Maria João Ramalhosa[1,2]
Paula Paíga[1]
Simone Morais[1]
Cristina Delerue-Matos[1]
Maria Beatriz Prior Pinto Oliveira[2]

Original Paper

Analysis of polycyclic aromatic hydrocarbons in fish: evaluation of a quick, easy, cheap, effective, rugged, and safe extraction method

QuEChERS method was evaluated for extraction of 16 PAHs from fish samples. For a selective measurement of the compounds, extracts were analysed by LC with fluorescence detection. The overall analytical procedure was validated by systematic recovery experiments at three levels and by using the standard reference material SRM 2977 (mussel tissue). The targeted contaminants, except naphthalene and acenaphthene, were successfully extracted from SRM 2977 with recoveries ranging from 63.5–110.0% with variation coefficients not exceeding 8%. The optimum QuEChERS conditions were the following: 5 g of homogenised fish sample, 10 mL of ACN, agitation performed by vortex during 3 min. Quantification limits ranging from 0.12–1.90 ng/g wet weight (0.30–4.70 μg/L) were obtained. The optimized methodology was applied to assess the safety concerning PAHs contents of horse mackerel (*Trachurus trachurus*), chub mackerel (*Scomber japonicus*), sardine (*Sardina pilchardus*) and farmed seabass (*Dicentrarchus labrax*). Although benzo(a)pyrene, the marker used for evaluating the carcinogenic risk of PAHs in food, was not detected in the analysed samples (89 individuals corresponding to 27 homogenized samples), the overall mean concentration ranged from 2.52 ± 1.20 ng/g in horse mackerel to 14.6 ± 2.8 ng/g in farmed seabass. Significant differences were found between the mean PAHs concentrations of the four groups.

Keywords: Fish / Liquid chromatography / Polycyclic aromatic hydrocarbons / QuEChERS

1 Introduction

Human health is largely determined by the diet. A recommendable diet should be able to provide sufficient nutrients and low levels of pathogenic microorganisms, as well as chemical contaminants. Fish constitutes an important source of proteins, minerals, vitamins and unsaturated essential fatty acids (PUFAs), especially omega-3 PUFAs. Epidemiological studies have shown over time beneficial effects of fish consumption in the prevention of coronary heart diseases [1]. In contrast to the potential health benefits of dietary fish intake, an issue of concern related with frequent fish consumption is the risk derived from exposure to chemical pollutants.

PAHs are a large group of organic compounds that are included in the European Union and US Environmental Protection Agency (US EPA) priority pollutant list due to their mutagenic and carcinogenic properties [2]. Excluding smokers and occupationally exposed populations, most individuals are exposed to PAHs predominantly from dietary sources [3]. In the marine environment, PAHs are bioavailable to marine species via the food chain, as waterborne compounds and from contaminated sediments. As lipophilic compounds they can easily cross lipid membranes and have the potential to bioaccumulate in aquatic organisms. Although for most people, fish and seafood represents only a small part of the total diet, the contribution of this food group to the daily intake of PAHs in some individuals may be comparatively important [4]. With the aim of minimising harmful effects on human health, recently, the European Union established a maximum level of 2 ng/g wet weight for benzo(a)pyrene (the marker used for the carcinogenic

risk of PAHs) in muscle meat of fish [5]. Recently, it was attributed to dibenzo(a,l)pyrene a carcinogenic potency that is approximately 100 times that of benzo(a)pyrene [6]. Until now, however, works that include the determination of dibenzo(a,l)pyrene in food are limited [7].

From the analytical point of view, isolation of PAHs from biological matrices most often involves complicated extraction and clean up procedures to provide extracts ready for the accurate analytical determination [8]. A large number of studies have been reported on PAHs extraction from fish products using Soxhlet based procedures [9], pressurized liquid extraction and supercritical fluid extraction [10]. Other techniques, such as microwave-assisted extraction, have been mainly used for environmental samples [11]. Most of the modern techniques use less organic solvent than conventional extraction, however they still involved considerable cleanup of glassware and extraction vessels before the next use. More recently, Anastassiades *et al*. [12] developed an approach that they named as quick, easy, cheap, effective, rugged, and safe (QuEChERS), which involves extraction with ACN partitioned from the aqueous matrix using anhydrous $MgSO_4$ and NaCl followed by a dispersive solid-phase extraction (dSPE) clean up with $MgSO_4$ and primary secondary amine (PSA). The method has already received worldwide acceptance and, with minor modifications, has become an Official Method of the Association of Official Agricultural Chemists (AOAC) International and of the Committee of European Normalization (CEN) [13, 14]. The QuEChERS procedure is being successfully applied for multi-residue analysis of hundreds of pesticides in fatty and nonfatty food matrices such as egg, avocado [15], milk [15], olive oil and olives [16], baby food [17], rice [18], honey [19], soils, tobacco [20] and several fruits and vegetables [21 – 23]. The extraction of pharmaceuticals and toxins in whole blood [24] and in bovine kidney tissue [25] by QuEChERS method has also already been reported.

The main objective of this study was to evaluate and, if appropriate, to validate the QuEChERS method for the extraction of 16 PAHs, 15 regarded as priority pollutants by the U.S. EPA and dibenzo(a,l)pyrene, in fish samples. To our knowledge, this method has not been tested yet for PAHs extraction. Extracts were analysed by LC with fluorescence detection (FLD). An additional goal was to assess the safety concerning PAHs contents of horse mackerel (*Trachurus trachurus)*, chub mackerel (*Scomber japonicus*) and sardine (*Sardina pilchardus*) caught in the North Atlantic Sea since they represent the three fish species more consumed in Portugal and are regularly eaten in Europe. One species from aquaculture, seabass (*Dicentrarchus labrax*), was also selected since its culture has increased considerably in the last years, reaching high production and commercial value in European countries [26].

