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Optimization of supercritical fluid extraction–gas chromatography of methylmercury in marine samples

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Abstract

Two-level factor designs were used to optimize the supercritical fluid extraction (SFE) of methylmercury (Me–Hg) in marine samples, which were subsequently analysed by gas chromatography–electron capture detection (GC–ECD). Several variables potentially affect to extraction efficiency and kinetics. The factors studied were: CO_2 flow-rate and density, extraction cell temperature, static extraction time, nozzle and trap temperature, amount of hydrochloric acid and contact time between the acid and the sample before to extraction. The extraction kinetics was studied in all experiments by splitting extracts at 2, 7, 17 and 37 min. The results suggest that only the extraction cell temperature is statistically significant. The optimized SFE procedure to extract Me–Hg was validated by means of three available reference materials having certified methylmercury content.

Keywords: Extraction methods; Factorial design; Methylmercury

1. Introduction

Supercritical fluid extraction (SFE) has become an attractive alternative to conventional solvent extraction for the recovery of organic compounds from environmental and biological samples because of several advantages, including increased speed, better recovery and the reduction in both solvent usage and solvent waste generation [1].

In recent years, SFE, particularly using carbon dioxide, has been applied successfully to the extraction of organic pollutants in solid samples. Among others, the extraction and determination of organometallic compounds from soils, sediments and other matrixes have been reported [2–4].

However, few reports have dealt with SFE of organic and inorganic mercury. Wai and coworkers [5] reported an in situ chelation method for the CH_3HgCl and $(CH_3)_2Hg$ from solid materials with supercritical CO_2 and methanol-modified CO_2, both containing lithium-bis-(trifluoroethyl) dithiocarbamate (LiFDDC) as a chelating agent. Liu and coworkers [6] showed the application of supercritical carbon dioxide modified with 5% of methanol to speciate individual organomercury compounds (CH_3HgCl, C_6H_5HgCl, $(C_6H_5)_2Hg$) and inorganic mercury compounds ($HgCl_2$, HgO, HgS) in soil and sediment samples which were determined by capillary gas chromatography with atomic emission detection (GC–AED). Wang and coworkers [7] proposed a selective extraction of Hg^{2+} by chelation combined with ionizable crown ethers in supercritical carbon dioxide modified with methanol. Biological matrixes, however, have been scarcely considered.

On the other hand, some developers optimize

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extraction methods one parameter at a time [8]. This sequential univariate strategy, however, is known to be scarcely effective for finding out the true optimum. Accordingly, formal optimization methods are generally preferred. Factor designs have been used for the simultaneous determination of various analytical SFE parameters including temperature, pressure, CO_2 density, fluid flow-rate, extraction time, etc [9–14]. Only two or three variables are considered in most cases, so few experiments must be performed in order to detect potential interactions between the experimental variables, which is unaffordable by the classical univariate method. In this paper, we tested a factor design to optimize 8 experimental variables: CO_2 flow-rate and density, extraction cell temperature, static extraction time, nozzle and trap temperature, amount of HCl (1:1) and contact time between HCl and the sample prior to extraction. With this aim, we chose a folded Plackett–Burman $2^8*3/32$ type IV Resolution design [15–17].

The optimization was targeted at both the overall extraction yield and the extraction kinetics; the statistical significance of each experimental variable studied was thus established in relation to both targets. The procedure was developed by using a spiked mussel homogenate. The recovery was assessed by using three available Certified Reference Materials.

2. Experimental

2.1. Reagents and apparatus

All reagents were of analytical-reagent grade. Methylmercury chloride (99%) and sodium acetate (99.5%) was obtained from Merck (Darmstadt, Germany). A stock solution 1000 μg Me-Hg ml^{-1} in toluene was used to obtain working standards solutions by dilution. A 1% solution of mercury chloride (99.5%) (Merck) in toluene was used as a column-conditioning solution [18]. Cysteine chlorhydrate (98.5%), 2-propanol, hydrochloric acid, anhydrous sodium sulphate (99%), toluene and acetone were obtained from BDH (Poole, UK).

