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Fast method for the simultaneous determination of monomethylmercury and inorganic mercury in rice and aquatic plants

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ABSTRACT

Recent investigations revealed that monomethylmercury (MMHg) can be absorbed and accumulated by plants, i.e. rice crops, thus becoming an important route of human exposure to MMHg through diet. The increasing concern about this fact makes that appropriate analytical methods for Hg speciation in these samples are urgently required. Therefore, the aim of this work has been the development of a fast and sensitive method which enables the simultaneous determination of MMHg and inorganic Hg in rice and aquatic plants. The proposed methodology is based on the extraction of Hg species by closed-vessel microwave heating, subsequent derivatization by ethylation and analysis by gas chromatography coupled to atomic fluorescence detection via pyrolysis (GC-pyro-AFS). A careful optimization of the extraction, using both acid (6 N HNO_3) and alkaline (tetramethylammonium hydroxide, TMAH) extractants, and derivatization conditions has been carried out. Spiked and unspiked aquatic plants (*Typha domingensis*) and CRMs certified for Total-Hg (BCR-60, BCR-482 and NCS ZC73027, corresponding to aquatic plant, lichen and rice, respectively) have been used. Under the final optimized conditions the simultaneous determination of MMHg and inorganic Hg can be carried out in less than 40 min with no tedious clean-up steps. Quantitative recoveries (from 92% to 101%) were obtained in aquatic plants (*Typha domingensis*) and CRMs spiked with known concentrations of MMHg. For unspiked BCR-60 and BCR-482, no statistically significant differences (p=0.05) were found in Total-Hg concentrations between those obtained by the sum of species and the certified values for both acid and alkaline extraction. For the analysis of low Hg polluted samples, an additional preconcentration step by evaporation under nitrogen stream was required but adequate blanks were only obtained for acid extraction. Detection limits in the low ng/g range (0.7–1.0 ng/g) were consequently achieved for both Hg species in the case of acid extraction and the analysis of NCS ZC73027 gave satisfactory results without statistically significant differences between the found and certified values (p = 0.05).

1. Introduction

Mercury (Hg) has been highly mobilized in the environment by different anthropogenic activities and once emitted it has a very long residence time in the atmosphere so it can be transported to very long distances from the sources. As a consequence it has been recognized as a global pollutant and it is now a matter of world concern [1]. Moreover, Hg can be in different chemical forms (mainly, elemental mercury, Hg°; inorganic mercury, IHg; monomethylmercury, MMHg) with different toxicity and biogeochemical behaviour [2]. Monomethylmercury is especially worrying because it is an important neurotoxin which can be bioaccumulated and biomagnified in the aquatic food webs leading to warning levels in fish. Nowadays, it is assumed that fish consumption is a major source of human exposure to this compound so there are specific regulations about it [2–5].

However, recent investigations have pointed out that MMHg can also be absorbed and accumulated by aquatic plants. This is especially troublesome in the case of rice due to its relevant role in the diet of millions of people [6–8]. Rice ingestion has been proposed as another potential important route of human exposure to MMHg in populations with a diet based on this product. Thus, China has been the first country to set a limit for Total-Hg concentration in rice (20 ng/g) [9] and additional regulations are probably underway [10]. Moreover, knowledge about absorption and subsequent distribution of Hg species

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in aquatic plants is still limited but it is also important for assessing their mobility in the environment. Therefore, there is a growing interest in the determination of Hg species in aquatic plants and in rice in particular.

From the analytical point of view, the determination of Hg species in this kind of samples is a challenge for different reasons. Firstly, the expected concentrations are in the low ppb range so high sensitive analytical techniques and preconcentration procedures are required. Secondly, the extraction of Hg species from the starch based matrix preventing losses and transformations is difficult. Moreover, there are no certified reference materials (CRMs) for Hg species in these samples. For that reason, the CRMs traditionally used for validation have been other biological samples which are mainly highly fatty fish tissues, but their use for validation of the methods in plants would not be an appropriate option.

