Sensitive Determination Method for Mercury Ion, Methyl-, Ethyl-, and Phenyl-mercury in Water and Biological Samples Using High-Performance Liquid Chromatography with Chemiluminescence Detection

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A sensitive determination method for mercury speciation analysis was developed. Four mercury species, mercury ion, methylmercury, ethylmercury, and phenylmercury, were complexed with emetine-dithiocarbamate (emetine-CS_2), and then injected onto a HPLC instrument coupled with a tris(2,2′-bipyridine)ruthenium(III) chemiluminescence detection system. The emetine-CS_2 complexing agent was effectively used to measure the concentration in addition to serving as a separation and detection reagent. The calibration curves for these mercury complexes were linear in the range of 0.050 – 10 μg L^{-1} (as Hg). The limit of detection for (emetine-CS_2)$_2$Hg, emetine-CS_2-methylmercury, emetine-CS_2-ethylmercury, and emetine-CS_2-phenylmercury were 30, 17, 21, and 22 ng L^{-1}, respectively. The sensitivity of this method enables the determination of mercury species in water samples at sub-ppb levels. Furthermore, the method was applied to biological samples in combination with acid leaching and liquid–liquid extraction using emetine-CS_2 as an extraction reagent. The determination results were in good agreement with the values of the certified reference materials.

Introduction

Mercury pollution is a major global environmental problem. Mercury is released through volcanic,[1,2] mining,[3] and industrial activities,[4-7] and then spreads through the environment in various chemical forms. The level of mercury contamination and the chemical form vary greatly depending on the surrounding environment. The main exposure pathway of mercury to humans is through the consumption of fish and shellfish, predominantly in the form of methylmercury (MeHg), which is known to be the causative agent of Minamata disease. Aquatic animals acquire mercury contamination *via* bioaccumulation through the food chain in their environment. Therefore, a simpler and more rapid speciation analysis method for the measurement of mercury species in water and biological samples is highly desirable.[8-10]

Various speciation analysis methods for the measurement of organo-mercury species have been developed; however, at present, most methods for mercury in water and biological samples are based on gas- or liquid-chromatography separation in conjunction with a mercury-specific detector, such as atomic absorbance spectroscopy (AAS),[11] atomic fluorescence spectroscopy (AFS),[12-15] or inductively coupled plasma mass spectrometry (ICP-MS).[16-18] The high performance liquid chromatography (HPLC) method for mercury speciation has the advantages of straightforward sample preparation and flexible separation conditions in comparison to GC.

A chemiluminescence detection system using tris(2,2′-bipyridine)ruthenium(III) (Ru(bpy)$_3$$^{3+}$) as a chemiluminescent reagent has been employed as a powerful tool in analytical chemistry.[19,20] Recently, we published a preliminary report on the determination of the mercury species methylmercury (MeHg), ethylmercury (EtHg), phenylmercury (PhHg), and the mercury(II) ion (Hg^{2+}) using HPLC coupled with Ru(bpy)$_3$$^{3+}$ chemiluminescence detection (HPLC-CL).[21] The determination method is based on strong complex formation between the emetine dithiocarbamate ligand (emetine-CS_2) and the mercury species, and subsequent chemiluminescence detection of the reaction of Ru(bpy)$_3$$^{3+}$ with alicyclic tertiary amine moiety of emetine-CS_2, as shown in Fig. 1. This method was first reported by Tsukagoshi *et al.* as a highly sensitive determination method for Ni^{2+} and Cu^{2+}, exploiting the *in-situ* complex formation of emetine, carbon disulfide, and the metal ions.[22] However, the method required approximately 1 h for quantitative complex formation. To reduce the reaction time, we used newly synthesized emetine-CS_2, which complexes with mercury species instantaneously upon mixing. In addition, we reinvestigated the chemiluminescent reaction conditions,
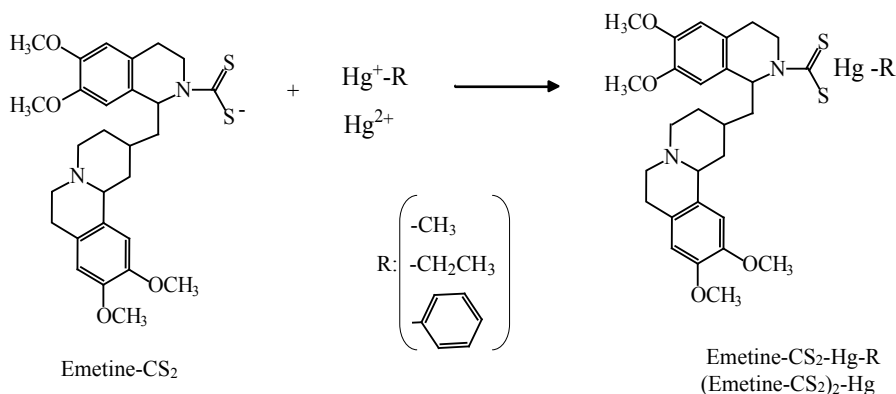
Fig. 1   Complexation reaction scheme of mercury species with emetine-CS$_2$.

