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Application of matrix solid-phase dispersion in the analysis of priority polycyclic aromatic hydrocarbons in fish samples

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Abstract

The performance of matrix solid-phase dispersion (MSPD) for the extraction of polycyclic aromatic hydrocarbons (PAHs) in fish tissue is described. The suitability of different solid supports was tested as well as the influence on the extraction efficiency of the natural fat content in samples. Under optimal conditions 0.6–0.8 g of tissue sample, are dispersed with 2 g of octadecylsiloxane (C_{18}) and 0.5 g of anhydrous sodium sulphate and transferred to the top of a polyethylene solid-phase extraction cartridge which already contains 2 g of florisil and 1 g of C_{18}. Cartridges were eluted with acetonitrile. The analysis of the extracts was carried out by high-performance liquid chromatography (HPLC) coupled with fluorescence detection. The proposed method provides detection limits between 0.04 and 0.32 ng/g for the different considered PAHs, below the maximum levels established by the some regulatory bodies for the six PAHs after recent oil spill episodes and European Union regulations. Recoveries over 80% were obtained for all compounds. Accuracy validation was carried out using the US National Institute of Standards and Technology (NIST) SRM 2977 reference material.

Keywords: Polycyclic aromatic hydrocarbons; Matrix solid-phase dispersion; Fish tissue

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are of major concern in all environmental compartments, since many of them have been labelled as probable carcinogens by the International Agency for Research on Cancer (IARC) [1]. These compounds are produced by natural and anthropogenic processes, so several analytical procedures have been developed for their analysis in water, soil, air and food matrices. However, their presence in food is quite serious, because food is an important and direct source of human exposure to PAHs.

PAHs reach seawater in variable amounts due to boat fuel (especially in ports) or in the highly impacting ship accidents causing oil spills. Moreover, due to PAHs persistence in the environment, contamination by spilled oil has often been assessed by analysing these compounds in the spill sites [2]. Occurrence of PAHs in seawater is limited by their low solubility. However, although their environmental concentrations are low, they tend to bio-accumulate in organic tissues due to their lipophilic character and resistance to degradation [3]. Introduction of PAHs in seafood chain is well demonstrated since high amounts of these compounds have been found in many aquatic species [4–6].

It has been generally assumed that PAHs play the fundamental role in the toxicity of oil to aquatic organisms [7]. In spite of the seriousness of oil spills consequences, national or international regulations of PAHs concentration in fish and seafood samples are very recent. The European Union (EU) has not established until February 2005 the admissible limits for PAHs in fish [8]. In that regulation, the EU highlighted the need of measuring the levels of benzo[a]pyrene (B[a]P) in foods where environmental pollution may cause high levels of contamination, and in particular, fisheries products subjected to contamination

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from oil spills. The limit was set at 2 ng/g wet weight only for B[a]P. Previously, after Prestige accident, the Spanish government established regulatory levels affecting six PAHs in fish and shellfish tissues, based on the same levels established by the French government 3 years before because of the Erika accident [9]. These levels, considering dry weight, were 20 ng/g of benzo[a]anthracene (B[a]A), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), B[a]P, dibenzo[a,h]anthracene (DB[ah]A), and indeno[1,2,3-cd]pyrene (I[123cd]P) in fish samples and 200 ng/g for seafood [10].

One of the most difficult tasks in the analysis of toxic wastes in the environment is to have and efficient, fast, low cost and uncomplicated process for their extraction from biological matrices. Traditional methods for isolation of fat-soluble analytes and, particularly PAHs, in biological matrices often involved the hydrolysis of lipids (e.g. alkaline digestion) and subsequent solvent extraction, followed by a more or less complicated cleanup process of the extracts. Soxhlet extraction was commonly used for biological tissues, such as meat [11,12], fish [6,13,14] and seafood [15] samples. Many authors have discussed Soxhlet disadvantages, a time-consuming preparation technique that consumes large volumes of solvents, but its efficiency, especially considering recoveries of analytes, still makes Soxhlet extraction the method of choice for many studies. Alternative techniques have been developed to the extraction of PAHs from solid matrices, including pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) [16–18], microwave-assisted extraction (MAE) [16,19–21] and supercritical fluid extraction (SFE) [16,22,23]. All these procedures have shown clear advantages in terms of time and solvent consumption, although for fatty samples the co-extraction of lipids with the low-polarity analytes, appears again unavoidable. Thus, a cleanup of the extracts is needed to separate PAHs from lipids and other organic compounds. This cleanup may be performed using gel permeation chromatography [17] or columns packed with alumina, silica, Florisil or C_{18}. In the case of SFE procedures an alternative approach to reducing the lipid content in extracts is to place some sorbent material in the extraction chamber [21].

