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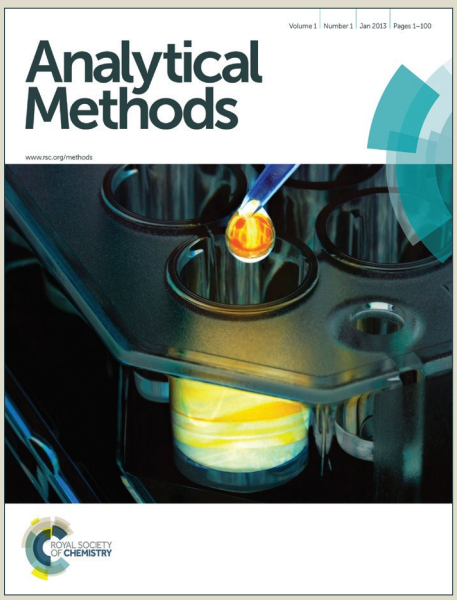

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Robust ultrasound assisted extraction approach using dilute TMAH solutions for the speciation of mercury in fish and plant materials by cold vapour atomic absorption spectrometry (CVAAS)

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Abstract

A simple and rapid ultrasound assisted extraction (UAE) protocol with dilute solutions of tetramethyl ammonium hydroxide (TMAH) for the speciation of mercury in fish and plant tissues was developed as an alternative to conventional methods which require intensive treatments. The main operational parameters such as extractant concentration (TMAH), sonication time and amount of sample were optimized using BCR ERM-CE 464 (tuna fish) and mercury loaded coriander powder, an in-house reference material, taken as representatives of fish and plant tissues respectively. Quantitative extraction of the inorganic mercury (iHg) and methylmercury (MeHg) species was obtained using 8 mL of 2% TMAH with a sonication time of 5 min for <0.5 g sample weight. After sonication, the supernatant obtained upon centrifugation was used directly for the determination of iHg by cold vapour atomic absorption spectrometry (CVAAS). Inorganic mercury was determined using $SnCl_2$ as reducing agent while total mercury was determined after oxidation of methyl mercury (MeHg) with $KMnO_4$ solution. Organic mercury, basically MeHg was obtained by difference. The analytical results were in good agreement with the certified reference values of iHg, MeHg and total mercury at a 95% confidence level. The method was further validated through the analysis of additional certified reference materials: BCR CE-463 (tuna fish), IAEA-350 (fish homogenate), BCR-60 (aquatic plant Lagarosiphon Major), BCR-482 (lichen). The detection limit of the overall procedure was found to be 0.014 µg g^{-1} for both inorganic and methyl mercury species.

Keywords: Ultrasound-assisted extraction, mercury speciation, TMAH, tuna fish, plants, CVAAS.

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Introduction

Mercury is a global pollutant and highly toxic among heavy metals because of its persistence, long range transport potential and bioaccumulation in the environment. Mercury is introduced in to the environment mainly as elemental mercury (Hg^0), inorganic mercury (iHg) and organic mercury species as a result of both natural and anthropogenic activities from where it re-enters the human food chain.[1-7] More than 2500 tons of mercury is emitted annually from global anthropogenic sources which are significantly contributing to elevated levels of mercury. It has been known that organomercury compounds, particularly methyl mercury (MeHg), are 50-100 times more toxic than inorganic mercury species.[8] These two are the common and predominant forms of mercury generally found in biological and environmental samples such as fish tissues and plant matrices.[9-13] Because of the accumulative properties and adverse toxic effects of mercury species even at ultra-trace levels, its accurate determination in fish and plant samples is very important for environmental protection and food safety.

As a consequence, considerable efforts and progress have been made in the development of sensitive and accurate sample preparation methods for the determination of total mercury and its speciation analysis in environmental and biological samples.[14-26] The most frequently used approaches for the extraction of mercury species from fish and plant samples are based on microwave [27-30] or ultrasound [31,32] assisted alkaline or acid leaching and solid phase extraction.[33-34] Despite excellent sensitivity and selectivity, most of the above mentioned approaches suffer from major limitations that include laboriousness of the procedures, use of high amount of acids along with complexing agents, lack of acceptable efficiency and time consuming.

TMAH and formic acid reagents have been extensively used as the most appropriate tissue solubilizers for various biological samples prior to analysis of various elements including mercury and its speciation.[35-40] Among these two solubilizers, the alkaline solubilization with TMAH offers a simple and rapid approach to the preparation of a homogenized sample solution which is a distinct advantage over conventional slurry preparation methods. Hence several methods for the determination of iHg and MeHg species using TMAH have been developed and reported in the literature.[35-38, 41-43] However, sample solutions

produced after solubilisation with TMAH are cloudy and also emit an unpleasant odour that requires adequate ventilation. Use of dilute TMAH solutions can minimize the odour, but quantitative extraction of species of interest may be affected.

Nowadays, there have been significant developments in green analytical methodologies aimed to reduce the amount of toxic chemical reagents as well as simplify and accelerate experimental procedures.[44-46] In this context, ultrasound assisted extraction (UAE) approach can be an excellent alternative to minimize the above mentioned limitations of conventional extraction procedures.[47-48] Being a clean technology, ultrasound energy has already been well exploited for a number of analytical applications such as speeding up solid-liquid extraction of elements/species of interest for the determination of total-element contents and speciation analysis, remediation, organic synthesis and a number of other analytical and industrial applications.[49-53] Based on these facts, ultra-sound assisted extraction protocol was utilized in the present work for the speciation of mercury in fish and plant materials using dilute TMAH solutions.

The most commonly and widely used techniques employed for determination of mercury species in a great variety of matrices including fish and plant tissues with and without applying chromatographic separation, are cold vapour atomic absorption spectrometry (CVAAS)[54] and atomic fluorescence spectrometry (CVAFS).[55] In the present study, CV-AAS was selected for Hg determination because of its high sensitivity, absence of spectral interferences, relatively low operational costs and simplicity as well as rapidity.

The main objective of the work has been to develop a simple, efficient and green analytical methodology for the determination of t-Hg, iHg and indirectly MeHg without the use of a chromatographic separation after treatment with dilute TMAH solutions with the aid of ultrasound probe energy which is suitable for both fish and plant tissues. Mercury loaded coriander powder (representative of samples of plant origin) and BCR CRM 464 (Tuna fish) (representative of fish) were used for optimization experiments. After extraction using optimized conditions, the concentration of iHg and tHg were determined using CVAAS after employing $KMnO_4$ treatment for the oxidation of organic mercury species to inorganic mercury. A closed microwave digestion procedure based on the use of

dilute nitric acid solutions and H_2O_2 was utilized for the dissolution of the test samples for subsequent determination of total mercury by CVAAS.