because it was reported that tetrabenazine, which has the same tertiary amine structure as emetine, exhibits maximum sensitivity at pH 1.5 – 2 even though the chemiluminescence intensities of tertiary amines typically increase with pH.[23]   Ru(bpy)$_3^{3+}$ is stable and has low background in acidic solution because it reacts with hydroxide, making it a more sensitive and selective determination reagent in low reaction pH.[24]   In our previous report, our proposed method achieved the determination of four mercury species (Hg^{2+}, MeHg, EtHg, and PhHg) in the linear range of 1 – 100 μg L^{-1}.

In this study, we report on an improvement of the HPLC-CL system for the measurement of mercury species in water and biological samples using emetine-CS$_2$ as an extraction, separation, and detection reagent.   The separation and detection conditions for the HPLC-CL system and the preparation procedures for water and biological samples were investigated in detail.

Experimental

Reagents and solutions

A 100 mg L^{-1} mercury chloride standard solution was purchased from Wako (Osaka, Japan).   A 10 mg L^{-1} standard solution of mixed methylmercury chloride and ethylmercury was purchased from Kanto Kagaku (Tokyo, Japan). Phenylmercury chloride was purchased from TCI (Tokyo, Japan).   The stock standard solution of phenylmercury (10 mg L^{-1} as Hg) was prepared in acetonitrile.   The standard solutions were stored under cool and dark conditions, and were diluted to 0.1 or 1 mg L^{-1} with 0.01 M HCl–acetonitrile (1:9, v/v) solution before use.   Tris(2,2′-bipyridine)ruthenium(II) chloride hexahydrate (Ru(bpy)$_3$Cl$_2$·6H$_2$O) was purchased from TCI.   Acetonitrile and dichloromethane were of HPLC grade, and hydrochloric, perchloric, nitric, and sulfuric acids were of poisonous metal analysis grade.   Emetine-CS$_2$ was prepared according to a published procedure.[21]   A 2 mM emetine-CS$_2$ stock solution was prepared in methanol containing 1% NH$_3$ and stored at –20°C; this stock solution was typically replaced after one month.   The emetine-CS$_2$ stock solution was diluted to 0.1 or 1 mM with acetonitrile before use.   The (emetine-CS$_2$)$_2$Ni complex was prepared according to a published procedure.[22] Water for all of the solutions was purified using an Elix 5 UV (Millipore, Tokyo, Japan) and a Milli-Q Advantage system (Millipore).   All other chemicals were of analytical reagent grade, and were used without further purification.
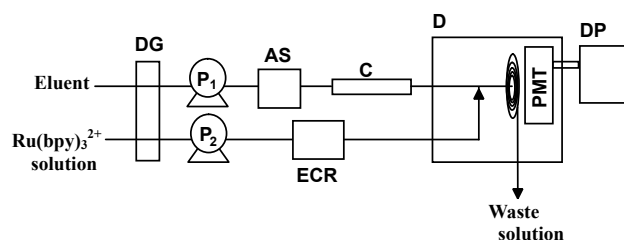

Fig. 2   Schematic diagram of the HPLC-chemiluminescent detection system.   DG, Degasser; P, pump; AS, autosampler; C, column; D, CL detector; ECR, electrochemical reactor; PMT, photomultiplier tube; DP, data processor.

