APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2005; **19**: 276–281
Published online in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/aoc.792

Analysis of diphenylarsinic acid in human and environmental samples by HPLC–ICP-MS

Yasuyuki Shibata[1]*, Katsuaki Tsuzuku[2], Sumiko Komori[1], Chieko Umedzu[1], Hiroe Imai[1] and Masatoshi Morita[1]

[1]National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan
[2]Shimadzu Techno-Research Co. Ltd, Kyoto, Japan

Received 10 January 2004; Accepted 7 June 2004

A simple, rapid and robust analytical method for determining diphenylarsinic acid in human and environmental samples was developed based on a combination of hydrophilic polymer-based gel-permeation high-performance liquid chromatography (HPLC) and inductively coupled plasma mass spectrometry (ICP-MS). Hair and nail samples were digested with alkali, and liberated diphenylarsinic acid (derivative) was extracted with diethyl ether, redissolved in water and injected for HPLC–ICP-MS analysis. Human urine, groundwater and water extracts from soils were injected for HPLC–ICP-MS directly after filtration. Using the method, diphenylarsinic acid in a solution was quantified in 7 min duration for an analysis with a detection limit of sub-nanograms per milliliter. The method has been applied to groundwater arsenic pollution recently uncovered in Japan. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: diphenylarsinic acid; groundwater pollution; HPLC–ICP-MS; arsenic speciation

INTRODUCTION

Arsenic in the environment has been studied extensively because of its notorious toxicity, and several tens of inorganic/organic arsenic compounds have been identified in nature, especially in marine organisms, which are found to be rich sources of a variety of organic arsenic compounds.[1–3] In addition to the natural arsenic compounds, a variety of arsenic compounds have been synthesized and used for different purposes, such as pharmaceuticals, agrochemicals, and food additives for poultry and swine.[4,5] The toxicity of arsenic, as well as its environmental cycling, is heavily dependent on the chemical species, and the development of methods for speciation analysis has been a key component in arsenic studies.[6,7]

In spring 2003, severe arsenic contamination of groundwater was uncovered in Kamisu-town, Ibaraki Prefecture, Japan. Several residents using the same well water fell ill with characteristic nervous symptoms. A medical doctor suspected contamination of well water by some toxic chemical as a common cause of this symptom,[8] and subsequent analysis of the water showed presence of up to 4500 ng ml^{-1} of arsenic.[9] Furthermore it was soon revealed that the arsenic in the well water was in an unusual organic form, i.e. diphenylarsinic acid (DPAA). The town and the surrounding area are rich in groundwater, and several thousands of personally owned tube wells are present and have been used daily by the residents. An extensive survey by the local government revealed that there were two highly polluted locations, 1 km apart, in the town, together with a couple of other contaminated wells in between. So far, the cause of the pollution has yet to be clarified.

Although there has been extensive use of some aromatic arsenic compounds, such as arsanilic acid, for poultry in some countries like the USA,[4,5] information on DPAA has been scarce. DPAA is known as an intermediate in both the synthesis and degradation of the two vomiting reagents, diphenylchloroarsine and diphenylcyanoarsine, which were produced for military purposes in some countries, including Japan, during the World Wars I and II.[10,11] In addition, information on the analysis of DPAA is also quite limited. Derivatization by thiol compounds followed by gas chromatography–mass spectrometry (GC–MS) analysis has been reported in the literature,[12,13] but the method is time consuming and does not seem to be suitable for the

*Correspondence to: Yasuyuki Shibata, Environmental Chemistry Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan.
E-mail: yshibata@nies.go.jp

analysis of hundreds or thousands of samples. Analysis of DPAA in human samples is even more difficult because its metabolism/excretion is virtually unknown, and the Japanese consume a variety of marine-organism-derived foods in daily life and metabolize and excrete their organic arsenic compounds. Therefore, the analytical method to be developed has to distinguish and separately quantify DPAA from other arsenic compounds and their metabolites commonly observed in normal Japanese people.