The most commonly used methods for MMHg in aquatic plants and rice require a sequence of several time-consuming steps [6,8,11–17]. They are all mainly based on alkaline digestion using KOH/methanol [6–8,14,15,18–20] or acid digestion using HBr-$CuSO_4$ [11–13,21,22] with several subsequent extraction and back-extraction steps. Moreover, they are selective for MMHg and do not allow the simultaneous determination of other Hg species. In order to get this information, a separate analysis for Total-Hg is required and IHg is estimated by difference [6–8,11–15,18–21,23]. Therefore, the aim of this work is to develop and validate a fast and sensitive method for the simultaneous determination of Hg species (MMHg and IHg) in aquatic plants and rice. The present method is based on closed-vessel microwave extraction of the Hg species, which avoids tedious and time-consuming sample preparation steps, followed by gas chromatography coupled to atomic fluorescence detection via pyrolysis (GC-pyro-AFS). This analytical approach has been satisfactorily used in previous studies with other biological or environmental samples, such as fish or sediments [24–28]. The most critical step is sample preparation, so a careful optimization has to be carried out for aquatic plants and rice. Taking into account the lack of appropriate certified reference materials, special attention will be paid to the method validation.

2. Materials and methods

2.1. Instrumentation

A gas chromatograph (GC, Shimadzu GC-2010) coupled to an atomic fluorescence detector (AFS, Millenium Merlin 10025 P.S. Analytical) via a pyrolysis unit was used for Hg speciation analysis. The GC was provided with a split/splitless injector and a non-polar capillary column (TRB-5, 30 m × 0.25 mm i.d. × 0.25 μm coating) from Teknokroma. The performance of this GC-pyro-AFS hyphenated system was previously described elsewhere [24]. The operating conditions used in this work are summarized in Table 1.

A LyoQuest-55 freeze-dried (Telstar), an electric mill model A 11 (IKA) and an ultrasonic bath (Ultrasons-H, Selecta) were used for the cleaning and preparation of field samples. Total-Hg concentration was determined in these samples by absorption atomic spectroscopy (AAS) using an Advance Mercury Analyser (AMA-254) from LECO Instruments.

Extraction of Hg species was conducted using an Ethos Plus laboratory microwave system from Milestone, equipped with temperature and pressure feedback control. This device enables the simultaneous extraction of 10 samples.

A heating module (Reacti-Therm from Pierce) with an evaporating unit was used for preconcentration.

2.2. Reagents and reference materials

Stock standard solutions of MMHg and IHg (1000 mg/L) were prepared by dissolving methylmercury chloride (Strem Chemicals) in methanol and Hg(II) chloride (Panreac) in 5% HNO_3. All stock solutions were stored in amber glass vials at −20 °C. Working standards were prepared daily by dilution with ultrapure water.

Table 1
Operating conditions for GC-pyro-AFS system.

Gas chromatograph	
Column	TRB-5, 30 m × 0.25 mm × 0.25 μm
Injector type	Split / Splitless
Injector volume (μL)	2.0 (in splitless mode)
Injector temperature (°C)	300
Temperature programme	40 °C (2.5 min), 20 °C/min to 90 °C, 100 °C/min to 200 °C (1 min).
He carrier gas flow (mL/min)	3.0
Pyrolyser	
Pyrolysis temperature (°C)	800
Atomic fluorescence detector	
Make-up gas flow (mL/min)	150
Sheath gas flow (mL/min)	300
AFS gain	1000
Filter factor	16

For Hg species extraction, tetramethylammonium hydroxide (TMAH, 25% in methanol) purchased from Sigma-Aldrich and ultrapure HNO_3 obtained from Scharlab were used. Sodium tetraethylborate 97% (Acros Organics, Thermo Fisher Scientific) and hexane (Scharlab) were used in the derivatization step. All chemicals were of analytical-reagent grade.

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

Argon C-50 was used as a make-up and sheath gas at the atomic fluorescence detector, helium C-50 was employed as a carrier gas and nitrogen C-50 was used for preconcentration. All gases were obtained from Carburos Metálicos.

Reference materials corresponding to aquatic plant, lichen and rice matrices with certified values for Total-Hg concentration were used for validation of the developed methodology. These reference materials were BCR-60 (*Lagarosiphon major* aquatic plant, 0.34 ± 0.04 μg/g of Hg) and BCR-482 (*Pseudevernia furfuracea* lichen, 0.48 ± 0.02 μg/g of Hg) supplied by the Institute for Reference Materials and Measurements, and NCS ZC73027 (*Oryza sativa* rice plant, 0.0048 ± 0.0008 μg/g of Hg) provided by the China National Analysis Center for Iron and Steel.