Experimental

Instrumentation

High intensity probe sonicator for ultra-sound assisted extraction

Extractions were performed using a 750 W power and 20 kHz frequency high intensity probe sonicator equipped with a 6 mm Ti probe (Sonic Vibra Cell, Sonics and Materials Inc., CT, USA, Model: VCX 750). According to manufacturer's recommendation, the amplitude of the ultrasonic processor for the ultrasonic vibrations at the probe was set at maximum allowable limit of 40%. Pre-cleaned polypropylene centrifuge tubes of 50 ml capacity (Tarson) were used as vessels for sonication experiments. After sonication, all the extracts were centrifuged at 8000 rpm (REMI Instruments Pvt. Ltd, Mumbai, India) for about 5 min for the rapid separation of the solid-liquid mixture.

Microwave Digestion system for total decomposition of samples

A microwave digestion system (CEM Mars 5, Matthews, NC, USA) was used for mineralization of the test samples for the determination of total mercury. The sample carousel was capable of holding 10 PTFE digestion vessels (XP-1500 Plus) with a capacity of 100 mL each which also includes a control vessel fitted with a fiber optic temperature sensor and a pressure transducer for controlling the microwave program and capable of withstanding pressure of 500 psi and temperatures up to 260 ^oC.

Determination of mercury and its species

Mercury was determined by cold vapour atomic absorption spectrometry (CV-AAS) using a mercury analyzer (Model MA 5840E, Electronics Corporation of India Ltd., Hyderabad, India). The information of organic and inorganic forms of mercury could also be obtained with the same instrumentation through changing reducing agents with different reducing powers. $SnCl_2$ is known to reduce only Hg^{2+} to Hg^0, whereas $NaBH_4$ is capable of reducing both iHg and MeHg to elemental mercury, albeit with different sensitivities.

Reagents and materials

All chemicals used in this work were at least of AR grade. High-purity water with a resistivity of >18 MΩ cm used for preparation of standards, samples

and for cleaning of vessels, was produced using a Milli-Q high purity water system, located in class 100 area of the Ultra-trace analysis laboratory of this Centre. Dilute solutions of TMAH, prepared from stock solution (25% in methanol, Aldrich, USA), was used as extractant. Tin (II) chloride ($SnCl_2$) (5%, w/v) used as reducing agent was prepared by dissolving the appropriate amount of $SnCl_2.2H_2O$ (Merck, India) in HCl and diluting with water. Sodiumborohydride ($NaBH_4$) (Merck, Darmstadt, Germany) (1%, w/v) was prepared fresh daily by dissolving the appropriate amount of solid in 0.3 % (w/v) NaOH solution. A carrier solution of 10% HCl was used along with $SnCl_2$ or $NaBH_4$ for reduction of mercury. Inorganic mercury standard solution (1000 mg L^{-1}) in 5% HNO_3 (SD Fine-Chem Ltd, Mumbai, India) traceable to NIST 3133 was used as a stock standard. A methyl mercury (CH_3Hg^+) stock standard solution (100 mg/L, Hg as MeHg) was prepared from methyl mercury iodide (Aldrich) by dissolving the appropriate amount of the solid in methanol and making up to required volume with high purity water. All the stock standard solutions were stored in a refrigerator at 4^0C and protected from light. Working standard solutions were prepared just before use by appropriate dilution of the stock standard solutions.

The following certified reference materials (CRMs) were analysed to evaluate the developed method; Lichen-482 from Community Bureau of Reference (BCR), BCR-60 (Lagarosiphon Major, aquatic plant), European reference Materials (ERM) CE-463 and 464 (Tuna Fish) and fish homogenate IAEA-350. All the solid reference materials were used as received, without further grinding and sieving.

Preparation of mixture of iHg and MeHg loaded coriander material (laboratory reference material)

In most of the certified reference materials (CRMs) either inorganic or methyl mercury is found to be at much higher concentrations relative to the other species. Particularly CRMs of plant origin containing high levels of Hg are scarce. To our knowledge no reference material is available, which is certified for higher contents (ppm) of both i-Hg and MeHg for the validation of methods for plant and fish samples. Another issue is large quantity of reference material required for optimization experiments. In view of this, coriander sample loaded with known content of mercury (iHg/MeHg separately) and a mixture of iHg and MeHg at high ppm level was prepared in the laboratory for use in the optimization experiments

related to samples of plant origin. In the present work we have chosen coriander material ***(Coriandrum sativum)*** (common edible plant in every house hold), for the preparation of in-house reference material because of its availability, ease of preparation, high uptake capacity for mercury species and cost effectiveness. In our earlier studies, the sorption capacities for iHg and MeHg were determined to be ~24 mg g^{-1} and ~7 mg g^{-1} respectively.[56]

A large quantity of coriander plants was obtained from the local market, washed thoroughly with water to remove all the adhering soil particles. The whole plant (roots, stem and leaves) was cut into small pieces and dried at 50°C in a conventional heating oven, ground in a planetary ball mill (Fritz, Germany) and sieved to get a particle size of ≤100 µm. After this step, about 10 g of the powdered coriander was placed in a glass beaker containing 200 mL of high purity water spiked with 100 µg of iHg and MeHg individually (designated as Cori-iHg and Cori-MeHg respectively) such that the amount of mercury species in coriander compounded to about 10 µg g^{-1} Hg in the solution.

The mixture was stirred continuously for about 1 hr for quantitative sorption and also to facilitate uniform loading of spiked mercury. After shaking, the mixture was separated by centrifugation (8000 rpm for 5 min) and the supernatant was drained. Then the sorbent was initially allowed to dry at room temperature and then dried in a conventional heating oven at ~40^0C to remove the residual moisture. Then the dried sample was finely ground and sieved to get 200-400 mesh size particles. In another set of experiments both iHg and MeHg (10 µg each) together were loaded on coriander powder (weight of coriander 10 g) using the similar procedure as described above such that total amount of mercury in coriander was about 20 µg g^{-1} (Cori-iHg-MeHg). In all the cases, the supernatant was analysed for the determination of residual mercury by CVAAS and results indicated the absence of mercury.

