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Ultrasound-assisted solvent extraction of total polycyclic aromatic hydrocarbons from mussels followed by spectrofluorimetric determination

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Abstract

An ultrasound-assisted solvent extraction procedure has been optimised to speed up total polycyclic aromatic hydrocarbons (T-PAHs) extraction from mussel soft tissue. The T-PAHs releases have been evaluated by spectrofluorimetry (excitation and fluorescence emission wavelengths of 300 and 382 nm, respectively, and using chrysene as calibrant). Variables such as sonication time, ultrasound frequency, *n*-hexane volume, dichloromethane volume, number of repeated extractions with *n*-hexane and number of repeated extraction with dichloromethane were simultaneously studied by applying a Plackett–Burman design (PBD) approach. Results showed that ultrasound frequency and *n*-hexane and dichloromethane volumes were statistically significant variables (confidence interval of 95%). These last two variables were finally optimised by using central composite designs (CCD), yielding optimum *n*-hexane and dichloromethane volumes of 2.5 and 6.5 ml, respectively. The lowest T-PAHs releasing at high ultrasound frequency (35 kHz) led to choice the lowest ultrasound frequency (17 kHz) to perform the extraction. Variables such as sonication time and number of repeated extraction with *n*-hexane or dichloromethane were statistically non-significant and they were fixed at 10 min and the extraction with *n*-hexane and dichloromethane were performed once. The limit of detection was 0.021 $\mu g\ g^{-1}$ (referred to dried mass), the repeatability of the overall method was 4.7% ($n = 9$) and the analytical recoveries were between 98 and 105%. The proposed method was finally applied to 16 mussel samples (*Mytilus galloprovincialis*) from *Ría de Arousa* estuary (Galicia, northwest Spain).

Keywords: Total polycyclic aromatic hydrocarbons; Mussel; Ultrasound-assisted solvent extraction; Spectrofluorimetry

1. Introduction

Nowadays, there is an increasing interest on hydrocarbons occurrence in the different compartments of the Environment, especially after oil spill episodes. Therefore, the knowledge of hydrocarbons levels and particularly polycyclic aromatic hydrocarbons (PAHs), in marine sediments and biota before and after oil spillages is important in order to elucidate the impact of such contamination episodes. Mussels are filter feeding bivalves molluscs that accumulate contaminants, such as hydrocarbons, in their soft tissues. As a result, mussels are cost-effective bioindicators of contaminants and have been used for decades to track patterns and trends of chemical contamination in coastal areas. In fact, since 1986 the American National Status and Trends (NS&T) Program has used mussels to assess contaminant trends [1].

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants derived from the combustion of fossil fuels. Most of these compounds have well-known mutagenic and carcinogenic activity and they have been included in environmental control legislation. The European directive 2455/2001/CE of the 20/11/01 includes PAHs as priority contaminants and establishes a maximum accept-

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able concentration (MAC) of 0.2 $\mu g\,l^{-1}$ in drinking water for a sum of six PAHs (fluoranthene, benzo(3,4)fluorantene, benzo(11,12)fluoranthene, benzo(1,12)perylene, benzo(3,4)-pyrene and indeno(1,2,3cd)pyrene) [2]. However, MAC values of PAHs in seafood products are not reported, although since *Erika* oil spill the *Agence Française de Sécurité Sanitaire* (AFSSA) established reference values for bivalve molluscs at 0.5 $mg\,kg^{-1}$ (dried mass) for the sum of 16 PAHs recognised as priority contaminants by the EPA (Environmental Protection Agency) [3]. The same reference values has been adopted after *Prestige* oil spill [4], 0.5 $mg\,kg^{-1}$ (dried mass) for the sum of 16 PAHs and 0.2 $mg\,kg^{-1}$ (dried mass) for the sum of six PAHs included in the WHO list [5].

There are several studies on PAHs determination in different environmental matrices, which use mainly gas chromatography (GC) and high performance liquid chromatography (HPLC) as it has been recently reviewed by de Boer and Law [6]. Most of these analytical methods allow the separation of the 16 priority PAHs. By other hand, the knowledge of total PAHs (T-PAHs) content is enough for many applications, such as the establishment of health status of biota after an oil spillage episode, or the determination of PAHs background levels [7,8]. Spectrofluorimetry is a simple and non-expensive technique which provides excellent sensitivity for PAHs quantification and it has been applied to assess T-PAHs in several environmental samples such as water [9] and seawater [10,11].

