


Optimization and comparison of miniaturized extraction techniques for PAHs from crude oil exposed Atlantic cod and haddock eggs

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Received: 14 October 2015 / Accepted: 26 November 2015 / Published online: 16 December 2015
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Abstract Two miniaturized extraction methods for a wide range of 2–6 ring polycyclic aromatic hydrocarbons (PAHs) and their alkylated homologues in small lipid-rich biota samples (≤ 100 mg) have been developed. Both methods utilize liquid extraction (LE) prior to a clean-up step using either normal phase solid phase extraction (SPE) or mixed-phase dispersive SPE (dSPE). Optimization of the methods was achieved by comparing the type and amount of sorbents, drying agents, and solvents used. In order to improve the limits of detection (LOD) of target PAHs under high sensitivity gas chromatography–tandem mass spectrometry analysis, specific emphasis was given to minimizing lipid co-extraction. The optimized LE–SPE method comprised extraction with dichloromethane/*n*-hexane (1:1, v/v) and clean-up by silica SPE, whereas the optimized LE–dSPE method comprised extraction with acetonitrile and clean-up with PSA and C18 sorbents. The methods were validated and directly compared through the analysis of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) eggs exposed to oil. The LE–SPE method resulted in lower levels of co-extracted lipids (14.1 ± 1.7 ng/ μ L) than the LE–dSPE method ($60 \pm$

14 ng/ μ L). Achieved PAH LODs for the LE–SPE method were typically an order of magnitude lower (< 5 ng/g) than for the LE–dSPE method (< 125 ng/g). The LE–SPE method offers the possibility for PAH analysis of small samples of fish eggs (~ 100 mg) exposed to small quantities of crude oil (~ 1 – 10 μ g/L total PAHs).

Keywords Polycyclic aromatic hydrocarbons · Crude oil · Fish eggs · Purification · Solid phase extraction · Dispersive SPE

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are considered the main compound group responsible for eliciting acute and sub-lethal toxic effects in marine organisms following oil spills or operational production discharges. Close correlations between the concentration of total dissolved PAHs and the toxicity of weathered oils to early life stages (eggs and larvae) of marine fish have been shown previously [1, 2]. More recently, concerns are being raised about the influence of micro-sized dispersed oil droplets on toxicity [3], especially towards early life stages of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) [4]. To understand this observed toxicity, accurate and sensitive measurement of low concentrations of PAHs and other oil-derived compounds accumulated in small samples of fish eggs is necessary.

Currently, there are few published methods that have successfully analyzed body residues of PAHs at low concentrations in small (< 0.1 g) samples [5]. A range of traditional high volume solvent extraction methods (e.g., microwave-assisted extraction, pressurized liquid extraction, and Soxhlet extraction) are suggested for miniaturization, but the main issue with most of these approaches is low recovery of volatile

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-9225-x) contains supplementary material, which is available to authorized users.

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compounds due to high temperature conditions during extraction [5]. More novel and sophisticated extraction methods (e.g., stir bar sorptive extraction and solid phase microextraction) have only shown to be applicable to certain types of analytes and concentration ranges [6–8]. Such approaches are limited when studying the toxicological effects of complex chemical mixtures such as crude oils, where it is necessary to extract and quantify a wide range of PAHs, alkylated PAHs, and potentially other compounds with varying physical and chemical properties (e.g., size, polarity, volatility) [9].

Adequate purification of PAH-containing extracts from complex environmental and biological matrices represents a critical aspect of the sample preparation process. Traditional clean-up techniques for such samples include normal phase solid phase extraction (SPE), using sorbents such as silica (Si), alumina (Al), or Florisil[®], and gel permeation chromatography (GPC), where SPE clean-up has typically been shown to provide a higher recovery of analytes than GPC clean-up techniques (reviewed in [5]). Dispersive SPE (dSPE) has recently emerged as a viable alternative to traditional SPE, offering higher sample throughput and simplicity, even for lipid-rich sample types [10, 11], and miniaturized techniques have been reported [12]. However, drawbacks exist with dSPE, including loss of certain analyte types and extracts with high levels of biogenic compounds. These drawbacks are often overlooked, as analyte concentrations reported in many studies are sufficiently high that they do not pose analytical problems [13, 14]. As gas chromatography–tandem mass spectrometry (GC–MS/MS) offers improved detection limits over standard GC–MS instruments, it is increasingly used for analysis of PAH extracts containing high concentrations of lipid matrix components and where preconcentration steps are not employed [14, 15]. However, when low limits of detection (LOD) are necessary, cleaner extracts and high analyte recovery remain crucial. Importantly, sample extracts with high levels of co-extracted lipid components have a negative impact on the quality of analytical results and instrument performance over time [15, 16].