Microwave-assisted digestion procedure using diluted acids for the determination of total mercury

It is still usual to digest the samples by adding large amounts of concentrated mineral acids which leads to the generation of large volumes of toxic wastes. At present, considering the excessive use of concentrated acids, environment-friendly strategies are being implemented without impairing

analytical performance aiming toward greener sample preparation methods.[57,58] In this context, dilute solutions of HNO_3 in the presence of auxiliary reagent H_2O_2 have been successfully developed and used in the complete digestion of bio-environmental samples for the determination of total mercury. The efficacy of the proposed extraction procedure was evaluated after decomposition of the test materials with closed microwave-assisted acid digestion procedure as described below.

For total Hg determination, an accurately weighed aliquot (~200 mg) of the target materials was placed in the PTFE microwave digestion vessel to which 1 mL of concentrated HNO_3 and 1 mL of H_2O_2 followed by 2 mL of high purity water was added. After closing, the vessels were clamped within a support module and placed inside the microwave digestion system. The following microwave program was used which comprises (i) the temperature was ramped to 100 ± 2^0C in 5 min (pre-digestion step) (ii) the temperature was ramped to 200 ± 5^0C in 10 min and held there for 10 min and (iii) 0 W for 20 min (cooling step). After cooling, the resultant clear sample digests were quantitatively transferred from the PTFE vessel to another pre-cleaned tube and diluted to desirable volume with water depending on the concentration level of mercury. After suitable dilution, all the sample solutions were analysed by CVAAS after VG of mercury by using $SnCl_2$ and/or $NaBH_4$ for the determination of total mercury present in each CRM. Corresponding process blank solutions were also subjected to the same procedure in the absence of sample.

Ultrasound-assisted extraction procedure

For the extraction of iHg and MeHg species by ultrasound energy, an accurately weighed aliquot (~200 mg) of the selected CRMs were placed in the polypropylene (PP) centrifuge tubes (50 mL volume) and 8 mL of desired extractant (2% v/v TMAH) solution was added. Then the sample-extractant mixture was sonicated for a chosen sonication time and amplitude settings. After sonication, the supernatant was separated from the solid phase by centrifugation for about 5 minutes at 8000 rpm. The known volume of the supernatant was then transferred to another pre-cleaned PP tube. The resultant solutions after suitable dilution were analysed for iHg and tHg by CVAAS as described below.

A known amount of iHg standard/sample solution was added into a reaction vessel (of CVAAS system) containing ~5 mL of 10% HCl carrier solution.

The reaction mixture was stirred well for a desired length of time (1-3 min) in a closed environment before passing the Hg^0 vapors to quartz cell of the AAS system for quantification. One part of the split samples was analysed for the determination of iHg by using $SnCl_2$ as selective reducing agent. To determine total Hg, it was necessary to add an appropriate amount of $KMnO_4$ solution to other part of the split sample for oxidation treatment in the presence of 5 % HNO_3 to convert MeHg to iHg which was followed by its determination by CVAAS using $SnCl_2$/or $NaBH_4$ as the reducing agents. Concentration of methylmercury was calculated as the difference between the total and iHg values.

Corresponding process blanks (with and without oxidative treatment) were also prepared in the same way without taking any sample material. Three different aliquots of each sample were used for the extraction process. All the analytical measurements were run in triplicate for each sample solutions. With each series of extractions, blank was also prepared and measured in parallel to determine cross-contamination of mercury. Quantifications of the mercury species in test samples are based on a 5 point calibration graph obtained with the standards of mercury in the concentration range of 0 (analytical blank)-100 ng/ml prepared using process blank solutions containing 2% TMAH and TMAH-extracted blank coriander sample solutions. These calibration plots were compared with those pure aqueous standards of mercury to test the matrix effects if any. Standard addition method was also applied, in order to look for other possible interferences, if any.

To test the volatility of mercury species, under ultrasound-assisted extraction conditions, a set of experiments was carried out in which standards containing known amounts of mercury species (iHg and MeHg) prepared in 8 mL of optimized extractant solution (2% TMAH) was subjected to the proposed ultrasound extraction procedure as in the case of samples. The resultant solutions (after suitable dilution) were analysed for the determination of species of mercury by CVAAS. Calibration plots were also obtained with these processed standard solutions and compared with the plots obtained for pure aqueous mercury standards.

After applying ultrasound-assisted extraction process, the extraction efficiency at each step was tested by calculating the percentage recovery of test mercury species in the samples using the following equation

$$\% \text{ Recovery} = \frac{Measured\ Concentration\ (mg\ kg^{-1})}{Certified\ Value\ (mg\ kg^{-1})}\ x\ 100$$

Results and Discussion

The main concerns in quantitative extraction of mercury species from solid matrices (in this case fish and plant tissues) should be the efficiency, volatility, inter-species conversion, contamination and amount of reagents. Extraction methods based on the use of ultrasound energy usually do not require intensive conditions such as high temperatures, pressures or concentrated acids. Based on this fact, the present work was initiated using dilute solutions of TMAH with the aid of ultrasound energy for the speciation of mercury. As mentioned in ealier sections, inorganic and methyl mercury (MeHg) species are the two common and predominant forms generally found in various biological and environmental samples. Hence, the present study was focussed on the determination of only inorganic and methyl mercury species.

Initially, a series of experiments were carried out to optimize these variables for quantitative recovery of both iHg and MeHg. Mercury loaded coriander powder (representative of samples of plant origin) and BCR CRM 464 (Tuna fish) (representative of fish tissue) were used for optimization experiments. In case of fish representative sample, the concentration of iHg was very low (represents only 2.3% of the total Hg concentration) hence the level of iHg was raised using standard addition to evaluate the stability of the both iHg and MeHg species during the USE process. Accordingly, ~0.2 g of ERM-CE464 was spiked with 100 µL of iHg standard (from 10 µL/mL stock standard), to which extraction solvent TMAH was added. After each extraction step, percentage recovery of both iHg and MeHg were determined during the method development.

