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Determination of mercury species in fish reference materials by gas chromatography-atomic fluorescence detection after closed-vessel microwave-assisted extraction

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Abstract

A simple and rapid method has been developed for speciation analysis of inorganic mercury and monomethylmercury (MMHg) in biological tissues. The procedure is based on the quantitative closed-vessel microwave-assisted leaching of mercury from biological samples with an alkaline extractant. The extracted mercury species are ethylated and analysed by capillary gas chromatography coupled to an atomic fluorescence detector via pyrolysis (CGC-pyro-AFS). The coupling between capillary gas chromatography and atomic fluorescence detector was optimized with the aim of minimizing the detection limits and time necessary for the species-selective determination of mercury compounds. The use of closed-vessel microwave-assisted extraction along with no clean-up steps before the ethylation leads to a significant total analysis time decrease with respect to similar procedures. The detection limit was 2 pg for MMHg (as Hg) and 1 pg for inorganic mercury. The method was validated by the analysis of DORM-2 (dogfish muscle) and DOLT-3 (dogfish liver) certified reference materials. The inorganic mercury and methylmercury concentrations found were in good agreement with the certified values. Recovery studies of fish muscle tissue spiked with inorganic mercury and MMHg were done to check the reliability of the method. In all cases satisfactory recoveries (92–105%) were obtained.

Keywords: Mercury speciation; Closed-vessel microwave-assisted extraction; Biological samples; Gas chromatography; Atomic fluorescence spectrometry

1. Introduction

The toxic effects and metabolic behaviour of mercury is largely dependent on its chemical form. Alkylmercury compounds are of special concern because of their easy penetration through biological membranes, efficient bioaccumulation, high volatility and long-term elimination from tissues [1]. Monomethylmercury (MMHg) is by far the most toxic and the most commonly occurring organo-mercury compound, and is recognized as a major environmental pollution issue and health hazard for humans. MMHg is biomagnificated through the trophic chain and, as a result, the average proportion of MMHg over total Hg in fish tissues can be up to 95% [2]. Contaminated seafood is the major route of exposure for humans to MMHg. Different guidelines have been established to regulate the mercury intake, especially in relation to seafood consumption (WHO, ECC) [3].

It is therefore clear that total mercury concentration determination is not far enough and mercury species levels in foodstuffs should be determined. To address this requirement, a range of procedures have been developed [4]. In general, the analytical methods used for speciation of mercury in environmental or biological samples include different

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sequential steps: (a) extraction and preconcentration (if necessary) to reach a final concentration over the determination limit, (b) separation of inorganic and organic species, and (c) determination of the species [5]. However, these speciation procedures are time consuming, and involve many steps in sample pre-treatment that reduce the procedure robustness and reliability. Thus, further studies are required to develop methods meeting the criteria of: ease of implementation, robustness, speed, accuracy and precision with low limits of detection [6].

The extraction and separation of mercury compounds in solid samples, such as sediments or biotissues, is one of the most key steps [7]. In this way, the conventional Westöö technique and further modifications have been extensively used, but only methylmercury is finally extracted by this procedure, not inorganic mercury. Most common drawbacks of these methods are the laboriousness of the procedures, risk of interferences and high solvent consumption. At present, the most popular extraction techniques are steam distillation [8], supercritical fluid extraction [9] and acid or basic liquid extraction either at room temperature [10] or using different heating sources such as sonication [7] or microwaves [11]. The use of microwave-assisted extraction technique has been confirmed as one of the best methods for mineralization and selective leaching of analyte compounds. There are two different microwave-assisted extraction systems: focused and closed-vessel. On one hand, focused microwave-assisted extraction (FMAE) technique to extract methylmercury has been implemented [11,12]. Most common drawbacks are related to difficulty to control extraction conditions and risk of losing the volatile compounds [13]. On the other hand, closed-vessel microwave-assisted extraction is free from the aforementioned problems and appears to be a good approach for mercury species extraction in biological samples.

