Fresenius J Anal Chem (2000) 366 : 453–460

SPECIAL ISSUE PAPER

Martina Logar · Milena Horvat · Ingrid Falnoga
Vekoslava Stibilj

A methodological study of mercury speciation using Dogfish liver CRM (DOLT-2)

Received: 1 August 1999 / Revised: 13 September 1999 / Accepted: 18 September 1999

Abstract The purpose of the study was to optimise analytical methods for determination of the chemical speciation of mercury in studies of protective mechanisms of selenium. Optimisation of the methods was performed using CRM DOLT-2 (Dogfish liver), both in its original form and after separation of various fractions. The sample was homogenised with 10 mM Tris-HCl buffer (pH 7.6) and ultracentrifuged. The soluble phase obtained was applied to a size exclusion chromatography column (Sephadex G-75 column) for separation of various protein fractions. Total mercury (total Hg), monomethyl mercury (MeHg) and selenium (Se) were determined in whole dogfish liver tissue and its soluble and insoluble phases (pellet). Different approaches for determination of total Hg and MeHg were compared. Simultaneous determination of MeHg and inorganic mercury (Hg^{2+}) was based on alkaline dissolution and/or acid leaching, followed by ethylation, room temperature precollection, isothermal gas chromatography (GC), pyrolysis and detection with cold vapour atomic fluorescence spectrometry (CVAFS). The sum of MeHg and Hg^{2+} was compared to total Hg results obtained by acid digestion and CVAAS detection. The accuracy of MeHg determination was checked by its determination using acid leaching at room temperature, solvent extraction, back extraction into Milli-Q water, ethylation, GC and CVAFS detection. For the insoluble phase it is recommended to use solvent extraction for MeHg and acid digestion CVAAS for total Hg. For determination of MeHg and Hg^{2+} in the lyophilised sample and water soluble fractions containing low concentrations of mercury species, the simultaneous measurement of MeHg and Hg^{2+} after alkaline dissolution is the most appropriate method.

1 Introduction

The capability of organisms to cope with harmful reactive metal ions in the environment is invariably linked to the existence of specific metal-binding proteins. Among them the low molecular weight proteins called metallothioneins (MTs) with SH-based clusters are believed to be important in short-term protection against toxic levels of some metal ions (Cd, Hg, Cu, Zn) [1], and in the case of mercury recent investigations have been focused particularly on selenoproteins [2].

For studies of metal metabolism the speciation of the element is very important. The total concentration of the element is of little value without knowledge of its chemical forms.

Mercury exists in a large number of different chemical and physical forms with a wide range of properties. The most toxic mercury compound is MeHg, the organic form of mercury. MeHg is mainly formed in the aquatic environment by biotic and/or abiotic processes. The accumulation of MeHg in biota, and its biomagnification in aquatic food chains are of particular concern due to its extreme toxicity and its ability to bioaccumulate in fish tissues.

During recent years many analytical techniques for determination of total Hg and MeHg in biological materials have been available. They involve, in most cases, a succession of analytical steps (extraction, derivatisation, separation, detection) which may all be prone to systematic errors [3]. Over ten years ago, these methods were poorly validated due to a lack of evaluation programmes and of certified reference materials. The situation improved recently thanks to the development of more sensitive and specific analytical techniques, the organisation of intralaboratory studies and the availability of certified reference materials (CRMs) [4].

The purpose of this work was to compare and optimise different analytical techniques in order to find the most suitable, simple, accurate and sensitive way of determining total Hg and MeHg in very diluted biological samples, where only small amounts of sample with a low concentration of MeHg are available. Experiments were performed on DOLT-2, Dogfish liver, a lyophilised certified reference material (CRM) produced by the National Research Council of Canada (NRCC). This reference material was selected in order to make our results comparable

M. Logar · M. Horvat (✉) · I. Falnoga · V. Stibilj
Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

with those obtained in other laboratories engaged in similar studies. DOLT-2 is certified for total Hg, MeHg and Se, and contains relatively high concentrations of these metals.

2 Experimental

Reagents

1% (w/v) sodium tetraethylborate solution: prepared from $NaBEt_4$ (Strem Chemicals, Newburyport MA, USA) in Milli-Q water containing 1% KOH (analytical grade, Merck, Darmstadt, Germany); 2 M potassium acetate buffer: prepared from potassium acetate (extra pure, Merck) and acetic acid (Suprapur, Merck) in Milli-Q water; 25% (w/v) KOH in methanol solution: prepared from KOH (analytical grade, Merck) and methanol (SupraSolv, Merck); 6 M, 8 M HCl solutions: prepared from HCl (30%, Suprapur, Merck) in Milli-Q water; 5% $SnCl_2$ solution: prepared from $SnCl_2 \cdot 2H_2O$ (analytical grade, Merck) in Milli-Q water containing 3 M H_2SO_4 (Suprapur, Merck); 5% (v/v) H_2SO_4 solution: prepared from H_2SO_4 (Suprapur, Merck) in Milli-Q water; 18% (w/v) KBr solution: prepared from KBr (analytical grade, Carlo Erba, Milano, Italy) in Milli-Q water; 1 M $CuSO_4$ solution: prepared from $CuSO_4 \cdot 5H_2O$ (analytical grade, Merck) in Milli-Q water; CH_2Cl_2 (SupraSolv, Merck); HNO_3 (65%, Suprapur, Merck); Tenax (20/35 mesh, Alltech, Deerfield IL, USA); OV-3 15% (Sigma-Aldrich, Deisenhofen, Germany); argon; nitrogen; Milli-Q deionised water (> 18 MΩcm, Millipore, Bedford MA, USA).