In the current study, we have optimized and evaluated two novel, miniaturized extraction and clean-up methods for the analysis of very low concentrations of a wide range of 2–6 ring PAHs and their alkylated homologues in small samples of cod and haddock eggs. As this matrix is dominated by high levels of lipids, such as polar phospholipids (mainly phosphatidylcholine, PC; phosphatidylethanolamine, PE), free fatty acids (FFAs), triacylglycerides (TAG) and cholesterol [17], the method development focused on minimizing lipid co-extraction. Both methods are based on liquid extraction (LE) followed by a clean-up step employing either normal phase SPE or mixed-phase dSPE. The performance of both methods is compared to a previously published sonication-assisted saponification (SAS) method [18]. An important focus of the current

work is the ability to both qualitatively and quantitatively describe the lipid removal success of each purification method and optimization step.

Experimental

Chemicals and materials

All solvents were of analytical grade (GC Suprasolv[®] or HPLC) and purity was verified in-house prior to use. PAHs and deuterated homologues were supplied by Chiron AS (Trondheim, Norway). PAH stock solutions for spiking (27 compounds, 10–1000 ng/mL in *n*-hexane) and calibration (63 compounds, 0.2–250 ng/mL in isooctane) were prepared in the laboratory (details available in Table S1 in the Electronic Supplementary Material, ESM). A PAH mixture concentration of 100 ng/g wet weight (ww) of egg sample was used during all method optimization studies. A surrogate internal standard (comprising 100 ng/g naphthalene-*d*₈, biphenyl-*d*₈, acenaphthylene-*d*₈, anthracene-*d*₁₀, pyrene-*d*₁₀, perylene-*d*₁₂, and indeno[1,2,3-*c,d*]pyrene-*d*₁₂) was added to the samples prior to extraction and a recovery internal standard (10 ng/g 9-methylanthracene-*d*₁₂) was added immediately prior to analysis. Bond Elut SPE columns (Si, Florisil[®] and Al; 500 mg, 3 mL) and bulk sorbents (primary secondary amine; PSA, 57.5 μm and C18; end capped, 57.5 μm) were supplied by Agilent Technologies. Bulk Florisil[®] (100–200 mesh), NaCl, MgSO₄, and Na₂SO₄ were supplied by Sigma-Aldrich and cleaned at 400 °C overnight. Ultrapure water was supplied by Millipore[®] or NanoPure[®] systems. Samples (clean and exposed) of fertilized Atlantic haddock (*Melogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*) eggs were collected from a local brood stock and kept frozen (–80 °C) in sterile vials prior to extraction.

Liquid extraction–solid phase extraction (LE–SPE)

Cod and haddock egg samples (100 mg ww) were liquid extracted using dichloromethane (DCM)/*n*-hexane (1:1, 9:1, or 1:9, v/v). Egg samples were homogenized in 2 mL of the selected solvent system using a Virtis Tempest IQ 2.0 Microprocessor fitted with a small stainless steel knife (0.5 cm diameter) and operated at 20,000 rpm (30 s). Na₂SO₄ (50–250 mg) was added and the sample vortex extracted (Labinco L46 instrument at maximum speed, 30 s). Following centrifugation (Beckman GS-15R, 2 min at 2000 rpm), the supernatant was transferred to a clean vial. The extraction procedure was repeated 1–3 times, and the combined extracts concentrated by solvent evaporation at 40 °C under a gentle flow of nitrogen prior to clean-up.

A face-centered ($k=1$) central composite design was applied to optimize the DCM/*n*-hexane extraction. Three factors

were tested: number of extraction cycles (1–3), amount of Na_2SO_4 (50–250 mg), and percentage DCM (10–90 %) in *n*-hexane. The experimental design was conducted and evaluated using Sirius 9.0 software (Pattern Recognition Systems, Bergen, Norway) and can be viewed in ESM Table S2.

In order to optimize clean-up of the DCM/*n*-hexane (1:9, v/v) extracts with respect to removal of lipids, whilst at the same time maximize recovery of 2–6 ring PAHs and their alkylated homologues, three different normal phase SPE sorbents were tested (Si, Florisil® and Al; 500 mg, 3 mL). For each sorbent, solvents of increasing elution strength were assessed (0–50 % DCM in *n*-hexane). The SPE columns were conditioned with 6 mL *n*-hexane immediately prior to use. Extracts (0.5–1 mL in *n*-hexane) were transferred to the column, followed by elution with 6 mL of the selected elution solvent. The eluate was evaporated under a gentle flow of nitrogen and the final volume was adjusted to 0.5 mL prior to analysis.

Liquid extraction–dispersive solid phase extraction (LE–dSPE)

Samples for dSPE clean-up assessment (LE–dSPE) were liquid extracted using pure acetonitrile (ACN) or cyclohexane/ethyl acetate (CE) (1:1, v/v). The extraction procedure was otherwise the same as described for LE–SPE above. Following extraction, the extracts were mixed with sorbents (PSA, C18, Florisil®, 50–150 mg) and salt (MgSO_4 or Na_2SO_4 , 75–225 mg), vortexed (10–60 s), and then centrifuged (5 min, 2000 rpm). The supernatant was collected and the sample volume reduced under a gentle flow of nitrogen prior to analysis.