Extractions were carried out with 99.995% pure carbon dioxide from Carburos Metálicos (La Coruña,

Fig. 1. Evaluation of the homogeneity of the material used to optimize the extraction procedure through the variation of the mean values and standard deviations obtained vs. the amount of sample subjected to extraction and analysis.

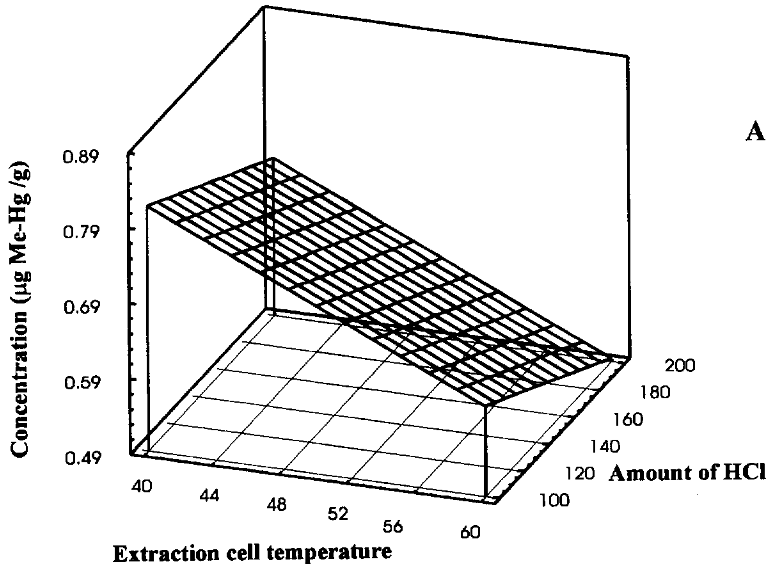
Table 1
Design matrix used and response values obtained in the folded Plackett–Burman factorial design

Run	A	B	C	D	E	F	G	H	Total response (μg Me–Hg g^{-1})
1	0	0	0	0	0	0	0	0	0.75
2	–	+	–	–	–	+	+	+	0.71
3	+	+	–	+	+	–	+	–	0.87
4	–	+	–	–	+	–	+	+	0.72
5	+	–	–	–	+	–	–	+	0.66
6	–	–	+	+	+	–	+	+	0.54
7	+	+	+	–	+	+	–	+	0.67
8	–	+	+	–	+	–	–	–	0.34
9	+	+	+	+	+	+	+	+	0.33
10	–	+	+	+	–	–	–	+	0.86
11	–	+	–	+	+	+	–	–	0.59
12	+	–	+	+	–	+	–	–	0.52
13	–	–	+	–	+	+	+	–	0.32
14	–	–	+	–	–	+	–	+	0.24
15	+	–	–	+	–	+	+	+	0.67
16	–	–	–	+	–	–	+	–	0.78
17	–	–	–	–	–	–	–	–	0.74
18	+	–	+	–	–	–	+	+	0.60
19	+	+	+	–	–	–	+	–	0.63
20	–	–	–	+	+	+	–	+	0.74
21	+	+	–	–	–	+	–	–	0.95
22	+	+	–	+	–	–	–	+	0.73
23	–	+	+	+	–	+	+	–	0.42
24	+	–	+	+	+	–	–	–	0.91
25	+	–	–	–	+	+	+	–	0.77
26	0	0	0	0	0	0	0	0	0.84

Spain). The pump and collection trap were cooled with industrially pure CO_2.

Experiments were performed on a Hewlett–Packard (Palo Alto, C.A. USA) Model 7680A supercritical fluid extractor using standard steel cells of 7.0 ml inner volume. The system, however, was altered as described later on. The collection trap (7 cm long×5 mm I.D., 540 μl inner volume) was packed with Hypersil (ODS) of 30 μm average particle size.

