Journal of Chromatography B, 867 (2008) 179–188

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Urine analysis of patients exposed to phenylarsenic compounds via accidental pollution

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ARTICLE INFO

Article history:
Received 10 October 2007
Accepted 30 March 2008
Available online 20 April 2008

Keywords:
Chemical warfare agents
LC-ICP-MS
Urine analysis

ABSTRACT

New methods involving high-performance liquid chromatography/inductively coupled plasma mass spectrometry were examined for the determination of phenylarsenic compounds derived from chemical warfare agents. Several methods were examined for the separation of diphenylarsinic acid (DPAA), phenylarsonic acid, phenylmethylarsinic acid (PMAA), phenyldimethylarsine oxide, and diphenylmethylarsine oxide. Analysis of the urine samples of the patients exposed to phenylarsenic compounds indicated that the main phenylarsenic components were DPAA and PMAA; moreover, some unknown arsenicals, which were also found in contaminated groundwater and rice samples, were also detected.

1. Introduction

In the spring of 2003, health damage was reported in Kamisu, Ibaraki Prefecture, Japan, and it was caused by drinking well water contaminated with a high concentration of arsenic, which was 450 times higher than the safety standard for drinking water in Japan. After a precise investigation, the main arsenic species was identified as diphenylarsinic acid (DPAA) [1]. Generally, arsenic exists in the environment in various chemical forms, but is not produced in nature. DPAA is considered to be a material for the synthesis of vomiting agents such as diphenylarsine chloride and diphenylarsine cyanide or a degradation compound of the agents [2]. Ministry of the Environment in Japan investigated the origin of the contamination, and subsequently DPAA that was solidified in cement was found to be illegally buried in the ground; it was supposed to be the origin. The patients in Kamisu showed disorders such as cerebellar symptoms, tremors, myoclonus, and sleep disturbance, which were not symptoms derived from inorganic arsenic intoxication [3].

Rapid speciation analysis without derivatization is necessary to analyze biological samples such as urine samples of patients. There are some reports on speciation analysis of phenylarsenic compounds derived from chemical warfare agents. Haas et al. [4] reported an analytical method involving HPLC for the determination of some phenylarsenic compounds of diphenylarsine hydroxide, adamsite, triphenylarsine, triphenylarsine oxide, phenylarsonic acid (PAA), and phenylarsine oxide. Smedts et al. reported methods involving HPLC and micellar electrokinetic capillary chromatography for the analysis of phenylarsine oxide, diphenylarsinehydroxide, and triphenylarsine [5,6]. However, they need a relatively long analysis time and the detection limits are fairly high. Furthermore, there were few methods for the analysis of DPAA before the contamination in Kamisu was reported. The authors examined an analytical method for the rapid determination of DPAA and PAA by using a hyphenated system coupled with high-performance liquid chromatography and inductively coupled plasma mass spectrometry (LC-ICP-MS) [7]. When DPAA and PAA were analyzed by a method described in the report, the detection limits of DPAA and PAA were 2.0 and 1.0 ng mL^{-1} of arsenic, respectively. Since the contamination was reported, analytical methods involving LC-ICP-MS [8] and LC-electrospray (ESI)-MS [9] have been reported by other researchers in Japan. However, as the number of objects to be analyzed has increased, more phenylarsenic compounds have been identified; Ministry of the Environment proclaimed that phenylmethylarsinic acid (PMAA) was discovered in environmental samples. This implied phenylarsenic compounds were methylated in the environment, and therefore the following phenylarsenic compounds were considered as the target of the analysis in this study: DPAA, PAA, PMAA, phenyldimethylarsine oxide (PDMAO), and diphenylmethylarsine oxide (DPMAO). The structures of these phenylarsenic compounds are shown in Fig. 1. The method we had established could not separate them satisfactorily. Therefore, several new analytical methods

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doi:10.1016/j.jchromb.2008.03.033

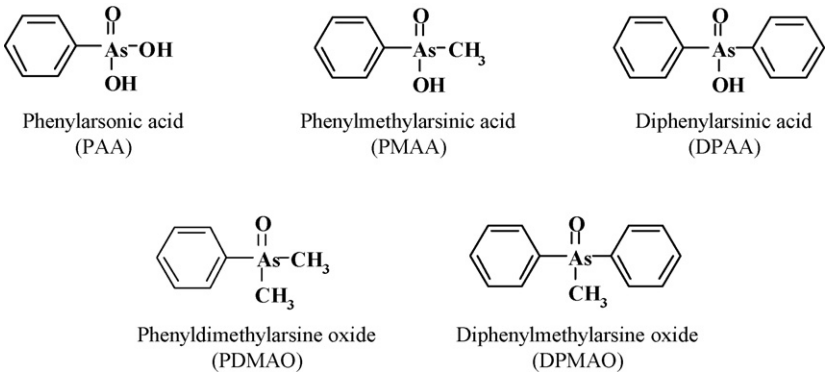

Fig. 1. Structures of phenylarsenic compounds.

were examined for the routine analysis of these phenylarsenic compounds, and subsequently urine samples of patients in Kamisu were investigated.

The major disadvantage of the LC-ICP-MS system is that the identification of peaks can be ambiguous. Qualitative analysis by using LC-ICP-MS depends on the comparison of the retention time of the analyte on the chromatogram with those of standard compounds. It seemed to be impractical to employ one method for the analysis of urine samples because they would contain unknown arsenicals. Recently, HPLC with a dual detection system employing ICP-MS and ESI-MS was applied for both quantification and identification [10]. In the present study, different chromatographic columns and mobile phases were tested for investigating the presence of phenylarsenic compounds in the urine of the patients, the urine of arsenic-administered mice and environmental samples, groundwater and rice samples, were analyzed. Additionally, inorganic and methylated arsenicals were also analyzed by an existing method.