A 2^{6-3} fractional factorial design with fold-over (ESM Tables S3 and S4) was applied to optimize ACN extraction prior to dSPE clean-up. Three factors were tested: extraction time, amount of salt and type of salt (NaCl , $\text{MgSO}_4/\text{Na}_2\text{SO}_4$). The number of extraction cycles (1–3) was evaluated individually. The experimental design was conducted and evaluated using Sirius 8.1 software (Pattern Recognition Systems, Bergen, Norway).

As solvent exchange is not possible between the two steps in the dSPE method, it is crucial that the solvent is compatible with the sorbents applied in the clean-up step. Furthermore, an important consideration in the choice of solvent for this type of method is its capacity for co-extraction of matrix lipids. Initially, a comparative study was done to investigate the effect of each sorbent type in dSPE clean-up using CE (1:1, v/v) and ACN as solvents. For each solvent a screening study was performed using 150 mg of MgSO_4 , 100 mg of PSA, and 50 mg of C18. Using ACN as the solvent, the clean-up effect of different sorbent types (PSA, C18 Florisil®) and sorbent combinations was tested according to an experimental study as described in ESM Table S5. Briefly, MgSO_4 (100 mg) and

PSA (100 mg) were used as sorbents in a baseline method. The increase in clean-up efficiency when adding C18 (100 mg) and/or Florisil® (100 mg) was tested, as well as the influence of performing the clean-up with polar sorbent (PSA) and nonpolar sorbent (C18) in two tiered steps. Final optimization was performed using an experimental design (2^{6-3} factorial design with fold-over) to determine the amount of sorbents and salts to be used (Tables S3 and S4 in ESM). The design also looked at how the clean-up efficiency was affected when exchanging MgSO_4 with Na_2SO_4 .

Sonication-assisted saponification (SAS–SPE)

The extraction was performed as described by Hansen et al. [18]. Briefly, cod or haddock eggs (100 mg ww) were saponified in an alkaline solution (6.5 % w/v KOH in methanol/water (3 mL 4:1, v/v)) for 2 h at 80 °C, followed by filtration and serial extraction with hexane (3×3 mL). The combined extracts were cleaned with a saturated aqueous solution of NaCl, dried with anhydrous sodium sulfate, and concentrated by solvent evaporation prior to clean-up with SPE (500 mg Si, 33 % DCM in *n*-hexane).

Method validation

All three methods (SAS–SPE, LE–SPE, and LE–dSPE) were subject to method validation and LOD assessment following the initial optimization of LE–SPE and LE–dSPE. For this purpose, cod or haddock egg samples (100 mg) were spiked either with a PAH mix at four levels (0.1, 1, 10, and 100 ng/g) or with a dissolved crude oil (~100 µg total oil dissolved in 20 µL DCM, with ~1 µg total target PAHs). Samples comprised three replicates of cod and three replicates of haddock (total $n=6$) at each spiking level, including laboratory and matrix blanks. Both laboratory (no matrix) and matrix (unexposed fish eggs) extraction blanks were assessed for the determination of method LODs (LOD=the average concentration in blanks+three times standard deviation). Where no increase in analytical signal was observed in blank samples, LODs were determined as 33 % of the instrumental limit of quantification (LOQ; lowest level in calibration curve where signal to noise (S/N) ratio was >10).

Analysis of crude oil exposed haddock eggs

The optimized LE–SPE and LE–dSPE methods were applied to a set of real samples from an exposure experiment where haddock eggs were exposed to mechanically dispersed crude oil over 9 days (total PAH concentration was ~0.7 µg/L water in low dose groups and ~7 µg/L in high dose groups). The exposure conditions are described by Sørhus et al. [4]. Replicate samples from each exposure tank (four tanks) were subsampled (2×100 mg) and treated using both methods.

Chemical analysis of PAHs

During method development and optimization, PAHs were analyzed by GC–MS, using an Agilent 6890 GC coupled to an Agilent 5973 MS fitted with an EI source operated in SIM mode. The GC column was either a DB-17MS (30 m × 0.25 mm × 0.25 μm) or a DB-5MS UI (30 m × 0.25 mm × 0.25 μm), and the carrier gas was helium at a constant flow of 1 mL/min. Samples (1 μL) were injected at 300 °C in splitless mode. Purge-off time was 2 min. For analysis using the DB-17 column, the oven temperature was held at 50 °C for 2 min, raised to 110 °C at 10 °C/min, raised to 290 °C at 6 °C/min and held at this temperature for 16 min. For analysis using the DB-5 column, the oven temperature was held at 40 °C for 1 min, raised to 315 °C at 6 °C/min, and held at this temperature for 5 min. Molecular masses and descriptive fragments of PAHs were monitored in seven selective ion monitoring (SIM) windows.

