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Approach for rapid extraction and speciation of mercury using a microtip ultrasonic probe followed by LC–ICP-MS

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ABSTRACT

A fast method for mercury extraction from biological samples based on the use of HCl leaching plus different enzymatic hydrolysis (with and without mercury complexing agents), and the use of focussed ultrasounds (2-mm microtip) is here proposed. Total mercury content in several biological samples was determined by FI–ICP-MS using a carrier solution consisting of 0.1% (v/v) HCl, 0.1% (v/v) 2-mercaptoethanol, to avoid memory effect, and 0.15% (w/v) KCl. For mercury speciation a RP18 chromatographic column coupled to ICP-MS was used. A mobile phase consisting of 0.1% (v/v) formic acid, 0.1% (v/v) HFBA, 2% (v/v) methanol, and 0.02% (w/v) mM L-cysteine at pH 2.1 was used for chromatographic separation of the mercury species in the sample extracts. Extraction procedures were validated by using 50 mg of tuna fish tissue CRM-463 (2.85 ± 0.16 mg kg^{-1} for methylmercury). The recoveries obtained were $99 \pm 3\%$ and $93 \pm 1\%$ after acid leaching (HCl 7 M) and enzymatic extraction (15 mg protease type XIV in 2.5% (v/v) 2-mercaptoethanol), respectively. The optimal sonication conditions (5 min of exposure time and 40% of ultrasound amplitude) were applied to 5 mg of CRM-463 ($88 \pm 5\%$), 5 mg of mussel tissue ($81 \pm 11\%$) and to 2 mg of zebra fish embryos ($90 \pm 10\%$) obtaining good recoveries in all cases. Methylmecury was found to be the most abundant Hg specie in all samples. The developed method is simple and rapid (5 min sample treatment); it is suitable for very small samples and does not alter the original form of the mercury species. Thus, it is of special interest in those cases in which validation of the results may often be hampered by lack of sample availability.

1. Introduction

Inorganic mercury (Hg^{2+}) and monomethylmercury (CH_3Hg^+) are the two most abundant Hg species generally found in biological samples [1]. Toxicity, biochemical behaviour and transport of mercury in the environment are clearly dependent on its chemical form [2]. In this context, it is well known that organomercurial compounds are more harmful than inorganic mercury. The fact that the latter is biomethylated by aquatic organisms to form methylmercury is the reason why fish constitutes the major source of MeHg for humans [3] (predatory species may preconcentrate 10,000–100,000 times the mercury concentration in water [4]). The most relevant aspects for bioaccumulation are fish size and/or fat content [5,6], the protein affinity mechanisms [7], and the content of dissolved oxygen in the fish habitat [8].

Mercury interacts with proteins and enzymes due to its strong affinity for sulphur, causing organ dysfunction and a devastating effect on the whole central nervous system of human [11]. In spite that toxicity of EtHg is lower than that of MeHg, both species cause similar symptoms [9,10].

Extraction of mercury species from a complex sample is recognized as one of the most crucial steps before their determination. A successful extraction procedure for speciation analysis requires high extraction efficiency, and more importantly, all original species must keep intact prior to analysis [12]. Various extraction procedures, such as distillation and acid and alkaline extraction, have a tendency to form artificial methylmercury from inorganic mercury during sample preparation [13,14]. To avoid that acid and alkaline digestion or solvent extraction at moderate temperatures and pH are the methods commonly used to extract mercury species from biological tissues [15]. The high affinity of mercury to sulphydryl groups makes L-cysteine and 2-mercaptoethanol two very useful reagents to extract mercury species in combination with ultrasounds. Several studies have shown that mercury extraction efficiency increases with increasing concentration of 2-mercaptoethanol [12]. Ultrasound-assisted extraction has already been shown as a very promising technique for the extraction of mercury species. In this way, Rio-Segade and Bendicho [16] developed an ultrasound-assisted extraction method using 2 and 4 mol L^{-1} HCl for mercury speciation in fish tissues.

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Furthermore, enzymatic hydrolysis has also been used due to the ability of enzymes to act on specific chemical bonds avoiding alteration of the chemical forms of mercury [17]. Rai et al. employed Protease XIV to extract inorganic mercury and methylmercury species from fish samples [14].

