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On-line speciation of inorganic and methyl mercury in waters and fish tissues using polyaniline micro-column and flow injection-chemical vapour generation-inductively coupled plasma mass spectrometry (FI-CVG-ICPMS)

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ABSTRACT

A simple and efficient method for the determination of ultra-trace amounts of inorganic mercury (iHg) and methylmercury (MeHg) in waters and fish tissues was developed using a micro-column filled with polyaniline (PANI) coupled online to flow injection-chemical vapour generation-inductively coupled plasma mass spectrometry (FI-CVG-ICPMS) system. Preliminary studies indicated that inorganic and methyl mercury species could be separated on PANI column in two different speciation approaches. At pH <3, only iHg could be sorbed and almost no adsorption of MeHg was found (speciation procedure 1). If the sample solution pH is ~7, both MeHg and iHg species could be sorbed on the PANI column. Subsequently both the Hg species were selectively eluted with 2% HCl and a mixture of 2% HCl and 0.02% thiourea respectively (speciation procedure 2).

The adsorption percentage of iHg on the PANI column was unchanged even with acidity of the sample solution increased to 6 mol L^{-1}. Therefore, an acidic solution (5 mol L^{-1} HCl), used for ultra-sound assisted extraction of the mercury species from biological samples, was used directly to separate MeHg from iHg in the fish tissues (tuna fish ERM-CE 463, ERM-CE 464 and IAEA-350) by PANI column using speciation procedure 1. The determined values were in good agreement with certified values. Under optimal conditions, the limits of detection (LODs) were 2.52 pg and 3.24 pg for iHg and MeHg (as Hg) respectively. The developed method was applied successfully to the direct determination of iHg and MeHg in various waters (tap water, lake water, ground water and sea-water) and the recoveries for the spiked samples were in the range of 96–102% for both the Hg species.

1. Introduction

The toxicological effects of inorganic and organic mercury species have been recognized for many years [1,2]. The two predominant pathways for human exposure to mercury are drinking water and dietary intake, the major source being methylmercury from fish and other seafood materials [3,4]. Mercury species, usually present in waters and fish are mercury (II) (iHg) and methylmercury (MeHg) which can be naturally produced by biomethylation of inorganic mercury in the aquatic environment by sulphate reducing bacteria which is subsequently bioaccumulated through aquatic food chains [5,6]. In the case of fish, high MeHg absorption occurs due to very high specificity of the intestine wall towards MeHg. Hence, total mercury concentrations up to 4000 μg kg^{-1} have been found in fish with methylmercury fraction up to 95% [7–10]. Therefore, regular consumption of marine based food supplements, especially in combination with fish, can result in high daily intake of mercury compounds; as high as several microgram per gram.

Recently, the UC Santa Cruz researchers reported that groundwater at Stinson Beach in Marin County and Elkhorn Slough in Monterey County, appears to contain 'surprisingly' high levels of methylmercury, the highly toxic form of mercury that accumulates in the marine food chain and poses a public health problem in most regions of the world [11]. More recently, U.S. Geological Survey (USGS) released a report on mercury contamination in fish, bed sediment, and water from streams across the United States (1998–2005) [12]. As stated in the report, mercury contamination was detected in every fish sampled in 291 streams across the country. About a quarter of these fish samples were found to contain mercury at levels exceeding the criterion for the protection of people who consume average amounts of fish, established by the U.S. Environmental Protection Agency.

As a safeguard for human health, World Health Organization (WHO) established a limit of 1 ng mL^{-1} of mercury in drinking water. The US EPA came up with stringent recommendations for

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the consumption of fish and shellfish, setting the limit as 300 μg methylmercury per kg of fish tissue (wet mass) [13]. At present, the maximum allowable level for total mercury in fish set by the European Union is 500 μg per kg of fish tissue (wet mass) with the exception of certain listed fish species for which 1000 μg per kg applies [14]. In 2003, the Joint FAO/WHO expert committee on food additives revised its provisional tolerable weekly intake for methylmercury to 1.6 μg per kg body weight [15].

Based on these facts, the development of highly efficient analytical methodologies for the speciation analysis of mercury in waters and fish tissues is very important for environmental protection and food safety. As drinking water and sea-food, in particular fish, are the major routes of ingestion of mercury species into the human body, routine monitoring of mercury species in these substances assumes increasing importance for toxicological assessment as well as to asses their mobility in the environment. In the case of mercury speciation in fish tissue, suitable extraction procedures must be used in conjunction with the best separation procedures and detection techniques in order to determine individual mercury species. To extract the mercury species from biological materials, acid or alkaline digestions methods are generally used [7].

Numerous analytical methods have been reported and reviewed in the literature for the separation of mercury species followed by their determination using element specific techniques [1,16–18]. Four main approaches have been employed for the separation of the mercury species in environmental and biological samples. These involve (i) the cold vapour technique by using $SnCl_2$ or $NaBH_4$ to reduce mercury species to volatile elemental form [19], (ii) separation by gas chromatography (GC) [20] or (iii) high performance liquid chromatography (HPLC) or (iv) capillary electrophoresis [21,22] with various spectrometric detection strategies such as atomic emission spectrometry (AES) [23–25], atomic absorption spectrometry (AAS) [26,27], atomic fluorescence spectrometry (AFS) [28–31] or inductively coupled plasma mass spectrometry (ICP-MS) [4,32–36]. The ability of particle beam/electron impact ionization-mass spectrometry (PB/EI-MS) to provide elemental and molecular information for a sample solution has been evaluated for the speciation of inorganic and organic mercury compounds [37]. Mercury cold vapour generation coupled to atomic absorption spectrometric detection (CVAAS) is one of the widely used analytical methods for the determination of mercury [38–41]. Combination with hydride/vapour generation techniques provide improved sensitivity and significantly reduces the matrix effects [42]. Among the methods stated above, the coupling of HPLC with ICP-MS is one of the most powerful methods for mercury speciation analysis because of the simplicity of the interface, high sensitivity and selectivity, availability of isotope ratio information [4,43]. However, problems associated with most of the hyphenated systems for routine mercury analysis are complexity, high cost of instrumentation and maintenance, high retention times and poor detection limits [42,44].

Determination of total and iHg and subsequent subtraction to determine MeHg content is a common indirect speciation approach found in most of the reported methods. But an analytical technique that can provide separation and direct determination of the species in a single step without using concentrated acids and/or organic reagents would be highly desirable for the metal speciation in various samples. To this end, the possibility of using PANI packed in a home made micro-column, for the on-line speciation of iHg and MeHg in various waters and fish tissues has been investigated using flow injection ICP-MS.

The physicochemical properties of polyaniline and its potential applications in diverse fields have been reviewed [45,46]. PANI has been used as base material for the preparation of mercury standard for use in neutron activation analysis [47]. The applicability of polyaniline for the determination of Cd, Cu, Pb and Sb in potassium iodide medium in biological matrices was also studied [48]. In our earlier studies, PANI was applied successfully for the removal of radioactive ruthenium (^{106}Ru) from actual radioactive waste solutions [49]. Fixed bed column studies were conducted to evaluate the performance of a PANI synthesized on jute surface for the removal of Cr(VI) in aqueous environment [50]. More recently, the applicability of PANI for the on-line preconcentration and recovery of palladium from various water samples has been reported [51]. Despite the extensive literature on various applications of PANI, no application of PANI for the on-line speciation of mercury in waters and fish has been reported.

In our earlier batch studies [52], it has been demonstrated that PANI is an excellent sorbent for the separation of inorganic and methyl mercury species from waters (with K_d values $\sim 8 \times 10^4$ and $\sim 7 \times 10^3$ for iHg and MM respectively). These studies were carried out in batch mode (off-line). Instead of separating the species in off-line mode, it would be most useful if the species can be separated with on-line approach. With several obvious advantages over batch or off-line procedures, the on-line mode using columns or micro-columns improves the detection power, precision and also allows a higher sampling throughput.