Standard solutions

1 ng/mL working standard solutions were used for MeHg (as Hg) and Hg^{2+} for calibration of the CVAFS system, and a 10 ng/mL Hg^{2+} working standard solution for CVAAS measurements. The standards were prepared in Milli-Q water by dilution of stock solutions. They were prepared daily and stored in the dark at 4 °C.

Cleaning procedure

Special precautions must be taken to avoid contamination. All glass and Teflon materials need to be cleaned carefully. All vessels were left to soak in a soap (Micro-90, Bioblock, Illkirch Cedex, France) solution overnight. They were thoroughly rinsed first with tap water then with Milli-Q water. The vessels were placed in 50% (v/v) concentrated HNO_3 solution and heated at 60 °C for 2 days. After being thoroughly rinsed with Milli-Q water, vials were transferred in 10% (v/v) concentrated HCl solution and left to soak for a further day at room temperature. They were thoroughly rinsed again with Milli-Q water, filled with 1% HCl and stored in polyethylene plastic bags. Vials were emptied just before use for sample processing.

Biochemical separation procedure

A schematic presentation of the biochemical separation is shown in Fig. 1. CRM DOLT-2 lyophilised liver tissue was used for separation of pellet and supernatant (cytosol) and for isolation of water soluble proteins with accompanying bound metals (Se, Zn, MeHg, Hg). Among the protein fractions, metallothionein protein was partly characterised by its molecular weight (MW), Zn content and the absence of a UV absorption peak at 280 nm.

For sample preparation, 2 g and 4 g of sample was homogenised (1 : 9 or 1 : 5.7 w/v) with a glass homogeniser and a motor-driven Teflon™ pestle (1000 rpm) in nitrogen-saturated Tris-HCl buffer (pH 7.6, at 1–4 °C), 10 mM dithiothreitol (DTT), 25% saccharose and 0.1 mM PMSF (phenylmethylsulfonyl fluoride). The 15 or 10% homogenate was centrifuged at 100 000 g for 90 min at

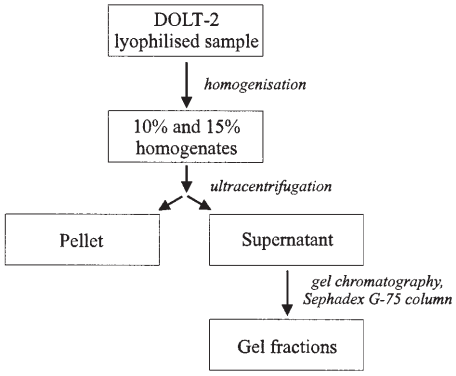


Fig. 1 Biochemical separation procedure (at 4 °C)

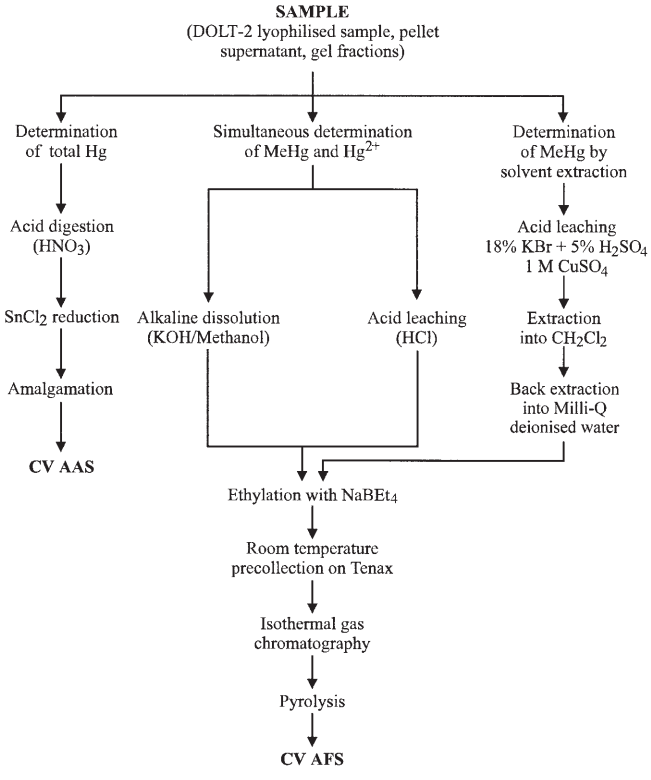


Fig. 2 Schematic presentation of methods for determination of total Hg and MeHg

4 °C in a Centrikon T-2070 ultracentrifuge (Kontron instruments; rotor TFT 70.38). Supernatant was decanted from pellet and both parts were frozen until further analysis (metal determination, gel filtration).

5 mL of the corresponding supernatant was applied to a calibrated 2.4 × 65 cm Sephadex G-75 column and eluted at 13.8 mL/h in a nitrogen atmosphere at 4 °C with 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM DTT. The UV absorption at 280 and 254 nm and the concentrations of metals (Se, Zn, Hg and MeHg) were determined in the column fractions (5 mL).

