Short communication

Selective Determination Method for measurement of Methylmercury and Ethylmercury in soil/sediment samples using high-performance liquid chromatography–chemiluminescence detection coupled with simple extraction technique

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ABSTRACT

A novel simultaneous determination method for measurement of monomethylmercury (MeHg) and monoethylmercury (EtHg) in soil/sediment samples has been developed. The method was based on the eluting MeHg from soil/sediment sample using 5M HCl containing 5 mM Pd^{2+} and 0.1 M Cu^{2+} solution and then the extraction of MeHg as MeHgCl to toluene. MeHgCl in toluene was back-extracted to an aqueous phase with an EDTA solution, creating a MeHg–EDTA complex. Finally, emetineCS_2 solution was added to the MeHg–EDTA complex solution to form an emetineCS_2–MeHg complex. The generated emetineCS_2–MeHg and emetineCS_2-EtHg complexes were effectively separated with reverse-phase HPLC and were detected with strong chemiluminescence reaction of tris(2,2′ -bipyridine)ruthenium(III) and emetineCS_2. The calibration curves for MeHg and EtHg with HPLC–CL, using the peak height, were linear from 0.5–20 ng (as Hg). The detection limits were 0.16 ng. The repeatability of the whole procedure using 1 ng of MeHg and 1 ng of EtHg in 20 mL HCl was 2.0% and 1.4% (n = 3). The sample throughput of the HPLC–CL system was 4/h. This procedure was validated by analyzing for certified reference material (ERM CC580, estuarine sediment). The MeHg concentration determined by the proposed method was in good agreement with the certified value. Furthermore, EtHg was detected in ERM CC 580. In addition, preliminary study concerning a relationship between mercury contamination level and production of MeHg were performed.

Key words: methylmercury, ethylmercury, HPLC, chemiluminescence, soil, sediment.

INTRODUCTION

Mercury is one of the most toxic metals in the environment. The harmfulness of mercury is known to be highly dependent on its chemical form. MeHg is more toxic than other chemical forms of mercury. The main exposure pathway of MeHg to humans is through the consumption of marine foods [1]. In general, it is considered that the contamination source of marine foods is bioaccumulation through the food chain, in which the primary producers of MeHg are microorganisms in sediment. Therefore, the accurate determination of MeHg in sediment is critical to understanding the environmental mercury cycle.

However, the determination of MeHg in soil/sediment is difficult because the concentration of inorganic mercury in soil/sediment samples are over 100–1000 times higher than that of MeHg and the chemical compositions of these samples vary widely according to locality and the surrounding environment [2, 3]. The main procedure for determining the amount of MeHg in soil/sediment samples at present is through elution of MeHg from the soil/sediment using an acid-leaching/alkaline digestion/distillation process, a solvent extraction for cleanup, an alkylation reaction for concentration of MeHg as volatile alkylated MeHg, and then determination by gas chromatography (GC) coupled with various mercury detection techniques such as atomic fluorescence spectroscopy (AFS) [4, 5] and inductively coupled plasma mass spectroscopy (ICP-MS) [6, 7].

Alkylation methods such as ethylation are effective concentration methods for the determination of MeHg. Although pg/L levels of MeHg in a water sample were determined with alkylation-purge-trap-GC-pyrolysis-AFS [8], this method presents a

problem when used to determine MeHg in soil/sediment. The problem is artifact formation of MeHg from inorganic mercury in soil/sediment samples through the alkylation reaction [6, 8-10]. Some HPLC methods were reported for the determination of MeHg from soil/sediment samples without using an alkylation reaction. However, those methods do not have sufficient sensitivity for determination of soil/sediment samples [11, 12]. Therefore, pre-concentration methods were also combined [13-15].

Recently, we proposed a novel sensitive HPLC-chemiluminescence detection system for mercury species in water and biological samples [16, 17]. Our proposed method is based on complex formation of mercury species and emetine-dithiocarbamate (emetineCS_2) ligand, HPLC separation, and chemiluminescent reaction detection. The absolute detection limits was <6 pg (as Hg). However, the effective measurement of MeHg in soil/sediment sample was difficult because the huge peak of Hg^{2+} was appeared in the chromatogram.