The objective of this work was to develop an on-line procedure for the preconcentration and separation of mercury species using a PANI micro-column followed by their determination using FI-CVG-ICPMS system without using any dervitizing reagents or buffer solutions. The retention and elution conditions for iHg and MeHg species on PANI column have been studied. In the developed procedure, mercury species were directly transformed into their hydrides or cold vapour using $NaBH_4$. The instrument and various parameters affecting the speciation analysis were optimized to obtain the best sensitivity and efficient separation of the test compounds. Analytical response functions were obtained for each of the test compounds. After optimization, the speciation procedure was then successfully applied to various real water samples and tuna fish reference materials (ERM-CE463, ERM-CE464 and IAEA-350). The mercury compounds were extracted from fish homogenate using an ultrasound-assisted extraction method reported in the literature.

2. Experimental

2.1. Instrumentation

A VG Plasma Quad 3 ICP-MS (VG Elemental, Winsford, Cheshire, UK) fitted with a standard double Scott quartz spray chamber and a concentric nebuliser was used for Hg detection. The mercury signal was monitored by the most abundant isotopes of mercury m/z 199, 200 and 202 using the peak jump mode. For the experiments with on-line preconcentration and speciation, a time resolved mode of data acquisition (TRA) was used for obtaining a chromatogram. The ICP-MS operating conditions that yielded the best sensitivity and chromatographic resolution of the mercury species after optimizing cold vapour generation conditions are summarized in Table 1.

Table 1
Instrumental and operating parameters of ICP-MS.

Instrumental parameters	Scanning parameters
Coolant gas: 13.4 L min^{-1}	Scanning mode: peak jump
Aux. gas: 0.97 L min^{-1}	Number of replicates: 3
Nebulizer gas: 0.85 L min^{-1}	Dwell time: 300 μs/channel
Sampler cone: 1.0 mm Ni	Sample delay: 30 s
Skimmer cone: 0.7 mm Ni	Stabilization delay: 20 s
Torch type: Fassel	Flow rate: 2 mL/min
Plasma FW power: 1350 W	Sample injection loop volume: 100 μL
Reflected power: <5 W	Isotopes monitored: m/z 199, 200, 202

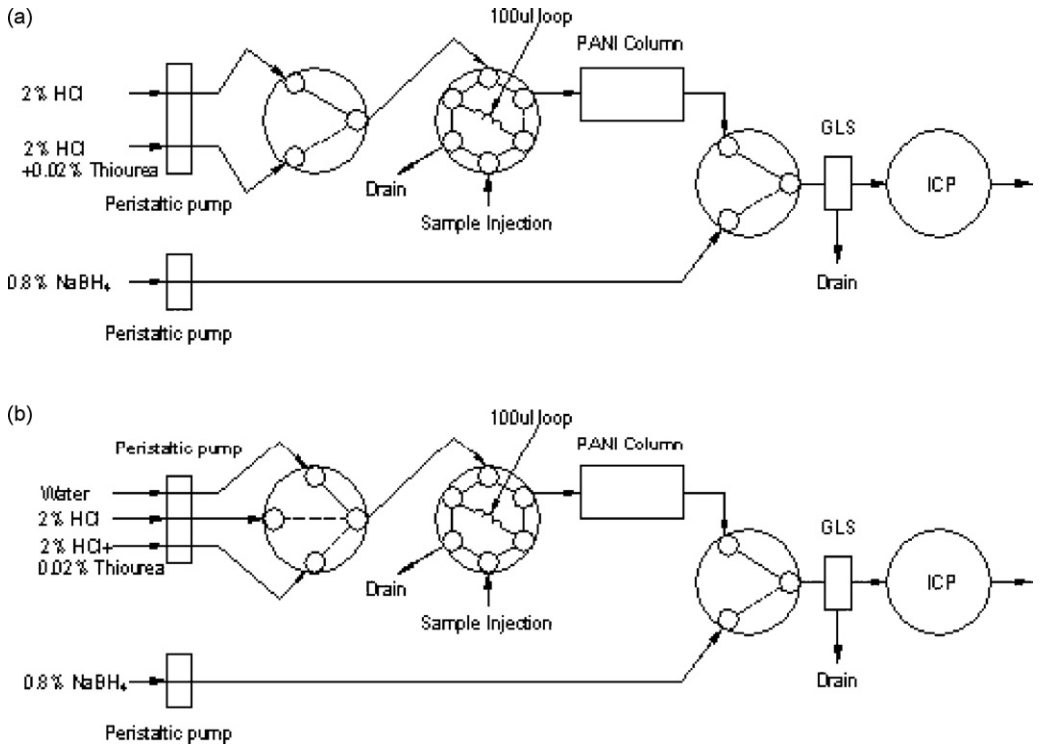

Fig. 1. Schematic diagram for the on-line speciation of iHg and MeHg species with PANI column by FI-CVG-ICPMS through (a) speciation procedure 1 and (b) speciation procedure 2.

Volatile species generated from the vapour generating system were transported to the ICP-MS for Hg determination. The on-line mercury speciation measurements were performed by FI-CVG-ICPMS system and the schematic diagram of the set-up is shown in Fig. 1a and b. In this case, micro-column packed with PANI was inserted immediately after the injection valve. The sensitivity of the instrument was checked each day before starting the experiments.

A 130 W power and 20 kHz (Cole Parmer Instruments, IL, USA, Model: CP 130PB-1) high intensity probe sonicator equipped with a 6 mm Ti probe was used for ultrasound-assisted extraction of mercury species from fish homogenate. The amplitude of the ultrasonic processor could be set to desired level in the 10–100% range of the nominal power.

2.2. Standards, reagents and materials

Analytical reagent grade chemicals were used without further purification.

Inorganic mercury (Hg^{2+}) stock standard solution (1000 mg L^{-1}) was prepared from mercuric chloride (Merck). A methyl mercury (CH_3Hg^+) stock standard solution (100 mg L^{-1}, Hg as MeHg) was prepared from methyl mercury chloride (Aldrich) by dissolving appropriate amount of the solid in acetone and making up to volume with high purity water. All the stock standard solutions were stored in a refrigerator at 4 °C and protected from light. Working standard solutions were prepared just before use by appropriate dilution of the stock standard solutions with ultra-pure water.

Sub-boiled HCl and HNO_3 were prepared in-house by sub-boiling distillation in quartz stills. Ultra-pure water (>18 M(cm resistivity), generated using a Milli-Q high purity water system, located in class 100 area. All containers were soaked in 20% HNO_3 and cleaned thoroughly with high purity water prior to use. Thiourea (NH_2CSNH_2) (Merck, Darmstadt, Germany) was used along with HCl to elute inorganic mercury. Syringe filters when used were 0.20 μm PTFE of 17 mm diameter.

2.3. Preparation of PANI micro-column

PANI, an oxidation product of aniline, was synthesized by chemical oxidation of doubly distilled aniline using ammonium peroxydisulphate in HCl medium. PANI was prepared in our laboratory by a method described by Syed and Dinesan [45]. The full details of the PANI preparation can be found in our earlier publications [51,52]. After preparation, the polymer was dried at 50–60 °C in an oven, powdered in a mortar and sieved to get 100–150 mesh size particles.

PTFE micro-columns of 30 mm length having three different internal diameters (2 mm, 3 mm and 4 mm i.d) were prepared for on-line speciation studies in the present work. Accurately weighed amount of PANI was packed evenly into the column by "tap and fill" method. A small portion of glass wool was introduced at both ends and end caps were fitted. The column was washed thoroughly with Milli-Q water and then conditioned to desired pH before passing the mercury-containing solutions. After use, the column was washed and maintained wet with Millipore water.

