— **Minireview** —

Laboratory Identification of the Nerve Gas Hydrolysis Products Alkyl Methylphosphonic Acids and Methylphosphonic Acid, by Gas Chromatography-mass Spectrometry after *tert*-Butyldimethylsilylation

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Alkyl methylphosphonic acids (RMPAs) and methylphosphonic acid (MPA), both nerve gas hydrolysis products, can be identified by gas chromatography-mass spectrometry (GC-MS). We adopted *tert*-butyldimethylsilylation (TBDMS) as a derivatization technique for the identification of these products by GC-MS. We found that it was difficult to detect RMPAs and MPA from evidence specimens such as soils and body fluids, and ascertained several causes for this. The first is the interference from the TBDMS derivatization. It was determined that the TBDMS derivatization of RMPAs and MPA was suppressed in the presence of divalent metal cations such as calcium and magnesium ions, as well as some neutral compounds like carbohydrates. A cleanup procedure using a strong anion-exchanger (SAX)-solid phase extraction (SPE) method was optimized to remove the interfering compounds. The second reason that detection was difficult was because of strong matrix adsorption. We elucidated that the aqueous extraction recoveries for all phosphonates were inversely correlated with the phosphate adsorption coefficient in the soil samples. To diminish the adsorption of phosphonates to soils, a method of alkali extraction was adopted. For human serum samples, we adopted acetonitrile or trichloroacetic acid deproteinization which worked to break the binding between the proteins and the phosphonates. The detection yields of RMPAs and MPA from soils and human serum were dramatically increased using these pretreatment procedures combined with extraction and/or SAX-SPE. The method of SAX-SPE was applied to various types of samples, such as seawater, drinks and human urine, and good detection yields of RMPAs and MPA were provided by GC-MS.

Key words —— alkyl methylphosphonic acid, methylphosphonic acid, solid phase extraction, *tert*-butyldimethylsilylation, gas chromatography-mass chromatography

INTRODUCTION

It is possible that chemical warfare agents (CWA) might be used as a means of terrorism, and detection and identification is required for incident management. Nerve gases including sarin are fast acting, lethal and easily decomposed to produce the characteristic compounds, alkyl methylphosphonic acids (RMPAs): pinacolyl methylphosphonic acid (PMPA), isopropyl methylphosphonic acid (IMPA), and ethyl methylphosphonic acid (EMPA) (Fig. 1).[1] Tabun decomposes in different ways. RMPAs can be further hydrolyzed to methylphosphonic acid (MPA). RMPAs are stable, non-toxic and synthetic compounds, and can be used as specific indirect markers of nerve gas exposure and usage. MPA is not only an RMPAs hydrolysis product, but is also a degradation product of methylphosphonyl dichloride, methylphosphonyl difluoride and dimethyl methylphosphonate, all of which are intermediates in the synthesis of nerve gases.[1] Therefore, the determination and identification of RMPAs and MPA in the evidence specimens is important for the verification of nerve gas exposure.

In the Matsumoto (1994) and Tokyo Subway (1995) sarin gas attacks, we were involved in the forensic investigation to detect sarin and its re-