Most of the studies carried out to assess PAHs contamination in marine ecosystems have been developed for marine sediment samples and Soxhlet extraction with non-polar solvents and alkaline saponification have been the most commonly used extraction techniques [6,12,13]. Additionally, accelerated solvent extraction (ASE) [14], supercritical fluid extraction (SFE) [15], microwave assisted solvent extraction (MASE) [16] and more recently, microwave assisted alkaline saponification (MAAS) [17,18] have been also applied to extract PAHs from environmental samples, specially marine sediments. The application of ultrasound energy for accelerating or assisting both inorganic and organic compounds extraction from solid materials is a current practice [19]. The speeding up is attributed to the induced cavitations process occurring in the liquid when applying ultrasound energy, which promotes an increase of pressure and temperature and allows a high analyte transport from the solid particles to the liquid phase [19]. Therefore, there are reported some applications of ultrasound-assisted solvent extractions to assess PAHs, which uses ultrasonic probe devices [20,21]. However, decreases in the PAHs yields when assisting the solvent extraction with ultrasounds are commonly reported and they are attributed to the aging of the ultrasound probe [22]. Another drawback of ultrasound probe devices is the increase in temperature as consequence of ultrasounds irradiation, which might produce analyte losses or analyte alterations. Recently, ultrasound water-bath devices have been demonstrated to be useful for assisting solvent extraction of both inorganic and organic compounds. The new ultrasound water-bath devices offer the possibility of temperature control, which compensates the increases in temperature for long sonication times.

Therefore, the aim of the current work has been studying of the possibilities of ultrasounds water-bath devices to assist solvent extraction of PAHs from biological materials (mussel soft tissue). Ultrasound-assisted solvent extraction has been performed at room temperature and all potential variables affecting the solvent extraction process have been optimised by an experimental design approach.

2. Experimental

2.1. Apparatus

Hitachi single-beam fluorescence spectrophotometer model F-2500 equipped with 10 mm quartz cells was used for all determinations. Raypa® Model UCI-150 ultrasonic bath with ultrasound frequencies at 17 and 35 kHz and programmable for temperature and time was from R. Espinar S.L. (Barcelona, Spain). Vibrating ball mill, Retsch (Haan, Germany), equipped with zircon cups (15 ml in size) and zircon balls (7 mm diameter) was used to pulverise dried mussel samples. A Lab Blender Stomacher 400 (Seward Med. Ltd., London, UK) was used to blend and homogenize fresh mussel samples. During the blending process samples are contained in Stomacher closure bags 6041/CLR (Seward). A LYPH-LOCK® 6 l freeze dry system, model 77530 from Labconco Corporation (Kansas City, USA) was used to lyophilise mussel samples. Centrifuge Centromix (Selecta, Barcelona, Spain) was used to separate solid and liquid phases. Glass chromatographic columns (35 cm length and 15 mm diameter) with integral sintered disc and PTFE stopcock (Selecta, Barcelona, Spain) were use for drying and purifying extracts. Chemometrics package was Statgraphics Plus V 5.0 for Windows, 1994–1999 (Manugistics Inc., Rockville, MD, USA). Surfer V 7.04 package (2001, Golden Software Inc., Colorado, USA) was used to obtain isoplot maps.

2.2. Reagents

Ultra-pure water, resistance 18 MΩ cm (Millipore Co., Bedford, MA). Chrysene stock standard solution (55.6640 $mg\,l^{-1}$) was prepared from chrysene 98% (Sigma–Aldrich, Steinhein, Germany). *n*-Hexane (PA-ACS) was obtained from Panreac (Barcelona, Spain). Dichloromethane (Uvasol) was supplied by Merck (Darmstadt, Germany). Active magnesium silicate (Florisil), 60–100 mesh was supplied by Sigma–Aldrich.