The main aim of this study is the evaluation of the extraction efficiency of both acid leaching and enzymatic hydrolysis (with and without 2-mercaptoethanol) assisted by ultrasounds for mercury determination and speciation in fish tissues samples. An ultrasonic probe has been selected as it drastically decreases the extraction time and has successfully been used for enzymatic hydrolysis of selenium species in a wide range of matrices [18]. The effect of several parameters affecting mercury extraction such as acid concentration, ultrasonic amplitude, sonication time, amount of extractant and sample size (50 and 5 mg) have been evaluated.

The optimized enzymatic hydrolysis with 2-mercaptoethanol have been applied to biological samples including mussels, swordfish and zebra fish embryos. The latter are particular relevant because as living organisms they are considered an excellent alternative model for the OECD 305 bioconcentration test, which is expensive, time consuming and requires the use of at least 108 juvenile or adult fish. These embryos have a similar genetic code to humans but they are not considered as laboratory animals by the Directive 86/609/EEC.

Finally, a chromatographic method for separation of mercury species coupled to ICP-MS have also been developed and validated using tuna fish tissue CRM-463 as reference material.

2. Experimental

2.1. Instrumentation

Samples for total mercury determination were digested in doubled-walled advanced composite vessels using a 1000 W MSP (microwave sample preparation system) microwave oven (CEM, Matthews, NC, USA). A Vibra cell VCx130 ultrasonic processors (Connecticut, USA) equipped with a titanium 2-mm-diameter microtip and fitted with a high-frequency generator of 130 W at frecuency of 20 KHz was used for extracting mercury species. An Eppendorf Centrifuge 5804 R (Hamburg, Germany) F34-6-38 was used for separation of solid residues from the soluble fraction.

A quadrupole ICP-MS Thermo X-Series (Thermo Electron, Windsford, Cheshire, UK) equipped with a Meinhard nebulizer, a Fussel Torch, and an Impact Bead Quartz Spray Chamber cooled by a Peltier system was used for mercury determination. The mass calibration of the ICP-MS instrument was tuned daily with a solution containing 1 $\mu g\,L^{-1}$ of Li, Be, Co, Ni, In, Ba, Ce, Pb, Bi and U in 5% (v/v) HNO_3.

The liquid chromatographic system used for mercury speciation consisted of a PU-2089 LC pump (JASCO, Tokio, Japan) fitted with a six-port injection valve (model 7725i, Rheodyne, Rohner Park, CA, USA) with a 100-μL injection loop and a reversed-phase analytical column (Symmetry Shield RP_{18} column, 150 mm × 3.9 mm, 5 μm, Waters). The outlet of the column was directly connected to the nebulizer of an ICP-MS system using PEEK tubing (⌀ = 0.13 mm). The optimal operation conditions and data acquisition parameters are summarized in Table 1.

2.2. Reagents and materials

All reagents used were of analytical grade. H_2O_2 (35%) from Panreac and HNO_3 (65%) from Merck were used for acid digestion of samples. Non-specific protease type XIV, 2-mercaptoethanol, lipase and L-cysteine monohydrochloric (98%) from Sigma (Sigma–Aldrich, Steinheim, Germany) were used for enzymatic hydrolysis. A 50 mM Tris–HCl buffer solution at pH 7.5 from Fluka were used as extractant media. For acid leaching, HCl (37%) from Merck was used.

Table 1
Operational parameters of the HPLC-ICP-MS system.

Plasma conditions	
Foward power	1250 W
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	0.73 L min^{-1}
Nebulizer gas flow rate	0.83 L min^{-1}
Nebulizer	Meinhard
Spray chamber	Impact Bead Quartz Spray
Measurement parameters	
Acquisition mode	Time resolved analysis
Isotope monitored	^{202}Hg, ^{201}Hg and ^{200}Hg
Dwell time per point	200 ms
Replicates	3
HPLC conditions	
Column	Symmetry Shield RP 18 (150 × 3.9 mm, 5 μm)
Injection volume	100 μL
Column temperature	25 °C
Mobile phase	0.1% (v/v) formic acid, 0.1% (v/v) HFBA, 2% (v/v) methanol, 0.02% (w/v) L-cysteine (pH 2.1)
Elution mode	Isocratic
Flow rate	1 L min^{-1}

The carrier solution for total mercury analysis contained KCl from Riedel-de Haën, HCl and 2-mercaptoethanol.

