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Experimental design of a microwave-assisted extraction–derivatization method for the analysis of methylmercury

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Abstract

A simultaneous microwave-assisted extraction–derivatization procedure was developed and optimized for methylmercury analysis from biological samples. The analyte was derivatized with sodium tetraphenylborate forming a more hydrophobic compound, methylphenylmercury, which was extractable in toluene. The microwave extraction–derivatization procedure was optimized using experimental design, 2^{5-1} fractional factorial. This chemometrical approach considers main effects as well as interactions of the influential parameters, indicating that temperature and its interaction with $NaBPh_4$ and acetic acid volumes were the variables that significantly affected methylmercury recoveries.

Keywords: Microwave-assisted extraction; Extraction methods; Derivatization, GC; Factorial design; Experimental design; Methylmercury; Organomercury compounds

1. Introduction

The determination of total mercury is not sufficient to assess the risks associated with consumption of mercury-containing foodstuffs since the toxicity of mercury is highly dependent on its chemical form, the organometallic compounds being more toxic than the inorganic mercury compounds. As a result, much attention has been given to speciation of mercury in environmental and biological samples, and the subject has been reviewed recently [1–3].

In recent years, metal speciation was possible with the use of modern high-tech hyphenated techniques. The highly sensitive and selective elemental detection systems such as atomic absorption spectrometry (AAS) [4,5], atomic fluorescence spectrometry (AFS) [6–8], inductively coupled plasma and microwave-induced plasma atomic emission spectrometry (ICP-AES, MIP-AES) [9–11] and inductively coupled plasma mass spectrometry (ICP-MS) [12–14] are coupled to modern chromatographic separation systems such as gas chromatography (GC).

Environmental analytical chemists are continuously seeking to improve procedures for the extraction–determination of methylmercury by reducing analysis time and increasing accuracy and sensitivity. To perform mercury speciation analyses, extraction methods must be capable of quantitatively extracting mercury from the matrix while not altering the individual mercury species in any way. Typically, mercury compounds are extracted from seafood samples using a standard solvent extraction method, based on organomercury acid leaching in an organic

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solvent [15,16] or an alkaline hydrolysis [17]. A simpler alternative is the steam distillation [18], that avoids a large number of matrix interferences when is coupled with the subsequent derivatization reaction. However, controversial discussions occurred with respect to the certified methylmercury contents in sediment reference materials owing to the suspicion of artifact MeHg formation in distillation-based methods [19]. A workshop organized to discuss this question in detail, concluded that the findings on artifact formation of MeHg are not sufficient to claim that MeHg results are overestimated and more research must be undertaken [20].

Supercritical fluid extraction (SFE) has been used to extract methylmercury from various types of matrices, including marine biological samples, sediments, soils, etc. [21–23]. The SFE of methylmercury from biological samples is hindered by the organomercurial binding to proteins, which entails pretreatment with HCl or degradation with NaOH to cleave the bonds.

Microwave-assisted extraction (MAE) provides a number of advantageous features that have recently started to be explored for a variety of purposes, including the extraction and speciation of organomercurial compounds [24–26]. Tseng et al. [25] used an ''open vessel focalized microwave system'', acid medium and ethylation with sodium tetraethylborate prior to quantitation. One other alternative is provided by a ''closed vessel microwave system'' which uses sophisticated pressure and temperature controls for the simultaneous extraction of the various samples. Vázquez et al. [27] used such a system to develop a rapid, efficient method for the extraction of methylmercury from a certified reference material (DORM-1). Extractions were carried out in toluene to which a small amount of HCl was previously added to facilitate cleavage of methylmercury bonds.

The vast majority of organometallic compounds of environmental interest are ionic or highly polar. However, most of them are unfit for direct separation by GC, and must be derivatized into volatile molecules prior to analysis. The methods commonly used to derivatize organomercuric compounds are formation of volatile hydrides in combination with cryotrapping of volatile species [5,28,29] and alkylation by Grignard reagents [30,31] or sodium tetraethylborate ($NaBEt_4$) [6,32–35]. Most researchers derivatize Hg(II) and methylmercury to diethylmercury and methylethylmercury, respectively, using $NaBEt_4$. New reagents, similar to $NaBEt_4$, such as sodium tetrabutylammonium tetrabutylborate [36], sodium tetraphenylborate [37,38] and sodium tetra(*n*-propyl)borate [39] can obviously be used as derivatization reagents. These alkylation reactions are compatible with modern extraction methods as solid-phase microextraction (SPME) [13,40–45], purge and trap [35,46] and MAE [47].