2.3. Mussel samples

Mussel samples (*Mytilus galloprovincialis*) were collected from 16 mussel farms at *Ría de Arousa* estuary, Galicia, northwest Spain (Fig. 1), in July 2002 and July 2003. Studies were performed using all soft mussel tissue (muscle and

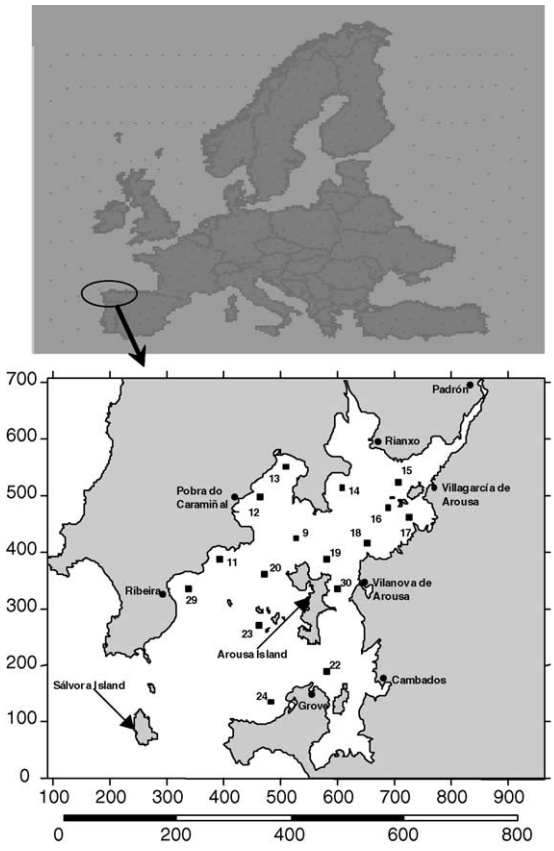

Fig. 1. Sampling farmed mussels stations in *Ría de Arousa* estuary.

gill) by preparing a mussel pool with all the mussels from each mussel farm. After mechanical blending, homogenization and freeze dry processes, the dry mussel were pulverized by using a vibrating ball mill and they were kept into pre-cleaned polyethylene bottles with hermetic seals.

2.4. Total polycyclic aromatic hydrocarbons ultrasound-assisted extraction

Around 0.5 g of mussel was weighted into glass centrifuge tubes and 6.5 ml of dichloromethane was added. The mixture was sonicated at 17 kHz and at room temperature for 10 min and after centrifugation at 3000 rpm for 10 min, the supernatant was separated by decantation and reserved. Then, 2.5 ml of *n*-hexane were added to the mussel residue and the mixture was again sonicated at 17 kHz and at room temperature for 10 min. After centrifugation at 3000 rpm for 10 min, the *n*-hexane supernatant was decanted and combined with the dichloromethane extract.

2.5. Clean-up procedure

The 6.5 + 2.5 ml extracts were concentrated by rotary evaporation to ≈1 ml and the residue was finally made up to 10 ml with *n*-hexane. The organic extracts were fractionated by adsorption chromatography by passing through a glass column packed with 4 g of Florisil. For elution, 20 ml of *n*-hexane/dichloromethane (60/40) were added and the column was mechanical stirring for about 5 min and allowed to settle for 20 min. After this time, the total PAHs were eluted from column and they were concentrated by rotary evaporation to ≈1 ml. The 1 ml residue was finally made up to 5 ml with *n*-hexane.

2.6. Spectrofluorimetric measurements

Preliminary studies were carried out in order to select the excitation and fluorescence emission wavelengths and excitation and fluorescence emission wavelengths of 300 and 382.0 nm, respectively, were chosen. Both excitation and emission slits were fixed at 10.0 nm. Fig. 2(a) and (b) shows the fluorescence spectra within 220 and 500 nm for chrysene standards at different concentrations and a mussel extract. It can be seen that the same of chrysene fluorescence spectra (standards in Fig. 2(a)) are similar than the T-PAHs fluorescence spectra (mussel extract in Fig. 2(b)). Therefore, calibration was performed using chrysene as calibrant covering concentrations within the 0.0–0.3 mg l^{-1} range and using *n*-hexane as solvent, as recommended by López de Alda-Villaizán et al. [23] when determining T-PAHs by spectroflu-