Heptafluorobutyric acid, formic acid, L-cysteine mono hydroclorhidric from Sigma and methanol (Scharlau, Barcelona, Spain) were used in the chromatographic mobile phases.

Standard solutions were prepared daily by making appropriated dilutions of a methylmercury chloride stock standard solution of 1000 mg L^{-1} (Alfa Aesar, Karlsruhe, Germany) in methanol (LC grade, Scharlau) with ultra-pure water (18.2 MΩ cm^{-1}) from a Milli-Q water purification system (Millipore, MA, USA). This solution was stored in the dark at −18 °C.

2.3. Mercury speciation by HPLC–ICP-MS

The resulting supernatants from the procedures detailed in Fig. 1 were analyzed by FI–ICP-MS and LC–ICP-MS for determination of total mercury and its species, respectively. The extracts obtained were centrifuged at 7600 rpm for 5 min and the supernatants were filtered through a 0.22 μm membrane before LC–ICP-MS analysis.

Quantification was performed in time resolved analysis mode and the operating conditions of LC–ICP-MS are summarized in Table 1.

The most abundant mercury isotope ^{202}Hg was used for data evaluation and the analytical precision was verified by replicate measurements (three injections of a single sample). Quantification was based on peak areas by standard addition method. The different extractions methods reported in this work were optimized using the certified reference material, tuna fish tissue CRM-463 ($2.85 \pm 0.16\,\mu g\,g^{-1}$ methylmercury), from the Community Bureau of Reference of European Commission (BCR).

3. Results and discussion

3.1. Determination of mercury by ICP-MS: memory effect

Determination of total mercury in biological samples using a classic wet digestion method followed by ICP-MS suffered from serious memory effects [12]. Mercury accumulates within the sample introduction system and it is slowly released over time; thus increasing the response signals for the same mercury concentration. This fact results in non-linear calibration curve, need for

