{f–j}$	408.26 ± 19.56$^{i–m}$	5.4 ± 0.08^{m}
Methanol	250	4.45 ± 0.59	21.19 ± 0.94^{a}	26.2 ± 3.12^{a}	0.74 ± 0.11^{a}
	500	9.46 ± 0.39	10.15 ± 0.57^{a}	15.3 ± 1.26^{a}	0.66 ± 0.06^{a}
	1000	21.45 ± 4.83	85.45 ± 30.42^{b}	115.77 ± 37.58^{b}	1.32 ± 0.16^{ab}
	1250	19.95 ± 2.19	139.14 ± 27.76^{c}	205.28 ± 39.61^{c}	1.96 ± 0.12^{bcd}
	1500	28.5 ± 6.71	211.18 ± 10.55^{d}	249.73 ± 23^{cde}	2.39 ± 0.31^{cde}
	2000	28.1 ± 5.34	236.18 ± 7.86^{de}	312.32 ± 7.58^{fg}	2.86 ± 0.13$^{d–g}$
	2500	32.124 ± 6.93	270.63 ± 8.03^{efg}	336.76 ± 9.45^{gh}	2.6 ± 0.09$^{c–f}$
	3500	30.35 ± 7.02	291.13 ± 14.55$^{f–j}$	360.61 ± 3.13$^{g–j}$	4.27 ± 0.74$^{i–l}$
	4500	31.11 ± 8.36	279.39 ± 6.83$^{e–j}$	387.53 ± 15.91$^{h–l}$	4.25 ± 0.48$^{i–l}$
	7500	24.49 ± 3.64	270.51 ± 7.68^{efg}	368.42 ± 14.07$^{h–l}$	5.16 ± 0.18^{lm}
Ethanol	250	7.42 ± 2.6	16.14 ± 2.91^{a}	25.4 ± 3.29^{a}	0.69 ± 0.17^{a}
	500	18.3 ± 2.78	89.93 ± 2.47^{b}	131.03 ± 9.02^{b}	0.61 ± 0.03^{a}
	1000	25.58 ± 4.41	206.52 ± 27.91^{d}	281.33 ± 20.68^{ef}	1.77 ± 0.13^{bc}
	1250	27.83 ± 1.56	276.76 ± 6.27$^{e–i}$	373.68 ± 12.01$^{h–l}$	3.21 ± 0.51$^{e–i}$
	1500	36.58 ± 5.56	280.25 ± 7.21$^{e–j}$	375.45 ± 10.93$^{h–l}$	3.23 ± 0.14$^{e–i}$
	2000	35.56 ± 2.73	264.36 ± 32.9^{ef}	369.12 ± 33.22$^{h–l}$	3.36 ± 0.36$^{e–i}$
	2500	36.48 ± 6	273.31 ± 6.35$^{e–h}$	371.45 ± 8.74$^{h–l}$	3.97 ± 0.14^{hij}
	3500	33.86 ± 5.78	271.75 ± 5.45$^{e–h}$	370.09 ± 4.45$^{h–l}$	3.59 ± 0.36$^{f–j}$
	4500	35.93 ± 6.06	313.29 ± 18.2$^{g–j}$	402.72 ± 17.13$^{i–l}$	4.58 ± 0.97$^{j–m}$
	7500	27.6 ± 7	277.55 ± 9.99$^{e–i}$	387.1 ± 8.35$^{h–l}$	5.1 ± 0.26^{klm}
Acetonitrile	250	11.63 ± 0.66	7.06 ± 1.33^{a}	8.78 ± 1.75^{a}	0.39 ± 0^{a}
	500	25.13 ± 6.68	165.97 ± 2.8^{c}	229.39 ± 8.8^{cd}	1.28 ± 0.52^{ab}
	1000	35.13 ± 5.88	269.35 ± 7.15^{efg}	357.58 ± 1.64^{ghi}	3.09 ± 0.13$^{e–h}$
	1250	38.45 ± 5.03	316.78 ± 12^{hij}	417.14 ± 14.96^{klm}	3.71 ± 0.42$^{g–j}$
	1500	30.28 ± 5.21	271.86 ± 11.5$^{e–h}$	362.21 ± 12.59$^{h–k}$	2.88 ± 0.13$^{d–g}$
	2000	34.94 ± 5.79	298.46 ± 2.49$^{f–j}$	380.01 ± 6.86$^{h–l}$	2.81 ± 0.12$^{d–g}$
	2500	28.42 ± 3.45	270.11 ± 7.31^{efg}	367.12 ± 8.68$^{h–l}$	3.05 ± 0.33$^{e–h}$
	3500	32.08 ± 3.94	270.94 ± 3.19^{efg}	374.86 ± 6.34$^{h–l}$	4.21 ± 0.56$^{i–l}$
	4500	27.56 ± 4.89	296.75 ± 24.08$^{f–j}$	401.78 ± 23.77$^{i–l}$	4.07 ± 0.19$^{h–k}$
	7500	20.98 ± 2.67	262.99 ± 14.23^{ef}	371.74 ± 2.98$^{h–l}$	5.36 ± 0.15^{m}

Table 3
Mean (±SE) concentrations (μM) of biliary 7,8-D BaP, 1-OH BaP, 3-OH BaP and BaP over treatment combinations in Experiment II. Values with different letters are significantly different (Duncan's multiple range tests, $P < 0.05$), $n = 3$.