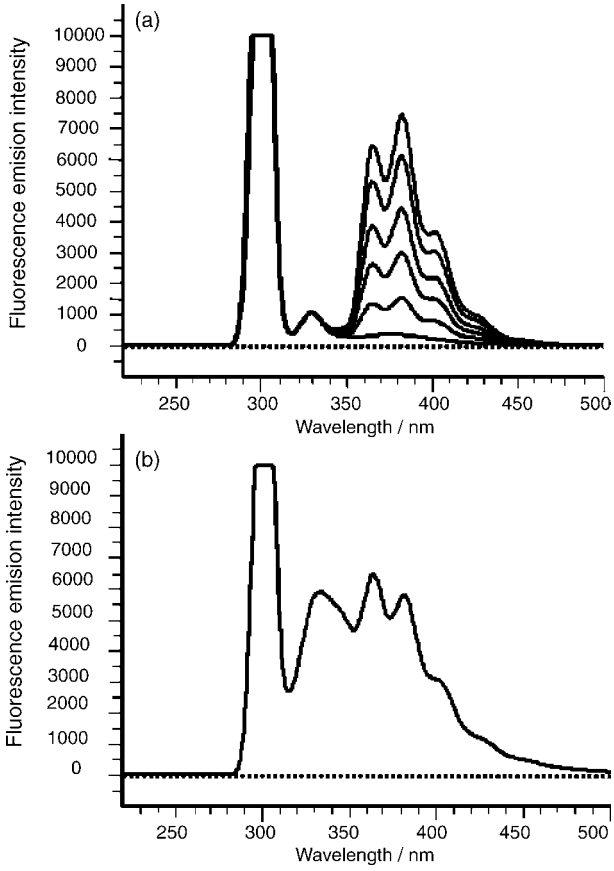

Fig. 2. Fluorescence spectra of chrysene standards (0.0–0.5 mg l^{-1}) in hexane (a) and un-spiked mussel extract (b).

orimetry. Mean standard addition slope ($n = 7$), expressed as mean ± S.D., was $9410.4 \pm 340.7\ mg^{-1}$.

3. Results and discussion

3.1. Statistically significant variables affecting T-PAHs ultrasound-assisted extraction from mussel soft tissue

Moreover the extracting volume (*n*-hexane and dichloromethane), solvents recommended when using spectrofluorimetric determinations [23,24], and the ultrasound frequency, other variables that could affect the solvent extraction process such as extraction (sonication) time, the number of repeated extraction with *n*-hexane and the number of repeated extraction with dichloromethane were considered in the current study. All variables are continuous except the frequency of the ultrasound energy, which can only adopt two discrete values (17 and 35 kHz), and the number of repeated extractions, which can only adopt natural numbers (1, 2, 3, and so on). A last variable, called *dummy factor*, was finally taken into account. Dummy factors are imaginary variables for which the change from one level to another is not supposed to cause any physical change [25]. These variables are commonly used in order to evaluate the possible systematic error and/or the existence of an important variable that was not been taken into account.

The significance of the variables commented above was simultaneously evaluated by applying a $2^7 \times 3/32$ type III resolution design Plackett–Burman design (PBD), for 7 variables, 4 degrees of freedom, 12 runs and 2 replicates. Table 1 lists the low (−) and high (+) levels of each variable, while the PBD experimental conditions and the values for the response variable are listed in Table 2. All runs listed in Table 2 were carried out with 0.5 g of a lyophilised mussel sample. After each experiment, the extracts were subjected to the clean-up procedure described in Section 2.5. The response variable was the concentration of the released T-PAHs, using 300 and 382 nm as excitation and fluorescence emission wavelengths, respectively. T-PAHs concentrations were obtained after *n*-hexane calibration with chrysene.

Table 1
Experimental field definition for the Plackett–Burman design[a]

Variable	Symbol	Low level (−)	High level (+)
Ultrasounds frequency (kHz)[b]	*A*	17	35
Sonication time (min)	*B*	10	30
n-Hexane volume (ml)	*C*	5	10
Dichloromethane volume (ml)	*D*	5	10
Number of repeated extractions with *n*-hexane[b]	*E*	1	3
Number of repeated extractions with dichloromethane[b]	*F*	1	3
Dummy factor	*G*	−1	+1

[a] The mussel sample mass was 0.5 g for all experiments.
[b] Non-continuous variable.