2.3. Procedures

2.3.1. Field aquatic plants pretreatment

Field aquatic plants used for optimization and validation were prepared as described elsewhere [29]. In brief, leaves from *Typha domingensis* plants were separated, cleaned in deionized water and sonicated (up to 8 cycles of 10 min) using an ultrasonic bath to remove any external source of pollution. After rinsing, samples were lyophilised by an ultrafast freezing (up to −50 °C kept during 2 h and a subsequent vacuum process at 0.2 mbar and −50 °C during 24 h). Then, plant samples were homogenized and ground with an electric mill to obtain a suitable particle size and stored in a desiccator until extraction.

Initial tests showed that real samples required a conditioning step before the addition of the extractant in order to avoid the absorption of the extracting agent by the matrix and assure the effectiveness of the extraction process. Thus, the required amount of lyophilised material was weighed and conditioned before the analysis by addition of ultrapure water (1.0 mL every 0.1 g of sample).

2.3.2. Determination of mercury species

Two different extractants (6 N HNO_3 and TMAH) were used. In both cases, 0.2 g of sample were weighed and an addition of a

minimum of 10 mL of extractant was required. The slurries were then exposed to microwave heating in a closed-vessel system. The maximum irradiation temperature was 80 °C and 100 °C for acid and alkaline extraction, respectively. These temperatures were reached 10 min after the unit was turned on and held for 10 min. The obtained suspensions were centrifuged at 3000*g* for 10 min and the supernatants were stored at 4 °C.

The derivatization was performed by ethylation with $NaBEt_4$. As a rule of thumb, 2 mL of the extracts were buffered with 5 mL of 0.1 M acetic acid / sodium acetate solution (pH 3.9). After the adjustment of pH to 3.9 with glacial acetic acid or ammonia, 2 mL of hexane and 500 µL of $NaBEt_4$ at 6% (w/v) were added. The mixture was manually shaken for 5 min and centrifuged at 600*g* for 5 min. Finally, the organic phase was recovered, transferred into a glass vial and stored at −20 °C until analysed by GC-pyro-AFS.

When necessary, the organic extract containing the ethylated Hg species were preconcentrated by evaporation to a minimum volume (25–50 µL) under a gentle stream of nitrogen at room temperature.

2.3.3. Quality control

Careful attention was paid to blank control and memory effects for Hg analysis. All glassware used was cleaned with detergent, thoroughly rinsed with tap water, soaked in a 10% HNO_3 solution for at least 3 days and finally rinsed with ultrapure water. Digestion vessels were submitted to an additional hot cleaning treatment with concentrate HNO_3 (irradiation for 10 min at 150 °C after a 10 min ramping time) in order to avoid potential risk of memory effects.

A procedural blank was prepared along with the samples in each extraction. These blanks were used not only to ensure the purity of chemical reagents but also to perform the required corrections when calculating the concentrations of Hg species in the analysed samples.

The quantification of the Hg species was performed by using the "standard-sample-standard" bracketing technique according to the response factors obtained for standards injected between samples. Each sample was triplicate analysed before and after a standard and the mean value was provided. Blanks were also analysed periodically between samples and standards.

2.4. Experimental strategy

The optimization of Hg species extraction was performed using field samples corresponding to leaves of *Typha domingensis*. Total-Hg concentration was previously analysed by AAS with an Advance Mercury Analyser (AMA). Samples spiked at 0.5 µg/g MMHg and unspiked samples were analysed for each experiment performed along the optimization.

Due to the current lack of CRMs for Hg species in plants or rice, orthogonal analytical strategies were used to cross-validate the optimized methodology. Hence, CRMs certified for Total-Hg were spiked with MMHg at different concentrations (0.5 µg/g MMHg spike for BCR-060 and BCR-482, and 0.01 µg/g MMHg spike for NCS ZC73027) to assess any potential MMHg degradation or matrix effects. Both unspiked and MMHg spiked CRMs were analysed.

Concerning the spiking procedure, the lyophilised field samples and/or CRMs were spiked before the extraction. The mixture was stirred for 2 h with a lab orbital shaker to ensure a homogeneous distribution of the spike solution.

3. Results and discussion

3.1. Optimization of the extraction procedures

The extraction of Hg species from plant tissues, as well as from other solid matrices, is the most critical step in the sample preparation procedure. This process must allow a quantitative extraction and avoid the degradation and/or interconversion of Hg species. The optimization of a method for Hg speciation in aquatic plants and rice involved a careful study of parameters affecting both extraction and derivatization procedures. For this purpose, both acid and alkaline extractants have been employed. The extraction reagent (6 N HNO_3 or TMAH), microwave heating (time program, irradiation temperature), and derivatization (volumes of extract and amount of the derivatization reagent) conditions have been evaluated. The leaves of the aquatic plant *Typha domingensis* spiked with MMHg, as described in Section 2.4, have been used for the optimization of extraction procedures.