2.4. Ultrasound assisted extraction of mercury species from fish tissues (tuna fish ERM-CE463, ERM-CE464, IAEA 350)

A simple and rapid ultra-sound assisted extraction method using 5 mol L^{-1} HCl as described by Reyes et al. [7] was used for the extraction of inorganic and methyl mercury species. A similar procedure was also used for mercury speciation in fish samples by Ortiz et al. [8]. Polypropylene centrifuge tubes of 50 mL capacity were used for sonication experiments.

Accurately weighed amounts (0.25 g) of ERM-CE463, ERM-CE464 and IAEA-350 were transferred to polypropylene centrifuge tubes (50 mL volume) and 4 mL of 5 mol L^{-1} HCl was added. Then the mixture was sonicated at room temperature for predetermined time (5 min) at desired sonication amplitude (50%) settings. After sonication, the supernatant was separated from the solid phase by

centrifugation for 5 min at 4000 rpm. A known volume of the supernatant was transferred to another pre-cleaned centrifuge tube for speciation analysis. Corresponding process blanks were prepared in the same way without any sample material. Each sample was processed in triplicate. With each series of extractions, blank was also measured in parallel.

2.5. Optimization of cold vapour generation conditions

The conditions for mercury vapor generation were optimized using a FI method. The PANI column was removed from the system during these studies. Samples were introduced into ICP with an in situ vapor generation sample introduction system. ICP conditions were optimized to maximize the mercury ion signal using a FI method. A simple FI system was assembled from a six-port injection valve (Cole-Parmer, UK) with a 100 μL sample loop. A schematic diagram of the vapor generating system is shown in Fig. 1a and b. Since iHg and MeHg show different behavior and different sensitivities in the CV generation process, mercury species were studied separately to obtain the best compromised operating conditions for the vapor generation system. Standard solutions of known concentrations of iHg and MeHg were loaded into the injection loop with a syringe and injected into the carrier solution and then mixed with a stream of $NaBH_4$. A PEEK 3 way valve was used for mixing the sample, $NaBH_4$ and HCl. Vapour generation reaction takes place at the merging zone of the valve and the mercury vapor (Hg^0/hydride) generated was then driven into the ICP-MS system through PEEK tubing by argon injected (850 mL min^{-1}) into the gas liquid separator (GLS) unit where mercury vapour was separated and introduced into ICP-MS system for mercury determination. All the speciation experiments were carried out at room temperature.

Several operating parameters such as concentration of $NaBH_4$, concentration of HCl and flow rate were studied to obtain the optimum conditions. To quantify mercury concentration, the area of the peak generated in TRA mode was used as analytical signal. The analysis of each sample was repeated at least twice. A blank was injected periodically after the analysis of sample to assess any possible memory effects, which were found to be insignificant. A control solution with a known mercury concentration was injected at regular intervals during the working day to assess the overall stability and sensitivity of the ICP-MS system.

2.6. General mercury speciation schemes

pH variation experiments with PANI for the quantitative removal of both i-Hg and MeHg had shown that the retention of iHg on PANI was almost independent of pH whereas a pH dependent binding trend was seen for MeHg, with maximum binding occurring at pH > 5. These studies clearly indicated that inorganic and methyl mercury species could be separated on PANI column in two different speciation approaches.

Speciation procedure 1: In this procedure, pH of the sample (water) was maintained <3. Under these conditions, methyl mercury is not retained on the PANI column while inorganic mercury species are completely retained on the column. Subsequently, inorganic mercury can be eluted using a mixture of HCl and thio-urea. Various waters and fish tissues were analyzed for mercury species using this procedure.

The micro-column filled with PANI powder, was conditioned for about 1 min with 2% HCl solution at a flow rate of 2 mL min^{-1}. Then the sample was loaded in to a 100 μL loop (load mode) and injected in to the 2% HCl carrier solution (inject mode). The iHg species is retained by the PANI column while the MeHg species comes out to react with $NaBH_4$ stream and generate CH_3HgH species. Subsequently, the iHg was eluted and mixed with the $NaBH_4$ solution to form volatile Hg^0 species. Schematic diagram for the on-line speciation of iHg and MeHg species with PANI column by FI-CVG-ICPMS using speciation procedure 1 is shown in Fig. 1a. After separation and determination of the mercury species, the column was regenerated with a 2% HCl solution before passing the next sample.

Speciation procedure 2: Both the mercury species get sorbed on PANI column when pH of the mobile phase was maintained at >5. The retained mercury species iHg and MeHg were selectively eluted from PANI column using HCl and a mixture of HCl–thiourea respectively. Real water samples were tested using the optimized speciation approach to determine the efficacy of the proposed speciation approach. Using this procedure, iHg and MeHg determinations were carried out in water samples. Schematic diagram for the on-line speciation of iHg and MeHg species using PANI column and FI-CVG-ICPMS system through speciation procedure 2 is shown in Fig. 1b.

3. Results and discussion

Keeping the advantages of "on-line approach" as compared to off-line (batch mode) in mind, on-line experiments for the speciation of iHg and MeHg were investigated in the present studies. Quantification of the two mercury species (iHg and MeHg) in fish tissues has also been carried out in addition to waters as these two matrices are the major contributors to mercury risk for humans and wildlife.

3.1. Optimization of the on-line mercury speciation procedures

3.1.1. Effect of acidity on the separation of Hg species

In order to perform optimum speciation of mercury species, experiments were conducted with individual species standard solutions of iHg and MeHg (5 ng mL^{-1}). Preliminary experiments indicated that separation of iHg and MeHg on PANI micro-column occurred, which was studied systematically by varying HCl concentration in the range of 0.1–6 mol L^{-1} as well as under different pH conditions – from 1 to 10 – using FI-ICPMS and the results are shown in Fig. 2. The flow rate of the mobile phase used for all the studies was 2 mL min^{-1}. Conventional pneumatic nebulization was employed in these optimization studies for speciation conditions without vapour species generation. As may be seen from Fig. 2, the retention of iHg and MeHg on PANI column was highly quantita-

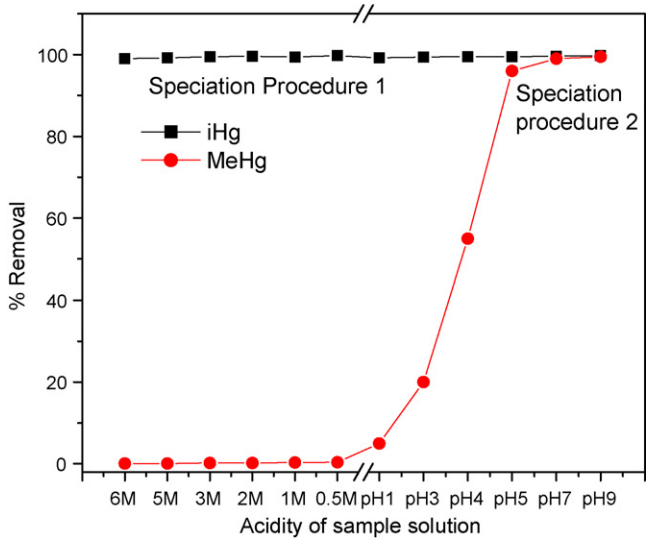

Fig. 2. Effect of acidity on the separation of iHg and MeHg species on PANI column: concentration of mercury species in the injected solution = 1 ng mL^{-1} each, sample injection volume = 100 μL.

tive (>99%) in the pH range of 5–10. However, with the increase in sample acidity, the sorption of MeHg on PANI decreases and almost no adsorption was found above pH 3 whereas the sorption of iHg remained unchanged even when the acidity of the sample medium was increased to 6 mol L^{-1}. This could make it possible for the separation of iHg and MeHg species by two ways.