2 Experimental

2.1 Reagents and materials

Certified EPA 610 Polynuclear Aromatic Hydrocarbons standard mixture containing naphthalene (Naph) 1000 μg/mL, acenaphthylene (Aci) 2000 μg/mL, acenaphthene (Ace) 1000 μg/mL, fluorene (Flu) 199.9 μg/mL, phenanthrene (Phe) 99.8 μg/mL, anthracene (Ant) 100.0 μg/mL, fluoranthene (Fln) 200.1 μg/mL, pyrene (Pyr) 99.9 μg/mL, benz(a)anthracene (B(a)A) 100.1 μg/mL, chrysene (Chry) 100.0 μg/mL, benzo(b)fluoranthene (B(b)Ft) 200.2 μg/mL, benzo(k)fluoranthene (B(k)Ft) 99.9 μg/mL, benzo(a)pyrene (B(a)P) 100.0 μg/mL, dibenz(a,h)anthracene (DB(a,h)A) 200.0 μg/mL, benzo(g,h,i)perylene (B(g,h,i)P) 200.0 μg/mL and indeno(1,2,3-cd)pyrene (InP) 100.1 μg/mL was provided from Supelco (Bellefonte, PA, USA). Individual standards of each referred PAHs and of dibenzo(a,l)pyrene (2000 μg/mL) were also purchased from Supelco (Bellefonte, PA, USA). PAHs purities were guaranteed between 97.9 and 99.9%. Working mixed standard solutions containing all the PAHs were prepared by dilution of the stock solutions with ACN and stored at −20°C in darkness to avoid volatilization and photodegradation.

Standard reference material SRM 2977 (mussel tissue) was supplied by NIST (Gaithersburg, ND, USA).

Acetone (Riedel-de Haën, Seelze, Germany, purity 99.8%) and ACN (Sigma-Aldrich, Steinheim, Germany, purity >99.9%) were the solvents used. Ultrapure water was produced by a Milli-Q simplicity 185 system (Millipore, Molsheim, France). Anhydrous sodium sulphate (purity 99%) was purchased from Panreac (Barcelona, Spain) and, before use, was previously dried for 4 h at 400°C in a muffle furnace.

The glassware was washed with detergent and water, rinsed with acetone and n-hexane (Vaz Pereira, Sintra, Portugal) and dried at 90°C before use.

QuEChERS columns (50 mL Teflon centrifuge tube containing 6 g $MgSO_4$ and 1.5 g $C_2H_3NaO_2$) and cleanup columns (15 mL Teflon centrifuge tube containing 900 mg $MgSO_4$, 300 mg PSA and 150 mg endcapped C18) were supplied by Unit Chemical Technologies, Inc. (Bristol, PA, USA).

2.2 Sample collection and characterization

Fresh samples of horse mackerel were purchased from different local markets in Oporto region (NW Portugal) during 2007 to 2008. Sample collection and biometric characterization were performed in accordance to the EPA Guide No 823-B-00-07 [2]. Specimens were separated in two groups: males and females. 89 individuals were manually headed, eviscerated and filleted. Each sample used for further analysis was constituted by the edible parts of, at least, 4 individuals and a minimum mass of

Table 1. PAHs spiking levels tested.

Compound	Spiking level (ng/g) I	Spiking level (ng/g) II	Spiking level (ng/g) III
Naphthalene	200.6	100.0	19.0
Acenaphthene	200.6	100.0	19.0
Fluorene	39.6	19.7	3.8
Phenanthrene	20.0	10.0	1.9
Anthracene	20.0	10.0	1.9
Fluoranthene	39.5	19.7	3.7
Pyrene	19.4	9.7	1.8
Benz(a)anthracene	19.7	9.8	1.9
Chrysene	19.8	9.9	1.9
Benzo(b)fluoranthene	40.2	20.0	3.8
Benzo(k)fluoranthene	20.0	10.0	1.9
Benzo(a)pyrene	20.1	10.0	1.9
Dibenzo(a,l)pyrene	40.0	19.9	3.8
Dibenz(a,h)anthracene	40.0	19.9	3.8
Benzo(g,h,i)perylene	39.8	19.8	3.8
Indeno(1,2,3-cd)pyrene	20.1	10.0	1.9

200 g. Fish muscles were mechanically homogenised with a kitchen blender (Brio 400 W_{MAX}, Ufesa, Spain). The homogenized samples were kept frozen in 125 ml polycarbonate containers at −20°C until analysis.

Moisture was evaluated using 10 g of homogenized sample according to the Portuguese standard procedure NP 2282 [27] and the official AOAC method [28]. For total fat content determination, the recommended AOAC method [28] was applied.

2.3 Extraction procedure

For recovery studies, 5 g of homogenised fish sample were fortified with 2 mL of working standard solution containing the 16 selected PAHs at three concentration levels ranging from 1.8 ng/g pyrene to 200.6 ng/g naphthalene (Table 1). Spiked samples were allowed to stand for 30 min before extraction, protected from light. Spiked and non-spiked (blank) fish samples were transferred to the QuEChERS column and 8 mL of ACN was added. Concerning mixing the column content with the homogenised sample, two methods were tested: vortex during 1, 2, 3 or 5 min using a vortex mixer (Nahita 681/5, Navarra, Spain); and ultrasonic bath (Fungilab, Barcelona, Spain) with a working frequency of 30 kHz during 3, 10 or 20 min. After centrifugation in a 2.16 Sartorius centrifuge (Sigma, Goettingen, Germany) during 3 min at 3400 rpm, the extract was recovered. Two aliquots corresponding to half volume of the extract each, without and after PSA clean-up, were separated for analysis. Prior to injection into the LC-FLD system, both supernatants were filtered through a 0.20 µm PTFE membrane filter from Teknokroma (Barcelona, Spain).

All experiments were performed, at least, in triplicate.

2.4 Liquid chromatography analysis

Extracts were analysed using a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan) equipped with a LC-20AB pump (high-pressure gradient solvent delivery module equipped with two dual-plunger tandem-flow pumps), DGU-20AS degasser and a fluorescence RF-10AXL (FLD) detector. Separation of the compounds was performed in a C18 column (CC 150/4 Nucleosil 100-5 C18 PAH, 150 × 4.0 mm; 5 µm particle size; Macherey-Nagel, Duren, Germany) maintained at room temperature (20 ± 1°C). The injected volume was 15 µL.