In this study, we focus on the development of a simple, rapid and robust analytical method of DPAA based on an high-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC–ICP-MS) technique. Previously, we have established an HPLC–ICP-MS method for the arsenic speciation of marine organisms and human urine and blood samples by the combination of three different HPLC column conditions.[14–16] We used them as a starting condition for the development of DPAA analysis, for plenty of information has been available on the separation of normal arsenic compounds from Japanese human and environmental samples using the system, which will be a good basis for the development of analytical method for DPAA.

EXPERIMENTAL METHODS

Fifteen previously reported arsenic standards[14] were employed for the development of the analytical method. DPAA was synthesized by Trichemicals Co. Ltd, Japan, and also by Dr K. Nakamiya and Dr J.S. Edmonds in our institute. Phenylarsonic acid (MPAA) was obtained from Tokyo Kasei Kogyo Co., Japan. All the reagents for treatment/chromatography were of reagent grade or liquid chromatography grade. Water was purified by a Milli-Q water purification system (Millipore Co. Ltd). A finely powdered human hair certified reference material, NIES CRM No. 13,[17] originally developed for the speciation of mercury and elemental analysis, was employed for the quality control during the routine analysis.

HPLC columns were purchased from Showa Denko (Asahipak GS220 7G, 7C) or GL Sciences (Inertsil ODS, Inertsil C8, Inertsil C4, Inertsil Ph, Inertsil CN, Nucleosil 5SA). Either a 10A (Shimadzu Co.) or 1100 (Agilent) HPLC system was used for the separation of arsenic compounds, and either an Agilent 4500 or 7500 ICP-MS system was used as an arsenic-specific detector. $m/z = 75$ was monitored, together with m/z 77 and 82, to identify ArCl interference; and the selenium peak was also monitored in the case of human samples. In the established condition, a short Asahipak GS220 7C (7.6 mm × 100 mm) gel-permeation column was used with aqueous buffer (25 mM tetramethylammonium hydroxide–25 mM malonic acid, pH 6.8 adjusted by ammonia; 1 ml min^{-1}). Typically, DPAA was eluted at around 4.5 min, whereas the majority of other arsenic compounds appeared between 2 and 3 min.

Human hair samples were placed into a glass or plastic centrifuge tube and then cut into pieces by scissors. After gentle mixing, around 100 mg of the hair powders were carefully weighed into a plastic tube, and 2 ml 2 M NaOH solution added and heated to 90 °C for 3 h. If a sufficient amount of hair was not available, then all the hair samples from a person were weighed carefully into a plastic tube, cut into pieces by scissors and then treated in the same manner as above. After cooling, the solution was transferred to a glass tube and acidified with 2 ml conc. HCl, and then 2 ml diethyl ether was added. After vigorous shaking, the diethyl ether layer was obtained by centrifugation (1500 rpm for 5 min). The extraction procedure was repeated twice, and the extracts were combined together, dried under a nitrogen stream, and 0.1 ml conc. HNO_3 added. After swirling the tube for a while with occasional warming, 10 ml of purified water was added to the tube. 4 ml of the solution was placed in a different tube and neutralized by ammonia; then, after evaporation, 0.4 ml of buffer was added. 20 µl of the final solution was injected to the HPLC column for speciation of arsenic. 1 ml was used for total arsenic determination and the remaining 5 ml was kept for future GC–MS or other analysis.

Around 100 mg of CRM No. 13 was carefully weighed into two plastic tubes. A known amount of standard mixture of MPAA and DPAA was added to one tube, and both tubes were treated in the same manner as above on each day. The analytical results were used for checking the recovery of MPAA/DPAA and for contamination level during the treatment.

In the case of nail samples, up to 100 mg of toenails and fingernails were separately weighed into a plastic tube and treated with NaOH at 90 °C for 3 h. Then the samples were treated in the same manner as above. Several hundred milligrams of nails from the normal population was collected and powdered under liquid nitrogen by a miniature air hammer. The resultant nail powders were mixed well together and a portion, *ca* 100 mg each, was used to check the recovery of MPAA/DPAA during the treatment procedure.