3.1.1. Acid extraction

Previous investigations suggested that some biological or environmental matrices could consume the bulk of the derivatization reagent before the reaction with the target Hg species takes place [24]. Thus, parameters affecting the derivatization step were previously optimized. Two concentrations of ethylating reagent ($NaBEt_4$ at 3% or 6%) and volumes of extract (1 or 2 mL) were tested. These experiments were performed in the acid extraction conditions described in a previous work developed for Hg speciation in soils and sediments [25] and based on a microwave-assisted acid extraction. This method involved an extraction of 0.2 g of sample using 6 N HNO_3 (10 mL) and irradiation up to 80 °C for 5 min after a 5 min ramping time. The recoveries of spiked MMHg were better (71.6 ± 5.9%) by increasing the concentration of $NaBEt_4$ up to 6% (w/v) compared with the recovery lower than 65% (62.4 ± 3.7%) obtained using $NaBEt_4$ at 3% (w/v). The volume of extract used for ethylation had no effect on the recovery. However, the higher volume of extract (2 mL) was selected for further analysis in order to improve the sensitivity of the method. Thus, the following experiments were performed using $NaBEt_4$ at 6% as ethylating reagent and 2 mL of extract.

Irradiation temperatures from 80 to 130 °C were then evaluated in steps of 10 °C using the same extractant (10 mL of 6 N HNO_3) and microwave time program (a 5-min ramping time up to the maximum temperature followed by a 5-min irradiation step at the maximum temperature). Temperatures higher than 100 °C induced a degradation of MMHg and subsequent conversion in IHg. Low temperatures provided the best recoveries (71.6 ± 5.9% at 80 °C) but the extraction of Hg species was not quantitative yet under these conditions.

The effect of the irradiation time was also checked. Thus, experiments at irradiation temperatures from 80 to 100 °C in 10 min irradiation steps instead of 5 min were carried out. Recoveries higher than 90% (92.5 ± 2.3%) were obtained with an irradiation temperature of 80 °C (Fig. 1a).

3.1.2. Alkaline extraction

A method previously developed for Hg speciation in fish tissues based on a microwave assisted alkaline extraction using methanolic TMAH and irradiation up to 180 °C for 10 min after a 10 min ramping time was firstly evaluated [24]. However, no quantifiable peaks were found in the spiked aquatic plant. A slight burning of the sample was observed so 180 °C appears to be an extremely high temperature for this application. Subsequent tests also demonstrated that temperatures higher than 120 °C were not adequate as they seem to induce an interconversion of Hg species. Therefore, as in the case of acid extraction, different irradiation temperatures from 80 to 100 °C were finally studied and the best results were obtained at 100 °C (Fig. 1b).

The amount of the alkaline extractant was also checked using different TMAH volumes (2, 5 and 8 mL). The results indicated that 5 mL of TMAH was enough to obtain a quantitative extraction of both MMHg and IHg (Fig. 1c). However, 8 mL of TMAH was the volume selected for the following experiments in order to guarantee quantitative recoveries of Hg species when using higher amounts of sample (up to 0.4 g). Thus, lower irradiation temperature (100 °C) and higher volume of alkaline reagent (8 mL of TMAH) are necessary to extract Hg species from aquatic plants in comparison to fish tissues when alkaline extraction is used.