The column was standardised with marker proteins of known molecular weight (Pharmacia AB, gel filtration kit): blue dextran (MW 2000000), bovine serum albumine (MW 67000), chymotripsinogen A (MW 25000), myoglobin (MW 17000) and cytochrome c (MW 12400). The exclusion range was from 70 kDa to 5 kDa.

Methods for determination of total Hg and MeHg

Various approaches for determination of total Hg and MeHg were used as presented in Fig. 2. Some of the methods were previously optimised and they were used in this study to compare the results obtained by modified methods. Most emphasis was placed on the first step, which releases MeHg and Hg^{2+} bound in tissues. Therefore, the efficiency of alkaline dissolution and acid leaching methods were compared. Experiments were performed on DOLT-2 lyophilised sample, pellet and supernatant solution. Gel fractions were analysed only by the most appropriate technique, because of the small quantities of sample available. In such experiments it is advantageous to use the CRM as all steps can easily be checked for accuracy.

Determination of total Hg

Approximately 0.2 g of sample was weighed directly in a Pyrex digestion vessel, acidified with 2 mL of concentrated HNO_3 and left to react at room temperature at least an hour. The ampoules were then sealed. The strong acid digestion decomposes the samples and oxidises and converts all organic forms of mercury into inorganic mercury. A complete digestion of organic matter was achieved by heating the ampoules in an aluminium block for 12 h on a hot plate at 70 °C. After digestion was completed, the tubes were cooled in liquid nitrogen and opened. The contents of the vessels were transferred to 50 mL volumetric flasks and diluted with Milli-Q water to the mark. An aliquot of the digest (0.02–2 mL) was added to the reduction vessel containing 10 mL 5% $SnCl_2$ solution. After reduction of Hg^{2+} with $SnCl_2$ to elemental mercury, Hg^0 was swept from the solution by aeration with N_2 and concentrated on a gold trap. The collected Hg was thermally desorbed from the gold wire by heating the trap to 600 °C and measured by cold vapour atomic absorption spectrophotometry (CVAAS). The limit of detection calculated on the basis of three times the standard deviation of blanks was 0.2 ng/g [5, 6].

Determination of MeHg

The preseparation step of the solvent extraction technique developed by Liang et al. [7] was modified. Acid leaching at room temperature instead of alkaline digestion at high temperature was used.

Approximately 0.1 g of sample was placed in a screw capped 30 mL Teflon vial for 15 min at room temperature with a mixture comprising 5 mL of 5% H_2SO_4 + 18% KBr and 1 mL of 1 M $CuSO_4$ solution. 10 mL of CH_2Cl_2 was added to each vial and the samples were shaken vigorously for 15 min. After this step, the samples were centrifuged for 5 min at 3200 rpm. The organic and aqueous phases were separated in a Teflon separating funnel. The CH_2Cl_2 phase was transferred to a 60 mL Teflon vessel. The extraction was repeated with additional 5 mL of CH_2Cl_2. 20 mL of Milli-Q water was added to the combined CH_2Cl_2 extracts, followed by solvent evaporation at about 80 °C on a water bath and completed by purging with N_2 for 5 min to remove remaining CH_2Cl_2. An aliquot (30 µL–2 mL) of the aqueous sample was added to 40 mL of Milli-Q water in a Teflon reaction vessel. The sample was buffered with an appropriate amount of acetate buffer (min. 0.2 and max. 2 mL) to reach a pH of 4.9 as necessary for the ethylation process and 50 µL of 1% of $NaBEt_4$ solution was added. The vessel was immediately closed, and mixture allowed to react without bubbling for 15 min. Ethylated MeHg as ethylmethylmercury was purged onto Tenax traps for 15 min with Hg-free nitrogen and thermally desorbed (200 °C) onto an isothermal GC column at 80 °C. Under a flow of argon the eluted Hg species were converted into Hg^0 by pyrolytical decomposition at 800 °C and then detected by a cold vapour atomic fluorescence detector. The limit of detection calculated on the basis of three times the standard deviation of blanks was about 0.02 ng/g, when 0.1 g of sample was analysed [8, 9].

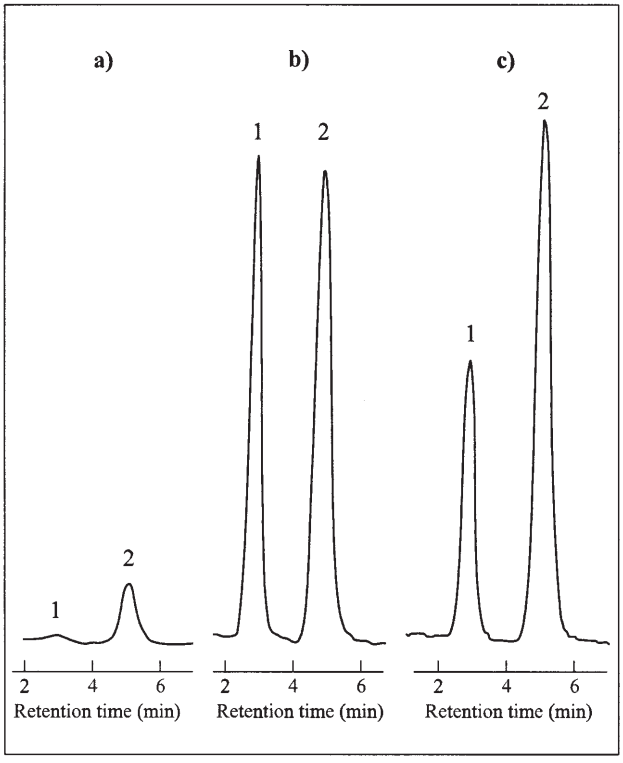


Fig. 3 Typical chromatograms obtained in simultaneous determination of MeHg and Hg^{2+} after acid leaching of DOLT-2 lyophilised sample. a) Reagent blank. b) Standards: 100 pg of MeHg (as Hg) and 100 pg of Hg^{2+}. c) 50 µL of acid leachate: 57.1 pg of MeHg (as Hg) and 114.1 pg of Hg^{2+}. Peaks: 1 = $CH_3HgCH_2CH_3$; 2 = $(CH_3CH_2)_2Hg$