Apparatus

HPLC experiments were conducted using the chemiluminescence detection (HPLC-CL) system shown in Fig. 2.   The system assembly consisted of a Shimadzu LC-20AD HPLC pump (Kyoto, Japan), a 320UP degasser (ERC, Saitama, Japan), an AS-3500 autosampler (DIONEX, Osaka, Japan) equipped with a 200-μL sample loop, an L-column ODS2 column (5 μm, 250 mm × 4.6 mm i.d., Chemical Evaluation and Research Institute, Tokyo, Japan), an HX-201 flow-through-type electrochemical reactor (Hokuto Denko, Tokyo, Japan), a Comet 2000 chemiluminescence detector (Comet, Kanagawa, Japan), and a Chromato-PRO data processor (Runtime Instruments, Kanagawa, Japan).   Total-Hg (T-Hg) analysis was performed with a semi-automated mercury analyzer (Model HG-201, Sanso Seisakusho Co., Ltd., Tokyo, Japan) based on cold-vapor atomic absorption spectroscopy (CV-AAS).   The moisture content was measured with an electronic moisture analyzer, MA35 (Sartorius, Goettingen, Germany).

Analytical conditions

The HPLC conditions were as follows: the eluent was 20 mM citrate buffer (pH 3.1)–acetonitrile (51:49, v/v) delivered at a flow rate of 1.5 mL min^{-1}.   A 0.25 mM Ru(bpy)$_3$Cl$_2$ was prepared in 0.1 M sulfuric acid, and was delivered at a flow rate of 0.3 mL min^{-1}.   The electrolytic current of the electrochemical reactor was set at 200 μA.

Standard emetine-CS$_2$-mercury species complex solutions (0.050 – 10 μg L^{-1} as Hg) were prepared from the mercury standard solutions, i.e., 500 μL of 2 M NaCl, 5 mL of acetonitrile, and 200 μL of 0.1 mM emetine-CS$_2$ solution in 10 mL volumetric flasks.   The solutions were made up to 10 mL

with water, and were shaken vigorously. It was confirmed that the complexation reactions of the mercury species and emetine-CS_2 occurred instantaneously upon the addition of emetine-CS_2 solution to the solution containing the mercury species at room temperature. Chloride ion was also added to the sample solution as a stabilization reagent of Hg^{2+}. A 200-μL aliquot was injected into the HPLC-CL system.

T-Hg was determined after mixed acid-digestion of a known amount of the sample in a 50-mL volumetric flask with 1 mL of pure water, 2 mL of nitric acid–perchloric acid (1:1, v/v), and 5 mL of sulfuric acid. The flask was heated on a hot plate at 230°C for 30 min.[25] This digested sample was diluted to 50 mL with water, and aliquot was analyzed with the CV-AAS system using 1 mL of a 10% stannous chloride solution as a reducing agent.

Water samples and preparation procedure

Water samples were collected from the Cikaniki River in the province of West Java, Indonesia, and the Idrija River in Slovenia. These two rivers have been contaminated with Hg through small-scale gold mining using an amalgam method and activity in the Idrija mercury mine, although the mining activity ceased in 1995. Seawater from Kagoshima Bay, Japan, was also measured to evaluate a high-salinity sample. These samples were filtered through a 0.45-μm hydrophilic PTFE membrane filter, and stored at 4°C.