Matrix solid-phase dispersion (MSPD) can be regarded as a valid alternative to the more classical sample preparation methods, especially for semisolid samples, because it allows the extraction and cleanup in a single step using solid sorbents with significant reduction in both sample size and solvent consumption [24–26]. An important practical advantage is that MSPD does not need special instruments or costly hardware. In fact, MSPD was applied to the analysis of contaminants in different food samples, as reported in literature [27–33], including B[a]P from fish muscle [30], although B[a]P was the only PAH studied in samples applying a procedure developed for the determination of chlorinated pesticide residues. Moreover, this study considered only high B[a]P concentration levels with rather inefficient recoveries (ca. 70%).

The aim of the present study was to develop and evaluate a simple and fast MSPD procedure for the analysis of the six PAHs included in the recent regulations for fish and seafood. The method was optimised for fish samples having very different fat contents. All extracts were analysed by LC using fluorescence detection. The analytical method was validated using US National Institute of Standards and Technology (NIST) SRM 2977 reference material.

2. Experimental

2.1. Reagents and materials

Acetonitrile and methanol (gradient-grade, Lichrosolv), *n*-hexane, dichloromethane and chloroform (Suprasolv), and sulphuric acid (96%) were obtained from Merck (Darmstadt, Germany). EPA-610 Polycylic aromatic hydrocarbons mixture was supplied by Supelco (Bellefonte, PA, USA). Anhydrous sodium sulphate was supplied by Panreac (Barcelona, Spain). Ultrapure water was produced by a Milli-Q system supplied by Millipore (Bedford, MA, USA). Durapore filters (Millex GV, 13 mm, 0.22 μm) were also supplied by Millipore. Isolute syringe barrels fitted with a single bottom frit and additional polyethylene frit were supplied by Isolute (Mid Glamorgan, UK). Standard reference material (SRM) 2977 (mussel tissue) was purchased from the NIST (Gaithersburg, MD, USA).

Florisil (60–100 mesh), C_{18} (70–230 mesh), and acidic silica gel (44% sulphuric acid) prepared in the laboratory mixing neutral silica with concentrated sulphuric acid, were all obtained from Aldrich (Milwakee, MI, USA). These materials were assayed as co-columns in experiments aiming to maximize the cleanup effectiveness in the MSPD extraction process.

2.2. Apparatus

The high-performance liquid chromatography system consisted of a 600E pump and gradient controller (Waters, Milford, MA, USA), and a fluorescence detector HP Series 1100 (Agilent, Waldbronn, Germany). The injector was a Rheodyne, Model 7725i (Cotati, CA, USA), fitted with a 20-μL external loop. The temperature of the column was controlled by means of a MetaTherm 9540 oven, supplied by MetaChem (Torrance, CA, USA).

Chromatographic separation was performed with a 250 mm × 2.1 mm I.D. Vydac 201 TP52 column, with a particle size of 5 μm, purchased from Agilent. A 30 mm × 2 mm guard column Vydac 201 TP was employed to protect the analytical column.

Data acquisition was carried out by means of Agilent Chemstation Software (Rev. A. 06.03 [509]).

Sample extracts were concentrated using a TurboVap II nitrogen evaporator supplied by Zymark (Hopkinton, MA, USA).

2.3. Chromatographic conditions

Acetonitrile and water were used as eluents at a flow-rate of 0.4 mL/min. The gradient elution program was: 47% acetonitrile initially until 2 min, then increasing linearly to 90% acetonitrile in 10 min, and then linearly to 100% in 8 min, holding in 100% for 10 min and finally back to initial conditions in 10 min. Column temperature was maintained at 35 °C. Fluorescence detector was programmed in order to optimise sensitivity for all the peaks while minimising interferences. The fluorescence intensity was measured at the following excitation/emission wavelengths pairs: 286/410 nm for B[a]A, 294/425 nm for B[b]F, B[k]F, B[a]P and DB[ah]A, and 245/500 nm for I[123cd]P.