The purpose of this work was to develop a microwave extraction–derivatization procedure capable of quantitatively extracting methylmercury from seafood samples in a closed-vessel system. A factorial design approach was used to optimize the extraction–derivatization parameters. The variables studied included volume of solvent (toluene), amount of acetic acid, amount of derivatizing reagent ($NaBPh_4$), extraction time and temperature. The statistical significance of each experimental variable studied was established in relation to the percentage of methylmercury recovery by analyzing a certified reference material (DORM-2). The extracts obtained were analyzed by GC–atomic emission spectrometric detection (AED) [9]. Final conditions were validated against two other available certified reference materials.

2. Experimental

2.1. Reagents, standards and solutions

Methylmercury chloride (99% purity), sodium tetraphenylborate (99.5%) and toluene (99.5%) were purchased from Merck (Darmstadt, Germany). Acetic acid (99.8%) and alumina were purchased from Sigma–Aldrich. Sodium hydroxide (98%) and anhydrous sodium sulfate (Analar grade, 99%) were purchased from BDH.

Methylmercury chloride stock solution of 1 g l^{-1} (as Hg^{2+}) was prepared in aqueous solution. Working standard solutions were prepared by appropriate dilution of the stock solution. All solutions were stored at 5°C on the dark when not in use.

Buffer solutions acetic acid–2 *M* acetate, pH 5, was prepared by mixing appropriate amounts of acetic acid and sodium hydroxide. The derivatization

reagent, 1% sodium tetraphenylborate ($NaBPh_4$) solution, was prepared daily in water.

Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Carrier and make-up gas was helium of 99.9995% purity (Carburos Metálicos, La Coruña, Spain). Reagent gases oxygen and hydrogen of 99.9995% purity (Carburos Metálicos) were used to enhance the combustion of the organic compounds and to improve the baseline stability, respectively.

Certified reference materials, DORM-2, CRM 463 and CRM 464, were obtained from the National Research Council of Canada (NRCC) and Standards Measurement and Testing Programme of the European Commission (BCR, Brussels, Belgium), respectively.

2.2. Apparatus

The microwave extractor system was a MES 1000 (CEM, Matthews, NC, USA) equipped with a solvent detector. The MES-1000 was able to extract 12 samples simultaneously in PTFE-lined extraction vessels under the same conditions (temperature and pressure). An inboard pressure control system was installed for monitoring and controlling pressure conditions inside the extraction vessels. The analyses of the extracts were performed on a Hewlett-Packard (Palo Alto, CA, USA) 5890A Series II gas chromatograph equipped with a Hewlett-Packard Model 5921A microwave-induced plasma atomic emission spectrometer. Acquisition and reprocessing data were carried out by means of a Hewlett-Packard Model 9144 Chemstation and the Chemstation software. Injections were made by means of a HP 7673 series automatic sampler into a split–splitless capillary injection port. The GC separations were performed on a 30 m×0.32 mm I.D. DB-5ms capillary column with a film thickness of 0.25 μm obtained from J & W Scientific (Folsom, CA, USA). Optimized GC parameters were: injection port, split/splitless; injection port temperature, 200°C; column flow, 3.2 ml min^{-1}; injection volume, 1 μl; column head pressure, 140 kPa; oven initial temperature, 90°C; initial time, 3 min; ramp rate, 30°C min^{-1}; oven final temperature, 270°C; final time, 10 min; transfer line temperature, 280°C.

Optimized AED parameters were: wavelength, 248 nm for carbon line and 254 nm for mercury line; helium make-up flow-rate, 180 ml min^{-1}; ferrule purge vent, 20 ml min^{-1}; scavenger gases, 350 kPa for hydrogen and 200 kPa for oxygen; helium supply purge, 205 kPa; spectrometer purge flow-rate, 2 ml min^{-1}; solvent vent off-time, 0–3.5 min; cavity temperature, 280°C.