An Agilent 7890 GC coupled with an Agilent 7010 triple quadrupole mass spectrometer fitted with electron ionization (EI) source and collision cell was used for the analysis of samples during the method validation studies. Briefly, two DB-5MS UI GC columns (15 m × 0.25 mm × 0.25 μm) were coupled in series through a purged ultimate union (PUU). The carrier gas was helium at constant flow (1.2 mL/min). Samples (1 μL) were injected at 300 °C in the splitless mode. The temperature gradient started at 60 °C for 1 min, raised to 120 °C at 40 °C/min, and finally raised to 310 °C at 5 °C/min. In post-run, the first column was backflushed at 310 °C for 5 min. The transfer line temperature was 280 °C, the ion source temperature was 300 °C, and the quadrupole temperatures were 180 °C. N₂ was used as collision gas at a flow of 1.5 mL/min and helium was used as a quench gas at a flow of 4 mL/min. Analytes were identified by two unique multiple reaction monitoring (MRM) transitions and quantified by the most intense peak (see Table S1 in ESM).

Lipid class characterization

In order to determine the extraction and clean-up efficiency of the selected methods, total lipid and lipid class analyses were performed on both the fish eggs and the extracts before and after clean-up. For the determination of lipids in the clean eggs, Folch extraction [19] was applied. Briefly, samples were homogenized in chloroform/methanol (2:1, v/v) and filtered through a glass filter. Non-lipid material was removed by mixing with KCl solution (0.88 %), followed by centrifugation. The supernatant was removed and the organic phase was dried over MgSO₄, followed by filtration and adjustment of volume. Lipid profiles (distribution of lipid classes) in all samples were investigated by thin-layer chromatography

(TLC) fractionation, followed by methanolysis of individual fractions.

TLC lipid class analysis was performed as described by Olsen and Henderson [20]. Briefly, extracts (10–100 μL) were applied to silica 60 HPTLC plates (10 × 10 cm, Merck®), along with standards of TAG, FFA, cholesterol, and fractions of PE and PC purified from herring roe. The plate was developed twice, first with methyl acetate/isopropanol/chloroform/methanol/0.25 % KCl (25:25:25:10:9, v/v) followed by vacuum drying, and second with hexane/diethyl ether/acetic acid (80:20:2, v/v). The plates were dried under vacuum and developed by spraying with a solution of 3 % cupric acetate in 8 % phosphoric acid, followed by heating for 20 min at 160 °C. TLC plates for use in quantitative TLC profile analysis were developed with 2',7'-dichlorofluorescein (0.1 % in ethanol), followed by identification of the spots by inspection under UV (366 nm). The individual spots were scraped off and subject to methanolysis followed by GC–flame ionization detector (FID) analysis.

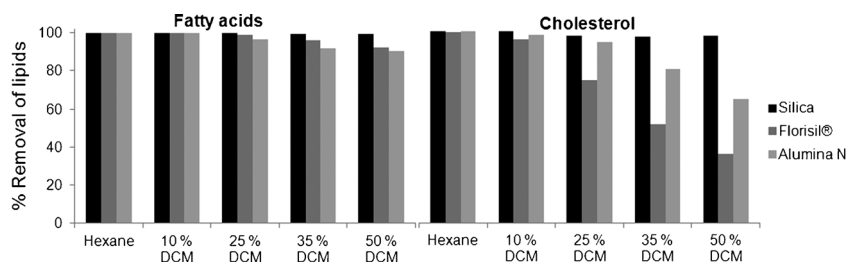
Samples for quantitative lipid analysis were derivatized by methanolysis to form the corresponding methyl esters (FAME) [21]. An Agilent 7890A GC fitted with a FID was used for analysis. Samples (1 μL) were injected at 280 °C in the splitless mode. The GC column was a CP-Wax 52 CB (25 m × 0.25 mm × 0.25 μm), and the carrier gas was helium at a flow of 1 mL/min for 45 min, followed by 3 mL/min for 23 min. The oven temperature was held at 90 °C for 2 min, then raised to 160 °C at 15 °C/min, raised to 225 °C at 2.5 °C/min and held for 3 min, and finally raised to 240 °C where the temperature was held for 50 min. The detector temperature was 300 °C.

Results and discussion

Lipid profile of cod and haddock eggs

The lipid profiles of cod and haddock eggs were investigated both quantitatively and qualitatively. The total extractable (Folch method) lipid content for clean cod and haddock eggs was 6.8 mg/g of eggs in both cases (wet weight), and quantifiable lipid (methanolysis followed by GC–FID) content was 0.5–0.7 % for both cod and haddock. Figure S1 (ESM) shows the developed TLC plates of lipid extracts from clean haddock and cod eggs (Folch method). The quantified lipid distribution based on TLC-separated fractions (ESM Table S6) was similar for cod and haddock, with the main difference occurring internally between different classes of phospholipids. These results are in line with literature reported values for cod eggs [22], whilst data on haddock eggs has, to the authors' knowledge, not previously been reported.