Table 2
$2^7 \times 3/32$ Plackett–Burman design for the significant variables determination

Run	Variables							
	A (kHz)	*B* (min)	*C* (ml)	*D* (ml)	*E*	*F*	*G*	[T-PAHs] ($mg\ kg^{-1}$)
1	35	10	10	5	1	1	+1	5.9
2	35	30	5	10	1	1	−1	5.8
3	17	30	10	5	3	1	−1	6.6
4	35	10	10	10	1	3	−1	8.5
5	35	30	5	10	3	1	+1	6.9
6	35	30	10	5	3	3	−1	7.1
7	17	30	10	10	1	3	+1	9.3
8	17	10	10	10	3	1	+1	6.1
9	17	10	5	10	3	3	−1	8.6
10	35	10	5	5	3	3	+1	5.9
11	17	30	5	5	1	3	+1	7.7
12	17	10	5	5	1	1	−1	9.0
13	35	10	10	5	1	1	+1	6.8
14	35	30	5	10	1	1	−1	6.5
15	17	30	10	5	3	1	−1	6.6
16	35	10	10	10	1	3	−1	7.4
17	35	30	5	10	3	1	+1	6.1
18	35	30	10	5	3	3	−1	6.6
19	17	30	10	10	1	3	+1	9.2
20	17	10	10	10	3	1	+1	6.5
21	17	10	5	10	3	3	−1	6.9
22	35	10	5	5	3	3	+1	5.2
23	17	30	5	5	1	3	+1	8.1
24	17	10	5	5	1	1	−1	9.9

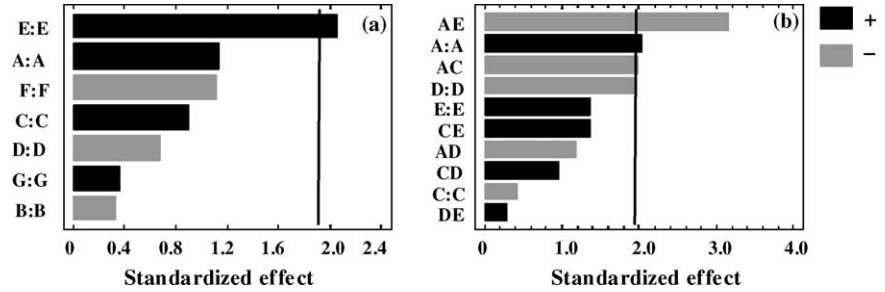

Fig. 3. Standardised ($P = 95\%$) main effects Pareto chart (a) and standardised ($P = 95\%$) two-factor interactions Pareto chart (b) for the $2^7 \times 3/32$ Plackett–Burman design.

The statistical evaluation of results was attained at a 95% confidence interval from which a minimum t value of 1.9, calculated by the Statgraphics routine trough an iterative process [26,27], was obtained. Variables which t values higher than ± 1.9 were considered as statistically significant factors. These results can be visualised by standardised ($P = 95\%$) main effect Pareto chart shown in Fig. 3(a). It can be seen that the variable *number of repeated extraction with n-hexane*, coded as *E*, is statistically significant, and the effect of this variable is negative, meaning a decrease in T-PAHs yields when increasing the number of repeated extraction from 1 to 3. It can be also noticed that the variable *sonication time*, coded as *B*, is non-significant. This supposes that solvent extraction can be considered complete after 10 min, which was the lowest sonication time tested. The dummy factor (*G*) was also non-significant, meaning that there are not systematic error neither unknown variables affecting the ultrasound-assisted solvent extraction process.

The effects of less significant variables were discharged and the two order interactions between factors were evaluated. Results are shown as standardised ($P = 95\%$) two-factor interactions Pareto chart in Fig. 3(b). It can be seen that the variable ultrasound frequency (*A*) is statistically significant, offering lower T-PAHs recoveries when using the high ultrasound frequency (35 kHz). In addition, the variable dichloromethane volume is also significant, leading higher T-PAHs concentrations for a large volume. The Pareto chart in Fig. 1(b) shows also that there are two statistically significant two-factor interactions, the combined effect of ultrasound frequency and number of repeated extractions with *n*-hexane (*AE*) and the two-factor interaction ultrasound frequency and *n*-hexane volume (*AC*).

From these results it can be concluded that the statistically significant variables affecting the ultrasound-assisted solvent extraction of T-PAHs from mussel soft tissue are the ultrasound frequency, the number of repeated extraction with *n*-hexane, the dichloromethane volume and the *n*-hexane volume. The fist two variables are non-continuous factors and both offer a negative effect on T-PAHs extraction. Therefore, the low values shown in Table 1 (17 and 1 kHz, for ultrasound frequency and number of repeated extraction with *n*-hexane, respectively) were chosen. The other two variables, dichloromethane volume and *n*-hexane volume, were optimised later applying an orthogonal central composite design.