In the first set of experiments, 2% HCl was used as carrier solution (Fig. 1a). Under these conditions, MeHg was not retained on the PANI column while inorganic mercury was completely retained on the PANI column. Subsequently, elution of retained iHg from PANI column was performed with an optimized mixture of 2% HCl and 0.02% thiourea. Once the retention and elution procedure was optimized with individual mercury species standards, mixtures of iHg and MeHg were used in further studies. Herein afterwards, this procedure is called "speciation procedure 1".

The difference in retention behaviour of the two species under acidic conditions could be explained as follows. The species iHg and MeHg exhibit different ionic character in HCl medium. It was reported that, at pH < 10, most of the inorganic mercury exists as anionic form such as $HgCl_3^-$ and $HgCl_4^{2-}$, while methylmercury remains as neutral species, CH_3HgCl [53]. Under these conditions, PANI which exhibits anion-exchange characteristics, retains the anionic species viz., $HgCl_3^-$ and $HgCl_4^{2-}$ while neutral CH_3HgCl species exhibit negligible retention.

A relatively large value of K_d for iHg species ($\sim 8 \times 10^4$), further confirms the higher affinity of Hg(II) species than MeHg species towards polyaniline. Gupta et al. [54] have proposed the probable mechanism of adsorption of Hg(II) ions on polyaniline in addition to ion-exchange process as follows

$$-[B\text{–}N\text{–}Q\text{–}N\text{–}B\text{–}N\text{–}Q]_n - \xrightarrow{iHg} -[B\text{–}N(Hg)\text{–}Q\text{–}N(Hg)\text{–}B\text{–}N(Hg)\text{–}Q]_n-$$

where B is the benzeinoid ring and Q is the quinoid ring.

The polyaniline has alternating benzenoid and quinoid rings connected by nitrogen. Nitrogen atoms in polyaniline act as adsorption sites for Hg(II) ions.

If the pH of the mobile phase is maintained >5, both the mercury species are sorbed on PANI column. The retained mercury species can be selectively eluted from PANI column using HCl and a mixture of HCl–thiourea respectively. This procedure is described in detail as follows.

3.1.2. Species selective elution (speciation procedure 2)

Since the retention of both mercury species was not possible in speciation procedure 1, the separation of iHg and MeHg through selective elution after loading on PANI column was investigated. The pH profile experiments suggested that both the mercury species are retained quantitatively (>99%) when pH of the mobile phase is >5. Hence, a method for the sequential elution of iHg and MeHg in aqueous samples was proposed using a neutral mobile phase (pH ~ 7). The neutral pH was selected so that water samples could be analysed directly without any pH adjustment. The aim of this procedure was first to load the mercury species on PANI micro-column, elution of MeHg with low acidic media (dilute HCl) while iHg required some complexing agent to strip it from the PANI column. To check the influence of HCl concentration on the selective elution of MeHg alone, on-line elution studies were performed with HCl in the concentration range of 0.2–5% at an optimized flow rate of 2 mL min^{-1}. From these results, it was observed that quantitative elution of MeHg from PANI micro-column could be achieved with 0.5% HCl where desorption of iHg was almost insignificant. The quantitative elution of iHg from the column could not be achieved even with 50% HCl. Based on the results obtained with various preliminary on-line column experiments, quantitative recovery (>99%) of the bound iHg was achieved using a 2% HCl–0.02% thiourea mixture. Herein afterwards this procedure is termed "speciation procedure 2". The greatest advantage of this method is that only a minimum of reagents and sample handling steps are required, which stands as a prerequisite for accurate results in routine analysis, particularly for the speciation of mercury.

3.2. Optimization of vapour/hydride generation conditions

Vapour generation of mercury species after column separation followed by introduction into plasma is found to give higher sensitivities than conventional direct nebulization due to improved transfer of the analyte [19]. Hence, mercury species were subjected to vapour generation for their determination by ICP-MS. The FI-CVG-ICPMS was optimized for mercury species sequential determination. The parameters optimized were reagent concentration ($NaBH_4$ and HCl), and flow rates of reagents and gases. Since iHg and MeHg show different bahaviour and different sensitivities in the CV generation process, operating conditions of the CV generation system was optimized by injecting individual mercury compounds using FI system.

3.2.1. Sodium tetrahydroborate ($NaBH_4$) concentration and input rate

The concentration of $NaBH_4$ and flow rate is critical in optimizing the mercury signal in ICP-MS. Therefore, the effect of $NaBH_4$ concentration on the generation of mercury vapour, i.e. signal sensitivity was studied keeping the concentration of HCl fixed at 2%. The concentration of the reductant was varied from 0.1 to 3% (w/v). In all cases, the solution was prepared in basic media (NaOH 0.3%, m/v), in order to stabilize this reagent. It is well known that inorganic mercury is reduced to its elemental form while MeHg forms CH_3HgH when reacted with $NaBH_4$. Peak areas of the Hg species as a function of the concentration of $NaBH_4$ were obtained. These results clearly indicate that as the $NaBH_4$ concentration increased, signal intensity of both the mercury species increased significantly in the range of 0.1–1% and reached a maximum when the $NaBH_4$ concentration was about 1.5%. Compared with the conventional nebulization, it was observed that the signal intensity of both the mercury species increased 10–15 fold when 1% $NaBH_4$ was used. Peak area of mercury species decreases when $NaBH_4$ concentration was increased further to 3%; this decrease is more prominent for MeHg species. At higher concentration of $NaBH_4$, however, the plasma becomes unstable with high-reflected power (>20 W) and reproducibility was poorer owing to excessive hydrogen gas being produced into the plasma. The enhancement factors of iHg and MeHg were different which may be attributed to the variation of the vapour generating efficiency of the mercury species (Hg^{2+}/CH_3Hg^+). Finally, it was chosen the reductant concentration of 1% of $NaBH_4$ in all the subsequent experiments.

Furthermore, the effect of the $NaBH_4$ flow rate (input rate) on the S/N ratios was also studied from 0.5 to 2 mL min^{-1}. The input rates that resulted in the maximum signal intensities were found to be 0.8 mL min^{-1} and 1.0 mL min^{-1} for MeHg and iHg respectively. As a compromise, the optimum flow rate for the 1% $NaBH_4$ was chosen to be about 1.0 mL min^{-1}.

3.2.2. HCl concentration and flow rate

To check the influence of HCl concentration and flow rate on vapour generation of test mercury species, different concentrations of HCl solutions were tested on individual standard solutions keeping $NaBH_4$ concentration at 1%. The concentration of the acid was optimized in the range of 0.5–5%. This study clearly established that, the best signals for both the mercury species were obtained

when HCl concentration was 2%. Low HCl concentration results in incomplete vapour generation of Hg species. Therefore, a 2% HCl was used in further studies.

Optimization of HCl flow rate was carried out between 0.5 and 2 mL min^{-1}. Trailing peaks of Hg species were found for both the Hg species at low flow rates of HCl (<1%). Considering the sensitivity and the consumption of HCl, a flow rate of HCl at 1 mL min^{-1} was selected.

3.3. Mercury speciation using FI-CVG-ICPMS in waters

Mercury speciation was performed by both the speciation schemes (speciation procedures 1 and 2) using the FI-CVG-ICPMS system as shown in Fig. 1a and b. The column, filled with the PANI powder, was conditioned for about a minute with a 2% HCl solution at a flow rate of 2 mL min^{-1}. The sample was loaded into the 100 μL loop (load position) and injected into the 2% HCl carrier solution (inject position). Then the separation of the Hg species was carried out using speciation procedure 1. After the species determination as described in Section 3.1, the micro-column was regenerated with 2% HCl at a flow rate of 2 mL min^{-1} for about a minute to remove residual thiourea from the column. After the regenerating the column, a new sample can be introduced after washing the sample line with Milli-Q water.