2. Experimental

2.1. Materials

Methanol, ethanol, and acetonitrile (HPLC grade) and nitric acid, citric acid monohydrate, trisodium citrate dihydrate, phosphoric acid, and potassium dihydrogenphosphate (analytical grade) were purchased from Kanto Chemical Co. (Tokyo, Japan). Tetramethylammonium hydroxide pentahydrate and sodium 1-butanesulfonate were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Malonic acid and trifluoroacetic acid (analytical grade) were obtained from Wako Pure Chemicals Co. (Osaka, Japan). Inorganic arsenicals such as arsenate and arsenite and methylated arsenicals such as arsenobetaine, arsenocholine, methylarsonic acid (MAA), dimethylarsinic acid (DMAA), trimethylarsine oxide, and tetramethylarsonium and phenylarsenic compounds such as DPAA, PMAA, PDMAO, and DPMAO were obtained from Tri Chemical Laboratories Inc. (Yamanashi, Japan). PAA was purchased from Acros Organics (Geel, Belgium). Groundwater samples were pumped out from a depth of 5 or 6 m in the Kizaki area of Kamisu, Ibaraki, Japan, in 2003. Rice samples were supplied by Prof. Nirei of Ibaraki University. Urine samples were collected from the residents in Kamisu and preserved in a deep freezer at −84 °C.

2.2. Instruments

The HPLC system comprised a carrier reservoir (CR670), a pump (PU611, GL Sciences Inc., Tokyo), and an autosampler with a built-in column oven (MIDAS, Spark Holland). Analytical columns such as Inertsil C4 (150 mm × 2.1 mm, 5 μm), Inertsil NH2 (250 mm × 1.5 mm, 5 μm), and Inertsil AS (150 mm × 2.1 mm, 3 μm) and guard columns such as Inertsil C4 (10 × 1.5 mm, 5 μm) and Inertsil NH2 (10 mm × 1.5 mm, 5 μm) were obtained from GL Sciences. The Inertsil AS column has almost the same features as an ODS column and it was improved for the separation of inorganic and methylated arsenicals. CHEMCOSORB 5CN-U (150 mm × 2.1 mm, 5 μm), whose silica gel possesses a high surface area (600 $m^2\ g^{-1}$), was purchased from Chemco Scientific Co., Ltd. (Tokyo, Japan). The ICP-MS system was ELAN DRC-e (Perkin-Elmer SCIEX Inc., Ontario, Canada), and the retention times and peak areas were determined with a TotalChrom Workstation Version 6.2.0 (Perkin-Elmer SCIEX Inc., Ontario, Canada).

2.3. Examination of methods for analysis of phenylarsenic compounds

LC-ICP-MS and three types of columns were utilized. In the method using the Inertsil C4 (C_4 column), the mobile phase comprised water and organic solvents. The separation of arsenicals was optimized by the adjustment of the pH with nitric acid and by adjusting the concentration of the organic solvent (acetonitrile, methanol, and ethanol). In the method using the CHEMCOSORB 5CN-U (CN column), the mobile phase comprised citrate buffer and organic solvents. The separation was optimized by the adjustment of the pH with phosphate buffer and by adjusting the concentration of the buffer and organic solvent (acetonitrile and methanol). In the method using the Inertsil NH2 (NH_2 column), the mobile phase comprised phosphate buffer and acetonitrile. The separation was optimized by the adjustment of the pH with phosphate buffer and by adjusting the concentration of the buffer and acetonitrile. Additionally, inorganic and methylated arsenicals were also examined.

For the detection of arsenic, the dynamic reaction cell (DRC) mode was employed and oxygen was selected as the reaction gas. Thus, AsO^+ (m/z 91) was monitored instead of As^+ (m/z 75) in order to prevent interference from $ArCl^+$ (m/z 75) as shown in reference [11]. ICP-MS was performed under the following conditions, although the conditions were optimized before every determination. The nebulizer gas flow was 0.86 L min^{-1}; auxiliary gas flow, 1.15 L min^{-1}; plasma gas flow, 18 L min^{-1}; cell gas flow, 0.9 mL min^{-1}; RPq, 0.6; and RF power, 1600 W.

2.4. Determination of phenylarsenic compounds in urine of patients in Kamisu

The urine samples were three-fold diluted with water, filtered using a 0.45-μm membrane filter (GL Sciences Inc., Tokyo), and

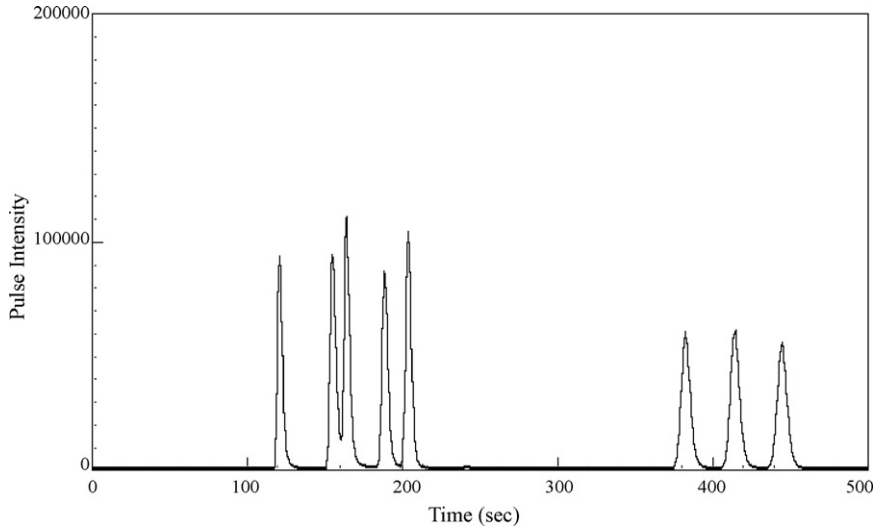

Fig. 2. Chromatogram of inorganic and methylated arsenicals. The separation was performed with the Inertsil AS column (150 mm × 2.1 mm); the mobile phase comprised 10 mM of sodium 1-butanesulfonate, 4 mM of tetramethylammonium hydroxide, 4 mM of malonic acid, and 0.05% methanol, the pH was adjusted to 3.0 with nitric acid, the flow rate was 0.2 mL min^{-1}, the temperature was 40 °C, and the injection volume was 5 μL. The elution order is arsenate, arsenite, MAA, DMAA, arsenobetaine, trimethylarsine oxide, tetramethylarsonium, and arsenocholine.