Solvent	Volume (μl)	Biliary FAC			
		7,8-D BaP	1-OH BaP	3-OH BaP	BaP
Acetone	250	0.23 ± 0.02^{abc}	0.07 ± 0.03^{a}	0.72 ± 0.03^{a}	0.49 ± 0.03^{a}
	500	0.39 ± 0.05^{def}	1.48 ± 0.29^{bc}	3.67 ± 0.49^{b}	0.76 ± 0.05^{a}
	1000	0.54 ± 0.07^{ghi}	3.3 ± 0.77^{f}	8 ± 0.73^{e}	1.88 ± 0.1^{de}
	1250	0.84 ± 0.03^{m}	4.55 ± 0.42^{g}	10.54 ± 0.47^{f}	2.58 ± 0.07^{f}
	1500	0.74 ± 0.06^{klm}	3.86 ± 0.1^{f}	9.01 ± 0.24^{e}	2.38 ± 0.09^{f}
	2000	0.83 ± 0.05^{m}	3.31 ± 0.14^{f}	8.89 ± 0.24^{e}	2.47 ± 0.06^{f}
Methanol	250	0.21 ± 0.04^{ab}	0.03 ± 0.01^{a}	0.83 ± 0.08^{a}	0.6 ± 0.06^{a}
	500	0.31 ± 0.02^{bcd}	0.3 ± 0.04^{a}	1.15 ± 0.02^{a}	0.78 ± 0.02^{a}
	1000	0.43 ± 0.04^{efg}	0.23 ± 0.15^{a}	1.43 ± 0.04^{a}	1.1 ± 0.1^{b}
	1250	0.64 ± 0.01^{ijk}	1.74 ± 0.21^{bcd}	3.7 ± 0.53^{b}	1.82 ± 0.08^{cde}
	1500	0.66 ± 0.02^{jkl}	1.55 ± 0.21^{bc}	4.14 ± 0.22^{bc}	1.74 ± 0.08^{cde}
	2000	0.77 ± 0.02^{lm}	1.53 ± 0.12^{bc}	3.8 ± 0.18^{b}	1.86 ± 0.02^{de}
Ethanol	250	0.15 ± 0^{a}	0.08 ± 0.02^{a}	0.85 ± 0.04^{a}	0.55 ± 0.02^{a}
	500	0.33 ± 0.05^{cde}	0.24 ± 0.02^{a}	1.03 ± 0.01^{a}	0.71 ± 0.01^{a}
	1000	0.43 ± 0.04^{efg}	1.23 ± 0.36^{b}	3.85 ± 1^{b}	1.17 ± 0.1^{b}
	1250	0.53 ± 0.02^{gh}	2.37 ± 0.16^{de}	5.89 ± 0.45^{d}	1.63 ± 0.1^{cde}
	1500	0.53 ± 0.01^{gh}	2.35 ± 0.04^{de}	5.83 ± 0.43^{d}	1.61 ± 0.16^{cd}
	2000	0.65 ± 0.01^{ijk}	2.17 ± 0.11^{cde}	5.04 ± 0.81^{bcd}	1.9 ± 0.19^{e}
Acetonitrile	250	0.27 ± 0.01^{bcd}	0.16 ± 0.03^{a}	0.91 ± 0.01^{a}	0.64 ± 0.02^{a}
	500	0.32 ± 0.02^{bcd}	1.48 ± 0.13^{bc}	4.09 ± 0.25^{b}	0.71 ± 0.08^{a}
	1000	0.45 ± 0.02^{fg}	1.62 ± 0.12^{bcd}	5.47 ± 0.11^{cd}	1.55 ± 0.05^{c}
	1250	$0.64 \pm 0.04^{h-k}$	2.57 ± 0.26^{e}	6.39 ± 0.46^{d}	1.77 ± 0.09^{cde}
	1500	0.61 ± 0.02^{hij}	2.12 ± 0.14^{cde}	6.21 ± 0.3^{d}	1.78 ± 0.06^{cde}
	2000	0.69 ± 0.03^{jkl}	$1.89 \pm 0.13^{b-e}$	6.17 ± 0.86^{d}	1.75 ± 0.07^{cde}

3.3. Experiment III: liquid–liquid extractions (L–LEs)

Total FACs recovered did not differ significantly between the two types of organic solvent compositions (ethyl acetate:acetone in the first step + ethyl acetate in the second and third steps; 10% 2-propanol: methylene chloride in the first step + methylene chloride in the second and third steps), or among different volumes tested (680, 1360, 2040 or 3400 μl) (before hydrolysis + after hydrolysis, $P > 0.05$; Fig. 3a–d). Recovered FAC concentrations prepared through L–LE procedures were significantly lower than those achieved in Experiments I + II with the addition of acetone (Student's t-test, $P < 0.01$ for the metabolites, and $P < 0.05$ for BaP; Fig. 4a–d). On average acetone addition provided 3.52, 9.17, 5.79 and 3.91 times higher 7,8-D BaP, 1-OH BaP, 3-OH BaP and BaP than the selected L–LE method (680 μl ethyl acetate:acetone in the first step + ethyl acetate in the second and third steps).

3.4. Experiment IV: impact of proteins on FAC detection

BSA additions (0, 0.5, 1, 2, 5 or 7 mg/ml of solution) did not significantly influence recovered FAC concentrations before or after acetone addition (Two-way MANOVA, $P > 0.05$, data not shown). Furthermore, in this experiment the correlations between the protein and FACs concentration were not significant (Table 4).

Table 4
Pearson's correlation coefficients between biliary FACs and protein concentration of the injected mixture in Experiments I, II, and IV. Double asterisk means statistically significant at $P < 0.01$.

Biliary FAC	Experiment I	Experiment II	Experiment IV
7,8-D BaP	−0.762**	−0.636**	0.298
1-OH BaP	−0.862**	−0.728**	−0.341
3-OH BaP	−0.892**	−0.740**	0.066
BaP	−0.657**	−0.585**	−0.22

4. Discussion

Different methods have been developed for preparation of fish bile samples prior to detection of FACs through HPLC/FL. The most applied methods involve addition of organic solvents or L–LE. Among organic solvents used, different volumes of methanol, acetonitrile and ethanol have been applied to the mixture of fish bile after hydrolysis. Though FACs are recognized as being an important fish biomarker, studies of fish bile preparation procedures are scarce. Furthermore, there is a high inter- and even intra-laboratory variation, especially in the application of organic solvents during preparation procedures [17–35].