River and seawater samples were prepared by combining a known amount of the water sample (maximum amount 4 mL), 500 μL of 2 M NaCl solution (river water samples only), 5 mL of acetonitrile, and 200 μL of 0.1 mM emetine-CS_2 solution in a 10-mL volumetric flask. The solution was diluted with ultrapure water until a marked line of the volumetric flask, and was shaken vigorously. A 200-μL aliquot was injected into the HPLC-CL system.

Biological samples and preparation procedure

To evaluate the accuracy of the method, four certified reference material (CRM) samples were measured. Tuna fish (ERM, CE 464) was purchased from Sigma-Aldrich (MO, USA). Cod fish (NMIJ, CRM 7402-a, Japan), swordfish (NMIJ, CRM 7403-a), and human hair (NIES, CRM No. 13, Japan) were purchased from Wako. In addition, fresh sea fish samples were purchased from a local supermarket. Tuna and swordfish were homogenized in a blender. The human hair sample was cut into 3 mm sections.

Acid leaching was employed to liberate the mercury species from the biological samples. A 10-mL volume of 5 M HCl was added to approximately 0.1 g of sample in a 50-mL centrifuge tube. The mixture was then placed in an ultrasonic bath for 10 min, and the suspension was centrifuged at 3000 rpm for 10 min. A 8-mL of supernatant was transferred into a 50-mL centrifuge tube, neutralized with 8 mL of 5 M NaOH, and adjusted to approximately pH 5 using 5 mL of 1 M citrate buffer (pH 5.0). The solution was cooled to room temperature, and 5 mL of dichloromethane and 50 μL of 1 mM emetine-CS_2 were added. The solution was shaken for 5 min, and was then centrifuged for 10 min at 3000 rpm. A 1-mL aliquot of the dichloromethane phase was transferred to a 10-mL centrifuge tube, which was then evaporated at 45°C under N_2 gas. The residue was dissolved in 1 mL of 20 mM borate buffer (pH 9.1)–acetonitrile (50:50, v/v). A 20-μL aliquot of this solution was injected into the HPLC-CL system.
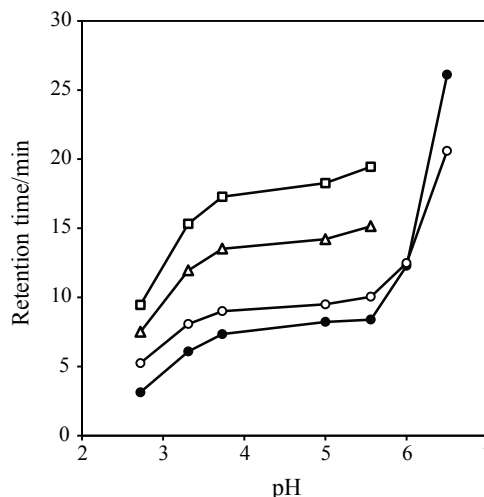

Fig. 3   Effect of the pH of the eluent on the retention times of complexes of mercury species. Sample: 10 μg L^{-1} standard emetine-CS_2-mercury species complexes solution; Hg (●), MeHg (○), EtHg (△), PhHg (□). Condition: eluent, 20 mM citrate buffer-acetonitrile (52:48, v/v).

Results and Discussion

Separation and detection conditions

We previously reported that our HPLC-CL system based on the detection of complexed mercury species with emetine-CS_2 suffered from interference peaks despite the high sensitivity of the method. The peak of (emetine-CS_2)$_2$Hg could not be completely separated from the emetine-CS_2-EtHg peak, and considerable peak broadening was observed. Therefore, we added sodium perchlorate to the eluent as an ion-pair reagent in order to suppress the peak broadening and to control the retention time. Although complete separation of four mercury species was achieved, various peaks appeared near the peaks of mercury complexes.[21] Therefore, we newly selected an ODS column that does not experience secondary interactions with residual silanol groups, since emetine-CS_2 has a tertiary amine moiety.