In this study, we propose a simple extraction technique coupled with HPLC-chemiluminescence detection system for measurement of MeHg and EtHg in soil/sediment samples. A developed extraction technique have allowed to selective measurement of MeHg and EtHg from the elution solution containing a high concentration of Hg^{2+}. In addition, preliminary study concerning a relationship between mercury contamination level and production of MeHg were also performed.

EXPERIMENTAL

Reagents and Solutions

A standard solution of mixed methylmercury and ethylmercury (10 ppm) was purchased

from Wako (Osaka, Japan). Tris(2,2′-bipyridine)ruthenium(II) chloride hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$) was purchased from TCI (Tokyo, Japan). EmetineCS_2 was prepared according to a published procedure [16]. Acetonitrile for the eluent was of guaranteed grade. All the other chemicals were of analytical reagent grade and were used without further purification. A 2 mM emetineCS_2 stock solution was prepared in methanol containing 1% NH_3 solution and stored in the freezer; this stock solution was diluted to 0.1 mM with acetonitrile before use. A 0.1 M Pd^{2+} solution and a 1 M Cu^{2+} solution were prepared from $PdCl_2$ and $CuSO_4 \cdot 5H_2O$ in a 5 M HCl solution, respectively. A 2 mM EDTA solution was prepared in a 20 mM borate buffer (pH 9.1). Water for all the solutions was purified using an Elix 5 UV (Millipore, Tokyo, Japan) and a Milli-Q Advantage system (Millipore).

Apparatus

The HPLC experiments were conducted using a chemiluminescence detection system. The system assembly consisted of two LC-20AD HPLC pumps (Shimadzu, Kyoto, Japan), a 320UP degasser (ERC, Saitama, Japan), an AS3500 autosampler (Dyonex, Osaka, Japan) equipped with a 200 μL sample loop, an *L*-column2 ODS (4.6 × 250 mm; i.d., 5 μm particle size, Chemical Evaluation and Research Institute, Tokyo, Japan), an HX-201 flow-through-type electrochemical reactor (Hokuto Denko, Tokyo, Japan), and a Comet2000 chemiluminescence detector. All connecting tubes were made of polyetheretherketone and had a 0.5 mm i.d. The column temperature was maintained at 25 °C using a CTO-10AC column oven (Shimadzu). Chromatograms were recorded with a Chromato-Pro data processor (Runtime Instruments, Kanagawa, Japan). Total-mercury (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)[2]. The total carbon content and inorganic carbon content were measured using a TOC-V analyzer equipped with a SSM-5000A module (Shimadzu), and the total organic carbon (TOC, %) content was calculated by subtracting the inorganic carbon value from the total carbon value. The chemical composition of the soil was measured using a wavelength dispersive X-ray fluorescence spectrometer (ZSX-mini, Rigaku Co. Tokyo, Japan). The moisture content was measured with an electronic moisture analyzer MA35 (Sartorius, Goettingen, Germany).

Soil/Sediment Samples

Soil/sediment samples were collected from five mercury-contaminated areas. Sediment samples were collected from Minamata Bay [2] and Kagoshima Bay [18]. Soil samples were also collected from locations near the Idria mercury mine (Slovenia) [3], the abandoned gold mine (Kagoshima, Japan), and a small-scale gold mining area (Indonesia) [19]. These samples were dried at 45 °C for 5 days and then finely ground in an agate mortar. The dried samples were used to measure the dry-base mercury concentration, TOC content, and chemical composition. To evaluate the accuracy of the method, a certified reference material (ERM CC580, estuarine sedimetnt) was also measured.