The initial composition of the mobile phase was 50% of ACN and 50% water and a linear gradient to 100% was programmed in 15 min, with a final hold of 13 min. Initial conditions were reached in 1 min and maintained for 6 min before next run. The total run time was 40 min with a flow rate of 0.8 mL/min.

Fluorescence wavelength programming was used to perform better sensitivity and minimal interference. Each compound was detected at its optimum excitation/emission wavelength pair: 315/260 nm (naphthalene, acenaphthene and fluorene), 366/260 nm (phenanthrene), 430/260 nm (anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene and dibenzo(a,l)pyrene and 505/290 nm (indeno(1,2,3-cd)pyrene). Acenaphthylene determination was not performed.

PAH identification was carried out with individual stock solution of each PAH by comparison of their retention times with those of the standards. The concentration of the studied compounds was determined by comparing peak areas in the sample with those found for mixtures of PAH standards of known concentration analysed in the same conditions. The effect of interfering matrix compounds in the chromatographic determination, which affected the signal of analytes causing bias, was tested by the use of calibrations using standards prepared in ACN and in blank fish extracts, *i.e.* matrix matched standard calibrations. External calibrations with PAHs mixed standards, using at least 6 calibration points, were performed.

Each analysis was run at least in triplicate.

2.5 Statistical analysis

Statistical significance was assessed using a one-way analysis of variance (ANOVA) (SigmaStat for Windows Version 3.1, SPSS Inc., USA). Differences between groups were considered significant when $p < 0.05$ (two tailed).

3 Results and discussion

3.1 Chromatographic analysis

For detection and quantification of the 15 PAHs considered by EPA as priority pollutants and dibenzo(a,l)pyrene, the programmed chromatographic conditions were

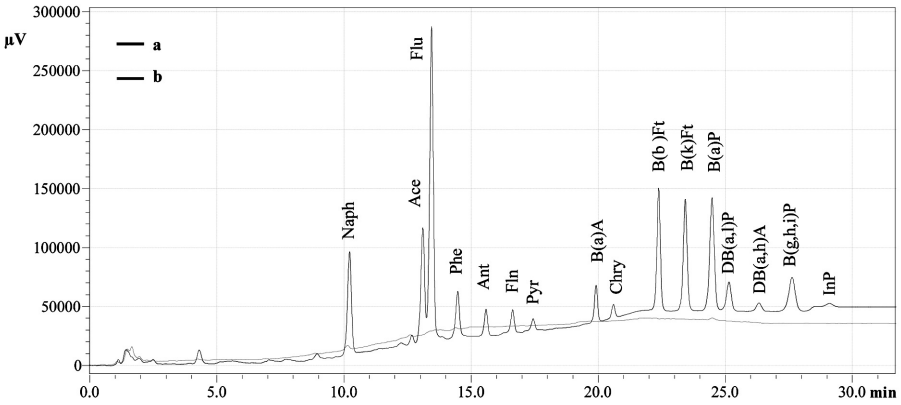

Figure 1. Overlay LC-FLD chromatograms of a matrix-matched standard PAHs mixture (a) containing 16 PAHs (Naph-100.3 μg/L, Ace-100.3 μg/L, Flu-19.8 μg/L, Phe-10.0 μg/L, Ant-10.0 μg/L, Fln-19.7 μg/L, Pyr-9.69 μg/L, B(a)A-9.84 μg/L, Chry-9.91 μg/L, B(b)Ft-20.1 μg/L, B(k)Ft-9.98 μg/L, B(a)P-10.0 μg/L, DB(a,l)P-20.0 μg/L, DB(a,h)A-20.0 μg/L, B(g,h,i)P-19.9 μg/L and InP-10.0 μg/L) and a blank horse mackerel extract (b).

Table 2. Average retention time and calibration data obtained using matrix matched standards for the selected PAHs.

Compound	Calibration range (μg/L; n = 6)	Retention time (min)	Regression equation[a] (n = 6)	R^2	LOD (μg/L)	LOD (ng/g wet weight)	LOQ (μg/L)	LOQ (ng/g wet weight)
Naphthalene	0.0 – 200.6	10.2	$y = 5464x + 70212$	0.99999	1.40	0.56	4.70	1.90
Acenaphthene	12.5 – 200.6	13.1	$y = 6893x - 3334$	0.99999	0.86	0.35	2.90	1.20
Fluorene	0.0 – 39.6	13.4	$y = 99029x + 9769$	0.99999	0.12	0.05	0.39	0.16
Phenanthrene	0.0 – 20.0	14.5	$y = 25204x - 2328$	0.99989	0.26	0.11	0.87	0.35
Anthracene	1.3 – 20.0	15.6	$y = 12912x - 654$	0.99966	0.46	0.18	1.50	0.61
Fluoranthene	2.5 – 39.5	16.6	$y = 7102x - 672$	0.99999	0.10	0.06	0.49	0.19
Pyrene	1.2 – 19.4	17.4	$y = 9076x - 306$	0.99995	0.17	0.07	0.55	0.22
Benz(a)anthracene	1.2 – 19.7	19.9	$y = 21737x - 1345$	0.99999	0.09	0.04	0.30	0.12
Chrysene	2.5 – 19.8	20.6	$y = 9568x - 14127$	0.99993	0.21	0.09	0.71	0.28
Benzo(b)fluoranthene	2.5 – 40.2	22.4	$y = 37349x - 6858$	0.99998	0.23	0.09	0.77	0.31
Benzo(k)fluoranthene	1.3 – 20.0	23.4	$y = 82530x - 8801$	0.99997	0.13	0.05	0.43	0.17
Benzo(a)pyrene	1.3 – 20.1	24.5	$y = 90305x + 8531$	0.99996	0.17	0.07	0.56	0.22
Dibenzo(a,l)pyrene	2.5 – 30.0	25.1	$y = 101645x - 9094$	0.99987	0.46	0.18	1.50	0.61
Dibenz(a,h)anthracene	2.5 – 40.0	26.3	$y = 2992x - 4301$	0.99996	0.35	0.14	1.20	0.47
Benzo(g,h,i)perylene	2.5 – 39.8	27.6	$y = 77722x - 7861$	0.99999	0.21	0.09	0.71	0.28
Indeno(1,2,3-cd)pyrene	1.3 – 20.1	29.1	$y = 5159x + 2701$	0.99983	0.33	0.13	1.10	0.44