Total mercury determination

Different volumes of HNO_3 and H_2O_2, different irradiation times and microwave power settings of CEM microwave system were tested to ensure total recovery of Hg. In each case ~200 mg of solid sample was taken and digested using the microwave program as described in the earlier section. The addition of a mixture of 1.5 mL HNO_3, 1 mL of H_2O_2 and 2.5 mL of water greatly improved the efficiency of digestion, providing a clear solution and quantitative recovery of mercury from the CRMs, selected in this work. The reduction of mercury was

carried out using $NaBH_4$ with a concentration of 2% w/v for subsequent determination of total mercury by CVAAS. Results obtained with the digestion performed with the proposed procedure were found to be in good agreement with certified values (recoveries higher than 98%). The use of diluted HNO_3 in the presence of H_2O_2 was proven to be a feasible and recommendable sample digestion procedure complying with the green chemistry recommendations.

Speciation analysis of mercury

Ultrasound assisted extraction of mercury species may not be equally effective for all solid samples, so maximizing the extraction yield requires the process variables to be optimized for each specific matrix (in this case plant and fish matrices). The extraction efficiency of ultrasound energy is essentially governed by various parameters that included extractant concentration (TMAH), sonication time and amount of sample. Hence these variables were optimized individually to achieve quantitative recovery of both the mercury species while the others were kept constant.

Optimization of concentration of TMAH

As mentioned in earlier sections, the TMAH is strongly alkaline, soluble in aqueous media, stabilizes volatile elements and does not require heating or only requires gentle heating and is thus promising for speciation analysis of mercury. In the present work, dilute solutions of TMAH were used to test its efficacy as an extractant to achieve quantitative extraction of the mercury species from plant and fish tissues with the aid of ultrasound energy. Based on the results obtained from various preliminary experiments, different concentrations of TMAH in the range of 0.5-3% were chosen for two representative materials keeping other parameters (sonication time-5 min, volume of extractant-8 mL and amount of sample-~200 mg) constant. An extractant volume of 8 mL was chosen in all the optimization experiments so that the required number of replicates could be performed without exhaustion of the sample solution.

As a compromise between sensitivity and reagent consumption, 5% w/v $SnCl_2$ in 10% v/v HCl solution was chosen as the reducing agent for the determination of iHg while 2% w/v $NaBH_4$ and 5% v/v HNO_3 was chosen as optimum conditions for tHg determination in final TMAH-sample extracts after oxidation treatment with $KMnO_4$.

Fig 1a&1b shows the effect of concentration of TMAH on the extraction efficiency of iHg and MeHg species from BCR CRM 464 and mercury loaded coriander representative materials. As shown in these figures, extraction efficiency (i.e., recovery of Hg species from solid matrix) with water (in the absence of TMAH) was very low (<10%) while the efficiency of TMAH for the extraction of both iHg and MeHg increased with concentration of TMAH up to 2%, reached plateau in the concentration range of 2 to 5%, the highest studied concentration. As seen from Figs 1a&1b, the optimum concentration of TMAH was found to be about 1.5% for quantitative extraction (>95%) of the two selected mercury species from BCR-464 while 2% of TMAH was required for mercury loaded coriander material (which is of plant origin). In general, fish tissues are soft compared to plants and hence fish tissue requires lower concentration of TMAH for the complete extraction of species of interest.

Both iHg and MeHg species show similar extraction behaviour with quantitative recoveries between 95-102% when dilute solutions of TMAH were used as extractant. After sonication, the colour of the final extractant solution resembled the original colour of the powdered sample. The effect of ultrasound energy on the stability of Hg species was also studied using the two representative materials by analyzing TMAH-extracted solutions at different time intervals. These studies clearly indicate that, after carrying out UAE with 2% TMAH, the two tested mercury species remained stable even after standing for a week in the laboratory at room temperature. A TMAH concentration of 2% v/v was adopted for further extraction experiments to make it suitable to both fish and plant tissues.

Optimization of sonication time

The sonication time of the sample is an important parameter because the dose of ultrasound sonication received by the matrix and extractant mixture determines the extent of cavitation phenomena followed by the efficiency of extraction. Sonication time of 5 min or less is usually reported when ultrasonic probes are used for solid liquid extraction. Fixing ultrasound amplitude (40%), extractant concentration (2% v/v TMAH), extractant volume (8 ml) and sample weight (~200 mg), the influence of sonication time on the extraction of Hg species was investigated in the range of 1 min to 6 min. In both the fish and coriander representative samples, extraction efficiency of the two Hg species

goes up from 45% to ~98% as the sonication time increased from 1 to 4 min and stays almost constant in time interval 5-7min. The results obtained from these studies indicated that sonication time of 4 min was found to be sufficient for the quantitative extraction of mercury species from both the representative materials which is advantageous to obtain a high sample throughput. A sonication time of 5 min was thus selected as optimum for further optimization studies since the species recovery was highly reproducible.

Evaluation of $KMnO_4$ concentration and reaction time for MeHg oxidation

Firstly, the concentrations of $KMnO_4$ and HNO_3 were optimized for the quantitative conversion of MeHg to Hg^{2+} followed by CVAAS determination. This oxidation treatment was performed before adding a reducing agent for VG of mercury. As mentioned above, iHg was determined using $SnCl_2$ as the selective reducing agent whereas tHg was determined after oxidation of organic mercury to iHg through reaction with $KMnO_4$ followed by reduction to elemental mercury. A variety of oxidizing agents viz., H_2O_2, $KMnO_4$, $K_2Cr_2O_7$ and $K_2S_2O_8$ in combination with strong acids (such as HCl and HNO_3), UV and microwave irradiation have been extensively used for the oxidation of organic mercury to iHg followed by the determination of tHg. In the present work, $KMnO_4$ was selected to decompose organomercury species (predominantly MeHg in this case) due to its ease of preparation, stability and low mercury blank. $KMnO_4$ also promotes efficient stabilization of mercury in solution until analysis.[41]

Since the extraction of mercury species was carried out using a 2% TMAH solution, it is necessary to add HNO_3 along with $KMnO_4$ so as to acidify the sample digest for the rapid oxidation of the organomercury species. Methylmercury loaded coriander sample (Cori-MeHg) and tuna fish (BCR-CE 464) were taken as representatives for optimizing the concentration of HNO_3 and $KMnO_4$ required for quantitative conversion of CH_3Hg^+ to Hg^{2+}. After taking through the general speciation procedure, a sample volume of 0.5 mL was taken for optimization studies. In order to optimize the composition of HNO_3 and $KMnO_4$, a factorial (two factors, three levels) experimental design approach was applied and the conversion efficiency of MeHg at each level of treatment was estimated. Based on the results obtained from various preliminary experiments, a mixture of 4.5 mL of 0.02% w/v $KMnO_4$ and 5% v/v HNO_3 (added to reaction vessel of CVAAS containing 0.5 mL TMAH-extracted sample) was selected as

base level for the two representative materials (the upper and lower levels were obtained using a difference of ±0.01% for $KMnO_4$ and ±2.5% for HNO_3). The mixture was stirred for about 1 min and then reducing agent added for the determination of mercury by CVAAS. At each optimization step, corresponding solutions were employed as blanks.