2.4. Fish tissue, spiking procedure and lipid determination

In order to produce comparative results, all the development of the extraction method was performed with stock sample of turbot (*Psetta maxima*) muscle. Additionally, a stock salmon (*Salmo salar*) sample was used to check the ability of the method to be applied to different kind of fish samples. Salmon was chosen as second sample material to test lipid content influence in the procedural performance. Both salmon and turbot were produced in aquaculture.

Optimization experiments were performed using stocks of spiked fish tissue. Both for salmon and turbot, 150 g of ground tissue were slurred with 15 mL of acetonitrile containing known concentrations of PAHs to obtain samples with concentrations of PAHs close to the regulated levels fixed by the EU and national food agencies. The mixtures were mechanically stirred and the samples were allowed to air-dry with occasional mixing at ambient temperature for several days. Aliquots of non-spiked materials were reserved as sample's blank. Then all spiked and non-spiked materials were frozen, lyophilised and stored in amber glasses under 0 °C. The percentage water of fresh fish tissues was gravimetrically determined, and resulted in 76.5% for turbot and 62.9% for salmon. Also the humidity of lyophilised samples were determined and resulted in 8.3% for turbot and 4.3% for salmon. Humidity of mussel tissue certified reference material was 8.2%.

The lipid content of each type of sample was determined by the Bligh and Dyer method [34], and using solvent extraction with a methanol-chloroform mixture. The percent of lipids expressed per wet weight was 1.7 and 16.5% for turbot and salmon muscle respectively. Lipid content of mussel tissue was also determined, and resulted in 1.3%. Lipids in sample extracts during method development were evaluated evaporating the extracts to dryness under nitrogen stream, and accurately weighing the solid residue.

2.5. Sample preparation

Accurately weighted 0.6–0.8 g of fresh fish sample (or 0.2 g of lyophilised sample reconstituted with 400 μL of ultrapure water), are thoroughly blended with 2 g of C_{18} in a glass mortar. Then 0.5 g of anhydrous sodium sulphate are added and the mixture blended again until visually homogeneous. Previously, a syringe barrel containing a frit at the bottom was filled (from bottom to top) with a layer of 2 g of Florisil and then another layer of 1 g of C_{18}. These materials will act as co-column or clean-up phases in the cartridge elution. Once the MSPD blend is homogeneous it was transferred to the syringe barrel. The material in the column is covered with another frit having 20 μm of pore size, and slightly compressed with a syringe plunger.

The cartridge is eluted with 10 mL of acetonitrile by gravity, allowing the eluate to drip slowly into a graduated receiving tube. Then the eluate is evaporated to 0.5 mL under nitrogen stream and transferred to a 2 mL volumetric flask. 0.5 mL of ultrapure water is added, and the flask volume made to the mark with acetonitrile. Extracts are filtered through 0.22 μm Durapore filters and 20 μL are injected in the HPLC system.

3. Results and discussion

3.1. Optimisation of extraction conditions

3.1.1. Clean-up efficiency

Lipids may be the main interference in the analysis of some contaminants in biological materials. HPLC columns are highly sensitive to trace amounts of lipidic material, which affect the active surface of the stationary phase and degrade the resolving power of the column. Thus, the presence of lipids in the extracts must be avoided or reduced as much as possible in order to extend the column lifetime and to improve detection and quantification limits [35].

Analytical literature suggests two destructive lipid removal methods, saponification and oxidative dehydration with sulphuric acid [35,36]. Obviously, the first one is not compatible with MSPD extraction, but dehydration with sulphuric acid may be combined with MSPD by the use of sulphuric acid impregnated silica gel layered in cartridges. To test this possibility some experiments were carried out placing 1 g of acidic silica as co-column in the MSPD extraction of turbot samples dispersed in 2 g of Florisil. Cartridges were eluted with 15 mL of *n*-hexane-dichloromethane (1:1) followed by 10 mL of dichloromethane. Only B[b]F was detected in the eluates showing that the co-column is clearly effective in lipid removal, but also in trapping most of the analytes. Similar observation has been reported by Wells and Echarri [37], in the extraction of polychlorinated biphenyls, explaining the observed effect by the formation of carbon as a sub product of lipid's dehydration, that retains non-polar compounds.