Figure 3 shows the effect of the eluent pH on the retention times of the four emetine-CS_2-mercury species complexes tested. The elution order of these complexes was Hg^{2+}, MeHg, EtHg, and PhHg below pH 6. The Hg^{2+} species eluted first because it complexed with two polar ligands, as opposed to the other mercury species that complex with only one polar emetine-CS_2 ligand. Conversely, the retention time of the Hg^{2+} complex drastically increased in comparison with the MeHg complex above pH 6, as the two ligands of the Hg^{2+} complex became neutrally charged. Briefly, the elution order of the mercury complexes was easily controlled with the pH and the content of the organic solvent in the eluent.

In our previous studies concerning the chemiluminescence reaction of Ru(bpy)$_3$$^{3+}$, the effect of dissolved oxygen in the reaction medium was not fully characterized. Under these reaction conditions, however, the peak height increased when a high-efficiency degassing device was introduced into the system. In addition, the use of HPLC-grade acetonitrile as an eluent led to low chemiluminescence background in comparison to experiments using a guaranteed grade solvent. Although not fully understood, the elimination of dissolved oxygen and the use of a higher purity solvent improved the signal-to-noise ratio
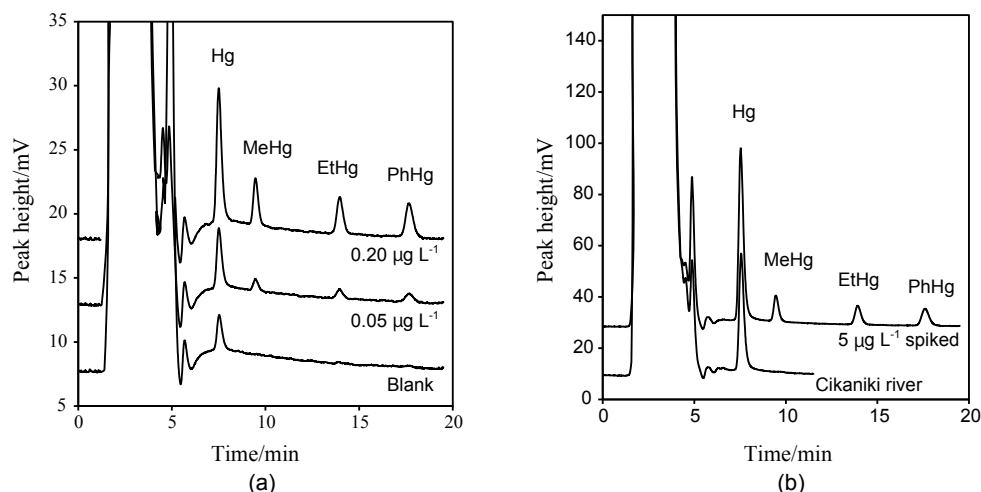
Fig. 4   (a) Typical chromatograms obtained from the analysis of a standard emetine-CS_2-mercury species complex solution. (b) Typical chromatograms obtained from the analysis of Cikaniki river water sample and Cikaniki river water spiked with 5 μg L^{-1} of four mercury mixed standard solution.

Table 1   Analytical results of the mercury species in water samples and of the spike-recovery tests

Water sample	Spiked/μg L^{-1}				Measured/μg L^{-1}, mean ± s.d., $n = 3$ (recovery value, %)			
	Hg	MeHg	EtHg	PhHg	Hg	MeHg	EtHg	PhHg
Idria River	—	—	—	—	nd	nd	nd	nd
	0.25	0.25	0.25	0.25	0.218 ± 0.001 (87)	0.252 ± 0.003 (101)	0.251 ± 0.003 (100)	0.251 ± 0.010 (101)
Cikaniki River	—	—	—	—	10.2 ± 0.5	nd	nd	nd
	5	5	5	5	16.1 ± 0.9 (106)	5.20 ± 0.06 (104)	4.89 ± 0.01 (98)	5.07 ± 0.08 (101)
Seawater	—	—	—		nd	nd	nd	nd
	0.5	0.5	0.5	0.5	0.403 ± 0.011 (81)	0.533 ± 0.008 (107)	0.528 ± 0.015(105)	0.515 ± 0.003 (103)

nd: not detected.