[a] y – area; x – concentration (μg/L).

optimized based on previous works [11, 29] and gave an acceptable separation of the compounds (Fig. 1), with retention times in the range of 10.2 to 29.1 min (Table 2). The occurrence of matrix effects is a known problem which may have a significant impact on the quantification of compounds by chromatography. Matrix effects can reduce or enhance the analytical response when compared to the same concentration in a matrix-free solution. In this study, the presence or absence of matrix effects was demonstrated by comparing the response produced from the analyte in an ACN solution with that obtained from the same quantity of analyte in the presence of the matrix extract at the same levels of calibration. Both types of external calibrations curves were linearly fitted with correlation coefficients always higher than 0.9997 for all PAHs. However, chromatographic responses had significant differences for six compounds for which the ratio between slopes of both types of calibration curves (R) were not equal to 1. Matrix effects were noted for naphthalene (R = 1.11), anthracene (R = 0.91), chrysene (R = 0.87), benzo(a)pyrene (R = 0.83), dibenzo(a,l)pyrene (R = 0.67) and dibenz(a,h)anthracene (R = 0.86). Therefore, matrix matched standard calibrations were used along this study. Table 2 presents the more relevant information.

LOD and LOQ were defined and determined as the minimum detectable amount of analyte with a signal-to-noise ratio of 3:1 and 10:1, respectively, from horse mackerel spiked extracts [30]. LODs between 0.04 ng/g wet weight (0.09 μg/L) for benz(a)anthracene and 0.56 ng/g wet weight (1.40 μg/L) for naphthalene were obtained, with corresponding LOQs in the range 0.12 – 1.90 ng/g wet weight (0.30 – 4.70 μg/L). Concerning the maximum level of 2 ng/g wet weight established by the European Union for benzo(a)pyrene, the LOQ attained is sufficiently low for the method to be used for monitoring purposes.

Table 3. Effect of extraction time using vortex (1, 2, 3 and 5 min) and the ultrasonic bath (3, 10 and 20 min) on the average recoveries (%, $n = 4$) and repeatability (RSD,%) obtained with the QuEChERS method in spiked homogenized fish samples analysed by LC-FLD. The fortification level used, calculated as mass of PAH per mass of sample, is indicated in Table 1.

Compound	Recovery ± RSD (%)						
	Vortex				Ultrasonic bath		
	1 min	2 min	3 min	5 min	3 min	10 min	20 min
Naphthalene	100.0 ± 3.6	102.0 ± 4.2	109.0 ± 1.5	102.0 ± 1.9	56.0 ± 3.8	61.4 ± 1.7	80.0 ± 5.7
Acenaphthene	94.1 ± 3.1	93.6 ± 3.4	94.2 ± 1.6	92.1 ± 0.6	48.5 ± 1.8	62.4 ± 1.1	78.1 ± 5.1
Fluorene	98.5 ± 5.0	97.1 ± 4.3	102.0 ± 1.8	101.0 ± 2.4	50.7 ± 3.5	61.4 ± 1.3	76.3 ± 5.5
Phenanthrene	75.8 ± 1.7	84.3 ± 1.3	92.0 ± 0.4	94.1 ± 1.6	42.5 ± 1.1	48.1 ± 2.0	69.4 ± 2.7
Anthracene	84.6 ± 2.4	88.1 ± 2.5	95.3 ± 1.1	93.6 ± 1.8	35.1 ± 2.3	32.6 ± 3.5	74.1 ± 4.0
Fluoranthene	90.4 ± 1.0	90.4 ± 1.8	91.8 ± 1.9	95.6 ± 2.3	50.5 ± 0.6	57.7 ± 1.1	77.1 ± 1.5
Pyrene	92.0 ± 6.6	91.4 ± 0.2	95.0 ± 1.0	100.0 ± 2.7	48.5 ± 1.1	55.7 ± 1.4	78.8 ± 1.6
Benz(a)anthracene	87.4 ± 1.8	88.7 ± 1.3	93.8 ± 1.5	92.1 ± 1.6	51.5 ± 0.9	58.4 ± 1.1	78.1 ± 2.7
Chrysene	90.7 ± 2.3	91.9 ± 1.5	92.9 ± 1.3	95.7 ± 2.3	50.5 ± 1.3	55.5 ± 1.5	77.9 ± 3.2
Benzo(b)fluoranthene	95.1 ± 3.3	95.6 ± 1.5	98.8 ± 1.1	94.9 ± 2.2	50.4 ± 0.5	57.9 ± 1.3	76.4 ± 3.7
Benzo(k)fluoranthene	89.7 ± 1.9	91.7 ± 1.2	93.9 ± 1.1	94.7 ± 1.4	50.6 ± 1.0	58.2 ± 1.2	76.2 ± 3.3
Benzo(a)pyrene	86.6 ± 2.1	88.4 ± 1.7	90.1 ± 1.5	92.5 ± 1.5	49.8 ± 0.9	57.5 ± 1.3	70.6 ± 4.1
Dibenzo(a,l)pyrene	91.2 ± 3.1	89.2 ± 1.7	93.2 ± 1.6	93.2 ± 1.8	46.5 ± 3.8	60.2 ± 1.8	73.0 ± 3.6
Dibenz(a,h)anthracene	86.3 ± 1.4	94.6 ± 5.1	100.0 ± 1.8	97.7 ± 1.6	50.5 ± 1.3	58.9 ± 0.9	75.0 ± 5.4
Benzo(g,h,i)perylene	82.2 ± 4.5	87.0 ± 1.7	94.5 ± 2.0	98.6 ± 1.4	49.0 ± 3.3	53.7 ± 3.9	70.1 ± 3.5
Indeno(1,2,3-cd)pyrene	87.5 ± 3.1	90.5 ± 3.8	89.6 ± 2.0	102.0 ± 1.9	48.3 ± 4.4	54.8 ± 1.1	69.2 ± 6.0

3.2 QuEChERS extraction

3.2.1 Preliminary considerations

Water content is considered to be a critical parameter to be controlled during QuEChERS extraction. QuEChERS was designed for pesticide residue analysis of fruits and vegetables that contain more than 75% moisture. However, it was already applied after proper validation to assess pesticides in non-aqueous samples such as olive oil [16] and in a hydroethanolic matrix (wines) [23]. In this work, the fresh fish species that were selected present high moisture contents varying across the seasons and with fish activities (mainly during reproduction and migration).