The extracts are later analysed by coupled techniques including separation by gas chromatography (packed or capillary column) or liquid chromatography and detection by electron capture (ECD) [14], atomic absorption spectroscopy (AAS) [12], atomic fluorescence spectroscopy (AFS) [6,7,15–17] or inductively coupled plasma mass spectrometry (ICP-MS) [18]. The GC-ECD was traditionally used. However, a major problem inherent to this technique is that the non-selective detector responds to any other halogen-bearing compounds, extracted together with organomercury. Therefore, extensive clean-up procedures are necessary to reduce potential interferences. This leads to time consuming and labour intensive procedures losing information about inorganic mercury. Atomic fluorescence spectrometry and inductively coupled plasma mass spectrometry have both been shown to be suitable detection techniques for organomercury speciation after separation by gas chromatography. Both techniques are extremely sensitive and selective detectors for mercury. The advantages of the emerging and underexploited GC-AFS coupling are its comparatively low cost and simple operation. Thus, its high sensitivity, high selectivity, low cost and simple operation make it one of the best techniques for mercury speciation.

The analysis of mercury species by gas chromatography requires a previous derivatization process to form the more easily volatile derivatives. Procedures based on Grignard reagents offer the possibility of selecting different alkyl groups [19,20]. However, owing to the fact that Grignard reagents are very sensitive toward water, the organomercury species have to be extracted into organic solvent prior to derivatization. As a result, the whole sample preparation can be tedious and time consuming. In order to circumvent this trouble, the use of sodium tetraethylborate has been also reported [21,22]. It acts as an aqueous-phase ethylating reagent, quantitatively transferring ethyl group to ionic mercury species. This reaction has significant advantages over Grignard reaction since the derivatization can be performed in aqueous phase, subsequently reducing the time of analysis and eliminating the need for organic solvent extraction. Thus, this reaction produces a fast and easy procedure for methylmercury determination.

The purpose of this work was to develop a simplified method combining the advantages of the above discussed approaches and eliminating their drawbacks. For the first time, closed-vessel microwave-assisted extraction has been used in conjunction with ethylation and analysis by capillary gas chromatography with atomic fluorescence spectrometry detection leading to a simple and rapid procedure for mercury speciation in biological samples.

2. Experimental

2.1. Instrumentation

A gas chromatograph (Varian 3900, Varian Ibérica, Spain) was coupled to an AFS detector (Millenium Merlin, P.S. Analytical, United Kingdom) via a pyrolysis unit. The instrumental configuration is schematically illustrated in Fig. 1 and the operating conditions used in the system are shown in Table 1. The chromatograph was provided with a non-polar capillary column (DB-5, 15 m × 0.25 mm × 0.25 μm, J&W Scientific, Folson, USA) and helium was used as carrier gas. The eluted mercury species were transferred to the detector through a pyrolysis oven which converts the different mercury species to atomic mercury vapour. The pyrolysis unit is provided with a temperature control module. The analytical column ending was linked to a deactivated silica capillary tube (50 cm long) through a deactivated universal press-tight connector (Teknokroma, San Cugat del Valles, Spain). This connection was used to preserve the life of the column as well as to interface the GC and detection system. After the pirolysis unit a T-connection (1/16″) was placed in the deactivated silica capillary tube to allow a make-up gas pathway for improving the transport efficiency. Argon was used as make-up and also as sheath gas for the AFS detector. Finally, data were acquired by Speciation Application Millenium Systems Soft-

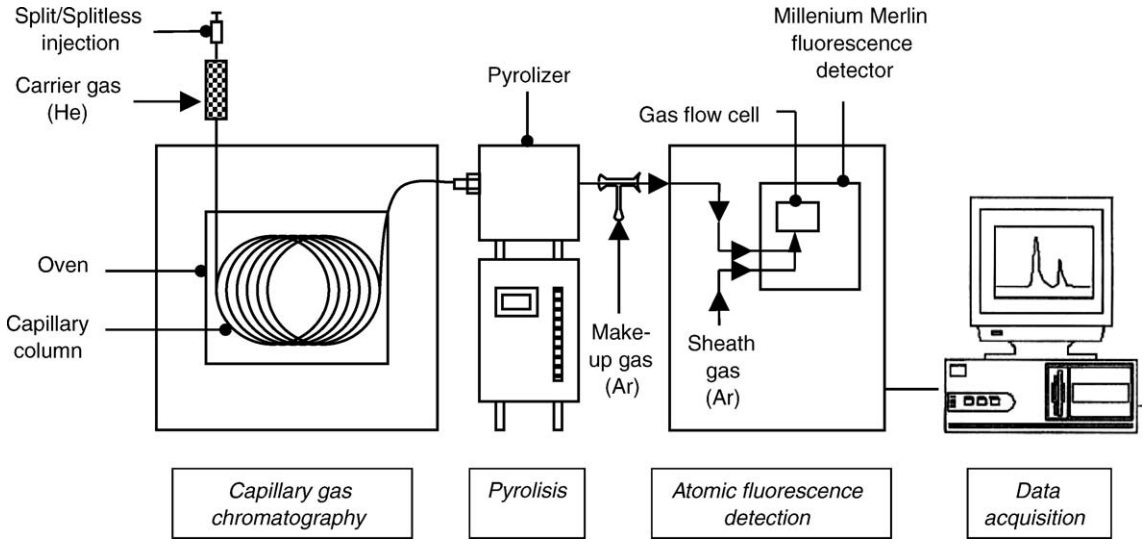

Fig. 1. Instrumental set-up for mercury speciation: CGC-pyro-AFS system.