2.3. Experimental design

The aim of this study was to verify that an experimental design permits one (A) to establish the effect of the variables (factors) involved in the extraction–derivatization step over the analytical response (methylmercury concentration) and, (B) to find the optimum values of those factors that give a maximum in the analytical response.

The optimization of the microwave extraction–derivatization procedure was carried out using a 2^{5-1} fractional factorial design (resolution V). Thus it is possible to obtain separate estimates of the main effects and their two-factor interactions assuming all higher-order interactions negligible. For all statistical calculations involved in this optimization process the software Statgraphics, version 6.0 was used (Manugistics, Rockville, MD, USA) [48]. In accordance with published studies [27,37,38], we observed that the five variables controlling the extraction and derivatization reaction were the acidity, concentration of derivatization agent, solvent volume, temperature and microwave extraction time. The variables and their respective ranges are listed in Table 1.

2.4. Microwave-assisted extraction–derivatization procedure

2.4.1. Standard solution derivatization

A 200-μl volume of buffer acetic acid–2 *M* acetate (pH 5), 2 ml aqueous solution of 1% $NaBPh_4$

Table 1
Variables and range selected for the screening design (2^{5-1})

Key	Variable	Screening range
A	HAc volume (μl)	200–2000
B	$NaBPh_4$ 1% volume (ml)	2–10
C	Toluene (ml)	5–15
D	Temperature (°C)	50–100
E	Extraction time (min)	2–10

and 5 ml of toluene were added to a volume of 10 ml of different methylmercury solutions (1–116 ng ml^{-1}, as Hg^{2+}). The solutions were maintained in the extraction vessel at 100°C for 5 min at 100% of power and at a pressure of 690 kPa.

2.4.2. Sample preparation

In the course of our optimization experiments, we varied 17 mol l^{-1} acetic acid, 1% $NaBPh_4$ and toluene amounts, as well as the respective reaction temperatures and reaction times. A 0.1-g portion of a biological material, DORM-2, with a certified methylmercury content of 4.47±0.32 mg kg^{-1} (as Hg^{2+}) was accurately weighed in the PTFE-lined extraction vessel. A 10-ml volume of water, aqueous 17 *M* acetic acid and 1% $NaBPh_4$ were added (the reagent volumes depending on the particular experiment to be carried out as dictated by the design) and allowed to equilibrate with the matrix before addition of the solvent (toluene). The extraction vessels were closed after ensuring that a new rupture membrane was used for each extraction. The extractions were performed at 100% microwave oven power at a temperature and time fixed for each individual experiment. Once the exposure to microwaves is completed; the sample carousel was removed from the microwave cavity and cooled in a water bath. After the centrifugation, 2 ml of the organic phase was passed through an activated Alumina column, 5 cm×0.5 cm, in order to remove lipids and then eluted with 3×1 ml toluene for clean-up. After clean-up, the eluate was transferred to 5-ml volumetric flask with toluene. Finally, an aliquot of the solution was transferred into a 1-ml septum-capped vial and 1 μl injected into the GC–AED system.

3. Results and discussion

3.1. Calibration

The GC conditions were adapted from the parameters previously optimized [9,16]. A five-point external standard calibration in the range 1–116 ng ml^{-1} (as Hg^{2+}) was performed daily. Correlation coefficients were between 1 and 0.9995. For the quantification, we used the average response factors from the multilevel calibration recorded from Hg at 254 nm. Evaluation of background signal-to-noise ratio ($S/N=3$) indicated an absolute detection limit for an injection volume of 1 μl (splitting ratio 5:1) of 0.04 pg for $MeHg^+$ (as Hg^{2+}). With a sample intake of 100 mg, the detection limit in biological sample was about 8 μg kg^{-1} for $MeHg^+$ (as Hg^{2+} and dry mass). The quantitation limits ($S/N=10$) were 0.250 pg and 53 μg kg^{-1}, respectively.

The repeatibility of the chromatographic procedure was assessed by performing eight consecutive injections of an 18 ng ml^{-1} derivatised standard solution and five injections of a derivatised biological sample. The relative standard deviations (RSDs) obtained were 5.5% and 3.5%, respectively.