Analyses were carried out on a HP-5890A Series



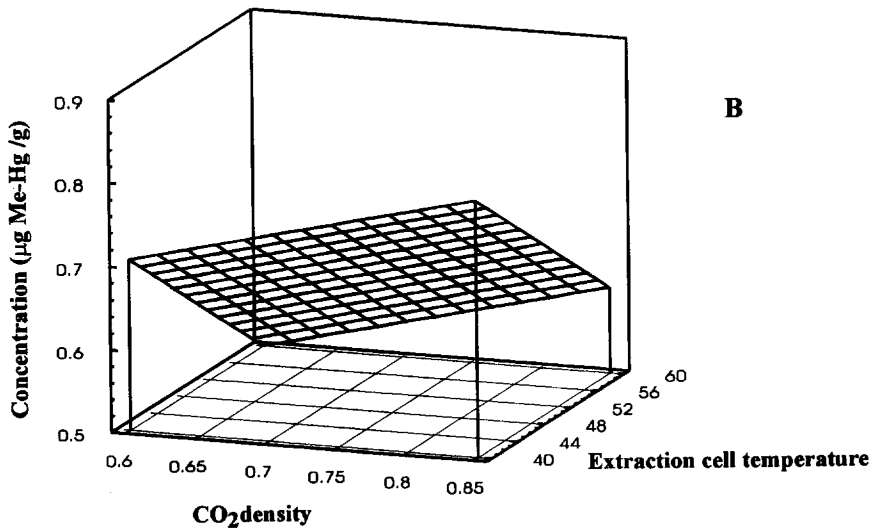


Fig. 2. Response surfaces estimated from the factor design. obtained by plotting: (A) extraction cell temperature–amount of HCl, and (B) extraction cell temperature–CO_2 density.

II gas chromatograph equipped with a nickel-63 electron capture detector and an HP-7673 automatic injector. Data were acquired using an HP ChemStation. Two types of fused-silica capillary columns were used: an Alltech AT-5 column (5% polymethyldiphenyl-siloxane), 30 m long×0.54 mm I.D.×5 μm phase thickness; and an HP-1 column (dimethylsiloxane) 5 m long×0.53 mm I.D.×2.65 μm phase thickness. The need for the chromatographic columns to be conditioned with $HgCl_2$ to obtain satisfactory separations and reproducible results has been sufficiently confirmed [18].

Optimum chromatographic conditions with the AT-5 column were: injector temperature, 200°C; detector temperature, 250°C; column head pressure, 75 kPa; nitrogen make-up flow-rate, 5.4 ml min^{-1}; for the first ramp, oven initial temperature, 90°C; ramp rate, 20°C min^{-1}; oven final temperature, 140°C and for the second ramp, oven initial temperature, 140°C; ramp rate, 2°C min^{-1}; oven final temperature, 150°C. With HP-1 column we use isothermal separations at 90°C and 30 kPa of column head pressure.

2.2. Sample preparation

Optimization experiments were carried out on a mussel homogenate, obtained from several mussel samples from Galician coast (Spain). An amount of several kilograms of mussels was triturated, homogenized, freeze-dried and then sieved in order to obtain a particle size below 60 μm. An amount of 50 g of homogenate was slurried with 250 ml of methanol containing a known amount of Me–Hg to form a dough that was mechanically mixed for a several min. The sample was allowed to air-dry in the dark for a week and stored in a dry, dark place for two months prior to analysis. On the assumption that no Me–Hg loss occurred during drying or storage, the expected final concentration was 2.4 μg Me–Hg g^{-1} on a dry-weight basis.