3.2. Optimisation of significant variables by central composite designs

The significant variables *dichloromethane volume* and *n-hexane volume* were simultaneously optimised by applying an orthogonal central composite design 2^2 + star with 4 error degree of freedom, 2 center points, 2 replicates and 20 runs. The new experimental field definition offered lower and upper values of 2.5 and 7.5 ml for *n*-hexane volume and lower and upper values of 5.0 and 15.0 ml for dichloromethane volume. Star and center points were calculated by the routine of the Statgraphics program and they were 2.3 ml (− star) and 7.7 ml (+ star) for the *n*-hexane volume and 4.7 ml (− star) and 15.4 ml (+ star) for the dichloromethane volume. The center points were 5.0 and 10 ml for *n*-hexane volume and dichloromethane volume, respectively. Experimental conditions for the orthogonal central composite design 2^2 + star are

Table 3
2^2 + star orthogonal central composite design

Run	Variables		
	C (ml)	*D* (ml)	[T-PAHs] (mg kg^{-1})
1	5.0	10.0	8.2
2	2.5	5.0	10.3
3	7.5	5.0	7.1
4	2.5	15.0	9.3
5	7.5	15.0	8.7
6	2.3	10.0	10.7
7	7.7	10.0	6.0
8	5.0	4.6	6.1
9	5.0	15.4	4.1
10	5.0	10.0	8.7
11	5.0	10.0	9.0
12	2.5	5.0	8.6
13	7.5	5.0	8.0
14	2.5	15.0	9.5
15	7.5	15.0	9.2
16	2.3	10.0	11.1
17	7.7	10.0	6.7
18	5.0	4.6	7.0
19	5.0	15.4	4.5
20	5.0	10.0	9.2

Table 4
ANOVA table obtained for 2^2 + star orthogonal central composite design

Variables	Sum of squares	Degrees of freedom	Main squares	*F*-ratio	*P*-value
C: *n*-hexane volume	16.92	1	16.92	7.13	0.019
D: dichloromethane volume	0.40	1	0.40	0.17	0.687
CC	13.21	1	13.21	5.57	0.035
CD	1.06	1	1.06	0.45	0.516
DD	7.84	1	7.84	3.30	0.092
Total error	30.86	13	2.37		

listed in Table 3, together with the response variable (T-PAHs concentration) after performing each experiment (0.5 g of a lyophilised mussel sample, clean-up procedure described in Section 2.5, spectrofluorimetry determination using 300 and 382 nm as excitation and fluorescence emission wavelengths, respectively).

The statistical evaluation of the ANOVA table, listed as in Table 4, shows that the quadratic term for the variable *n*-hexane volume is statistically significant, which implies a response surface with curvature, such as shown in Fig. 4. The study of this response surface reveals that high T-PAHs recoveries are reached when using small low volumes of *n*-hexane, while the highest T-PAHs yields are obtained when using volumes of dichloromethane around 7.5 ml. Since these results, optimum volumes of 2.5 and 7.5 ml were considered for *n*-hexane and dichloromethane, respectively.

3.3. Analytical performances

n-Hexane calibration and the standard addition technique using chrysene as calibrant were statistically compared in order to observe matrix effects. The *n*-hexane calibration was performed covering the 0.0–0.5 mg l^{-1} range and a mean slope, expressed as mean ± S.D., of 13811 ± 760 mg $^{-1}$ l ($n = 7$) was obtained. Similarly, standard addition graphs were performed by adding chrysene concentration within the 0.0–0.3 mg l^{-1} range to an extract from a mussel sample. The mean slope, also expressed as mean ± S.D., was 9410 ± 341 mg $^{-1}$ l ($n = 4$).

A statistical comparison of slopes for the calibration and standard addition graphs was carried out checking the variance by applying the Cochran's and Bartlett's tests at a 95% confidence level ($P = 0.05$) [28]. Results indicate that there is statistical significant difference amongst the S.D., for calibration and standard addition graphs and the Multiple range test was applied to compare means. Results from multiple range test have revealed that there is a statistically significant difference between slopes of both calibration and standard addition graphs and, therefore, measurements must be carried out using the standard addition technique.