Analytical Conditions and Preparation Procedure of Soil/Sediment Samples for MeHg and EtHg Determination

Seventeen milliliters of 5 M HCl, 2 mL of a 1 M Cu solution, and 1 mL of a 0.1 M Pd solution were added to 0.1–1 g of the sample or a needed volume of mercury standard

144 solution in a 50 mL centrifuge tube. The mixture was shaken for 60 min and then
145 centrifuged for 10 min at 3000 rpm. The supernatant was decanted into a 40 mL conical
146 bottom centrifuge tube for solid–liquid separation and then 5 mL of toluene was added.
147 The mixture was shaken for 10 min and then centrifuged for 5 min at 3000 rpm. The
148 aqueous phase was removed with suction apparatus and then centrifuged for 5 min at
149 3000 rpm again. A 4 mL aliquot of the toluene phase was transferred to a 10 mL
150 centrifuge tube and then 1 mL of a 2 mM EDTA solution was added. The mixture was
151 shaken for 30 s and then centrifuged for 5 min at 1200 rpm. A 500 μL aliquot of the
152 aqueous phase was transferred to a 1.8 mL vial of auto-sampler and then 500 μL of
153 acetonitrile and 20 μL of 0.1 mM emetineCS_2 solution were added. A 200 μL aliquot
154 was injected into the HPLC-CL detection system.

155 The HPLC conditions used for soil/sediment sample analysis were as follows:
156 The eluent was a 20 mM citrate buffer (pH 3.1)–acetonitrile (50:50, v/v) solution; this
157 eluent was delivered at a flow rate of 1.5 mL/min. The $Ru(bpy)_3^{2+}$ solution was
158 composed of 0.25 mM $Ru(bpy)_3Cl_26H_2O$ and 0.1 M sulfuric acid and was delivered at a
159 flow rate of 0.3 mL/min. The electrolytic current of the electrochemical reactor was set
160 at 200 μA. These conditions were determined in a previously published paper [16, 17].
161
162
163 **RESULTS AND DISCUSSION**
164 ***Selective Extraction of Methylmercury and Ethylmercury***
165 An organic solvent extraction step was necessary to reduce the amount of inorganic
166 mercury from the sample extract because direct determination using acid leaching was
167 difficult because of the high concentration of Hg^{2+}. We first employed an HCl

168 leaching-toluene extraction system. A summary of our work is as follows: The method
169 was based on the extraction of MeHg as MeHgCl to toluene phase, while Hg^{2+} was not
170 extracted to form the $HgCl_4^{2-}$ complex. MeHgCl in toluene was back-extracted to an
171 aqueous phase with an EDTA solution, creating a MeHg–EDTA complex. Finally,
172 emetineCS_2 solution was added to the MeHg–EDTA complex solution to form an
173 emetineCS_2–MeHg complex. Figure 1(A) shows typical chromatograms obtained from
174 three different concentrations of HCl solutions containing 10 mg L^{-1} of Hg^{2+}, 0.2 μg L^{-1}
175 of MeHg, and 0.2 μg L^{-1} of EtHg as a model eluted solution. Clearly, the use of a 5 M
176 HCl solution led to the effective elimination of Hg^{2+} with toluene extraction. Recent
177 reports have described a microwave-assisted nitric acid–leaching method employed for
178 the determination of MeHg in soil/sediment samples [5, 20, 21]. However, Hg^{2+} was
179 extracted to toluene as $Hg(NO_3)_2$ and EtHg was decomposed in nitric acid solution, as
180 shown in Fig. 1(B). Therefore, the nitric acid leaching-solvent extraction system was
181 not suitable from the points of view of sample cleanup to reduce the amount of Hg^{2+} and
182 determination of EtHg.

183 To establish the optimal conditions for a sensitive, stable, and simple procedure,
184 the effects of various parameters on the extraction procedure were investigated. First,
185 the effect of extraction time on the extraction efficiency of MeHg was investigated.
186 Because the extraction efficiencies remained constant in the range of 5–120 min, an
187 extraction time of 10 min was selected. A 5 mL volume of toluene was employed to
188 make the sample handling easier. EDTA was selected as a complexing agent for
189 back-extraction for the following reasons: The stability of an EDTA–mercury complex
190 is reasonably high for back-extraction and an emetineCS_2–mercury complex is easily
191 formed with the addition of emetineCS_2 to the solution containing the EDTA–mercury