Fig. 1 Fatty acid and cholesterol removal (%) as a function of SPE sorbent and eluent strength



Sonication-assisted saponification

Alkaline saponification followed by LE with *n*-hexane efficiently removed FFAs, TAGs, and PC/PE, but was not successful at removing cholesterol from the final extracts (data not shown). Further clean-up of the extracts by SPE (Si, 33 % DCM in *n*-hexane as eluent) was required to provide samples amenable to GC–MS analysis.

Liquid extraction–solid phase extraction (LE–SPE)

The face-centered central composite design for optimization of the LE procedure prior to SPE clean-up revealed that three extraction cycles, addition of 150 mg Na₂SO₄, and the use of 50 % DCM in *n*-hexane offered the highest PAH recovery. The difference in average percentage recovery when increasing from two extraction cycles (~85 %) to three extraction cycles (~90 %) was not large. No discrimination based on mass, alkylation or other properties was observed.

Several normal phase SPE materials (Si, Florisil®, and Al) and elution solvents were compared for clean-up efficiency of the liquid extracts (LE–SPE). Using 100 % *n*-hexane as eluent, all tested sorbents provided clean extracts (Fig. 1), with approximately 100 % of fatty acids (FAs) and cholesterol removed. However, with increasing eluent strength (% DCM), Si outperformed both Florisil® and Al, especially in terms of TAG and cholesterol removal. Combined sorbent and eluent systems that provided acceptably clean extracts were further tested for PAH recovery (Fig. 2). Florisil® failed to adequately recover the higher PAHs (4 rings+), even at 10 % DCM. Both Si and Al offered better recoveries of the higher PAHs at 10 % DCM than *n*-hexane alone (Fig. 2). No added benefit was observed from increasing the DCM to *n*-hexane ratio beyond 10 % (data not shown). Si with an eluent of 10 % DCM in *n*-hexane offered the best performance for both lipid removal (13.3±1.2 ng/μL FAs and 0.8±0.5 ng/μL cholesterol) and analyte recovery (75–120 %) and was therefore selected for further use.

Liquid extraction–dispersive solid phase extraction (LE–dSPE)

Initially, two solvent systems were tested for the LE–dSPE approach (ACN and CE, 1:1, v/v). Data (ESM Fig. S2)

showed that CE extracted significantly more lipid material than ACN, and CE also caused the sorbents to function less efficiently. For this reason, ACN was applied in the further method optimization.

The 2⁶⁻³ factorial design with fold-over for the ACN–dSPE approach identified the type and amount of salt as the most important factors in optimizing PAH recovery. Na₂SO₄ performed better than MgSO₄ with less (75 mg) as opposed to higher quantities of salt being more beneficial. However, Na₂SO₄ led to a significantly higher co-extraction of PC/PE than MgSO₄, although no significant difference in removal of cholesterol or other lipid classes was observed between either salt type (ESM Fig. S3). Magnesium oxide (MgO), a potential impurity present in MgSO₄, has previously been described as a sorbent for the enrichment of PAHs from water samples [23] and in normal phase HPLC separation of a variety of compounds, including PAHs [24]. Jin et al [23] observed greater interaction with MgO for larger PAHs, but this was not observed in the current study. Despite exhibiting a lower PAH extraction efficiency compared to Na₂SO₄, MgSO₄ was selected for use in the LE–dSPE method owing to the superior removal of lipid compounds, which is necessary to maintain low analytical instrumental LODs.

Increasing the number extraction cycles from one to two yielded a significantly higher recovery of PAHs (from ~60 to ~80 % average recovery), with only a small added benefit by increasing to three extraction cycles (ESM Fig. S5). However, the co-extraction of lipids also increased linearly with number of extraction cycles. After just two cycles, the quantity of

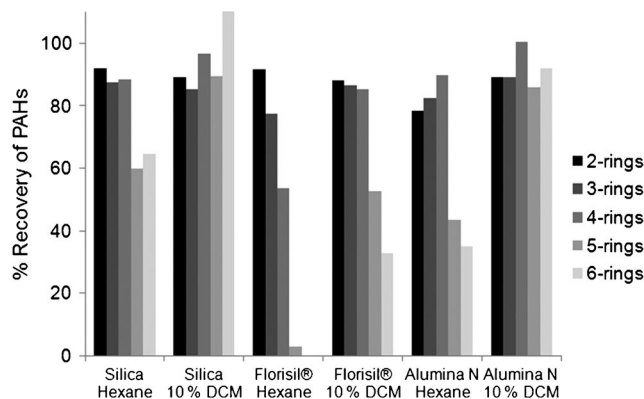


Fig. 2 PAH recovery for different sized PAHs (2–6 rings, including alkylated compounds) as a function of eluent strength and SPE sorbent