then analyzed by employing the newly established methods. For some samples containing a large amount of hydrophilic arsenicals that could not be separated by these methods, in order to examine whether unknown arsenic species existed in the urine samples, further analysis was carried out with the Inertsil AS column, which can separate inorganic and methylated arsenicals. The analytical conditions selected in this study were established by Shibata and Morita [12]. The mobile phase comprised 10 mM of sodium 1-butanesulfonate, 4 mM of tetramethylammonium hydroxide, 4 mM of malonic acid, and 0.05% methanol, and the pH was adjusted to 3.0 with nitric acid. The flow rate was 0.2 mL min^{-1}; the temperature, 40 °C; and the injection volume, 5 μL. The chromatogram of eight hydrophilic arsenicals, including arsenate, arsenite, MAA, DMAA, trimethylarsine oxide, tetramethylarsonium, arsenobetaine, and arsenocholine, is shown in Fig. 2.

2.5. Analysis of urine of arsenic-administered mouse

Three types of diets containing approximately 1 μg g^{-1} of arsenic of DPAA, PAA (main component in contaminated groundwater), and PMAA (main component in contaminated rice) were prepared as follows. Ten grams of commercial dietary supplement food (CalorieMate, Otsuka Pharmaceutical Co., Ltd.) was powdered; after 20 μg of each phenylarsenic compound was added, they were lyophilized and then mixed with 10 g of flour.

Mice that were four weeks old (SPF/VAF Crlj:CD1 (ICR); Charles River Japan, Inc., Kanagawa, Japan) were fed a seafood-free diet for a week and then fed a prepared diet for one month. Subsequently, urine was collected overnight and the collected samples were two-fold diluted with water; this was followed by filtration. The metabolites were then analyzed by LC-ICP-MS.

2.6. Analysis of contaminated groundwater and rice

The groundwater samples were diluted to a tenth of their original concentration and filtered. The preparation of the rice samples was performed as follows. Rice was pulverized and 0.2 g of rice was weighed. Phenylarsenic compounds were extracted with 5 mL of a mixture of water/methanol (1:1) at 80 °C, and the extract was dried under reduced pressure. It was reconstituted in 500 μL of water and filtered. Subsequently, the arsenic species were analyzed by LC-ICP-MS.

3. Results

3.1. Methods for analysis of phenylarsenic compounds

Three kinds of mechanisms were employed for the separation. Firstly, separation on the basis of the hydrophobicity of the arsenicals was examined and then the C_4 column was selected. The mobile phase of the method that the present authors established was water (pH 2.0 by nitric acid)/acetonitrile = 70:30. However, the satisfactory separation of compounds with weak interaction, such as PAA, PMAA, and PDMAO, could not be accomplished; especially PAA cannot be separated with PMAA. Therefore, those analytical conditions were improved. When trifluoroacetic acid was added instead of nitric acid, it resulted in undesirable effects. When the pH was lowered to 1.5, a slight separation was observed. Next, the proportion of the organic solvent was adjusted. A reduction in acetonitrile and the addition of methanol improved the separation, but not to a sufficient extent. When the proportion was set to 5% of acetonitrile and 15% of ethanol, a remarkable improvement was achieved. The temperature of the column oven was set to 40 °C to reduce the pump pressure and improve the peak shape. However, the analysis time increased considerably and the flow rate was then adjusted to 0.3 mL min^{-1}. The injection volume was set at 20 μL. The complete chromatogram is shown in Fig. 3(a). Inorganic and methylated arsenicals were hardly retained on the C_4 column and they were eluted almost at the same retention time as the arsenate in the chromatogram. The detection limits at a signal-to-noise ratio of three for phenylarsenic compounds were as follows. For PAA, it was 0.25; for PMAA, 0.25; for DPAA, 0.5; for PDMAO, 0.1; and for DPMAO, 0.3 ng mL^{-1} of arsenic. The detection limits were lower than those by a method described in our previous report [7].

Secondly, the CN column with dipole–dipole interaction was selected and the selectivity of separation was changed. The pH was adjusted from 4 to 6 with citrate buffer. At a pH of 6, the retention of PAA, PMAA, and DPAA decreased. In contrast, at a pH of 4, the retention of PAA, PMAA, and DPAA increased. When the pH was set at 5.5, a satisfactory level of separation was achieved. Although