2.3. Extraction procedure

Irrespective of the working conditions imposed by the particular factor design, all samples were prepared by following the same procedure prior to extraction. In order to minimize contamination and plugging of sintered disks, the top and the bottom caps of extraction thimble were fitted with two filter paper disks of same diameter as the cap I.D. Also, a piece of teflon tubing of the same outer diameter as the thimble I.D. was placed in the thimble to avoid potential interactions between its steel walls and the analyte.

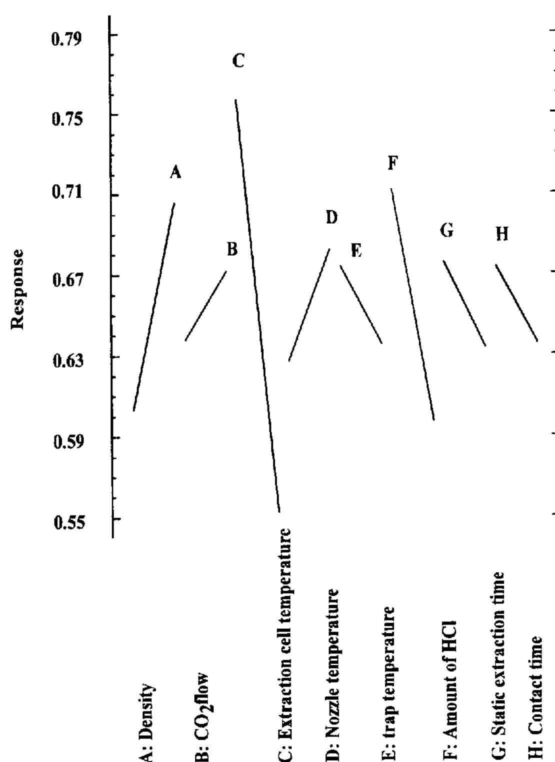


Fig. 3. Graph showing the influence of main effects on the extraction of Me–Hg. The lines indicate the magnitude and sign (increase or decrease) of the variation of the extraction efficiency with the factor level (from low to high).

In a typical experiment, an amount of mussel homogenate spiked (to which the required amount of hydrochloric acid (1:1) was added) was mixed and homogenized with Celite before it was transferred to the tube. Then, the thimble was sealed with the top cap and placed in the extraction chamber.

The static and dynamic SF CO_2 extraction program was then started under conditions dictated by the particular factor design tested. Finally the Me–Hg was eluted from trap with 1+1 ml of toluene and collected in two 2-ml vials.