Table 1
Amount of lipids in the extracts of turbot samples, as a function of the type of sorbent used as dispersant and for clean-up

MSPD solid support	Co-column	Fat in the extract (mg/mL)
2 g Florisil	2 g Florisil	0.30
2 g Florisil + 0.5 g Na_2SO_4	2 g Florisil	0.23
2 g C_{18}	2 g Florisil	0.18
2 g C_{18} + 0.5 g Na_2SO_4	2 g Florisil	0.12
2 g C_{18} + 0.5 g Na_2SO_4	1 g C_{18}	0.42
2 g C_{18} + 0.5 g Na_2SO_4	2 g Florisil + 1 g C_{18}	0.08

Elution solvent: 10 mL of acetonitrile.

Some other co-columns are possible to assist in sample cleanup and fractionation. Among available materials, Florisil and C_{18} have been frequently applied as MSPD material and simultaneously packed in the bottom of the MSPD column or as an additional external column [24]. Some exploratory experiments were carried out using Florisil as co-column and mixtures of *n*-hexane:dichloromethane in proportions 9:1, 7:3 and 1:1 to elute the cartridges. Solvent mixtures with higher proportions of dichloromethane produced extracts having high concentration of lipids that produced the formation of solid residues after solvent evaporation and exchange to acetonitrile. Additionally it was shown that significant amounts of PAHs are entrapped with this lipidic residue, causing low recoveries. On the other hand, larger amounts of eluting solvent are needed to extract the analytes from the cartridge when mixtures of high content in *n*-hexane (mixtures 9:1 and 7:3) are applied. The consequence is that these eluting mixtures also produced important lipid coelution and similar problems to those described above. The best compromise conditions in this series of experiments were produced by eluting the cartridges with 15 mL of the 1:1 mixture of *n*-hexane:dichloromethane. In those conditions recoveries were c.a. 80% except for B[a]P (62%). This observation may justify the low recoveries reported in the pioneering paper of Crouch and Barker [30] for B[a]P, the only PAH studied. In that paper the appearance of precipitates in solvent evaporation is mentioned and centrifugation of extracts recommended.

Experiments with C_{18} as co-column gave better results to those described above. Then we considered the combination of both Florisil and C_{18} as solid supports, using acetonitrile as eluting solvent. Also the possibility of drying the blended sample with anhydrous sodium sulphate was considered in further experiments. Table 1 summarizes the results in these experiments, showing the amount of lipids present in the extracts. It can be seen that C_{18} as dispersant material improves the results of Florisil. Also the use of anhydrous sodium sulphate was shown as a significant factor to improve the ability of both adsorbents to retain the fats. Based on these results, 2 g of C_{18} with 0.5 g of sodium sulphate anhydrous as solid support, and two co-columns of Florisil (2 g) and C_{18} (1 g) were chosen to develop the extraction method, as described in Section 2.5.

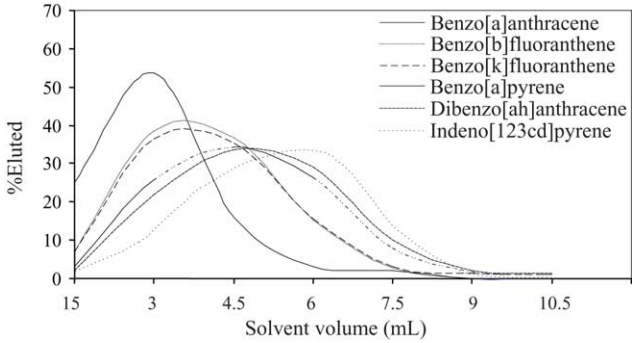

Fig. 1. Elution profiles of the six analytes from the MSPD cartridge.

3.1.2. Elution profile of PAHs

Once fixed the solid phases to perform the matrix dispersion, the next step in the optimisation of the extraction method is to determine the volume of acetonitrile necessary to recover PAHs in samples. Again, the focus is obtaining clean extracts reducing the solvent volume to a minimum.

To evaluate the elution volume of acetonitrile, staged elutions of turbot samples MSPD were performed by collecting up to seven 1.5-mL fractions of eluate into 2-mL volumetric flasks containing 0.5 mL of water. These extracts were filtered as described in Section 2 and injected directly in the chromatograph. Fig. 1 shows the normalized profiles of each PAH elution in this study, indicating that 9 mL of acetonitrile allows the quantitative elution of all the analytes from the cartridge. Thus, the elution volume was established in 10 mL to ensure reproducible results even for the most retained PAH.