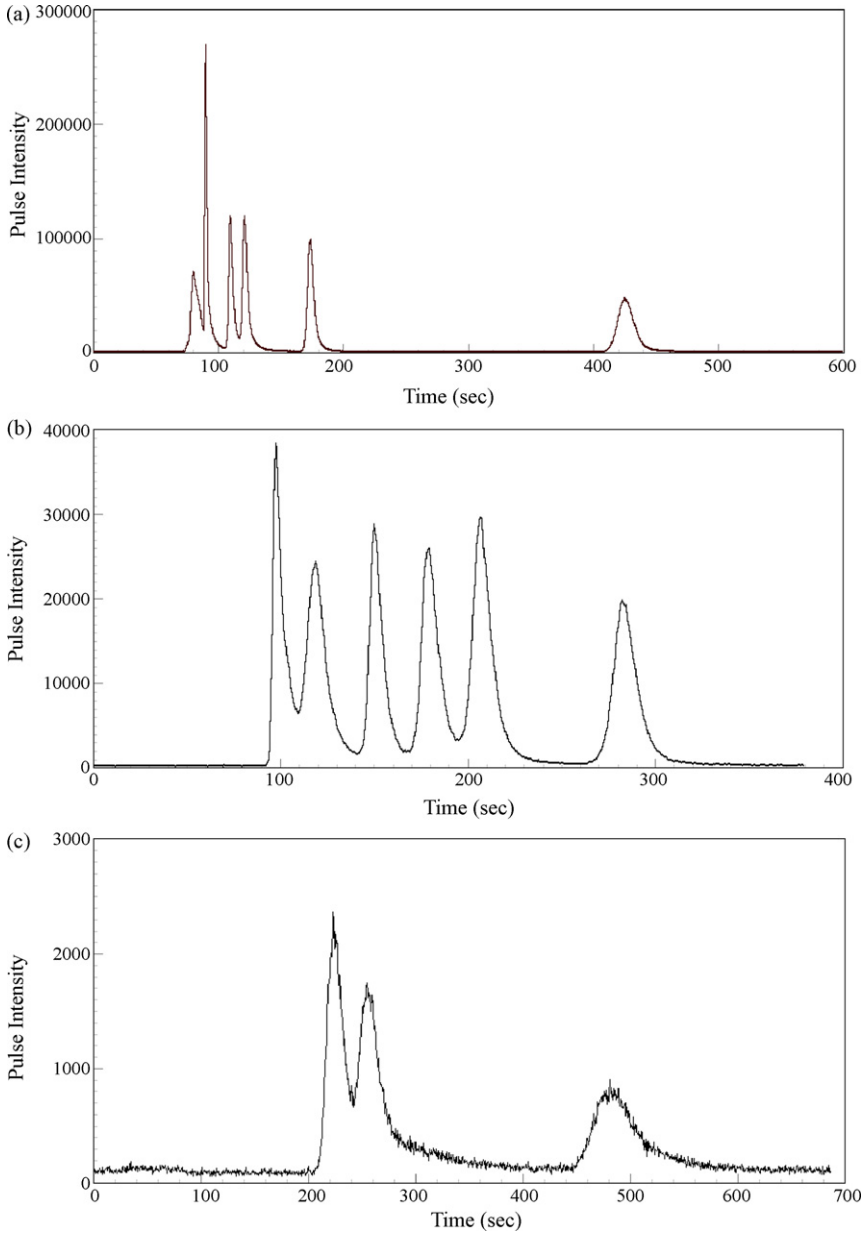

Fig. 3. Chromatograms of standard phenylarsenic compounds. (a) C_4 column (150 mm × 2.1 mm) with guard column (C_4; 10 mm × 1.5 mm); the mobile phase was water (pH 1.5 by nitric acid)/ethanol/acetonitrile = 80:15:5, the flow rate was 0.3 mL min^{-1}, the temperature was 40 °C, and the injection volume was 20 μL. The elution order is arsenate, PDMAO, PMAA, PAA, DPMAO, and DPAA. Arsenate is separated on behalf of inorganic and methylated arsenicals. (b) CN column (150 mm × 2.1 mm) with guard column (C_4; 10 mm × 1.5 mm); the mobile phase was citrate buffer (pH 5.5)/methanol/acetonitrile = 70:20:10, the flow rate was 0.2 mL min^{-1}, the temperature was 40 °C, and the injection volume was 10 μL. The elution order is arsenate, PAA, PMAA, PDMAO, DPAA, and DPMAO. Arsenate is separated on behalf of inorganic and methylated arsenicals. (c) NH_2 column (250 mm × 1.5 mm) with guard column (NH_2; 10 mm × 1.5 mm); the mobile phase was phosphate buffer (pH 2.5)/acetonitrile = 50:50, the flow rate was 0.1 mL min^{-1}, the temperature was the ambient temperature, and the injection volume was 5 μL. The elution order is DPAA, PMAA, and PAA.

the peak shape tended to be considerably broad, an increase in the buffer concentration up to 50 mM brought about an improvement. When the organic solvents acetonitrile and methanol were used, satisfactory separation was observed, and the proportion was then set to 10% of acetonitrile and 20% of methanol. The temperature of the column oven was set to 40 °C to reduce the pump pressure and improve the peak shape. When over 20 μL of the sample was injected, the peak shape of PAA collapsed and the injection volume was then set to 10 μL. The complete chromatogram is shown in Fig. 3(b). However, the CN column method had drawbacks in that PAA was not separated from DMAA and arsenobetaine; these compounds exist in seafood and seaweed and are often detected in human urine [13]. The detection limits at a signal-to-noise ratio of three for phenylarsenic compounds were as follows. For PAA, it was 1.0; for PMAA, 0.5; for DPAA, 0.5; for PDMAO, 0.5; and for DPMAO, 1.0 ng mL^{-1} of arsenic. The detection limits were not significantly lower than those by a method described in our previous report [7].

Thirdly, separation on the basis of the functional group of the arsenicals was examined and the NH_2 column was then selected. The pH considerably affected the separation. When the pH was adjusted to around 2.5, the arsenicals tended to be eluted in the order cationic species (arsenicals having an oxide group, tetramethylarsonium, and arsenocholine), hydrophobic species, and anionic species (arsenicals having a hydroxyl group), although the separation of five phenylarsenic compounds was not achieved.