Human urine samples were either treated similarly as hair, or were centrifuged and filtered through a 0.45 µm filter (Zartorius) without pretreatment, and 20 µl of the filtrate was injected for HPLC–ICP-MS. Well water samples were simply filtered and injected. Soil extracts, after centrifugation, were treated in the same way as well water samples.

RESULTS

Development of HPLC–ICP-MS condition for DPAA analysis

Although DPAA is fairly soluble in water, it shows a considerably stronger hydrophobic interaction with column materials than other arsenic compounds in our hands. In fact, we frequently encountered unexpected broadening or delay of the DPAA peak during development of the analytical system. Also, we basically decided to exclude

organic solvent from the buffer in order to minimize maintenance/other troubles caused by the deposition of carbon on the sampling/skimmer cones during ICP-MS. As many of the arsenic compounds, including DPAA, become anionic at neutral to slightly alkaline pH, ion-exchange chromatography seemed to be a choice for the separation. We did not obtain promising results by a brief survey of silica-gel-based ion-exchange columns–organic buffer combination, possibly because of unexpected interaction of phenyl groups with either hydrophobic linker or end-capping materials. Polymer-based anion-exchange columns based on styrene–divinylbenzene co-polymer, which have been used frequently for speciation of arsenic,[18] were not checked because of expected strong hydrophobic interaction between benzene rings. In our previously established aqueous ion-pair condition for the separation of organic arsenic compounds in marine life (LC-1[14]), DPAA could not be eluted at all from a C18 column. The situation was the same even using columns with lower hydrophobicity, including C8, C4 and phenyl columns with the same buffer. DPAA was eluted from a silica-gel column with CN residues, but the separation quality was poor.

On the other hand, DPAA was eluted from the GS220 7G (7.6 mm × 500 mm) gel-permeation column as a single peak at around 22 min, which is considerably later than the other arsenic standards. Although the separation capability of gel permeation in general is lower than the other ion-pair chromatographies due to its broader peak width, the LC-3 system[14] based on GS220 has been found to be insensitive to the matrix of the samples and durable to larger injection volumes. Therefore, we selected GS220 as a base separation system and selected a shorter column, GS220 7C with 100 mm length, to speed up the speciation procedure per sample.

Figure 1 shows the separation of the standard mixture of MPAA and DPAA, together with a chromatogram of groundwater from the most contaminated well. The limit of detection of DPAA with an injection volume of 20 µl was found to be around 0.1 ng ml^{-1} as arsenic, or 0.3 ng DPAA in 1 ml, in the solution. As shown Fig. 1, DPAA was eluted at around 4.5 min, and 6 min was enough to detect and quantify both MPAA and DPAA. However, an unknown peak with a longer retention time of around 5.5 min was found in some groundwater samples and soil extracts (data not shown), and so we selected 7 min for the duration time in routine analysis. Also, on rare occasions, another compound with an even longer retention time, around 13 min, was noticed. Identification of these compounds, as well as the retention times of vomiting reagents and their first decomposition product, bis(diphenylarsine)oxide,[10] are among the urgent tasks to be conducted in the near future.

Although MPAA is clearly detected in the chromatogram, other arsenic peaks, such as dimethylarsinic acid and arsenobetaine, and chloride interference were partially overlapped in the peak. It should be noted that the present condition has this drawback and, thus, will be applicable only for the DPAA analysis routinely; the method will give us a maximum estimate of MPAA, except in those cases where the absence of the above interferences to MPAA is evident, which, however, will be easily demonstrated by the application of the other HPLC condition, including LC-1 and LC-2.