From Fig 2, it can be seen that the conversion efficiency of MeHg varied significantly with different concentrations of $KMnO_4$ and HNO_3 added to the TMAH-extracted coriander sample solution. From these studies, it was observed that the best efficiency of conversion was obtained with a mixture of 4.5 mL of 0.02% w/v $KMnO_4$ and 5% v/v HNO_3 for 0.5 mL of sample solution. This is believed to be a result of the efficient conversion of MeHg to Hg^{2+} in the standard and samples as well as due to stabilization of mercury in the standard/sample solution in its oxidized form. Similar results were obtained for fish representative sample and hence data not shown here.

In the case of MeHg standard, the addition of a mixture of 4.5 mL of 0.01% w/v $KMnO_4$ and 5% v/v HNO_3 allowed quantitative conversion to iHg whereas the conversion efficiency was only 70-80% for TMAH-extracted sample solutions. Hence, it was felt that more oxidizing agent is required for test samples in comparison with the MeHg standard solution, because of the presence of other sample components which competed with the MeHg species during the oxidation process. This may be mainly due to the consumption of a major part of $KMnO_4$ by the sample matrix thereby reducing the availability of oxidizing agent for oxidative conversion of CH_3Hg^+ to Hg^{2+}. Based on these results, a mixture of 4.5 mL of 0.02% w/v $KMnO_4$ and 5% v/v HNO_3 was added to the reaction vessel (of CVAAS) containing 0.5 mL of sample solution prior to reduction to elemental mercury. However, for treating higher volume of TMAH-extracted sample solutions (>0.5mL) (depending on the concentration of MeHg), an increased amount of $KMnO_4$ solution is required to be added for quantitative conversion.

After optimization of the concentration of oxidizing agent $KMnO_4$, it was necessary to optimize the reaction time (stirring time) required for complete oxidation of the CH_3Hg^+ to Hg^{2+} in the tested samples. Based on a series of experiments, a reaction time of one minute was chosen as optimum, since recovery of mercury was quantitative and mercury signal was highly reproducible. No significant improvement in sensitivity could be obtained with

longer reaction periods (1- 3 min). Hence, a reaction time (i.e., stirring time) of 1 min was used in all subsequent experiments.

Tao et al[37] had to use reagents such as L-cysteine and $KMnO_4$ for the determination of iHg and tHg respectively. They added L-cysteine to sample solutions to liberate iHg from protein-bound mercury or other molecules in the TMAH-extracted solutions. In this work, however, addition of L-cysteine did not enhance the iHg indicating that reducing agent ($SnCl_2$ or $NaBH_4$) alone was found to be sufficient (without need of L-cysteine) for the quantitative recovery of iHg in the sample solutions after UAE using dilute TMAH (~2%) solutions.

Figures of merit

The whole analytical procedure proposed for the speciation of mercury in plant and fish tissues is presented schematically in Fig 3. Calibration curves were obtained across the concentration range 0 (analytical blank) to 100 ng/mL for iHg and MeHg species prepared in different solvent media (aqueous, 2% TMAH and TMAH-extracted solutions of blank coriander powder). Analytical response characteristics of iHg and MeHg species spiked in different solvent media are presented in Table 1. In all the cases, the correlation coefficients were >0.995. The slopes of the calibration curves corresponding to Milli-Q water, 2% TMAH solutions and TMAH-extracted sample solutions spiked with iHg and MeHg did not differ significantly, showing no matrix effect in TMAH medium demonstrating the efficacy of the developed UAE procedure using dilute solutions of TMAH. This allows the use of aqueous standard calibration curve for quantification purposes. As both the external and standard addition approaches provided comparable results, all mercury measurements were subsequently carried out using only external calibration method.

Analytical results of the mercury loaded coriander sample and various CRMs together with the certified/reference values are presented in Table 2 and 3 respectively. The determined values for total iHg obtained by both UAE and MAD digestion methods agree with the certified values (at 95% confidence level). The organic mercury concentration, calculated as the difference between the total and iHg values also agrees with the certified MeHg concentration. This demonstrates that most of the organic mercury obtained by arithmetical difference is mainly MeHg. The detection limit of the method determined as the concentration corresponding to three times the standard deviation of the blank

was 0.014 µg g^{-1} based on 0.4 g of sample and 8 mL of extractant solution. The precision, evaluated as the relative standard deviation (RSD%), was better than 10% in most of the cases.

The proposed analytical procedure reduces markedly the concentration of TMAH required for extraction by more than 10 times compared to reported solubilisation methods and also time needed for sample preparation (total 10 min including centrifugation time). In addition, keeping the number of analytical steps to a minimum, considerably reduces the sources of analytical errors.

Conclusions

An effective analytical method based on the use of dilute TMAH (2%) solution as extractant with the aid of ultrasound energy for the speciation analysis of mercury by CVAAS in plant and fish tissues was developed. The developed extraction procedure and Hg-species determination was validated by the analysis of various certified reference materials. After ultrasound-assisted extraction, TMAH-extracted sample solutions were directly analysed for iHg by CVAAS while tHg was determined after oxidation with a solution of $KMnO_4$. This method also provides very important information on the toxic organomercury content, mainly MeHg (determined as difference between tHg and iHg) in fish and plant tissues without handling highly toxic methyl mercury standard. If, in the event of sample containing other organic species such as phenyl mercury, dimethyl mercury, then the present method shall be suitable only for the identification of inorganic and organic forms of mercury. The developed method can, not only significantly reduce sample preparation time, but also provide quantitative recoveries (in the range of 95-102%) and preserve the integrity of the species. In addition, extra-reagents (such as L-cysteine) and concentrated reagents (TMAH) are not required for the determination of iHg and total mercury. In the proposed UAE approach, speciation analysis of mercury was achieved without using any chromatographic technique, requiring only ultrasound probe and CVAAS instruments. The main features of the present UAE method are; no matrix separation, reduction in time and solvent consumption, easy implementation, efficacy, reproducibility and safety of the procedure.