Acetone is a good protein precipitant [43,44], however, to our knowledge the hydrolyzed bile sample has never been diluted with acetone. In Experiments I and II 2000 and 1250 μl of acetone, respectively, produced the highest BaP FAC concentrations highlighting the importance of optimizing the type and volume of organic solvents during preparation stages.

Several studies have reported higher concentrations of 7,8-D BaP, 1-OH BaP, and 3-OH BaP compared to other biliary FACs (e.g., quinone metabolites and some of the diol and hydroxylated metabolites) [28,38,40,45]. In this study only the three biliary metabolites and the parent compound were detectable in all the treatment combinations. Though 2000 μl acetone was the best choice to liberate 1-OH BaP, 3-OH BaP, and BaP, 2000 μl of acetonitrile or ethanol performed as well for 7,8-D BaP. Nevertheless, choice of optimal organic solvent is specific to the parent compound and metabolite of interest. Therefore, to select the best treatment suite of evidences procedure was followed and we selected 2000 and 1250 μl of acetone for the first and second stage of organic solvent addition, respectively, due to almost the highest performance in liberating all the investigated FACs.

Experiment II demonstrated the presence of biliary BaP FACs residues in the pellets which are trapped during centrifugation of the biliary mixture in stage one, and/or are the unhydrolyzed compounds of the first stage of preparation. This study showed that virtually all biliary BaP FACs become available for quantification

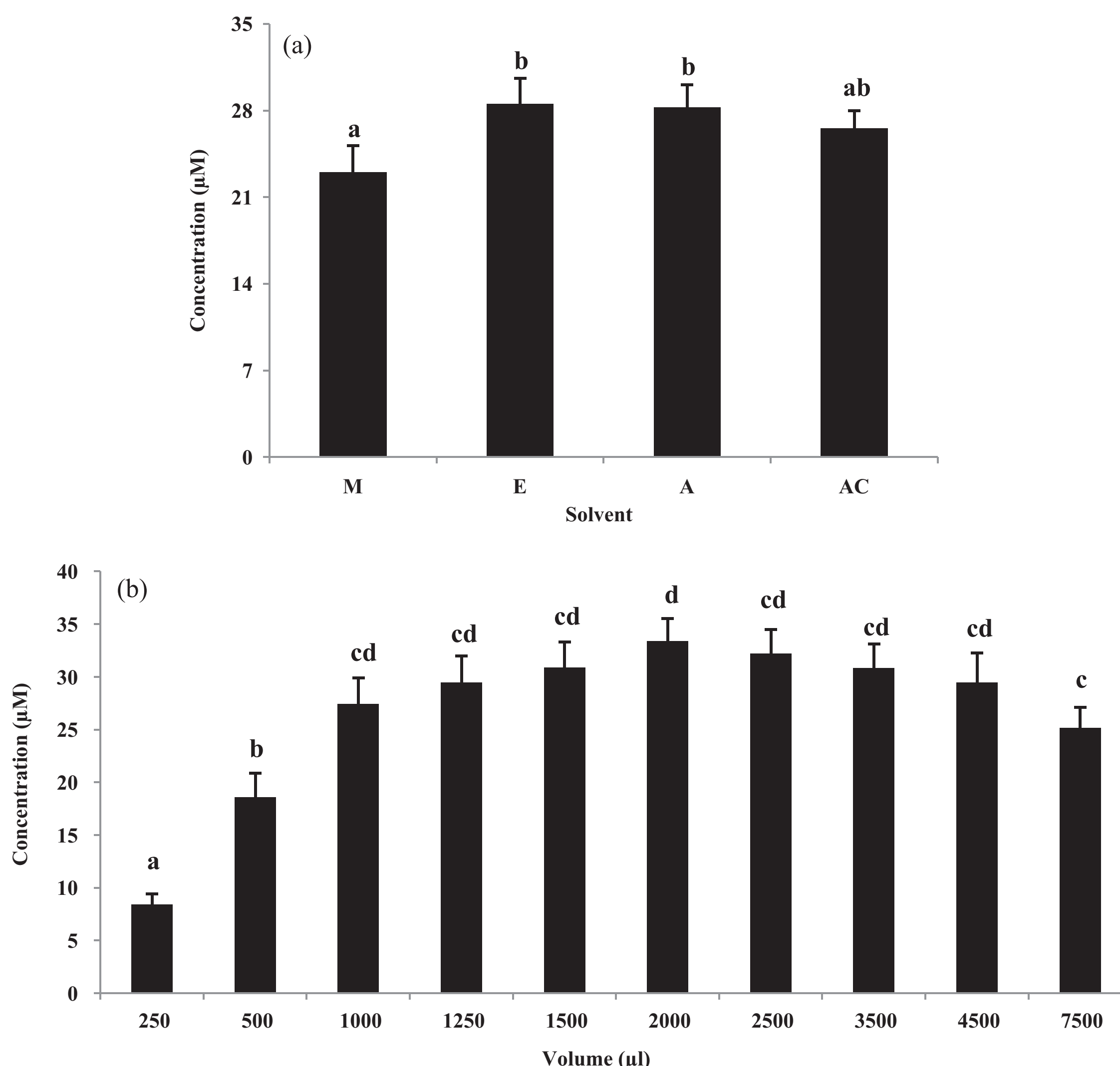

Fig. 2. Mean (±SE) concentrations (μM) of 7,8-D BaP over (d) solvent and (e) volume (μl); AC = acetone, A = acetonitrile; M = methanol; E = ethanol. Values with different letters are significantly different (Duncan's multiple range tests, $P < 0.05$), $n = 30$ for (d), and $n = 12$ for (e); *note*: range of scales is different between figures.

through the two-stage preparation method and that nothing significant is to be gained by further extraction from the pellets. However, this may not be the case when working with the fish in highly PAHs polluted areas. Therefore, when working with the fish from highly polluted areas it may be worth testing a few samples with one more stage. Since only 2.12, 1.17, and 2.25% of total quantified 7,8-D BaP, 1-OH BaP, and 3-OH BaP were recovered from pellet, in the studies with limited time and budget, the second stage of organic solvent addition may be waved. However, due to the large recovery of parent compound by the second stage (32.14%) this decision is not appropriate for the studies which the amount of unbiotransformed biliary FACs (parent compounds) is of concerns.