Simultaneous determination of MeHg and Hg^{2+}

For simultaneous determination of MeHg and Hg^{2+} two methods were compared: alkaline dissolution and acid leaching.

Alkaline dissolution. For the alkaline dissolution method at elevated temperature (80 °C), 0.05 g of dry tissue, 0.1 g of wet tissue (pellet) or 0.2–1 g of fluids (supernatant and column fractions) were dissolved with 2 mL of 25% KOH/methanol solution in a 30 mL screw capped Teflon vial for 3 h in an oven at 80 °C. The digest was cooled and according to the sample size and concentration of the Hg species, diluted with methanol prior to analysis. If samples were not analysed the same day as digested, they were reheated for 1 h at 80 °C in an oven before analysis to release MeHg and Hg^{2+} bound to particles [10].

Acid leaching. The same amount of sample as in alkaline dissolution was used. Acid leaching was performed in precleaned Teflon vials as for alkaline dissolution. Various concentrations of hydrochloric acid (6 M, 8 M and 11 M HCl) were applied and two different volumes of HCl (5 and 10 mL) were tested. Isolation of mercury species was performed at room temperature and after heating the samples for 5 min at 105 °C. HCl leaching at room temperature was performed by 24 h shaking on an automatic shaker. The samples were diluted to 25 mL with Milli-Q water prior to analysis. In hot acid leaching the diluted sample was heated at 105 °C for 5 min, left to cool and then analysed. In Fig. 3 some typical chromatograms obtained by simultaneous determination of MeHg and Hg^{2+} after acid leaching of DOLT-2 lyophilised sample are presented.

MeHg and Hg^{2+} in the alkaline dissolved sample and acid leachate were determined directly from solution and the ethylation and

measurement procedure described above for MeHg followed (the ethylation reaction results in the formation of ethylmethylmercury from MeHg and diethylmercury from Hg^{2+}). The limit of detection calculated on the basis of three times the standard deviation of blanks for 50 μL samples was about 1 pg for MeHg (as Hg) and 3 pg for Hg^{2+} [10].

Determination of Se

Selenium was determined by optimised atomic fluorescence spectrometry (AFS) coupled with the hydride generation technique [11]. 0.5–1 g of fluids (supernatant and column fraction) were placed in a 30 mL Teflon vial, and 1–0.5 mL of concentrated HNO_3 was added. The vials were closed and the samples were digested for 10 min on a sand bath at the temperature of boiling HNO_3. After cooling the samples, twice 0.5 mL of H_2O_2 was added and then evaporated to half volume. Then 1–0.5 mL of 6 M HCl was added and heated another 10 min on a sand bath. The digest was finally diluted to 5.0–2.0 mL with Milli-Q water prior to analysis. For selenium determination in tissue and pellet was used radiochemical neutron activation analysis (RNAA) described elsewhere [12, 13].

Analytical quality control

Analytical quality control of the results obtained for total Hg and MeHg was performed by analysis of certified reference materials (CRMs). CRMs should be of similar composition and concentration range to the sample analysed to ensure the accuracy of the results. TORT-1 (Lobster Hepatopancreas) and DOLT-2 (Dogfish liver) produced by the National Research Council of Canada (NRCC) were employed. These materials were analysed daily. Results for CRMs fell within acceptable ranges.

3 Results and discussion

The aim of this study was to optimise the most suitable methods for determination of total Hg and MeHg in small quantities of biological samples and with low contents of MeHg. As shown in Fig. 2 various approaches for simultaneous determination of MeHg and Hg^{2+} were tested.

In the case of alkaline dissolution for simultaneous determination of MeHg and Hg^{2+} a previously employed and validated method was used [10]. The data obtained for MeHg and Hg^{2+} in DOLT-2 lyophilised sample agreed well with the certified values. From previous experiments it was known that the amount of sample may affect the results for MeHg and Hg^{2+}. Possible matrix interferences were checked by the standard addition method. Samples were spiked before dissolution with 35.7 ng MeHg (as Hg) and 75 ng Hg^{2+} of standard solution. Various aliquots of alkaline dissolved sample were injected for ethylation. The results obtained are shown in Fig. 4. The average recovery for MeHg is 93.6% and 107.8% for Hg^{2+}. The high recovery for Hg^{2+} is most probably related to the decomposition of spiked MeHg. This is suggested by the lower values for MeHg and the fact that the sum of MeHg and Hg^{2+} spiked equals about 100%. This further suggests that spiked MeHg is not equivalent to endogenous MeHg and special precautions are necessary when the quality of data is checked by spiking experiments. In practical terms this means that 20–100 μg of the sample can be taken for ethylation without significant matrix interferences. Figure 5 shows that the slopes of standard addition curves for MeHg and Hg^{2+} are similar to the slopes obtained for calibration standards, indicating no matrix interferences for the conditions applied.