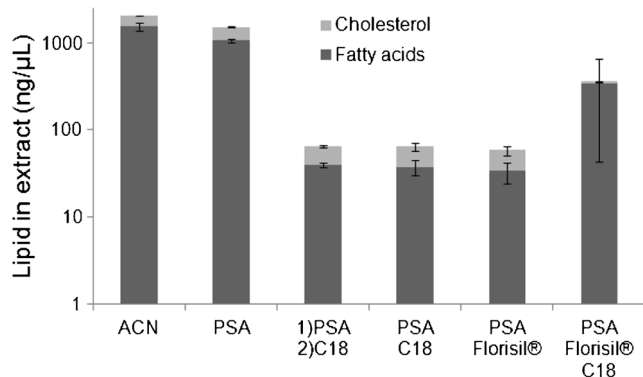


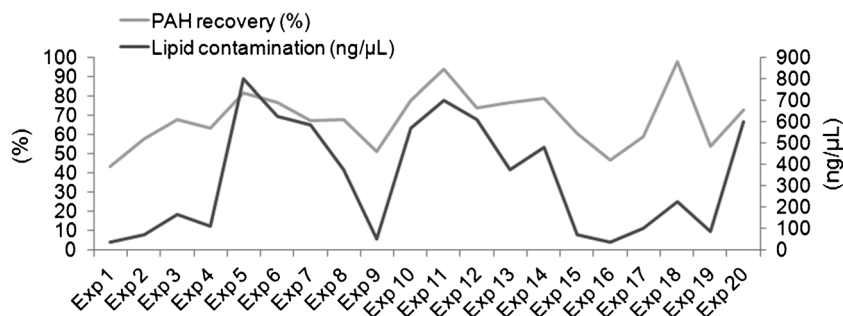
Fig. 3 Remaining lipids in dSPE extracts after ACN extraction and clean-up by various combination of sorbents. Two-step extraction is indicated by 1), 2) ($n=3$). Axis is logarithmic

lipids in the extracts was above acceptable levels (100 ng/μL). For this reason, only one extraction cycle was applied in the final method. The final method therefore comprised ACN (2 mL), MgSO₄ (75 mg), and only one extraction cycle (10 s).

The clean-up efficiency of different dSPE sorbents (PSA, C18, and Florisil®) and MgSO₄ combinations for the removal of lipid contaminants from the egg samples was studied. It was observed that MgSO₄ and PSA alone were able to remove only a small amount of FAs, and no cholesterol (Fig. 3). When C18 was added, a greater removal of FAs and cholesterol was observed. A third approach, suggested by Molina-Ruiz et al. [10], is a two-step clean-up consisting of (1) MgSO₄ and PSA, and (2) C18. No significant difference in clean-up was observed between performing clean-up in one or two steps (Fig. 3). Although Florisil® has previously been observed to yield positive clean-up effects [25], in the current study no additional benefits were observed when applied with PSA and C18. Therefore, Florisil® did not serve as a viable replacement for C18 (Fig. 3).

Ultimately, no specific sorbent or sorbent combination offered a significantly better clean-up efficiency. As it offered the highest degree of simplicity, a one-step clean-up method using a mixture of C18 and PSA was selected for further optimization. Implementing the selected extraction and clean-up parameters in a 2⁶⁻³ factorial design with fold-over, PAH analyte recovery was observed to be inversely correlated with matrix (lipid) removal (Fig. 4). The two parameters

Fig. 4 Relationship between PAH recovery and lipid co-extraction as a function of extraction and clean-up conditions in LE-dSPE



observed to be of importance for PAH recovery (type of salt and amount of C18 sorbent) were also the most important for lipid removal, but with opposite optimal conditions. In order to be able to optimize the PAH recovery under such contradicting conditions, a total lipid (FAs and cholesterol) cutoff level at 100 ng/μL was chosen. The optimized method is described in Table 1. Sample extracts using the final dSPE method typically contained 18.5±4.0 ng/μL FAs and 41±10 ng/μL cholesterol. Recovery of analytes with this method was in the range 30–98 %.

Method validation

The SAS-SPE method and the optimized LE-SPE and LE-dSPE methods were subject to a full method validation at four concentration levels (0.1, 1, 10, and 100 ng/g individual PAHs), and a dissolved crude oil (~5 mg/mL in DCM). Background levels of target PAHs in laboratory extraction blanks (no biotic matrix) and matrix blanks had a significant influence on the achievable PAH analyte LODs for each of the three techniques (ESM Table S7). The range of LODs based on matrix blanks were 0.07–5 ng/g (LE-SPE), 0.07–121 ng/g (LE-dSPE), and 0.07–5000 ng/g (SAS-SPE). In general, volatile components were the most problematic, exhibiting high LODs compared to other components in the standard mixture. The LE-dSPE method also exhibited issues with some of the larger PAH compounds, possibly related to the presence of lipid co-extractants.