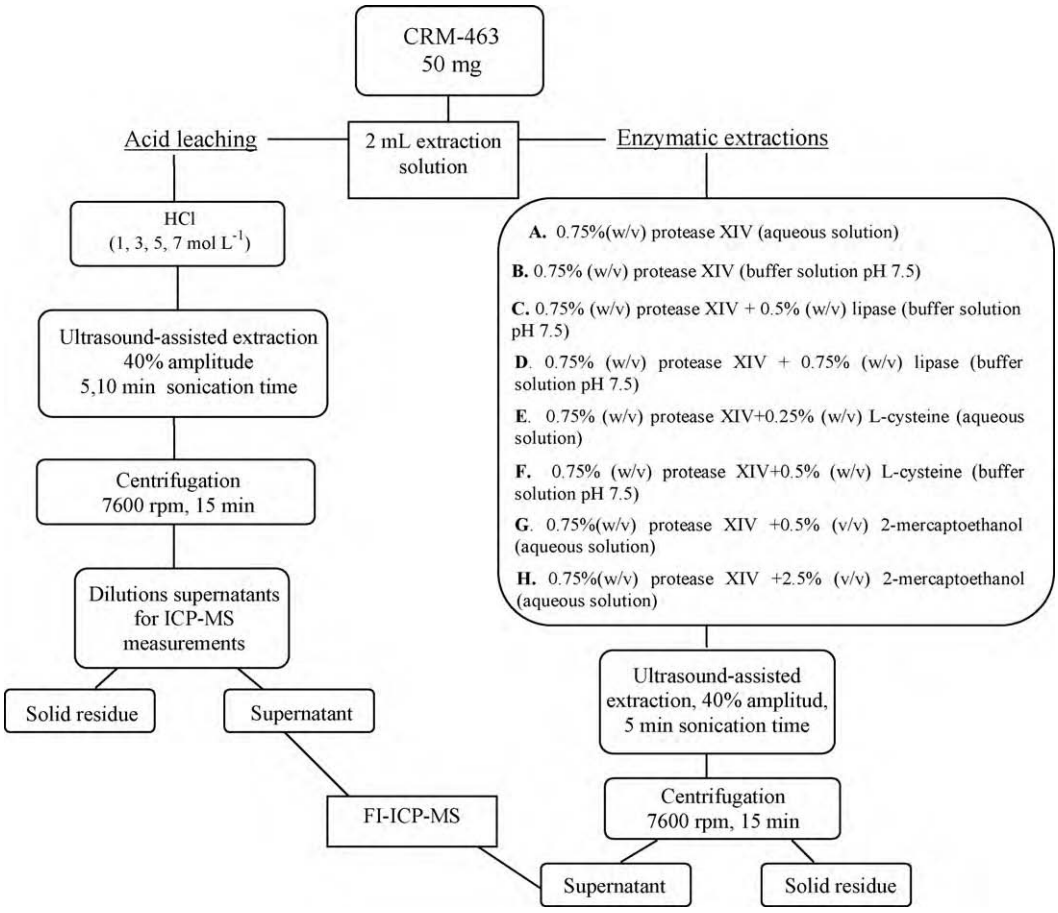

Fig. 1. Scheme showing the different extraction methods used for sample treatment.

long washing times and poor sensitivity [19]. Since the memory effect can be overcome by the addition of Hg(II) ligands [20], a carrier solution containing 0.1% (v/v) HCl, 0.1% (v/v) 2-mercaptoethanol and 0.15% (w/v) KCl was used with this purpose in the present study. HCl was added to the carrier solution in order to maintain the pH at approximately 2, since quantitative analysis of mercury species in biological matrices is favoured at acid medium [12].

3.2. Evaluation of the extraction efficiency

Several extraction procedures based on the use of ultrasonic probe sonication were compared. For this purpose, acid leaching and enzymatic hydrolysis with and without mercury complexing reagents (L-cysteine and 2-mercaptoethanol) were used. In order to optimize the acid leaching, several variables such as concentration of HCl, 1, 3, 5 and 7 mol L^{-1}, ultrasonic amplitude, 40 and 60%, and sonication time, 5 and 10 min, were investigated. In the case of enzymatic extractions several types of enzymes were tested using the optimal ultrasonic parameters found for acid leaching. Besides the extraction of mercury species with 15 mg of protease XIV, several amounts of lipase were added (0.5% (w/v) and 0.75% (w/v)), plus different extraction media such as phosphate buffer solution at pH 7.5 and Tris–HCl buffer solution at pH 7.5. Finally, two mercury complexing reagents, L-cysteine (0.25% (w/v) and 0.5% (w/v)) and 2-mercaptoethanol (0.5% (v/v) and 2.5% (v/v)) were tested to improve the extraction efficiency. Extraction procedures were optimised by using the tuna fish tissue CRM-463 certified for methylmercury. A summary of the different strategies employed is compiled in Fig. 1.

3.2.1. Acid leaching

Acid hydrolysis is one of the most popular methods for mercury extraction [17]. Releasing of protein-bound mercury species with hydrochloric acid has been shown as more efficient than nitric or acetic acid-based leaching [16]. In general, the extraction efficiency is essentially governed by acid concentration, sonication time and ultrasonic amplitude. Thus, four hydrochloric acid concentrations, 1, 3, 5 and 7 mol L^{-1}, were used for sonication during 5 and 10 min at 40 and 60% ultrasonic amplitude. As it has been commented before, optimization of the different parameters was performed using tuna fish tissue CRM-463.

Mercury recovery increases with increasing HCl concentration obtaining a maximum recovery of $99 \pm 3\%$ with 7 mol L^{-1} hydrochloric acid (Fig. 2). These results could be due to the retention of mercury within fish tissue particles at HCL concentration lower than 7 mol L^{-1}. Regarding exposure time, shorter sonication periods have a favourable effect on mercury extraction. Recoveries were considerably lower at a longer exposure time (10 min) as compared to 5 min, which may be caused by volatilization of mercury species due to the creation of the oxidative energy during the sonication process. Thus, 5 min was chosen as optimal treatment time.