ware (P.S. Analytical, United Kingdom) and processed by Microcal Origin 5.0 (Microcal Software Inc., Northampton, MA, USA).

A laboratory microwave system (Ethos Plus; Milestone, Monroe, CT, USA), equipped with temperature and pressure feedback control was used in this study. This device is accurate in sensing temperature within ±2.0 °C of set temperature, and automatically adjusts the microwave field output power. This device is prepared for extracting 10 samples simultaneously. The high pressure closed digestion vessels used for extraction are made of high purity TFM (a thermally resistant form of Teflon) and have a capacity of 100 mL.

2.2. Reagents, standards, samples and certified reference materials

Stock standard solutions of 1000 μg ml^{-1} of Hg^{2+} and MMHg were prepared by dissolving mercury(II) chloride (Panreac) in 5% HNO_3 (Merck) and methylmercury chloride (Strem Chemicals) in methanol, respectively. A 1000 μg ml^{-1} (as mercury) solution of Ph_2Hg (Strem Chemicals) was also prepared by dissolution with hexane (Merck). All stock solutions were stored in amber glass bottles in a cold room at 4 °C. Working standards were prepared daily by proper dilution with ultrapure water.

Table 1
Operating conditions for GC-pyro-AFS system

Gas chromatography	
Column	DB-5, 15 m × 0.25 mm × 0.25 μm
Injector type	Split/splitless
Injector volume (μl)	1 (splitless)
Injector temperature (°C)	300
Temperature programme	40 °C (2 min), 40 °C/min, 200 °C (2 min)
He carrier gas flow (ml min^{-1})	3
Pyrolyser	
Pyrolysis temperature (°C)	800
Atomic fluorescence detector	
Make-up gas flow (ml min^{-1})	150
Sheath gas flow (ml min^{-1})	300
AFS gain	1000
Filter factor	16

For the sample extraction methanolic tetramethylammonium hydroxide (25%, w/w) was obtained from Sigma–Aldrich (Steinheim, Germany). Sodium tetraethylborate 98% was purchased from Strem Chemicals (Bischheim, France). Hexane and isooctane for gas chromatography (Merck) were used as organic solvents. All chemicals were of analytical-reagent grade.

Ultrapure water (18.2 MΩ cm) was obtained from an Elga Purelab Ultra Analytic water purification system.

Helium C-50 was used as a carrier gas and Argon C-50 was used as a make-up and sheath gas at the transfer line and the AFS detector, respectively (Carburos Metálicos, Spain).

The certified reference materials used were DORM-2 (dogfish muscle) and DOLT-3 (dogfish liver) from National Measurement Standard of Research Council of Canada. The certified reference materials were used as provided.

For the spike experiments, a commercially available fish sample (*Cynoscion* sp.) was acquired. Muscle tissue was separated for analysis and stored at −80 °C, and then it was lyophilised. Finally, the sample was homogenised to a powder and kept at 4 °C until analysis.

2.3. Procedures

A schematic diagram of the procedure is shown in Fig. 2.

Mercury extraction for speciation analysis was carried out with 0.2 g of sample and 2.0 ml of tetramethylammonium hydroxide (TMAH) following a previously optimized procedure [23] (microwave programme in Fig. 2). The final volume