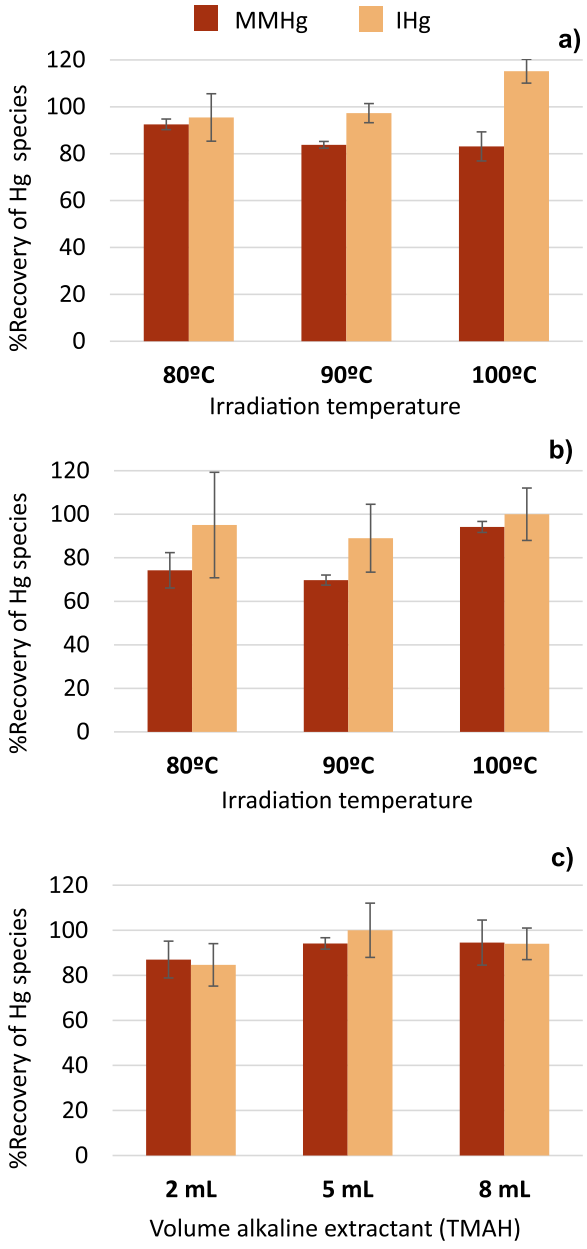

Fig. 1. Recoveries of Hg species in leaves from *Typha domingensis* spiked with MMHg: a) Acid extraction with 10 mL of 6 N HNO_3 at different irradiation temperatures; b) Alkaline extraction with 5 mL of TMAH at different irradiation temperatures; c) Alkaline extraction at an irradiation temperature of 100 °C using different volumes of TMAH.

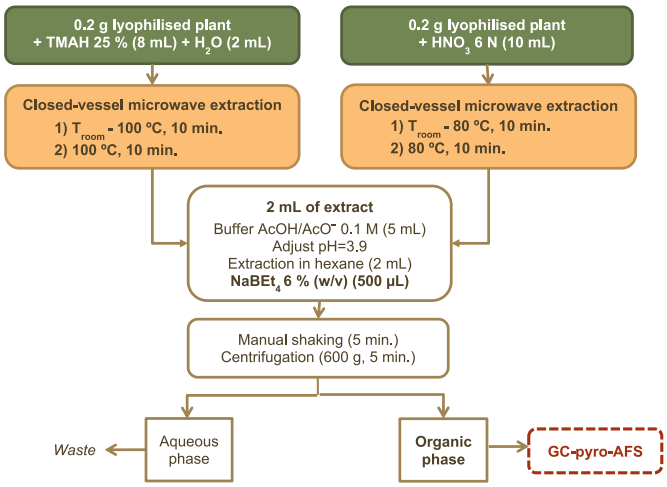

Fig. 2. Schematic flow diagram of the proposed methods for Hg species determination in vegetal matrices.

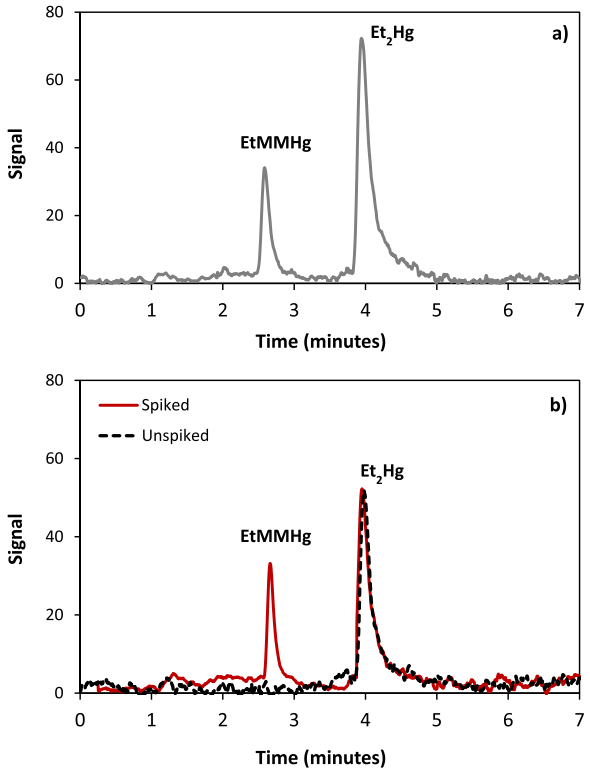

Fig. 3. Chromatograms of (a) a standard (MMHg at 10 µg/L and IHg at 25 µg/L), and (b) unspiked and spiked (MMHg at 0.5 µg/g) real aquatic plant (leaves of *Typha domingensis*).