Extraction efficiency decreased with increasing amplitude from 40 to 60% (data not shown) because a high vibrational amplitude generates a great number of cavitation bubbles in the solution, which may dampen the passage of ultrasound energy through the liquid.

Based on these results, the optimal conditions found for quantitative extraction of mercury were 7 mol L^{-1} hydrochloric acid and 5 min of sonication time at 40% ultrasonic amplitude.

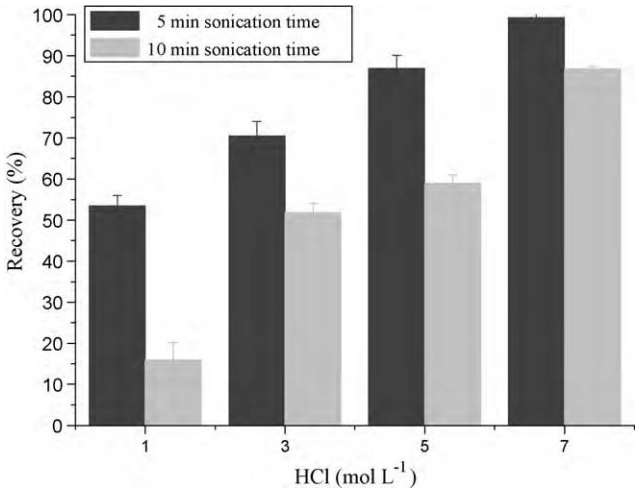

Fig. 2. Effect of hydrochloric acid concentration on the total mercury recovery for 50 mg of sample using 1, 3, 5 and 7 mol L^{-1} HCl as extractant agent at two sonication times, 5 and 10 min.

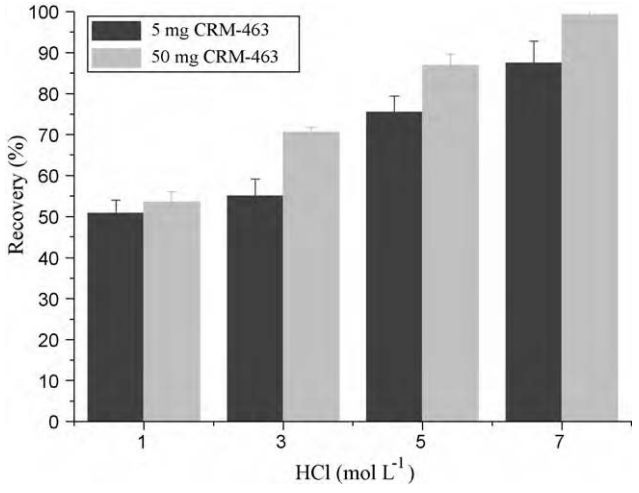

Fig. 3. Comparison of the effect of hydrochloric acid concentration on the total mercury recovery for 5 and 50 mg of sample using 1, 3, 5 and 7 mol L^{-1} HCl as extractant agent and a sonication time of 5 min.

One of the advantages of ultrasonic probe sonication is its ability to handle small amounts of sample in volumes as low as microliters. For this purpose, the optimised extraction procedure was applied to 5 mg of tuna fish reference material and, as shown in Fig. 3, the mercury recovery from CRM-463 varying the HCL concentration was almost independent of the amount (5–50 mg) of sample used. The recovery of mercury and the precision of the results for 5 mg of samples, 88 ± 5%, were lower than with 50 mg; however, this lower value could be considered satisfactory as it could be attributed to a lack of homogeneity of CRM-463 when the amount of sample is too small.