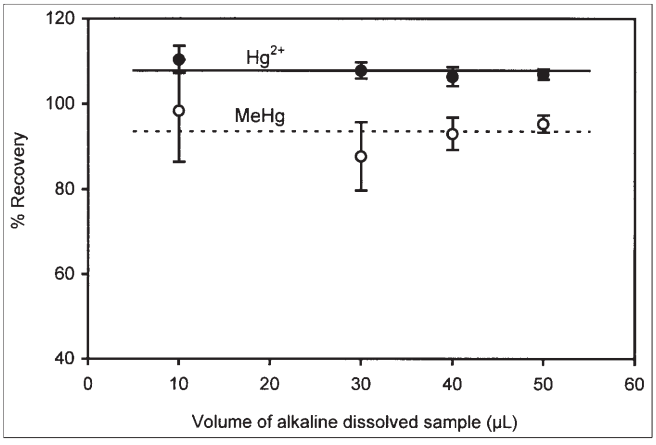


Fig. 4 The effect of addition of various aliquots of alkaline dissolved sample (50 mg/diluted to 25.3 mL) spiked with 35.7 ng MeHg (as Hg) and 75 ng Hg^{2+} on recoveries obtained in simultaneous determination of MeHg and Hg^{2+}

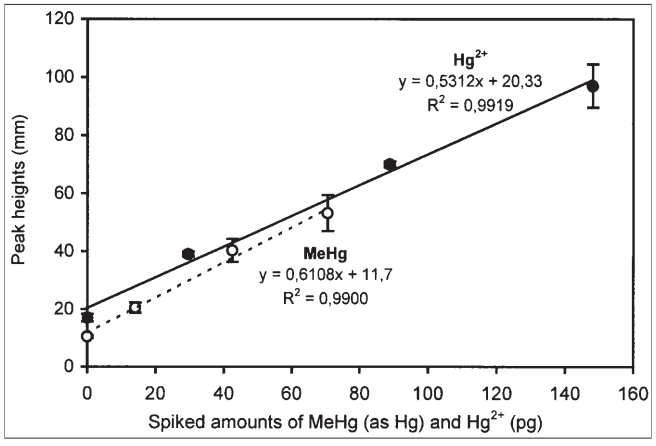


Fig. 5 Standard addition curves for MeHg and Hg^{2+} for alkaline dissolution of DOLT-2 lyophilised sample. The slopes obtained for calibration standards without sample are for MeHg y = 0.63 x and for Hg^{2+} y = 0.47 x

The present solvent extraction method (Fig. 2) differs from that described by Liang et al. 1996 [14] in the very first step [15]. Acid leaching at room temperature, commonly used for sediments, was used instead of alkaline digestion at high temperature. Our samples were digested with a mixture of H_2SO_4, KBr and $CuSO_4$ solution at room temperature instead of using KOH in methanol solution, acidification with concentrated HCl and heating the samples for 3 h at 80 °C. Copper (II) ions were added to the sample in order to increase the release of MeHg from bound sites. Use of H_2SO_4, KBr and copper (II) ions resulted in good recovery (about 95%) and repeatability of data for the samples used. Various CRMs were tested to

Table 1 Comparison of MeHg results for CRMs obtained by solvent extraction method with certified values

CRM			MeHg (as Hg) ng/g	
Producer	Code No.	Matrix	Measured values	Certified values
NRCC	DOLT-2	Dogfish Liver	660 ± 20	693 ± 53
NRCC	TORT-1	Lobster Hepatopancreas	124 ± 15	121 ± 14
NIST	SRM 2974	Mussel Tissue	74.0	77.2 ± 3.8
NIST	SRM 1566a	Oyster Tissue	15.8, 16.0	17.6 (inf.value)

Results are expressed as the mean value ± standard deviation of at least three or more independent determinations, on a dry weight basis
NRCC: National Research Council of Canada; NIST: National Institute of Standards and Technology

Table 2 Comparison of the results for MeHg obtained by solvent extraction method with simultaneous determination of MeHg and Hg^{2+} after alkaline dissolution

Sample	Solvent extraction	Simultaneous determination of MeHg and Hg^{2+}; alkaline dissolution
DOLT-2	660 ± 20	652 ± 40
10% Homogenate		
Supernatant	2.44 ± 0.21	2.38 ± 0.11
Pellet	426 ± 40	318 ± 10
15% Homogenate		
Supernatant	3.16 ± 0.21	4.20 ± 0.21
Pellet	409 ± 27	383 ± 10

Results are given in ng/g MeHg (as Hg) on a wet weight basis, expressed as the mean value ± standard deviation of at least three or more independent determinations, except for DOLT-2 on a dry weight basis
Certified value: MeHg (as Hg) 693 ± 53 ng/g

check this method and the results obtained are in good agreement with certified values (Table 1).

A comparison of the results for MeHg and total Hg in DOLT-2, supernatant solution and pellets obtained by different methods is presented in Tables 2 and 3.

The results for MeHg obtained by the solvent extraction method and by simultaneous determination of MeHg and Hg^{2+} after alkaline dissolution agree well for DOLT-2 lyophilised sample and for supernatant solution (soluble phase). For pellet (insoluble phase) much lower results were obtained by the second approach.