Analytical performance

Typical chromatograms obtained from the HPLC-CL analysis of standard emetine-CS_2-mercury species complex solutions are shown in Fig. 4(a). Because Hg^{2+} is present in various reagents as an impurity, detectable levels of (emetine-CS_2)$_2$Hg were found in blank solutions (0.06 ± 0.01 μg L^{-1}, as Hg, $n = 4$). The calibration curves for these mercury complexes were linear in the range 0.050 – 10 μg L^{-1} (as Hg). The detection limits obtained from the analysis of standard solutions of (emetine-CS_2)$_2$Hg, emetine-CS_2-MeHg, emetine-CS_2-EtHg, and emetine-CS_2-PhHg were 30, 17, 21, and 22 ng L^{-1}, respectively. The limit of detection of (emetine-CS_2)$_2$Hg was calculated as three-times the standard deviation of the peak height obtained from the blank solution. The limits of detection of the other three complexes were calculated as three-times the signal from the baseline noise. The precisions of the peak height calculated from four injections of 0.1 μg L^{-1} standard solutions of (emetine-CS_2)$_2$Hg, emetine-CS_2-MeHg, emetine-CS_2-EtHg, and emetine-CS_2-PhHg were 4.6, 3.3, 3.1, and 2.8%, respectively. The present method achieved a 20-times greater sensitivity and more effective separation in comparison to our previous method.[21]

Determination of sub-μg L^{-1} level contaminated water samples

The performance of our proposed method suggested that the sub-μg L^{-1} levels present in contaminated water samples can be measured only through the utilization of emetine-CS_2. We therefore approached the determination of mercury species in various water samples using this method. Table 1 shows our results. Hg^{2+} was detected at μg L^{-1} levels from the Cikaniki River sample, as shown in Fig. 4(b). Mercury species in the other samples were not detected, because the concentration of T-Hg in water samples obtained from non-contaminated sites was at a sub-ng L^{-1} level,[10] and T-Hg concentrations in Idrija River water have been reported to be at a ng L^{-1} level.[26]