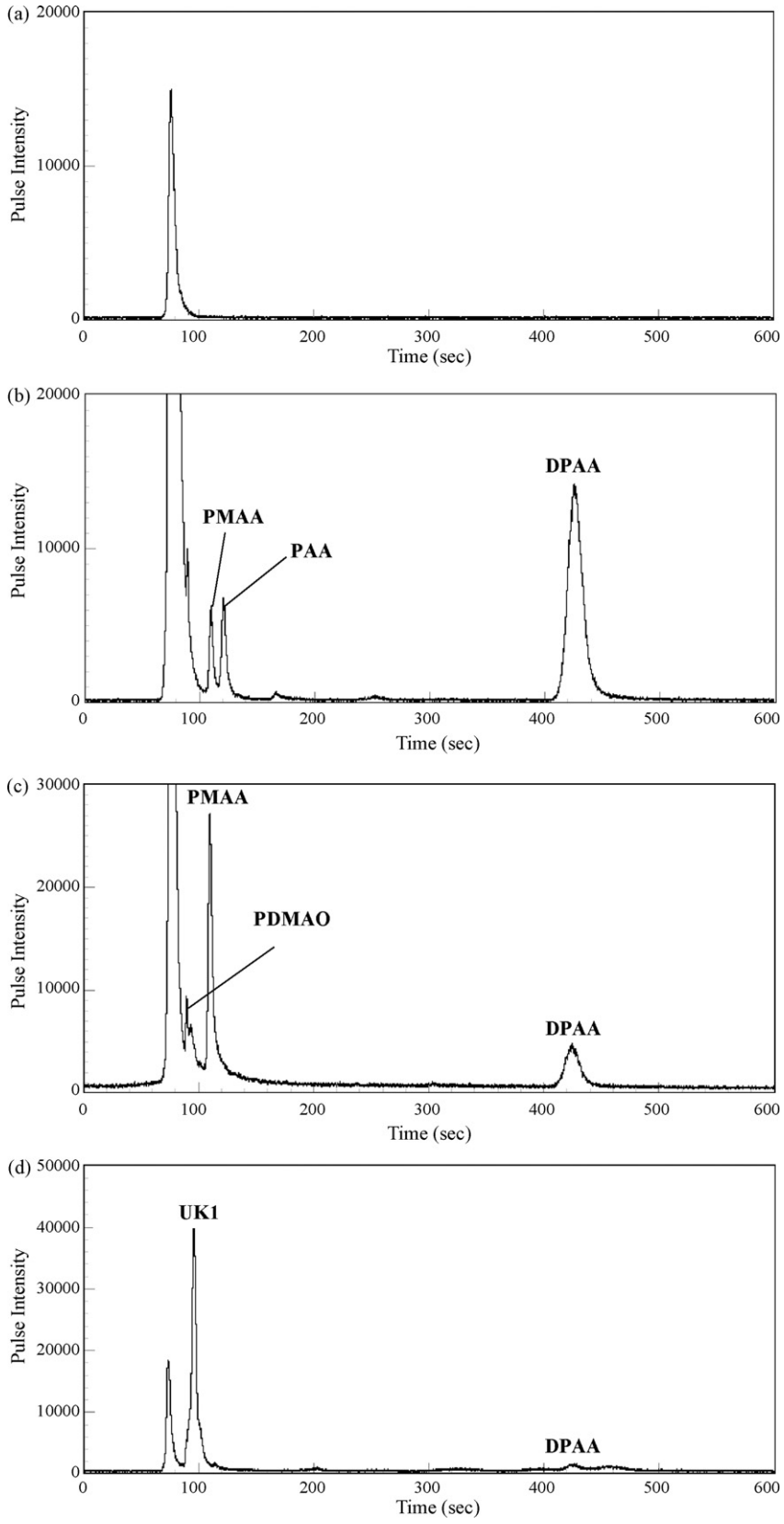

Fig. 4. Chromatograms of urine of several patients in Kamisu. The measurement was performed with the C_4 column. (a) Urine of a person who was not exposed, (b)–(d) urine of the patients.

Phosphate buffer was appropriate as the buffer and the concentration was set to 20 mM because higher concentrations of the buffer weakened the interaction of the arsenicals. Regarding the organic solvent, methanol weakened the interaction but acetonitrile strengthened it, which indicated that hydrophilic interactions could exist [14]. The concentration of acetonitrile was set to 50%, but large amount of acetonitrile would impose a burden on ICP-MS. Hence, in order to reduce the amount of acetonitrile entering the plasma, a column with a small internal diameter was selected and the flow rate was adjusted to 0.1 mL min^{-1}. However, the peaks were not sharp and good sensitivity was not obtained; thus, it was inferred that it was not possible to separate the arsenic compounds. The chromatogram is shown in Fig. 3(c).

Ultimately, the C_4 and CN columns were used for the determination of phenylarsenic compounds in urine and environmental samples; however, the NH_2 column was not used.

3.2. Determination of phenylarsenic compounds in urine of patients in Kamisu

The urine samples were determined by the newly established methods using the C_4 and CN columns. There were diverse samples; some samples contained no arsenicals and other samples contained phenylarsenic compounds with an arsenic concentration greater than 100 ng mL^{-1}. DPAA and PMAA were often detected, as shown in Fig. 4(b) and (c). Various unknown arsenicals were also found in Fig. 4 and highly contaminated samples tended to have many kinds of arsenicals. A noticeable unknown arsenical was observed around 95 s (Fig. 4(d)); this is represented as UK1. Moreover, some samples contained larger amounts of UK1 as compared to phenylarsenic compounds, although unknown arsenicals cannot be identified.

Furthermore, there are first eluted large peaks in all the chromatograms of Fig. 4. Therefore, several samples were analyzed by a method using the Inertsil AS column to investigate the highly hydrophilic components that could not be separated with a C_4 column. The main arsenicals in the hydrophilic components were DMAA and arsenobetaine (Fig. 5), which are largely contained in seafood. This suggests the first eluted peaks in the chromatograms of Fig. 4 are mainly composed of DMAA and arsenobetaine. However, some unknown arsenicals were also found, as shown in Fig. 5(b), although some of them might be arsenosugars. Thus, the main phenylarsenic compounds in the urine samples were DPAA and PMAA.

3.3. Analysis of urine of arsenic-administered mice

To determine the unknown arsenicals in the urine of the patients, the urine samples of mice were analyzed after administering DPAA, PAA, or PMAA. When PAA was administered, approximately 91% of arsenic components in the urine was PAA, but weak methylation was observed; PMAA and a very small amount of PDMAO were produced (Fig. 6(b)). Although some hydrophobic compounds were also detected, they were contained in the standard reagent, as shown in Fig. 7; the hydrophobic impurity