192 complex. In addition, the chemiluminescence reaction between $Ru(bpy)_3^{3+}$ and EDTA
193 was weak under our employed reaction conditions (pH 1.8) [22]. The effect of the
194 back-extraction solution pH on the extraction efficiency was also investigated. A
195 constant back-extraction rate was obtained in the pH range of 7–10 when 1 mL of a 2
196 mM EDTA solution was added to 4 mL of a 1 μg L^{-1} MeHg–toluene solution. Therefore,
197 a 2 mM EDTA solution prepared with a 20 mM borate buffer (pH 9.1) was selected as a
198 back-extraction solution. In addition, the back-extraction time of 30 s was selected
199 because the extraction efficiency was constant with hand shaking at 5 s. The volume of
200 back-extraction solution employed was 1 mL in this study, although the small volume of
201 back-extraction solution led to a high-concentration of final sample solution because
202 liquid handling of such a small volume was difficult and directly affected repeatability.
203
204 ***Effect of Masking Metal Ions for Sample Matrixes***
205 Recovery tests were performed with seven different soil or sediment samples, as listed
206 in Table 1. A 5 M HCl solution of 160 mL was added to 2.0 g of the samples except for
207 ERM CC580 (1.0 g). The mixture was shaken for 60 min and then centrifuged for 10
208 min at 3000 rpm. The supernatant was decanted for solid-liquid separation and then the
209 supernatant was divided into six portions at 20 mL each in 40 mL conical bottom
210 centrifuge tube. Three portions were spiked with 6 ng MeHg and other three portions
211 were kept as blank sample. These sample solutions were treated with the preparation
212 procedure as described above. As shown in Fig. 2, recovery values were considerably
213 different for each sample when only 5 M HCl was used. This difference could be
214 attributed to the eluted sample matrix because the components of the sample were
215 different. These results suggested that eluted matrix compounds in soil/sediment

samples intercept the extraction and/or back-extraction process of MeHg. To eliminate the effect of matrix compounds, various soft-metal ions were added to the 5 M HCl solution. Although the most effective masking reagent would have been Hg^{2+}, its use may have caused an artifact problem [23]. The addition of Cu^{2+} has been recommended to release MeHg from the adsorbed Hg species in the solid samples [8]. The addition of 0.1 M Cu^{2+} to 5 M HCl improved the recovery values of samples except for the marine sediment samples. Furthermore, we found that the addition of Pd^{2+} in this solution significantly improved the recovery values of all samples. These results suggested that eluted MeHg from the solid samples can be detected quantitatively with the preparation procedure.

Next, the role of Cu^{2+} and Pd^{2+} was investigated using two different back extraction reagents, EDTA and cysteine. When cysteine was employed as a complexing reagent for back-extraction, complex formation of emetineCS_2 and MeHg did not occur. Therefore, concentrations of MeHg in back-extracted solutions were measured as T-Hg. A 5 M HCl solution or a 5 mM Pd^{2+} and 0.1 M Cu^{2+} containing 5M HCl solution of 200 mL was added to 2.5 g of ERM CC580. The mixture was shaken for 60 min and then centrifuged for 10 min at 3000 rpm. The supernatant was decanted for solid-liquid separation and then the supernatant was divided into six portions at 20 mL each in 40 mL conical bottom centrifuge tube. 5 mL of toluene was added each. The mixtures were shaken for 10 min and then centrifuged for 5 min at 3000 rpm. The aqueous phase was removed with suction apparatus and then centrifuged for 5 min at 3000 rpm again. A 4 mL aliquot of the toluene phase was transferred to a 10 mL centrifuge tube and then 1 mL of a 2 mM EDTA solution or a 0.1% cysteine solution was added. The mixture was shaken for 30 s and then centrifuged for 5 min at 1200 rpm. A 800 μL aliquot of the

aqueous phase was measured as sample solution.

Figure 3 shows the obtained results. When a cysteine solution was used, the same amount of mercury was detected in both 5 M HCl alone and 5 M HCl containing Cu^{2+} and Pd^{2+}. When the EDTA solution was used, a small amount of mercury was detected in the 5 M HCl solution, although the same level of mercury compared with that of cysteine was detected in the 5 M HCl containing Cu^{2+} and Pd^{2+}. These results suggested that the extracted form of MeHg to toluene was not only the MeHgCl form. MeHg was extracted to the toluene phase with matrix compounds, which were more stable complexes in comparison with the EDTA–MeHg complex but weaker complexes than the cysteine–MeHg complex. Briefly, the role of Cu^{2+} and Pd^{2+} is masking of the matrix compounds to form a MeHgCl complex only.