The complex formation of dithiocarbamate ligands with various transition metal ions, such as Ni^{2+}, Cu^{2+}, Pd^{2+}, Pb^{2+}, and Zn^{2+}, is well known, and these reactions have been used in various analytical applications such as detection, separation, and concentration.[27-29] In our mercury determination system, the presence of these metal ions could cause interference with the detection of mercury complexes, and so we tested for emetine-CS_2 complexes with various transition-metal ions. Although peaks for (emetine-CS_2)$_2$Ni and (emetine-CS_2)$_2$Pd were specifically confirmed, these two complex peaks eluted before (emetine-CS_2)$_2$Hg, at 4.9 and 5.1 min, respectively; (emetine-CS_2)$_2$Hg elutes at 7.5 min. Consequently, interference of mercury determination from the formation of emetine-CS_2 complexes with other transition metal ions was not confirmed
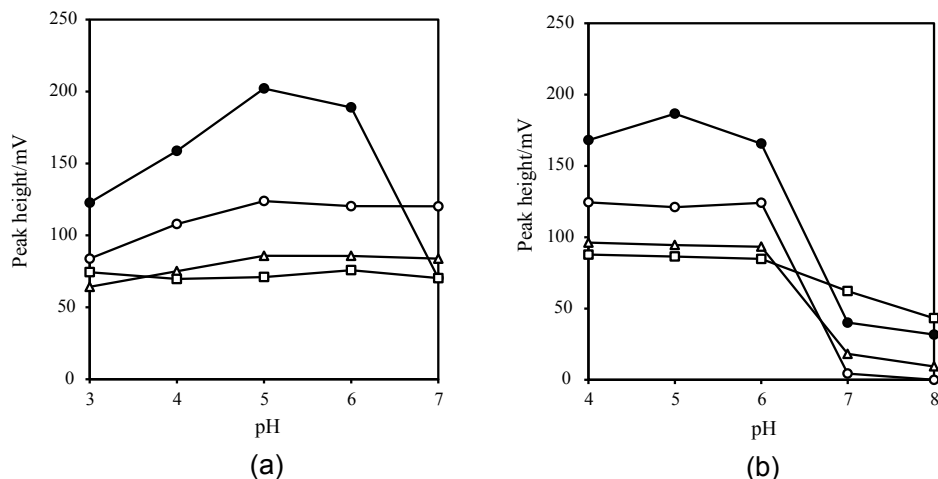
Fig. 5   Effect of the extraction pH on the peak heights of emetine-CS$_2$-mercury species complexes using (a) emetine-CS$_2$ and (b) (emetine-CS$_2$)$_2$Ni as the extraction reagent. Sample: 50 ng of four mercury species in 20 mL of BR buffer; Hg (●), MeHg (○), EtHg (△), PhHg (□). Values are mean of three operations.
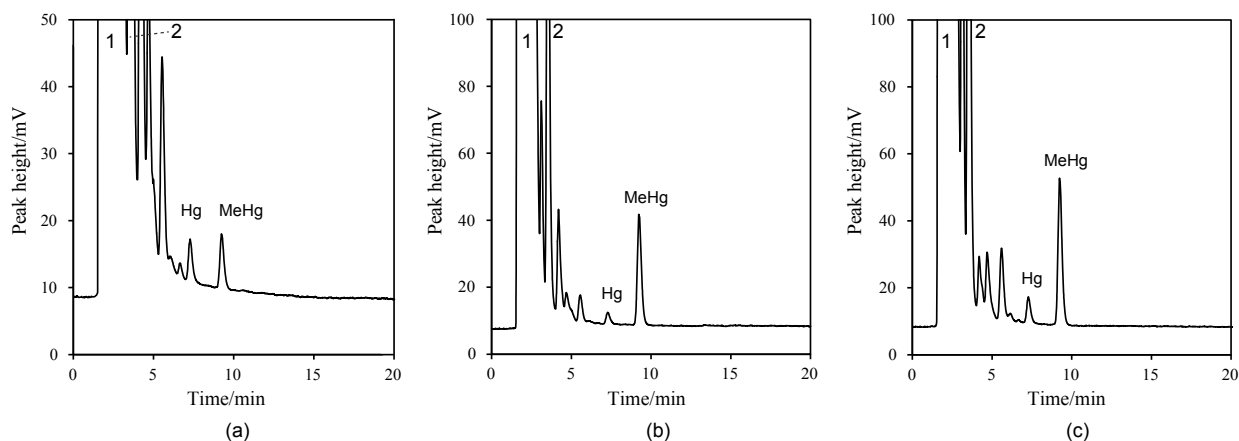

Fig. 6   Typical chromatograms from the analysis of (a) CRM 7402-a (cod fish), (b) CE 464 (tuna fish), and (c) CRM No. 13 (human hair). Peak identification: 1, emetine-CS$_2$; 2, oxidation product of emetine-CS$_2$.

under our separation conditions. These results suggested that the proposed method is selective against a matrix containing other metal ions.

Determination of biological samples

For the measurement of mercury species in biological samples, a series of procedures concerning elution, clean-up, and formation of the emetine-CS$_2$ complex were developed. In this study, emetine-CS$_2$ was used not only as the detection and separation reagent, but was also as a solvent-extraction reagent for clean-up. Various transition-metal ions react with emetine-CS$_2$, as discussed above.   In samples containing high concentrations of metal ions, emetine-CS$_2$ as an extraction reagent may be exhausted. However, too high a concentration of emetine-CS$_2$ as an extraction reagent would interfere with the detection of mercury complex peaks by a huge peak of itself and other metal ion complex peaks. Hence, the effective usage of a small quantity of emetine-CS$_2$ reagent was desired. The metal-exchange reaction of the dithiocarbamate complex has