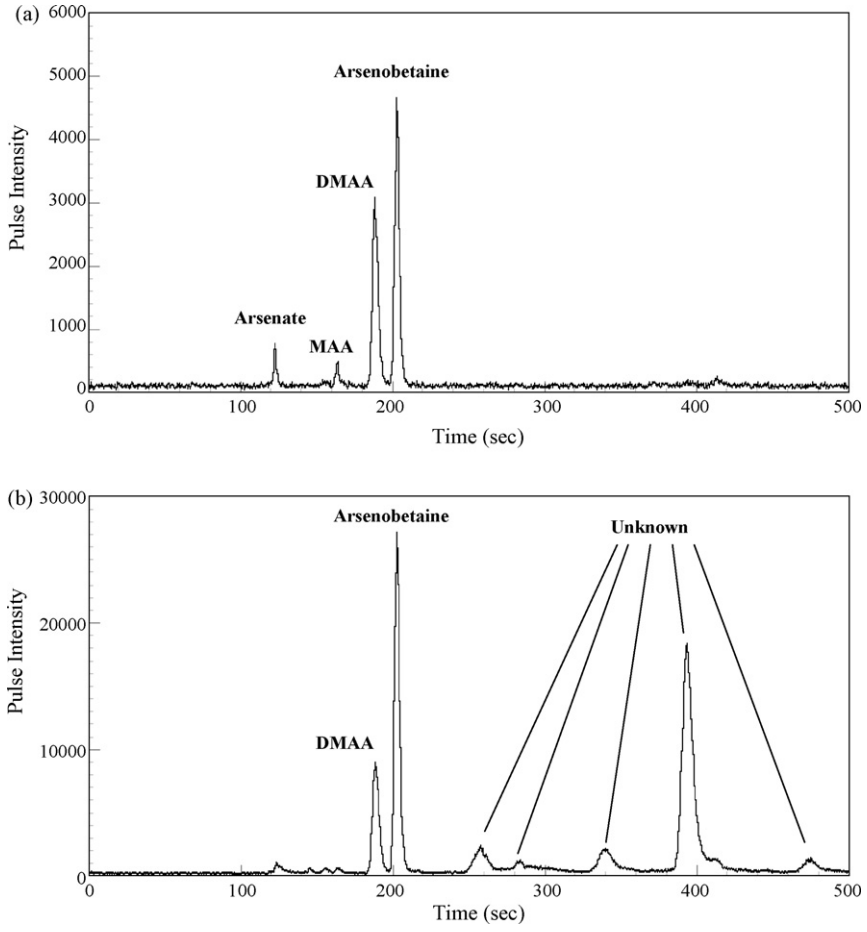

Fig. 5. Hydrophilic arsenicals in the urine of several patients in Kamisu. The measurement was performed with the Inertsil AS column.

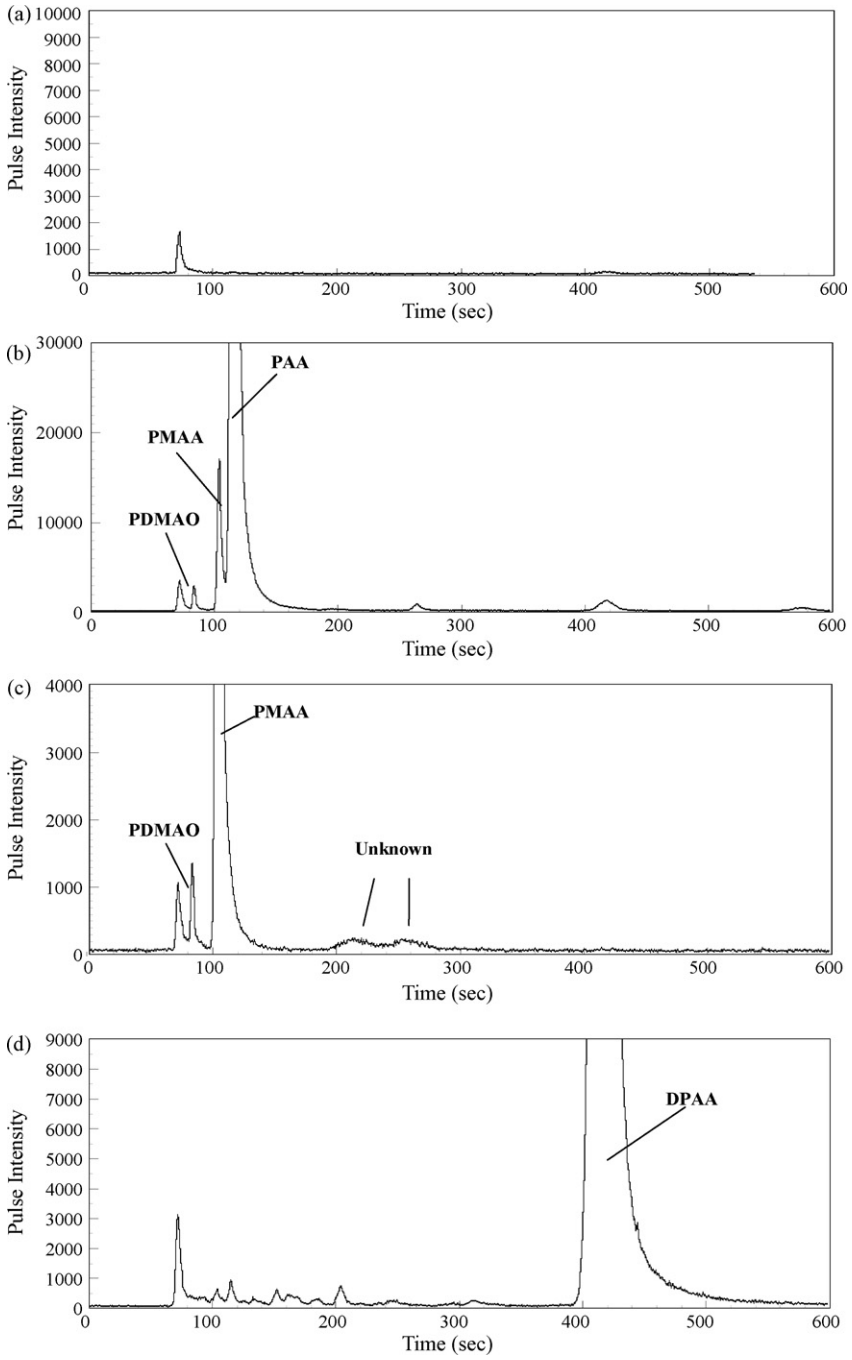

Fig. 6. Metabolites in urine of mice. The measurement was performed with the C_4 column. (a) Blank sample, (b) urine of PAA-administered mouse, (c) urine of PMAA-administered mouse, and (d) urine of DPAA-administered mouse.

content that was detected was approximately 1.6%. After the administration of PMAA, approximately 88% of arsenic components in the urine were not metabolized; however, a small amount of PDMAO and two more hydrophobic metabolites were found at 215 and 250 s in the chromatogram of Fig. 6(c). The administration of DPAA resulted in the production (less than 1%) of some hydrophilic compounds, but methylated DPMAO was not produced (Fig. 6(d)).