Analytical Figure of Merits

The typical chromatograms obtained from standard solution which prepared with a series of sample preparation procedure were shown in Fig.4a. The calibration curves for MeHg and EtHg, using peak height, were linear from 0.5–20 ng (as Hg; data points were at least five; coefficients of determination were over 0.995). The detection limits were 0.16 ng (signal-to-noise ratio of 3). The repeatability of the whole procedure was 2.0% and 1.4% (1 ng, n = 3). The sample throughput of the HPLC system was 4/h. Our proposed method is a very simple device configuration compared with previously reported other HPLC methods because it does not require the decomposition of mercury compounds followed by HPLC separation and the high purity gas for detection process [11-15].

Determination of Various Soil/Sediments Samples

To evaluate the accuracy of the developed method, a certified reference material CC580 (estuarine sediment, ERM) was analyzed (Fig. 4b). Although 77% of the contained mercury (T-Hg, 132 mg kg^{-1}) was eluted with 5 M HCl leaching, the peak of Hg^{2+} is considerably smaller than that of MeHg. The results using the proposed method were in good agreement with the values of the certified reference material. Determination results of a standard sample listed in Table 3. In the results of CC580 and soil 3 (near abandoned gold mine, Kagoshima, Japan) samples, EtHg were detected at the μg kg^{-1} level (Fig. 4b). There are few reports about the existence of EtHg in soil/sediment [24]. The existence of EtHg in natural soil can give us important information to elucidate the Hg cycling in the environment.

The relationship between inorganic mercury pollution levels and the concentration of MeHg was confirmed using Indonesian soil/sediment samples. As shown in Fig. 5, the MeHg concentration was not simply related to the inorganic mercury pollution level. Although the chemical composition, TOC content, and mercury contamination level were almost same, the MeHg concentrations in soil samples from the paddy field were extremely high in comparison with the land soil samples. The paddy fields may therefore play an important role for MeHg formation.

Conclusion

A novel determination method for MeHg and EtHg in soil/sediment samples using an HPLC–CL system coupled with simple extraction techniques has been described. It was

found that the addition of Cu^{2+} and Pd^{2+} in the eluted solution became an effective masking reagent to extract MeHg to the organic phase in a simple form. The developed selective extraction technique for organomercury compounds would be applied with other determination methods because it is simple and effective. Further investigation on various elution methods, such as ultrasonication and microwave, at various elution solutions are needed for the confirmation of the complete elution of MeHg from various soil/sediment samples. In addition, the presence of EtHg in soil was confirmed using our proposed method. The presence of EtHg in the environment has scarcely been reported. Because our proposed method can easily detect EtHg and measure MeHg at the same time, a breakthrough regarding EtHg in the environment is anticipated.

Acknowledgments

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313 **References**

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360

361 **Figure Captions**

362 Fig. 1 Typical chromatograms obtained for (A) three different concentrations of HCl
363 solution and (B) 5 M HCl and HNO_3 as extraction solutions. Sample, 4 ng MeHg and
364 EtHg/20 mL of extraction solutions containing 10 ppm Hg^{2+}.

365

366 Fig. 2 Recovery values of seven different samples with three different extraction
367 solutions. The details of the sample are listed in Table 1. Extraction solution, 5 M HCl
368 only (dark gray), 5 M HCl containing 0.1 M Cu^{2+} (light gray), 5 M HCl containing 0.1
369 M Cu^{2+} and 5 mM Pd^{2+} (white). Recovery value = (Spiked - Blank)/6 × 100 (mean ±
370 s.d., n = 3, %) .

371

372 Fig. 3 Back-extraction efficiencies of EDTA and cysteine back-extraction solutions
373 with two different extraction solutions. Extraction solution, (a) 5 M HCl only (b) 5 M
374 HCl containing 0.1 M Cu^{2+} and 5 mM Pd^{2+}. Back-extraction solution, EDTA: 2 mM
375 EDTA prepared with 20 mM borate buffer (pH 9.1), Cysteine: 0.1% *L*-cysteine prepared
376 with 20 mM borate buffer (pH 9.1). Obtained values are the mean of three
377 determinations ± s.d..