The most applied L–LE method was the method of Steward et al. [14] (ethyl acetate:acetone in the first step + ethyl acetate in the second and third steps) which has been slightly modified by other authors. In this study to ascertain the extraction efficiency of L–LE methods, biliary compounds were extracted with two organic solvent combinations (ethyl acetate:acetone and ethyl acetate, 2-propanol:methylene chloride and methylene chloride) and each with four different volumes. Results showed the two L–LE methods, and different volumes, produced similar BaP FAC concentrations which were lower than those produced by the two-stage acetone addition (Fig. 4a–d). The lower efficiency of L–LEs could be due to the relatively low solubility of the FACs in the organic solvents applied. In addition to being less efficient, the L–LE procedures are more expensive and time- and labor-intensive than the two-stage organic solvent method.

Samples being analyzed for pharmaceuticals are commonly cleaned of protein due to protein–drug binding [46]. In proteomic studies different precipitants including acids, salts, metal ions, and organic solvents have been widely used to precipitate proteins. For example trichloroaceticacid (TCA) and ammonium sulfate have good efficiencies in precipitation of proteins [47,48]. However, due to the sensitivity of most of UPLC and HPLC columns to a wide range of salts and acids the most applied family of precipitants remains organic solvents. Organic solvents cause aggregation of proteins through expediting electrostatic protein interactions and reducing hydrophobic interactions between proteins [46].

PAHs are known to bind with some proteins [49,50] and possibly become less available for analysis through HPLC. In Experiments I and II, the strong negative correlation between protein content and biliary FACs (Table 4) suggested potential prohibitive function of proteins during FACs detection. Correlations were weaker with pellets (Experiment II) than supernatants (Experiment I), possibly due to denaturation of proteins during the first preparation stage (Experiment I) which might have caused the errors in