been used for various analytical applications, and it is known that its reaction is pH-dependent.[30] If the effective extraction pH condition for the metal-exchange reaction between mercury species and the emetine-CS$_2$ which already formed with other metal ion is confirmed, the effective usage of emetine-CS$_2$ would be achieved for mercury analysis in a sample solution containing large amount other metal ions.   Therefore, we optimized the extraction pH for the extraction of mercury species using two types of extraction reagents.   Figure 5 illustrates the effect of the pH on the extraction performance using (a) emetine-CS$_2$ and (b) (emetine-CS$_2$)$_2$Ni as extraction reagents. As shown in Fig. 5(b), the metal-exchange reaction proceeded efficiently below pH 6. On the basis of this finding, we maintained the extraction solution at around pH 5 using a citrate buffer solution.

In order to establish a preparation procedure for biological samples, we investigated the stability of organomercury species in the elution solvent (5 M HCl), the elution method and time, the extraction time, and the amount of extraction reagent needed.

Table 2   Analytical results of the MeHg concentration in biological samples

Sample	MeHg/mg kg⁻¹ᵃ	Certified value/mg kg⁻¹	Recovery, %	T-Hg/mg kg⁻¹	Condition
Tuna fish (CE 464)	5.00 ± 0.13	5.50 ± 0.17	—	5.24 ± 0.10[b]	Dry
Cod fish (CRM 7402-a)	0.57 ± 0.02	0.58 ± 0.02	—	0.61 ± 0.02[b]	Dry
Swordfish (CRM 7403-a)	4.50 ± 0.26	5.00 ± 0.22	—	5.34 ± 0.14[b]	Dry
Human hair (CRM No.13)	3.89 ± 0.08	3.8 ± 0.4	—	4.42 ± 0.20[b]	Dry
Tuna fish	0.053 ± 0.002	—	96.9 ± 4.9[c]	0.076 ± 0.008[a]	Wet
Swordfish	1.11 ± 0.07	—	100 ± 10[d]	1.80 ± 0.1[a]	Wet
Human hair	0.25 ± 0.02	—	—	0.47 ± 0.02[a]	Wet

a. Mean $\pm$ s.d. ($n = 4$).   b. Certified value.   c. 50 ng spiked.   d. 200 ng spiked.

Although MeHg and EtHg were stable in 5 M HCl for at least 1 h, PhHg half decomposed to Hg^{2+} in 5 M HCl after ~30 min. Two elution methods, namely, the ultrasonication and shaking methods, were investigated with different elution times of MeHg from biological samples to the solution phase. The times of ultrasonication and shaking were tested at 5, 10, 30, and 60 min and 30, 60, 90, and 120 min, respectively, using cod fish samples. The obtained peak heights were nearly constant, except those for samples that were ultrasonicated for 30 and 60 min; these solutions became visibly cloudy. This result suggested that extended ultrasonication caused the elution of matrix compounds, which prevented efficient extraction of MeHg. The amount of emetine-CS₂ reagent used was varied within the range of 5 – 200 nmol. The peak height of MeHg was constant at over 40 nmol. The extraction time against the extraction efficiency was found to be constant at 1, 5, 10, and 30 min. From these results, we established the preparation procedure.

Figure 6 shows typical chromatograms obtained from the HPLC-CL analysis of CRM samples. The simple chromatograms were confirmed. Analytical results for all biological samples tested are listed in Table 2. EtHg was not detected in our measured samples. The determination values of CRM samples using the proposed method were in good agreement with the certified values. The recovery values of MeHg using fresh fish samples were found to be almost 100%. These results suggested that the proposed method is also selective against matrix extracts of biological samples.

Conclusion

A determination method for the measurement of four mercury species, Hg^{2+}, MeHg, EtHg, and PhHg, using emetine-CS₂ as a separation reagent for reverse phase HPLC and a detection reagent for $Ru(bpy)_3^{3+}$ chemiluminescence detection has been developed. The method was applied to the determination of mercury species in water and biological samples. It provides simple and straightforward analysis for mercury speciation.

Acknowledgements

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