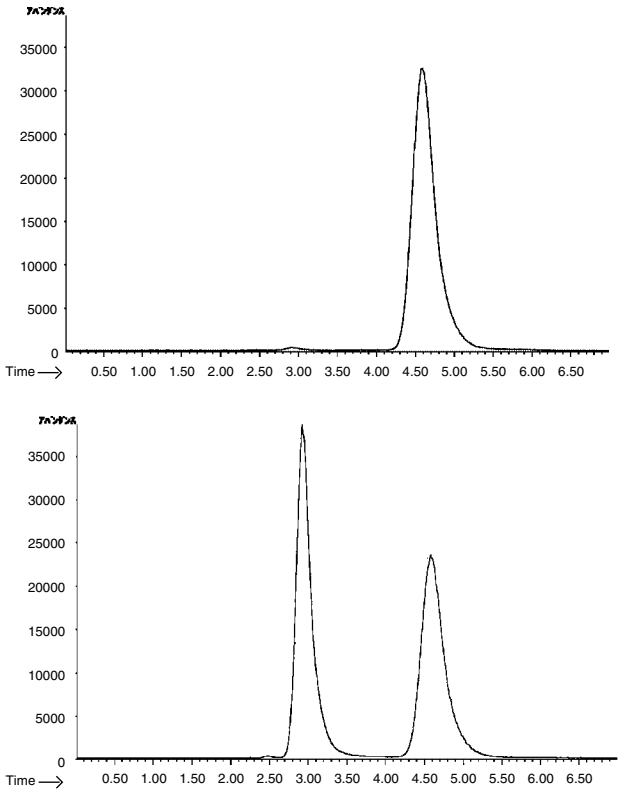

Figure 1. HPLC–ICP-MS chromatograms: (bottom) standard containing MPAA (58 ng ml^{-1}) and DPAA (55 ng ml^{-1}); (b) groundwater from the most polluted well.

Establishment of hair/nail pretreatment method

In the case of human hair and nail analysis, we postulated that DPAA will behave in a similar manner to dimethylarsinic acid, i.e. be bound to proteins in hair and nail probably through thiol groups. The carbon–arsenic bond is strong, and methylated organic arsenic compounds were reported to be resistant to decomposition even under boiling with conc. HNO_3.[19] The same report also showed a similar resistant property for MPAA, although partial decomposition of MPAA was inferred. We first checked the possibility of applying nitric acid treatment, but found that DPAA was partially decomposed to MPAA during the treatment. These data suggest that the carbon–arsenic bond in the case of the aromatic group is a bit weaker than that in the alkyl group. So, we focused our efforts on the conventional alkali treatment procedure, which has been used successfully for the speciation analysis of inorganic and methylated organic arsenic compounds in human hair samples in combination with hydride generation–cold trap methods.[20,21]

At first, stabilities of DPAA and MPAA were tested under the treatment condition. There were no signs of decomposition of either of the compounds during 2 M NaOH treatment at 90 °C for 3 h. Then, the standard hair powder, CRM No. 13, was spiked with a known amount of DPAA standard and treated in the same manner. Direct HPLC–ICP-MS analysis of the treated solution, however, gave us several additional peaks, possibly because of the interference by the matrix (mixtures of decomposed protein fragments). So, we decided to try to extract DPAA from the alkali-treated solution before HPLC–ICP-MS analysis. By the acidification of the alkali-treated solution followed by toluene extraction, the majority of spiked DPAA was recovered in the toluene fraction. The extraction efficiency did not show a clear dependence on the acid concentration from 1 to 6 M. Interestingly, the same extraction procedure against DPAA standard solution (without hair) did not work well, irrespective of the acidity, suggesting that DPAA is present in a different chemical form (in a different valence state, or derivatized possibly by some sulfur-containing fragments) in the alkali-treated hair samples. This hypothesis is further supported by the findings that DPAA could not be back-extracted to water phase by a simple pH neutralization procedure, and that conc. HNO_3 treatment after evaporation of organic solvent is effective to detect DPAA in the final solution. Based on these results, we set the pretreatment procedure as written in the Experimental section. Diethyl ether was selected as an extraction solvent instead of toluene in the final procedure.