A peak eluting around 70 s (first peak) was also observed in all the chromatograms of Fig. 6. When the samples were analyzed by using the Inertsil AS column, it was identified as corresponding to DMAA. It is also detected in blank samples, and was probably not derived from the metabolism in the mice.

Consequently, unknown metabolites detected in the urine of the mice did not correspond to unknown arsenicals in the urine of the patients which are shown in Figs. 4 and 5.

3.4. Analysis of contaminated groundwater and rice

For further investigation, contaminated groundwater and rice samples were analyzed. In the groundwater, DPAA and PAA were the main species detected (Fig. 8(a)), and some unknown arsenicals were also found. Highly contaminated samples contained DPAA and PAA at concentrations higher than 1 $\mu g\,mL^{-1}$ of arsenic, and more unknown compounds tended to be contained in more contaminated samples. Further, there was a peak eluting at the same

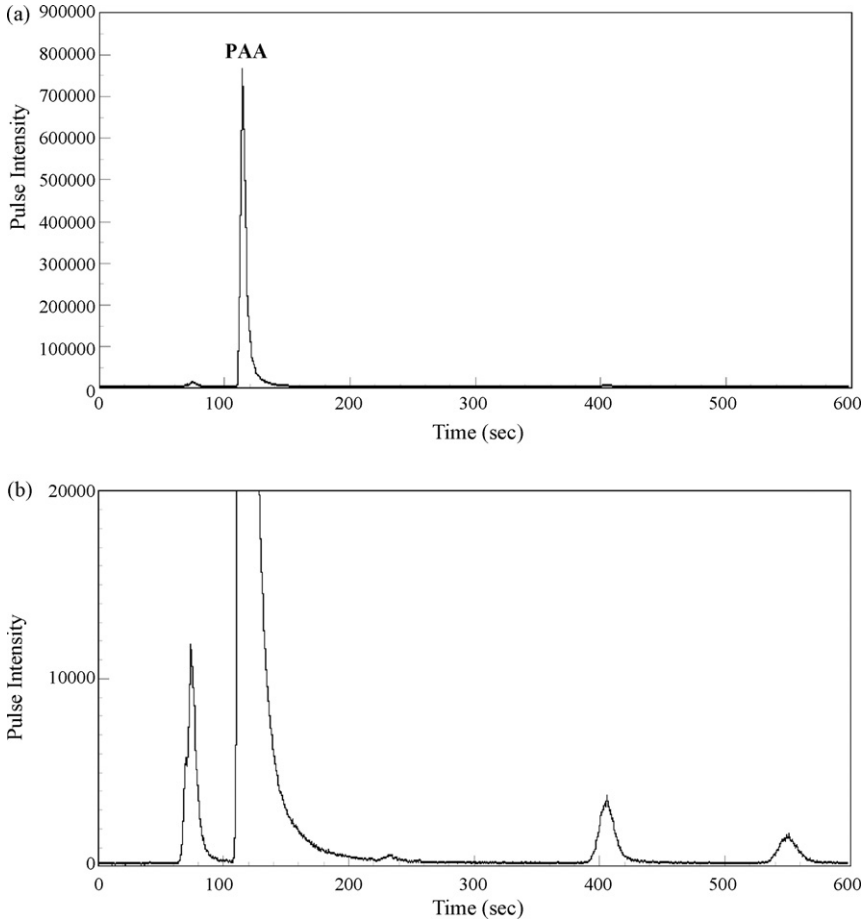

Fig. 7. Chromatogram of PAA for administration. The measurement was performed with the C_4 column. (b) The low-count region of chromatogram (a). Impurity peaks are detected at 75, 405, and 550 s. The impurity content is approximately 4%.

retention time as UK1 in a certain sample (Fig. 8(b)), but the other unknown arsenicals in the groundwater samples did not correspond with those in the urine samples of the patients. The rice samples contained large amounts of PMAA and small amounts of DPAA and PDMAO, as shown in Fig. 9(a). The amount of PMAA in rice samples ranged from hundreds to 1500 ng g^{-1} of arsenic (dry weight), and it accounted for approximately 41–73% of arsenicals in rice samples. There is a peak eluting just before PDMAO, and it was identified to be mainly due to arsenate by the analysis using the Inertsil AS column. DPMAO was also detected in some samples. Additionally, one unknown arsenical was also found, as shown in Fig. 9(b). A peak with the same retention time and peak shape as this unknown peak was found in some urine samples of the patients.

4. Discussion

In the urine of arsenic-administered mice, we found a few metabolites, all of which were not only methylated compounds (Fig. 4); this implies that moreover phenylarsenic compounds undergo other metabolic processes. Although PAA produced methylated metabolites, DPAA was not methylated, but produced hydrophilic compounds; this suggests that some functions can strengthen the hydrophilicity of molecules via metabolism. When PMAA was administered, PDMAO and arsenicals that were more hydrophobic than PMAA were detected, which suggests that PMAA might have undergone multiple metabolic processes. Therefore, it is suggested that the metabolic processes of phenylarsenic compounds differ from those of inorganic arsenicals [15] and arsenicals containing the same hydroxyl groups do not necessarily undergo the same metabolism. It is possible that the difference results from the water solubility of the phenylarsenic compounds. PAA and DPAA are slightly soluble but PMAA is very soluble; PAA and DPAA cannot be resolved at concentrations greater than 3.7 and 0.5 mg mL^{-1}, respectively, while PMAA is easily resolved even at concentrations greater than 10 mg mL^{-1}.