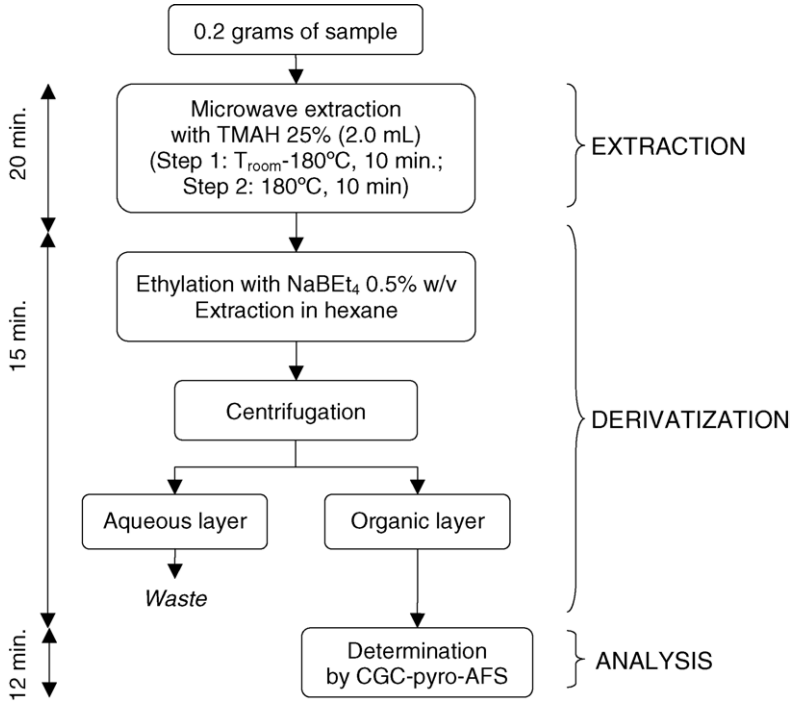

Fig. 2. Schematic flow diagram of the proposed procedure for the mercury speciation in fish tissues.

was adjusted to 10 ml with ultrapure water for microwave requirements. A clear solution was obtained after microwave irradiation. Then the vessels were cooled down to room temperature, made-up to a known volume and stored in the cold room until analyzed. Blanks were prepared along with the samples in each batch.

Volumes of 2 ml of the alkaline extracts were used for derivatization. The pH was adjusted to 3.9 using concentrated acetic acid and 5 ml of 0.1 M acetic acid–sodium acetate buffer. Then, 5 ml of sodium tetraethylborate (0.5%, w/v) and 2 ml of hexane were added and the mixture was manually shaken for 5 min. The sample was centrifuged for 5 min at 3000 rpm. The organic layer was transferred to a glass vial and stored at 4 °C.

Finally, portions of the hexane extracts (100 μl) were accurately transferred to fresh 1.1 ml screw cap vials and spiked with diphenylmercury in hexane (5 μl, 1000 μg l^{-1} (as Hg)). An aliquot of 1 μl was analysed by CGC-pyro-AFS system. The optimized separation and detection conditions are summarized in Table 1.

For the recovery experiments, the fish muscle tissue was spiked before the extraction with standard solutions of inorganic mercury, MMHg and a mixture of both mercury species at different concentration levels. The masses of spike were roughly the same as those extracted.

3. Results and discussion

Preliminary experiments were carried out to select the derivatization and organic solvent extraction conditions. The use of non-polar capillary columns for the separation requires a previous derivatization of the ionic mercury species. Although derivatization of mercury by ethylation with $NaBEt_4$ has been relatively popular prior to purge-and-trap analysis, its combination with solvent extraction was rare. The ethylation conditions initially selected were based on those proposed for a capillary GC-ICP-MS hyphenated system [18]. Thus, the derivatization was carried out at pH 3.9 (controlled with acid acetic/acetate 0.1 M buffer solution) with $NaBEt_4$ at a 0.10% (w/v) concentration. Two organic solvents were tested for extraction of derivatizated species, isooctane and hexane. However, hexane was selected for this purpose because the peaks of analytes were 15–20% higher than those obtained for isooctane.

The stability of these species was evaluated during 1 week by comparing the results obtained versus those of freshly prepared extracts ($t = 0$):

$$R = \frac{X}{X(t=0)}$$

The combined uncertainty (U_t) of the measurements is given by the expression:

$$U_t = (\mathrm{CV}^2 + \mathrm{CV}^2_{(t=0)})^{1/2} \frac{R}{100}$$

where X is the mean value of the measurements made at different times and CV is the coefficient of variation of X.