Recoveries of spiked DPAA against CRM No. 13 and a nail powder are summarized in Table 1. Recoveries of around 80% or more were obtained for DPAA by the procedure. Also, the linearity of the peak response versus spiked amount of MPAA/DPAA was found to be quite good. The data in Table 1, however, suggest that there may be a tendency to have better recovery in samples having smaller amounts of DPAA, possibly because of the underlying mechanism that DPAA was recovered only after modification by some components in the hair digests, the ratio of which versus DPAA might affect the efficiency of DPAA modification and, hence, it's recovery. The data suggest that the method tends to underestimate DPAA concentration in the samples containing higher amounts of DPAA, though it will give us reliable data in the samples containing lower concentrations of DPAA. Figure 2 shows the chromatogram of the extract from the CRM No. 13 hair sample spiked with a mixture of MPAA and DPAA together with an example of hair obtained from a person exposed to the contaminated well water. As expected, a clear peak of DPAA was observed in the extract for the person. The typical detection limit of the whole procedure is around 1 ng of arsenic per gram, or a few nanograms of DPAA in 1 g, when 100 mg of hair/nail samples are used as starting material.

Table 1. Recovery of spiked DPAA from standard hair, CRM No. 13, and nail powder samples (n: total number of data)

DPAA added (ng g^{-1})	Sample	DPAA obtained (ng g^{-1})		Recovery (%)
		Average	SD	
5.5 ($n = 3$)	Hair	5.8	0.11	106
11.0 ($n = 3$)	Hair	10	0.11	94
27.5 ($n = 3$)	Hair	24	0.99	87
55 ($n = 3$)	Hair	47	1.5	85
55 ($n = 2$)	Nail	40		73
55 ($n = 45$)	Hair[a]	42	3.8	77

[a] Compilation of data of all quality assurance samples analyzed so far during the analysis of the human samples.

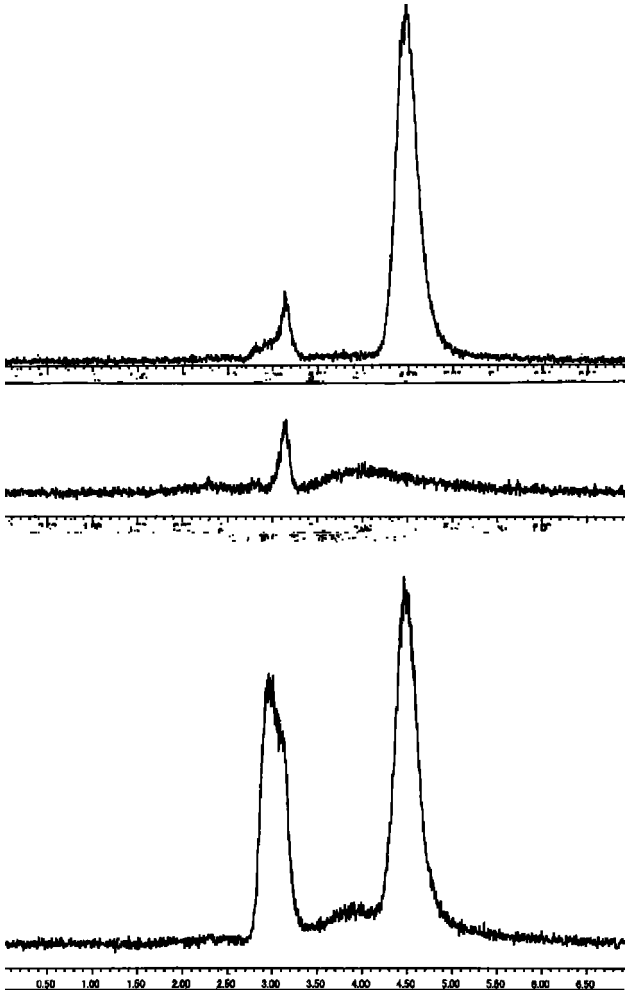

Figure 2. HPLC–ICP-MS chromatograms of the extracts from spiked human hair SRM No. 13 (bottom), hair without spike (middle), and hair of an affected person (top).