Fig. 3 shows a typical chromatogram of a mixed standard solution of 1 ng mL^{-1} of iHg and MeHg using speciation procedure 1 recorded by monitoring different isotopes of mercury. As seen from here, the naturally occurring isotopic pattern of mercury is seen for both species. Very clearly, the mercury species were fully resolved and the separation was complete in less than 5 min. The background at all the isotopes of mercury (m/z 199, 200, 202) increased when CV generation sample introduction system was used. This behaviour is possibly due to the trace mercury contamination of the reagents used and/or the memory of mercury from the spray chamber. Similar observations were noticed by Wan et al. [55] during the mercury speciation studies in water samples. Peak area measurements indicated that the response for mercury was different for iHg and MeHg which may be attributed to variation in the CV generation efficiency as mentioned earlier. Fig. 4a is the FI-CVG-ICPMS chromatogram showing the non-adsorption of MeHg and adsorption of iHg species on the PANI micro-column using speciation procedure 1 when a 3 ng mL^{-1} mixed standard solution containing both the Hg species was injected. The recoveries of the Hg species from spiked

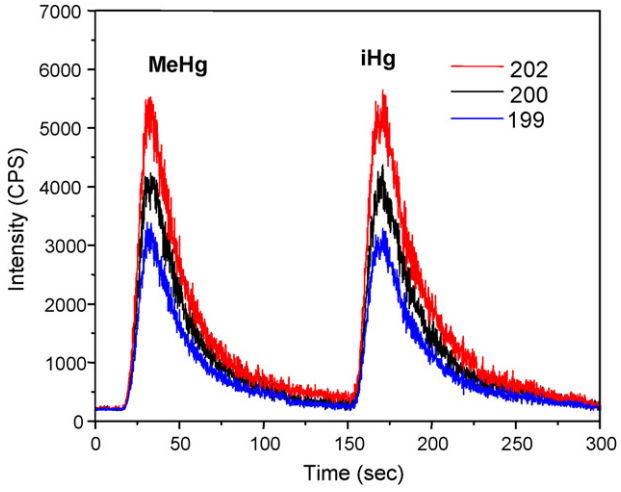

Fig. 3. FI-CVG-ICPMS chromatogram for MeHg and iHg species showing naturally occurring isotope pattern of mercury. Concentration of mercury species in the injected solution = 1 ng mL^{-1} each, sample injection volume = 100 μL.

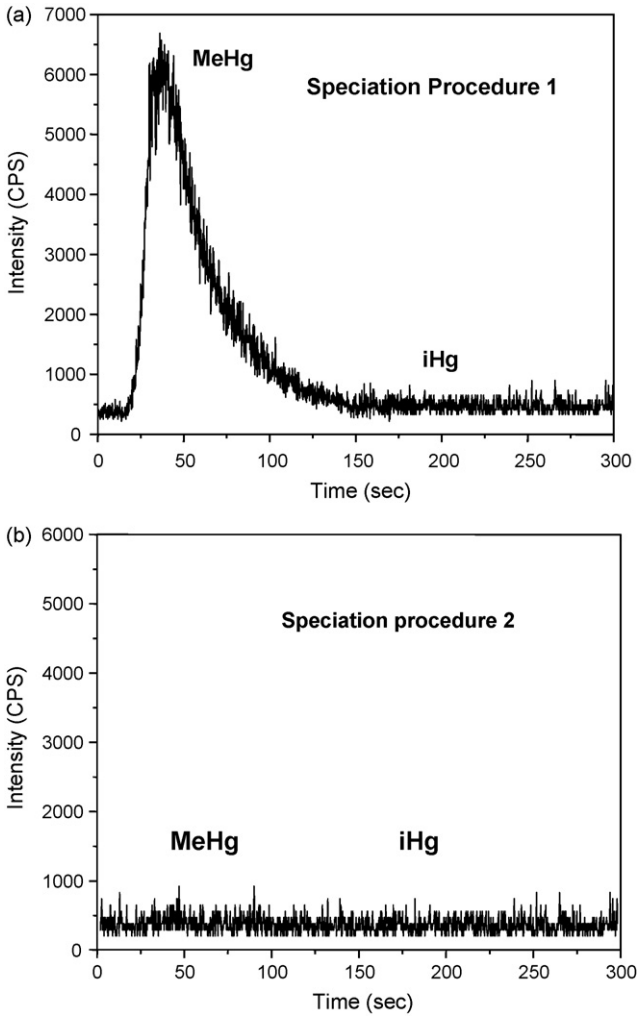

Fig. 4. FI-CVG-ICPMS chromatogram showing. (a) Non-adsorption of MeHg and adsorption of iHg species on the PANI micro-column using speciation procedure 1. Concentration of mercury species in the injected solution = 1 ng mL^{-1} each, sample injection volume = 100 μL, acidity of sample solution = 2%. Eluent used: 2% HCl + 0.02% thio-urea for MeHg. (b) Adsorption of both MeHg and iHg species on the PANI micro-column using speciation procedure 2. Concentration of mercury species in the injected solution = 1 ng mL^{-1} each, sample injection volume = 100 μL, pH of sample solution ~7. Eluent used: 2% HCl for iHg and 2% HCl + 0.02% thio-urea for MeHg.

water samples were also investigated using optimized conditions of speciation procedure 1. The recoveries obtained are presented in Table 2. As may be seen from Table 2, quantitative recoveries (>96%) were obtained for both the Hg species with added concentrations ranged from 1 to 10 ng mL^{-1} (as Hg).

In another set of experiments, PANI micro-column was conditioned to pH ~ 7 with Milli-Q water which is used as carrier solution.

Table 2
Recovery of iHg and MeHg from synthetic water samples with PANI column using speciation procedure 1.

Added (ng mL^{-1})		Found (ng mL^{-1})[b]		Recovery (%)	
iHg	MeHg	iHg	MeHg	iHg	MeHg
1	–	0.97 ± 0.03	–	97	–
–	1	–	0.98 ± 0.04	–	98
1	1	0.96 ± 0.02	1.02 ± 0.05	96	102
5	5	4.9 ± 0.1	4.85 ± 0.20	98	97
10	10	9.9 ± 0.2	9.95 ± 0.22	99	99.5
5	1	4.95 ± 0.19	0.96 ± 0.03	99	96
1	5	1.01 ± 0.03	4.9 ± 0.02	101	98

Table 3
Recovery of iHg and MeHg from synthetic water samples with PANI column using speciation procedure 2.

Added (ng mL^{-1})		[b]Found (ng mL^{-1})		Recovery (%)	
iHg	MeHg	iHg	MeHg	iHg	MeHg
1	–	0.97 ± 0.02	–	97	–
–	1	–	0.99 ± 0.03	–	99
1	1	1.03 ± 0.01	0.96 ± 0.01	103	96
5	5	4.8 ± 0.1	4.9 ± 0.2	96	98
10	10	9.8 ± 0.3	9.6 ± 0.2	98	96
1	5	0.96 ± 0.03	4.88 ± 0.12	96	97.6
5	1	4.9 ± 0.2	0.97 ± 0.03	98	97

Then the sample was loaded into 100 μL sample injection loop and injected into the carrier stream for the separation of Hg species under the conditions optimized in speciation procedure 2. Typical chromatogram showing adsorption of both MeHg and iHg species on the PANI micro-column using speciation procedure 2 by FI-CVG-ICPMS is shown in Fig. 4b. As in the case of speciation procedure 1, the quantitative recoveries were obtained for both the iHg and MeHg species (Table 3).

Calibration plots (Fig. 5a and b) of mercury species were obtained with the two proposed speciation approaches (speciation procedures 1 and 2) by injecting different amounts (0.1–0.4 ng each as Hg) of mercury species into the system with the PANI column using FI-CVG-ICP-MS system with 100 μL loop. In order to test recovery of the mercury species from the PANI column, similar calibration plots were also obtained by directly injecting into the FI-CVG-ICPMS system but without the PANI column as done in the case of experiments with PANI column. The peak areas were compared for the plots obtained with and without PANI column and results were found to be in good agreement. Analytical response characteristics of test mercury species obtained with PANI micro-column are presented in Table 4. As seen from the results, good linearity with correlation coefficients ($R^2 > 0.99$) for both the Hg species was obtained with this proposed speciation approaches.