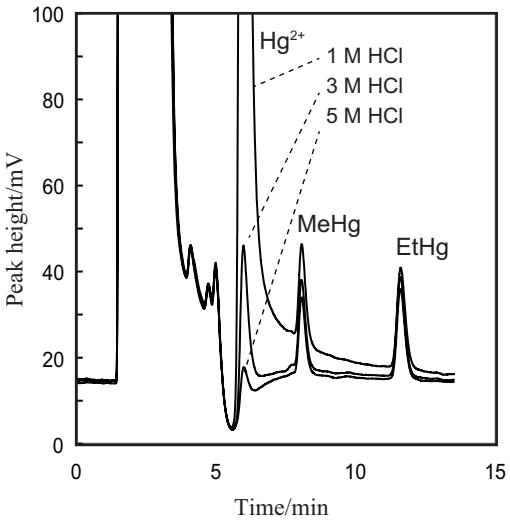
378

379 Fig. 4 Typical chromatograms obtained of (a) standard solution and (b) estuarine
380 sediment (ERM CC580). Inset of (b): 20-times expansion of chromatograms.

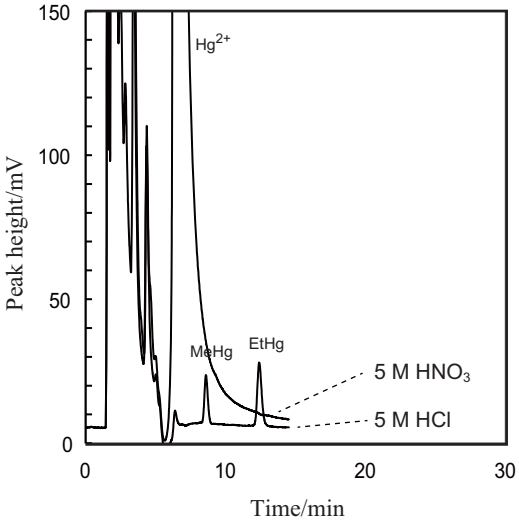
381

382 Fig. 5 Relationship between T-Hg concentrations and MeHg concentrations. Sample,
383 land soils (n = 28) (■), paddy field soils (n = 40) (□), river sediments (n=9), (Δ) and

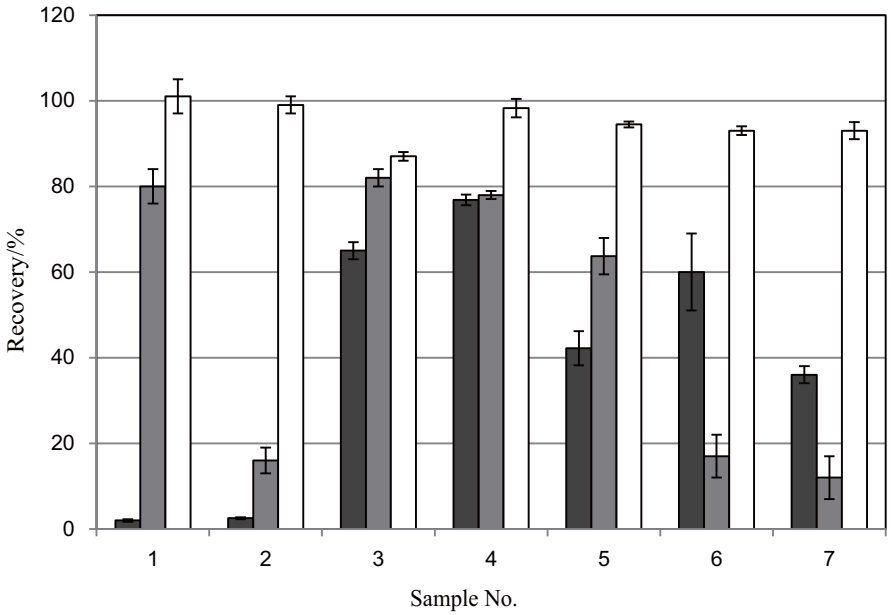
384 pond sediments (n = 2) (▲) of Indonesia.