The optimum conditions for both alkaline and acid extraction are summarized in Fig. 2. These conditions enable the simultaneous extraction and determination of both Hg species of concern. Furthermore, the chromatograms represented in Fig. 3 (corresponding to the analysis of a standard and spiked and unspiked real aquatic plant samples) show that, in all cases, it is possible to separate and detect both MMHg and IHg in less than 5 min.

3.2. Method validation

3.2.1. Limits of detection and quantification

The procedural limits of detection (LOD) and quantification (LOQ) were estimated in accordance with the base line noise. The LOD and LOQ correspond to the analyte concentrations that caused a height 3-fold or 10-fold the base line noise level, respectively. They were in all cases in the ng/g range (25 ng/g for MMHg and 22 ng/g for IHg) but these limits were not low enough for the application to unpolluted aquatic plant samples. Therefore, additional preconcentration step was required for samples with low MMHg concentrations. Based on our previous experience, preconcentration was performed by evaporation under a nitrogen stream without heating of the organic extract obtained after the derivatization [25]. The evaporation was conducted up to a minimum volume (25–50 µL) but not to dryness. Thus, a preconcentration factor up to 30-fold could be reached. Spurious IHg was found in some preconcentrated blanks of TMAH hindering the application of this preconcentration step for this Hg species, since this observation revealed that alkaline extraction is not good in terms of detection limits. No problem was found for acid extraction and detection limits in the low ng/g range were consequently achieved for both Hg species (0.7–1.0 ng/g) after the preconcentration step.

Table 2
Comparative analytical performance of proposed acid and alkaline extraction methods for BCR-60 spiked at 0.5 μg/g MMHg. Experimental values for *F*-Snedecor or *t*-Student (F_{exp} or t_{exp}) were in all cases lower than the theoretical values (F_{th} or t_{th}) indicating that no statistically significant differences were found in terms of precision (*F*-test) or accuracy (*t*-test). The statistical tests were conducted using the IBM SPSS Statistics software (version 22.0) from IBM Corporation.

	MMHg		IHg	
	Acid	**Alkaline**	**Acid**	**Alkaline**
Repeatability (%RSD, n = 8)	4.7	4.2	5.5	7.5
Inter-days precision (n = 8, *F*-test, F_{th}: 4.995, *p* = 0.05)	F_{exp}: 1.614	F_{exp}: 2.775	F_{exp}: 1.757	F_{exp}: 2.290
Inter-methods precision (n = 14, *F*-test, F_{th}: 3.153, *p* = 0.05)	F_{exp}: 2.024		F_{exp}: 1.012	
Inter-methods accuracy (n = 14, *t*-test, t_{th}: 2.06, *p* = 0.05)	t_{exp}: 1.56		t_{exp}: 1.82	

3.2.2. Precision

A thorough evaluation of both acid and alkaline extraction methods in terms of analytical performance was carried out using the BCR-60 reference material spiked with MMHg (0.5 μg/g). The main results are summarized in Table 2.

The repeatability of each method was evaluated by the analysis of 8 independent samples in the same day. The relative standard deviations (% RSD) for both Hg species were slightly lower for the acid extraction than for the alkaline extraction (Table 2).

The inter-days precision was assessed by analysing the same sample in two consecutive days (n = 8). The variances of the concentrations obtained by both methods were found to be not statistically different according to Snedecor´s *F*-test (p = 0.05).

Additionally, both extraction methods were compared in terms of precision (Snedecor´s *F*-test) using the results obtained in two consecutive days. No statistically significant differences were found between the variances of the concentrations found in both cases at a 95% confidence level (n = 14).

3.2.3. Accuracy

Firstly, the accuracy of both methods were compared by using the spiked BCR-60 (Table 2). No statistically significant differences (Student *t*-test, p = 0.05) were found in Hg species concentrations (spiked MMHg and native IHg, n = 14), which confirmed that both acid and alkaline extraction methods can be considered equally accurate at the chosen confidence level.