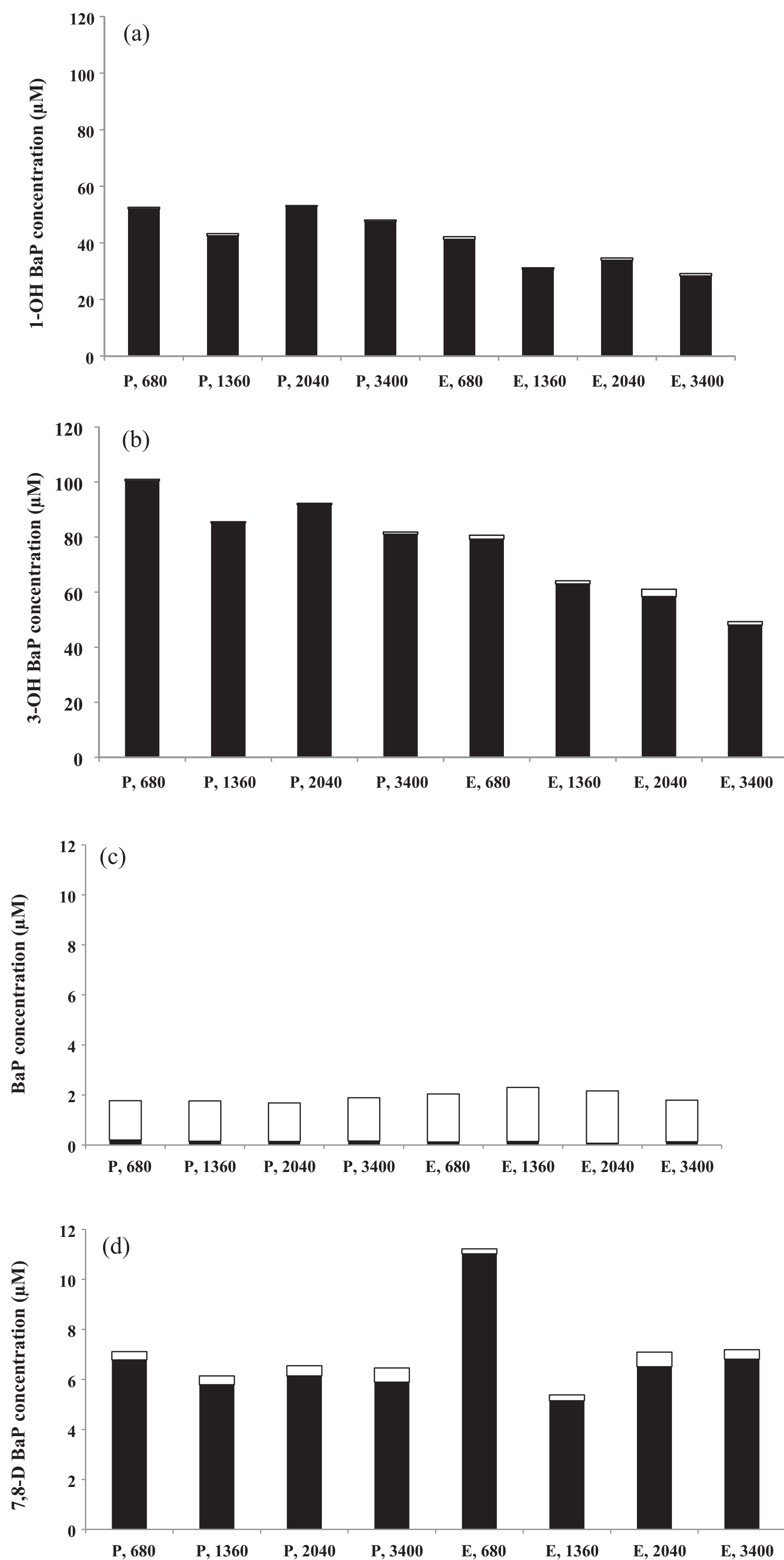

Fig. 3. Mean concentrations (µM) of: (a) 1-OH BaP; (b) 3-OH BaP; (c) BaP; and (d) 7,8-D BaP extracted with different volumes (µl, the values after comma) of ethyl acetate:acetone in the first step + ethyl acetate in the second and third steps (E), and 10% 2-propanol: methylene chloride in the first step + methylene chloride in the second and third steps (P). White area shows the concentrations of biliary FACs before hydrolysis, black area shows concentrations of biliary FACs after hydrolysis; *note*: range of scales is different between figures.

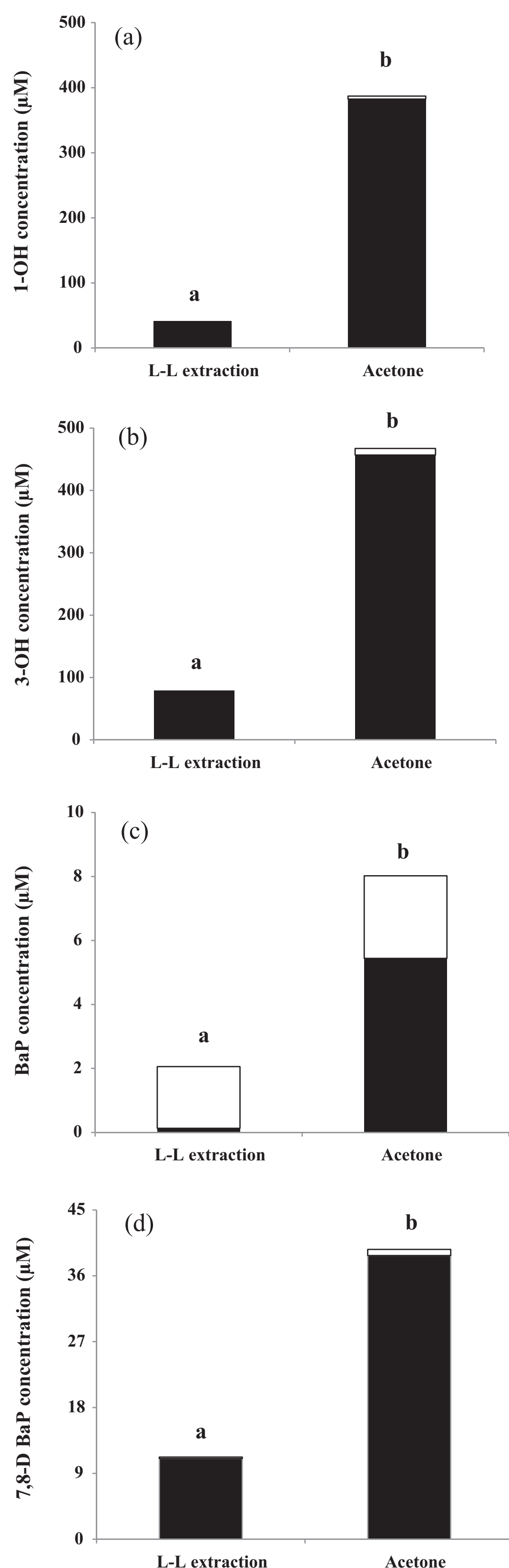

Fig. 4. Mean concentrations (µM) of: (a) 1-OH BaP, (b) 3-OH BaP, (c) BaP and (d) 7,8-D BaP. White area shows the concentrations of biliary FACs before hydrolysis (L–LE, 680 µl ethyl acetate:acetone, ethyl acetate), or in the centrifuged pellets (1250 µl acetone, Experiment II). Black area shows biliary FACs concentration after hydrolysis (L–LE, 680 µl ethyl acetate:acetone, ethyl acetate), or in the supernatants (2000 µl acetone, Experiment I). Bars labeled with different letters are significantly different (Student's *t*-test, $P < 0.01$ for (a–c) and $P < 0.05$ for (d); *note*: range of scales is different between figures.

quantification or function of biliary proteins. However, other than the quantitative differences between protein content of the mixtures in Experiments I and II, proteins precipitated may be specific to the precipitant used [51,52]. Conceivably, in the present experiment, acetone removed biliary proteins with high binding affinities to the biliary BaP FACs allowing a higher concentration of free FACs to be detected by HPLC. This is in agreement with findings that acetone is able to precipitate a wide range of proteins in biological samples [53,54]. It is also possible that the other bile compounds such as salts, ions, pigments, and cholesterol [55] influence the detection of biliary FACs. In this study we did not run any qualitative tests on proteins. Further studies are necessary to identify the role of different proteins in detection of biliary FACs.

In order to bind BSA with PAHs, incubation times of several hours up to several days have been applied [56,57]. Therefore, in this study by not incubating BSA with the biliary mixtures, no PAH–BSA complex is expected. Though in Experiments I and II the role of proteins during the biliary FACs quantification was stressed, addition of BSA prior or after the addition of acetone to the mixture (Experiment IV) did not significantly influence the concentration of biliary FACs. These results prove the solely presence of proteins cannot interfere the detection of PAH biliary metabolites and the mechanism might be through specific binding of some proteins with PAH compounds [58,59]. For further study, we propose hydrolyzing proteins with trypsin to determine the impact of protein bindings on recovery of biliary FACs and using more selective analysis such as liquid chromatography–mass spectrometry (LC/MS).