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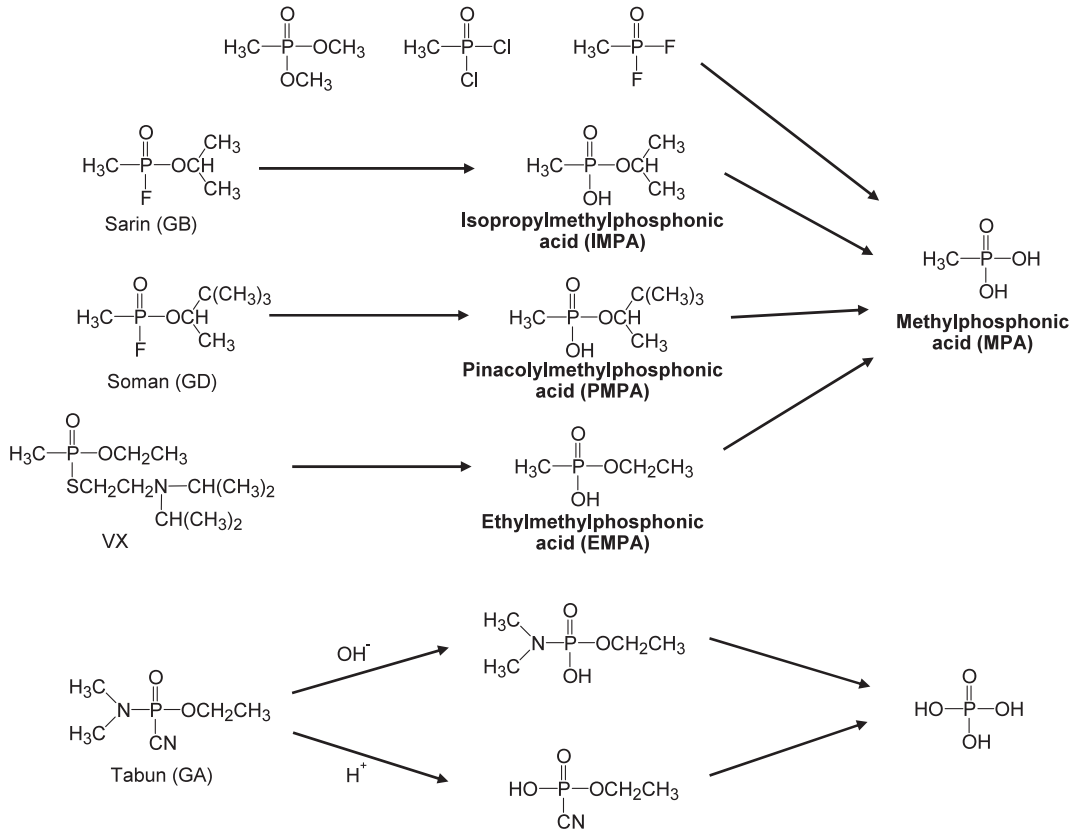

Fig. 1. Hydrolysis Pathway of Nerve Gases and Their Synthetic Intermediates

lated compounds from the evidence specimens.[2)] We adopted gas chromatography-mass spectrometry (GC-MS) as an identification technique for nerve gases and related compounds. However, it was difficult to detect both RMPAs and MPA in some specimens. We examined the cause of the poor detection of them from evidence samples, and established a pretreatment method prior to *tert*-butyldimethylsilylation (TBDMS) derivatization for GC-MS. We applied this to various types of environmental and biological aqueous samples and confirmed the effectiveness of these procedures.

In this review we report the chromatographic determination of nerve gases and their related compounds combined with MS. Of these, the determination of nerve gas hydrolysis products, RMPAs and MPA, by GC-MS after TBDMS derivatization including sample preparation is additionally reported.

NERVE GAS HYDROLYSIS PRODUCTS FOR GC-MS

A variety of analytical methods especially based on chromatographic techniques have been reviewed for the determination of CWA and related compounds.[3–8)] Among them, MS has been recommended as the standard technology for chemical weapon inspection,[8)] and GC-$MS_{(n)}$,[9,10)] capillary electrophoresis (CE)-MS[11)] and high performance liquid chromatography (LC)-$MS_{(n)}$[12–15)] have also been reported. LC-MS is utilized for the analysis of the non-volatile, polar and high molecular weight compounds, and has become one of the major analytical means in forensic science.[16)] By using LC-MS with a soft ionization technique such as atmospheric pressure ionization, $[M+H]^+$ or $[M-H]^-$ fragments usually appear in the mass spectra, and LC-MS has been applied for a rapid method of screening nerve gases and related compounds from aqueous samples.[8,17,18)] CE is an analytical method for CWA hydrolysis compounds requiring only simple preparation of dilution and filtration and easy equipment operation and maintenance.[19)] However,