In Fig. 3, R is plotted versus storage time for mercury species. The U_t data ranged from 0.002 to 0.092. These results clearly indicate that ethylated species remain stable for the storage time of 4 days, which is of special interest

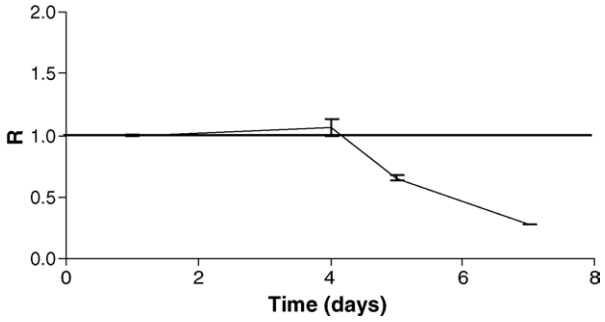

Fig. 3. Stability of ethylated mercury species extracted in hexane. *R* defined as ratio between stored to fresh extracts.

when storage is required before analysis. For a longer time a significant signal decreasing is observed, more than 50% for a week of storage.

3.1. Optimization of GC-pyro-AFS system conditions

The multitude of non-interdependal parameters involved required a systematic approach to the optimization of the system. These parameters are related to chromatographic separation conditions, pyrolysis and AFS detection. The AFS instrument was optimised for maximum sensitivity, and the chromatography was also optimised to achieve best resolution. The evaluated parameters were injection mode, injector temperature, programme of temperature, carrier gas flow-rate, pyrolyser temperature, data acquisition settings, make-up gas flow rate and sheath gas flow rate. The 50 $\mu g\,l^{-1}$ mixed standards of $HgEt_2$, MeHgEt and Ph_2Hg (this latter as internal standard) in hexane were used in the method optimization.

3.1.1. Chromatographic parameters

The objective of the optimization was therefore to minimize the peak width, reducing the run time of the chromatographic separation while preserving the baseline resolution of the species (MeEtHg, Et_2Hg and Ph_2Hg). The basic parameters of concern included the carrier gas flow rate and the injector and column temperature.

A non-polar capillary column (15 m × 0.25 mm × 0.25 μm) was used. The proximity of the boiling-point of hexane to that of MeEtHg makes it necessary to use an oven temperature programme to obtain a baseline separation between MeEtHg and hexane and between MeHgEt and Et_2Hg. The finally selected temperature program with an initial step at 40 °C and final temperature of 200 °C is shown in Table 1. Heating of the transfer line was not necessary. All compounds were baseline separated within 7.5 min.

The effect of the carrier gas flow rate on the separation was checked from 3 up to 7 $ml\,min^{-1}$. The lowest value (3 $ml\,min^{-1}$) was selected as suitable because the best baseline resolution was obtained for all the compounds under these conditions.

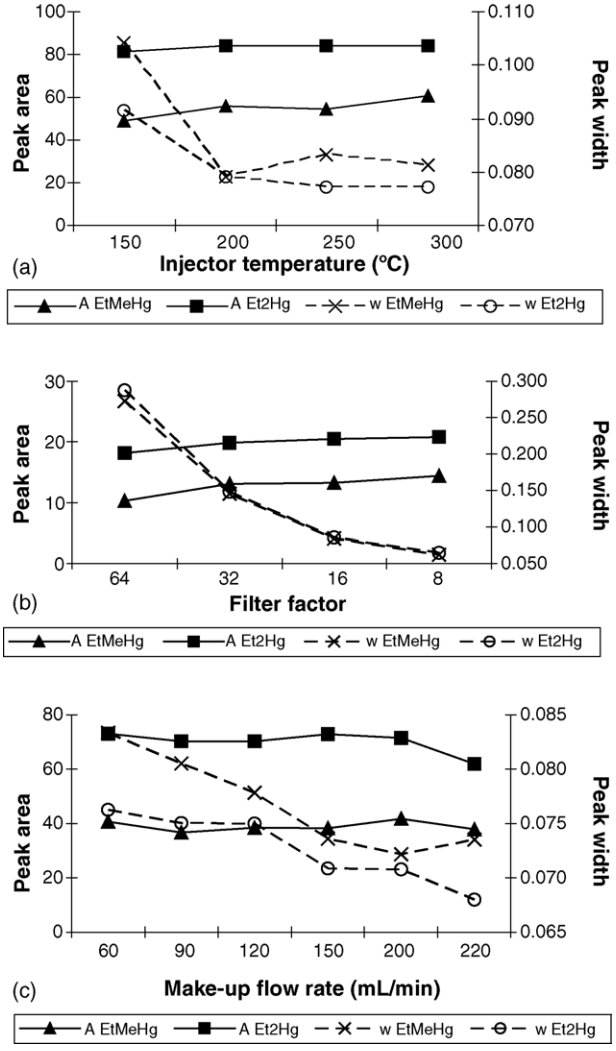

Fig. 4. Optimisation of parameters for GC-pyro-AFS system. Effect of peak area and peak width for 50 $\mu g\,l^{-1}$ of MeEtHg and Et_2Hg of: (a) injector temperature; (b) filter factor and (c) make-up flow rate.