5. Conclusions

1. To avoid underestimation of biliary FACs, it is necessary to add an appropriate volume and type of organic solvent to the bile samples during the preparation procedure.
2. A two-stage preparation of bile samples is necessary for accurate biliary FACs quantification. Therefore, during the preparation stages all fractions (supernatant and centrifuged solids) must be checked for the presence of FACs. The two-stage preparation method showed the addition of 2000 and 1250 µl of acetone was the most efficient choice for liberating biliary BaP FACs from the supernatant and pellet, respectively.
3. All liquid–liquid extraction methods may not be appropriate for preparation of fish bile samples containing FACs.
4. Proteins have a significant negative influence on the detection of biliary FACs. However, the inhibition mechanism requires further study.

Acknowledgments

Special thanks go to Professor Mad Nasir Shamsudin, Professor Mohamad Pauzi Zakaria, Professor Ahmad Makmom b. Hj Abdullah, Mr. Jasni bin Md. Yusoff and Mr. Tengku Shahrul Tengku Md. Yusoff for their helpful arrangements during preparation of fish bile samples and HPLC analyses. We are grateful to Professor Ahmad Bin Selamat and Dr. Saeid Nikbin for their kind assistances during statistical analyses.

References

[1] B. Fouillet, P. Chambon, R. Chambon, M. Castegnaro, N. Weill, Effects of ozonation on mutagenic activity of polycyclic aromatic hydrocarbons, Bull. Environ. Contam. Toxicol. 47 (1991) 1–7.
[2] J. Blahova, K. Kruzikova, K. Hilscherova, R. Grabic, J. Halirova, J. Jurcikova, T. Ocelka, Z. Svobodova, Biliary 1-hydroxypyrene as a biomarker of exposure to polycyclic aromatic hydrocarbons in fish, Neuro Endocrinol. Lett. 29 (2008) 663–668.
[3] U. Kammann, J. Gercken, Monitoring von PAK-Metaboliten in Aalmuttern (*Zoarces viviparus*) aus der Wismar-Bucht= Monitoring of PAH metabolites in