3.3.1. *Optimization of the pre-concentration procedure for waters*

To improve the sensitivity in order to detect mercury species at environmental levels, it was decided to include a preconcentration step. In order to estimate the achievable preconcentration factor for very dilute solutions, the maximum volume of sample that can be passed through the column loaded with PANI must be determined. In our earlier studies, we reported the use of PANI to preconcentrate mercury species off-line. This has now been modified to an on-line method so that the pre-concentrated volume would be injected into the ICP-MS. The maximum amount of iHg and MeHg that could be retained by the PANI column was evaluated by using breakthrough (BT) capacity curves. From these studies it was estimated that the PANI column (30 mm × 3 mm) was able to accumulate up to 100 mg iHg/g and 2.5 mg MeHg/g.

Different amounts sample solutions containing 1 ng each of MeHg and iHg were passed through column after being introduced into the carrier stream (Millipore water) through injection valve in 'load' mode with the help of a peristaltic pump using the optimized experimental conditions of speciation procedure 2. The column was then washed by passing the water through it for about 1 min. After passing a known volume of the sample solution, elution of MeHg and iHg was achieved using 2% HCl and a mixture of HCl–thiourea respectively. The eluates (mercury species) from the PANI column were transported to ICP-MS after vapour generation for quantification. The data for obtaining the elution profile (i.e. chromatogram) of Hg species was acquired in time resolved acquisition (TRA) mode. The recovery was evaluated by comparing the signal with that of signal obtained with mixed standard solution containing 1 ng of each of iHg and MeHg initially. Fig. 6 shows FI-CVG-ICPMS chromatogram obtained after preconcentration of 50 mL sample solution containing absolute amount of 1 ng each of the Hg species on the PANI column using the optimized conditions of speciation procedure 2.

Finally, the carrier solution was pumped through the column for about 3–4 min for the removal of residual eluent mixture in

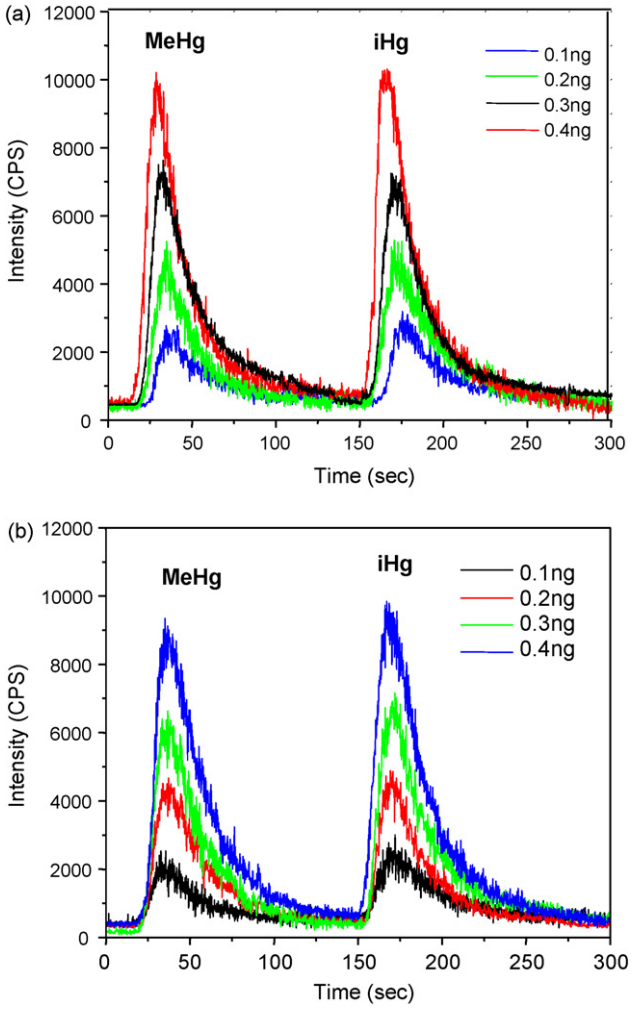

Fig. 5. Calibration plots obtained for mixed standard solution of MeHg and iHg species after passing through the PANI column by FI-CVG-ICPMS using (a) speciation procedure 1. Sample injection volume = 100 μL, acidity of sample solution = 2%. Eluent used: 2% HCl + 0.02% thio-urea for MeHg. (b) Speciation procedure 2. Sample injection volume = 100 μL, pH of sample solution ~7. Eluent used: 2% HCl for iHg and 2% HCl + 0.02% thio-urea for MeHg.

Table 4
Analytical response characteristics of Hg species with FI-CVG-ICPMS.

Species	Response function	R^2	Limit of detection (pg mL^{-1})	Absolute limit of detection[a] (pg)
iHg	$y = 1.00E^{+6}x - 2300$	0.9995	25.2	2.52
MeHg	$y = 7.46E^{+5}x - 2440$	0.9994	32.4	3.24

[a] Obtained with 100 μL sample injection loop.

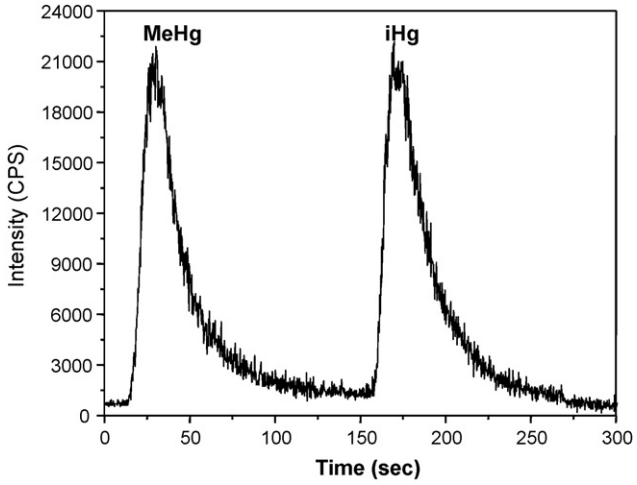

Fig. 6. Typical chromatogram showing the separation of MeHg and iHg species after preconcentration on PANI micro-column using speciation procedure 2 with FI-CVG-ICP-MS detection. Volume of the sample solution = 25 mL, pH of sample solution ~7, Sample flow rate 3 mL min^{-1}. Concentration of mercury species in the sample solution = 1 ng each. Eluent used: 2% HCl for iHg and 2% HCl + 0.02% thio-urea for MeHg.

Table 5
Characteristic data of different PANI micro-columns under the conditions of speciation procedure 2.

Dimension of the PANI micro-column	Weight of PANI in the column (mg)	Preconcentration factor obtained with PANI micro-column	
		iHg	MeHg
30 mm × 2 mm id	~50	120	60
30 mm × 3 mm id	~75	180	85
30 mm × 4 mm id	~100	240	120

order to restore the initial conditions before starting the next retention–elution cycle. Similar preconcentration studies were carried out on other PANI micro-columns (30 mm × 3 mm id and 30 mm × 4 mm id). The preconcentration factors obtained for Hg species with PANI column of different dimensions are presented in Table 5.

3.3.2. Effect of other ions on the separation of Hg species

The efficacy of the proposed speciation approach was tested in the presence of various cations and anions commonly co-exist in various natural and wastewaters using FI-CVG-ICPMS. For this purpose, the effect of diverse cations such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Cr^{3+} and anions Cl^-, NO_3^-, SO_4^{2-}, at a range of concentrations (1–200 μg mL^{-1} each) which could influence the separation of Hg species was studied. For this purpose, a standard solution containing 1 ng mL^{-1} each of both the Hg species spiked with a mixture of diverse ions was injected under optimized conditions as described above using both the speciation procedures. As the results indicate, none of the cations or anions interfered with the separation of the Hg species by PANI. This study clearly indicates that the proposed speciation approaches with PANI is robust and making the method very promising for the speciation analysis of Hg.