3.2.2. Enzymatic extraction

Quantification of mercury by ICP-MS using HCl as extractant reagent suffers of several drawbacks: signal depression, changes in the viscosity of the aspirated solutions, variations on the nebulization efficiency and in aerosol transport [21]. Furthermore, the use of high concentration of HCl hampers the good performance of the ultrasounds tip. Thus, besides the almost quantitative recovery achieved with HCl, different enzymatic mixtures following the procedures detailed in Section 2 were applied. Table 2 shows the recoveries of mercury obtained using different enzymatic extraction assisted by ultrasounds. Recoveries for procedures A–D, only based on enzymatic hydrolysis with protease type XIV, did not reached 40%. In order to improve this value, the use of additional enzymes in the extraction medium, Hg complexing reagents and different conditions were tested: (1) use of lipase enzyme (procedures C and D), due to high lipid content of the fish samples, (2) use of buffer solutions at pH close to 7.5 (procedures C, D and F), for optimal enzyme activity [22] and (3) use of a sulphydryl reagent (L-cysteine and 2-mercaptoethanol) because its high affinity for mercury. From the results, addition of lipase had a negative effect on the recovery and was not dependent of the amount added (Table 2).

Recovery achieved with the addition of 0.25% (w/v) L-cysteine in aqueous solution or 0.5% (w/v) L-cysteine in buffer solution at pH 7.5 as mercury complexing reagent (procedures E and F) was about 40%. This recovery could be improved by adding HCl to the extraction solution and heating. Hight and Cheng doubled the amount of mercury extracted with the use of heated HCl, until reaching quantitative extraction [23]. Other authors have achieved a quantitative extraction of mercury by using 0.10% (v/v) HCl, 0.05% (m/v) L-cysteine, 0.10% (v/v) 2-mercaptoethanol mixture [24,25], however, the extraction times needed were higher than the 5 min employed in the method developed in this work. The use of 2-mercaptoethanol had, as expected, a positive effect on the recovery. The efficiency increased with higher concentrations. The best recovery (91 ± 4%) was obtained when the extractant contained 2.5% (v/v) of 2-mercaptoethanol and 0.75% (w/v) of protease type XIV (procedure H). According to De Souza et al., a significant increase in mercury recoveries was observed when using 2-mercaptoethanol instead of L-cysteine [25]. No significant improvement in terms of efficiency was obtained neither by increasing 2-mercaptoethanol percentage (5% (v/v)), nor by applying a second hydrolysis to the solid residue. According to the data shown in Table 2, the most suitable methodology for total mercury extraction was the enzymatic extraction using 2.5% (v/v) of 2-mercaptoethanol and 0.75% (w/v) of protease type XIV. Thus, this procedure was selected for further applications to other fish tissue samples such as mussel and swordfish. To check the applicability of the method to microsample analysis, mercury extraction was carried out in zebra fish embryos (sample size about 2 mg),

Table 2
Extraction of total mercury from CRM-463 (2.85 ± 0.16 μg g^{-1}) using different enzymatic hydrolysis and measured by FI-ICP-MS.

Type enzymatic extraction		Recovery, $x \pm s$[a] (%)
A	0.75% (w/v) Protease type XIV (aqueous solution)	36 ± 2
B	0.75% (w/v) Protease type XIV (Buffer solution pH 7.5)	39 ± 13
C	0.75% (w/v) Protease type XIV + 0.5% (w/v) Lipase (buffer solution pH 7.5)	11.0 ± 0.2
D	0.75% (w/v) Protease type XIV + 0.75% (w/v) Lipase (buffer solution pH 7.5)	5 ± 1
E	0.75% (w/v) Protease type XIV + 0.25% (w/v) L-cysteine (aqueous solution)	40 ± 3
F	0.75% (w/v) Protease type XIV + 0.5% (w/v) L-cysteine (buffer solution pH 7.5)	41 ± 2
G	0.75% (w/v) Protease type XIV + 0.5% (v/v) 2-mercaptoethanol (aqueous solution)	80 ± 1
H	0.75% (w/v) Protease type XIV + 2.5% (v/v) 2-mercaptoethanol (aqueous solution)	91 ± 4

[a] Average value ± standard deviation (n = 3).

Table 3
Results of total mercury measurements by FI-ICP-MS.