The limit of detection (LOD) and the limit of quantification (LOQ), expressed as $\boldsymbol{3}\sigma$ or $\boldsymbol{10}\sigma$, were 0.021 and 0.047 μg g^{-1}, respectively. Precision has been assessed by determining 11 time a same mussel extract (within-run precision) and also evaluating the repeatability of the over-all procedure, after 11 ultrasound-assisted solvent extraction from a same mussel sample and spectrofluorimetric measurement of each extract. Results, expressed as R.S.D., were 2.5% for the within-run precision and 4.7% for the repeatability of the over-all procedure. Finally, accuracy of the proposed method was studied through the analytical recovery. Different subsamples from a same mussel sample were spiked with different chrysene solutions to reach chrysene concentrations of 0.1, 0.2 and 0.3 mg l^{-1} in the final extracts. Each level of fortification was carried out three times. After spiking, the samples were kept at room temperature for 24 h, performing the ultrasound-assisted solvent extraction and the spectrofluorimetric determination. Analytical recoveries of 94 ± 1%, 100 ± 3% and 99 ± 2% were achieved for mussel samples spiked with 0.1, 0.2 and 0.3 mg l^{-1}, respectively.

3.4. Applications

Thirty-two farmed mussel samples from *Ría de Arousa* estuary (Fig. 1), collected in July 2002 (before *Prestige* oil

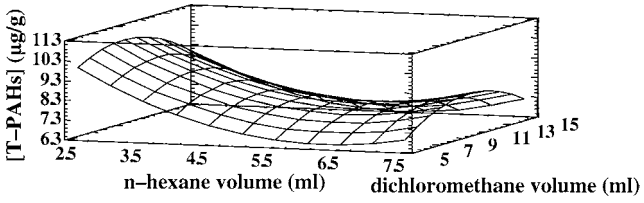

Fig. 4. Estimated response surface from the 2^2 + star orthogonal central composite design.

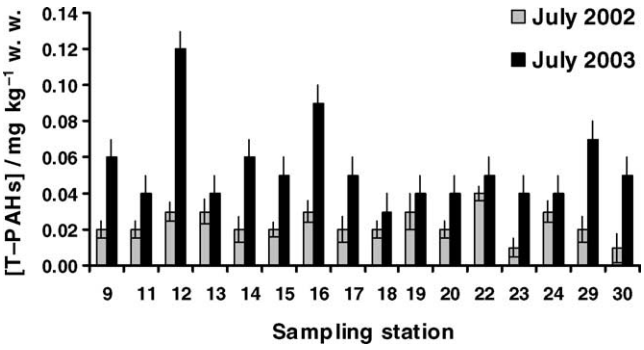

Fig. 5. T-PAHs concentration (mg kg^{-1} wet weight) in farmed mussels collected on July 2002 and July 2003.

spill) and July 2003 (after *Prestige* oil spill), were analysed for T-PAHs. Around 0.5 g of each pool was subjected by duplicate to the proposed ultrasound-assisted solvent extraction and each extract was analysed twice by spectrofluorimetry. T-PAHs concentrations, expressed as a dried weight, were varied from 0.82 to 3.27 mg kg^{-1} for farmed mussels collected in July 2002 and within the 2.45–9.80 mg kg^{-1} range for farmed mussel collected in July 2003. Taking into account the moisture of mussel samples, around 81.7%, T-PAHs concentrations, expressed as wet weight, were within the 0.01–0.04 and 0.03–0.12 mg kg^{-1} ranges for July 2002 and July 2003 samplings, respectively. All T-PAHs levels found in mussels are higher than the reference value, 0.5 mg kg^{-1} as dried weight. This difference is because the reference value is referred to the sum of 16 recognised priority contaminants by the EPA. Results expressed as wet weight are plotted in Fig. 5 where it can be seen a significant increase in T-PAHs levels after *Prestige* oil spill. Studies carried out in wild mussels from different places along the Galician coast after the *Aegean Sea* oil spill have revelled T-PAHs concentration, for the sum of 12 PAHs between 0.02 and 0.56 mg kg^{-1} as wet weight (approximately 1.6 and 45.7 mg kg^{-1} as dried weight) [29]. These data are far higher than those found in the current study. This is because the *Prestige* oil spillage did not directly go into the *Ría de Arousa* estuary.