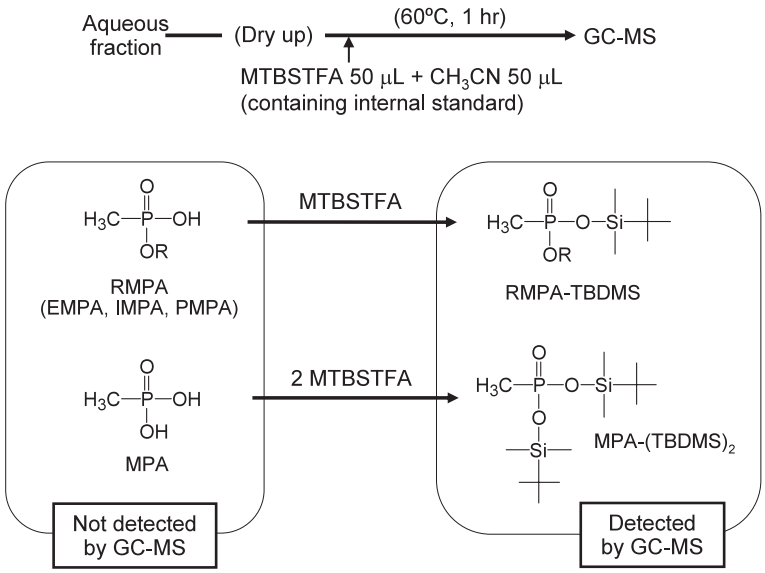

Fig. 2. *tert*-Butyldimethylsilyl Reaction of RMPAs and MPA

CE, especially by indirect UV-visible detection with visualizing agents, has somewhat lower detection sensitivity than GC-MS and LC-MS, and detection and migration time of the target compounds is susceptible to a sample matrix, so that the environmental samples like soil aqueous extract and river water have usually been subjected to CE analysis.[19] CE is therefore considered adaptable for rapid screening rather than for the quantitative determination of the nerve gas hydrolysis products.[20]

GC-MS is the standardized analytical method for detecting CWA and their related compounds for chemical weapon inspection.[8] In Japanese forensic science, GC-MS has also been mainly utilized as an analytical method for volatile organic compounds. Though nerve gases can be detected by GC-MS, RMPAs and MPA cannot be directly applied to GC-MS because of their polarity and water-soluble properties. Therefore, a derivatization procedure for conversion of RMPAs and MPA into volatile compounds is necessary prior to GC-MS analysis. In particular, derivatization reactions are used for the chromatographic analysis of degradation products of chemical warfare agents.[21] Several derivatization techniques for nerve gas hydrolysis products have been reported: trimethylsilylation,[22] methylation,[23] TBDMS[24] and pentafluorobenzylation.[25,26] TBDMS derivatives are comparatively stable and easily prepared, and have good GC peak resolution,[24] and Tsunoda[27] also applied the TBDMS derivatization procedure of the herbicides glyphosate, glufosinate and bialaphos and their metabolites by GC-MS and GC using flame ionization detection. We adopted the TBDMS derivatization technique of nerve gas hydrolysis products to GC-MS (Fig. 2). Figure 3 shows a total ion chromatogram and mass spectra of TBDMS-derivatized nerve gas hydrolysis products. Derivatives were well-separated from each other, and the mass spectra showed typical fragmentation patterns. Calibration curves for peak area ratios of TBDMS derivatives of RMPAs and MPA to the internal standard (anthracene) peak were linear for the amounts in the reaction vial, which ranged from 0.15 to 20 μg/ml, with correlation coefficients better than 0.975. The repeatability (n = 7) for the determination of 0.8 μg phosphonates ranged from 9.8–12.0% expressed as the relative standard deviation. Detection limits for RMPAs and MPA derivatives were 100 ng/ml in the reaction vial (S/N = 3) for extracted ion chromatograms.[28]

INFERFERING FACTORS FOR THE TBDMS OF NERVE GAS HYDROLYSIS PRODUCTS

Some compounds which suppress TBDMS derivatization of RMPAs and MPA are included in aqueous extracts from samples. It has been reported that metal cations form insoluble complexes with anionic phosphonates.[29,30] We examined the influence of metal cations on the TBDMS deriva-