When phenylmethylmercury standards are injected and recorded at 254 nm (mercury line), as shown in Fig. 1A, two or more weaker peaks usually appear next to it. When the chromatograms were recorded at 248 nm (carbon line) various peaks appear corresponding to an excess of derivatization reagents (Fig. 1B). The presence of high-intensity carbon peaks in the 248 nm chromatogram can be clearly seen. The closeness of both spectral lines and the fact that the instrument cannot completely separate them explains the interfering peaks recorded at 254 nm. The extent of derivatization of the standards was also determined. A second extraction–derivatization procedure was applied to the remaining aqueous phase to quantify the extent of the derivatization reaction. The results obtained suggest that only about the 1% of the initial amount of methylmercury remained underivatized (Fig. 1C).

3.2. Factorial design. Evaluation of the extraction–phenylation process using microwave energy

The microwave extraction–derivatization conditions were optimized using 0.1 g of the reference material DORM-2. The screening design used was a two-level fractional factorial design with resolution V. Five variables were included: the amounts of 17 mol l^{-1} acetic acid and 1% $NaBPh_4$, the volume of toluene, the temperature and microwave extraction time. Table 2 summarizes the design matrix and the recoveries obtained in each run, expressed as percentages vs. the certified value (4.47±0.32 mg kg^{-1} as Hg).