The study of accuracy was then extended to the other available CRMs (BCR-482 and NCS ZC73027) apart from BCR-60. All of them are only certified for Total-Hg and orthogonal validation procedures were used to confirm the effectiveness of the developed methods for MMHg extraction without interconversion between Hg species. It should be pointed out that the analysis of NCS ZC73027 required an additional pre-concentration step (see experimental Section 2.3.2)

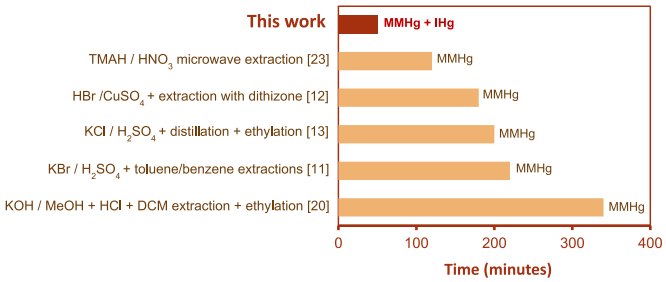

Fig. 4. Comparison of the total analysis time for Hg species determination in plants and/or rice using different procedures reported in the literature.

since this material presents a low concentration of Total-Hg. No traces of MMHg were found in the direct analysis and the CRMs were spiked with MMHg (0.5 μg/g MMHg spike for BCR-060 and BCR-482, and 0.01 μg/g MMHg spike for NCS ZC73027). Recoveries close to 100% were obtained for the spiked MMHg (Table 3).

The analysis of CRMs without MMHg spike was also performed. No statistically significant differences (Student *t*-test, p = 0.05) were found in Total-Hg concentrations between those calculated as a sum of Hg species and the certified values when applying either acid or alkaline extraction (without preconcentration) procedures for BCR-60 and BCR-482 and acid extraction (with preconcentration) for NCS ZC73027 (Table 3). Thus, it was demonstrated that both acid and alkaline extraction methods could be applied with accurate results for the determination of Hg species in polluted samples and acid extraction is the best option for low Hg-polluted samples.

Finally, it is also remarkable that the total time of analysis has been significantly reduced in comparison to the currently proposed methods in literature mainly because no tedious clean-up steps are necessary (Fig. 4). Moreover, most of these methods did not allow the simultaneous determination of I Hg. In those cases, it is necessary to perform an independent analysis of Total-Hg and, thus, IHg would be calculated by difference. In the proposed method, information about both Hg species could be simultaneously obtained in a single analysis.

4. Conclusions

In this work, we have developed two fast and adequate sensitive methodologies based on microwave assisted extraction using both acid and alkaline extractants for the simultaneous determination of Hg species in aquatic plants and rice. These methods considerably reduce the total time of analysis in comparison with the methods traditionally used up to the moment. Both optimized extraction methods could be applied with satisfactory results for Hg polluted samples and are comparable in terms of precision and accuracy. However, the analytical performance of the alkaline extraction presented some limitations especially in terms of blanks. Therefore, for low Hg polluted samples the method based on acid extraction including an additional preconcentration step would be the best option.

Table 3
Total-Hg concentrations (calculated by sum of Hg species in the direct analysis) and recoveries of spiked MMHg obtained in certified reference materials from vegetal matrices analysed directly and spiked at different levels of MMHg (0.50 μg/g for BCR-60 and BCR-482 and 0.010 μg/g for NCS ZC73027). The uncertainty of the results corresponds to standard deviation of 5 independent replicates.

Certified reference material	**Certified value (Total-Hg in μg/g)**	**Found value (Total Hg in μg/g)**		**Recovery of spiked MMHg (%)**	
		Acid extraction	**Alkaline extraction**	**Acid extraction**	**Alkaline extraction**
BCR-60 (aquatic plant)	**0.340 ± 0.040**	**0.333 ± 0.018**	**0.344 ± 0.049**	**101 ± 6**	**98 ± 4**
BCR-482 (lichen)	**0.480 ± 0.020**	**0.484 ± 0.013**	**0.458 ± 0.050**	**98 ± 2**	**100 ± 15**
NCS ZC73027 (rice)*	**0.0048 ± 0.0008**	**0.0047 ± 0.0003**	–	**99 ± 5**	**81 ± 6**

* Using preconcentration.

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