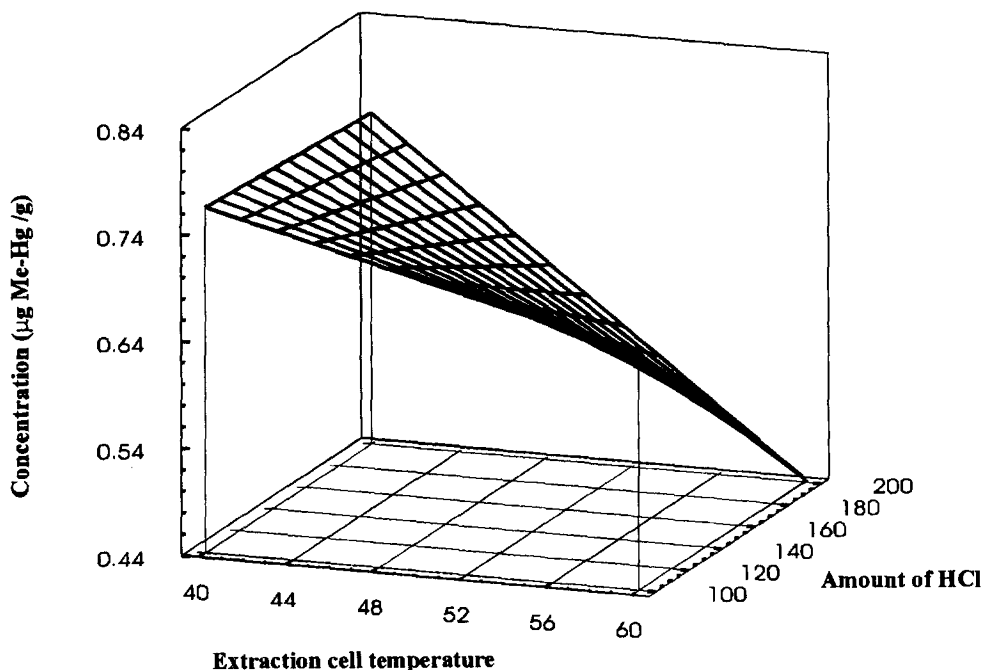


Fig. 4. Response surface estimated from the reduced model obtained by plotting: extraction cell temperature–amount of HCl.

2.4. Clean-up of sample extract

When HP-1 column had to be used, the SFE extracts were cleaned by complexation of the Me–Hg with 3 ml of 1% aqueous solution of cysteine acetate. Other compounds coextracted by SFE, which could overlap methylmercury peak remain in the organic layer. Then, the aqueous layer was back-extracted with 4 ml of HCl (1:1) and 5 ml of toluene.

2.5. Evaluation of the homogeneity of the laboratory-spiked mussel material

The homogeneity of the spiked material as regards analyte distribution was evaluated after a little over two months storage. The procedure described by Hight and Corcoran [19] was used for the manual liquid–liquid extraction of Me–Hg, with modifications as detailed elsewhere [20]. Such conditions were used on samples masses from 0.05 to 0.3 g.

As can be seen in Fig. 1, the results were highly disperse for sample size of 0.3 g and inaccurate for sample sizes of 0.15 and 0.2 g. This is logical because this type of sample containing lipids requires the use of 2-propanol [19,20] during the toluene extraction to destroy the relatively strong emulsions formed. When the amount of sample increases, emulsions become stronger, thus making difficult to obtain phase separation with logical consequences both in accuracy and precision. We chose 0.1 g as the optimum sample size for subsequent experiments in order to prevent the variability between sample portions from masking the influence of experimental variables.

3. Results and discussion

3.1. Calibration

In the firsts experiments it was observed that manual extraction produces much cleaner extracts as compared to SFE. Thus, homogeneity study was carried out by means of an HP-1 column, whereas an AT-5 column was used in all of quantitative determinations during the factorial design. This type of column allows the analysis of relatively dirty extracts by using programmed temperature elutions [18,20].