At both the 10 and 100 ng/g spiking levels, all three methods yielded extracts containing the target PAH analytes at concentrations above their LODs. However, only the LE-SPE technique permitted the determination of most target PAH analytes at the 1 ng/g spiking level. At the lowest validation level tested (0.1 ng/g) none of the methods yielded extracts containing target PAH analytes above the LODs. Background levels of target PAHs in the laboratory blanks were subtracted from the measured values in the spiked samples prior to reporting. The precision in quantification of PAHs in the spiked egg samples was overall very good for all methods (ESM Table S8) at any level above the LODs. At

Table 1 Final extraction and clean-up methods

	LE-SPE	LE-dSPE
Extraction		
Solvent	50 % DCM in <i>n</i> -hexane	Acetonitrile
Drying agent	Na ₂ SO ₄ (150 mg)	MgSO ₄ (75 mg)
Extraction cycles	3 (30, 10, 5 s)	1 (10 s)
Final extract volume	6 mL	2 mL
Extract concentrated prior to clean-up	Yes, to 1 mL	No
Clean-up		
Technique	SPE	Dispersive SPE
Sorbent	Si (500 mg)	PSA (50 mg) C18 (150 mg) MgSO ₄ (75 mg)
Solvent	10 % DCM in <i>n</i> -hexane (6 mL)	Acetonitrile

the lowest acceptable spiking level for the LE-dSPE and SAS-SPE methods (10 ng/g), the %RSDs were in the range 1–10 % (LE-SPE), 1–21 % (LE-dSPE), and 2–19 % (SAS-SPE). At the lowest acceptable spiking level for LE-SPE (1 ng/g), the %RSD was in the range 4–25 %.

The accuracy in determination of the concentration of spiked samples was assessed for all three methods at all four spike levels (ESM Table S9). All three methods show a general linearity between spiked and determined concentration, indicating good extraction efficiencies over the target PAH analyte range studied (0.1–100 ng/g) above their respective

LODs. However, there is some systematic deviation (bias) in the determination for most analytes, with least bias observed for LE-SPE (75 % of analytes are within 25 % bias), followed by LE-dSPE (65 % of analytes are within 25 % bias). SAS-SPE is mostly wrong, proving it not to be a good fit for analysis at the low concentration levels applied in the current study.

In addition, the accuracy in determination of the target PAH analytes in a spiked crude oil (100 µg total oil dissolved in DCM) was assessed. In a crude oil, the relative concentrations of the target PAH analytes can vary greatly, and the accuracy in determination may be influenced by the presence of other, non-target crude oil components. Total PAHs determined using the three extraction methods were 9.09±0.33 µg/g (LE-SPE), 9.19±0.94 µg/g (LE-dSPE), and 6.59±0.27 µg/g (SAS-SPE). The true total PAH value in the spiked oil was 9.84±0.62 µg/g. Detailed results are given in Table S10 (ESM). The deviation in determined values for both LE-SPE and LE-dSPE methods was less than 10 %, whilst the SAS-SPE underestimated the PAH content by 33 %. A summary of the performance in terms of PAH profile (alkylated PAHs are grouped by number of aromatic rings for simplicity)

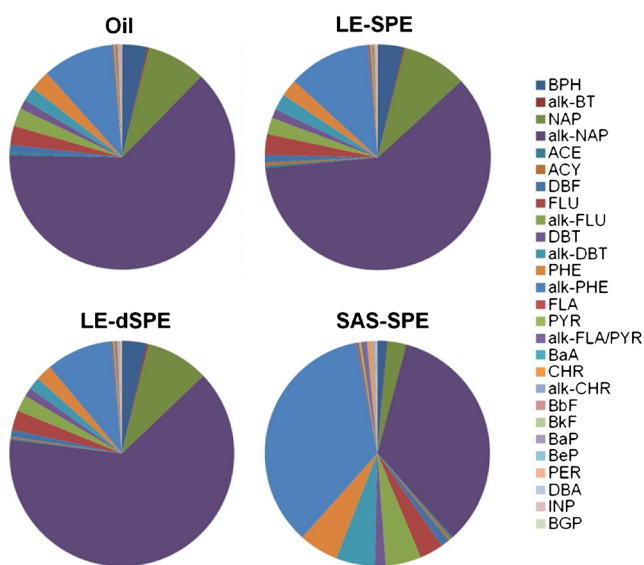


Fig. 5 Determination of crude oil derived compounds from a spiked sample, plotted as a profile of total PAH determined (*BPH* biphenyl, *BT* benzothiophene, *NAP* naphthalene, *ACE* acenaphthene, *ACY* acenaphthylene, *DBF* dibenzofuran, *FLU* fluorene, *DBT* dibenzothiophenes, *PHE* phenanthrene, *FLA* fluoranthene, *PYR* pyrene, *BaA* benz[*a*]anthracene, *CHR* chrysene, *BbF* benzo[*b*]fluorene, *BkF* benzo[*k*]fluorene, *BaP* benzo[*a*]pyrene, *BeP* benzo[*e*]pyrene, *PER* perylene, *DBA* dibenz[*a,h*]anthracene, *IND* indeno[1,2,3-*c,d*]pyrene, *BGP* benzo[*ghi*]perylene, *alk* alkylated)