DISCUSSION

As of the end of November 2003, around 300 hair/nail samples, several tens of urine samples and 2000 water/soil

samples from the region have been analyzed by the method, proving the simple and robust nature of the method for real human/environmental sample analysis. The analytical method of DPAA developed in the present study is based on the previous HPLC–ICP-MS condition for the speciation of arsenic in marine organisms established in our laboratory in the late 1980s.[14] At that time, 15 inorganic and organic arsenic compounds had been identified in marine organisms, and a sensitive and reliable speciation method of these arsenic compounds was needed. As is well known, using high concentrations of organic solvents and/or inorganic salts is difficult with ICP-MS due to the deposition of these materials on the interface and causing long-term instability in sensitivity. Therefore, we employed and established ion-pair chromatographic conditions, LC-1 and LC-2, by using aqueous buffers containing ion-pairing reagents having only small hydrophobicities, i.e. tetraethylammonium or 1-butane sulfonate, together with organic buffer using malonic acid.[14] Under these aqueous buffers, many of the ODS columns available at that time showed inferior separation power, probably because of mutual interaction of the octadecyl chains and subsequent loss of effective surface area for interaction with ion pairs.[3,22] We found that a specific column, Inertsil ODS, maintained separation capability even in pure aqueous buffer, and also that the addition of a trace amount of methanol (0.05% v/v) was quite effective in preventing a reduction in surface of the octadecyl moiety on the column in the long term.

Although these ion-pairing conditions were found to be effective for separating major water-soluble arsenic compounds in marine life and for MPAA, they were found to interact too strongly with DPAA. Suitable conditions for DPAA were not found even by decreasing the number of carbon chains from C18 to C4 (butyl) or C6 (phenyl). On the other hand, the polymer-based gel-permeation column GS220 was found to work well for DPAA separation. GS220 was reported to be composed of polyvinyl alcohol resin. Although it is called a 'gel-permeation column', the resin has carboxyl groups on the surface, and also double bonds in its backbone structure to give it mechanical stiffness. Therefore, the separation mechanism is fairly complex, including size exclusion, charge interaction and hydrophobic interaction. In fact, we found that GS220 was quite effective not only in separating low-molecular-weight arsenic compounds,[14] but also in separating halide anions from halo-oxoanions,[23] and that iodide interacts with the resin much more strongly than bromide and chloride, probably because of its hydrophobic character. Silica-gel-based ion-exchange columns, on the other hand, did not work well, probably because of interaction of the phenyl group in DPAA with either the hydrophobic linker or end-capping materials of the columns. Ion-exchange columns based on hydrophilic polymer resins may be an alternative choice for separation of both MPAA and DPAA from other arsenic compounds.

In addition to the conventional alkali treatment method, a simple hot-water extraction method of arsenic in human hair was reported recently.[24,25] Although good recovery was reported on CRM No. 13, a finely ground hair powder with virtually no intact cuticles,[17] which are barriers for efficient extraction, the recoveries of the method on the real samples were not always good,[24] probably because of the inferior extraction efficiency of the method with samples having intact cuticles. It is impractical to grind each of the samples finely to destroy cuticles completely for the extraction. Therefore, we selected the traditional alkali digestion method for the extraction.

Alkali digestion has been used and established for the quantitative speciation of arsenic in hair samples.[20,21] Although the true extraction efficiency of DPAA from hair/nail samples by the method has not yet been clarified, we suppose the recovery of spiked samples will give us good estimates of the true recovery values. We detected DPAA from some of the samples from people living in the area. There is, on the other hand, virtually no information on the possible metabolism and/or interaction of DPAA with other biomolecules within the body. We found that DPAA recoveries in some urine samples seem to be affected by the pretreatment procedures (data not shown). Clearly, more research is needed on the metabolism and fate of DPAA/MPAA in the human/animal body, as well as on their mechanisms of toxicity and on the development of analytical methods based on the information.