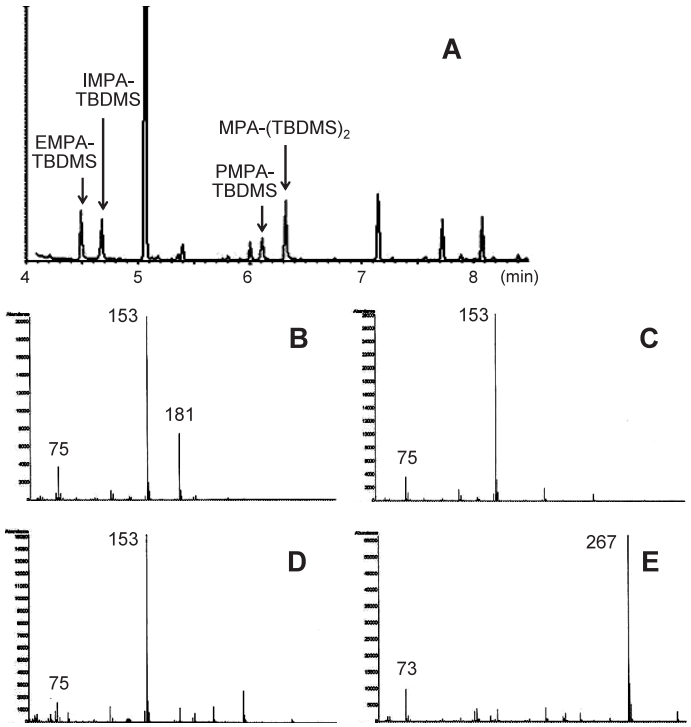

Fig. 3. GC-MS of TBDMS Derivatives of RMPAs and MPA

GC: HP-5 MS (30 m × 0.25 mm i.d., 0.25 μm film thickness), 90°C (1 min)–(20°C/min)–290°C (5 min), He 0.8 ml/min, split (250°C) 50 : 1, 1 μl inject; MS: quadrupole, EI (34.6 μA, 69.9 eV, positive, m/z 50–550). A: Total ion chromatogram of TBDMS derivatives of RMPAs and MPA (each 25 μg/ml); B–E: EI mass spectra of TBDMS derivatives of EMPA (B), IMPA (C), PMPA (D) and MPA (E).

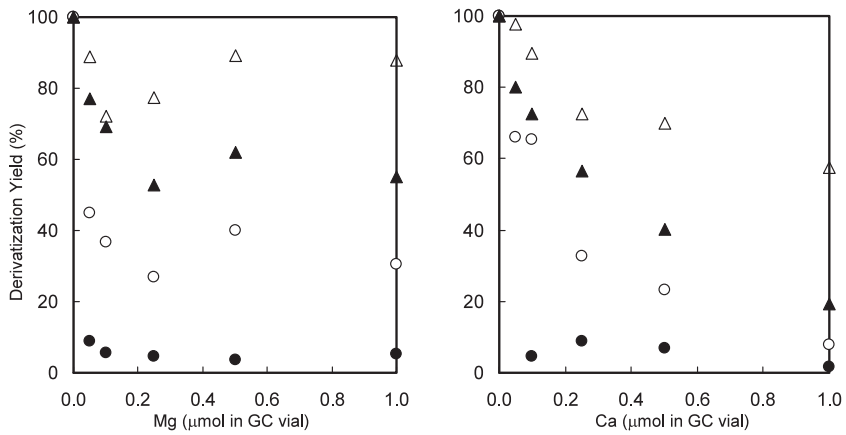

Fig. 4. Effects of Alkaline Earth Metals on the Detection Yields of TBDMS Derivatives of RMPAs and MPA

A 0.5 ml aqueous solution containing 4.5 μg/ml RMPAs and MPA, and known concentration of cations (calcium chloride or magnesium chloride) was dried and derivatized by TBDMS followed by GC-MS.

tization of phosphonates, and found that both calcium and magnesium ions (Ca^{2+}, Mg^{2+}) suppressed the derivatization (Fig. 4). Moreover, some neutral compounds, such as carbohydrates, which contain hydroxide groups competed against the phosphonates in the silylation reaction and suppressed the TBDMS derivatization.[28,31]

Several investigations on cleanup of nerve gas hydrolysis products using solid phase extraction (SPE) have been reported,[23,25,32–34] and some drawbacks were found such as low recoveries of RMPAs and MPA and insufficient information about SPE procedures. We attempted some types of SPE including strong cation-exchanger,[28] anion-exchanger and reversed-phase[31,35] followed by TBDMS derivatization. We selected strong anion-