Similarly, for total Hg good agreement between CVAAS acid digestion and simultaneous determination of MeHg and Hg^{2+} for DOLT-2 lyophilised sample and supernatant solution was obtained. The concentration of total Hg found in pellet was much lower for simultaneous determination of MeHg and Hg^{2+}, which can be explained by lower recoveries for both species. In order to improve the recoveries for MeHg and Hg^{2+} the applicability of HCl leaching instead of alkaline dissolution was investigated.

Initially, alkaline dissolution was compared with HCl leaching using 5 mL of 6 M HCl. The results obtained are

Table 3 Comparison of the results for total Hg obtained by CVAAS acid digestion method with simultaneous determination of MeHg and Hg^{2+} after alkaline dissolution

Sample	CVAAS acid digestion	Simultaneous determination of MeHg and Hg^{2+}([a]); alkaline dissolution
DOLT-2	1960 ± 30	2040 ± 100
10% Homogenate		
Supernatant	7.91 ± 0.27	7.73 ± 0.07
Pellet	1510 ± 20	1170 ± 10
15% Homogenate		
Supernatant	11.9 ± 1.40	10.7 ± 0.19
Pellet	1510 ± 14	1200 ± 30

Results are given in ng/g on a wet weight basis, expressed as the mean value ± standard deviation of at least three or more independent determinations, except for DOLT-2 on a dry weight basis
Certified value: total Hg 1990 ± 100 ng/g
([a]) Sum of MeHg and Hg^{2+} gives total Hg value

shown in Table 4. Evidently, HCl leaching did not show any improvement of recoveries for MeHg and Hg^{2+}. Moreover, results for Hg^{2+} in pellet show much lower recoveries as compared to alkaline dissolution. From previous experience [16, 17] it is known that HCl should release MeHg and Hg^{2+} efficiently from biological samples, therefore further optimisation of acid leaching was done.

Acid leaching was performed under different experimental conditions. Various concentrations of HCl (6 M, 8 M and 11 M HCl), volumes of acid (5 and 10 mL) and temperature of leaching (at room temperature and after heating for 5 min on 105 °C) were used to evaluate the robustness of the method. In Table 5 results for DOLT-2 lyophilised sample are presented.

As evident, the volume of acid added at room temperature did not significantly affect MeHg data. Elevated temperature, a higher concentration of acid and smaller volume of acid gave lower MeHg results. After heating the samples the MeHg results were generally lower than data obtained by HCl leaching at room temperature. The difference is more pronounced at higher concentrations of HCl.

The results for Hg^{2+} are slightly elevated at higher concentration of added acid. Also, the volume of acid used affects the Hg^{2+} data; that is higher results in the case of 10 mL of HCl compared to 5 mL. The temperature does not significantly affect the Hg^{2+} concentration.

The sum of MeHg and Hg^{2+} corresponds to the total Hg value when simultaneous measurement was used. The sum shows good agreement with the certified values when 10 mL of HCl was used. Also, with this volume of HCl the temperature has no effect on total Hg data. When 5 mL of acid was used lower results for total Hg were obtained. In addition, the data obtained at higher temperature are lower compared to room temperature leaching. Close examination of these data shows that the reason for the lower total Hg data is the lower concentration of MeHg, particularly when a higher concentration of acid was used. If the lower results for MeHg were related to decomposition of MeHg, a higher concentration of

Table 4 Comparison of results for MeHg and Hg^{2+} obtained by simultaneous determination of MeHg and Hg^{2+} after alkaline dissolution and acid leaching

Results are given in ng/g on a wet weight basis, expressed as the mean value ± standard deviation of at least three or more independent determinations, except for DOLT-2 on a dry weight basis

Sample	MeHg		Hg^{2+}	
	Alkaline dissolution	Acid leaching 5 mL, 6 M HCl	Alkaline dissolution	Acid leaching 5 mL, 6 M HCl
DOLT-2	652 ± 40	662 ± 37	1390 ± 80	1203 ± 31
10% Homogenate				
Supernatant	2.38 ± 0.11	2.69 ± 0.13	5.31 ± 0.12	5.10 ± 0.10
Pellet	318 ± 10	300 ± 21	852 ± 26	511 ± 55
15% Homogenate				
Supernatant	4.20 ± 0.21	3.33 ± 0.0	6.51 ± 0.36	5.40 ± 0.70
Pellet	383 ± 10	327 ± 17	820 ± 30	531 ± 47

Table 5 Comparison of the results for DOLT-2 lyophilised sample obtained by simultaneous determination of MeHg and Hg^{2+} after acid leaching under different experimental conditions (temperature, concentration and volume of added acid)

	Volume of acid (mL)	6 M HCl		8 M HCl		11 M HCl	
		Room temperature	105 °C 5 min	Room temperature	105 °C 5 min	Room temperature	105 °C 5 min
MeHg	5	662 ± 37	577 ± 50	560 ± 50	483 ± 80	623 ± 20	437 ± 0
	10	646 ± 50	580 ± 80	640 ± 55	600 ± 15	713 ± 25	628 ± 10
Hg^{2+}	5	1203 ± 31	1193 ± 100	1390 ± 52	1314 ± 80	1530 ± 50	1329 ± 20
	10	1343 ± 90	1362 ± 69	1460 ± 20	1479 ± 60	1440 ± 20	1495 ± 60
total Hg[a]	5	1865 ± 93	1778 ± 90	1950 ± 95	1798 ± 80	2150 ± 54	1763 ± 39
	10	1990 ± 100	1920 ± 106	2100 ± 102	2112 ± 94	2150 ± 60	2100 ± 80