Biological sample	Acid digestión, $x \pm s$[a] (mg Hg kg^{-1} sample)	Enzymatic hydrolysis with 2-mercaptoethanol, $x \pm s$[a] (mg Hg kg^{-1} sample)	Recovery*, $x \pm s$[a] (%)
Mussel (50 mg)	0.18 ± 0.02	0.16 ± 0.04	89 ± 6
Mussel (5 mg)		0.15 ± 0.02	81 ± 11
Swordfish (50 mg)	0.47 ± 0.01	0.39 ± 0.02	83 ± 4
Zebra fish (2 mg)	4.5 ± 0.6	4.0 ± 0.6	90 ± 10

* Recovey of total mercury obtained after enzymatic hydrolysis as compared to acid digestion.
[a] Average value ± standard deviation ($n = 3$).

incubated with 200 μg L^{-1} of methylmercury, and mussel tissue (sample size about 5 mg). The results obtained are shown in Table 3. The contents of mercury in samples (even microsamples) treated by enzymatic hydrolysis were in good agreement with those obtained by a classical acid digestion treatment. As expected, the precision of the results when using microsamples (a few mg) was lower, than that obtained when using higher amounts of sample.

3.3. Mercury speciation by HPLC–ICP-MS

The results showed before demonstrate the suitability of using of ultrasonic probe sonication (2 mm microtip) for the rapid and efficient extraction of mercury from fish tissues of different nature and sample size. However, since species interconversion caused by ultrasonication has been reported for several authors [26], the extracts resulting from enzymatic hydrolysis with 2-mercaptoethanol were analysed by LC–ICP-MS. Based on previous works published in the literature [27], the chromatographic column selected was a reverse phase column (Symmetry Shield RP18, 150 mm × 3.9 mm, 5 μm) and the mobile phase used contained 0.1% (v/v) formic acid, 0.1% (v/v) HFBA, 0.02% (w/v) L-cysteine and 2% (v/v) methanol.The addition of 0.02% (w/v) L-cysteine to the mobile phase was found to be critical in preventing retention of mercury in the reverse phase column and long tailing in the ICP-MS [28] chromatographic peaks. As it can be seen in the elution profiles of methylmercury and inorganic mercury standards shown in Fig. 4 (using separation conditions given

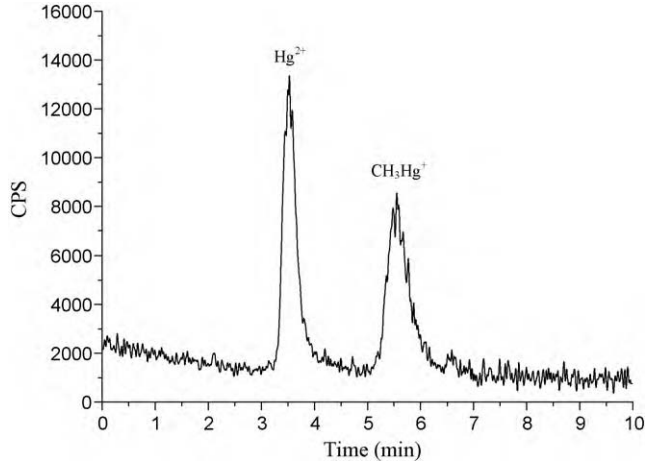

Fig. 4. Chromatogram obtained for 50 μg L^{-1} of Hg^{2+} and $MeHg^{+}$ standards using reverse phase chromatography and ICP-MS.