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Figure Captions

1. Effect of concentration of tetramethylammonium hydroxide on the recovery of mercury species from the two representative samples (a) Tuna Fish (BCR-464) and (b) Coriander sample loaded with iHg and MeHg

   Extraction conditions-Weight of representative sample = ~200 mg, Concentration and volume of TMAH = 2% and 10 mL respectively, Sonication time = 5 min; Mercury was determined by CVAAS after reduction with $SnCl_2$ (iHg) or $NaBH_4$ (total Hg).

2. Effect of concentration of $KMnO_4$ and HNO_3 on the oxidation of methyl mercury;

   Extraction conditions-Weight of coriander sample loaded with iHg and MeHg = ~200 mg, Concentration and volume of TMAH = 2% and 8 mL respectively, Sonication time = 5 min; Mercury was determined by CVAAS after reduction with $SnCl_2$ (for iHg) or $NaBH_4$ (for total Hg).

   Conditions used for oxidative treatment: TMAH-extracted sample volume taken for oxidation treatment = 0.5 mL and volume of $KMnO_4$ and HNO_3 mixture = 4.5 mL

3. Schematic flow diagram of the proposed ultrasound-assisted extraction method for the analysis of total mercury and its species from various fish and plant matrices

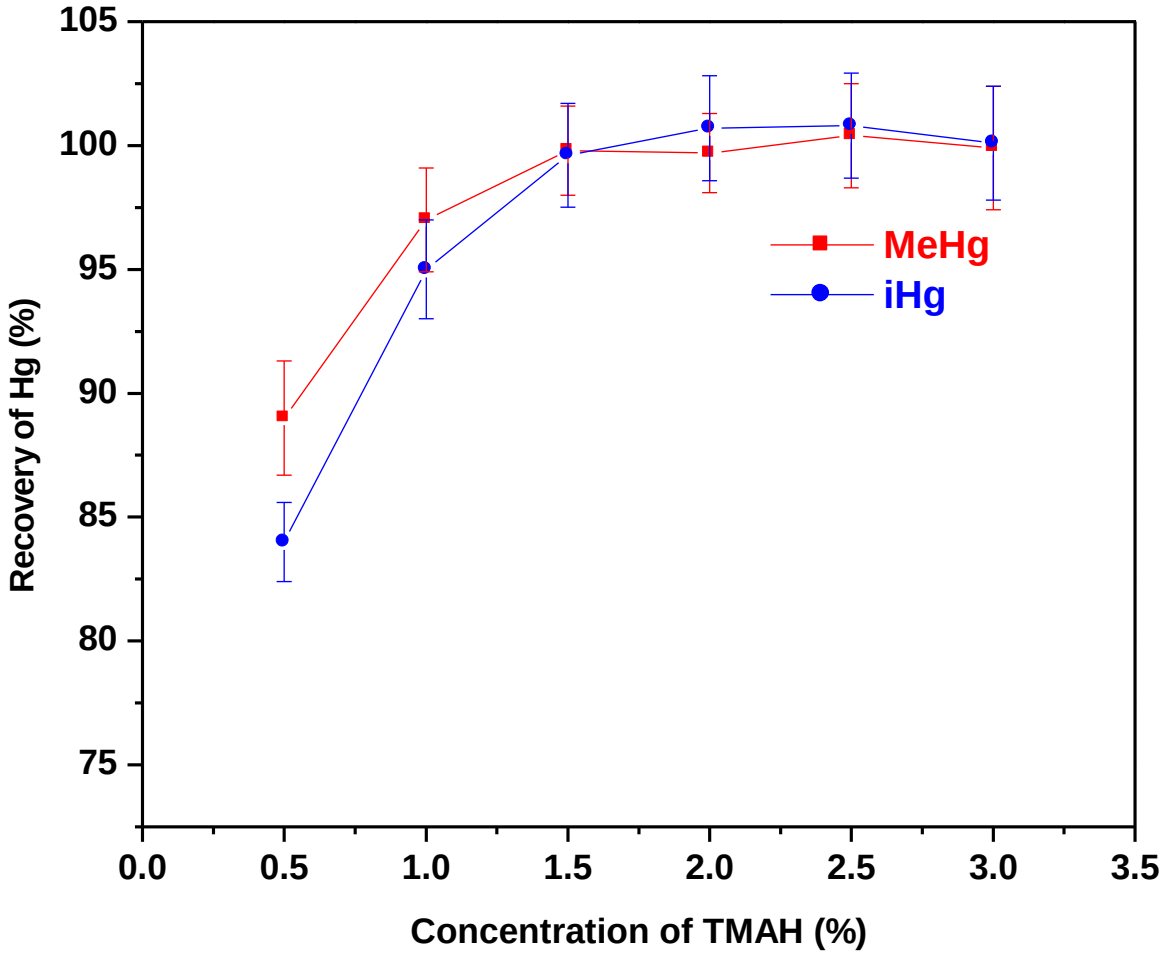

Fig 1a

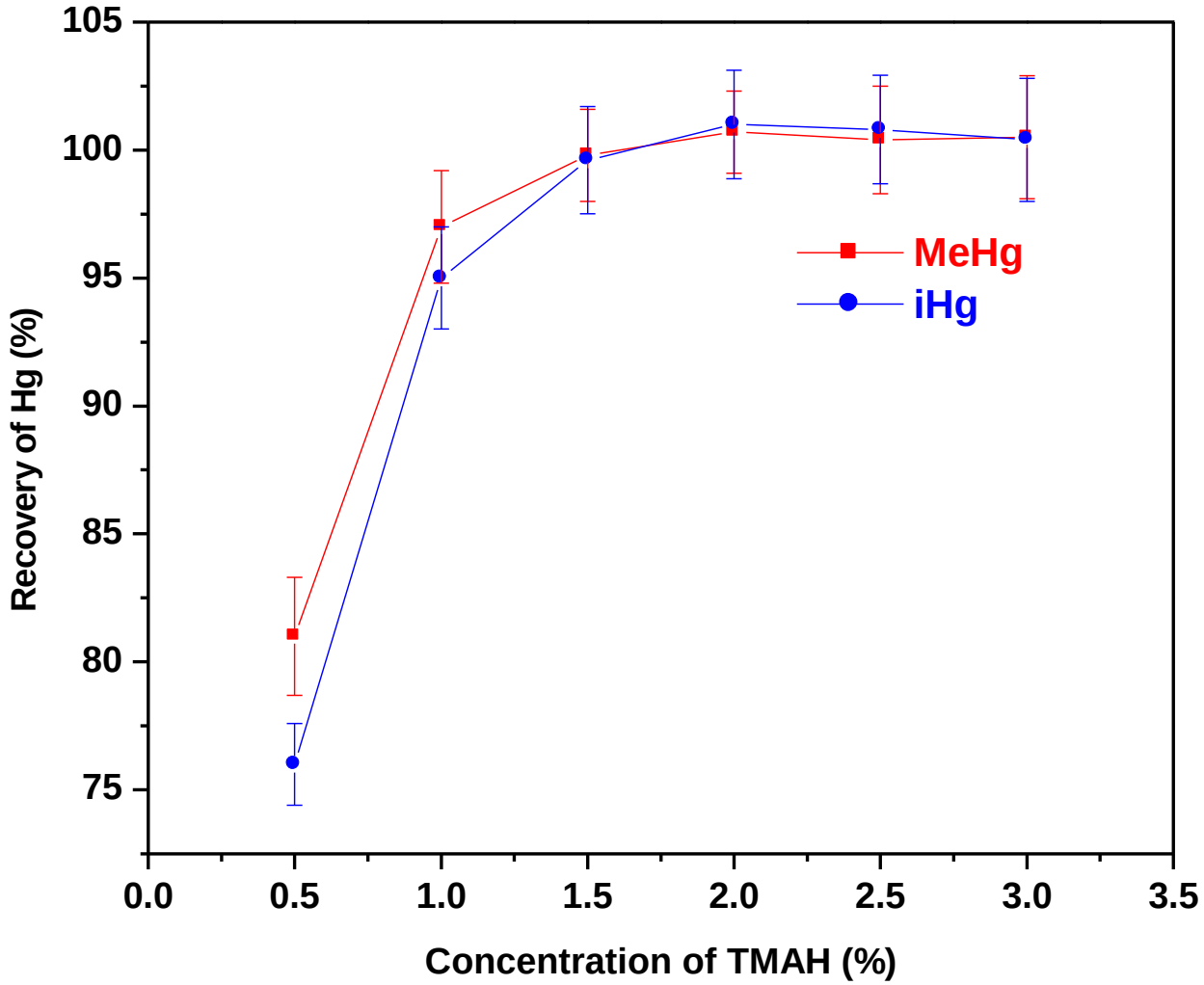

Fig 1b

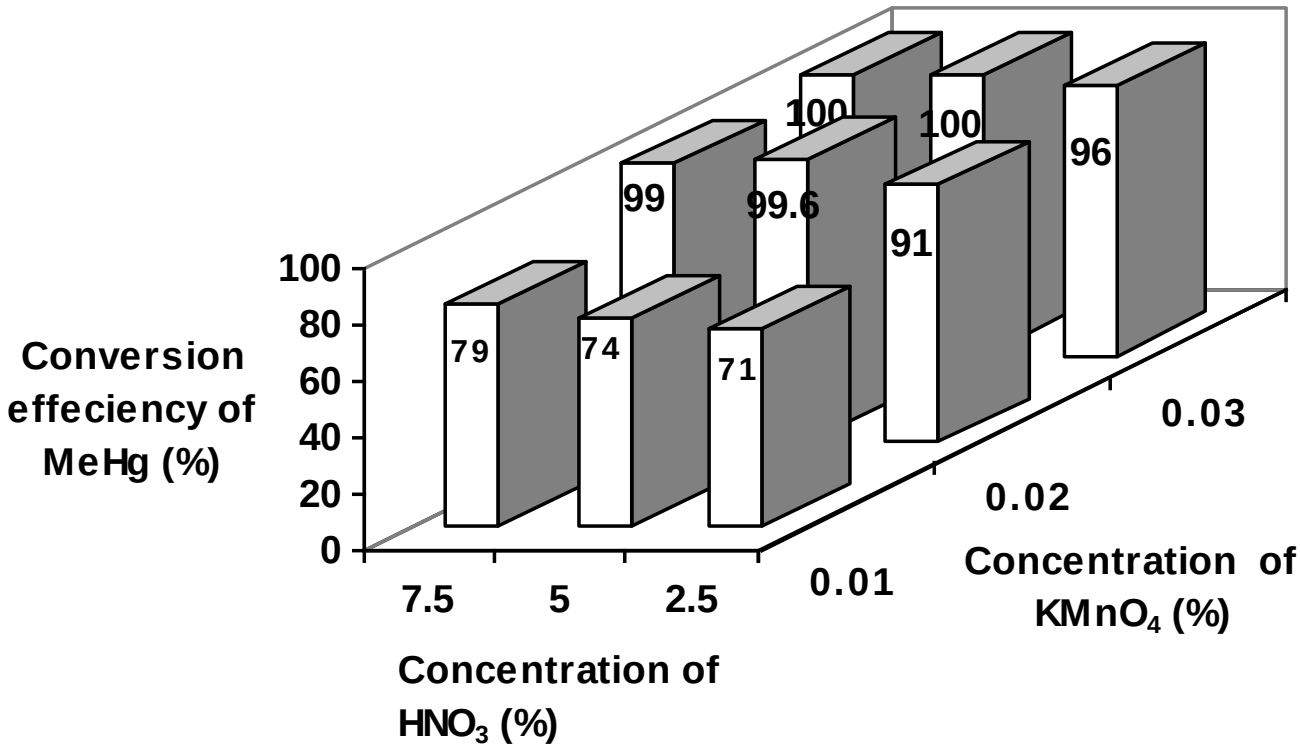

Fig 2

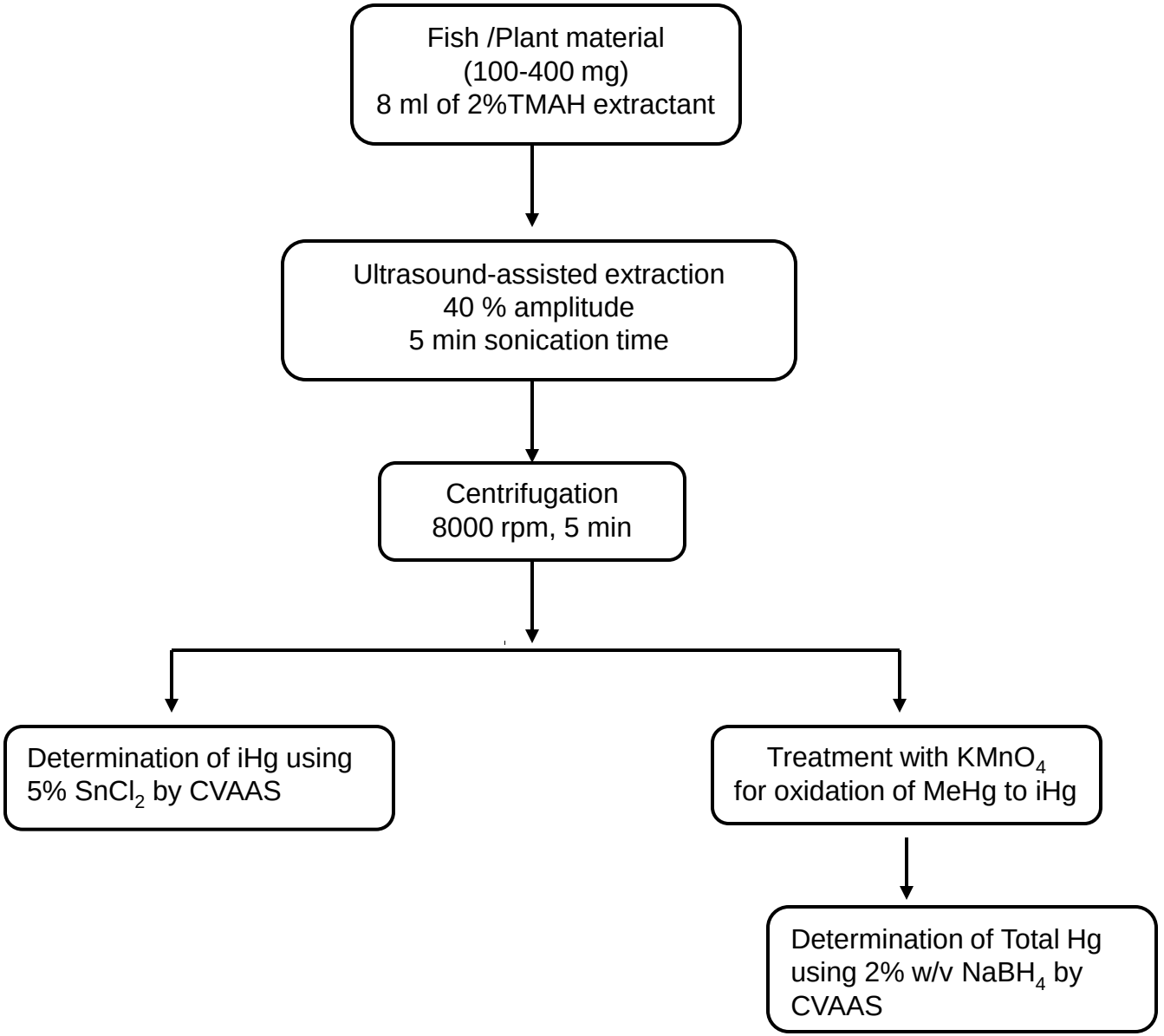

Fig 3

Table 1. Slopes corresponding to various calibration methods after spiking with inorganic and methyl mercury in different media with CVAAS

Medium	Response function	
	Hg^{2+} spiked	**$^{\#}CH_3Hg^{+}$ spiked**
Aqueous medium	y = 0.048x- 0.079 **R^2 = 0.996**	y = 0.047x- 0.067 **R^2 = 0.998**
2% TMAH medium	y = 0.050x + 0.037 **R^2 = 0.995**	y = 0.049x + 0.029 **R^2 = 0.996**
TMAH-extracted coriander sample (blank) solution	y = 0.047x + 0.029 **R^2 = 0.997**	y = 0.046x + 0.033 **R^2 = 0.995**

Calibration points- 10, 25, 50, 75, 100 ng/mL

$^{\#}$ Determined after $KMnO_4$ treatment

Table 2. Analytical results obtained for mercury loaded coriander powder samples with the proposed ultrasound assisted extraction (UAE) method (n=3)

Sample type	Loaded values (mg Kg^{-1})		Values obtained with the developed UAE method (mg Kg^{-1})			MW digestion (mg Kg^{-1})
	Hg^{2+}	CH_3Hg^+	Hg^{2+}	$^{\#}CH_3Hg^+$	Total-Hg	Total mercury