The injection temperature was evaluated from 150 to 300 °C using the splitless mode (Fig. 4a). The effects on peak area and peak width showed that, the higher the temperature the best results were obtained. As the column upper working limit was 325 °C, the injector temperature was selected to be 300 °C.

3.1.2. Data acquisition

The spectrometer has a reference cell to check lamp emission. The measurements were performed in Ratio mode, which is a compensation mode between the emission and reference cell readings as internal baseline correction. The calibration range selected was 1000 (the most sensitive).

The filter factor for data acquisition was found to be a critical parameter for detection of the fast transient chromatographic signal. In fact, filter factor (between 8 and 64) had a significant influence on the peak width (Fig. 4b). The peak widths decreased with the filter factor, but also a noise

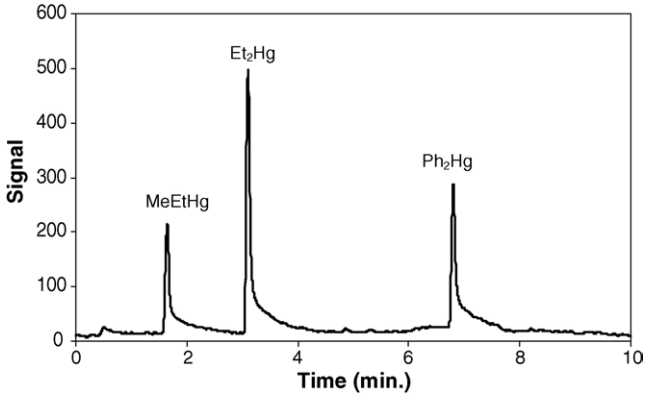

Fig. 5. Chromatogram of 50 μg l^{-1} of MeEtHg, Et_2Hg and Ph_2Hg (as internal standard) in hexane.

increase was observed. Thus, the filter factor for data acquisition finally selected was 16 as a compromise between peak width and noise.

3.1.3. Pyrolyser temperature

Before AFS detection, the mercury species should be atomised. A home-made pyrolysis unit (described in Section 2) was used. The optimum pyrolysis temperature reported in the literature ranged between 700 and 900 °C, no adequate pyrolysis is obtained for lower temperatures [24]. Thus, in this work the pyrolysis temperature was tested in the range 700–850 °C to provide minimal peak broadening with maximum area. A compromise situation between peak area and width was reached for 800 °C.

3.1.4. Make-up and sheath gas flow-rate

In the interface GC-AFS an additional gas flow (make-up) is necessary to complement the He flow. In the AFS, a sheath gas was used in order to achieve an optimum conformation of the mercury vapour at the excitation cell. Argon was selected to minimise the damage caused on the detector by He [22]. The make-up gas flux was tested in the range 60–220 ml min^{-1} and an intermedial value (150 ml min^{-1}) was found as optimum for peak area and widths values (Fig. 4c). It is not possible to modify the sheath gas flow in our instrument, so it was kept constant to 300 ml min^{-1}.

3.1.5. Analytical figures of merit

A typical chromatogram obtained using the optimal operating conditions is shown in Fig. 5. For a 50 μg l^{-1} (as Hg) MeHgEt, $HgEt_2$ and Ph_2Hg in hexane, the separation is reached in less than 8 min and the retention times are 1.65, 3.11 and 6.80 min, respectively.

The relative standard deviation of 10 repeated 1 μl injections of 50 μg l^{-1} MeHgEt, Et_2Hg and Ph_2Hg (as Hg) standard in hexane were 4.7, 5.9 and 8.9%. Calibrations curves in the range 5–200 μg l^{-1} for MeHgEt exhibit linear regression slopes of 0.721(±0.026) and determination coefficients of $R^2 = 0.995$. The same parameters for inorganic mercury (as Et_2Hg) were 1.148(±0.036) and 0.996, respectively. In both cases, the intercepts were considered as negligible by using the Student's t-test ($\alpha = 0.05$). Limits of detection, LOD, were estimated in accordance to the base line. The base line noise was evaluated by recording the detector response over a period about 10 times the peak width. The LOD was obtained as the sample concentration that caused a peak with a height three-fold the base line noise level. The obtained values were 2 pg for MMHg (as Hg) and 1 pg for inorganic mercury.