The original procedure consists of extracting the homogenised sample by hand-shake or Vortex during 1 min with the same amount of ACN (1 g sample/mL final extract) in order to have a final extract, concentrated enough without the need of a solvent evaporation step. The use of ACN in QuEChERS procedure has already showed to provide high recoveries for many pesticides from different classes [16] and alone, it is often sufficient to perform excellent extraction efficiency without the need to add nonpolar co-solvents. Concerning the extraction of PAHs in a previously reported optimization study, ACN was compared with other low toxicity solvents and proved to attain the highest extraction yields [11]. Consequently, no other extraction solvent or mixture was tested. Furthermore, ACN is compatible with the optimised LC-FLD procedure and consequently no solvent exchange is required reducing the loss of analytes during sample preparation. This is particularly important for PAHs since some of these compounds are extremely volatile and if, during an evaporation step, the extract is taken to complete dryness, naphthalene, acenaphthene and fluorene can be totally lost [31].

A preliminary study was realized testing different ratios of mass of sample per volume of solvent, ranging from 1 (defined in the original QuEChERS procedure) to 0.5. In order to maximize sensitivity and to obtain a suitable dispersion and extraction of the pollutants, 10 mL of solvent are required to treat 5 g of sample (ratio 0.5).

3.2.2 Optimization study

The optimization experiments concerning the selection of the technique used for mixing the sample and solvent with the QuEChERS column content, and the influence of extraction time were performed in horse mackerel homogenized samples fortified at level I (spiking concentration of each PAH is given in Table 1). Table 3 shows recovery data attained using matrix-matched calibrations for quantification prepared with fish blanks extracted with QuEChERS. Regarding the utilization of vortex, recoveries improved when extraction time increased from 1 to 3 min, being constant thereafter. The overall mean recoveries reached at 1, 2, 3 and 5 min were 89.6 ± 2.8%, 91.5 ± 2.4%, 95.8 ± 1.4% and 95.9 ± 1.9%, respectively. Yields of phenanthrene, anthracene, benz-(a)anthracene, benzo(b)fluoranthene, dibenzo(a,l)pyrene, dibenz(a,h)anthracene and indeno(1,2,3-cd)pyrene were significantly enhanced by the increase of extraction time. Using ultrasonic bath, recoveries were always

Table 4. PAHs recoveries (mean ± relative standard deviation; $n = 4$ from homogenized horse mackerel samples using the optimal QuEChERS extraction conditions, at three fortification levels (I, II and III, defined in Table 1).

Compound	Recovery ± RSD (%)		
	Spiking level I	Spiking level II	Spiking level III
Naphthalene	108.8 ± 1.5	105.9 ± 1.9	110.5 ± 1.2
Acenaphthene	94.2 ± 1.6	96.0 ± 3.8	97.3 ± 3.1
Fluorene	101.7 ± 1.8	97.3 ± 1.1	100.7 ± 2.9
Phenanthrene	92.0 ± 0.4	99.2 ± 2.3	92.9 ± 2.2
Anthracene	95.3 ± 1.1	94.5 ± 1.4	95.0 ± 1.9
Fluoranthene	91.8 ± 1.9	94.4 ± 0.5	91.7 ± 4.8
Pyrene	95.0 ± 1.0	93.1 ± 0.4	96.3 ± 3.7
Benz(a)anthracene	93.8 ± 1.5	91.0 ± 0.4	90.0 ± 1.9
Chrysene	92.9 ± 1.3	92.0 ± 0.9	91.6 ± 1.6
Benzo(b)fluoranthene	98.8 ± 1.1	90.5 ± 0.3	93.7 ± 3.5
Benzo(k)fluoranthene	93.9 ± 1.1	90.2 ± 0.4	89.8 ± 3.6
Benzo(a)pyrene	90.1 ± 1.5	87.5 ± 0.5	87.3 ± 1.0
Dibenzo(a,l)pyrene	93.2 ± 1.6	86.6 ± 0.2	89.3 ± 1.1
Dibenz(a,h)anthracene	103.6 ± 1.5	96.8 ± 1.9	101.5 ± 1.0
Benzo(g,h,i)perylene	94.5 ± 2.0	90.0 ± 0.5	86.0 ± 1.6
Indeno(1,2,3-cd)pyrene	89.6 ± 2.0	84.8 ± 0.4	89.2 ± 3.4

lower than those obtained with vortex (Table 3). Quantitative extraction (average value >75%) was only achieved after 20 min; recoveries range between 69.2 ± 6.0% for indeno(1,2,3-cd)pyrene and 80.0 ± 5.7 for naphthalene. Comparing both methodologies, vortex allowed the efficient extraction of all compounds being three minutes the optimum time. This value is significantly higher than the one applied in the original procedure but can still be considered a short time.

Clean up procedure was also studied using aliquots of the same extract without and after PSA clean-up. No significant influence was detected in the LC-FLD chromatograms obtained which are mainly free of interfering peaks (Fig. 1; the peak shape of indeno(1,2,3-cd)pyrene became less well defined), thus allowing low detection and quantification levels to be reached (Table 2). Then, the approach of using a cleanup column after extraction was discarded minimising sample preparation.