Results are given in ng/g (for MeHg as Hg) on a wet weight basis, expressed as the mean value ± standard deviation of at least three or more independent determinations, except for DOLT-2 on a dry weight basis

Certified values: MeHg (as Hg) 693 ± 53 ng/g, total Hg 1990 ± 100 ng/g, Hg^{2+} 1250–1344 ng/g (calculated)

[a] total Hg: sum of MeHg and Hg^{2+}

Table 6 Comparison of total Hg and MeHg results for DOLT-2 lyophilised sample obtained by CVAAS acid digestion method for total Hg and solvent extraction method for MeHg with simultaneous determination of MeHg and Hg^{2+} after alkaline dissolution and acid leaching

Total Hg			MeHg		
CVAAS acid digestion	Simultaneous determination of MeHg and Hg^{2+}([a])		Solvent extraction	Simultaneous determination of MeHg and Hg^{2+}([a])	
	Alkaline dissolution	Acid leaching		Alkaline dissolution	Acid leaching
1960 ± 30	2040 ± 100	1990 ± 100	660 ± 20	652 ± 40	646 ± 50

Results are given in ng/g (for MeHg as Hg), expressed as the mean value ± standard deviation of at least three or more independent determinations, on a dry weight basis

Certified values: total Hg 1990 ± 100 ng/g, MeHg (as Hg) 693 ± 53 ng/g

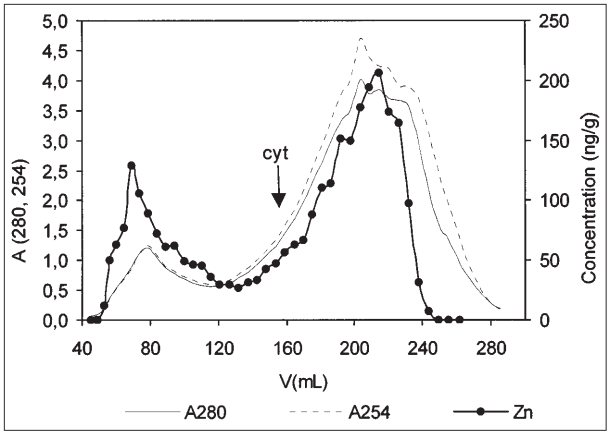
[a] Sum of MeHg and Hg^{2+} gives total Hg value

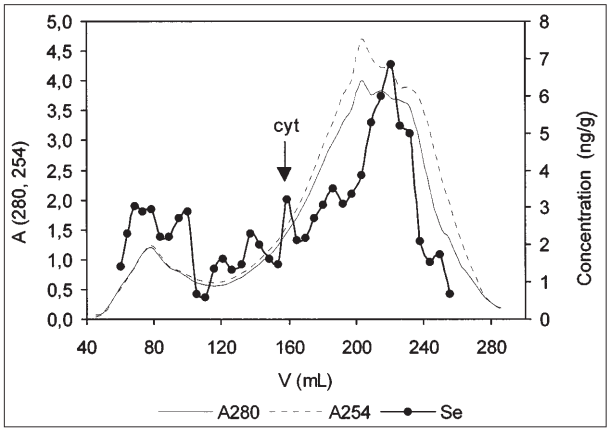
Table 7 MeHg, Hg^{2+}, total Hg, and Se distribution between water soluble phase (supernatant) and pellet from certified reference material DOLT-2

	Sample		% MeHg	% Hg^{2+}	% total Hg	% Se
DOLT-2	10% Homogenate	Supernatant	4	4	4	12
		Pellet	96	96	96	88
	15% Homogenate	Supernatant	4	3	3	10
		Pellet	96	97	97	90

Hg^{2+} would be obtained. However, the results presented in Table 5 show that Hg^{2+} results are also lower. This indicates that lower data are not related to decomposition of MeHg but due either to matrix interferences during the ethylation step and/or incomplete leaching.

It can be concluded that acid leaching with HCl could be a useful method for simultaneous determination of MeHg and Hg^{2+}. However, special attention should be paid to the volume of acid added and the temperature of leaching. It is recommended to use 6 M HCl at room temperature, and the volume of acid should be optimised in accordance with the amount of sample taken for analysis to prevent possible matrix interferences during the ethylation step and/or incomplete recoveries.





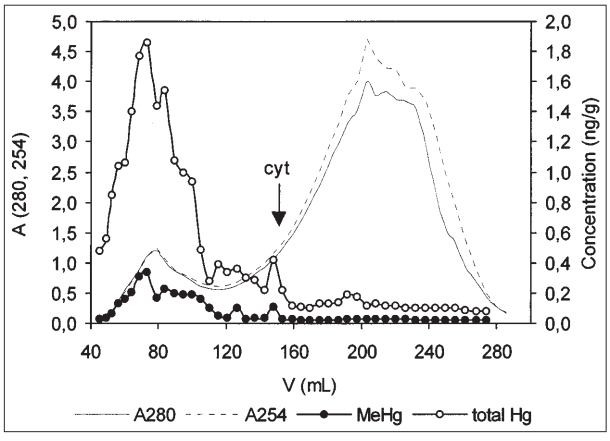


Fig. 6 Elution profiles of UV absorbances (254, 280 nm) and metals (Zn, Se, MeHg and total Hg) in water soluble protein fractions after size exclusion chromatography (Sephadex G-75 column) of supernatant from DOLT-2 lyophilised sample. The position of cytochrome (cyt) with MW 12 000 is shown by an arrow

In summary, the comparison of results for MeHg and total Hg for DOLT-2 lyophilised sample presented in Table 6 shows good agreement for MeHg and total Hg, regardless of the method used. We concluded that alkaline dissolution is the most appropriate method for simultaneous determination of MeHg and Hg^{2+} in supernatants (soluble phase) and DOLT-2 lyophilised sample. For pellets it is recommended to use solvent extraction for determination of MeHg and acid digestion CVAAS for total Hg determination.