Table 2
Effect of ethylation reagent amount on the response obtained for the determination of MMHg and Hg^{2+} in fish tissues

$NaBEt_4$ concentration (%, w/v)	Recovery (%)	
	MMHg	Hg^{2+}
0.1	94.0 ± 2.5	61.7 ± 6.4
0.3	100.0 ± 7.6	102.4 ± 4.0
0.5	93.0 ± 3.0	101.9 ± 6.9

Values were obtained as the percentage of the certified values of the DOLT-3 ($n = 4$).

3.2. Analysis of biological materials

The procedure applied for sample preparation was based on a previously optimized closed-vessel microwave-assisted extraction of biomaterials [23]. The extractant used was TMAH (2.0 ml of TMAH for 0.2 g of sample). The initially

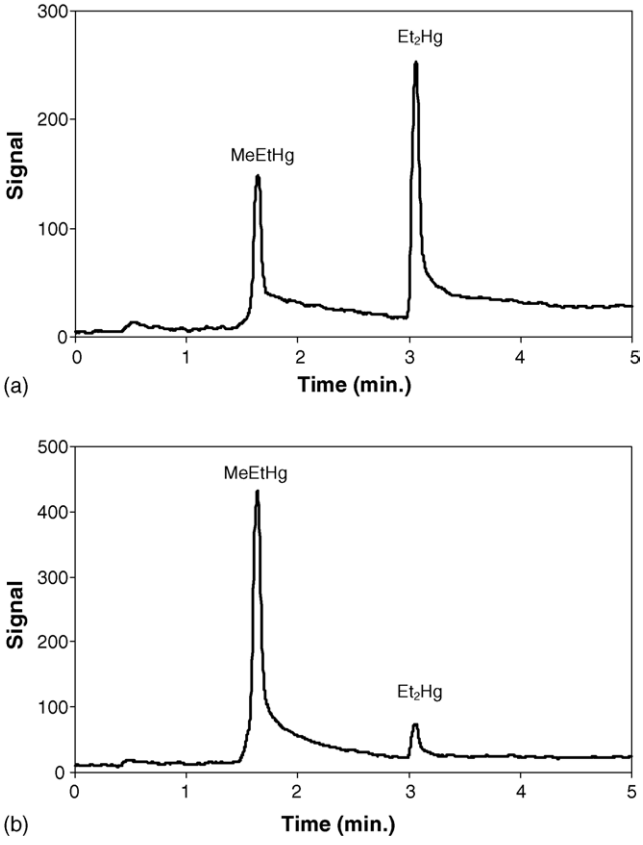

Fig. 6. Typical chromatogram obtained by Et-GC-pyro-AFS after closed-vessel microwave-assisted extraction of: (a) DOLT-3 and (b) DORM-2.

Table 3
Results for the determination of mercury in biological certified reference materials

CRM	Methylmercury (as Hg) (μg/g)		Inorganic mercury (μg/g)	
	Certified value	Found value	Proposed value	Found value
DORM-2	4.47 ± 0.32	4.54 ± 0.32 (n = 6)	0.17	0.17 ± 0.04 (n = 6)
DOLT-3	1.70	1.71 ± 0.12 (n = 7)	1.67	1.71 ± 0.06 (n = 7)

Table 4
Recoveries of mercury species added to fish muscle tissue

Added (μg/g)		Found (μg/g)		Recovery (%)	
MMHg	IHg	MMHg	IHg	MMHg	IHg
0	0	0.63 ± 0.06	0.16 ± 0.02	–	–
0	1.0	0.68 ± 0.07	1.11 ± 0.13	–	93.3 ± 10.2
0	2.0	0.69 ± 0.06	2.05 ± 0.11	–	94.0 ± 5.4
2.0	0	2.64 ± 0.09	0.26 ± 0.05	99.9 ± 4.3	–
4.0	0	4.82 ± 0.16	0.22 ± 0.03	104.4 ± 5.8	–
2.0	1.0	2.60 ± 0.19	1.12 ± 0.06	97.6 ± 8.4	95.9 ± 6.1
4.0	2.0	4.44 ± 0.29	2.21 ± 0.09	95.1 ± 7.1	102.3 ± 4.7

Mean ± SD (n = 4).

extraction and ethylation conditions selected (pH 3.9 and 0.1% $NaBEt_4$) were applied to the hydrolisate of the biomaterial (2.0 ml of extract neutralized with acetic acid in the presence of buffer of pH 3.9), but the recoveries obtained for both species were not satisfactory. These microwave extraction conditions had been successfully used previously for mercury analysis, though. They were checked again and the extractant volume was increased up to 5 ml of TMAH, but no recovery improvements were obtained. Then, the effect of derivatizing reagent amount was checked and the results are shown in Table 2. Apparently, an unidentified constituent(s) of the sample consume(s) the bulk of the derivatization reagent before it reacts with the target species. A three to five-fold increase in the concentration of ethylation reagent was necessary.