eelpout (*Zoarces viviparus*) from Wismar Bight, Umweltwissenschaften und Schadstoff-Forschung 22 (2010) 541–546.
[4] C.D. Klaassen, J.B. Watkins Iii, Mechanisms of bile formation, hepatic uptake, and biliary excretion, Pharmacol. Rev. 36 (1984) 1–67.
[5] J.B. Ortiz-Delgado, H. Segner, J.M. Arellano, C. Sarasquete, Histopathological alterations, EROD activity, CYP1A protein and biliary metabolites in gilthead seabream *Sparus aurata* exposed to benzo(a)pyrene, Histol. Histopathol. 22 (2007) 417–432.
[6] N. Wessel, R. Santos, D. Menard, K. Le Menach, V. Buchet, N. Lebayon, V. Loizeau, T. Burgeot, H. Budzinski, F. Akcha, Relationship between PAH biotransformation as measured by biliary metabolites and EROD activity, and genotoxicity in juveniles of sole (*Solea solea*), Mar. Environ. Res. 69 (2010).
[7] X. Yang, P.C. Baumann, Biliary PAH metabolites and the hepatosomatic index of brown bullheads from Lake Erie tributaries, Ecol. Indic. 6 (2006) 567–574.
[8] R. van der Oost, L. van Gastel, D. Worst, M. Hanraads, K. Satumalay, F.-J. van Schooten, H. Heida, N.P.E. Vermeulen, Biochemical markers in feral roach (*Rutilus rutilus*) in relation to the bioaccumulation of organic trace pollutants, Chemosphere 29 (1994) 801–817.
[9] U. Varanasi, W.L. Reichert, B.T. Le Eberhart, J.E. Stein, Formation and persistence of benzo[a]pyrene-diolepoxide-DNA adducts in liver of English sole (*Parophrys vetulus*), Chem. Biol. Interact. 69 (1989) 203–216.
[10] L.D. Arcand Hoy, C.D. Metcalfe, Biomarkers of exposure of brown bullheads (*Ameiurus nebulosus*) to contaminants in the lower Great Lakes, North America, Environ. Toxicol. Chem. 18 (1999) 740–749.
[11] E.P. Johnston, P.C. Baumann, Analysis of fish bile with HPLC—fluorescence to determine environmental exposure to benzo (a) pyrene, Hydrobiologia 188 (1989) 561–566.
[12] J. Beyer, G. Jonsson, C. Porte, M.M. Krahn, F. Ariese, Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: a review, Environ. Toxicol. Pharmacol. 30 (2010) 224–244.
[13] A. Hillenweck, C. Canlet, A. Mauffret, L. Debrauwer, G. Claireaux, J.P. Cravedi, Characterization of biliary metabolites of fluoranthene in the common sole (*Solea solea*), Environ. Toxicol. Chem. 27 (2008) 2575–2581.
[14] A.R. Steward, C. Kandaswami, C. Ziper, J.P. Rutkowski, H.C. Sikka, S. Chidambaram, Disposition and metabolic fate of benzo [a] pyrene in the brown bullhead, Environ. Toxicol. Chem. 9 (1990) 1503–1512.
[15] K.L. Willett, P.R. Gardinali, L.A. Lienesch, R.T. Di Giulio, Comparative metabolism and excretion of benzo(a)pyrene in 2 species of ictalurid catfish, Toxicol. Sci. 58 (2000) 68–76.
[16] S. Zhu, L. Li, C. Thornton, P. Carvalho, B.A. Avery, K.L. Willett, Simultaneous determination of benzo[a]pyrene and eight of its metabolites in *Fundulus heteroclitus* bile using ultra-performance liquid chromatography with mass spectrometry, J. Chromatogr. Technol. Biomed. Life Sci. 863 (2008) 141–149.
[17] U. Kammann, PAH metabolites in bile fluids of dab (*Limanda limanda*) and flounder (*Platichthys flesus*): spatial distribution and seasonal changes, Environ. Sci. Pollut. Res. Int. 14 (2007) 102–108.
[18] F. Ariese, J. Beyer, D. Wells, E.A. Maier, B.M. Gawlik, A. Lamberty, The Certification of the Mass Fractions of PAH Metabolites (1-hydroxyphenanthrene, 1-hydroxypyrene and 3-hydroxybenzo [a] pyrene) in Two Fish Bile Materials: BCR-720 (sediment-exposed Flounder Bile), BCR-721 (oil-exposed Plaice Bile), in, European Commission, Luxembourg, 2004, pp. 49.
[19] F. Ariese, J. Beyer, D. Wells, Two fish bile reference materials certified for PAH metabolites, J. Environ. Monit. 7 (2005) 869–876.
[20] D.M. Richardson, M.J. Gubbins, I.M. Davies, C.F. Moffat, P.M. Pollard, Effects of feeding status on biliary PAH metabolite and biliverdin concentrations in plaice (*Pleuronectes platessa*), Environ. Toxicol. Pharmacol. 17 (2004) 79–85.
[21] F. Ariese, I. Burgers, K. Oudhoff, T. Rutten, G. Stroomberg, D. Vethaak, Comparison of Analytical Approaches for PAH Metabolites in Fish Bile Samples for Marine and Estuarine Monitoring, Vrije Universiteit, Institute for Environmental Studies, 1997, p. 29.
[22] A.L. Pikkarainen, Ethoxyresorufin-O-deethylase (EROD) activity and bile metabolites as contamination indicators in Baltic Sea perch: determination by HPLC, Chemosphere 65 (2006) 1888–1897.
[23] F. Ariese, S.J. Kok, M. Verkaik, C. Gooijer, N.H. Velthorst, J.W. Hofstraat, Synchronous fluorescence spectrometry of fish bile: a rapid screening method for the biomonitoring of PAH exposure, Aquat. Toxicol. 26 (1993) 273–286.
[24] C. Harman, T.F. Holth, K. Hylland, K. Thomas, M. Grung, Relationship between polycyclic aromatic hydrocarbon (PAH) accumulation in semipermeable membrane devices and PAH bile metabolite levels in Atlantic cod (*Gadus morhua*), J. Toxicol. Environ. Health A 72 (2009) 234–243.
[25] W.M. Ruczynska, J. Szlinder-Richert, M. Malesa-Ciecwierz, J. Warzocha, Assessment of PAH pollution in the southern Baltic Sea through the analysis of sediment, mussels and fish bile, J. Environ. Monit. 13 (2011) 2535–2542.
[26] P.J. Ruddock, D.J. Bird, D.V. McCalley, Bile metabolites of polycyclic aromatic hydrocarbons in three species of fish from the Severn Estuary, Ecotoxicol. Environ. Saf. 51 (2002) 97–105.
[27] P.J. Ruddock, D.J. Bird, J. McEvoy, L.D. Peters, Bile metabolites of polycyclic aromatic hydrocarbons (PAHs) in European eels *Anguilla anguilla* from United Kingdom estuaries, Sci. Total Environ. 301 (2003) 105–117.
[28] A. van Schanke, J.P. Boon, Y. Aardoom, A. van Leest, F.J. van Schooten, L. Maas, M. van den Berg, J.M. Everaarts, Effect of a dioxin-like PCB (CB 126) on the biotransformation and genotoxicity of benzo [a] pyrene in the marine flatfish dab (*Limanda limanda*), Aquat. Toxicol. 50 (2000) 403–415.
[29] A. van Schanke, F. Holtz, J. van der Meer, J.P. Boon, F. Ariese, G. Stroomberg, M. van den Berg, J.M. Everaarts, Dose- and time-dependent formation of biliary benzo[a]pyrene metabolites in the marine flatfish DAB (*Limanda limanda*), Environ. Toxicol. Chem. 20 (2001) 1641–1647.