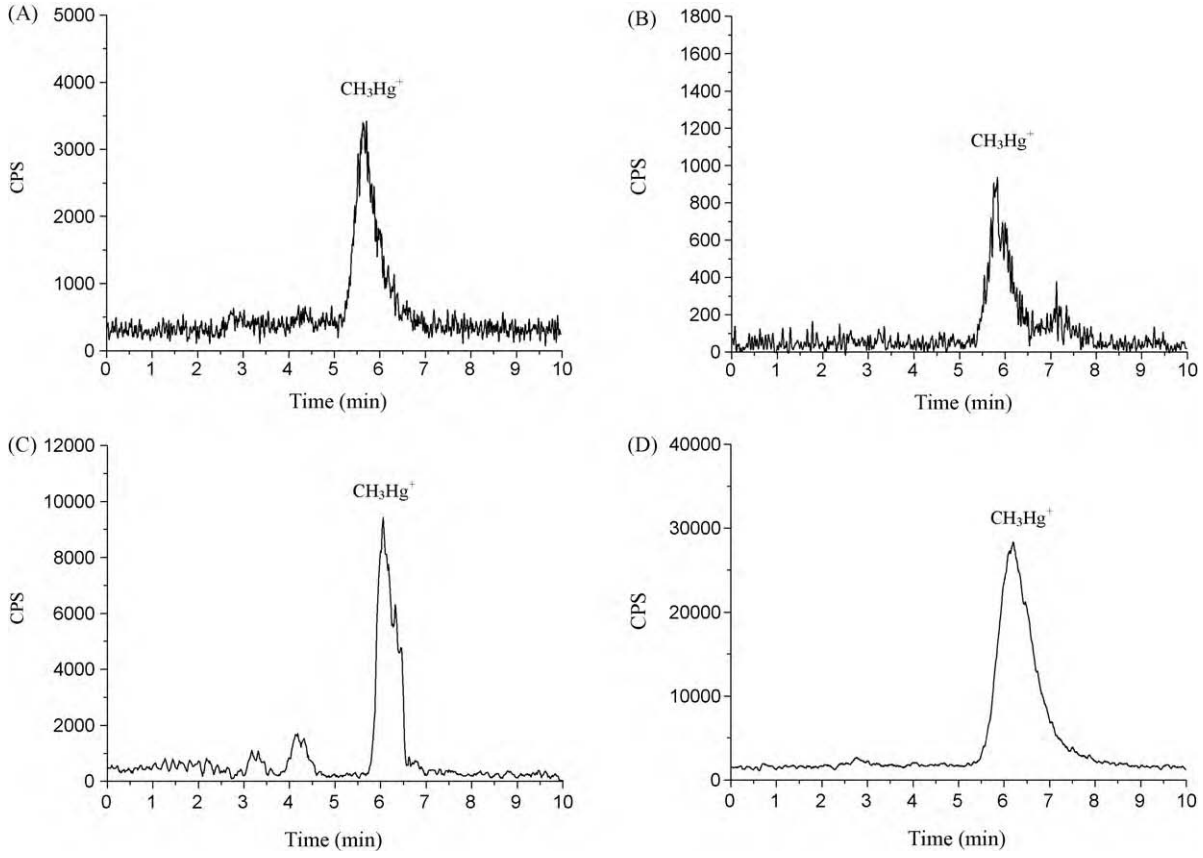

Fig. 5. Chromatograms of mercury species found in (A) tuna fish CRM-463, (B) mussel, (C) swordfish tissue, and (D) zebra fish embryos using reverse phase chromatography coupled to ICP-MS.

in Table 1), separation of Hg species is very fast; it only takes around 7 min.

Chromatographic analyses were performed on enzymatic extracts of tuna fish CRM-463, mussel and swordfish tissue and zebra fish embryos. The chromatograms obtained (Fig. 5(A)–(D)) showed a single peak in each of the evaluated sample, which was identified (by spiking the extract) as $MeHg^+$.

The chromatographic profile of the CRM-463 revealed that interconversion of methylmercury did not occur during ultrasound-assisted extraction of mercury species. As expected, the main mercury specie found in all the analyzed samples was $MeHg^+$, which is in good agreement with the studies reported by Cabañero et al. [29]. This may be explained due to the MeHg biomagnification phenomena through the trophic chain and the high affinity of fish intestine wall toward MeHg absorption [30]. Moreover, the selected methods allow extraction of mercury species in a few minutes from small size samples.

4. Conclusions

Quantitative extraction of mercury has been achieved using enzymatic extraction (15 mg protease XIV) plus 2.5% (v/v) 2-mercaptoethanol as complexing agent for mercury combined with the use of a titanium ultrasonic probe of 2 mm. The developed method is simple and rapid (10 min sample treatment), and no transformations between mercury species were detected. One novelty of the procedure is its ability to be applied to small size samples, which is significantly relevant for those cases in which big amounts of samples are not available or when they are highly valuable.

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