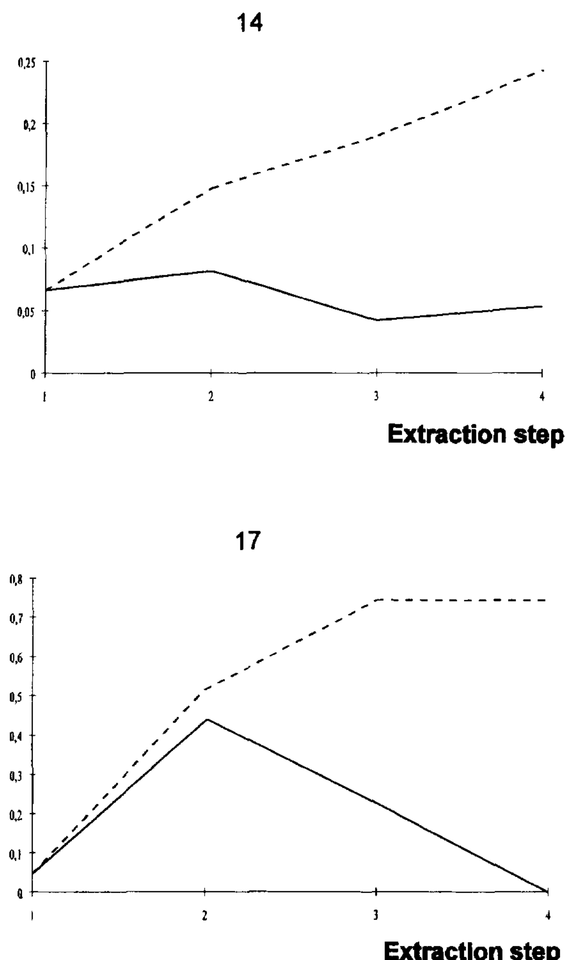


Fig. 5. Me–Hg extraction kinetics in two experiments selected from Table 1. Run number corresponds to the sequential number in Table 1. Solid lines represent the proportions of Me–Hg recovered in each successive extraction at the stated time value. Dashed lines indicate the cumulative concentration found (μg Me–Hg g^{-1}) during the consecutive extractions.

Since it was possible that the extracts obtained in the different experiments dictated by the factorial designs would imply appreciable differences in coextracted species from the matrix, the use of an AT-5 column appears advantageous in order to guaranty selectivity in the Me–Hg determination along the optimization study.

Calibration lines were drawn at four concentration levels in the range 5–40 ng Me–Hg ml^{-1}. Chromatographic peak areas were fitted by linear regression obtaining correlation coefficients of 1.000 with the HP-1 column and 0.998 with the AT-5 column.

3.2. Optimization of SF extraction process. Factorial designs

The number of variables potentially affecting the extraction efficiency and kinetics was very large (eight variables). Factors such as CO_2 flow-rate and density, extraction cell temperature, nozzle and trap temperature, amount of hydrochloric acid (1:1), static extraction time and contact time between HCl and the sample before to extraction were, in principle, influential. A full, two-level factor design would involve an overall $2^8=256$ experiments, in addition to the replicates needed for statistical evaluation of the coefficients for the fitted model and the degree of coincidence of the hyperplane obtained. We therefore chose a folded Plackett–Burman $2^8*3/32$ type IV Resolution design [15–17], allowing 15 degrees of freedom for error estimation, which involves 24 randomized runs plus 2 centred points. This design possesses an alias structure such that main effects are clear of two-factor interactions but these are partially confounded with other two-factor interactions. The upper and lower values given to each factor were as follows: CO_2 density (Factor A), 0.60–0.85 g cm^{-3}; CO_2 flow-rate (Factor B), 1.0–2.0 ml min^{-1}; extraction cell temperature (Factor C), 40–60°C; nozzle temperature (Factor D), 45–60°C; trap temperature (Factor E), 15–40°C; amount of HCl (Factor F), 100–200 μl; static extraction time (Factor G), 5–15 min; and contact times before extraction (Factor H), 0–60 min. Table 1 shows the experimental design matrix developed and the overall Me–Hg extraction yield obtained in each duplicate experiment.

The analysis of the results of Table 1 lead to the conclusion that only the extraction cell temperature was statistically significant. Since Plackett–Burman designs often underestimate medium effects the amount of HCl (1:1) and CO_2 density, were also considered. Fig. 2 shows the response surfaces obtained for the model using the variables: (Fig. 2A) extraction cell temperature vs. amount of HCl (1:1) and (Fig. 2B) extraction cell temperature vs. CO_2 density. As can be seen the extraction efficiency was directly proportional to high CO_2 density and low thimble temperature. On the other hand, we should

Table 2
Comparative results of the determination of Me–Hg in marine samples (μg Me–Hg g^{-1})

Sample	*n*	Certified Me–Hg content	GC–ECD Manual extraction		SFE–GC–ECD		GC–AED Manual extraction	
			Me–Hg found	Recovery (%)	Me–Hg found	Recovery (%)	Me–Hg found	Recovery (%)
DORM-1	6	0.785±0.06	0.69± 0.03	88±3	0.76± 0.02	96±3	0.70±0.03	89±4
CRM no. 464	6	5.500±0.17	5.20±0.13	88±5	4.53±0.06	82±1	–	–
CRM no. 463	6	3.04±016	2.83±0.28	90±4	2.64±0.09	87±3	2.6±0.3	84±9
Cockle	6	–	–	–	0.64±0.02	–	(0.66±0.07)[a]	–
Clam	6	–	–	–	0.83±0.05	–	(1.0±0.1)[a]	–