Coriander powder (blank)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Inorganic mercury loaded coriander powder	10	-	10.8±0.5	<LOD	10.3±0.2	10.5±0.4
Methylmercury loaded coriander powder	-	10	<LOD	10.1±0.5	10.6±0.3	9.7±0.5
Mixture of inorganic and methylmercury loaded coriander powder	10	10	10.1±0.4	9.7±0.3	19.8±0.4	20.3±0.8

Table 3. Analytical results obtained for CRMs of plant and fish tissues with the developed ultrasound assisted extraction (UAE) method (n=3)

Type of Reference Material	Certified values (mg Kg^{-1})		Obtained in this work (mg Kg^{-1})			MW digestion (mg Kg^{-1})
	Total-Hg	CH_3Hg^+	Total-Hg	$^{\#}CH_3Hg^+$	Hg^{2+}	Total-Hg
Lagarosiphon Major BCR-60 Aquatic plant	0.34±0.04	<LOD	0.33±0.03	<LOD	0.35±0.02	0.35±0.03
Lichen BCR-482	0.48±0.02	<LOD	0.50±0.04	<LOD	0.49±0.03	0.47±0.05
Fish Homogenate IAEA-350	4.68±0.28	3.65±0.35	4.65±0.21	3.74±0.19	0.91±0.05	4.65±0.22
Tuna Fish ERM-CE 463	2.85±0.16	3.04±0.16	2.92±0.13	2.88±0.12	0.04±0.01	2.93±0.12
Tuna Fish ERM-CE 464	5.24±0.10	5.50±0.17	5.36±0.12	5.24±0.11	0.12±0.02	5.28±0.13

values calculated as difference between total mercury and inorganic mercury

LOD = Limit of detection