Fig. 6 shows the T-PAHs isoplot concentrations (mg kg^{-1} as wet weight) map in farmed mussels collected in July 2002 (Fig. 6(a)) and July 2003 (Fig. 6(b)). Results shows that the highest T-PHAs concentrations are in mussels harvested in inner-left part of the estuary, near to *Pobra do Caramiñal* port, and decrease toward the mouth of the estuary, although high T-PAHs levels are also found in the mouth-right part of the estuary, near *Cambados* and *O Grove* urban nucleus. In general, ocean currents go into the estuary from the mouth-right part of the estuary with a high flow and go out from the inner-left part with a low flow.

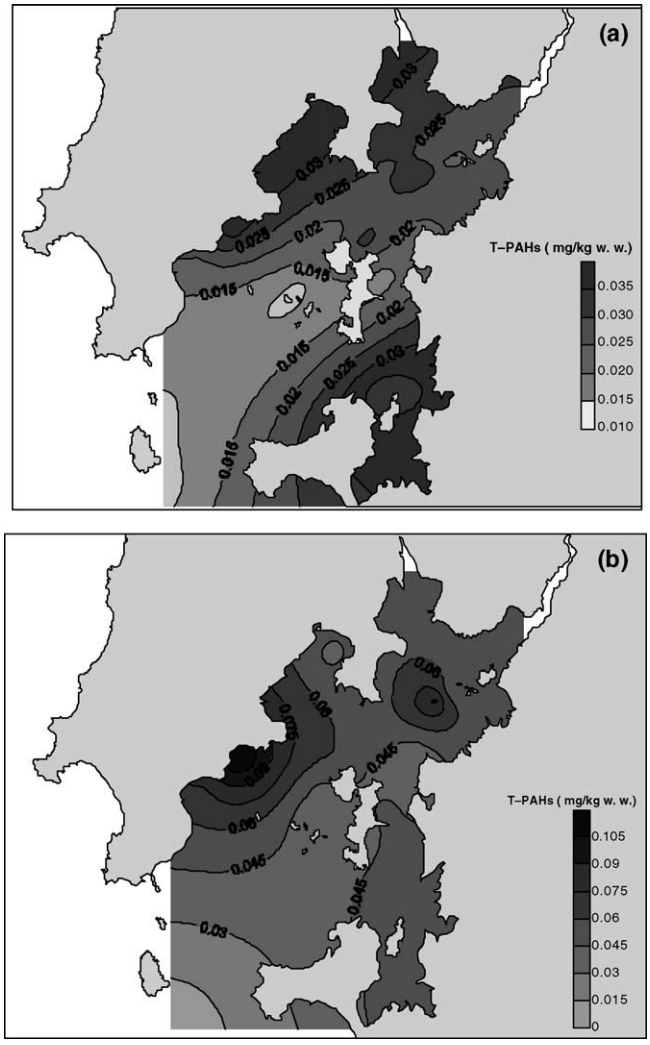

Fig. 6. T-PAHs isoplot concentrations (mg kg^{-1} wet weight) map in farmed mussels collected on July 2002 (a) and July 2003 (b).

4. Conclusions

The proposed ultrasound-assisted solvent extraction by means of ultrasound water-bath with controlled temperature avoids problems associated with ultrasonic probes and allows an efficient assisting of T-PAHs solvent extraction from mussels. The extraction is carried out in two stages, the first one by using *n*-hexane and second one, which uses dichloromethane. Both successive solvent extractions are subjected to ultrasound energy remaining at 17 kHz for 10 min. The use of a non-expensive analytical technique such as spectrofluorimetry allows a fast T-PAHs screening in mussel samples. Each individual PAH in those mussel samples with high T-PAHs contents must be analysed by more sophisticated analytical techniques.

Since the T-PAHs determination in farmed mussels harvested in *Ría de Arousa* estuary, it can be said that the T-PAHs levels have been increased after *Prestige* oil spill, although the T-PAHs levels are far lower than those reported after other oil spillage episodes. Therefore, it could be though a moderate low effect of the *Prestige* oil spill on the ecosystem of *Ría de Arousa* estuary. Taking into account water circulation in *Ría de Arousa* estuary, mussels from farms located at the inner-left part of the estuary are exposed to the dissolved contaminants for a longer time than those mussels harvested in farms in the central and mouth part of the *Ría de Arousa* estuary.

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