The analytical performance of the methodology proposed in this work was evaluated by the analysis of two certified reference materials (DOLT-3 and DORM-2). Typical chromatograms obtained for fish tissues using aqueous ethylation are shown in Fig. 6. The found amounts and recoveries were achieved by comparing with test solutions containing the same concentrations than expected for the reference materials according to their claimed levels. The test solutions were prepared from the stock solutions after convenient dilutions. The concentrations obtained for the reference materials are given in Table 3. An excellent agreement was found between the found and certified values for inorganic and methylmercury compounds.

In order to complete the study, several concentrations of both mercury species were added to a fish muscle tissue. Recovery data for spiked samples are listed in Table 4. The recoveries were closed to 100% for both species within the precision of analysis. These results allow us to confirm that

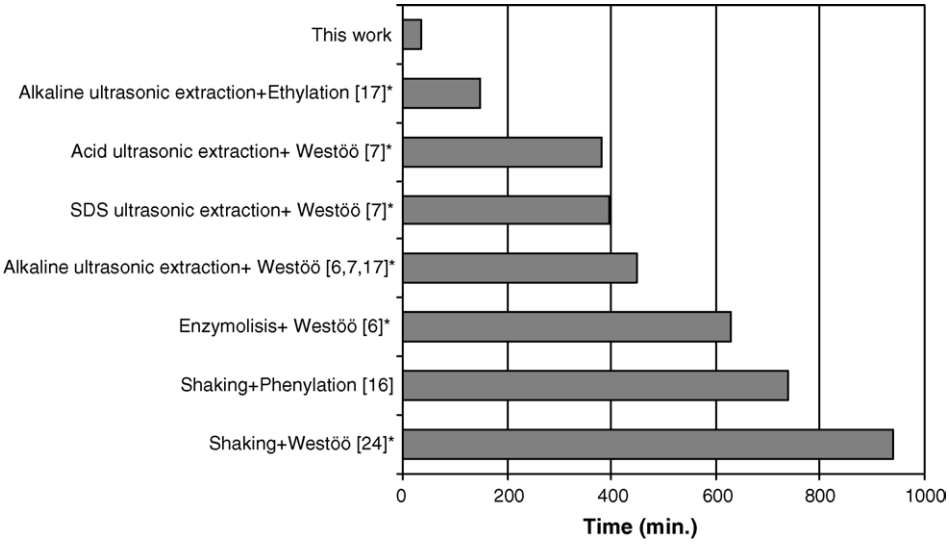

Fig. 7. Comparison of the time required for mercury speciation with capillary gas chromatograph coupled to atomic fluorescence detector using different procedures reported in the literature. (*) To indicate that only monomethylmercury is determined.

the microwave extraction procedure does not alter the chemical form of mercury and that mercury species can be quantitatively extracted from real samples with adequate recoveries.

In comparison with other sample preparation procedures reported in the literature using CGC-pyro-AFS systems, the one proposed in this work (including both extraction and adequation for analysis) allows a significant analysis time reduction. A comparison is shown in Fig. 7. Additionally, it should be noticed the possibility of 10 microwave extractions being accomplished simultaneously. Also it should be pointed out that both inorganic and monomethylmercury species, and not only monomethylmercury, can simultaneously and quantitatively be determined.

4. Conclusion

Routine mercury speciation is a difficult task due to numerous and cumbersome manipulations necessary for sample preparation, which are time consuming and affect the method precision. In the present work, a simple, fast and accurate method for sample preparation and mercury speciation in biological tissues has been developed and, for the first time, after closed-vessel microwave-assisted extraction with TMAH solution, both inorganic mercury and methylmercury concentrations can be directly determined by a hyphenated GC-pyro-AFS system. The proposed method offers the following advantages: (I) possibility of simultaneous determination of methylmercury and inorganic mercury; (II) a noticeable reduction of solvent volume; (III) considerable time savings in the procedure of sample preparation (Fig. 7); and (IV) the possibility of simultaneously extracting up to 10 samples, resulting in increased sample output compared with conventional extraction techniques.

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