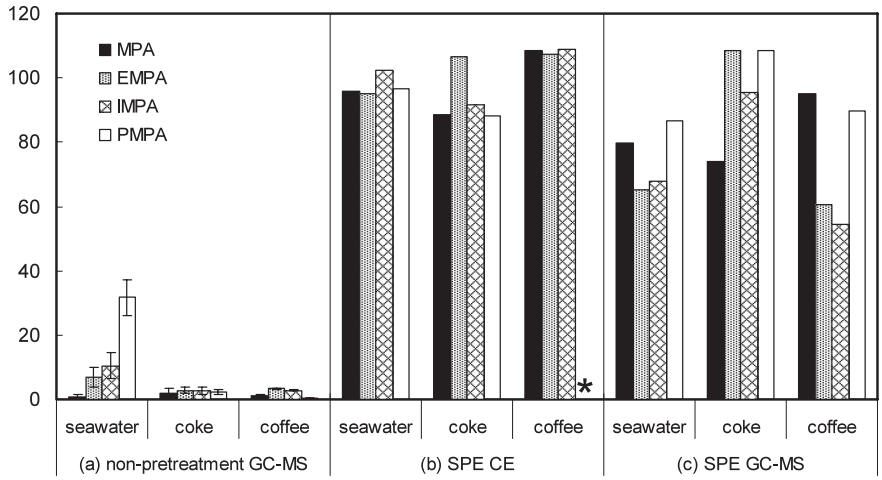

Fig. 5. Recoveries and Detection Yields of RMPAs and MPA from Seawater and Beverage Samples

(a) A 0.5 ml sample spiked with RMPAs and MPA (each 0.8 μg) was dried, TBDMS derivatized, and analyzed by GC-MS. (b) A 2 ml sample spiked with RMPAs and MPA (each 150 μg) was pretreated with SAX-SPE, and analyzed by CE. (c) A 2 ml sample spiked with RMPAs and MPA (each 3 μg) was pretreated with SAX-SPE, TBDMS derivatized, and analyzed by GC-MS. * Not determined because of interference by unknown compounds contained in the canned coffee. The values were from a single or an average of three determinations ±S.D.

exchanger (SAX)-SPE for cleanup of RMPAs and MPA from the aqueous samples,[35] and the cleanup methodology was optimized. As shown in Fig. 5, the detection yields of RMPAs and MPA by GC-MS were dramatically increased when a SAX-SPE cleanup procedure for seawater and drinks was used prior to analysis.[31,35]

DETECTION OF NERVE GAS HYDROLYSIS PRODUCTS FROM SOILS

For environmental evidence samples, especially soils, it was a problem that the GC-MS detectability of RMPAs and MPA was very low even when using a SAX-SPE pretreatment was used. Daughton *et al.*[36] indicated that RMPAs, especially MPA, was bound onto spodosol which contains rich oxidative products and humic substances, and we are not able to disregard the adsorption of RMPAs and MPA onto soils. We examined the aqueous extraction recoveries of RMPAs and MPA from various types of soil samples. To accurately evaluate the aqueous extraction recoveries of RMPAs and MPA, CE was adopted for their quantitative determination.[20,37] RMPAs were extracted from all types of soils, and it was found that the aqueous extraction recovery of MPA was much lower than that of RMPAs (Fig. 6). The hydrophobicity level of RMPAs and MPA was positively correlated with the aqueous extraction recoveries. Their low levels by TBDMS GC-MS were attributed to the adsorption of phosphonates to the soils as well as interference in the derivatization procedure. From a multivariate analysis between pedological characteristics and extraction recoveries of the phosphonates, the phosphate absorption coefficients showed a strong negative correlation with the aqueous extraction recoveries of both RMPAs and MPA.[38] Within the soil matrix, the positively charged polyvalent cations, adsorbed on clays and humic substances such as activated aluminum and ferric ions, are the main phosphate adsorption sites. Because phosphonates are structurally and physicochemically similar to phosphate, they might be adsorbed onto the phosphate adsorption sites. It is reported that polymerized hydroxyaluminum ion, activated aluminum and ferric ions become factors for the adsorption and stabilization of phosphate onto soils.[39]

To improve the extraction recovery of RMPAs and MPA from soils, several extraction procedures were reported.[40,41] Chaudot *et al.*[40] reported that the superfluidic extraction of EMPA and 68–83% of EMPA was recovered from soil samples, but MPA was not mentioned. Vermillion and Crenshaw[41] demonstrated the extraction of RMPAs and MPA from soils with carbonate, but incomplete improvement in extraction recovery occurred for some kinds of soils. We attempted to break the binding of phosphonates onto the soil under elevated pH conditions by 0.1 M sodium hydroxide (NaOH).[42] The soil alkaline extract was neutralized with