Under the optimal conditions described for cleanup and elution, Fig. 2 shows a typical chromatogram for an extract

Table 2
Regression analysis, LODs and LOQs of the proposed analytical procedure

Compound	Calibration range (ng/g)	Calibration equation	Slope standard error	Intercept standard error	Correlation coefficient	LOD (S/N = 3) (ng/g)	LOQ (S/N = 10) (ng/g)
B[a]A	1–20	$y = 6.935x + 0.313$	0.127	1.423	0.998	0.16	0.52
B[b]F	2–40	$y = 2.631x + 0.045$	0.027	0.620	0.999	0.30	0.99
B[k]F	1–20	$y = 15.695x + 3.648$	0.241	2.705	0.999	0.04	0.13
B[a]P	1–20	$y = 8.694x + 0.297$	0.145	1.631	0.998	0.07	0.25
DB[ah]A	2–40	$y = 4.758x - 0.459$	0.053	1.184	0.999	0.14	0.46
I[123cd]P	1–20	$y = 1.012x - 0.038$	0.003	0.035	0.999	0.32	1.07

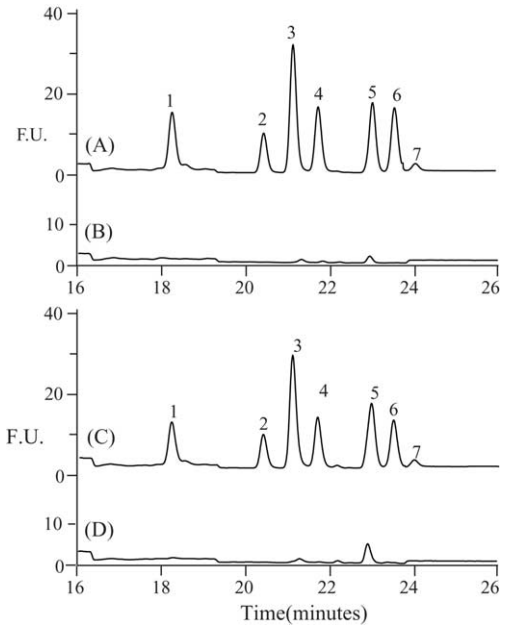

Fig. 2. Chromatograms of the extracts from spiked turbot (A), non-spiked turbot (B), spiked salmon (C) and non-spiked salmon (D). Key for peaks: (1) B[a]A; (2) B [b]F; (3) B[k]F; (4) B[a]P; (5) DB[ah]A; (6) benzo[ghi]perylene (present in the standard solution used in the spiking procedure although not considered in regulations nor in this study); (7) I[123cd]P.

of a spiked turbot muscle sample (Fig. 2A), and the corresponding sample blank (Fig. 2B).

3.2. Performance of the procedure

Calibration curves were constructed by injecting diluted standards in acetonitrile:water (3:1). A four-point calibration was performed. Each level was injected twice. Chromatographic peak heights were fitted by linear regression. Table 2 shows the concentration range and other data for calibration curves. Also detection and quantification limits of the analytical MSPD procedure are given, considering signal-to-noise ratios of 3 (LOD) and 10 (LOQ). As can be seen, quantification limits are by far below the regulatory levels established by the French government after Erika oil spill [9] and the Spanish government after Prestige episode [10], and also below the limit for B[a]P established recently by the EU [8].

To assess the reproducibility of the extraction method, five individual experiments were carried out in different days using the lyophilized stock turbot material. Average concentrations obtained for each compound and between days precision are also shown in Table 3. All those results are expressed on dry weight basis. Standard deviations lower than 6% also indicate that the stock material used is homogeneous enough for a dry sample weight of 0.2 g and that blending and manipulation of samples may be carried out in practice with reasonable variance.

Recoveries were determined by freshly spiking 0.2-g portions of lyophilised sample with standard solutions in acetonitrile. Each PAH was spiked at concentrations ca. 0.5, 1, 1.5 and 2 times the actual concentration measured in the original lyophilised samples. After spiking, samples were allowed to air-dry at room temperature before extraction for one day. Standard addition graphs were constructed from the representation of spiked concentration versus measured concentration calculating recoveries from these graphs as the slope ($\times 100$). Recoveries (between 88 and 106%) and standard deviations obtained are also given in Table 3.