[30] H. Vuontisjärvi, M. Keinänen, P.J. Vuorinen, K. Peltonen, A comparison of HPLC with fluorescence detection and fixed wavelength fluorescence methods for the determination of polycyclic aromatic hydrocarbon metabolites in fish bile, Polyc. Arom. Comp. 24 (2004) 333–342.
[31] P.J. Vuorinen, M. Keinänen, H. Vuontisjärvi, Bile PAH-metabolite concentrations in perch (*Perca fluviatilis*) exposed to crude oil and sampled in the Gulf of Finland near an oil refinery and in Baltic salmon (*Salmo salar*), in: ICES Annual Science Conference, Biological Effects Monitoring in the Baltic Sea, Tallinn, Estonia, 2003.
[32] P.J. Vuorinen, M. Keinänen, H. Vuontisjärvi, J. Barsiene, K. Broeg, L. Förlin, J. Gercken, J. Kopecka, A. Köhler, J. Parkkonen, Use of biliary PAH metabolites as a biomarker of pollution in fish from the Baltic Sea, Mar. Pollut. Bull. 53 (2006) 479–487.
[33] X.H. Wang, H.S. Hong, J.L. Mu, J.Q. Lin, S.H. Wang, Polycyclic aromatic hydrocarbon (PAH) metabolites in marine fishes as a specific biomarker to indicate PAH pollution in the marine coastal environment, J. Environ. Sci. Health A: Tox. Hazard. Subst. Environ. Eng. 43 (2008) 219–226.
[34] G. Jonsson, I.C. Taban, K.B. Jorgensen, R.C. Sundt, Quantitative determination of de-conjugated chrysene metabolites in fish bile by HPLC-fluorescence and GC–MS, Chemosphere 54 (2004) 1085–1097.
[35] G. Jonsson, J. Beyer, D. Wells, F. Ariese, The application of HPLC-F and GC–MS to the analysis of selected hydroxy polycyclic hydrocarbons in two certified fish bile reference materials, J. Environ. Monit. 5 (2003) 513–520.
[36] J.S. Brown, S.A. Steinert, DNA damage and biliary PAH metabolites in flatfish from Southern California bays and harbors, and the Channel Islands, Ecol. Indic. 3 (2004) 263–274.
[37] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[38] A. Karami, A. Christianus, Z. Ishak, M.A. Syed, S. Courtenay, The effects of intramuscular and intraperitoneal injections of Benzo[*a*]pyrene on selected biomarkers in *Clarias gariepinus*, Ecotoxicol. Environ. Saf. 74 (2011) 1558–1566.
[39] J.L. Plummer, B.R. Smith, L.M. Ball, J.R. Bend, Metabolism and biliary excretion of benzo[a]pyrene 4,5-oxide in the rat, Drug Metab. Dispos. 8 (1980) 68–72.
[40] U. Varanasi, J.E. Stein, M. Nishimoto, W.L. Reichert, T.K. Collier, Chemical carcinogenesis in feral fish: uptake, activation, and detoxication of organic xenobiotics, Environ. Health Perspect. 71 (1987) 155–170.
[41] C.M. Chen, H.C. Chang, Determination of berberine in plasma, urine and bile by high-performance liquid chromatography, J. Chromatogr. B: Biomed. Sci. Appl. 665 (1995) 117–123.
[42] R. Hubrecht, J. Kirkwood, The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, 8th ed., Wiley-Blackwell, 2010.
[43] L. Jiang, L. He, M. Fountoulakis, Comparison of protein precipitation methods for sample preparation prior to proteomic analysis, J. Chromatogr. A 1023 (2004) 317–320.
[44] B. Huang, C. Chen, Detection of protein S-nitrosation using irreversible biotinylation procedures (IBP), Free Radic. Biol. Med. 49 (2010) 447–456.
[45] F. Telli-Karakoç, P.J. Ruddock, D.J. Bird, A. Hewer, A. Van Schanke, D.H. Phillips, L.D. Peters, Correlative changes in metabolism and DNA damage in turbot (*Scophthalmus maximus*) exposed to benzo[a]pyrene, Mar. Environ. Res. 54 (2002) 511–515.
[46] C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 785 (2003) 263–275.
[47] J.S. Kim, Y.H. Je, A novel biopesticide production: attagel-mediated precipitation of chitinase from *Beauveria bassiana* SFB-205 supernatant for thermotolerance, Appl. Microbiol. Biotechnol. 87 (2010) 1639–1648.
[48] S.P. Ramachandrarao, M.A. Shaw, K. Sharma, 2-D difference in-gel electrophoresis (2-D DIGE) and urinary proteomics, in: Renal and Urinary Proteomics, Wiley-VCH Verlag GmbH & Co. KGaA, 2009, pp. 253–269.
[49] C. DeStefano-Shields, D. Morin, A. Buckpitt, Formation of covalently bound protein adducts from the cytotoxicant naphthalene in nasal epithelium: species comparisons, Environ. Health Perspect. 118 (2010) 647.
[50] K. Hemminki, L.F. Zhang, J. Kruger, H. Autrup, M. Tornqvist, H.E. Norbeck, Exposure of bus and taxi drivers to urban air pollutants as measured by DNA and protein adducts, Toxicol. Lett. 72 (1994) 171–174.
[51] E. Fic, S. Kedracka Krok, U. Jankowska, A. Pirog, M. Dziedzicka Wasylewska, Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis, Electrophoresis 31 (2010) 3573–3579.
[52] D. Isola, G. Marzban, L. Selbmann, S. Onofri, M. Laimer, K. Sterflinger, Sample preparation and 2-DE procedure for protein expression profiling of black microcolonial fungi, Fungal Biol. 115 (2011) 971–977.
[53] D. Barritault, A. Expert-BezanÇOn, M.-F. GuÉRin, D. Hayes, The use of acetone precipitation in the isolation of ribosomal proteins, Eur. J. Biochem. 63 (1976) 131–135.
[54] V. Thongboonkerd, K.R. McLeish, J.M. Arthur, J.B. Klein, Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation, Kidney Int. 62 (2002) 1461–1469.
[55] F. Creed, C. Spiers, Care of the Acutely Ill Adult: An Essential Guide for Nurses, Oxford University Press, 2010.

[56] N.E. Geacintov, T.J. Flamer, T. Prusik, J.M. Khosrofian, Triplet excited states of polycyclic aromatic compounds as probes of their microenvironment in serum albumin complexes, Biochem. Biophys. Res. Commun. 64 (1975) 1245–1252.
[57] B.N. Srinivasan, E. Fujimori, Benzo [a] pyrene-serum: albumin/cysteine interactions: fluorescene and electron spin resonance studies, Chem. Biol. Interact. 28 (1979) 1–15.
[58] A. Raha, V. Reddy, W. Houser, E. Bresnick, Binding characteristics of 4S PAH-binding protein and Ah receptor from rats and mice, J. Toxicol. Environ. Health A 29 (1990) 339–355.
[59] Y. Wei, A. Brandazza, P. Pelosi, Binding of polycyclic aromatic hydrocarbons to mutants of odorant-binding protein: a first step towards biosensors for environmental monitoring, Biochim. Biophys. Acta: Proteins Proteomics 1784 (2008) 666–671.