3.3.3. Application to water samples

In order to asses the applicability of this proposed preconcentration and speciation method, experiments were carried out with different real water samples-ground waters, lake (Hussain Sagar, Hyderabad, India), water samples collected from borewell near battery making factory and sea-water using both the speciation approaches (speciation procedures 1 and 2) as previously described. The water samples were collected in pre-cleaned polyethylene bottles and samples were analysed immediately after collection. After loading the samples on the PANI column through 100 μL sample loop, Hg species were subsequently eluted with optimized composition of eluent containing HCl and thiourea followed by measurement with FI-CVG-ICPMS.

3.3.3.1. Ground water sample. A ground water sample was collected within the premises of our center and filtered through Whatman filter paper. Direct analysis by FI-CVG-ICPMS indicated that the Hg level in the collected water sample was well below the detection limit of the instrument. The Hg levels were below the detectible levels even after 50 times precocentration of the ground water. Then the water sample was spiked with a known amount of Hg species (1 ng mL^{-1} each) and then injected through the column without adjustment of sample pH (the pH of sample solution as received was ~6.8). Quantitative recovery of both the mercury species was obtained even in the presence of high amount of total dissolved solids (~500 μg mL^{-1}) (Table 6).

3.3.3.2. Hussain Sagar lake water. The Hussain Sagar Lake in Hyderabad, India is an enchanting lake and is the largest man-made Lake in Asia [56]. Presently the lake water is in advanced state of eutrophication due to pollution with various industrial effluents. A water sample was collected from Hussain Sagar lake to identify and determine the levels of Hg species using the PANI column. Sample was loaded on the PANI column without any pH adjustment. Then the

Table 6
Determination of selected mercury species in natural water samples (injection volume -100 μl).

Type of water sample	Added (ng mL^{-1})		Found (ng mL^{-1})[a]			
	iHg	MeHg	Speciation procedure 1		Speciation procedure 2[c]	
			iHg	MeHg	iHg	MeHg
Tap water	0	0	BDL	BDL	–	–
	1	1	0.96	0.98	0.95	0.97
Lake water	0	0	0.76	BDL	0.74	BDL
	5	5	5.75	4.9	5.5	4.8
Ground water 1[b]	0	0	2.92	BDL	3.01	BDL
	5	5	7.7	4.8	8.1	4.9
Ground water 2[b]	0	0	3.9	BDL	4.2	BDL
	5	5	8.8	4.75	8.9	4.8
Sea-water	0	0	BDL	BDL	BDL	BDL
	1	1	0.95	0.94	0.93	0.95

BDL: Below detection limit. [a]Average values (n = 3). [b]Collected near battery making factory. [c]Sample injection volume 100 μL. [d]After eluting from the column.

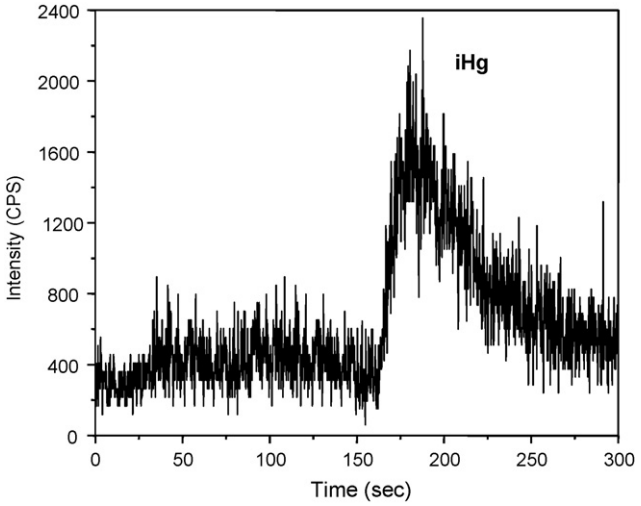

Fig. 7. Typical chromatogram of Hussain Sagar lake water sample showing inorganic mercury species after injecting through PANI column using speciation procedure 2 and FI-CVG-ICPMS detection. PH of sample solution ~7, sample injection volume = 100 μL, eluent used = 2% HCl for iHg and 2% HCl + 0.02% thio-urea for MeHg.

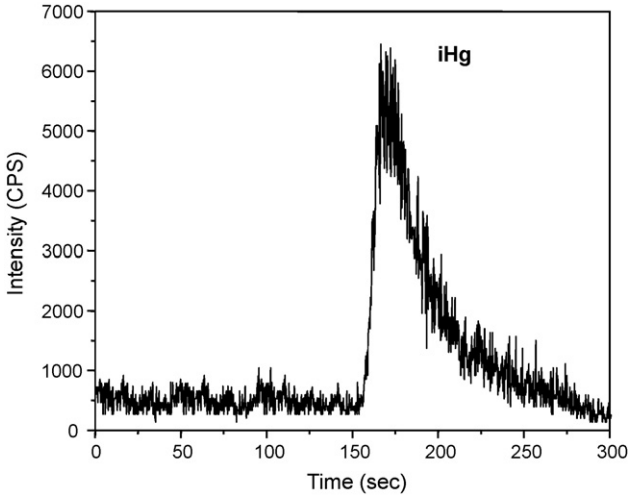

Fig. 8. Typical chromatogram of a water sample collected near battery making factory showing inorganic mercury species after injecting through PANI column using speciation procedure 2 and FI-CVG-ICPMS detection. pH of sample solution ~7, sample injection volume = 100 μL, eluent used: 2% HCl for iHg and 2% HCl + 0.02% thio-urea for MeHg.

retained Hg species were selectively eluted from the PANI column using 2% HCl and a mixture of 2% HCl and 0.02% thio-urea for iHg and MeHg respectively. Fig. 8 shows the chromatogram of Hussain Sagar lake water sample showing inorganic mercury species after injecting through PANI column using speciation procedure 2 with FI-CVG-ICPMS detection. As may be seen from Fig. 7, no methyl mercury was detected in this sample while the concentration of iHg was determined to be about 0.75 ng mL^{-1}.

3.3.3.3. Water samples collected near a battery making factory. Metallic, mercury has properties that have led to its use in many different consumer and commercial products and industrial sectors. Manufacturers around the world have long used mercury in batteries to prevent the buildup of hydrogen gas, which can cause the battery to bulge and leak in mercuric oxide batteries, mercury is used as an electrode rather than an additive to control gas buildup [57]. The mercury accounts for up to 40% of the battery weight and cannot be reduced without reducing the energy output of the battery. Due to the content of mercury, and the resulting environmental concerns, the sale of mercury batteries is banned in many countries.

To see the mercury levels in ground waters near a battery making factory located in Hyderabad, India, two water samples were collected outside the factory. After collection of the samples, water samples were filtered through 0.2 μm filter and speciation procedure was carried out. Typical chromatogram of a water sample collected near battery making factory showing inorganic mercury species after injecting through PANI column using speciation procedure 2 and FI-CVG-ICPMS detection is shown in Fig. 8. As seen from Table 6, the concentration of iHg was determined to be 2.92 ng mL^{-1} and 3.01 ng mL^{-1} for samples 1 and 2 respectively. But there was no detectable levels of MeHg species in the collected samples. The concentration levels of mercury species in the water samples studied except for samples 1 and 2 were below the permissible levels set by WHO where as water samples collected near battery making factory contain elevated levels of mercury. In all the cases including sea-water sample, the recoveries of iHg and MeHg species in spiked water samples were in the range of 95–102%.