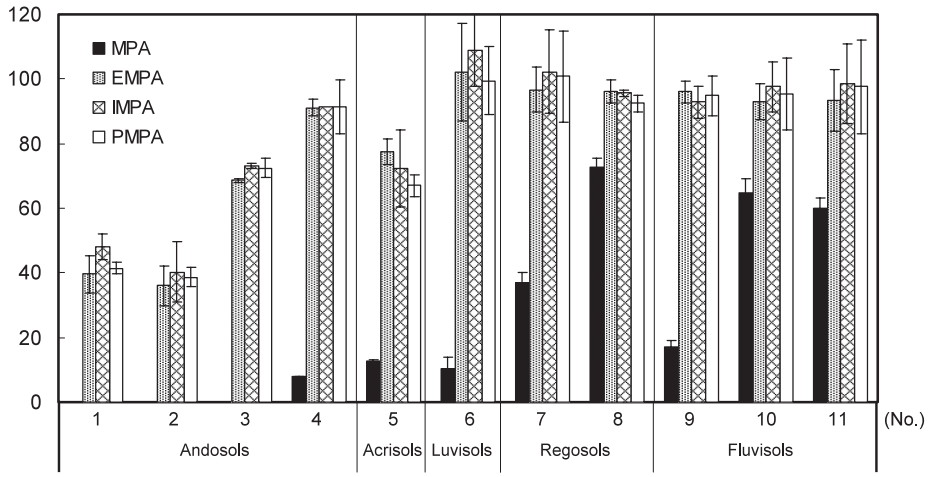

Fig. 6. Recoveries of RMPAs and MPA by Aqueous Extraction from Soil Samples

The recovery values were obtained after aqueous extraction and directly subjected to CE analysis and represent averages (n = 3).

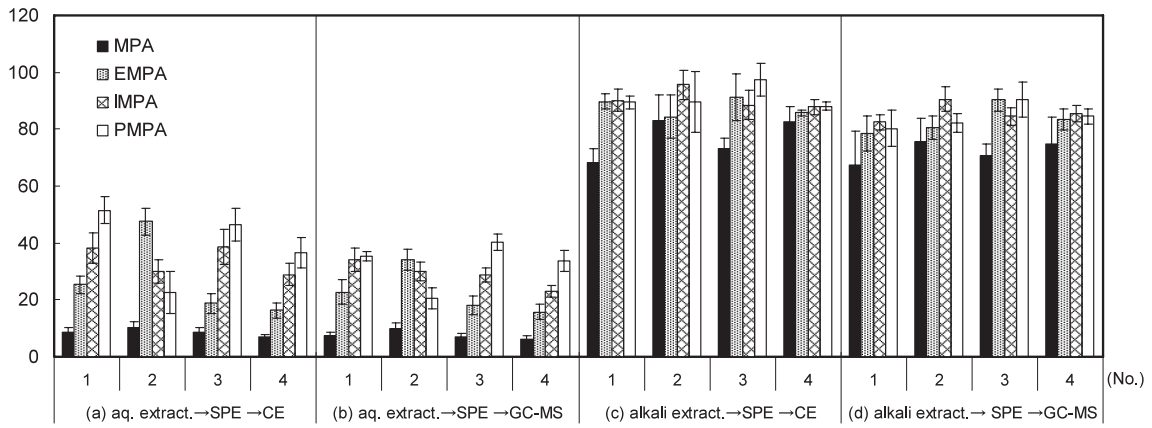

Fig. 7. Aqueous and Alkali Extraction Recoveries and Detection Yields of RMPAs and MPA from Soil Samples

A 2 g soil sample spiked with RMPAs and MPA was extracted with water [(a), (b)] or 0.1 M NaOH solution [(c), (d)], and the extract was treated by SAX-SPE, and analyzed by CE [(a), (c)] or analyzed by GC-MS after TBDMS derivatization [(b), (d)]. Spiked levels of RMPAs and MPA were 136–175 μg [(a), (c)] or 27–35 μg [(b), (d)]. The values were an average of three determinations ± S.D. Sample numbers do not correspond with those in Fig. 6.

hydrofluoric acid, subjected to SPE, and analyzed by TBDMS GC-MS. Figure 7 indicates the yields of RMPAs and MPA by TBDMS GC-MS detection from soil samples combined with neutral (water) or alkali aqueous soil extraction and SPE pretreatment. Using the established method combined with alkali soil extraction and SPE, extraction recoveries were dramatically increased, and the detection yields of RMPAs and MPA were over 65%.