For the optimization of time and selection of mixing method, the spiking level I (20 ng/g of benzo(a)pyrene) was used. Afterwards, two other fortification levels (II and III, Table 1) were chosen in order to test the recovery values over a certain range in concentration using the optimum conditions. The lower spiking level was selected in order to include the maximum admissible concentration for benzo(a)pyrene in fish muscle fixed at 2 ng/g wet weight by the European Regulation 208/2005/EC [5]. These results are displayed in Table 4, together with the data previously obtained for the spiking level used in the optimization study of the extraction time. The extraction efficiency was consistent over the entire range being benzo(g,h,i)perylene the most affected compound by the diminution of the fortification level from I to level III (94.5 ± 2.0% to 86.0 ± 1.6%, respectively). The overall mean recoveries obtained at level I, level II and level III were 95.8 ± 1.4%, 92.5 ± 5.2% and 93.8 ± 6.3%, respectively. No significant dispersion of results was observed and recovery did not differ substantially at the lowest and the highest concentrations. The reported results provided evidence that the adapted QuEChERS method achieved for all PAHs good recoveries (between 84.8 ± 0.4 for indeno(1,2,3-cd)pyrene and 110.5 ± 1.2% for naphthalene) and repeatability (between 0.2% for dibenzo(a,l)pyrene and 4.8% for fluoranthene). The reproducibility (between-day precision) of the analysis was also evaluated by repeating the analysis of spiked samples on three consecutive days. Values of RSD ranging from 1.1% for indeno(1,2,3-cd)pyrene and 7.6% for acenaphthene were reached.

3.3 Analysis of NIST-SRM 2977

It is well known that compounds spiked on a matrix will be extracted much easier than compounds naturally incurred in the material. For that reason, validation of the optimized QuEChERS extraction method for determination of PAHs in fish fillets was carried out analyzing the certified reference material NIST-SRM 2977 mussel tissue. This standard reference material is a freeze-dried tissue homogenate prepared from mussels collected in Guanabara Bay, Brazil. It is intended for use in evaluating analytical methods for the determination of fourteen PAHs (benzo(k)fluoranthene and dibenzo(a,l)pyrene are not present) in marine bivalve mollusc tissue and similar matrices. Reference values for 16 additional PAHs are provided. Actually, no SRM of fish naturally contaminated with PAHs is available.

Table 5. Certified, reference (non certified) and measured concentrations of native PAHs (mean, SD and recovery ± RSD) in the NIST reference material SRM 2977 ($n = 4$).

Compound	Mass fraction, mean ± SD (ng/g)		Recovery ± RSD (%)
	[a]Certified or [b]reference values	Measured values	
Naphthalene	19 ± 5[b]	n.q.	n.q.
Acenaphthene	4.2 ± 0.4[b]	0.46 ± 0.30	11 ± 65
Fluorene	10.24 ± 0.43[a]	10.20 ± 0.22	99.9 ± 2.2
Phenanthrene	35.1 ± 3.8[a]	38.0 ± 0.9	108.0 ± 2.2
Anthracene	8 ± 4[b]	5.1 ± 0.2	63.5 ± 4.3
Fluoranthene	38.7 ± 1.0[a]	40.4 ± 0.7	104.0 ± 1.6
Pyrene	78.9 ± 3.5[a]	87.2 ± 2.2	110.0 ± 2.5
Benz(a)anthracene	20.34 ± 0.78[a]	16.10 ± 0.92	79.0 ± 5.7
Chrysene	49 ± 2[b]	34.5 ± 2.7	70.4 ± 7.7
Benzo(b)fluoranthene	11.00 ± 0.28[a]	11.50 ± 0.49	104.0 ± 4.3
Benzo(a)pyrene	8.35 ± 0.72[a]	8.20 ± 0.13	98.2 ± 1.6
Dibenz(a,h)anthracene	1.41 ± 0.19[a]	1.36 ± 0.03	96.5 ± 1.8
Benzo(g,h,i)perylene	9.53 ± 0.43[a]	9.86 ± 0.05	104.0 ± 0.6
Indeno(1,2,3-cd)pyrene	4.84 ± 0.81[a]	4.73 ± 0.20	97.7 ± 4.1

n.q., not quantified due to peak overlapping

Table 6. Average biometric data, moisture and fat content of the homogenized fish samples.

Sample	n	Gender	Average size ± SD (cm)	Average weight ± SD (g)	Humidity ± SD (%)	Fat content ± SD (g/100 g)
Horse mackerel	20	Female	32.1 ± 2.5	296 ± 76	74.4 ± 0.6	3.83 ± 0.37
	8	Male	31.4 ± 1.9	243 ± 37	77.5 ± 1.0	3.62 ± 0.30
Chub mackerel	7	Female	29.5 ± 1.2	254 ± 29	63.7 ± 1.7	11.8 ± 0.5
	6	Male	29.9 ± 1.2	288 ± 29	64.1 ± 1.9	9.84 ± 0.68
Sardine	19	Female	20.2 ± 1.0	94.0 ± 14	49.2 ± 1.1	22.2 ± 2.0
	21	Male	19.3 ± 1.0	86.4 ± 11	52.5 ± 1.2	21.5 ± 1.1
Seabass	4	Female	30.6 ± 0.6	320 ± 20	66.9 ± 0.8	9.04 ± 0.38
	4	Male	32.1 ± 0.1	371 ± 35	69.5 ± 0.1	8.73 ± 0.32

n: number of individuals

Bearing in mind that experiments to confirm accuracy must be performed in triplicate, or more, and due to the limited quantity (10 g) of NIST SRM-2977, a mass of 0.8 g was extracted by the QuEChERS method instead of 5 g. To compensate the reduction in sensitivity due to this alteration, extracts were evaporated to dryness and immediately before chromatographic analysis, the residue was redissolved in 1000 μL of ACN. Table 5 summarizes the data obtained. The first eluting compound, naphthalene, was affected by the presence of co-extracted compounds and was impossible to quantify due to the occurrence of peak overlapping. The very low recovery attained for acenaphthene, 11 ± 65% was possibly caused by the evaporation step that was introduced in the analytical protocol since this PAH is extremely volatile. The recoveries of all the other targeted contaminants were in the range of 63.5 (for anthracene) to 110.0% (for pyrene) with variation coefficients not exceeding 8%. The method provided consistent results with the certified and reference concentrations, except for naphthalene and acenaphthene, in line with other studies using much more complicated extraction procedures [32–35]. Concerning the quantification of acenaphthene from the same SRM, Liguori *et al*. [32] applied accelerated solvent extraction technique followed by a purification step with gel permeation chromatography and also found a value that did not match the certified value. This was identified as a small limitation of the proposed approach [32]. Furthermore, Sanz-Landaluze *et al*. [35] reported in their study that application of the same analytical procedure (including evaporation and reconstitution) to standard solutions can enable correction for losses in quantification of highly volatile PAHs in analysed samples.