100
80
60
40
20
0
Peak height/mV
0
5
10
15
Time/min
Hg2+
1 M HCl
3 M HCl
5 M HCl
MeHg
EtHg

(A)


150
100
50
0
Peak height/mV
0
10
20
30
Time/min
Hg2+
MeHg
EtHg
5 M HNO3
5 M HCl

(B)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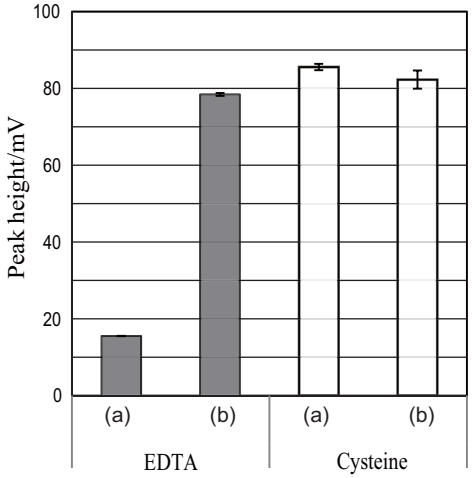
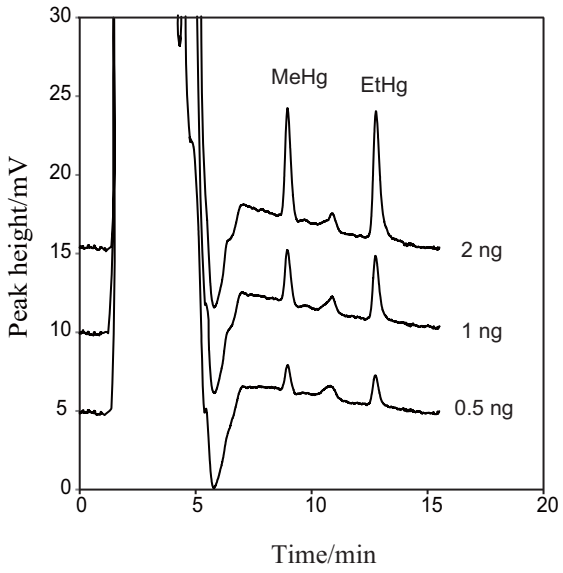
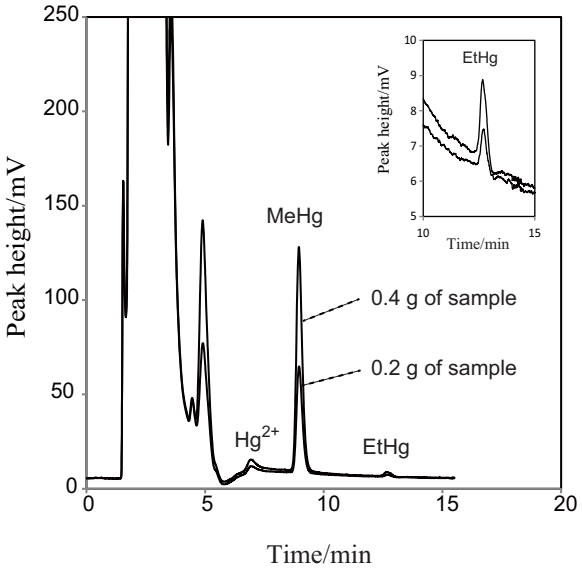
100
80
60
40
20
0
Recovery/%
1
2
3
4
5
6
7
Sample No.