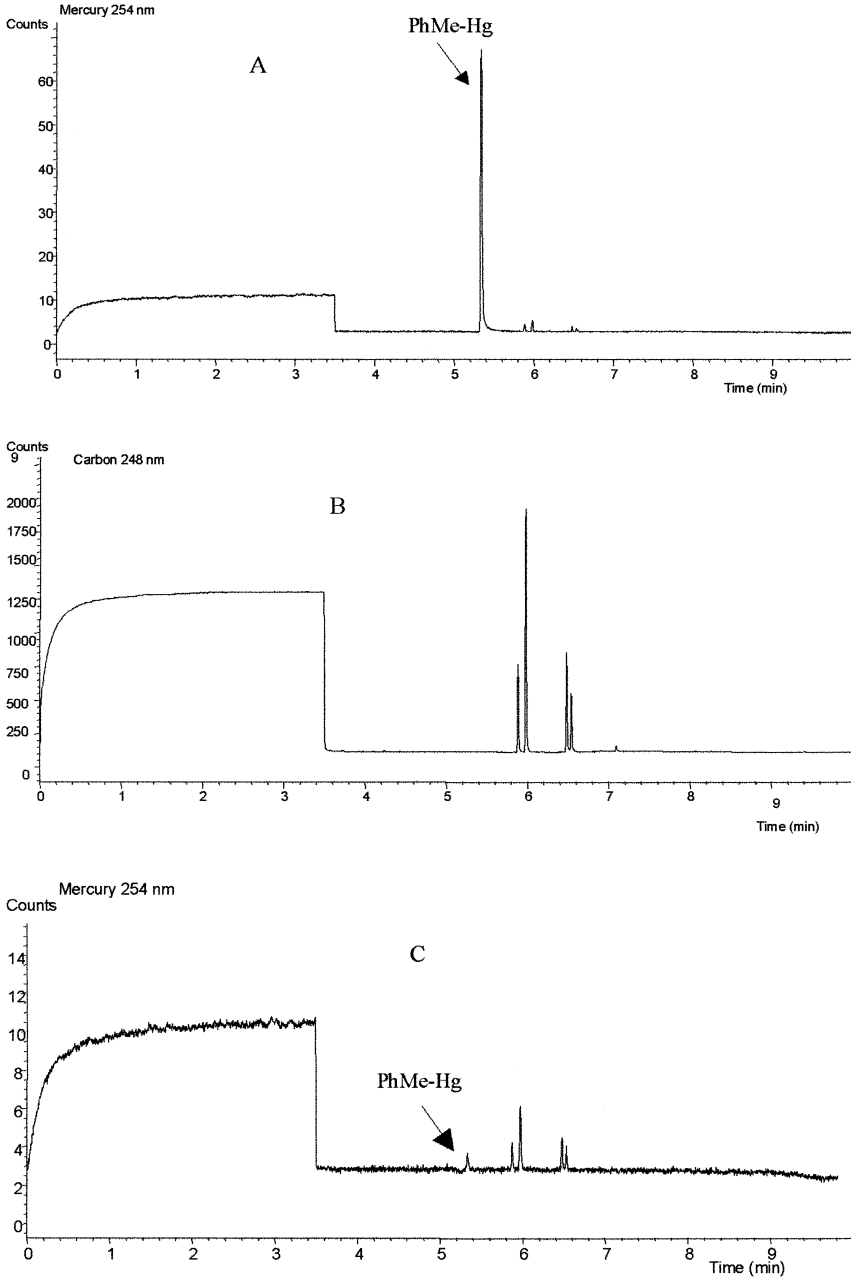

Fig. 1. Chromatograms of (A) derivatized standard methylmercury (40 ng ml^{-1}) recorded at 254 nm (Hg line); (B) derivatized standard methylmercury (40 ng ml^{-1}) recorded at 248 nm (C line) and (C) underivatized standard methylmercury after a second extraction–derivatization procedure recorded at 254 nm (Hg line).

An analysis of main effects obtained from the results in Table 2 shown that the extraction temperature, amount of 17 mol l^{-1} acetic acid and of 1% $NaBPh_4$ had a significant positive main effect on the yield of extraction–derivatization of methylmercury. This indicates that their values should be set at a

Table 2
Design matrix and response values in the screening design (2^{5-1})

Run No.	HAc volume (ml)	1% $NaBPh_4$ (ml)	Toluene (ml)	Temperature (°C)	Time (min)	Recovery (%)
1	200	2	5	50	10	75.3
2	2000	2	5	50	2	76.7
3	200	10	5	50	2	36.4
4	2000	10	5	50	10	72.7
5	200	2	15	50	2	51
6	2000	2	15	50	10	73
7	200	10	15	50	10	48.2
8	2000	10	15	50	2	71.4
9	200	2	5	100	2	95.8
10	2000	2	5	100	10	40.2
11	200	10	5	100	10	99
12	2000	10	5	100	2	86.4
13	200	2	15	100	10	66.3
14	2000	2	15	100	2	71.7
15	200	10	15	100	2	88.6
16	2000	10	15	100	10	101.9
17	1100	6	10	75	6	91.2
18	1100	6	10	75	6	87.4

high level to achieve good recoveries. On the other hand, the volume of toluene and the extraction time were not significant.

These less significant factors were excluded and the other three main factors (A, B and D) were considered to and evaluate two-factor interactions. In this reduced model, the results of the analysis of variance (ANOVA) carried out on the data are shown in Table 3. It can be deduced that factor D (extraction temperature) and the interactions between factor A–factor D and factor B–factor D were significant ($P<0.05$). It is important to note that factors A and B and their interaction were not significant. Likewise, the standardized effects shown that at 5% level with 11 degrees of freedom, values above $t=2.20$ must be considered significant.

We should note in this table the adverse effect of the interaction between AD factors. This interaction is affected by a negative sign, which means that the extraction–derivatization efficiency decreases when amount of acetic acid is at a lower level and the temperature changes from the higher (100°C) to the lower (50°C) level (Fig. 2A). This correlation can be explained by the fact that, using high acetic acid level the pH is low (pH≈3) and the temperature does not affect the efficiency of the extraction; recoveries

Table 3
Analysis of the data given in Table 2

Variable	ANOVA[a]					Standardized effects
	SS	DF	MS	*F*-ratio	*P* level	
A (acetic acid, ml)	69.72250	1	69.7225	0.38	0.5569	0.61
B ($NaBPh_4$, ml)	186.32250	1	186.3225	1.01	0.3356	1.00
D (temperature)	1317.69000	1	1317.6900	7.17	0.0215	2.68
AB	473.06250	1	473.0625	2.57	0.1369	1.60
AD	1095.61000	1	1095.6100	5.96	0.0327	−2.44
BD	1391.29000	1	1391.2900	7.57	0.0188	2.76
Total error	2021.04250	11	183.7311			
Total	6555.65	17				

[a] SS=Sum of squares; DF=degrees of freedom; MS=mean squares; *P* level=probability level.