The optimised methods were then used to study the distribution of MeHg, Hg^{2+}, total Hg and Se between the soluble and insoluble phase from DOLT-2. Water solubility was tested by preparing two different homogenates (10% and 15%). As shown in Table 7 the proportion of water soluble Se and Hg compounds was very low (~ 4% for mercury species and 10–12% for Se).

Further, the soluble phase obtained from 15% homogenate was applied to a size exclusion chromatography column (Sephadex G-75) for separation of various protein fractions. The elution profiles of UV absorbances (254, 280 nm) and metals (Zn, Se, MeHg and total Hg) are shown in Fig. 6. Elution profiles of Zn and Se are very similar, particularly in that the highest metal peak is at the end of the chromatogram. A completely different situation was found for Hg distribution. The highest peak is in the region of the void volume (MW ≥ 70 000), and a small peak near the range of cytochrome (MW 12 400, the identification point for MT-like proteins) was also observed. At the end of the chromatogram the Hg peak is almost absent.

Regarding the literature data on UV and Zn elution profiles after Sephadex G-75 chromatography of liver supernatants of human [18] and different animal species [19], and our own experience with hen livers (wet or lyophilised), it seems most unusual to have such low absorbances and Zn peaks at the region of the high MW proteins relative to the much higher values at the end of chromatogram. Regarding these differences in the size exclusion chromatography profiles between typical native samples and those from DOLT-2, we suppose that degradation of proteins occurred during preparation of this CRM, but it seems that the high molecular weight proteins binding Hg were preserved from degradation.

Based on these results it appears that DOLT-2 could be suitable for the assessment of the comparability of data in studies of metal distribution between water soluble and insoluble phases, but inappropriate for later protein separations. Such a judgement should be further confirmed by performing the same study on fresh dogfish liver.

This reinforces the desirability of having available reference materials which are true representatives of the original matrix and hence most suitable for biological and chemical speciation studies.

Acknowledgments We acknowledge the financial support of the Ministry of Science and Technology of Slovenia for funding this work through the project J1-7366 and J1-8907. Special thanks are due to Dr. A. R. Byrne for useful suggestions in the preparation of the manuscript.

References

1. Yoshikawa H, Ohta H (1982) Dev Toxicol Environ Sci 9:11–22
2. Yoneda S, Suzuki KT (1997) Biophys Res Comm 231:7–11

3. Horvat M (1996) Mercury analysis and speciation in environmental samples. In: Baeyens W et al (eds) Global and Regional Mercury Cycle. Sources, Fluxes and Mass Balances. Kluwer, The Netherlands, pp 1–31
4. Horvat M (1999) Chemosphere 39 : 1167–1179
5. Horvat M, Zvonaric T, Stegnar P (1986) Vestn Slov Kem Drus 33 : 475–487
6. Horvat M, Lupsina V, Pihlar B (1991) Anal Chim Acta 243 : 71–79
7. Liang L, Horvat M, Bloom NS (1994) Talanta 41 : 371–379
8. Horvat M, Liang L, Bloom NS (1993 a) Anal Chim Acta 281 : 135–152
9. Horvat M, Liang L, Bloom NS (1993 b) Anal Chim Acta 281 : 153–168
10. Liang L, Horvat M, Bloom NS (1994) Clin Chem 40 : 602–607
11. Cestnik D, Stibilj V, Falnoga I (1998) Speciation of selenium in biological samples. Abstract, Intelligent Analytical Solutions, Jülich, Germany
12. Stibilj V, Dermelj M, Franko M, Byrne AR (1994) Anal Sci 10 : 789–793
13. Dermelj M, Hancman G, Gosdar A, Byrne AR, Kosta L (1985) Vestn Slov Kem Drus 32 : 27–35
14. Liang L, Horvat M, Cernichiari E, Gelein B, Balogh S (1996) Talanta 43 : 1883–1888
15. Horvat M, Benedik L, Byrne AR, Dermelj M, Jacimovic R, Jereb V, Logar M, Mandic V, Milacic R, Scancar J (1999) Determination of trace and major elements and methylmercury in NIST RM 8046 III Mussel and SRM 1566b Oyster. Working report of the Jozef Stefan Institute, IJS-DP-8040
16. May K, Stoeppler M, Reisinger K (1987) Toxicol Environ Chem 13 : 153–159
17. Horvat M, May K, Stoeppler M, Byrne AR (1988) Appl Organomet Chem 2 : 515–524
18. Lui E, Fisman M, Wong C, Diaz F (1990) J Am Geriatr Soc 38 : 633–639
19. Hidalgo J, Tort L, Flos R (1985) Comp Biochem Physiol C 81 : 159–156