As a result of the analysis of urine and environmental samples, it was found that DPAA and PAA were mainly derived from water and methylated arsenicals such as PMAA, PDMAO, and DPMAO were mainly derived from rice. It is probable that most of the unknown arsenicals in the urine of the patients were not derived from the metabolites of the patients but from contaminated water. This is inferred on the basis of the following reasons. There were no common metabolites in the urine of the mice (Fig. 6), various unknown arsenicals were present in highly contaminated urine samples of the patients (Fig. 4) and groundwater samples, and only one unknown arsenical could be detected in rice (Fig. 9). Therefore, the existence of various unknown arsenicals in the urine of the patients suggested that many species of arsenicals were present around the source of contamination, particularly in the early period before the diffusion of arsenicals by the flow of groundwater. This theory is further supported by the fact that researches at sites contaminated by chemical warfare agents in Löcknitz,

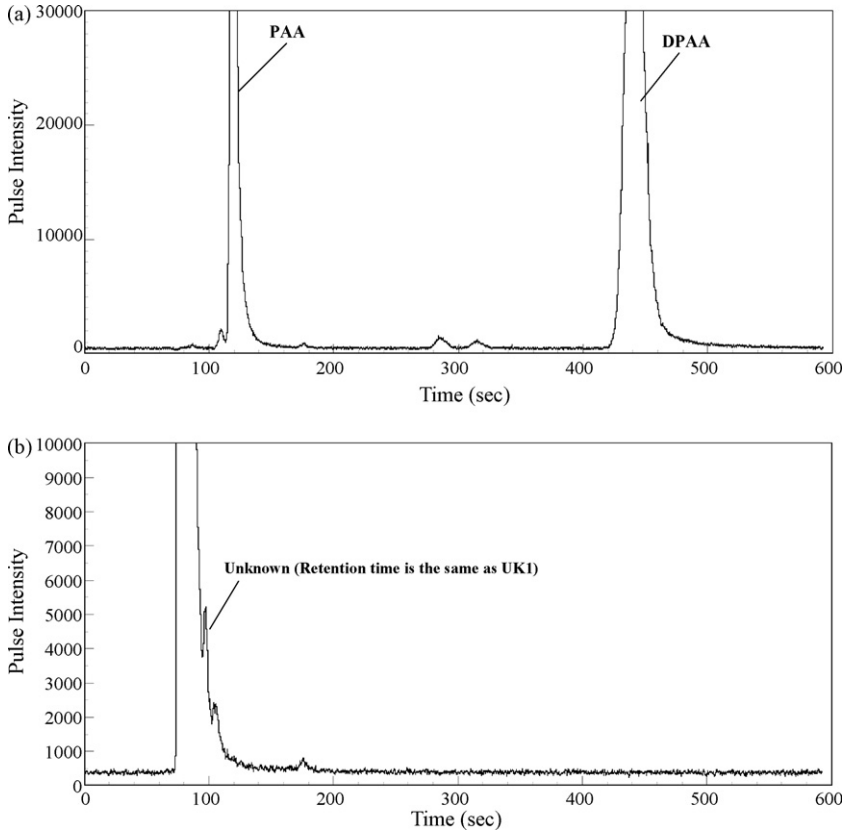

Fig. 8. Unknown compounds in contaminated groundwater. The measurement was performed with the C_4 column.

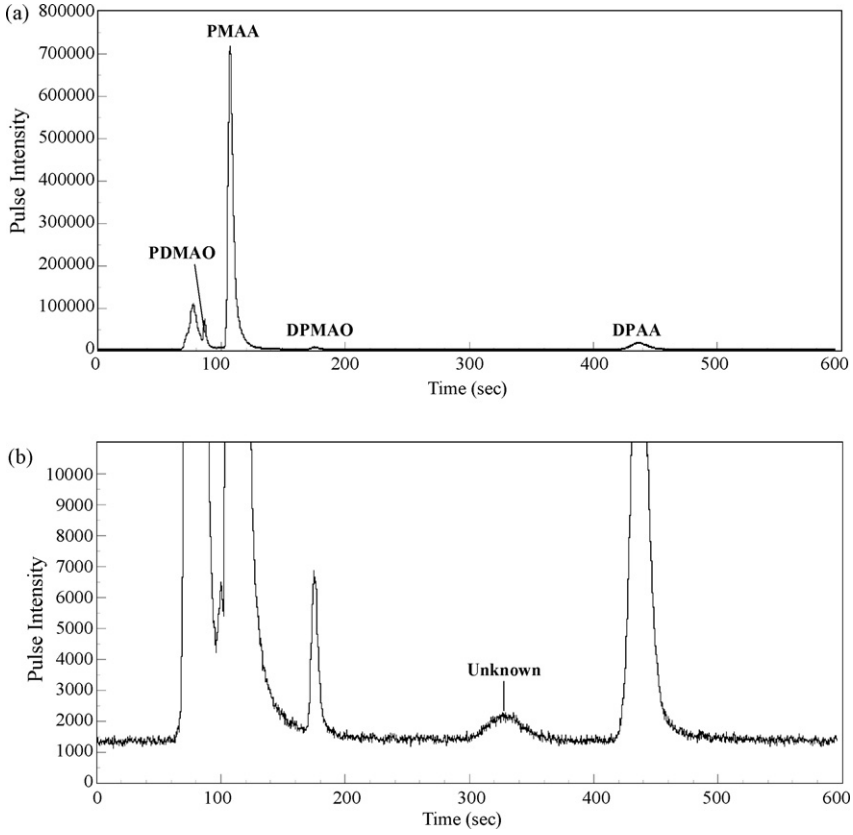

Fig. 9. Chromatogram of contaminated rice. The measurement was performed with the C_4 column. (b) The low-count region of chromatogram (a).

Germany, have identified as many as 32 arsenicals in soil and plants [16].

5. Conclusion

In this study, urine samples of patients exposed to phenylarsenic compounds were analyzed by newly established methods. The analysis indicated that most of the arsenic species in the urine of the patients were not derived from metabolism but depended on the substances ingested by them, which implied that arsenic species in the urine of the patients depended on the exposure conditions. Risk assessment of arsenic exposure under various conditions is important for accurate results, and speciation analysis is particularly vital for monitoring arsenic in the blood and urine of laborers for the appropriate disposal of abandoned chemical warfare agents. The newly established methods enable the rapid determination and speciation analysis of various phenylarsenic compounds. Therefore, they will be useful for the analysis of biological samples obtained from such laborers.

Acknowledgement

We would like to express our thanks to B. Sc. Miyashita for his analytical assistance.

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