DETERMINATION OF NERVE GAS HYDROLYSIS PRODUCTS FROM BIOLOGICAL SAMPLES

In the human body, most nerve gases are readily hydrolyzed to RMPAs, and become partially bound to proteins such as cholinesterases, resulting in the formation of nerve gas adducts.[43,44] The level of nerve gas hydrolysis products and adducts in biological fluids can reflect the extent of the exposure to nerve gases. Therefore, to verify a victim's exposure to a nerve gas, it is necessary to measure cholinesterase activity, and detect RMPAs and MPA, or verification of nerve gas protein adducts.[43–46] Measuring cholinesterase activ-

ity is not specific for the identification of nerve gases, but it is helpful as a complementary technique in organophosphorus compound-related poisoning cases. Nerve gas protein adducts were stable in blood during the lifetime of a protein,[44] and they are able to be detected long after exposure to nerve gases. However, some disadvantages should be addressed for complicated analytical procedure.

Several reports have appeared relative to GC-MS analysis of nerve gas hydrolysis products from serum[25,47–49] and urine samples,[25,47,50,51] and RMPAs but not MPA has been analyzed. However, the determination of MPA is important because it is not only an RMPAs hydrolysis product, but is also a degradation product of nerve gas synthesis intermediates. Blood plasma also contains a high concentration of proteins which interfere with a GC-MS analysis, and with which RMPAs and MPA are adsorbed. We applied a deproteinization and SPE pretreatment procedure for the preparation of human plasma samples. Considering the difference in the hydrophobicity pattern of RMPAs and MPA, they were separately quantitated using the two different cleanup pretreatments.[52] For RMPAs, CH_3CN was used for the deproteinization of human plasma, followed by SAX-SPE cleanup. The detection yields of RMPAs by GC-MS were increased from 69% to 99%. The detection limits of RMPAs (S/N = 3 on the extracted ion chromatogram of m/z 153) were 30 ng per 1 ml of plasma, and the calibration curves were linear in the concentration range of 75 ng to 2.6 μg per ml of plasma. With the detection limits, the lower exposure level can be detectable by adopting splitless injection or selected ion monitoring mode during the GC-MS analysis. For MPA, we adopted trichloroacetic acid (TCA) as the deproteinization reagent, followed by extraction with diethyl ether in order to remove most of the TCA which interfered with the TBDMS derivatization. Only MPA remained in the aqueous layer during the TCA extraction with ether, and the SAX-SPE procedure was adopted for the cleanup of the TCA deproteinization fraction. TCA and MPA were separately eluted with ammonia methanolic solution from the SAX-SPE cartridge by altering the ammonium concentration. The detection limit of MPA (S/N = 3 on the extracted ion chromatogram of m/z 267) was 33 ng per ml of plasma. The calibration curve was linear in the concentration range of 50 ng to 5 μg per ml of plasma, and the detection yield of MPA was quantitative.

From urine samples taken from sarin incident patients, RMPAs and MPA were detected during several days after sarin exposure.[51] Accordingly, urine is one of the most useful biological samples in nerve gas terrorism cases. We applied urines to SAX-SPE directly prior to GC-MS analysis of RMPAs and MPA, and the TBDMS derivatives of RMPAs and MPA in urine were quantitatively detected. The retention time of TBDMS-derivatized sulfate derived from urine constituent was similar to that of TBDMS-derivatized MPA, and the base peaks of TBDMS-derivatized sulfate and MPA are m/z 269 and 267, respectively. Consequently, the detection limit of MPA (S/N = 3 on the extracted ion chromatogram of m/z 267) was 700 ng per ml of urine. If possible, application of GC-MS/MS will resolve this interference for MPA. The detection limits of RMPAs by GC-MS were 60 ng per ml of urine. The calibration curve was linear in the concentration range of 140 ng–5.6 μg (for RMPAs) and 700 ng–5.5 μg (for MPA) per ml of urine.[52]