Acknowledgements

We thank Dr M. Nishikawa, Dr K. Nakamiya and Dr J.S. Edmonds for providing us with the DPAA standard, and Dr K. Jin, Hokkaido Institute of Public Health, and Ms K. Kamiya, Ministry of the Environment, for providing us with the opportunity to present part of the work at the occasion of the *11th Arsenic Symposium*, 14–15 October 2003, in Sapporo, Japan. YS also thanks Mr H. Murakami, Dr J.A. Tornes and Dr A. Waleiji for providing information on the production and analysis of chemical warfare and related reagents.

REFERENCES

1. Francesconi KA, Emonds JS. *Adv. Inorg. Chem.* 1997; **44**: 147.
2. Cullen WR, Reimer KJ. *Chem. Rev.* 1989; **89**: 713.
3. Shibata Y, Morita M, Fuwa K. *Adv. Biophys.* 1992; **28**: 31.
4. National Research Council. *Medical and Biologic Effects of Environmental Pollutants. Arsenic*. National Academy of Sciences: Washington, 1977.
5. Woolson EA (eds). *Arsenical Pesticides*. ACS Symposium Series 7. American Chemical Society: Washington, DC, 1975.
6. Morita M, Edmonds JS. *Pure Appl. Chem.* 1992; **64**: 575.
7. Benramdane L, Bressolle F, Vallon JJ. *J. Chromatogr. Sci.* 1999; **37**: 330.
8. Ishii K. In *11th Arsenic Symposium*, 14–15 October 2003, Sapporo, Japan; 1 (abstract, in Japanese).
9. Ishizaki M, Yanaoka T, Nakamura M, Hakuta T, Ueno S, Komuro M, Shibata M, Kitamura T, Suzuki Y, Sasamoto A, Honda A, Hanaoka S, Ogata T, Doi M. In *11th Arsenic Symposium*, 14–15 October 2003, 43 (abstract, in Japanese).
10. Tornes JA, Voie OA, Ljones M, Opstad AM, Bjerkeseth LH, Hussain F. Investigation and risk assessment of ships loaded with

chemical ammunition scuttled in Skagerrak. FFI/RAPPORT-2002/04951. Norwegian Defense Research Establishment, Kjeller, 2002.
11. Murakami H. *History of Noxious Gas Island (Okuno-jima)*. 1992; 81 (in Japanese).
12. Haas R, Schmidt TC, Steinbach K, von Loew E. *Fresenius J. Anal. Chem.* 1998; **361**: 313.
13. Pergantis SA, Heithmar EM, Hinners TA. *Analyst* 1997; **122**: 1063.
14. Shibata Y, Morita M. *Anal. Sci.* 1989; **5**: 107.
15. Shibata Y, Morita M. *Anal Chem.* 1989; **61**: 2116.
16. Shibata Y, Yoshinaga J, Morita M. *Appl. Organometal. Chem.* 1994; **8**: 249.
17. Yoshinaga J, Morita M, Okamoto K. *Fresenius J. Anal. Chem.* 1997; **357**: 279.
18. Madsen AD, Goessler W, Pedersen SN, Francesconi KA. *J. Anal. At. Spectrom.* 2000; **15**: 657.
19. Jin K, Ogura H, Taga M. *Bunseki Kagaku* 1983; **32**: E171.
20. Yamato N. *Bull. Environ. Contam. Toxicol.* 1988; **40**: 633.
21. Yamamura Y, Yamauchi H. *Ind. Health* 1980; **18**: 203.
22. Shibata Y, Shibata K, Morita M. Chemodynamics of arsenic in marine environment. In *Comparative Evaluation of Environmental Toxicants*, Inaba J, Nakamura Y (eds). Kodansha Scientific: Tokyo, 1998; 97–105.
23. Salov VV, Yoshinaga J, Shibata Y, Morita M. *Anal. Chem.* 1992; **64**: 2425.
24. Mandal BK, Ogra Y, Suzuki KT. *Toxicol. Appl. Pharm.* 2003; **189**: 73.
25. Shraim A, Hirano S, Yamauchi H. *Anal. Sci.* 2001; **17**(Suppl.): i1729.