3.4 Application to fish fillets

The developed analytical protocol was applied for quality control, concerning PAHs, of horse mackerel, chub mackerel and sardine. Three homogenized samples of cultured seabass were also analysed since there is little information regarding pollutant levels in farmed fish. Biological parameters of sampled fish are listed in Table 6. Individuals were males and females and rather homogene-

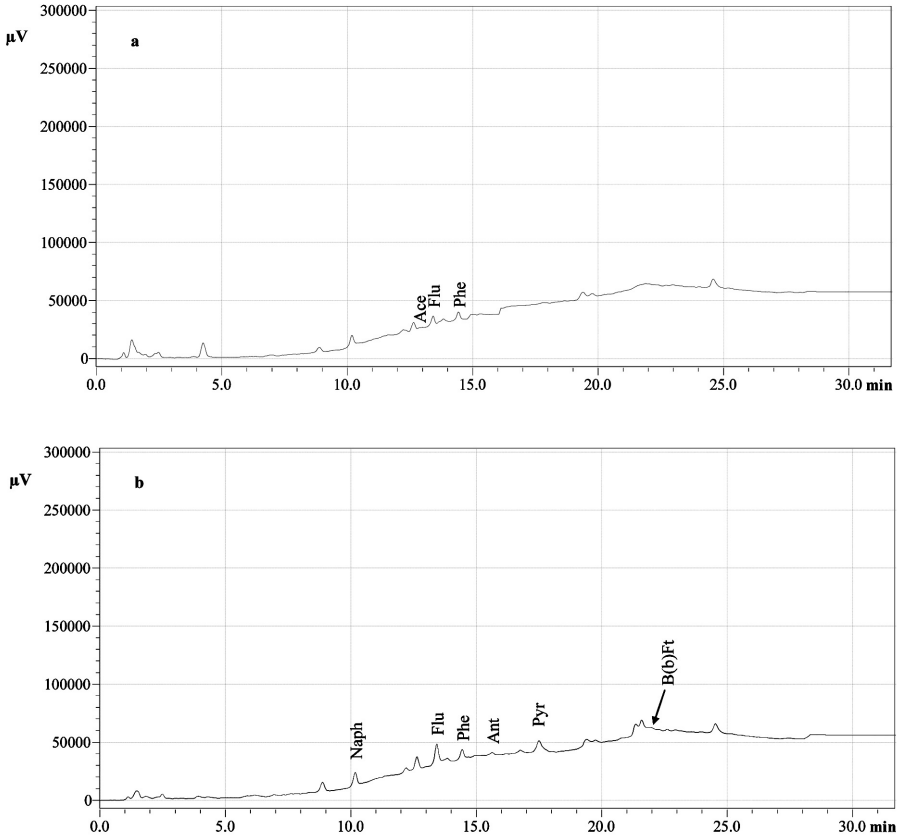

Figure 2. LC-FLD chromatograms of QuEChERS extracts of unspiked homogenized (a) sardine and (b) cultured European seabass samples.

ous in size and weight in each species. Moisture and total fat content vary inversely and considerable differences were identified between species being richer in fatty acids, by descending order, sardine, chub mackerel, seabass and horse mackerel. The knowledge of the total lipid content of fish and fish products is important to evaluate the nutritional value of diets and, in environmental monitoring programs, concentrations of persistent organic contaminants are often related on lipid content.

Representative chromatograms of sardine and seabass extracts are shown in Fig. 2 (a-b). Average concentrations of the detected PAHs, as well as, the sum of the compounds expressed as mass of PAH per wet weight of homogenized fish fillets are summarized in Tables 7–8. Significant differences were found between the mean PAHs concentrations of the four groups. No significant differences between genders of the same species were observed. Although benzo(a)pyrene, the marker used for evaluating the carcinogenic risk of PAHs in food, was not detected in the analysed samples (89 individuals corresponding to 27 homogenized samples), the sum of PAHs ranged from 0.73 ± 0.05 to 4.80 ± 1.00 ng/g in horse mackerel, 3.34 ± 0.96 to 5.03 ± 0.98 ng/g in chub mackerel, 3.64 ± 0.81 to 6.60 ± 1.40 ng/g in sardine, and increased drastically in the analysed seabass species from aquaculture, namely 11.63 ± 1.20 to 17.04 ± 4.00 ng/g. Concentrations of individual compounds are in a range up to 4.99 ± 0.51 ng/g (naphthalene in seabass). The analytes that are common in the four different fish species were acenaphthene, fluorene and phenanthrene. These PAHs accordingly with the International Agency for Research on Cancer [36] are included in Group 3: Unclassifiable as to carcinogenic in humans. The detection in seabass of naphthalene (corresponding to *ca*. 33% of total PAHs in SB1 and SB2 samples, and 22% in SB3 sample), benz(a)anthracene and benzo(b)fluoranthene, although their low levels determined is of special interest since they are classified by IARC as Group 2B: Possible carcinogenic to humans [36]. PAHs are soluble in fatty and lipid-rich tissues, where they accumulate preferentially. However they diffuse also into the muscles, where they may be bound to some structural elements. In this sense, the quantification of such compounds, particularly in fat rich wild fishes (such as sardine and chub mackerel) or farmed fishes (such as seabass) is needed to evaluate their safety. The present results for chub mackerel (overall mean concentration of 4.06 ± 0.69 ng/g) and sardine (overall mean concentration of 5.22 ± 1.09 ng/g) are comparable with those found in previous surveys conducted in Catalonia, Spain (total PAHs of 9.4 and 5.3 ng/g in chub mackerel and sardine, respectively) [37–39]. For the other species, PAHs concentrations in edible tissues were

Table 7. Average concentrations of PAHs measured in horse mackerel, chub mackerel and sardine samples (n ≥ 3) purchased in the city of Oporto (NW Portugal).