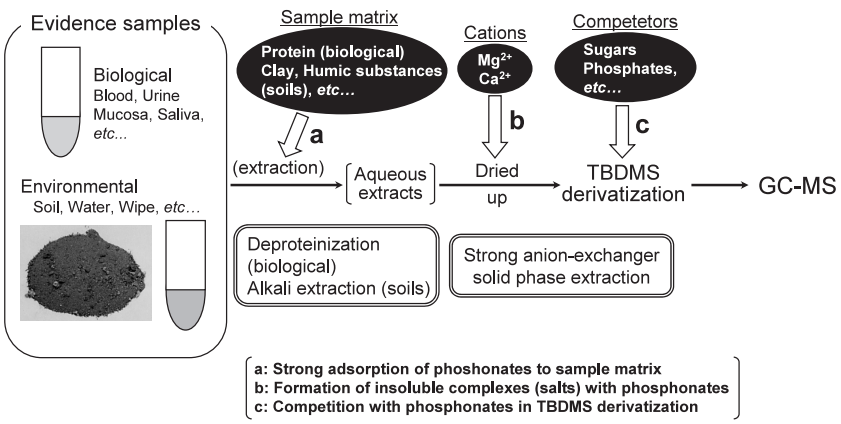

Fig. 8. The Schematic Overviews of the Sample Preparation, Interference Factors and Improvement Measurement for the Cleanup of RMPAs and MPA from Evidence Samples

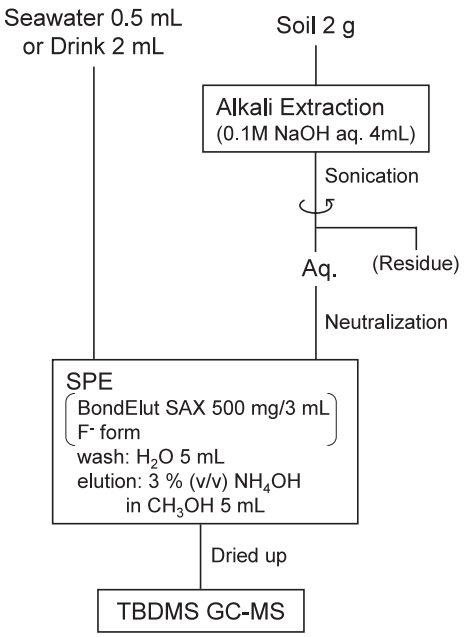

Fig. 9. Schematics Showing the Methodology Used in Environmental and Aqueous Sample Preparation

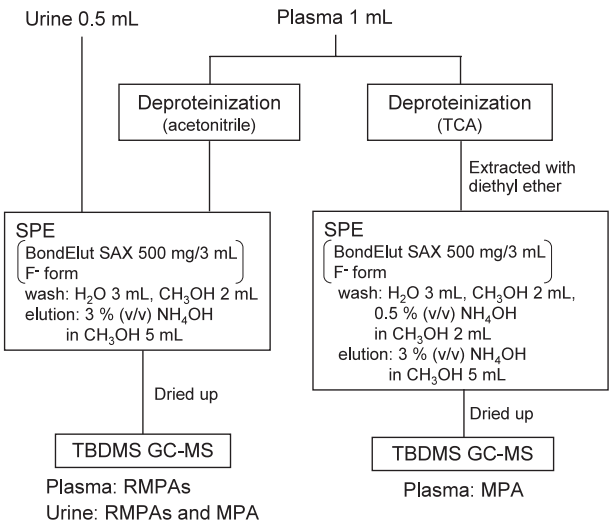

Fig. 10. Schematics Showing the Methodology Used in Biological Sample Preparation

CONCLUSIONS

We have presented in this review the laboratory identification method for the nerve gas hydrolysis products, RMPAs and MPA, by GC-MS after TBDMS. The schematic overviews contained in this summary, including the interfering factors in the TBDMS derivatization of phosphonates and the pretreatment improvement strategies to clean up the samples, are shown in Fig. 8. Figures 9 and 10 show the schematically quantitative determination methodology of RMPAs and MPA used in a wide variety of aqueous sample preparation. The TBDMS derivatization procedure has also been applied to thiodiglycol and ethanolamines, which are sulfur and nitrogen mustard hydrolysis products, respectively.[53, 54]

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