[a] Results obtained with n=4.

note the adverse effect of the thimble temperature and the amount of HCl; and the positive effect of the CO_2 density in Fig. 3. Excluding the less significant factors (CO_2 flow, nozzle and trap temperature, static extraction time and contact time before extraction) and keeping the other three allowed two-factor interactions to be evaluated. In this reduced model the extraction cell temperature was logically the statistically significant factor, but the interaction between the extraction cell temperature and the amount of HCl (1:1) appears also close to the significance boundary. This reduced model (only three main factors A,C and F) provided the response surface shown in Fig. 4. The overall conclusions are quite similar to those drawn from the response surfaces of Fig. 2. The amount of HCl (1:1) appears only influential when high extraction cell temperatures are used, being the extraction cell temperature the most influential factor. High CO_2 density values also improve extraction efficiency. In view of these results, it was decided to carry out a systematic study of the mutual influence of factors A and C. These experiments were conducted starting from 0.75 g cm^{-3} of CO_2 density to the maximum value allowable of 0.95 g cm^{-3} also fixing the thimble temperature at the minimum level allowed for the extractor used (40°C). The results show that 0.95 g cm^{-3} provides maximum extraction yield for Me–Hg. However, it was quite difficult to maintain this value in the extractor used so we adopted the following optimal conditions for extraction of Me–Hg from the material tested: CO_2 density, 0.90 g cm^{-3}; CO_2 flow-rate, 1.5 ml min^{-1}; extraction cell temperature, 40°C; nozzle temperature, 53°C; trap temperature, 15°C; amount of HCl, 100 μl; static extraction time, 10 min; contact time before extraction, 0 min. For the less significant factors we adopted medium values.

Repeatability and reproducibility of the optimized experimental procedure were evaluated by means of a series of five extractions performed the same day and another nine carried out on different dates. All these extractions were carried out using the laboratory spiked mussel sample. The average recoveries obtained were 85.2±2.3% (within-day extractions) and 83.5±4.0% (between-day extractions).

3.3. Extraction kinetics

In order to investigate the extraction kinetics, the dynamic extraction programme was split into four steps in such a way that extracts were collected at 2, 7, 17 and 37 min for separate analysis. Total recovery in each experiment was calculated by adding the amount of the analyte found in each collected fraction. Logically, the Me–Hg concentration vs extraction steps plots obtained in different experiments performed with the first factor design tested were widely divergent. In most cases, the extracts collected at the fourth time (37 min) contained no Me–Hg. On the other hand, some samples exhibited a much slower kinetics (Fig. 5). Obviously, the optimal extraction conditions were those producing the maximum extraction yield at the shortest possible extraction time. In the finally optimized conditions 17 min can be considered time enough to complete the extraction.