3.4. Mercury speciation using FI-CVG-ICPMS in fish tissues (Tuna Fish ERM-CE463, ERM-CE464 and IAEA-350)

The proposed speciation procedure 1 was carried out on various tuna fish certified reference materials (ERM-CE463, ERM-CE464, IAEA-350). Mercury species were extracted from the fish tissue using ultra-sound assisted extraction procedure as described in Section 2.4. The supernatant was diluted by another 15-fold with mobile phase solution and filtered through 0.2 μm filter and then subjected to the proposed speciation procedure 1. The concentration of iHg and MeHg species were determined by an external calibration method based on peak area. The spike recoveries of individual compound were determined by spiking the extracts with suitable amount of iHg and MeHg standard solutions. The recoveries of the test mercury compounds were in the range of 95–99%. The extraction efficiency was verified by comparing the total mercury concentrations in the extracts using (i) the present speciation procedure with certified values, (ii) those obtained from total digestion method wherein the fish tissues was digested by closed micro-wave digestion with HNO_3 and H_2O_2. The chromatogram of ERM-CE464 extract with well resolved peaks due to MeHg and iHg species is shown in Fig. 9. The results obtained with the developed FI-CVG-ICPMS method for different extracts of tuna fish CRMs are presented in Table 7. As seen here from this table, the values obtained for iHg and MeHg species were in good agreement (at 95% confidence level) with the certified/determined values of the tuna fish reference materials tested here. This shows the capability of the PANI

Table 7
Determination of mercury species in various tuna fish certified reference materials using speciation procedure 2.

Tuna fish	Certified value (μg g^{-1})		Determined value (μg g^{-1})		Recovery of MeHg (%)
	Total Hg	MeHg	iHg	MeHg	
ERM-CE 464	5.24 ± 0.16	5.12 ± 0.16	0.13 ± 0.02	4.89 ± 0.14	95.5
ERM-CE 463	2.85	3.04 ± 0.16	0.03 ± 0.01	2.87 ± 0.09	94.4
IAEA-350	4.68	3.65 ± 0.35	0.88 ± 0.06	3.48 ± 0.12	95.3

Table 8
Comparison of detection limits for Hg species in waters by various speciation and detection methods.

Speciation method	Detection method	Detection limit (pg/mL)		Reference
		iHg	MeHg	
Solid phase extraction using dithiozone immobilized on a reverse phase C18 catridge	Photodiode array detector	140	140	[58]
Staphylococus aureus loaded Dowex Optipore V-493 micro-column	CVAAS	2.5	1.7	[38]
Solid-phase extraction using PTFE turnings	CVAAS	40	80	[44]
Weakly basic anion exchangers Dowex M-41, PuroliteA-100, Lewatit MP-64, and AmberlistA-21	FI-CVAAS	400	400	[53]
Dowex 1x8 resin and Methylthymol Blue as complexing agent	CVAAS	0.8	0.8	[3]
Chlorella vulgaris immobilised on silica gel	FI-CVAAS	500	2000	[5]
Prodigy ODS(3) column	HPLC-CV/HG-mAFS-AFS	11000	8000	[59]
Stir bar sorptive extraction	GC-MS	200	20	[60]
Cloud point extraction	RPHPLC-ICPMS	6	13	[61]
Sodium diethyldithiocarbamate immobilized in polyurethane (PU-NaDDC)	HPLC-ICPMS	4.6	5.2	[33]
Reverse phase column ODS-2	LC-ICPMS	110	30	[55]
C8 column	LC-CVG-ICP-MS	4	3	[4]
PANI micro-column[a]	FI-CVG-ICPMS	0.21	0.54	Present method

[a] After applying preconcentration factors of 120 and 60 for iHg and MeHg respectively.

column for the separation of Hg species from complex biological matrices.

3.5. Analytical figures of merit

Reproducibility was determined using ten replicate injections of a test mixture containing 1 ng mL^{-1} of iHg and MeHg. This study revealed that the RSDs of the peak areas were 2 and 2.5% for iHg and MeHg, respectively. Calibration curves based on peak heights were linear for each of the mercury species in the range tested (0.1–10 ng mL^{-1}). The detection limits were calculated from these calibration curves and based on the amount (or concentration) necessary to yield a net signal equal to three times the standard deviation of the background. The detection limits (as Hg) were calculated to be 25 pg mL^{-1} for iHg and 32 pg mL^{-1} for MeHg (as Hg), which corresponded to absolute detection limits of 2.5 pg, and 3.2 pg respectively with sample injection volume 100 μL (see Table 4). The use of more purified reagents should lower the detection limits further.

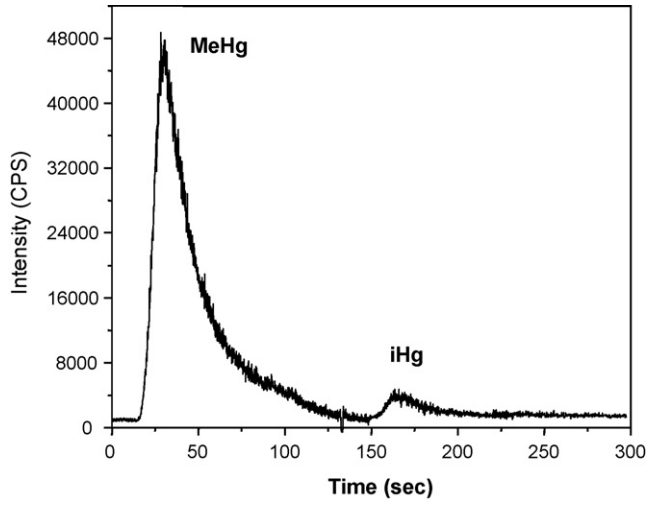

Fig. 9. Chromatographic separation of MeHg and iHg species from a tuna fish (ERM-CE464) extract on PANI micro-column using speciation procedure 1 and FI-CVG-ICPMS detection. Sample injection volume = 100 μL, eluent used: 2% HCl + 0.02% thio-urea for MeHg.

In the present on-line speciation approaches, the time needed for the speciation analysis of one sample was only about 6 min (5 min for separation of the Hg species and 1 min for column regeneration) and hence it is possible to analyse as many as 70–80 samples in an 8 h working day. While in the case of off-line mode, it requires 25–30 min for each sample (including sample loading, elution of the Hg species and their determination by CVAAS which restricts the analysis of samples to very few. Hence the sample throughput is limited in off-line mode by total analysis time and by the more number of analytical steps. The greatest advantage of present on-line speciation approach with FI compared to off-line method, is fast and simpler as the minimum number of analytical steps involved that minimizes contamination risks.

Table 8 presents a comparison of detection limits for a range of atomic spectroscopy methods for the test species evaluated here. As can be seen, the values obtained for the proposed FI-CVG-ICPMS method are very competitive with various hyphenated techniques with the added advantage of providing high preconcentration factors of PANI column and its cost effectiveness.

3.6. Conclusions

An on-line speciation method for the determination of iHg and MeHg species in waters and fish tissues using PANI micro-column and FI-CVG-ICPMS has been demonstrated. The use of PANI micro-column in place of the sample loop in the injection valve allowed the rapid and reproducible pre-concentration of mercury species in various waters prior to their determination. The proposed speciation approaches allow for easy and rapid separation and direct determination of both the species that avoid concentrated acids and organic solvents. The detection limits obtained for iHg and MeHg species (2.52 pg and 3.24 pg respectively with 100 μL sample loop) with this proposed speciation method are low enough for the mercury speciation of many real samples without using any complicated sample pre-treatment procedures such as dervitization of the species and conversion of the column using complexing agents which simplifies the instrumentation. Primary advantages of the proposed speciation approach include ease of synthesis of PANI in the laboratory and its cost effectiveness. The results of these studies suggest great promise in terms of performing preconcentration and speciation of Hg species in environmental and biological specimens.

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