3.3. Method validation: extraction of other kind of fish and seafood samples

As described before, the optimized MSPD procedure exhibit important advantages for the extraction of PAHs in turbot samples, not only in terms of reproducibility and recovery of analytes, but also in terms of time and solvent consumption. However, from the practical point of view there is a need to evaluate that the procedure is equally applicable no matter the kind of fish and seafood sample considered. Especially, the influence of fish fat content must be investigated to fully assess the procedure.

With this goal, the extraction method was applied to the extraction of PAHs in salmon muscle. Salmon can be considered as one of the fishes with highest fat content (salmon muscle contains about ten times more fat than turbot muscle). Fig. 2C and D depicts typical chromatograms of spiked and non-spiked salmon samples clearly showing the performance of the proposed procedure no matter the fat content of the samples.

The study of reproducibility and recovery described in previous section was repeated using the stock salmon mate-

Table 3
Reproducibility and recovery study for lyophilised stock samples

Compound	Turbot sample				Salmon sample			
	Average (ng/g) ($n=5$)	RSD (%) ($n=5$)	Recovery (%) (slope × 100)	Slope SD (%)	Average (ng/g) ($n=5$)	RSD (%) ($n=5$)	Recovery (%) (slope × 100)	Slope SD (%)
B[a]A	22.15	4.32	105.4	4.2	15.20	5.80	91.4	1.8
B[b]F	42.49	4.38	97.6	4.1	31.20	3.41	88.7	2.1
B[k]F	20.69	4.99	103.4	4.8	14.79	2.38	93.8	2.5
B[a]P	20.06	4.71	99.6	4.2	14.37	7.87	88.7	1.8
DB[ah]A	36.98	4.57	97.4	4.1	28.90	3.82	86.6	1.9
I[123cd]P	19.70	2.71	88.6	5.9	15.69	2.14	80.4	1.9

Table 4
Measured concentrations and confidence intervals vs. certified ones in NIST SRM 2977

	B[a]A	B[b]F	B[a]P	Db[ah]A	I[123cd]P
Certified (ng/g)	20.34 ± 0.78	11.01 ± 0.28	8.35 ± 0.72	1.41 ± 0.19	4.84 ± 0.81
This study (ng/g)	20.16 ± 3.62	8.94 ± 0.36	6.70 ± 1.14	1.44 ± 0.10	3.81 ± 0.46

rial and the results have been also summarized in Table 3. Homogeneity of materials and procedural precision appears similar for both types of samples. Although slightly lower than those calculated for low fat fish sample, recoveries for salmon sample ranging between 80 and 94% show the ability of the proposed procedure to quantitatively extract the analytes from highly fatty fish samples.

Finally, the extraction method was validated using a certified reference material obtained from the NIST, consisting on a lyophilised mussel tissue (SRM 2977). This material has certified concentrations for all the analysed PAHs except for Benzo[k]fluoranthene, at concentrations below the regulated levels. Five different extractions of the certified material were carried out and the extracts were analysed by HPLC. Average concentrations obtained (considering dry weight) with confidence intervals have been shown in Table 4, compared to certified concentrations for each PAH and its corresponding expanded uncertainty. Measured concentrations were not corrected with recovery percentages, providing a further confirmation of recovery values shown in Table 3. As can be seen, concentrations obtained for B[a]A, DB[ah]A and I[123cd]P are in agreement with the certified values. Only B[b]F appear below the certified concentrations, although the difference is really small and probably insignificant considering that the homogeneity limit declared for this reference material is 3 g although aliquots of 0.2 g have been taken for the analysis. Thus, expanded uncertainties showed in Table 4 are probably optimistic.

4. Conclusions

MSPD has been shown as an excellent alternative for the extraction of polycyclic aromatic hydrocarbons from fish tissues. This technique is really faster when compared to techniques such as microwave, Soxhlet or supercritical fluid extraction, inexpensive, and with very low solvent consumption. Because good reproducibility and recoveries are obtained, MSPD may be applied for processing a large number of samples in a short time with very low costs, thus being a practical choice for routine control and environmental campaigns monitoring the impacts of oil spill accidents on medium and long range scales. Detection and quantification limits are by far below the maximum allowable levels defined by the EU and national regulations.

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