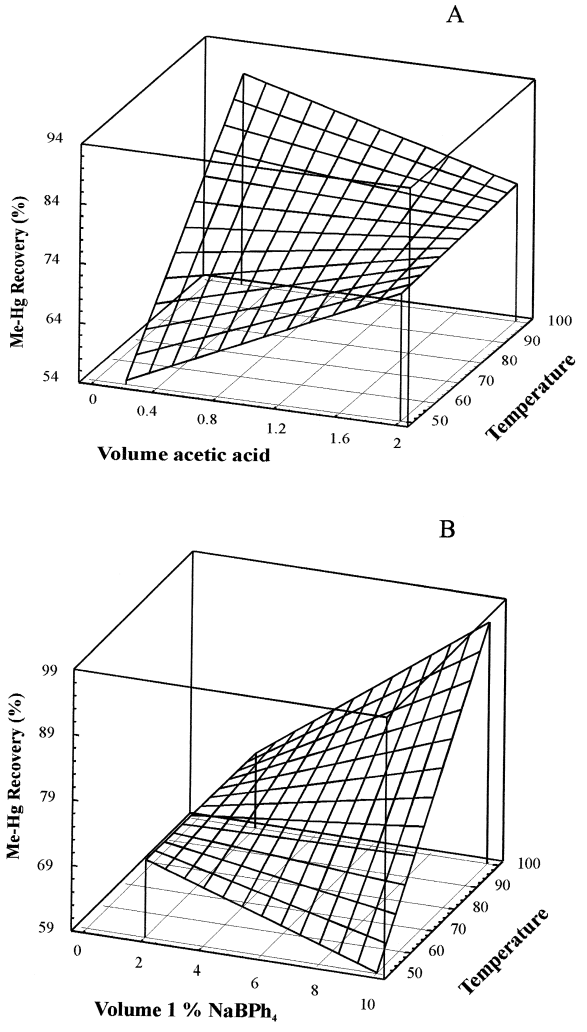

Fig. 2. Response surfaces estimated for the design in Table 2, obtained by plotting (A) microwave extraction vs. acetic acid volume and (B) extraction temperature vs. derivatizing agent ($NaBPh_4$).

were about 75–88%. However, low acetic acid levels favor the derivatization reaction (pH≈5), but high temperature is required to break the methylmercury–protein bonds and, in this way, to achieve maximum recoveries. Fig. 2B shows the estimated interaction surface obtained for the experimental model developed using the temperature and derivatizing agent (1% $NaBPh_4$) variables. As can be seen, the extraction efficiency was directly proportional to both factors, and it peaked at the highest levels tested. Under these conditions, natural methylmercury is released from the reference material, extracted and derivatised. Then, higher temperatures favor the extraction and this experimental design gives the optimum conditions to achieve maximum recoveries.

As a result of these observations, the following optimal working conditions were chosen: volume 17 mol l^{-1} acetic acid: 200 μl; volume 1% $NaBPh_4$: 10 ml; volume toluene: 10 ml; microwave extraction temperature: 100°C; microwave extraction time: 6 min.

The selection of this temperature was based on a consideration of practical attainment of maximum temperature when working with small amounts of biological material, as well as the matrix. It should be noted that, depending on the matrix and the quantity of sample analyzed, the amount of acetic acid could be higher.

Under these conditions, the reproducibility of methylmercury recovery from the reference material (DORM-2) was evaluated. The average recovery obtained for $n=6$, was 93.5±6.4%, and the RSD 6.8%.

3.3. Simultaneous extractions and sample size

Using the same certified reference material (DORM-2) and the optimal conditions developed above, single and multiple extraction–derivatization experiments were performed. The average recoveries obtained were 93.5±6.4% (single extraction) and 87.6±3.3 (simultaneous extractions, $n=6$). The results showed that both extraction procedures were comparable in terms of efficiency concerning the recoveries of methylmercury.

Another set of experiments were also performed working with sample sizes ranging between 0.05 and 1 g using a 6-min extraction–derivatization period, once the temperature had reached 100°C. The results including the experimental error are summarized in Table 4. When a sample size of 0.05 g is used in the extraction–derivatization process the recovery obtained is close to 100%, but standard deviation is high. This is probably because 0.05 g is below the sample size that guarantees the homogeneity for the DORM-2 material. When samples sizes in 0.2 to 1 g are used, the recoveries are a bit lower (≈80%).