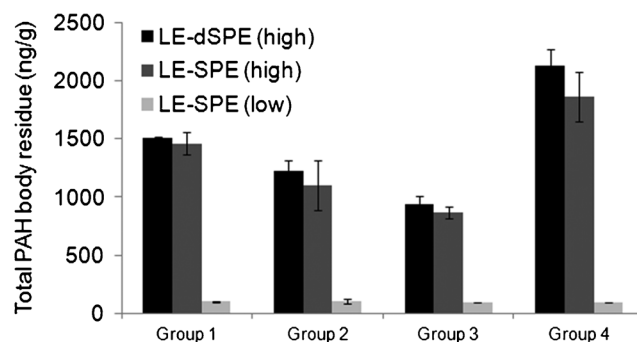


Fig. 6 Determined PAH body residue (63 compounds) in haddock eggs from four low and four high exposure groups. Both LE-dSPE and LE-SPE was applied to samples from the high groups. Error bars represent measurement range ($n=2$)

is provided in Fig. 5. The plot clearly demonstrates that both LE-SPE and LE-dSPE provide a much more accurate determination of the PAH profile of a crude oil than SAS-SPE. SAS-SPE significantly underestimates the more volatile compounds and overestimates the heavier compounds. On the basis of these results, both the LE-SPE and LE-dSPE methods clearly outperform the reported SAS-SPE method. LE-SPE and LE-dSPE are both considered suitable for analysis of PAH body residue in small biotic samples from organisms exposed to low concentrations of crude oil.

Analysis of crude oil exposed haddock egg samples

On the basis of their determined suitability, the LE-SPE and LE-dSPE approaches were used to analyze the PAH body residue in a set of haddock egg samples from a real exposure study with dispersed crude oil. The samples were replicates from four treatments at two doses (low dose: total PAH ~ 0.7 $\mu\text{g/L}$, high dose total PAH ~ 7 $\mu\text{g/L}$ water over 9 days). As a result of the previously described LODs, only LE-SPE was applied to the low dose samples. The results (total PAH body residue, measured by 63 individual compounds) are presented in Fig. 6. The body residue in low dose samples was consistent at ~ 100 ng/g for all groups, while body residue in high dose groups varied from 900 to 2200 ng/g between the individual groups. The observed variation is consistent for both the LE-SPE and LE-dSPE methods, demonstrating that the cause for the observed variation originates in the experimental design of the exposure study. The results clearly demonstrate the applicability of both methods for the analysis of real fish egg samples exposed to crude oil. Whilst both methods are comparable for the higher dose exposures, only LE-SPE can be used for body residue analysis in low dose exposures.

Conclusion

The LE-SPE and LE-dSPE methods developed and optimized in the current study offer a significant improvement over more traditional approaches (e.g., SAS-SPE) for the analysis of low concentrations of PAH compounds associated with small samples of lipid-rich biota. Both methods lend themselves to miniaturization, which has been previously shown to be a limitation for other established approaches. The most appropriate combination of extraction and clean-up techniques is dependent upon the target analytes, the type of biota sample, and, importantly, the analytical chemical instrumentation employed for analysis and quantification. Optimal conditions are a delicate balance between target analyte extraction efficiency and the degree of lipid co-extraction.

These miniaturized methods offer the possibility for directly linking body residue to the observed acute and sublethal toxicological effects resulting from low dose exposure of crude oil.

If extracts containing low concentrations of lipid co-extractants and high PAH analyte recoveries are necessary (because of low PAH body residue), LE-SPE offers the best results. Such requirements are typical when working with small volume samples which contain low concentrations of the target analytes. The time-saving ACN-dSPE approach can be implemented when target analyte concentrations are high and/or the sample volume is sufficiently large. A clear advantage of SPE in relation to dSPE is the possibility for using different solvent systems during the extraction and clean-up steps, meaning each of these steps can be optimized for the target analytes of interest. Furthermore, SPE permits either solvent reduction or solvent exchange prior to clean-up. With a large number of sorbent types and solvent systems available, miniaturized LE-SPE also has the potential for wider application than PAH analytes.

Acknowledgments This work is part of the project “Assessment of long-term effects of oil exposure on early life stages of haddock”, which is funded by the Norwegian Research Council (grant no. 234367). Marta Silva has received financial support from the Erasmus Mundus program EMQAL. The authors wish to acknowledge Bjørn Grung (University of Bergen) for guidance with experimental designs, Elin Sørhus and Ørjan Karlsen (IMR) for providing samples of fish eggs, and Marianne Unaas Rønsberg (SINTEF) for assistance in the laboratory.

Compliance with ethical standards All animal experiments within the study were approved by NARA, the governmental Norwegian Animal Research Authority (<http://www.fdu.no/fdu/>, reference number 2012/275334-2). The Austevoll Aquaculture Research Station has the following permission for catch and maintenance of Atlantic haddock: H-AV 77, H-AV 78, and H-AV 79. These are permits given by the Norwegian Directorate of Fisheries. Furthermore, the Austevoll Aquaculture Research Station has a permit to run as a research animal facility using fish (all developmental stages), with code 93 from the national Animal Welfare and Institutional Animal Care and Use Committee (IACUC); NARA.

Conflict of interest The authors declare no competing interest.

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