100
80
60
40
20
0
Peak height/mV
(a)
(b)
(a)
(b)
EDTA
Cysteine


Peak height/mV
Time/min
MeHg
EtHg
2 ng
1 ng
0.5 ng

(a)


Peak height/mV
Time/min
MeHg
EtHg
Hg2+
0.4 g of sample
0.2 g of sample
Peak height/mV
Time/min

(b)

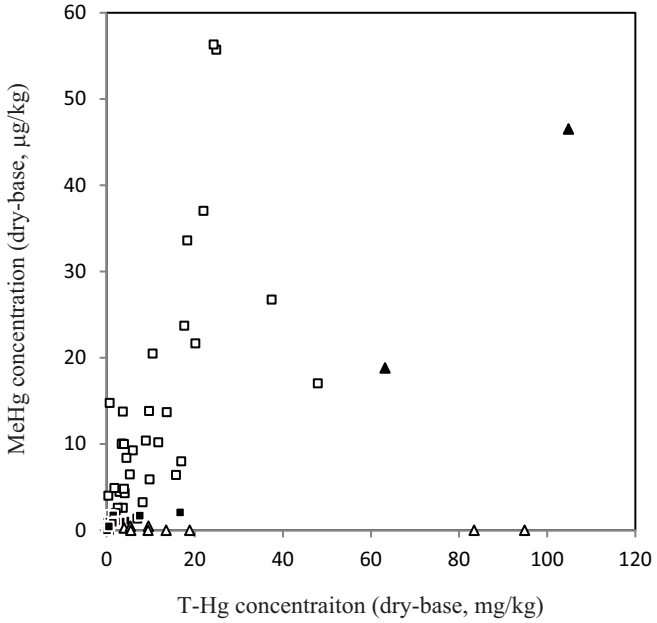

60
50
40
30
20
10
0
MeHg concentration (dry-base, µg/kg)
0
20
40
60
80
100
120
T-Hg concentraiton (dry-base, mg/kg)

Table 1 T-Hg concentration and properties of standard seven different samples

No	Sample	T-Hg (mg/kg)[a]	Acid-labile Hg (%)[b]	TOC (%)	Main chemical composition (%, as oxide)			
					SiO_2	Al_2O_3	CaO	Fe_2O_3
1	Estuarine Sediment (ERM CC580)	132 ± 3*	77	5.8	14.6	5.4	64.7	4.0
2	Soil 1 (near Idria Mercury Mine)	516 ± 34	8	1.1	42.0	14.5	26.1	5.8
3	Soil 2 (near abandoned Gold Mine)	74 ± 5	34	0.9	78.3	10.6	0.4	4.0
4	Soil 3 (near abandoned Gold Mine)	138 ± 4	23	2.5	68.5	16.2	1.4	6.5
5	Soil 4 (Paddy field in Indonesia)	7.7 ± 1	77	2.3	58.1	23.3	1.6	11.5
6	Marine Sediment 1 (Minamata Bay)	2.98 ± 0.03	77	1.2	53.8	19.0	6.9	10.5
7	Marine Sediment 2 (Kagoshima Bay)	1.57 ± 0.02	98	1.7	59.6	13.0	3.8	6.3

[a] Determination by CV-AAS (Mean ± s.d., n = 2) *Certified value.

[b] (Concentration of eluted Hg with 5 M HCl leaching/T-Hg concentration) × 100

Table 2 Determination results obtained from a proposed HPLC-CL method

No	Sample	Amount (g)	MeHg (μg/kg)	EtHg (μg/kg)
1	Estuarine Sediment (ERM CC580)*	0.1	72.2 ± 0.4	n.d.
		0.2	72.7 ± 0.8	1.42 ± 0.07
		0.4	-	1.26 ± 0.08
2	Soil 1 (near Idria Mercury Mine)	0.5	3.3 ± 0.6	n.d.
		1.0	3.44 ± 0.04	n.d.
3	Soil 2 (near abandoned Gold Mine)	1.0	0.48 ± 0.02	n.d.
4	Soil 3 (near abandoned Gold Mine)	0.5	9.04 ± 0.03	1.62 ± 0.01
5	Soil 4 (Paddy field in Indonesia)	0.25	7.59 ± 0.05	n.d.
6	Marine Sediment 1 (Minamata Bay)	0.5	0.98 ± 0.11	n.d.
		1.0	1.08 ± 0.05	n.d.
7	Marine Sediment 2 (Kagoshima Bay)	1.0	0.42 ± 0.02	n.d.

Obtained values are the mean of three determinations ± s.d.

*Certified value of MeHg is (75 ± 4) μg/kg