Sample		Mean concentration ± SD (ng/g wet weight)				
		Gender	Acenaphthene	Fluorene	Phenanthrene	∑ PAHs ± SD
Horse mackerel	HM1	Female	1.81 ± 0.12	0.32 ± 0.06	0.12 ± 0.01	2.25 ± 0.92
	HM2	Female	n.d.	n.d.	0.73 ± 0.05	0.73 ± 0.05
	HM3	Female	2.01 ± 0.18	0.59 ± 0.01	0.78 ± 0.16	3.37 ± 0.77
	HM4	Female	n.d.	0.49 ± 0.01	0.91 ± 0.17	1.40 ± 0.30
	HM5	Male	2.17 ± 0.12	0.66 ± 0.11	0.57 ± 0.02	3.40 ± 0.90
	HM6	Male	2.70 ± 0.23	0.64 ± 0.04	1.46 ± 0.07	4.80 ± 1.00
	HM7	Female	1.48 ± 0.12	0.60 ± 0.10	1.33 ± 0.06	3.40 ± 0.47
	HM8	Female	n.d.	0.59 ± 0.07	1.40 ± 0.12	1.99 ± 0.57
	HM9	Male	n.d.	0.75 ± 0.03	1.25 ± 0.11	2.00 ± 0.35
	HM10	Female	n.d.	0.46 ± 0.05	1.40 ± 0.13	1.86 ± 0.67
Chub mackerel	CH1	Female	2.56 ± 0.21	0.62 ± 0.01	1.85 ± 0.06	5.03 ± 0.98
	CH2	Female	2.85 ± 0.43	0.58 ± 0.11	0.72 ± 0.09	4.14 ± 1.30
	CH3	Female	2.56 ± 0.22	0.48 ± 0.03	0.47 ± 0.07	3.51 ± 1.20
	CH4	Male	2.82 ± 0.05	0.57 ± 0.08	1.31 ± 0.11	4.70 ± 1.10
	CH5	Male	2.22 ± 0.38	0.49 ± 0.03	0.63 ± 0.02	3.34 ± 0.96
	CH6	Male	2.20 ± 0.36	0.67 ± 0.05	0.76 ± 0.09	3.63 ± 0.86
Sardine	SA1	Female	2.42 ± 0.22	0.80 ± 0.04	2.26 ± 0.48	5.48 ± 0.90
	SA2	Male	2.24 ± 0.03	0.76 ± 0.08	1.97 ± 0.14	4.97 ± 0.79
	SA3	Female	2.27 ± 0.19	0.56 ± 0.07	1.37 ± 0.05	4.19 ± 0.86
	SA4	Female	2.09 ± 0.26	0.48 ± 0.10	1.07 ± 0.02	3.64 ± 0.81
	SA5	Male	2.53 ± 0.24	0.57 ± 0.09	1.36 ± 0.07	4.45 ± 0.98
	SA6	Female	3.70 ± 0.46	0.93 ± 0.09	1.97 ± 0.18	6.60 ± 1.40
	SA7	Male	3.73 ± 0.43	0.99 ± 0.01	1.73 ± 0.22	6.45 ± 1.40
	SA8	Male	3.81 ± 0.25	0.83 ± 0.07	1.32 ± 0.16	5.96 ± 1.60

n.d. – not detected

Table 8. Average concentrations of PAHs detected in cultured seabass (n ≥ 3) purchased in the city of Oporto (NW Portugal).

Sample	Gender	Mean concentration ± SD (ng/g wet weight)										
		Naphthalene	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benz(a)anthracene	Benzo(b)fluoranthene	Benzo(g,h,i)perylene	∑ PAHs ± SD
SB1	Male	4.99 ± 0.51	2.42 ± 0.36	1.89 ± 0.22	2.82 ± 0.36	n.d.	1.62 ± 0.22	n.d.	0.21 ± 0.03	0.32 ± 0.03	0.75 ± 0.04	15.02 ± 1.60
SB2	Female	3.76 ± 0.25	2.23 ± 0.40	1.22 ± 0.12	1.28 ± 0.02	n.d.	2.25 ± 0.32	n.d.	0.18 ± 0.01	n.d.	0.71 ± 0.12	11.63 ± 1.20
SB3	Female	3.77 ± 0.29	n.d.	0.80 ± 0.02	0.96 ± 0.04	0.47 ± 0.06	n.d.	10.90 ± 1.20	n.d.	0.14 ± 0.01	n.d.	17.04 ± 4.00

n.d. – not detected.

not found in literature. However, the attained results are in the same order of magnitude or lower than those from various studies with other marine organisms [40]. Furthermore, in some species, nearly all the 16 PAHs except benzo(a)pyrene, dibenz(a,h)anthracene and benzo(g,h,i)-perylene were detected [40]. Bioaccumulation of compounds in fish is a result of the different uptake and elimination processes of the compounds combined with metabolic clearance (biotransformation).

4 Conclusion

QuEChERS method enabled efficient extraction of a selected group of PAHs from homogenized fish samples. Results found were very promising and further studies will be conducted with food matrices having high levels of moisture and naturally contaminated with PAHs. As already reported for pesticides, this extraction technique for PAHs is also clearly advantageous over those involving intensive treatments since accurate results can be achieved with minimal sample preparation in a short time. This method is suitable for laboratories engaged daily in routine analysis of a large number of samples.

The authors declare that they have no conflict of interest.

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