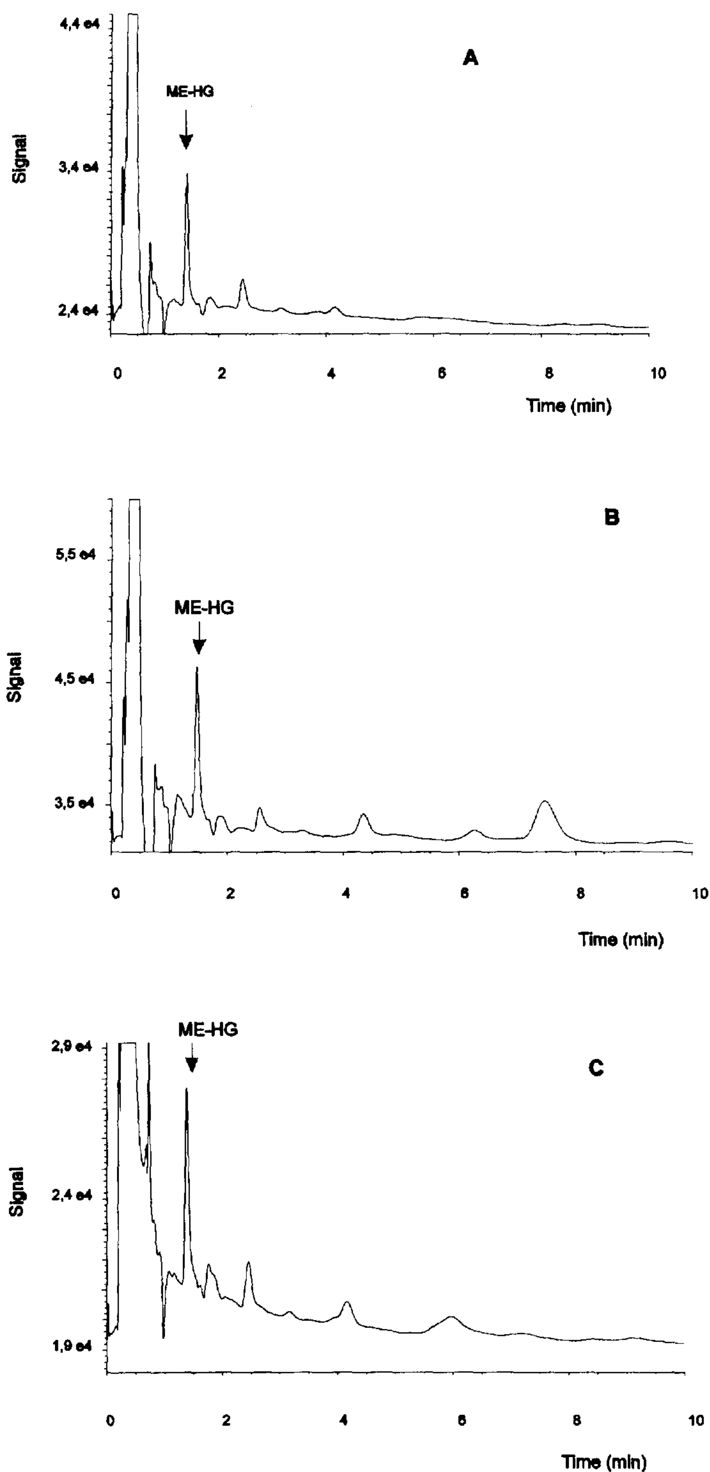


Fig. 6. Chromatograms using the SFE–GC–ECD system for (A) tuna CRMś 464 sample, (B) a clam sample and (C) a cockle sample.

3.4. Application to real samples and procedure validation

The optimized SFE procedure was validated using three available reference materials (DORM-1, supplied by the National Research Council of Canada, and tuna CRM's 464 and 463, supplied by the Community Bureau of Reference (BCR-UE)). These materials have very different methylmercury content (Table 2) and can be considered useful to validate any extraction or analysis procedure for methylmercury in biological materials.

The obtained results are given in Table 2 where recoveries are compared not only with certified values but also with the obtained recoveries by manual extraction. Also, polluted cockle and clam samples obtained near a chlor-alkali factory waste dump were analysed. Since no reference values exist for these type of samples comparative results obtained using manual extraction and GC–Atomic Emission Detection (reported elsewhere [21]) have been also included in the two last columns of Table 2 for comparative purposes. In Fig. 6 some real sample chromatograms are depicted showing the selectivity of the proposed procedure.

It is clear that SFE recoveries are comparable to the widely accepted manual extraction procedure, being slightly more precise. In addition, practical benefits including reduced sample manipulation, procedural automatization and low time consumption makes the SFE procedure clearly advantageous when compared with manual extraction.

Acknowledgments

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