Table 4
Percent recovery for the methylmercury vs. sample size

Sample size (g)	Average recovery for six simultaneous extractions (%)	Average recovery for one extraction[a] (%)
0.05	100±12	–
0.1	87.6±3.3	93.5±6.4
0.2	77.2±6.6	–
0.4	84.7±1.8	–
1	78±10	–

[a] $n=6$.

3.4. Validation of the procedure and application to real samples

The efficiency of the microwave procedure to extract methylmercury has been tested on other certified matrices, the tunas CRM 464 and CRM 463, supplied by the Community Bureau of Reference (BCR-UE). These materials have different methylmercury content and can be considered useful to validate the microwave assisted extraction–derivatization or analysis procedure for methylmercury in biological materials. Four simultaneous extractions have been performed with each material. Table 5 shows the results obtained for the certified reference materials investigated by microwave assisted extraction–derivatization which are compared with the certified values [49] and the results obtained by other procedures previously proposed [9,16,22].

Also, polluted cockle and mussel samples obtained near a chlor–alkali factory waste dump were analyzed. Since no reference values exist for these types of samples, comparative results obtained by manual extraction and GC–AED [9] have also been included in Table 5. Fig. 3 shows a chromatogram of a cockle sample after microwave assisted extraction–derivatization by the proposed procedure.

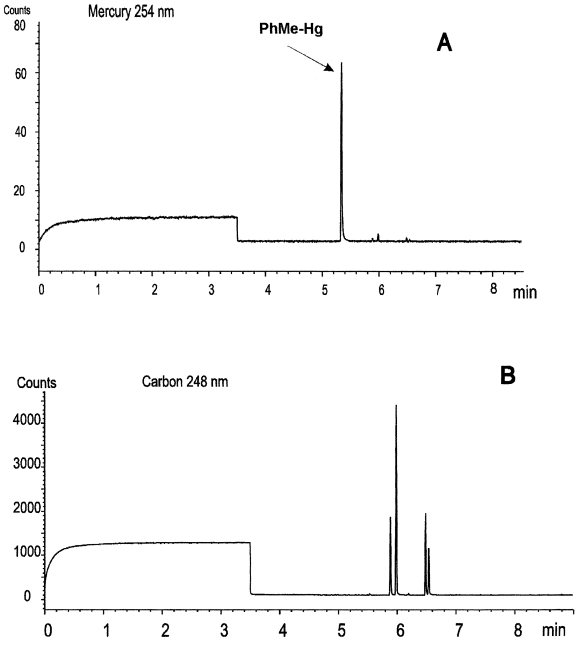

Fig. 3. Chromatograms obtained using the MAE–D–GC–AED system for a cockle sample. (A) Hg line, 254 nm and (B) C line, 248 nm.

In conclusion, the recoveries from the matrices analyzed were similar. The use of microwave energy in the extraction–derivatization of methylmercury in difficult matrices (biological samples) offers the following advantages: (1) with the chemical derivatization of methylmercury to obtain methylphenylmercury, column conditioning with inorganic salts is avoided prior to analysis by GC; (2) a notable reduction of solvent volume; (3) higher efficiency of extraction achievable under optimized conditions simultaneously with the reaction of derivatization; (4) considerable time saving in the procedure of sample preparation, and finally (5) the

Table 5
Comparative results for the extraction of biological samples using microwave extraction–derivatization (MAE–D), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and manual extraction

Sample	Mean±SD (mg kg^{-1} as Hg)				
	MAE–D	MAE	SFE	Manual extraction	Certified value
CRM-464	5.49±0.13	5.16±0.10	4.53±0.05	5.81±0.11	5.12±0.17
CRM-463	2.76±0.17	2.94±0.08	2.64±0.08	2.58±0.27	3.04±0.16
Cockle	1.43±0.13	1.66±0.04	–	1.31±0.05	–
Mussel	0.655±0.07	1.08±0.11	–	0.65±0.06	–

possibility of simultaneously extracting up to 12 samples resulting in increased sample output over conventional extraction techniques.

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